# Identification of epithelial alarmins that promote activation of primary human lung fibroblasts

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# Contents

**LIST OF FIGURES**

**Figure 32** Influence of selected Pattern Recognition Receptor ligands on IL-6 gene expression in primary human lung fibroblasts.

**Figure 33** Validation of the efficacy of a ribonuclease, benzonase, in a functional assay using Poly I:C as an analog of double stranded RNA and IL-6 gene expression as a marker of innate immune signaling activation.

**Figure 34** Influence of a Benzonase on IL-6 gene expression in MRC5 cells treated with conditioned media from damaged 16HBE14o- cells.

**Figure 35** Validation of the efficacy of the IL-1α neutralizing antibody (IL-1α NAb), IL-1β neutralizing antibody (IL-1β NAb) and IL-1 receptor antagonist (IL-1Ra).

**Figure 36** Influence of IL-1 antagonists on IL-6 expression in MRC5 cells treated with conditioned media from 16HBE14o- cells damaged with 200μM $H_2O_2$.

**Figure 37** Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on IL-6 gene expression (A) and IL-6 protein secretion (B) by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with $H_2O_2$.

**Figure 38** Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on IL-8 gene expression (A) and IL-8 protein secretion (B) by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with $H_2O_2$.

**Figure 39** Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on GM-CSF gene expression (A) and GM-CSF protein secretion (B) by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with $H_2O_2$.

**Figure 40** Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on MCP-1 gene expression (A) and MCP-1 protein secretion (B) by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with $H_2O_2$.

**Figure 41** Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on IL-1α gene expression by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with $H_2O_2$.

**Figure 42** Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on IL-1β gene expression by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with $H_2O_2$.

**Figure 43** Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on proinflammatory cytokine gene expression by primary human lung fibroblasts stimulated with conditioned media from 16HBE14o- cells (HBE) damaged with 50μM thapsigargin (TG).

**Figure 44** Influence of IL-1α (IL-1α Nab) and IL-1β (IL-1β Nab) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on phosphorylation of downstream TLR signaling molecules in primary human lung fibroblasts induced following treatment with conditioned media from $H_2O_2$ damaged 16HBE-14o- cells (HBE).

**Figure 45** Influence of IL-1α and Poly I:C on selected proinflammatory cytokine gene expression in primary human lung fibroblats in the course of time.

**Figure 46** Influence of IL-1α and Poly I:C on of IL-6 protein acumulation in primary human lung fibroblasts.

**Figure 47** Influence of IKK2i on IL-1α and Poly I:C induced IL-8 protein secretion by primary human lung fibroblasts.

**Figure 48** Influence of IKK2i on IL-1α and Poly I:C induced IL-6 protein secretion by primary human lung fibroblasts.

**Figure 49** Influence of TAK1i on IL-1α and Poly I:C induced IL-8 protein secretion by primary human lung fibroblasts.

**Figure 50** Influence of TAK1i on IL-1α and Poly I:C induced IL-6 protein secretion by primary human lung fibroblasts.

**LIST OF TABLES**

**Table 1** List of cytokines belonging to the IL-1 family.

**Table 2** List of alarmins implicated in chronic inflammatory lung diseases.

**Table 3** Selected Pathogen Associated Molecular Patterns and pathogens producing them.

**Table 4** Major cytokine families.

**Table 5** List of primary antibodies.

**Table 6** List of secondary antibodies.

**Table 7** List of qRT-PCR primers.

**Table 8** Composition and concentrations of reagents and antibodies used in ELISA.

**Table 9** List of components used to prepare polyacylamide gels.

**Table 10** List of buffers and reagents used for western blotting.

**Table 11** Concentration of reagents used in the HMGB1 ELISA.

## ABBREVIATIONS

ABTS -  2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

AIM2 - Interferon-inducible protein2

AP-1 - Activator protein 1

ARAD - Azithromycin reversible allograft dysfunction

ATF6 - Activating transcription factor 6

ATP - Adenosine-5'-triphosphate

BAFF - B-cell activating factor

BAL - Bronchoalveolar lavage

BCA - Bicinchoninic acid *assay*

Bcl-2 - B-cell lymphoma 2

bFGF - Basic Fibroblast Growth Factor

BIP - Binding immunoglobulin protein

BLC - B lymphocyte chemoattractant

BMDC - Bone marrow derived dendritic cells

BAMBI - BMP and activin membrane-bound inhibitor homolog

BOS - Bronchiolitis Obliterans Syndrome

BSA – Bovine serum albumine

CAFs - Cancer-associated fibroblasts

CCL20 - Chemokine (C-C motif) ligand 20

CCL5 - Chemokine (C-C motif) ligand 5

CD – Claster of differentiation

cDNA - Complementary DNA

CF – Cystic fibrosis

c-GCS - c-glutamylcysteine synthetase

CMV - Cytomegalovirus

COPD - Chronic obstructive pulmonary disease

COX2 - Cyclooxygenase 2

CSE - Cigarette smoke extract

CXCL10 - C-X-C motif chemokine 10

DAMP - Damage associated molecular pattern

DMSO - Dimethyl sulfoxide

DMTU - Dimethylthiourea

DTT - Dithiothreitol

ECM - Extracellular matrix

EGF - Epidermal Growth Factor

ELC - EBI1 ligand chemokine

EMT- Epithelial to mesenchymal transition

ER - Endoplasmic reticulum

ERAD - stress associated protein degradation

ERK - Extracellular signal-regulated kinases

FACS - Fluorescence-activated cell sorting

fBOS - Fibroproliferative BOS

FCH - Ferritin heavy chain

FEV1 - Forced expiratory volume in 1 second

fMLP - N-formyl-methionine- leucine-phenylalanine

FSL-1 -  Bacterial-derived TLR2/6 agonist

Gal-1 - Galectin-1

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GCP-2 - Granulocyte chemotactic protein GCP-2

GRP78 - Glucose regulated protein 78

$H_2O_2$ - Hydrogen peroxide

$H_2SO_4$ – Sulfuric acid

HCl - Hydrochloric acid

HMGB-1 - High-mobility group box B1

HNP1 - Human Neutrophil Peptide1

HO-1 - Haemoxygenase-1

HSP - Heart Shock Proteins

HSP - Heat shock protein

ICAM-1 - Intercellular Adhesion Molecule 1

IFN - Interferon

IGF-1 - Insulin-like Growth Factor 1

IGFBP - Insulin-like growth factor binding protein

IKK1 (IKKα) - Inhibitor of nuclear factor kappa-B kinase subunit alpha

IKK2 (IKKβ) - Inhibitor of nuclear factor kappa-B kinase subunit beta

IKK2i - IKK2 inhibitor

IKKγ (NEMO) - Inhibitor of nuclear factor kappa-B kinase subunit gamma

IKKε - IκB Kinase-epsilon

IL - Interleukin

IL-1R - Interleukin 1 receptor

IL-1Ra - Interleukin 1 receptor antagonist

iNOS - Inducible nitric oxide synthase

iNOS - Nitric oxide synthase

IPF - Idiopathic pulmonary fibrosis

IRAK1 - Interleukin-1 receptor-associated kinase 1

IRE1α - Inositol-requiring-1α

IRF - Interferon regulated factor

JAK/STAT - Janus kinase /signal transducer activator of transcription

JNK - c-Jun N-terminal kinases

LPS - lipopolysaccharide

MAPK - Mitogen-activated Kinase

MCP-1 - Monocyte chemoattractant protein-1

MDA5 - Melanoma Differentiation-Associated protein 5

MIF - macrophage migration inhibitory factor

MIP-1α - Macrophage inflammatory protein-1α

MK5 - MAPK-Activated Protein Kinase 5

MnSOD - Manganese superoxide dismutase

MSD - Meso Scale Discovery

MyD88 - Myeloid differentiation primary response gene (88)

NADPH - Nicotinamide adenine dinucleotide phosphate

NFATC4, NFATC1 - Nuclear factor of activated T-cells, cytoplasmic 1 and 4

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NLR - Nod-like Receptors

NO - Nitric oxide

OVA - Ovalbumin

PAMP - Pathogen associated molecular pattern

PAN - Persistent airway neutrophilia

PBEC - Primary bronchial epithelial cells

PCR - Polymerase chain reaction

PDGF - Platelet derived growth factor

PDGF - Platelet derived growth factor

PERK - RNA-dependent protein kinase (PKR)-like ER kinase

PF - Primary human lung fibroblasts

PMNs - Polymorphonuclear cells

PRR - Pattern Recognition Receptors

qRT-PCR - Real-time reverse transcription-PCR

RA - Rheumatoid arthritis

RAGE - Advanced glycation endproducts

RANKL - Nuclear factor kappa-B ligand

RAS - Restrictive allograft dysfunction

RIG-1 - Retinoic acid-inducible gene 1

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RSV - Respiratory Syncytial Virus

S100A - S100 calcium binding protein A

SA - Synovial fibroblasts

SAPK - Stress-activated Protein kinases

SDF-1 - Stromal cell derived factor

SDS - Sodium monododecyl sulfate

SdV - Sendai virus

SERCA - Sarco/endoplasmic reticulum Ca2+-ATPase

SLC - Secondary lymphoid chemokine

SLPI - Secretory leucocyte protease inhibitor

ssRNA - Single stranded RNA

TAK1i - TAK1 inhibitor

TBK1 - TANK-binding kinase 1

TBS - Tris-buffered saline

TGF-β1 - Transforming growth factor-β1

TICAM1 - Toll-like receptor adaptor molecule 1

TLR - Toll-like receptor

TMB - 3,3',5,5'-Tetramethylbenzidine

TRAF6 - TNF receptor-associated factor 6

TRAM - TRIF-related adaptor molecule

TRIF - TIR-domain-containing adapter-inducing interferon-β

TRIS - Tris(hydroxymethyl)aminomethane

UPR - Unfolded Protein Response

VCAM-1 - Vascular cell adhesion molecule 1

VEGF-A - Vascular endothelial growth factor A

XBP-1 - X-box binding protein 1

XTT - 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

α-SMA - α-Smooth muscle actin

**DECLARATION**

I declare that the work presented in this thesis is my own work unless acknowledged in the text. The BAL samples from post-transplant patients were provided by Dr Rahul Mahida and Dr Bart Vanaudenaerde, who also performed statistical analysis of some of the clinical data. In vitro work was performed at the Institute for Cellular Medicine in collaboration with GlaxoSmithKline, Stevenage, under the supervision of Prof. Derek Mann, Prof. Andrew Fisher and Prof. Stuart Farrow and with assistance of Dr Lee Borthwick and Dr Jelena Mann, who provided me with technical support in the early stages of my Phd.

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**ABSTRACT**

**Background**

Activation of the innate immune system plays a key role in exacerbations of chronic lung disease, yet the role of fibroblasts in innate immune responses and the identity of danger signals (alarmins) that may contribute to their activation are still to be unraveled. The objectives of this study were to identify epithelial alarmins released during environmental insults which induce innate immune responses in lung fibroblasts and dissect the mechanisms responsible for this.

**Methods**

Primary human lung fibroblasts (PHLF) were treated with conditioned media from primary bronchial epithelial cells (PBECs) exposed to oxidant injury or endoplasmic reticulum stress (ER stress) and fibroblast responses assessed. The relevance of alarmins in-vivo was assessed clinically by measurement of relevant alarmins longitudinally in patients developing Bronchiolitis Obliterans Syndrome (BOS) after lung transplantation.

**Results**

Conditioned media from PBEC cells subjected to oxidant injury and ER stress contained elevated levels of alarmins. Treatment of PHLFs with conditioned media from damaged cells significantly upregulated IL-6, IL-8, MCP-1, GM-CSF, IL-1α and IL-1β expression ($p < 0.05$). This effect was reduced with anti-IL-1α or IL-1Ra but not anti-IL-1β antibody. Co-stimulation with Poly I:C significantly accentuated the IL-1α induced inflammatory phenotype in PHLFs. Clinically, IL-1α was increased in BAL of lung transplant recipients with infections and within 3 months of developing bronchiolitis obliterans syndrome (BOS) ($p < 0.001$). Additionally, IL-1α levels positively correlated with elevated IL-8 ($p < 0.001$) and neutrophil counts ($p < 0.001$).

**Conclusions**

IL-1α plays a pivotal role in triggering proinflammatory responses in fibroblasts and this process is accentuated in the presence of viral pathogen associated molecular patterns. This novel pathway warrants further evaluation of its therapeutic potential to limit the repeated cycles of injury and exacerbation in chronic lung diseases.

**CHAPTER 1 INTRODUCTION**

**1.1 Wound healing and tissue fibrosis**

Wound healing is an immensely important physiological process, essential for maintaining tissue integrity and protection against various extrinsic factors including toxic chemicals and pathogens. Tissue injury very often leads to damage of the endothelial-epithelial barrier which triggers a cascade of events including platelet aggregation, formation of a protective fibrin-fibronectin meshwork, removal of dead cells by neutrophils and macrophages, angiogenesis, re-epithelization and reconstitution of the extracellular matrix (ECM) by activated fibroblasts secreting substantial amounts of collagen type III which is later replaced by the more robust collagen type I. In the final stage of the wound healing process – the remodeling phase - the excessive collagen is removed by matrix metalloproteases and the cells which are no longer required, such as the activated fibroblasts, undergo apoptosis [1] [2] [3]. However in chronic tissue injury the process of remodeling often becomes dysregulated leading to further activation and proliferation of fibroblasts which may result in scar tissue formation and development of fibrosis [4] [5] [6].

Fibrosis is a chronic disease characterized by progressive loss of epithelium that is replaced by fibroblasts producing excessive collagen leading to distortion of tissue architecture and function [7] [8]. Fibrosis has been recognized as a major cause of morbidity and mortality in many chronic diseases including liver cirrhosis [9], Crohn's disease [10], type II diabetes [11], chronic inflammatory lung diseases including chronic obstructive pulmonary disease (COPD) [12] and bronchiolitis obliterans syndrome (BOS) [13] and certain ageing-associated diseases eg. chronic renal disfunction [14]. In organs such as liver, heart, lung and kidney development of fibrosis is accompanied by progressive loss of organ function which may lead to serious clinical complications or even prove fatal. It is estimated that over 45% of deaths in the western world are attributed to some sort of fibrotic disease [15].

Fibrosis is associated with neutrophil and macrophage influx into the organ and upregulation of proinflammatory cytokines resulting in chronic inflammation. However it is unclear if prolonged inflammation is a trigger for fibrosis or whether these two processes only co-exist in chronically injured tissues. Martin *et al.* using

PU.1 mice lacking functional neutrophils and macrophages demonstrated that the fibrogenetic process may take place even in the absence of immune cell, which indicates that fibrosis may also develop without ongoing inflammation [16]. Some fibrotic diseases such as Crohn's disease [17], rheumatoid arthritis [18] and scleroderma [19] are responsive to immunosupressants. Corticosteroids, however, proved to be ineffective in the treatment of fibrotic diseases such as idiopathic pulmonary fibrosis (IPF), which develops in the environment of oxidative stress which suppresses corticosteroid activity [20].

Fibrotic lung diseases, such as IPF, COPD and BOS – a clinical manifestation of chronic post-transplant lung rejection, are characterized by chronic inflammation, degeneration of the respiratory epithelium and intensive fibroproliferation. The epithelial damage in these diseases may be triggered by oxidative stress caused by ROS released by activated neutrophils [21], viral [22] and bacterial [23] infections, cigarette smoke [24], air pollution [25] or allogenic injury [26]. Recent studied revealed that damaged cells may release danger signals (alarmins, damage associated molecular patterns – DAMPs), which may activate myeloid cells such as neutrophils and macrophages [27], however the interactions between epithelial alarmins and fibroblasts which become one of the most abundant cell type in fibrotic lung, have not been studied before. Therefore, the objective of this study was to investigate, if danger signals released by damaged human bronchial epithelial cells may activate human lung fibroblasts, to identify alarmins involved in fibroblast activation and to verify the clinical relevance of the potential findings by measuring the levels of the relevant alarmins in bronchoalveolar lavage (BAL) of lung transplant recipients who developed BOS.

A diagram representing a simplified process of wound healing and tissue fibrosis is shown in figure 1.

# Wound healing and tissue fibrosis
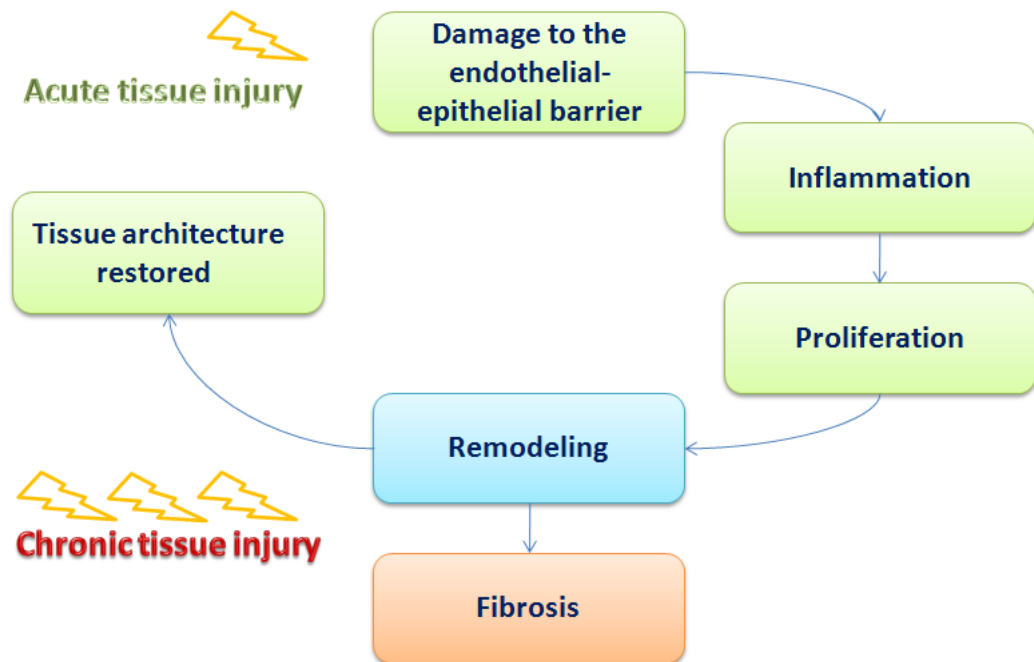


**Figure1 A diagram representing a simplified process of wound healing and tissue fibrosis**

Acute tissue injury which may be caused by infection, irritating substances or allogenic factors results in damage to the endothelial-epithelial barrier, followed by inflammation and fibroproliferation required for the remodeling and restoration of the tissue architecture. Repetitive tissue injury which may be caused by unresolved infection or prolonged exposure to irritating substances distorts the wound healing process, leading to chronic inflammation and tissue fibrosis.

## 1.2 Cellular mediators of fibrosis and inflammation

## 1.2.1 Structural cells

### *Fibroblasts*

Fibroblasts, which are the key drivers of fibrogenesis, may originate from different sources including increased proliferation of resident fibroblasts or epithelial cells which upon activation by profibrotic agents such as transforming growth factor-β1 (TGF-β1) and platelet derived growth factor (PDGF) may undergo a process called Epithelial to Mesenchymal Transition (EMT) and gain a new mesenchymal phenotype, characterized by upregulation of specific markers such as α-smooth muscle actin (α-SMA), desmin, vimentin and fibronectin [28]. The pool of fibrogenic cells may also be supported by bone marrow derived fibrocytes [29] that may be attracted to the site of injury by chemokines such as CXCL-12 [30]. Fibrocytes cultured in vitro can differentiate into myofibroblasts and gain the ability to produce collagen [31].

Once the process of wound closure has been completed, the activated fibroblasts which are no longer required should enter the apoptotic pathway. However, in fibrosis, this process becomes distorted and the activated fibroblasts continue to proliferate and produce excessive collagen deposits. Shalamit *et al.* showed that in the murine model of bleomycin-induced fibrosis, myofibroblasts present in the lung become resistant to Fas-ligand mediated apoptosis and gain the ability to counteract Fas+ lymphocytes [32]. This observation may suggest that the environment of injured tissue provides factors promoting survival of the activated fibroblasts. Cytokine profiling conducted on BAL from IPF patients showed that a correlation exists between the disease and upregulation of type 2 cytokines, including IL-4, which stimulates fibroblast proliferation. Furthermore, a proteomic analysis revealed that tissue samples from IPF patients expressed increased levels of proteins possessing anti-proteolitic activity such as α-2 Macroglobulin which is an inhibitor of metalloproteases involved in the ECM remodeling [33]. In addition, a gene expression analysis of pulmonary fibrosis revealed upregulation of several antiapoptotic genes including Twist-1 which is activated by profibrotic factors such as PDGF, Basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF)

[34]. These observations indicate that the environment in chronically injured organs selectively promotes fibroblast proliferation and survival.

Until quite recently fibroblasts have been regarded as cells whose role was limited mainly to the restoration of tissue architecture. However an increasing number of studies have suggested that fibroblasts may also function as mediators of the innate immune responses and that they may act to modulate the switch from acute response to chronic inflammation which coexists with fibrosis [35]. The influence of a microenvironment determined by damaged epithelium, which develops in organs exposed to chronic injury, such as post-transplant lung, on fibroblast behavior has not been studied before, and therefore one of the objectives of the study was to determine if danger signals released from damaged human bronchial epithelial cells may modulate fibroblast phenotype.

### *Epithelial cells*

Respiratory epithelium is built up with a single layer of ciliated, columnar cells based on a fibrous basement membrane. Three different cell types, namely ciliated cells, goblet cells and basal cells, can be distinguished in the epithelial layer. During epithelial injury, basal cells migrate to cover a site where epithelial cells get damaged, and differentiate to restore the epithelial structure.

Depending on the location, the respiratory epithelium can be divided into three groups: nasal, bronchial and alveolar epithelial cells, amongst whom, type I and type II pneumocytes have been distinguished. Type I pneumocytes make up around 96% of the alveolar surface and their main function is $CO_2/O_2$ gas exchange [36]. Type II pneumocytes make up the remaining 4% of ciliated epithelial cells and are mainly involved in the production of pulmonary surfactant which reduces surface tension and is required to increase pulmonary compliance (ability of the lung to expand) and prevent atelectasis (collapse of the lung) [37].

Respiratory epithelium provides the first line of defense against invading pathogens and airborne microparticles by formation of a physical barrier, expressing a vast array of Pattern Recognition Receptors (PRR) and production of proinflammatory

factors involved in mobilization and activation of immunocytes including macrophages, neutrophils and T cells [38].

Chemotaxis of inflammatory cells can be mediated by a plethora of chemotactic factors produced by bronchial cells, including monocyte chemotactic protein-1 and 4 (MCP-1 and MCP-4), IL-8, eotaxin, and arachidonic acid metabolites which act as attractants for neutrophils, eosinophils, and monocytes and increase mucus production [39].

Moreover, the airway epithelium actively participates in pathogen clearance and maintaining tissue homeostasis by producing mediators such as anti-microbial agents including lactoferrin, lysozyme, mucins, cationic proteins and anti-proteases such as alpha1-protease inhibitor, secretory leukoprotease inhibitor, alpha1-anti-chymotrypsin, alpha2-macroglobulin and tissue inhibitors of metalloproteases [40].

Bronchial epithelial cells are also a source of adhesion molecules such as integrins, selectins and cadherins which provide contact between adjacent cells and between cells and ECM [41].

Additionally, bronchial epithelium secrets a variety of growth factors such as Epidermal Growth Factor (EGF), TGF-ß, insulin-like growth factor (IGF) and PDGF, which play important roles in the process of wound healing and may contribute to the pathogenesis of fibrotic lung diseases such as IPF or BOS [42] [43].

Due to their location, bronchial epithelial cells are extremely prone to injury which may be caused by exposure to airborne chemicals and microparticles such as cigarette smoke, asbestos, or smog. Epithelial damage may also occur due to chronic oxidative stress which may cause rupture of the cell membrane, DNA damage and ATP depletion. Red-ox balance is respiratory epithelium is maintained by anti-oxidant agents such as glutathione, superoxide dismutase, and catalase [44].

Previous reports provide evidence that damaged cells may be a source of alarmins [27], however, the influence of oxidative stress on alarmin release and viability of primary human bronchial epithelial cells has not been investigated before. The interactions between primary human bronchial epithelial cells damaged with

oxidative stress and primary human lung fibroblast has not been studied before either.

## 1.2.2 Immunocytes

### *Macrophages*

Monocyte-derived phagocytes, macrophages, are involved both in innate and adaptive immunity. The main role of macrophages is phagocytosis of pathogens and cellular debris. Moreover, macrophages are a powerful source of proinflammatory cytokines and growth factors which support responses of lymphocytes and other immune cells to pathogens. Macrophages can be identified by specific markers including CD11b, CD14 and CD68. They also express a vast array of PRRs which enable them to recognize numerous PAMPs and DAMPs [45].

Macrophages derive from monocytes which circulate in the blood or reside in the spleen or bone marrow. Upon activation by certain proinflammatory cytokines or PAMPs, monocytes can differentiate into macrophages or dendritic cells.

Recently it has been suggested that two subsets of macrophages, termed as M1 and M2, can be distinguished. The concept of M1/M2 cells derived from an observation that macrophages from C57BL/6 mice, which are considered as prototypical Th1 strains, are more responsive to IFN-γ or LPS stimulation resulting in NO production than macrophages derived from BALB/c mice (prototypical Th2 strain). Upon LPS stimulation M2 macrophages start to metabolize arginine to ornithine while in M2 cells this metabolic pathway remains switched off. In vitro, macrophages can be differentiated into M1 phenotype using IFNγ and M2 differentiation can be achieved using IL-13 and IL-4.

M1 type of macrophage activation can also be elicit by cytokines such as TNFα and GM-CSF and certain PAMPs, including LPS, and is characterized by high expression of IL-12 and IL-23, low expression of IL-10 and enhanced production of reactive oxygen species and nitrogen intermediates. The biological functions of M1 macrophages include stimulating Th1 responses and mediating resistance against tumors and intracellular parasites.

M2 macrophage phenotype is characterized by low expression of IL-12, IL-23, IL-1β and caspase1 and high expression of IL-10, IL-1Ra and IL-1RII. M2 cells are involved in promoting Th2 responses, tissue repair, remodeling and tumorigenesis [46].

Pulmonary macrophages are a heterogeneous population comprising of alveolar, interstitial and intravascular macrophages and dendritic cells. The only macrophage type that is exposed directly to air, are alveolar macrophages, which are located in the surfactant film, produced by type 2 alveolar cells. Alveolar macrophages show great phagocytic potential and provide defense against invading pathogens and airborne microparticles.

Pulmonary interstitial macrophages are located in the lung connective tissue and are involved both in innate immune responses and adaptive immunity functioning as antigen-presenting cells. Compared to the alveolar macrophages, the interstitial macrophages show lower expression of cytokines such as TNFα, IFNα and IFNβ.

Dendritic cells, which also may belong to the macrophage lineage, in the lung are located mainly in the connective tissue. They are characterized by low phagocytic potential but high expression of class II antigens and function mainly as antigen presenting cells. Moreover, dendritic cells express a range of PRRs and are able to recognize and respond to selected DAMPs and PAMPs

Intravascular macrophages are highly phagocytic cells, located on the endothelium of capillaries, which participate in removing foreign bodies and pathogens entering the lung via bloodstream [47].

Macrophages are considered to play pivotal role in modulating innate immune responses in fibrotic lung, however their responsiveness to epithelial alarmins, released by human bronchial epithelial cells has not been studied before. And therefore one of the objectives of this study was to compare the PRR epression profile between macrophages and lung fibroblasts and verify the responsiveness of alveolar macrophages to alarmins which are able to trigger innate immune responses in fibroblasts.

*Neutrophils*

Neutrophils are granulocytes which along with basophils and eosinophils form the family of polymorphonuclear cells (PMNs). Neutrophils show a distinctive morphology that is characterized by a presence of a nucleus divided into 2-5 lobes.

Neutrophils normally circulate in the bloodstream, however in response to chemoatractants such as IL-8, leukotriene B4, IFNγ and N-formyl-methionine-leucine-phenylalanine (fMLP), they are also able to migrate through blood vessels towards the side of infection or injury in the interstitial tissue.

Following activation, neutrophils show a relatively short life span of approximately 5 days in the blood stream and up to 2 days in interstitial tissues.

Like macrophages, neutrophils participate in pathogen clearance by phagocytosis and production of ROS which are generated in the respiratory burst process. It has been suggested that ROS generated by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) by activated neutrophils and macrophages may be involved in the pathogenesis of some chronic inflammatory diseases such as chronic allograft rejection or arthritis. Following digestion of pathogens, neutrophils can be phagocytosed by macrophages which may also lead to increased production of ROS [48].

Neutrophils may be a source of proteases including elastase and cathepsin G. Neutrophil derived proteases are believed to participate in pathogen clearance, however recent studied indicate that they may also be involved in proteolitic activation of some cytokines and growth factors such as insulin-like growth factor binding protein (IGFBP) [49]. It has also been suggested that elastase may contribute to tissue damage in chronic degenerative diseases such as emphesyma, sarcocidosis and IPF. Moreover Gardi et al. [50], demostrated that elastase may cause breakdown of collagen which may have stimulatory activity on collagen production by fibroblasts.

Neutrophils express a variety of PRR, complement receptors, as well receptors for cytokines and chemokines and actively participate both in adaptive and innate

immune responses. Moreover, upon activation by DAMPs and PAMPs they can produce a number of cytokines and antimicrobial peptides.

The influx of neutrophils is associes with chronic inflammatory lung diseases such as COPD [51] and BOS [52], and therefore one of the objectives of the study was to investigate if neutrophil-derived factors such as elastase and hydrogen peroxide ($H_2O_2$) may cause damage of human bronchial epithelial cells and to establish if there exist a correlation between relevant epithelial alarmins and neutrophil number  in vivo.

## 1.3 Immune system

The underlying cause of chronic inflammatory diseases is deregulation of the responses of the innate and adaptive immune system, caused by repetitive tissue injury, prolonged exposure to irritating substances, chronic infection or allogenic immune responses.

The immune system is a complex set of structures and processes within a living organism that provides protection against and counteracts pathogenic or potentially hazardous agents, including viral and bacterial pathogens, parasites as well as injured and tumorogenic cells. Immune defense mechanisms evolved in all living organisms including plants and unicellular prokaryotes. Numerous studies indicate that basic defense mechanisms function even in bacteria, which protect themselves against bacteriophages with restriction enzymes that are able to cleave viral nucleic acids [53]. Interestingly, even some viruses developed sophisticated mechanisms to increase their chances of survival. It has been discovered for example that cholera viruses express cytosine and adenine DNA methyltransferases to methylate DNA which protect it from being cleaved by bacterial restriction enzymes [54]. Multicellular organisms, including animals and plants, developed far more advanced mechanisms of counteracting invasive pathogens, called the innate immune system. The innate immune system is orchestrated by a specialized set of intra- and extracellular receptors that gained the ability to recognize microbial and endogenous danger signals. Activation of the innate immune system leads to immediate but non-specific response against the invading pathogens. Target-

orientated defense mechanisms are present only in vertebrates and are referred to as the adaptive immune system. Immune defense mechanisms are essential to counteract pathogens and tumorogenic cells. However, deregulation of these processes may be detrimental for the organism and may provide foundation for development of autoimmune and chronic degenerative diseases.

### 1.3.1 Innate immune system

The innate immune system is an evolutionary conserved set of mechanisms and structures that evolved to provide the first line of defense against invading pathogens. Unlike adaptive immune system, generating immunity to specific pathogens via target-orientated antibodies, innate immune responses provide non-specific defense in a form of mechanical barriers and a rapid production of proinflammatory cytokines causing inflammation that is essential for wound healing and pathogen clearance. The innate immune system consists of three major components: anatomical barriers, activation of the complement system and activation of innate immune cells leading to inflammation [55].

Both DAMPs and PAMPs are able to activate the innate immune system via PRR expressed on structural cells and immunocytes, however the identity of danger signals playing povital role in the activation of the innate immune responses in the lung are yet to be established.

### 1.3.2 Anatomical barriers

Physical protection against pathogens is provided by anatomical barriers and mechanical removal of invading microorganisms. Mechanical removal mechanisms include the cough and sneeze reflex, vomiting and diarrhea and physical flushing action of body fluids eg. tears, saliva or perspiration. Anatomical barriers comprise of tough to penetrate structures that prevent microorganisms from migration and colonization. Examples of anatomical barriers in animals include skin, mucous membranes of respiratory tract and gastrointestinal system and bony encasements.

Mucous membranes are composed of a layer of epithelium secreting substantial amounts of glycoprotein, proteoglycans, peptydoglycans and lipids, providing a

physical barrier to trap microorganisms and chemical particles. Moreover, epithelial cells building mucous membranes, secrete lysozyme, an enzyme degrading peptidoglycal in bacterial cell walls, lactoferrin, binding iron and preventing it from being used by microorganisms and lactoperoxidase catalyzing red-ox reactions resulting in generating toxic superoxide radicals. Furthermore, epithelial cells in mucous membranes are constantly sloughed to remove microorganisms and nanoparticles that have attached to mucus.

Protection provided by mucous membranes is of great importance in organs such as the lung, that are constantly exposed to the outside environment. The alveolar mucous membrane is the largest surface in the human body to remain in contact with the external environment. Due to its location and function the respiratory epithelium is constantly exposed to a variety of airborne bacteria, fungi, spores and nanoparticles. To enable removal of microorganisms and foreign bodies from the respiratory tract, bronchial and nasal epithelium has been equipped with cilia, that move actively to encourage mucus flow. Respiratory epithelium provides the first line of defense against airborne pathogens and pollutants, however its ability to fight danger agents is limited and chronic infection or prolonged exposure to irritating substances may result in progressive loss of the epithelial cells and intensive fibroproliferation causing airway obliteration. The loss or dysfunction of respiratory epithelium is characteristic for many chronic inflammatory and degenerative lung diseases including asthma, COPD, IPF and BOS [56].

### 1.3.3 Pattern Recognition Receptors (PRRs)

PRRs are the main mediators of the innate immune responses. The main PRR families have been described below.

***Toll-like receptors (TLRs)***

The TLRs recognize a vast array of ligands including endogenous DAMPs, (alarmins) such as High Mobility Group box1 (HMGB-1), S100 calcium binding protein A (S100A) and Heart Shock Proteins (HSPs) and PAMPs such as bacterial lipopolysaccharide (LPS), nonmethylated CpG-containing DNA from bacteria and viruses and viral RNA (34). So far 11 members of the TLR family have been

identified: TLR 1-10 and IL-1R. All of them contain conserved leucine-reach repeat domain (LRR) and a Toll/Interleukine-1 receptor domain (TIR). TLR3, 9, 7 and 8 are localized intracellularly in the endosome membrane whilst TLR1, 2, 4, 6 and IL-1R are expressed on the cell surface. All TLRs with the exception of TLR3 which activates TRIF kinase, signal via an adaptor molecule MyD88. TLR4 activation additionally requires TRAM protein. Most TLR function as homodimers, although TLR2 is believed to form heterodimers with TLR1 or TLR6. Moreover, TLR4 cooperates with MD12 and CD14 co-receptors in bacterial LPS recognition. TLRs are upstream coordinators of several molecular pathways including Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Mitogen-activated Kinase (MAPK) pathway and regulate phosphorylation and activation of kinases such as c-Jun N-terminal kinases (JNK), p38 and Extracellular signal-regulated kinases (ERK) which are involved in cellular responses to various stress stimuli, including starvation, oxidative stress, radiation and infection. TLR activation leads to upregulation of numerous proinflammatory gene expression including IL-6, IL-8, IL-1β, TNFα, IFNα, IFNβ, CD40 and CD80 which play important roles in immune response and inflammation [57].

Accumulated evidence suggests that TLRs may serve important functions in the development of fibrotic diseases. Yang et al. showed that targeting TLR2 with specific blocking antibodies significantly decreased animal death rate and inhibited progression of bleomycin-induced pulmonary fibrosis in mice. Bleomycin-induced fibrosis is associated with upregulation of immunosuppressive cytokines such as Interleukin-13 (IL-13) and TGF-β1 and profibrotic alarmins including HMGB-1. The authors suggest that blocking TLR2 signaling may alleviate the disease by reversion of the immunosuppressive microenvironment brought about by bleomycin [58].

Seki et al. using TLR4-chimeric mice demonstrated that TLR4 is engaged in progression of liver fibrosis by enhancing TGF-β1 signaling. Inhibiting TLR4 signaling resulted in attenuated secretion of chemokines by hepatic stellate cells (HSC), the prime precursors for the profibrotic cells in the liver, and led to downregulation of the decoy receptor BMP and activin membrane-bound inhibitor homolog (BAMBI) which functions as a TGF-β1 inhibitor. Experiments on MyD88 deficient mice

confirmed that BAMBI suppression is mediated by the TLR4-MyD88–NF-κB axis [59].

Brentano et al. showed that TLR3, which recognizes single stranded RNA (ssRNA), was expressed in rheumatoid arthritis (RA) synovial fibroblasts (SA) and that these cells when stimulated with necrotic RA synovial fluid in vitro secreted elevated levels of IL-6, IFNβ, Chemokine (C-C motif) ligand 5 (CCL5) and C-X-C motif chemokine 10 (CXCL10). Nuclease treatment suppressed the production of these molecules, suggesting that endogenous RNA released by damaged cells in rheumatoid arthritis may stimulate proinflammatory cytokine and chemokine production via TLR3 [60].

TLR3 also plays an important role in immune responses to viral infections which are believed to be one of the factors enhancing the risk of IPF. Rudd et al. demonstrated that TLR3-/- mice challenged with Respiratory Syncytial Virus (RSV) showed increased pulmonary mucus production and enhanced type 2 cytokines expression in comparison to WT mice. Moreover, RSV promoted eosinophil accumulation in TLR3-/- but not WT mice, suggesting that TLR3 is implicated in maintaining homeostasis in the immune environment upon infection [61].

All cells have specific TLR expression profile, which determines their responsiveness to PAMPs and DAMPs. The TLR expression profile of primary human lung fibroblasts and their role in mediating innate immune responses to PAMPs and DAMPs have not been investigated before and therefore establishing this profiles was one of the objectives of this study.

 TLR signaling is shown in figure 2.
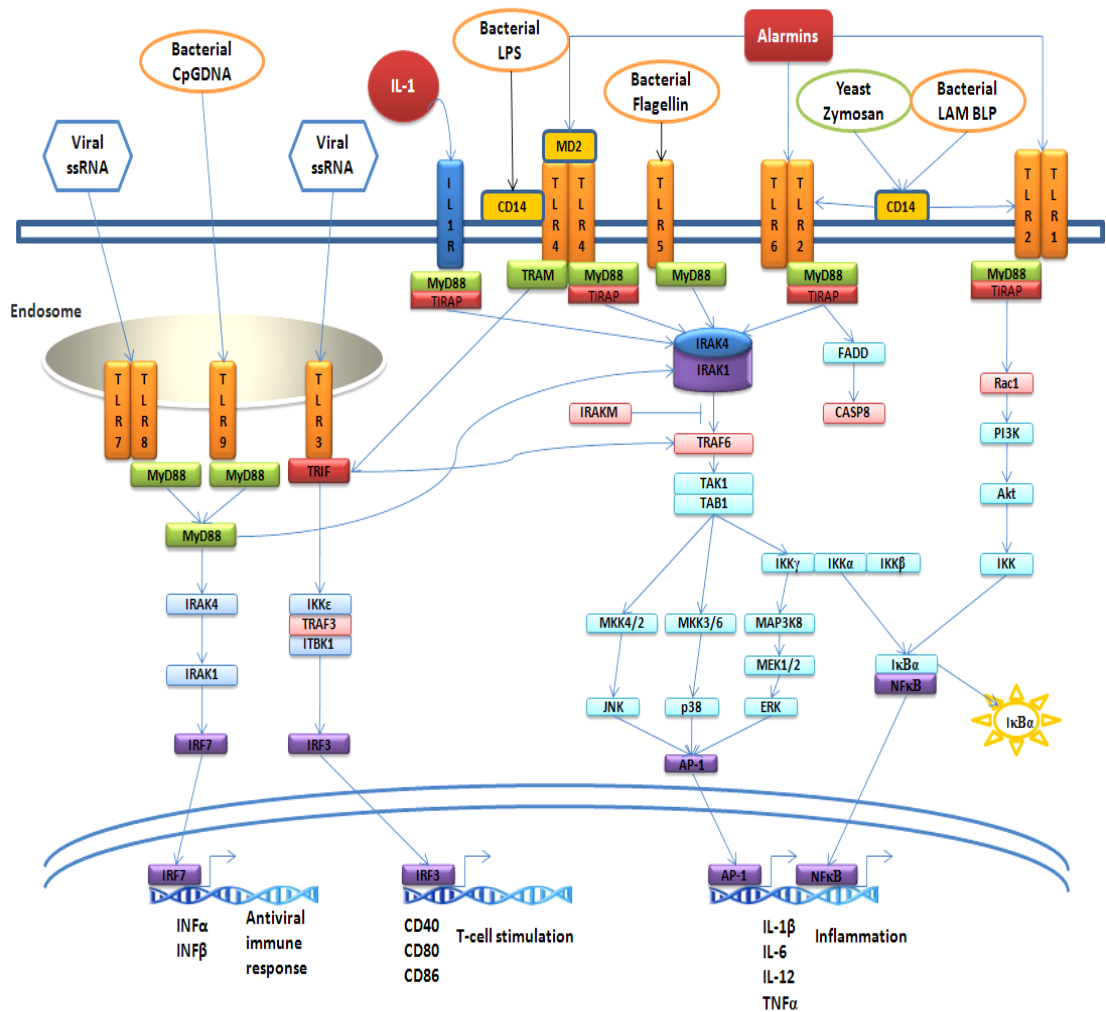
**Figure 2 Toll-like receptor signaling**

Toll-like receptors (TLR) may be activates by numerous damage associated molecular patterns such as interleukin-1alpha (IL-1α) and pathogen associated molecular patterns (PAMPs) including bacterial LPS, CpGDNA and flagellin, yeast zymosan and viral double stranded RNA (ssRNA). Ligand binding to TLRs results in activation of several signaling pathways inclusing NF-κB, MAP-kinase and IRF pathways and triggering gene expression of numerous cytokines and chemokines involved in processes such as inflammation, antiviral and antibacterial immune responses and T cell activation. Adapted from [62].

### Nod-like receptors

The Nod-like Receptors (NLRs) are a family of cytoplasmic proteins characterized by the presence of a series of tandem leucine-rich repeats and central nucleotide-binding oligomerization domain. NLRs recognize a wide array of PAMPs including lipopolysaccharide (LPS), mannan, zymosan, bacterial and viral nucleic acids, flagellin and lipoproteins and DAMPs such as Adenosine-5'-triphosphate (ATP), uric acid, heparin sulfate, silica, asbestos and amyloidβ. NLR activation leads to assembling of a Caspase-1 activation protein complex defined as an 'Inflammasome'. Activated Caspase-1 processes the cleavage of pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 respectively, which are then secreted from the cell [63].

So far, over 20 *Nlr* genes have been identified in the human genome. Mutations in Nlr genes have been associated with numerous autoimmune diseases. Autosomal dominant mutation in *Nlrp3* causes several disorders collectively termed cryopyrin-associated periodic syndromes [64], characterized by increased secretion of IL-1β even in the absence of any stimulation. *Nlr* gene mutations have also been linked to psoriasis and fibrostenosing disease in patients with Crohn's syndrome [65].

NLR are also postulated to be involved in the immune response to asbestos and silica which have been implicated as important risk factors in pulmonary fibrosis. Perkins et al showed that prolonged exposure to asbestos and silica fibers upregulates IL-1β secretion in human alveolar macrophages in vitro and in vivo. [66].

NLRP3 has also been recognized as an important sensor for DAMPs in bleomycin-indced pulmonary fibrosis in mice. Gasse et al showed that bleomycin-induced DNA damage leads to release of uric acid which is sensed by NLRP3 and stimulates IL-1β secretion. Inhibition of uric acid with uricase reduced IL-1β production, lung inflammation and fibrosis, suggesting that targeting danger signals released by damaged cells may be an important therapeutic target in fibrotic lung diseases [67].

### Receptor for advanced glycation endproducts

Receptor for advanced glycation endproducts (RAGE) is a 35kDa transmembrane receptor belonging to the immunoglobulin family. RAGE has the ability to recognize advanced glycation endproducts (AGE) and certain DAMPs such as HMGB1, S100b proteins and amyloidβ. RAGE ligands upregulate NADPH-dependant reactive oxygen species production, stimulate expression of numerous genes involved in modulating immune responses, cell adhesion and cell communication and activate a number of signaling molecules including phosphoinositol-3 kinase, NF-κB and Mitogen-activated Kinases (MAPK) including p38, Stress-activated Protein kinases (SAPK), Janus kinase /signal transducer and activator of transcription (JAK/STAT), Extracellular signal-regulated kinases 1/2 (ERK1/2; p44/p42) (59). RAGE is strongly expressed in the lung and it has been implicated to play an important role in certain chronic inflammatory diseases including IPF [68]. In vivo studies revealed that bleomycin caused more severe form of pulmonary fibrosis in RAGE-/- mice than in WT animals [69], which suggests that RAGE may play a protective function in chronically injured organs. Moreover, an immunohistochemical study performed on lung tissue specimen from IPF patients revealed diminished RAGE protein expression in comparison to control tissue [70].

### IL-1 receptor (IL-1R)

So far, two isoforms of IL-1 receptor have been identified: IL-1RI and IL-1RII. Both receptors recognize IL-1α and IL-1β but only IL-1RI is capable of transmitting intracellular signals initiated by IL-1. Binding of IL-1α or IL-1β to the IL-1RI results in the recruitment of a co-receptor IL-1RAcP which forms heterodimers with IL-1RI which leads to the activation of TIR domain of each receptor and is followed by phosphorylation of MyD88 and Interleukin-1 receptor-associated kinase 1 (IRAK1). Activation of IL-1RI results in mobilization of several signaling pathways including NF-κB, MAP kinases and IRF pathway and leads to upregulation of numerous cytokines mediating processes such as inflammation, T-cell stimulation and anti-viral responses.

IL-1RII, which is expressed mainly on macrophages and B cells, cannot pass signals mediated by IL-1α or IL-1β, because it lacks a cytoplasmic TIR domain and therefore cannot bind to MyD88 and hence it functions as a decoy receptor. Interestingly, IL-1RII has higher affinity to IL-1β than IL-1RI which makes it an efficient negative regulator of IL-1 signaling. Moreover, IL-1RII inhibits IL-1RI signaling by recruiting and therefore decreasing the pool of free interleukin-1 receptor accessory protein (IL-1RAcP). IL-1RII is also expressed in a soluble form consisting only with the extracellular domain. In order to efficiently bind IL-1, the soluble form of IL-1RII requires a soluble IL-1RAcP co-receptor. Different variants of IL-1 receptors are translated from different splice variants of the same RNA [71].

The balance between IL-1, IL-1Ra and soluble IL-1RII is believed to affect the severity of many chronic inflammatory conditions. It has been reported that some anti-inflammatory drugs eg. glucocorticoids, may reduce inflammation by upregulating the production of IL-1RII [72], suggesting that anti-IL-1 therapies could be considered in the treatment of patients who do not tolerate steroid drugs .

The importance of IL-1 antagonists in maintaining immune homeostasis can also be emphasized by the observations that animals immunized against their own IL-1Ra develop spontaneous diseases such as colitis, arthritis, psoriatic-like skin eruption or cancer [73].

Moreover, it has been shown that there is an association between IL-1Ra single-nucleotide polymorphism rs4251961, resulting in decreased levels of IL-1Ra and certain chronic diseases including type 2 diabetes and colon carcinoma [74].

IL-1β was the first member of the IL-1 protein family to be identified. IL-1α was discovered shortly after that, however the family is still expanding and so far, in total, eleven members have been distinguished. Apart from IL-1α and IL-1β, the following proteins have been included into the IL-1 family: IL-1Ra, IL-18, IL-36Ra, IL-36α, IL-37, IL-36β, IL-36γ, IL-38 and IL-33. The majority of the IL-1 family members, except for IL-1Ra and IL-36Ra, function as proinflammatory cytokines [75]. The role of IL-37 has not been fully described yet, however some reports indicate that it may exhibit anti-inflammatory properties. The members of IL-1 family have been listed in table 1.

| Name | Receptor | Co-receptor | Functions |
|------|----------|-------------|-----------|
| IL-1α | IL-1RI | IL-1RacP | Proinflammatory |
| IL-1β | IL-1RI | IL-1RacP | Proinflammatory |
| IL-1Ra | IL-1RI | NA | Antagonist for IL-1α, IL-1β |
| IL-18 | IL-18Rα | IL-18Rβ | Proinflammatory |
| IL-36Ra | IL-1Rrp2 | NA | Antagonist for IL-36α, IL-36β, IL-36γ |
| IL-36α | IL-1Rrp2 | IL-1RAcP | Proinflammatory |
| IL-37 | ? IL-18Rα | Unknown | Anti-inflammatory |
| IL-36β | IL-1Rrp2 | IL-1RAcP | Proinflammatory |
| IL-36γ | IL-1Rrp2 | IL-1RAcP | Proinflammatory |
| IL-38 | Unknown | Unknown | Unknown |
| IL-33 | ST2 | IL-1RAcP | Th2 responses, proinflammatory |

**Table 1 List of cytokines belonging to the IL-1 family**

### 1.3.4 Damage associated molecular patterns

Endogenous danger signals able to activate PRR are called damage associated molecular patterns (DAMPs) or alarmins. Alarmins are a heterogeneous group of molecules that are rapidly released following cell death to orchestrate so called 'sterile inflammation' via recruitment and activation of innate immune cells. Alarmins may be secreted by many different cell types including immunocytes as well as by endothelial and epithelial cells that provide structural support for organs and which, due to their location, are largely prone to injury. Alarmins signal via a range of receptors including TLR, Interleukin-1 Receptor (IL-1R) and RAGE and play a very important role in modulating innate immune response by activating lymphocytes and antigen presenting cells. Recent studies revealed that some alarmins may also promote proliferation and activatation of fibroblasts however their mechanisms of action remain unclear [27]. Characterization of selected alarmins has been described below and summarized in in table 1.

### Interleukin 1 alpha (IL-1α)

Interleukin 1 alpha (IL-1α) is a small 18kDa protein that is formed by a cleavage of a 31kDa precursor protein by a calcium activated cystein potease called Calpain [76]. IL-1α, similarly to IL-1β and IL-18, lacks a signal peptide fragment and is released via an unconventional secretory pathway. IL-1α is stored in intracellural compartments and may be released from damaged cells to act as a ''danger signal'' required to initiate wound healing and angiogenesis. Recent studies however suggest that IL-1α may also be secreted from uninjured cells upon exposure to stress stimuli such as viral infection [77], heat shock or serum starvation via a non-classical mechanism requiring a formation of a tetrameric IL-1α –S100A13 complex. The IL-1α –S100A13 complex formation requires Ca+ ions and is followed by its direct translocasion across the plasma membrane. Uninjured cells expressing dominant negative S100A13 are unable to export IL-1α upon exposure to a stress stimulus [78].

The role of IL-1 in the pathophysiology of fibrotic diseases was confirmed by a study on IL-R1 deficient mice which demonstrated that IL-1 is required for the development of pharmacologicaly induced hepatic [79] and pulmonary [80] fibrosis. Furthermore Couillin *et al.* showed that IL-1R/MyD88 signaling upregulates IL-6, TNFα and IL-1β secretion and is important in elastase-induced lung inflammation and emphysema  [81].

IL-1α is also believed to play an important role in T cell polarization. A study by Cauciq *et al.* performed using a mouse model of Ovalbumin (OVA)-induced allergic asthma demonstrated that mice treated upon sensitization with IL-1α showed reduced levels of IL-5 and OVA-specific IgE as well as decreased number of eosinophils and significantly increased percentage of neutrophils in BALs, suggesting that IL-1α may attenuate Th2 responses and shift T cell polarization towards Th1 [82].

IL-1α may function both as a positive and negative regulator of proliferation or apoptosis [83] [84]. The end result depends on cell type, dose and interactions with other factors. A natural inhibitor of IL-1 is IL-1 receptor antagonist (IL-1Ra). A synthetic variant of IL-1Ra is known under commercial name Anakinra and has been

successfully used in the treatment of arthritis [85]. IL-1Ra is recognized by IL-1R however it does not initiate signal transmission and therefore it acts as a negative regulator of IL-1 signaling. Upregulation of IL-1Ra as well as anti-IL-1α autoantibodies have been reported in several chronic conditions including IPF [86] which suggests that IL-1 may be one of the factors playing an important role in the pathogenesis of fibrotic lung diseases. IL-1R signaling is presented in figure 3.
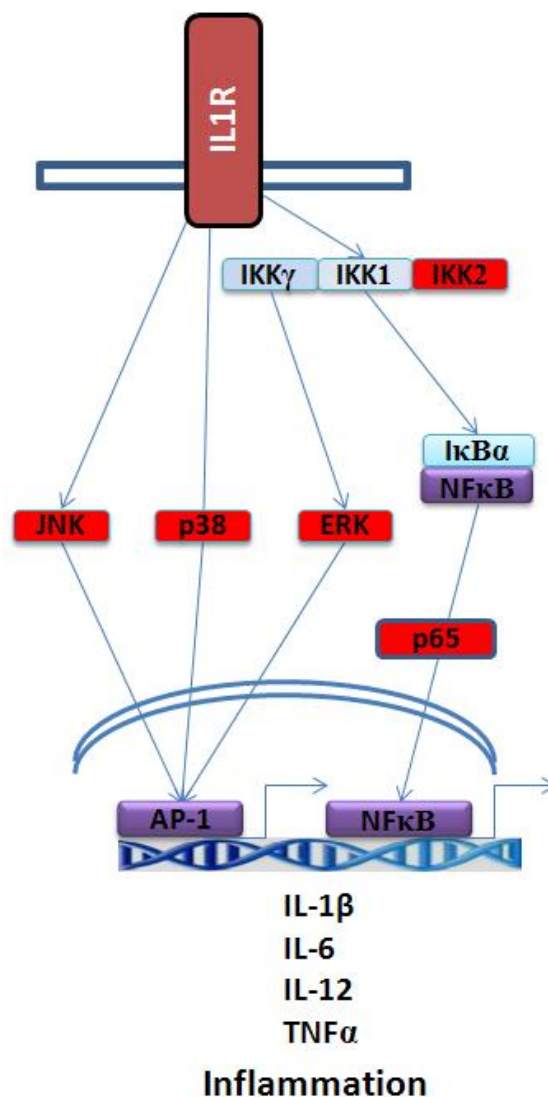


**Figure 3 IL-1 receptor signaling**

Activation of Interleukin 1 receptor (IL-1R) results in phosphorylation of downstream signaling molecules including NFκB and MAP-kinases (JNK, p38 and ERK) and activation of AP-1, which results in triggering expression of proinflammatory cytokines including IL-1β IL-6, IL-12 and TNFα. Adapted from [87].

## Other alarmins implicated in chronic inflammatory and fibrotic lung diseases

So far several alarmins have been identified as potential mediators of inflammation and fibrosis. Rottoli *et al.* [33] in a study of proteomic profiling of IPF distinguished Calgranulin-1 and Galectin-1 (Gal-1) as alarmins whose expression was strongly upregulated in the fibrotic lung. Gal-1 belongs to a family of carbohydrate-binding proteins which play important roles in many processes including T-cell homeostasis regulation, allergies and pathogen-host interactions. Gal-1 has also been recognized as an important factor involved in the regeneration of neural tissue after injury [88]. Calgranulin-1 is a member of the calcium-binding S100 protein family, expressed in many different cell types including neutrophils and epithelial cells. S100 proteins participate in immune responses by stimulating chemokine and cytokine production and are upregulated in disorders such as psoriasis, multiple sclerosis and cystic fibrosis [89].

Hamada *et al.* recognized High Mobility Group Box-1 Protein-1 (HMGB-1), one of the widest described DAMPs, as an alarmin whose levels were elevated in BAL from IPF patients. Moreover, a study on bleomycin-induced pulmonary fibrosis in mice revealed that upregulation of HMGB-1 gene expression in bronchiolar and alveolar epithelial cells was associated with the progression of the fibrogenic process. Furthermore, in vitro experiments showed that HMGB-1 stimulated fibroblast proliferation which is in agreement with previous reports suggesting that the environment of fibrotic organs, despite the presence of numerous proapoptotic molecules, promotes fibroblast survival [90].

Another group of alarmins that may be implicated in pulmonary fibrosis are α-Defensins, small antimicrobial proteins which act by binding to pathogen cell membranes and disrupt their integrity. Yoshioka *et al.* discovered that IPF patients had elevated plasma levels of α-Defensines and showed that Human Neutrophil Peptide1 (HNP1), a member of the α-Defensine family, expressed mainly in neutrophils but found also in the epithelia of the intestine and respiratory and urinary tract, promoted Collagen type-1 production by human fibroblasts in vitro [91]. HNP-1 was also found to promote NCI-H292 (human lung mucoepidermoid carcinoma cell line) cell migration and proliferation and to stimulate mucin gene

expression in vitro, indicating that α-Defensins may be also engaged in maintaining the integrity of the airway epithelium [92].

Heat shock proteins (HSP) are the next class of alarmins whose role in fibrosis is under investigation. HSP are a diverse group of proteins whose expression is transcriptionaly upregulated in response to various stress stimuli such as heat, osmotic stress, toxins, starvation, infection, inflammation, hypoxia or ER stress [93]. They also play an important role as molecular chaperons, orchestrating protein folding and preventing the accumulation of unfolded and misfolded proteins by modulating the Unfolded Protein Response (UPR). Recent studies revealed that there is a correlation between pulmonary fibrosis and activation of certain markers of UPR, including ATF-6 and XBP-1. One of the current hypopthesis suggests that UPR may be activated as a result of increased production of ECM proteins in fibrotic tissue or due to a mutation in a highly expressed protein, such as alveolar surfactant [94]. It has also been shown that certain molecular chaperons, such as HSP60 may be released from dying cells and act as putative ligands of the TLR 4 complex – a target of endogenous alarmins. In addition, HSP27 and HSP70 are strongly upregulated in response to oxidative stress which has been proposed as a major factor causing epithelial cell damage in IPF. Blocking HSP27 and HSP70 increases epithelial cell susceptibility to $H_2O_2$-induced damage and therefore HSPs are believed to serve a protective function in chronic tissue injury [93].

The alarmins believed to be implicated in chronic inflammatory lung diseases has been listed in table 2.

| Alarmin | Function in inflammation | Other functions | Receptor | Role in fibrosis |
|---|---|---|---|---|
| HMGB1 | Neutrophil recruitment , chemotaxis, cytokine induction, dendritic cell activation, | Maintaining chromatin architecture, gene expression regulation | RANGE TLR2 TLR4 | + |
| Uric acid | cytokine induction, dendritic cell activation, neutrophil recruitment | Final product of purine metabolism | TLR2 TLR4 CD14 | |
| Chromatin, nucleosomes and DNA | dendritic cells maturation and activation, neutrophil recruitment, B cell activation, cytokine induction | Maintaining chromosome structure, gene expression regulation | TLR9 | |
| Heat shock proteins | migration of dendritic cells to lymph nodes (gp96), dendritic cells maturation (HSP70), cytokine induction (HSP60, HSP70, HSP 90) | Stress response regulation, protein folding control | CD14 (HSP60, HSP70) CD91 (HSP90, HSP70, gp96, calreticulin) TLR4 (HSP60) Scavenger receptors (HSP70, gp96, calreticulin) | |
| Adenosine and ATP | chemotaxis, exacerbation of nephrities and bronchial astma | Energy transfer Secondary messenger Coenzyme | P1, P2X, P2Y (ATP) A1, A2A, A2B, A3 (adenosine) | |
| Galectins | dendritic cels maturation, monocyte recruitment, chemotaxis | Cell adhesion, glycoprotein synthesis and transport | CD2 | + |
| Thioredoxin | Chemotaxis | Redox reactions regulations | ND | |
| S100 proteins | Chemotaxis, neutrophil recruitment | Protein phosphorylation, gene expression regulation, Ca2+ homeostasis | RAGE | + |
| Cathelicidins | dendritic cells maturation, chemotaxis | Antimicrobial response | FPRL1 | + |
| Defensins | DC maturation, Adjuvant activity | Antimicrobial response | CCR6, TLR4 | + |
| N-formylated peptides | Chemotaxis, neutrophil recruitment | Products of bacterial and mitochondrial protein degradation | FPR, FPRL1 | |
| IL-1α | Innate immune responses, Inflammation, hematopoesis, | | IL-1R | ? |

**Table 2 List of alarmins implicated in chronic inflammatory lung diseases.** Adapted from [95].

Although the majority of alarmins are proteins, other molecules such as uric acid, ssRNA and methylated DNA have also been classified in that group. ssRNA and methylated DNA which may be of exogenous (viruses, bacteria) or endogenous (mitochondria) origin signal via TLR3 and TLR9 respectively. Cavassani *et al.* showed that lysates from necrotic cells stimulated TLR3-mediated chemokine generation by macrophages and that this effect was suppressed following nuclease treatment, suggesting that ssRNA released by injured cells may play a role in sterile inflammation [96].

## 1.3.5 Pathogen associated molecular patterns

Pathogen Associated Molecular Patterns (PAMPs) are pathogen derived danger signals, able to activate the innate immune system via specific PRRs in both plants and animals. From the structural point of view, PAMPs represent a heterogenous group of molecules comprising of sacharydes, proteins and nucleic acids. Selected PAMPs and their source of origin has been described below and listed in table 3. The role of DAMPs and PAMPs in activation of the innate immune system has been summarized in figure 4.

### *Lipoplysacharide (LPS)*

Lipoplysacharide (LPS), a glycolipid composed of a hydrophilic polysaccharide and a hydrophobic lipid A moiety is the major constituent of the outer membrane of Gram-negative bacteria. LPS is recognized by the lipopolysaccharide binding protein (LBP) which facilitates its transfer to CD14, that is then translocated to TLR4-associated LY96. Activation of TLR4 by LPS leads to activation of NF-κB signalling pathway, which results in activation of expression of numerous proinflammatory genes including IL-6, IL-8, TNFα and IL-1.

Additionally, LPS may interact with several other proteins including HSP70, growth differentiation factor 5 (GDF5), CD11/CD18 and CXCR4 [97]

*Poly(I:C) (Double-stranded RNA analog)*

Double stranded RNA is a genetic material in many viruses including Alternaviridae, Birnaviridae, Chrysoviridae, Cystoviridae, Endornaviridae, Hypoviridae, Partitiviridae, Picobirnaviridae, Reoviridae and Totiviridae. Poly I:C is a synthetic analog of dsRNA that is often use to induce anti-viral responses in eucariotic cells in vitro. dsRNA and Poly I:C are recognized by three receptors: TLR3, Melanoma Differentiation-Associated protein 5 (MDA5) and retinoic acid-inducible gene 1(RIG-1). The anti-viral responses mediated by dsRNA are characterized by activation of several signaling pathways engaging numerous signaling molecules including PI3 kinase, toll-like receptor adaptor molecule 1 (TICAM1), TNF receptor-associated factor 6 (TRAF6), IκB Kinase-epsilon (IKKε) and TANK-binding kinase 1 (TBK1) and Interferon regulatory factors (IRFs), which result in the production of ant-viral cytokines such as IFNα/β [98].

Other PAMPs believed to play a role in chronic inflammatory lung diseases include zymosan, beta glucan, muramyl dipeptide and flagellin. Selected Pathogen Associated Molecular Patterns and pathogens producing them have been listed in table 3. Role of DAMPs and PAMPs in activation of the innate immune system has been summarized in figure 4.

| | | | |
|---|---|---|---|
| **Bacteria** | **Gram-negative bacteria** | Lipopolysaccharide (LPS) | CpG unmethilated DNA |
| | **Gram-positive bacteria** | Lipoteichoic acid Lipopeptide Lipoarabinomannan | Flagellin (flagellated bacteria) |
| **Fungi** | | Chitin Glucans | |
| **Viruses** | | Double and single-stranded RNA | |

**Table 3 Selected Pathogen Associated Molecular Patterns and pathogens producing them.** Adapted from [99].
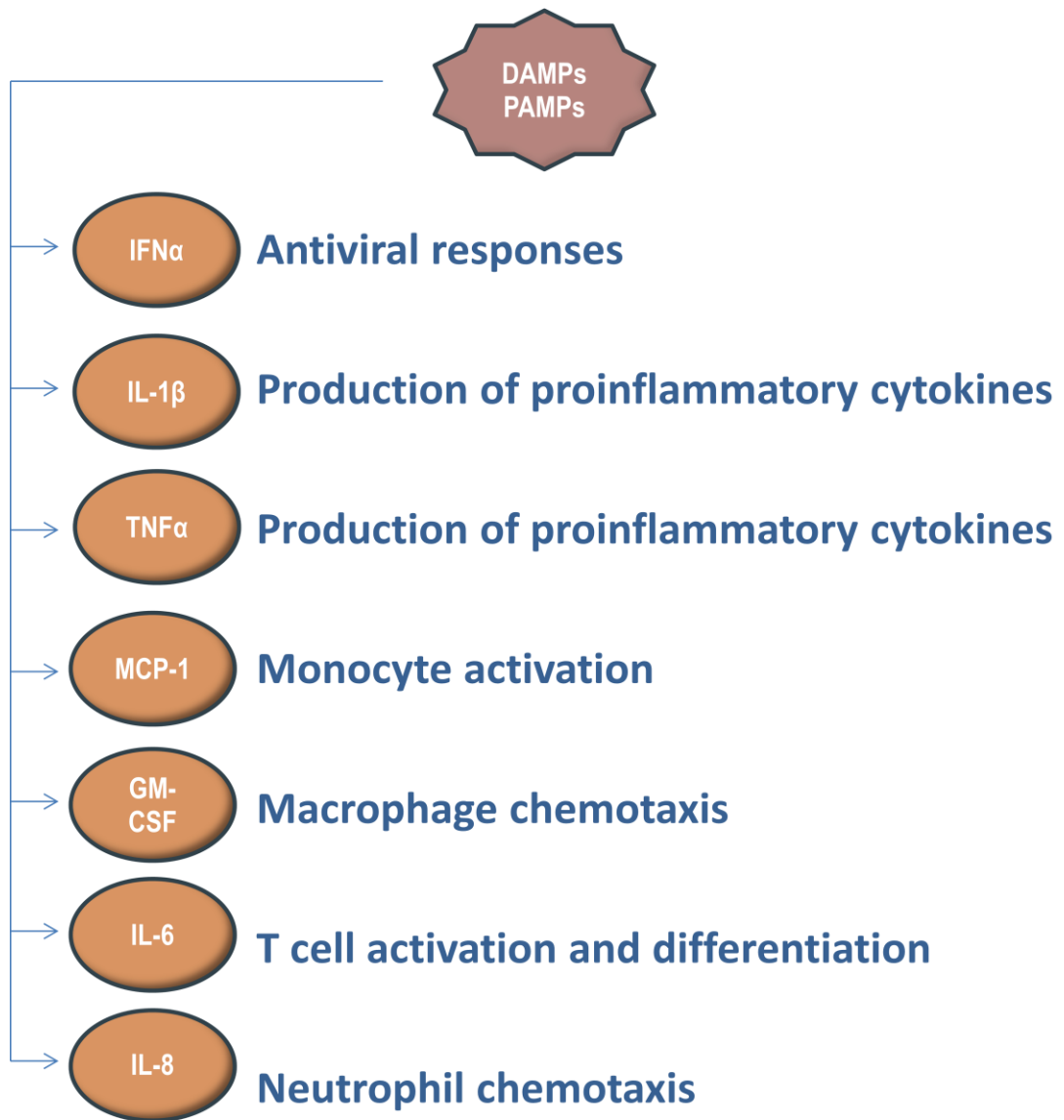
**Figure 4 Role of Damage (DAMP) and Pathogen (PAMP) associated molecular patterns in activation of the innate immune system.** Adapted from [99].

## 1.3.6 Intracellular mediators of the Innate immune signaling

### *NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)*

NF-κB is a protein complex functioning as a transcription factor stimulating expression of genes involved in processes such as inflammation, apoptosis, cell proliferation and red-ox homeostasis. NF-κB is present in the majority of animal cell types and is activated in response to numerous stimuli including proinflammatory cytokines, DAMPs and PAMPs, ROS, oxidized LDL and UV radiation. Deregulation of NF-κB signaling has been implicated in several diseases including cancer, chronic inflammatory diseases, autoimmune diseases and septic shock.

NF-κB can be activated via two routes known as the classical and the alternative pathway. Activation of the classical pathway usually takes place in response to inflammatory cytokines such as IL-1, TNFα and DAMPs and PAMPs of bacterial and viral origin and it involves phosphorylation of IKK complexes followed by phosphorylation, ubiquitination and degradation of an NF-κB inhibitor – IκB. Activation of the canonical pathway is mainly dependant on IKKβ and leads to activation of p50:RelA and p50:c-Rel dimers which are next translocated to the nucleus where they function as transcription factors stimulating expression of genes encoding cytokines (eg. TNFα, IL-1β, IL-1α, GM-CSF, IL-6), chemokines (IL-8, MCP-1, RENTES, MIP-1α), adhesion molecules (ICAM-1 VCAM-1) and enzymes (iNOS, COX2).

The alternative pathway is triggered by stimuli involved in cell growth and differentiation such as lymphotoxin-α, B-cell activating factor (BAFF) or nuclear factor-kappaB ligand (RANKL) which cause phosphorylation of IKKα homodimers, followed by p52:RelB dimer activation, which is next transported to the nucleus where it activates expression of genes such as cytokines (BAFF), chemokines (B lymphocyte chemoattractant (BLC), secondary lymphoid chemokine (SLC), EBI1 ligand chemokine (ELC), stromal cell derived factor (SDF-1)).

In order to maintain cell homeostasis NF-κB activity must be scrupulously regulated. One of the main regulators of NF-κB activity are IκB kinases called IKK proteins. So far three IKK proteins have been indentified, namely IKK1 (IKKα), IKK2 (IKKβ) and IKKγ (NEMO). As mentioned above, IKK1/2/NEMO complexes are involved in

regulation of the canonical pathway, while IKK1 homodimers participate in the alternative pathway activation [100-102].

An important role in NF-κB pathway activation plays ubiquitination which is required for IκB degradation.

Ubiquitination is involved in regulation of the canonical as well as the alternative pathway. Ubiquitination is a covalent, reversible modification that is catalyzed by a group of specialized enzymes, leading to ligation of ubiquitin residues to protein. It is an ATP-dependant, multi-step process requiring involvement of several enzymes including a ubiquitin-activating enzyme causing ubiquitin phosphorylation, ubiquitin-conjugating enzyme (E2 or Ubc) forming an E2-Ub thioester and a ubiquitin-protein ligase (E3), enabling the binding of ubiquitin to the target protein.

Proteins labeled with a polyubiquitin chain linked through lysine-48 (K48) undergo proteosomal degradation. Chains linked with K-63 lysine of ubiquitin have been found to be involved in DNA repair and activation of protein kinases – a process that does not include proteasome-mediated degradation.

The mechanism of ubiquitin-dependant prteosomal degradation of proteins is not yet fully understood. According to the current model, degradation of polyubiquitinated proteins is catalyzed by 26S proteasome which is a protein complex, consisting of approximately 60 subunits, and is found in the nucleus and cytosol of all cells. The 26S complex comprises of a 20S core structure that is linked to 19S regulatory subunits. Proteolitic degradation mediated by proteasome is a highly energy-consuming process requiring ATP hydrolysis.

Following binding to the 19S component, the protein-polyubiquitin complex becomes disassembled and the protein is unfolded. The linearization of protein is necessary to enable its transport via the pore in the core structure in the 20S subunit. Within the 20S subunit, the linearized protein is cleaved by six proteolitic sites. As a result small 3-25 aminoacid peptides are generated which then further cleaved into aminoacids by cytosolic endopeptidases and aminopeptidases [103].

The main functions of NF-κB include production of proinflammatory mediators and regulation of cell death/survival.

The ratio of dying to proliferating cells is one of the most important aspects of homeostasis in tissue. Increased proliferation of cells is required in processes such as tissue regeneration and wound healing, however uncontrolled and intensified cell division rate may also lead to cancer or fibrosis.

Inflammation – the end effect of NF-κB activation – may be associated with diseases characterized by increased cell proliferation such as inflammatory cancer as well as degenerative disorders characterized with progressive tissue damage and cell death such as chronic post-transplant organ rejection.

Cell death may be either apoptotic or necrotic. Apoptosis is characterized by membrane rupture, shrinking of the cells and condensation of the intracellular organelles. Necrosis causes cell swelling, membrane disruption and cell lysis.

Apoptosis is mainly induced in a receptor-dependant manner eg. following activation of TNFR while necrosis is usually mediated by ROS produced by mitochondria. In both cases it comes to activation of caspases which are cysteine proteases. Caspases are involved in protein digestion and degradation during programmed cell death, however they may also play regulatory functions proceeding cleavage of pro-proteins.

Increased activation of caspase 1, which is required to cleavage and activation of IL-1β, may result in enhanced production of proinflammatory cytokines and chemokines. Caspase 1 activation is not always associated with cell death and in some cells such as macrophages it is constitutively expressed which results in ability of these cells to produce active IL-1β following stimulation with a proinflammatory stimulus eg. LPS [104].

NF-κB may act as an inducer and inhibitor of cell death

A study on RelA knockout mice revealed that the animals die in the mid-digestion age due to enhanced apoptosis of liver cells, suggesting that NF-κB suppresses programmed cell death [105]. The control of cell death and survival by NF-κB may be mediated by a cross-talk between NF-κB and JNK. JNK activates caspases and stimulates production of ROS – two factors that contribute to and regulate programmed cell death. NF-κB may inhibit JNK function [106] and stimulate

expression of anti-apoptotic genes including B-cell lymphoma 2 (Bcl-2) [107], antioxidant genes such as Manganese Superoxide Dismutase (MnSOD) [108] and Ferritin heavy chain (FHC) [109].

*__Mitogen-activated protein (MAP) kinases (MAP-kinases)__*

MAP kinases are a group of serine/threonine-specific protein kinases that may be activated by numerous stimuli including proinflammatory cytokines eg. TNFα, IL-1, mitogens, heat shock and osmotic stress. MAP kinases regulate numerous intracellular processes including gene expression, cell differentiation, mitosis, cell survival and apoptosis.

To date several MAP kinase proteins have been identified including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38 isoforms. Extracellular signal-regulated kinases are a diverse family comprised of several ERK isoforms including ERK1,2,3,4 and 5.

ERK1 and 2 (p44/p42) are activated in response to stimuli such as mitogens, growth factors, cytokines and phorbol ester and are involved in regulation of numerous processes including cell differentiation mitosis and meiosis. ERK1/2 are activated by phosphorylation that is preceded by initiation of a three-part protein cascade involving activation if a MAP kinase kinase kinase (MAPKKK or MAP3K), a MAP kinase kinase (MAPKK or MAP2K), and a MAP kinase (MAPK). So far multiple proteins, including Raf, Mos and Tpl have been included into the MAP3K family and MEK1 and MEK2 has been recognized as the main kinases within the MAKK subgroup. MEK1 and MEK2 activate ERK1/ERK2 by phosphorylating Thr202/Tyr204 and Thr185/Tyr187, respectively. ERK1/ERK2 phosphorylation leads to activation of its downstream target molecules including 90 kDa ribosomal protein S6 kinase 1 (p90RSK) and E twenty-six (ETS)-like transcription factor 1 (Elk-1). p90RSK is a serine/threonine protein kinase functioning as ATP and magnesium ion binding protein. It acts as caspase inhibitor and therefore is a negative regulator of apoptosis. p90RSK is phosphorylated following activation of TLR1,2,3,4 and other receptors whose signaling is mediated by MyD88 including IL-1R. Elk-1 functions as a transcription factors and plays an important role in stimulating several genes

including c-fos – a cellular proto-oncogene whose upregulation has been associated with oncogenesis [110, 111].

ERK3 is constitutively localized in the nuclear and cytoplasmic compartments and in contrast to other MAP kinases, has only a single phosphorylation site at serine 189. The function of ERK3 still remains to be discovered however some reports suggest that it may play a role in cell cycle regulation.

Unlike ERK3, ERK4 is a relatively stable protein, which amongst other, is involved in activation of MAPK-Activated Protein Kinase 5 (MK5) – a nuclear enzyme which upon activation is translocated to the cytoplasm where it interacts with p38. Increased activation of MK5 occurs in response to proinflammatory cytokines and stress stimuli. It has been shown that ERK4 may interact and form heterodimers and oligomers with ERK3 [112].

Recently identified ERK family members include ERK5,6 and 7. Their function is still to be investigates however some reports indicate that they may play a role in regulating cell proliferation [113].

Another class MAP kinases that are activated in response to proinflammatory cytokines, and stress stimuli such as UV light or heat shock, are c-Jun N-terminal kinases (JNKs). JNKs are activated by phosphorylation of threonine (Thr) and tyrosine (Tyr) residues within a co called 'Thr-Pro-Tyr motif' and are involved in processes such as T cell differentiation and apoptosis. JNK phosphorylation is mediated by MKK4 and MKK7 while protein Ser/Thr and Tyr phosphatases can lead to their inactivation.

So far ten JNK isoforms have been identified: JNK1 (consisting of four isoforms), JNK2 (consisting of four isoforms) and JNK (consisting of two isoforms). JNK1 and JNK2 are expressed constitutively in all tissue while JNK3 expression so far has been confirmed in the brain, heart and testes.

JNK activates numerous downstream molecules including c-Jun, ATF2, ELK1, SMAD4, p53 and HSF1 which were found to participate in processes such as cell proliferation, apoptosis and differentiation, tumorogenesis, embryo development, fibrogenesis and cell survival in response to stress stimuli. JNK may also function as

inhibitor of several signaling molecules including Nuclear factor of activated T-cells, cytoplasmic 1 and 4 (NFATC4, NFATC1) which are involved in T cell differentiation and STAT3, mediating signaling of factors belonging to IL-6 cytokine including IL-6, oncostatin M and leukemia inhibitory factor [114, 115].

p38 are a group of MAP kinases that, alike JNK and ERK, are activated by multiple proinflammatory cytokines, UV radiation and stress stimuli such as osmotic stress or heat shock. p38 proteins participate in numerous processes related to cell differentiation and survival. p38 proteins described to date include p38α (MAPK14), -β (MAPK11), -γ (MAPK12 / ERK6), and -δ (MAPK13 / SAPK4).

MAPK14 (p38α) acts as a signaling molecule bridging several molecular pathways regulating processes such as proliferation, transcription, differentiation and development. MAPK14 can be activated by phosphorylation by MAP kinase kinases (MKKs) or as a result of autophosphorylation that may be triggered by interaction with MAP3K7IP1/TAB1. MAPK14 phosphorylation leads to activation of its downstream target molecules including ATF2, involved in regulation of transcription, CDC25B, functioning as a cell cycle regulator and a tumor suppressor - p53 protein. p38α has also been shown to play a role regulation of proliferation and differentiation of progenitor and stem cells in the lung. A study on mice revealed that inactivation of this protein kinase results in hyperproliferative epithelium that is highly prone to tumorigenesis. Moreover it has been shown that p38α plays an important role in morphogenesis in lung epithelial brunching, probably by regulating expression of proteins involved in regulation of cell adhesion and cell-cell interactions such as cadherin [116] [117].

MAPK11 (p38β) plays similar biological functions as MAPK14 and can be activated by MKKs, mainly MKK6. One of the targets of MAPK11 discovered so far, is ATF2/CREB2, which functions as a transcription factor and histone acetyltransferase [118].

MAPK12 is also known as ERK6 or p38γ, similarly to other MAP kinases responds to proinflammatory cytokines and environmental stress and has been reported to play a role in several processes related to cell differentiation, including development of myotubes from myofibroblasts, and downregulation of cyclin D1 in response to

stress stimuli eg. hypoxia. Moreover some studies suggest that MAPK12 may act as an inhibitor of proliferation during cell differentiation [119].

MAPK13 / SAPK4 also known as p38 δ is activated by dual phosphorylation on Thr-180 and Tyr-182, which can be mediated by MAP2K3 and MAP2K6. MAPK13 has been reported to play a role in activation of a ATF2 transcription factor and stathmin, which functions as a regulator of microtubule dynamics [120].

Since NF-κB and MAP-kinase pathways may be activated both by DAMPs and PAMPs, the assessment of phosphorylation state of protein kinanses signaling within these two pathways is often used as a marker of activation of the innate immune system.

## 1.4 Inflammation

The consequence of PRR, NFκB and MAP-kinase signaling pathway activation is triggering inflammatory responses. Inflammation can be defined as the end response of the body to an injurious agent such as mechanical or chemical trauma or infection. Inflammation comprises of various physiological reactions including increased release of a vast plethora of cytokines causing leucocytic migration and activation, seepage of plasma proteins, proximal vasodilation and distal constriction of blood vessels and it is required to wound healing and pathogen clearance. External symptoms of inflammation include swelling, redness, pain and increase in the body temperature. In inflamed tissues influx of immunocytes including neutrophils and macrophages is often observed [121].

Inflammation can be classified as acute or chronic.

### 1.4.1 Acute inflammation

Acute inflammation is the initial reaction of the organism to an injury or infection. Symptoms of acute inflammation become apparent within minutes post exposition to trauma. Acute inflammation is initiated by increased movement of plasma and leukocytes through endothelium of blood vessels into the injured tissue and is mediated mainly by neutrophils migrating through blood vessels in response to the chemokine IL-8 which can be released by injured cells as well as activated

macrophages, fibroblasts, endothelial and epithelial cells. Acute inflammation is also mediated by cells residing in tissues, including macrophages, dendritic cells, and structural cells which may produce inflammatory cytokines upon activation by DAMPs and PAMPs. Acellular components involved in acute inflammation include elements of the complement system and coagulation factors present in blood plasma, causing blood clot formation. Recently it has also benn proposed that acute inflammation may be released by alarmins released from damaged epithelium.

Acute inflammation is resolved within a few days and usually does not cause further complications [122].

## 1.4.2 Chronic inflammation

Chronic inflammation is a consequence of persistent acute inflammation which cannot be resolved due to non-degradable pathogens, continuous injury which may be caused by prolonged exposure to toxic factors, presence of foreign bodies or autoimmune reaction. In the context of chronic degenerative lung diseases, chronic inflammation is most often caused by chronic infection, cigarette smoking and exposure to asbestos, coal dust or smog. Chronic inflammation may also develop in organ transplant recipients as a result of an allogenic reaction.

Chronic inflammation is mediated mainly by mononuclear cells such as monocytes, macrophages, lymphocytes, but also by structural long-living cells, such as fibroblasts, and is manifested by production of a vast array of proinflammatory factors including IFN-γ, TNFα, IL-1 and other cytokines, growth factors, reactive oxygen species and hydrolytic enzymes.

Chronic inflammation may last up to months or even years and may vastly contribute to tissue damage and fibrosis. Chronic inflammation has been linked to multiple degenerative diseases including: COPD [123], IPF [124], asthma [125], chronic post-transplant organ rejection [126], arthritis [127], multiple sclerosis [128] and scleroderma [129].

### 1.4.3 Cytokines and chemokines

Proinflammatory cytokines and chemokines can be released by a variety of cells including immunocytes and structural cells, encompassing fibroblasts, epithelial and endothelial cells. Their production may be triggered in response to various stimuli, including DAMPs and PAMPs, small signaling molecules such as nitric oxide (NO) and other cytokines. The main role of cytokines is providing communications between cells both on the paracrine and autocrine level. Cytokines are a diverse group of molecules encompassing proteins, peptides and glycoproteins. From the functional point of view they may be divided into two major groups: pro- and anti-inflammatory. Additionally, some cytokines may function and chemotactic agents and growth factors [130].

The main cytokines believed to play important roles in chronic inflammatory lung diseases has been listed in table 4 and described in more detail below.

| Cytokines | | | |
|---|---|---|---|
| Chemokines | CCL CXCL CX3CL XCL | | |
| TNF | TNFA Lymphotoxin TNFB/LTA TNFC/LTB TNFSF4 TNFSF5/CD40LG TNFSF6 TNFSF7 TNFSF8 TNFSF9 TNFSF10 TNFSF11 TNFSF13B EDA | | |
| Interleukins | Type I | $\gamma$ chain | IL2/IL15 IL4/IL13 IL7 IL9 IL21 |
| | | B chain | IL3 IL5 GM-CSF |
| | | IL-6 family | IL6 IL11 IL27 IL30 IL31 |
| | | IL-12 family | IL12 IL23 IL27 IL35 |
| | Type II | IL-10 family | |
| | | IFN family | Type 1: IFN1A IFNA2 IFNA4 IFNA5 IFNA6 IFNA7 IFNA8 IFNA10 IFNA13 IFNA14 IFNA16 IFNA17 IFNA21 IFNB1 IFNK IFNW1<br><br>Type 2: IFNg |
| | IL-1 family | IL1A/IL1F1 IL1B/IL1F2 IL1Ra/IL1F3 IL1F5 IL1F6 IL1F7 IL1F8 IL1F9 IL1F10 33/IL1F11 18/IL1G | |
| | IL-17 family | IL17/IL25 (IL17A) | |

**Table 4 Major cytokine families.** Adapted from [131].

### 1.4.4 Main cytokines implicated in chronic inflammatory lung diseases

*Interleukin 8 (IL-8)*

**IL-8** is an 8 kDa non-glycosylated protein that is expressed and released by a variety of cells including macrophages, endothelial and epithelial cells and fibroblasts. IL-8 is recognized by and able to activate two G protein-coupled serpentine receptors: CXCR1 and CXCR2. CXCR2, apart from IL-8, is able to recognize several other CXC chemokines, while CXCR1 binds specifically to IL-8. Upon ligand binding, both receptors undergo phosphorylation, internalization and re-expression. IL-8 can exist in a monomeric form or it can form homodimers. IL-8 functions as a chemokine stimulating migration of cells such as neutrophils and granulocytes and as an angiogenic factor promoting blood vessel growth. Moreover IL-8 increases intracellular $Ca^{2+}$ concentration and is involved in respiratory burst and exocytosis. $Ca^{2+}$ mobilization, which is required for chemotactic cell movement is regulated both by CXCR1 and CXCR1, while respiratory burst activity and therefore the key anti-bacterial functions of neutrophils are mediated only by CXCR1 [132].

Binding of IL-8 to cell surface receptors leads to upregulation of CD11b/CD18 (Mac-1), a complement receptor able to bind to C3b and C4b. Mac-1, which is expressed mainly by phagocytic cells such as neutrophils, natural killer cells (NK) and macrophages, functions also as PRR and can recognize many molecules expressed on the surfaces of bacteria. Mac-1 upregulation upon IL-8 stimulation increases phagocytic ability of cells and plays an important role in pathogen and cell debris clearance [133].

In patients with Cystic Fibrosis unopposed proteolitic activity causes cleavage of CXCR1 receptor which disables neutrophil bacteria-killing capacity. Interestingly, glycosylated fragments of CXCR1 may function as DAMPs and may stimulate IL-8 production by activation TLR-2 expressed for example by bronchial epithelial cells [134].

IL-8 gene expression is upregulated upon activation of a variety of receptors including TLRs, IL-1R and TNFR. Increased levels of IL-8 have been reported in many diseases including chronic inflammatory diseases including chronic post-transplant

organ rejection [135], autoimmune diseases eg. psoriasis [136] and some metastatic diseases eg. lung cancer [137].

Upregulation of IL-8 is often accompanied by oxidative stress that may be caused by reactive oxygen species (ROS) produced by activated neutrophils. ROS contribute to pathogen clearance, however their uncontrolled overproduction may result in damage of the host tissue [138].

Expression of IL-8 is mediated via NF-κB signaling pathway and can be effectively inhibited by corticosteroids which block inflammation induced by factors such as TNFα, IL-1 and LPS. In vivo IL-8 induced neutrophil influx can be also abolished by CXCR2 antagonists eg. SB 225002 [139].

*Interleukin 6 (IL-6)*

**IL-6** is a 22-27 kDa protein whose molecular mass may vary depending on its glycosylation state. IL-6 may function both as pro- and antiinflammatory cytokine. The proinflammatory functions of IL-6 include involvement in B cell maturation, monocyte activation, T-cell differentiation and its pyrogenic activity manifested by ability to induce fever during infection. IL-6 may also function as anti-inflammatory cytokine by inhibiting IL-1 and TNFα activity and upregulating production of IL-1Ra and IL-10. IL-6 may be secreted by several different cell types including macrophages, T-cells, muscle cells and fibroblasts. Increased production of IL-6 has been observed during infection, after trauma eg. caused by tissue damage or during muscle contraction. Enhanced levels of IL-6 are associated both with acute and chronic inflammation. During muscle contraction IL-6 is released to blood stream and acts to enhance the breakdown of fats which stimulates energy mobilization and increases body temperature.

Expression of IL-6 can be triggered by DAMPs and PAMPs and some proinflammatory cytokines such as IL-1 and TNFα, suggesting that this cytokine may be implicated both in pathogen-induced and autoimmune inflammatory diseases. So far the role of IL-6 has been investigated mainly in chronic diseases and its importance importance been recognized in diseases such as atherosclerosis,

depression, diabetes, prostate cancer, Alzheimer's Disease, systemic lupus erythematosus and rheumatoid arthritis.

Apart its function in inflammation IL-6 plays also an important role in liver and neuronal regeneration haematopoiesis, fertility and embryonal development [140].

IL-6 signals through a receptor complex consisting of IL-6Rα chain (CD126) which has the ability to recognized and bind the ligand and gp130 component (CD130) responsible for signal transduction. CD130 is ubiquitously expressed while CD126 expression is restricted to certain cell types.

Binding of IL-6 induces receptor dimerization and leads to activation of associated proteins called JAKs (Janus Kinases) which undergo autophosphorylation and cause the receptor phosphorylation. Next, the phosphorylation sites on JAKs and the receptor bind other signaling molecules called STATs (Signal Transducers and Activators of Transcription) which initiates a cascade of phospharylation events leading to activation numerous intracellular signaling pathways including MAP-kinase pathway and PI3K/Akt pathway [141] .

***Monocyte chemotactic protein-1 (MCP-1)***

**MCP-1** also known as Chemokine (C-C motif) ligand 2 (CCL2) is a small 13 kDa proinflammatory chemokine endoced by CCL2 gene, involved in recruitment of monocytes, memory T cells and dendritic cells. MCP-1 is upregulated both during injury and infection and can be secreted by monocytes, macrophages, dendritic cells and some structural cells such as fibroblasts.

MCP-1 is bound by two receptors: CCR2 and CCR4. CCR2 (CD129) is expressed in the surface of monocytes, macrophages, activated memory T cells, basophiles and B cells and apart from MCP-1 it can also interact with CCL8 and CCL16. CCR4 expressed on Th2 T cells and is able to recognize several cytokines including CCL3, CCL5, CCL17 and CCL22. CCR2 and CCR2 are G-protein coupling receptors and their activation leads to several intracellular reactions including release of intracellular calcium, formation of  inositol triphosphat, and activation of PKC [142] [143].

MCP-1 expression can be triggered by a variety of factors including growth factors eg. PDGF, VEGF, cytokines eg. IL-1, IFNγ ,IL-4, TNFα, and bacterial molecules eg. LPS and it has been implicated in the pathogenesis of several diseases including atherosclerosis, psoriasis, rheumatoid arthritis, epilepsy, brain ischemia and traumatic brain injury [144].

Moreover, recent studies indicate that MCP-1 may be also implicated in pathogenesis of BOS where it may play an important role in leukocyte recruitment. Elevated levels of MCP-1 in human bronchial lavage fluid (BALF) have been associated with increased risk of transformation from acute to chronic inflammation and development of chronic allograft rejection post lung transplantation [145]. The role of MCP-1 in lymphocyte recruitment is additionally supported by recent studies demonstrating that mice treated with neutralizing antibodies to MCP-1, showed significantly reduced number of macrophages following tracheal transplantation [146].

MCP-1 has also been proposed as a biomarker that could be used to predict the risk of developing COPD in heavy smokers [147]. Increased levels of MCP-1 were also observed in BALs of asthmatics following allergen exposure [148] as well as patients suffering from chronic degenerative lung diseases such as IPF [149].

### *Granulocyte-macrophage colony-stimulating factor (GM-CSF)*

GM-CSF is a 14 kDa glycosylated protein cytokine that is produced by several cell types including macrophages, fibroblasts, mast cells and endothelial cells. Extracellular GM-CSF forms homodimers which are involved in stimulating stem cells into granulocytes (neutrophils, eosinophils, and basophils) and monocytes. GM-CSF concentrations in blood serum increase following bacterial, fungal, viral and protozoal infections. Enhanced blood levels of GM-CSF have been also detected in tumor-bearing animals animals. In vitro, production of this cytokine can be induced by proinflammatory cytokines such as IL-1 and TNFα. Increased GM-CSF levels were also noted following irradiation damage in bone marrow, where the cytokine plays an important role in the process of hematopoiesis [150].

GM-CSF signals through granulocyte macrophage colony-stimulating factor receptor (GM-CSFR) also known as CD116 (Cluster of Differentiation 116) which is expressed by mature neutrophils and myeloblast. CD116 is a momodimer composed of two subunits namely α chain responsible for ligand recognitions and binding and a β chain involved in signal transduction. α chain is specific to CD116 while β chain is also a component of receptors for IL-3 and IL-5. Ligand binding to the α chain leads to dimerisation of the α and β subunits and phosphorylation of the the β chain on tyrosine residues by JAK kinases. β subunit phosphorylation is followed by association with a Shc adaptor protein and which leads to activation of other downstream signaling molecules [151].

GM-CSF is involved in macrophage activation and is a strong chemoattractant for neutrophils. It stimulates microbicidal and phagocytotic activity, oxidative metabolism and improves cytotoxicity of both macrophages [152] and neutrophils [153].

In the lung GM-CSF is believed to play an important role in surfactant homeostasis regulation and host defense. In mice GM-CSF deficiency or disruption in its signaling pathway leads to pulmonary alveolar proteinosis (PAP) which may caused by impaired capacity of macrophages to surfactant metabolism, phagocytosis, cell adhesion and pathogen killing [154].

***Interleukin 1β (IL-1β)***

**IL-1β** in humans is synthesized as a 31kDa precursor and it is activated by cleavage by a protease, caspase-1 between Asp116 and Ala117 resulting in a production of an active, 17kDa, product. Caspase-1 is a component of a multiprotein oligomer called inflammsome whose composition may vary depending on the activation factors initiating its assembly. The main molecules building inflammsome, apart from caspase-1, include PYCARD (Apoptosis-associated speck-like protein containing a CARD (C-terminal caspase-recruitment domain) or ASC (Apoptosis signal-regulating kinase 1) a NOD-like receptor NALP and caspase5 [155].

ASK, which acts as an adaptor protein, recruits caspase-1 via CARD activation, which leads to cleavage of IL-1β and IL-18 precursor proteins causing their activation.

Inflammasome can be activated via multiple ligands including bacterial RNA, triphosphate (ATP), uric-acid, asbestos and silica [156].

IL-1β is a powerful cytokine able to trigger expression of other proinflammatory factors including IL-6, IL-8 and TNF-α via activation of IL-1R. It can also upregulate expression of its own gene resulting in formation of a positive feedback loop enhancing inflammatory responses. The main source of IL-1β are macrophages which show constitutive caspase-1 activation. IL-1β release from macrophages in considerably enhanced upon infection. In vitro, IL-1β release from macrophages can be achieved eg. via stimulation with TLR4 ligands such as bacterial lipopolisacharide (LPS) [157].

Anti-IL-1β therapies has been proposed as a clinical target in many chronic inflammatory and degenerative diseases including familial pyogenic arthritis [158], rheumatoid arthritis [159], urate crystal arthritis (gout) [160] and type 2 diabetes [161].

The cytokines described above may be produced by fibroblasts of different origin, and therefore in the current study, their expression has been used as a marker of innate immune signalling activation in human lung fibroblasts.
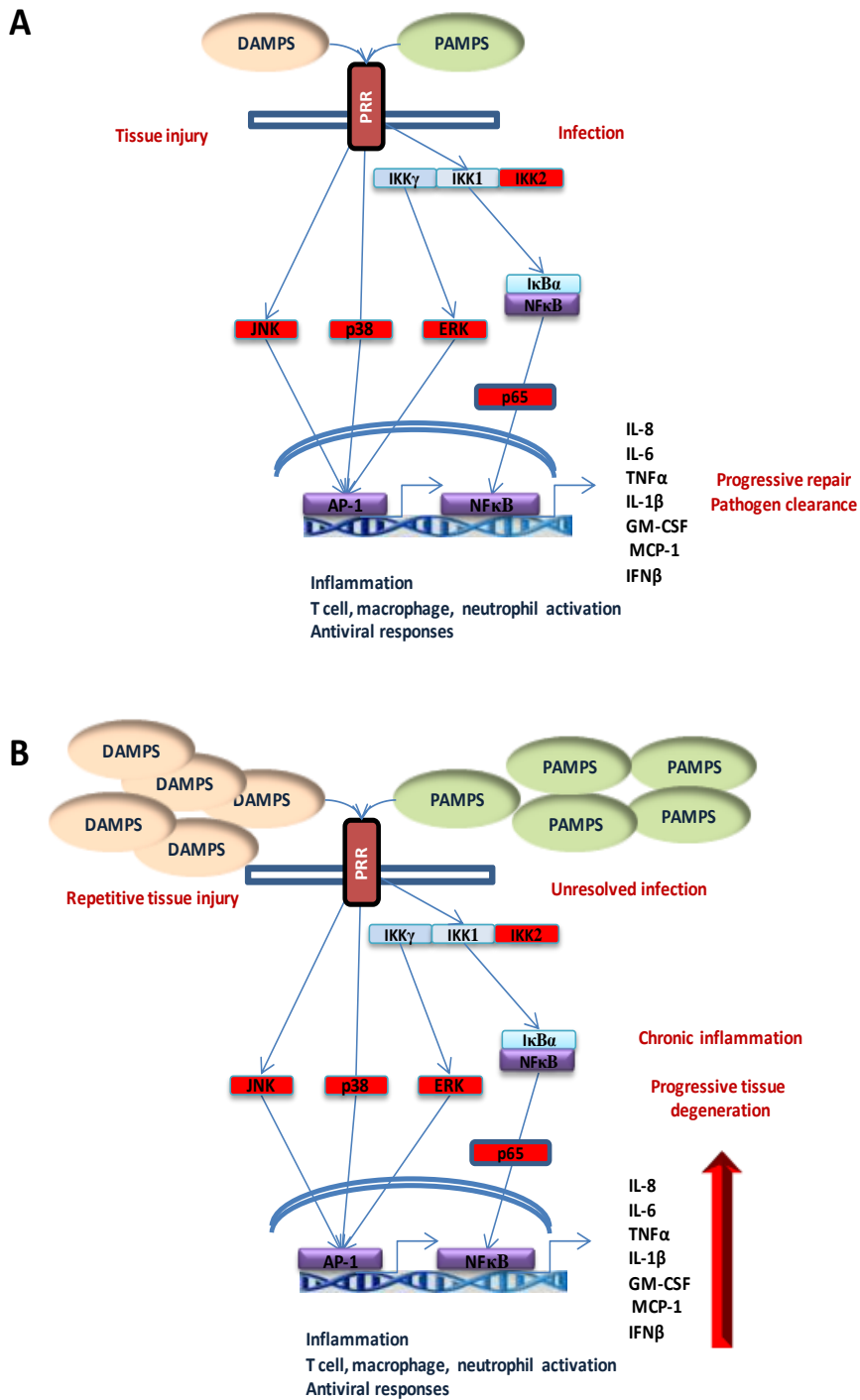
**Figure 5 Signaling in the innate immune system.** (A) Activation of the innate immune system is required for efficient tissue repair and pathogen clerance. (B) Repetitive dissue injury or unresolved infection may cause dysregulation of this process leading to chronic inflammation and progressive tissue degeneration. Adapted from [87].

## 1.5 Chronic degenerative lung diseases

Chronic degenerative lung diseases characterized by progressive degeneration of the respiratory epithelium, intensive fibroproliferation and chronic inflammation include idiopathic pulmonary fibrosis (IPF), bronchiolitis obliterans syndrome (BOS) and chronic obstructive pulmonary disease (COPD). Although, all these diseases are associated with epithelial damage, the profile of alarmins in BALs of patients affected by these conditions, has not been investigated before.

### 1.5.1 Bronchiolitis Obliterans Syndrome

Bronchiolitis Obliterans Syndrome (BOS), also referred to as chronic post transplant rejection, is a non-reversible obstructive lung disease characterized by chronic inflammation and fibrosis in the small airways which is often accompanied by chronic lower respiratory tract infection. BOS affects approximately 50% patients who survive 5 years after lung transplantation and accounts for 30% of deaths occurring within the first three years post transplantation [162].

BOS is characterized by progressive loss of epithelium, excessive fibroproliferation, and an influx of neutrophils and macrophages that are a source of ROS and proteolitic enzymes which may cause damage to the bronchial epithelium. In addition, macrophages secrete numerous fibrogenic cytokines including TGF-β1, PDGF and Insuline-like Growth Factor 1 (IGF-1) that promote fibroblast activation and proliferation [163]. Lung biopsies from BOS patients showed presence of eosinophilic plagues and polyps consisting of fibromyxoid granulation tissue, which cause the airway obliteration [163].

Numerous studies revealed that changes in the balance between type 1 and type 2 cytokines may play an important role in allograft airway remodeling. Type 1 cytokines such as IL-2 and Interferon gamma (IFNγ) are associated with cell-mediated immunity while type 2 cytokines, represented for example by IL-4, IL-5 and IL-15, are involved in humoral and allergric immunity. It has been postulated that the type 1 immune response promotes acute rejection while the type 2 response is implicated in chronic allograft rejection [162]. The balance between

type 1 and type 2 cytokines may have influence on expression pattern of TLR which recognize pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). TLR are involved in modulating innate immune response and have been proposed to play an important role in both acute and chronic transplant rejection. Palmer et al. showed that heterozygosity for either of two functional single nucleotide polymorphisms (SNPs), Asp299Gly or Thr399Ile in Tlr4 gene was associated with hyporesponsivness to endotoxin and decreased the risk of lung allograft rejection within 3 years post transplantation. TLR are also engaged in immune responses in chronic infections which have been implicated as an important risk factor for BOS [164].

The cellular mechanisms driving chronic rejection remain poorly elucidated but current consensus suggests that both alloantigen-dependent [165, 166] and alloantigen-independent insult to airway epithelium leads to an influx of inflammatory cells, chronic neutrophilic inflammation, dysregulated repair and resultant fibrotic plugging or the small airways presenting in the typical obliterative bronchiolitis (OB) lesions [167, 168]. A multitude of risk factors have been identified which include; HLA-mismatch [169], acute rejection episodes [170], recipient age [171], CMV [172], community respiratory viral infections [173], pseudomonal colonisation [174], lymphocytic bronchitis/bronchiolitis [175], gastro-oesophageal reflux [176], air pollution [177] and ischemia-reperfusion injury [178]. Additionally, recent observations suggest that pro-inflammatory cytokines such as IL-1 family members, TNFα [179] and IL-17 [180, 181] may be key pathways driving these insults, via subsequent induction of the potent neutophilic chemoattractant protein IL-8. One of the characteristics of chronic rejection is damage to the bronchial epithelium [163]. Previous studies have demonstrated that damage to epithelium may result in release of alarmins, including IL-1α, which in a paracrine manner may trigger expression of proinflammatory cytokines in lung fibroblasts and other epithelial cells [182]. However, the levels of IL-1α in BALs of patients with BOS and its role in the development of chronic lung rejection have not been studies before.

Clinically, chronic rejection manifests with persistent cough, increased sputum production, dyspnea, coarse crackles and can be quantified functionally by a progressive loss of forced expiratory volume in one second (FEV1). Traditionally this clinical presentation would have been labeled as Bronchiolitis Obliterans Syndrome

(BOS) but more recently has been termed Chronic Lung Allograft Dysfunction (CLAD) [13, 183].

Surgical biopsies from normal and BOS lung has been shown in figure 6.
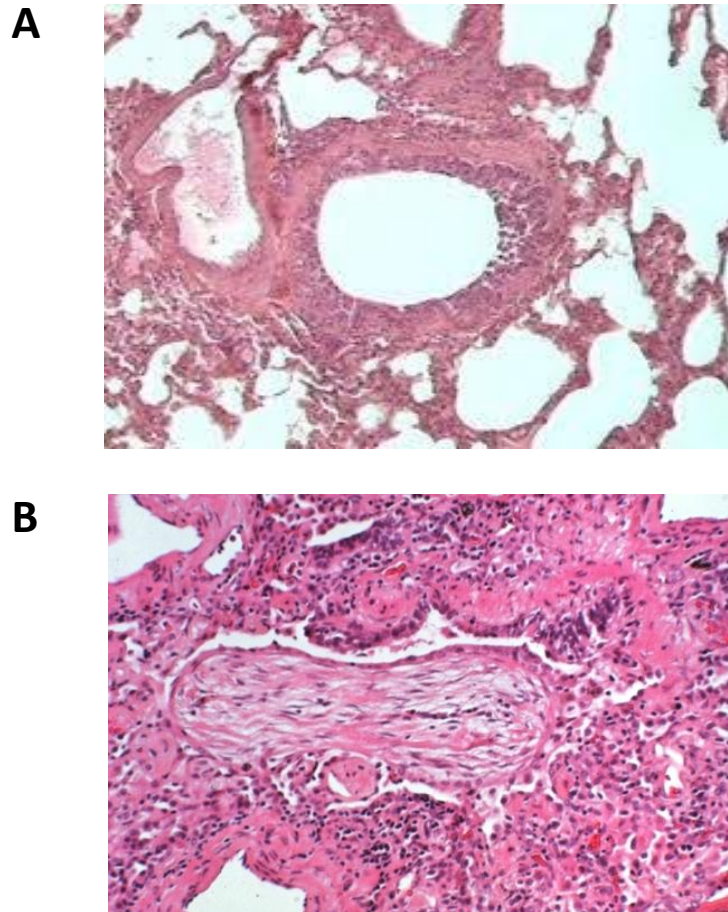


**Figure 6 Surgical biopsies from normal (A) and BOS (B) lung. H&E staining.**

## 1.5.2 Idiopathic Pulmonary Fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is a disease affecting approximately 70/100 000 people worldwide [184], characterized by progressive damage to the lung endothelium and epithelium, resulting in the loss of the alveolar structure and integrity (figure 58). The life expectancy of a patient with IPF is thought to range from 2 to 5 years and the only effective treatment is lung transplant [185]. IPF is associated with intensive fibroblast proliferation and infiltration of inflammatory cells including macrophages and neutrophis which not only cause chronic inflammation but also release a vast array of proteolotic factors including Cathepsin G, Elastase and Proteinase 3 which may distort cell-cell adhesion and damage cell membrane integrity [186]. Surgical lung biopsies from normal and IPF tissue has been shown in figure 7.
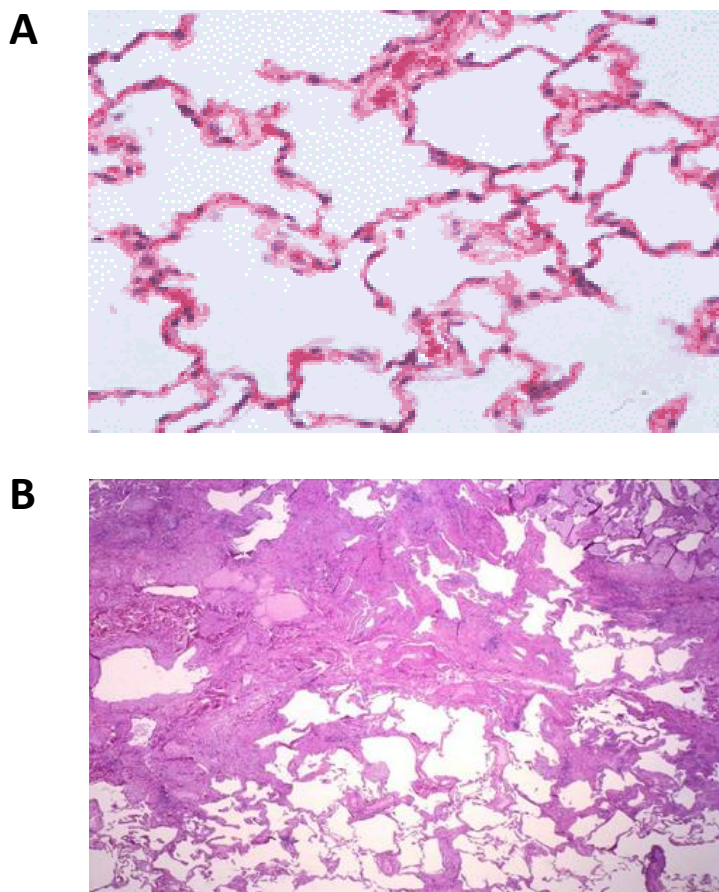


**Figure 7 Surgical lung biopsies from normal (A) and IPF (B) tissue. H&E staining.**

Factors that may contribute to IPF include cigarette smoking, exposure to pollutants and irritants (asbestos, silicon fibers), viral infections and genetic disorders (mutation in Sftpc gene) [187], however in many cases the underlying cause of the disease remains unknown.

One of the factors postulated to play an important role in provoking epithelial cell damage in IPF is oxidative stress. The concept of reactive oxygen species (ROS) as a factor playing a crucial role in the pathogenesis of this disease is supported by the fact that free radicals and other oxidants such as $H_2O_2$, peroxynitrate, and peroxynitrite have been found to upregulate expression of TGF-β1, metalloproteases and proinflammatory cytokines that are implicated in IPF [188]. Furthermore, antioxidants such as dimethyl sulfoxide (DMSO), dimethylthiourea (DMTU) and dithiothreitol (DTT) attenuated release of IL-6, IL-8, IL-1β and Tumor Necrosis Factor alpha (TNF-α) in fibrotic lung, suggesting that oxidative stress plays an important role in the patophysiology of IPF [189].

### 1.5.3 Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) refers to two often co-existing diseases: emphysema and chronic bronchitis and is characterized by chronic inflammation, progressive degradation of lung alveolar epithelium and airway narrowing. The underlying cause of COPD is cigarette smoking, however heavy air pollution, exposure to chemicals (cadmium, coal dust) and certain genetic disorders (α-1-antitrypsin deficiency, Sftpd polymorfism) have also been associated with the disease. It is estimated that between 8 to 11% of the British population are affected by COPD and the mortality rates for this disease in the UK are 634/million in males and 323/million in females [190].

It has been proposed that COPD, which similarly to IPF and BOS is characterized by increased number of fibroblasts, may be mediated by EMT which refers to a pathological conversion of epithelial cells into α-SMA expressing fibroblast. EMT is association with upregulation of specific markers such as fibronectin and vimentin and increased mobility of cells which gain a new mesenchymal-like phenotype. Kamitani *et al.* showed that EMT in primary bronchial epithelial cells can be triggered by TGF-β1 in SMAD-dependent manner and that this effect may be

accentuated by TNFα and to a lesser degree by IL-1β [191]. However, a role of other factors including BMPs and IL-8 in this process is also under investigation.

## 1.6 The relationship between chronic inflammatory lung diseases, oxidative stress and the stress in the endoplasmic reticulum (ER stress)

A common pathological feature of chronic inflammatory lung diseases is epithelial damage, which may lead to release of alarmins (damage associated molecular patterns, DAMP) and trigger further inflammation and mediate recruitment of immunocytes to the site of injury. Epithelial damage may result from a variety of causes including microbial attack, proteolytic damage, the effects of bystander ROS [192, 193] and endoplasmic reticulum (ER) stress [194, 195]. Two factors that may contribute to ER stress in chronic inflammatory and fibrotic lung diseases are oxidative stress induced by cigarette smoke [196] and viral infections which trigger ER stress and unfolded protein response in an IRE1-dependant manner [197]. On the cellular level, ER stress is caused by accumulation of unfolded or misfolded proteins, which may be a consequence of a mutation or intensified translation of proteins, which cannot be folded in an efficient manner [198].

Additionally, recent studies revealed that oxidative stress may also trigger the unfolded protein response (UPR) by upregulating glucose regulated protein 78 (GRP 78) and protein ubiquitination [199]. ER stress activates UPR, which may trigger pro-survival mechanisms such as ER stress associated protein degradation (ERAD) or may promote apoptotic mechanisms, including caspase-1 activation [199]. Although the activation of UPR has been demonstrated in several chronic inflammatory diseases including COPD [200], it is unclear as to how ER stress may contribute to inflammatory responses.

The unfolded and misfolded proteins are recognized and bound by molecular chaperons, including Binding immunoglobulin protein (BIP) and HSPs. The chaperon-protein complexes are recognized by transducers of unfolded protein response (UPR), including PREK, IRE1α and ATF6, which are located in the ER membrane.

PREK, IRE1α and ATF6 trigger intacellular mechanism leading to activation of transcription factors involved in the UPR, whose main function is translation and transcription attenuation and degradation of accumulated unfolded proteins in a process called ER stress associated protein degradation (ERAD), which involves protein ubiquitination and their proteosomal degradation.

In vitro, ER stress may be triggered with several drugs, including thapsigargin, which is a blocker of SERCA channels, tunicamycin, inhibiting protein glycosylation and DTT, which blocks formation of di-sulfide bond in proteins.

Induction of ER stress may be measured by various methods, including assessment of activation of proteins involved in UPR and splicing of XBP-1 mRNA, which occurs following IRE1α activation.

ER stress activates UPR, which may trigger prosurvival mechanisms such as ERAD or may promote apoptotic mechanisms, including caspase-1 activation [201].

**GENERAL HYPOTHESIS AND MAIN AIMS**

**General hypothesis:**
Alarmins released from damaged lung epithelial cells promote fibroblast activation.

**Main aims**
1. Establish an in vitro model of human bronchial epithelial cell injury
2. Investigate the influence of conditioned media from damaged epithelial cells on fibroblast activation, manifested by upregulation of proinflammatory and fibrogenic genes and activation of PRR signaling pathways (NFκB, MAP-kineses)
3. Establish Pattern Recognition Receptor expression profile in primary human lung fibroblasts
4. Identity epithelial alarmins responsible for lung fibroblast activation
5. Establish the responsiveness of primary human lung fibroblasts to selected Damage and Pathogen Associated Molecular Patterns
6. Measure concentrations of alarmins involved in fibroblast activation in BALs of post-transplant patients who developed BOS.
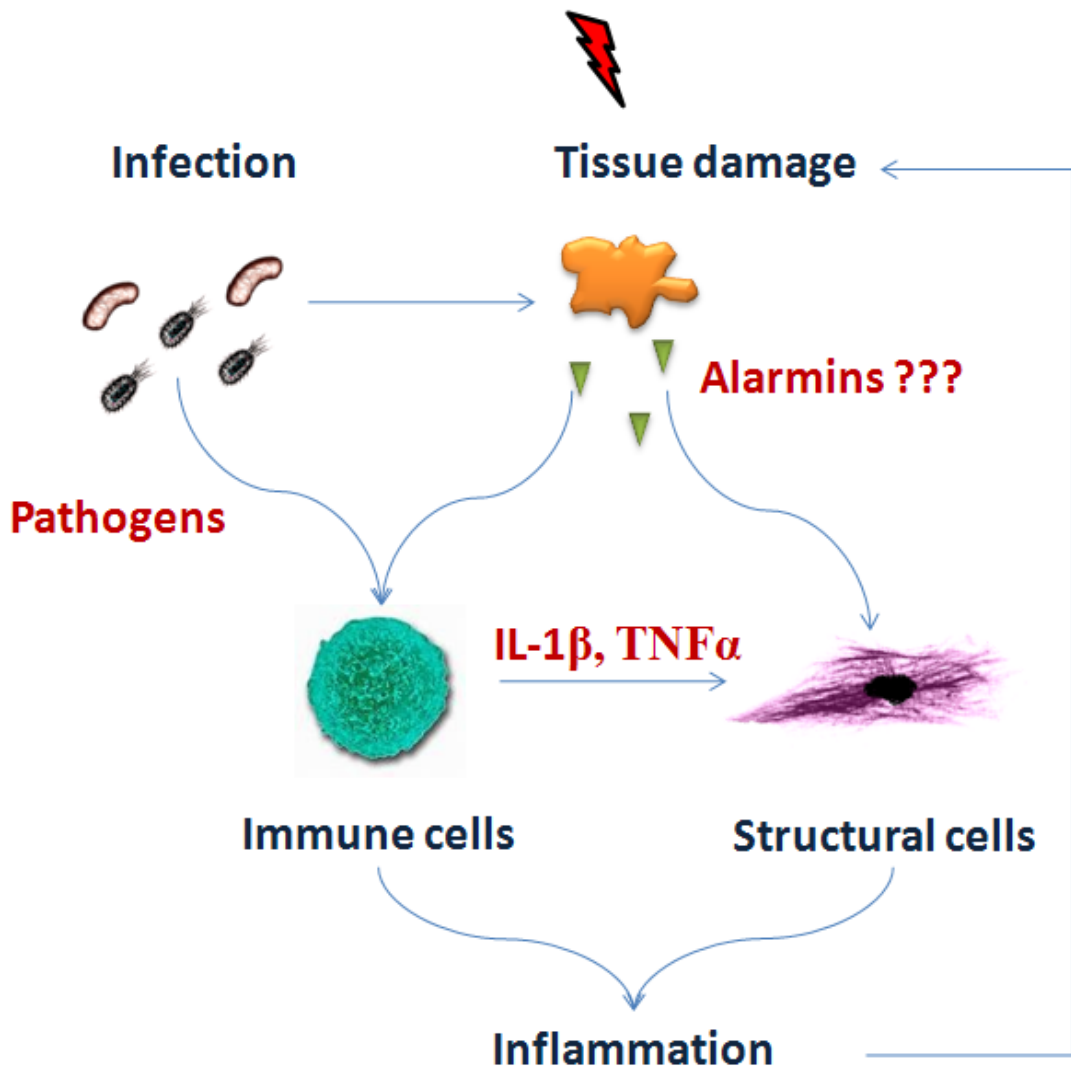
**Figure 8 A diagram showing a hypothetical sequence of events following tissue injury.**

Tissue damage caused by infection, chemical, machanical or allogenic injury leads to release of alarmins which activate innate immune responses in immunocytes and structureal cells resluting in increased production of proinflammatory factors. Prolonged and unresloved inflammation may cause further tissue damage .

## CHAPTER 2 MATERIALS AND METHODS

### 2.1 Patients

Bronchoalveolar lavage (BAL) was obtained from 52 lung transplant recipients within 3 years of transplantation, identified from a cohort under follow up at the Institute of Transplantation, Freeman Hospital, Newcastle. An aliquot of BAL was sent for formal microbiological culture. The remaining BAL fluid was filtered to remove mucus and the cellular fraction was separated from the supernatant by centrifugation. A total and differential cell count was performed and the supernatant was aliquoted and stored at -80°C for subsequent cytokine quantification by ELISA or MSD cytokine assay. Demographics of patients participating in the study is shown in table 12.

### 2.2 Reagents

### 2.2.1 Primary antibodies

| Protein | Company | Cat. No | Host | Isotype | Clonality | Application |
|---------|---------|---------|------|---------|-----------|-------------|
| p38 | Santa-Cruz | sc-7149 | Ra | IgG | pc | WB @ 1:2000 5%Mv |
| p38p | Santa-Cruz | sc-17852 | Ra | IgG | pc | WB @ 1:500 3%BSA |
| JNK-1/2 | Cell-signalling | 9252 | Ra | IgG | pc | WB @ 1:1000 3%BSA |
| JNK-1/2p | Cell-signalling | 4668 | Ra | IgG | mc | WB @ 1:1000 3%BSA |
| Erk-1/2 | Cell-signalling | 4695 | Ra | IgG | mc | WB @ 1:1000 3%BSA |
| ERK-1/2p | Cell-signalling | 4370 | Ra | IgG | mc | WB @ 1:1000 3%BSA |
| IKKα/β | Santa-Cruz | sc-7607 | Ra | IgG | pc | WB @ 1:1000 3%Mv |
| IKKα/βp | Cell-signalling | 2681 | Ra | IgG | pc | WB @ 1:1000 3%BSA |
| P65 | Santa-Cruz | sc-109 | Ra | IgG | pc | WB @ 1:1000 3%BSA |
| P65p | Cell-signalling | 3031 | Ra | IgG | pc | WB @ 1:1000 3%BSA |
| TLR3 | Imgenex | IMG-315A | Ms | IgG | mc | WB @ 1:250 5% Mv |
| IL-1RI | R&D | AF269 | Go | IgG | pc | CF @ 1μg/ml 1% BSA<br>CF @ 1:100 3%BSA |
| IL-1α | R&D | AF-200-NA | Go | IgG | pc | WB @ 0.1μg/ml Mv<br>CF @ 10μg/ml 1% BSA<br>Block @ 4μg/ml |
| IL-1β | R&D | AF-201-NA | Go | IgG | pc | Block @ 4μg/ml |
| Fibronectin | Sigma | F3648 | Ra | IgG | pc | CF@ 1:100 1%BSA |
| IL-6 | R&D | DY206 | Ms | IgG | mc | CF@ 2μg/ml 1%BSA |

Ms- mouse; Ra- rabbit; Do-donkey; Go- goat; mc- monoclonal; pc- polyclonal; WB- western blott; CF- immunocyto/histochemistry; Block- neutralization.

**Table 5 List of primary antibodies**

## 2.2.2 Secondary antibodies

| Company | Cat. No | Host | Antigen | Conjugate |
|---|---|---|---|---|
| **Sigma** | F2012 | Go | Mouse IgG | FITC |
| **Sigma** | T6778 | Go | Rabbit IgG | TRITC |
| **Sigma** | F7367 | Ra | Goat IgG | FITC |
| **Sigma** | A3673 | Go | Mouse IgG | HRP |
| **Abcam** | ab6721 | Go | Rabbit IgG | HRP |
| **Abcam** | ab98513 | Do | Goat IgG | HRP |

Ra- rabbit; Do-donkey; Go- goat; HRP- horseradish peroxidase; FITC- Fluorescein isothiocyanate; TRITC- Tetramethyl Rhodamine Isothiocyanate

**Table 6 List of secondary antibodies**

## 2.2.3 Chemical inhibitors

TAK1i ((5Z)-7-Oxozeaenol) (Cat No. 499610-1MG) and IKK-2i IV (5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide) (Cat No. 401481-500UG ) were purchased from Calbiochem.

hrIL-1Ra was obtained from R&D systems (Cat No. 280-RA-050).

**Primers (5'-3')**

| GENE NAME | FORWARD PRIMER SEQUENCE | REVERSE PRIMER SEQUENCE |
|---|---|---|
| TLR1 | CCTAAAGACCTATCCCAGAA | ACAGTAGGGTGGCAAGAAAT |
| TLR2 | TTGTGGATGGTGTGGGTCTT | AGGTCACTGTTGCTAATGTA |
| TLR3 | TATTTCCCTTGCCTCACTCC | TGGTTAGGTTGAGTATGTGT |
| TLR4 | TTTTTCTAATCTGACCAATC | TCATAGGGTTCAGGGACAGG |
| TLR5 | TACCCCCTTGACTATTGACA | ATAACCATCTTTCAATACAG |
| TLR6 | TTCCATTTTGTTTGCCTTAT | TTATGGGAAAGTCTCAAAAC |
| TLR7 | GATTTACTCCATTCAACAGC | TGTCGTTCATCATCAGTTTC |
| TLR8 | ATGTTCCTTCAGTCGTCAAT | TTTTGCTTTTTCTCATCACA |
| TLR9 | TACCTTGCCTGCCTTCCTAC | TGTCACCAGCCTTTCCTTGT |
| TLR10 | TTATGACAGCAGAGGGTGATG | GGAGTTGAAAAAGGAGGTTA |
| IL-1R1 | CCTAAAGACCTATCCCAGAA | ACAGTAGGGTGGCAAGAAAT |
| RAGE | TTGTGGATGGTGTGGGTCTT | AGGTCACTGTTGCTAATGTA |
| NLPR3 | TATTTCCCTTGCCTCACTCC | TGGTTAGGTTGAGTATGTGT |
| IL-6 | TTTTTCTAATCTGACCAATC | TCATAGGGTTCAGGGACAGG |
| IL-8 | TACCCCCTTGACTATTGACA | ATAACCATCTTTCAATACAG |
| IL-1β | TTCCATTTTGTTTGCCTTAT | TTATGGGAAAGTCTCAAAAC |
| MCP-1 | GATTTACTCCATTCAACAGC | TGTCGTTCATCATCAGTTTC |
| GM-CSF | ATGTTCCTTCAGTCGTCAAT | TTTTGCTTTTTCTCATCACA |
| IL-1α | AATGACGCCCTCAATCAAAG | TGGGTATCTCAGGCATCTCC |
| XBP-1 | GGAGTTAAGACAGCGCTTGG | ACTGGGTCCAAGTTGTCCAG |
| GAPDH | GAGTCAACGGATTTGGTCGT | GACAAGCTTCCCGTTCTCAG |

**Table 7 List of qRT-PCR primers**

## 2.3 Cell culture

### 2.3.1 Primary cell isolation and culture

#### *Human primary bronchial epithelial cells (PBEC)*

Human primary bronchial epithelial cells (PBEC) were obtained from by bronchial brushings from anonymous post-transplant patients and were cultured on 0.5% Purecol (Invitrogen) coated dishes in SAGM (Lonza) medium containing 2% FBS (Lonza), 100U/ml penicillin (Sigma) and 100µg/ml streptomycin (Sigma). All post-transplant patients participating in the study gave informed consent and the study was approved by the local regional ethics committee (Ref. 2001/179).

#### *Human primary lung fibroblasts*

Human primary lung fibroblasts were isolated using collagenase type 2 digestion technique from parenchyma of lungs that were not used for transplantation. Lung tissue pieces from anonymous human donors were washed in DMEM (containing 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine) and chopped using a mezzaluna. The tissue pieces were washed on a 40µm filter with DMEM to remove debris and red cells and resuspended in 10ml serum free DMEM containing 0.1% BSA and 0.2% collagenase type 2. The tissue-collagenase suspension was incubated on a roller at room temperature for 2hrs. The tube was shaken occasionally during the incubation to stop the tissue from clumping. The suspension was filtered through a 100µm filter and the filtrate was spun down at 1200rpm for 5mins. The cell pellet was washed by resuspending in DMEM and spun down again. Following washing, the cells were resuspended in complete DMEM, counted and plated at 80 000 cells/cm$^2$. The cells were left to attach for 1hrs after which they were washed with PBS and fresh culture medium was added to the culture. The ICC analysis showed that the isolated cells expressed high levels of fibronectin (a marker of fibrogenic cells) and had characteristic for fibroblasts spindle-like shape. Additionally, a qRT-PCR analysis revealed that the isolated cells expressed transcripts for other fibroblast markers including Col1A1, Col3A3, Col4A1 and α-SMA (data not shown). The cells were propagated in the culture and used for experiments at passage 1-5.

### 2.3.2 Cell line culture

MRC5 cells (human fetal lung fibroblast cells) were cultured in DMEM/F12 (Sigma) containing 10% FBS, 1% L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin.

16HBE14o- (SV40 large T-antigen transformed human bronchial epithelial cell line) was maintained in HiClone medium (Pierce) supplemented with 10% FBS, 1% L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin.

BEAS2B (human bronchial epithelial cells transformed with polyomavirus DNA) were maintained in BEGM (Lonza) media containing 2% FBS (Lonza), 100U/ml penicillin (Sigma) and 100μg/ml streptomycin (Sigma).

A549 (carcinomic human alveolar basal epithelial cell line) were cultured in DMEM Medium (Sigma) supplemented with 10% FBS, 1% L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin.

THP-1 (monocytic leukemia cell line) was cultured in RPMI1640 medium (Sigma) supplemented with 10% FBS, 1% L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin.

 All cells were maintained at 37°C at an atmosphere of 5% $CO_2$.

When the cells reached 80-90% confluence, the monolayers were subcultured as follows. The growth medium was removed, cells were washed with phosphate buffered saline (PBS) and trypsinised with 0.05% Trypsin solution (Sigma) at 37°C. After cell dissociation, trypsin was neutralized with culture medium and the cells were pelleted by centrifugation. The cell pellets were resuspended in culture medium and seeded at an appropriate density. All experiments were performed on cell monolayers that reached 80-90% confluency.

### 2.3.3 Cell count

In order to determine the cell number, 10µl of cell suspension was pipetted onto a hemocytometer chamber and the count was performed in 5 large size squares (1mm x 1mm). The cell density was determined according to the following equation:

Cell density [cells/ml] = $\frac{nC}{nB} \times d \times 10^4$

where:

nC – cell number

nB – number of large boxes (1mm x 1mm)

d – dilution factor

### 2.4 Assessment of cell viability by flow cytometry using propidium iodide (PI) staining.

**Assay principle**

Propidium iodide (PI) is a fluorescent dye (excitation wavelength 488nm) able to bind DNA by intercalating bases. PI is incorporated by cells whose cell membrane has been discontinued and it can be used to distinguish necrotic and apoptotic from viable cells.

**Assay procedure**

Flow cytometric analysis was performed to assess the viability of epithelial cells following treatment with $H_2O_2$, freeze-thaw stress, ER stress, elastase and cigarette smoke extract (CSE). The conditioned media from cell cultures were collected and stored at -80°C for ELISA analysis. The cells were pelleted and resuspended in 300µl DMEM phenol red free media (Gibco) and incubated for 30mins at 37°C. Immediately prior to analysis the cells were stained with PI at a final concentration

of 10 µg/ml. The analysis was performed using FACScan Cytometer and the data were processed with CellQuest software. All samples were analyzed in biological triplicates. The mean values were used to compare cell viability between samples.

### 2.5 Assessment of cell viability using Trypan Blue staining

**Assay principle**

Trypan blue is a blue diazo dye, commonly used to distinguish between dead and viable cells. Trypan blue is incorporated by damaged cells. As a consequence their cytoplasm is colored blue and dead cells can be recognized by observation under a light microscope. Cells with intact membranes remain uncolored.

**Assay procedure**

The cells were pelleted and resuspended in 300µl medium. Immediately prior to analysis the cells were stained by addition of equal volume of with trypan blue (300µl). The number of dead (stained blue) and viable (unstained) cells was counted under a light microscope using hemocytometer and the cell viability was expressed as a percentage of viable cells. All samples were analyzed in biological triplicates. The mean values were used to compare cell viability between samples.

### 2.6 XTT cytotoxicity assay

**Assay principle**

XTT cytotoxicity test (Roche) is based on the cleavage of the yellow tetrazolium salt XTT to form an orange fermazan dye by metabolically active cells.

**Assay procedure**

XTT assay was used to assess the influence of BALs from post-transplant patients on PBEC cell viability. PBEC cells were seeded at $2x10^4$ cells/ml in the medium volume of 100ul/well in a 96 well culture plate. The cells were allowed to grow for 24hrs after which they were treated for 24hrs with 100µl 1:1 BAL/medium. Following the treatment, 10µl of XTT reagent (prepared by mixing XTT (1mg/ml) with electron coupling reagent (1.25mM)) was added to each well. Following 4hrs incubation the

formazan dye formation corresponding to the relative cell number was quantified using a multiwell spectrophotometer. All measurements were performed at the wave-length of 450nm.

## 2.7 Enzyme-linked immunosorbent assay (ELISA)

IL-1α, HSP60, IL-8, IL-6, IL-1β and GM-CSF concentrations were measured using ELISA DuoSet kit (RnD Systems) according to the manufacturer's instructions. MCP-1 concentrations were measured using a sandwich ELISA kit from Peprotech. Briefly, 96 well plates were coated with an appropriate capture antibody diluted to the working concentration in PBS without carrier protein. Following overnight incubation, the plates were blocked with 1% BSA and 100μl of an appropriate dilution of sample or protein standard was added to each well. Seven point standard curves were prepared using 2-fold serial dilutions of an adequate recombinant protein. All samples were analyzed in biological triplicates and technical duplicates. Antigen detection was performed by incubation with an appropriate biotynylated antibody and the plates were developed by adding HRP-linked streptavidin producing visible signal upon addition of the substrate - tetramethylbenzidine. The reaction was stopped with 2M $H_2SO_4$ and the optical density at the wavelength of 450nm was measured using a microplate reader. The replicate readings for standards and samples were averaged and the zero standard optical density was subtracted. The standard curves were created using EXCEL software by plotting the mean absorbance for each standard against the concentration. The concentrations of samples were determined by comparing the absorbance to the standard curve and multiplying by the dilution factor.

The composition of blocking buffers, wash buffers, dilution reagents and concentrations of the capture and detection antibodies used has been listed in table 8.

| Protein name | Capture antibody origin and concentration | Detection antibody origin and concentration | Reagent diluent | Substrate | Detection range | Supplier |
|---|---|---|---|---|---|---|
| IL-1α | Mouse anti-human IL-1α 2µg/ml | Biotinylated goat anti-human IL-1α 12.5ng/ml | 1% BSA in PBS, pH 7.2-7.4 | TMB | 7.81-500pg/ml | R&D Systems |
| HSP60 | Mouse anti-human HSP60 2µg/ml | Biotinylated mouse anti-human HSP60 1µg/ml | 1 mM EDTA, 0.5% Triton X-100 in PBS, pH 7.2 - 7.4. | TMB | 0.625-40ng/ml | R&D Systems |
| IL-6 | Mouse anti-human IL-6 2µg/ml | Biotinylated goat anti-human IL-6 200ng/ml | 1% BSA in PBS, pH 7.2-7.4 | TMB | 31.24-2000pg/ml | R&D Systems |
| IL-8 | Mouse anti-human IL-8 4µg/ml | Biotinylated goat anti-human IL-8 20ng/ml | 0.1% BSA in TBS-T (0,05% Tween20 in TBS), pH 7.5-7.6 | TMB | 7.81-500pg/ml | R&D Systems |
| IL-1β | Mouse anti-human IL-1β 4µg/ml | Biotinylated goat anti-human IL-8 200ng/ml | 0.1% BSA in PBS-T (0,05% Tween20 in TBS), pH | TMB | 3.9-250pg/ml | R&D Systems |
| MCP-1 | Rabbit anti-human MCP-1 0.5µg/ml | Biotinylated rabbit anti-human IL-8 0.5µg/ml | 0.1% BSA in PBS-T (0,05% Tween20 in TBS), pH | ABTS | 31.24-2000pg/ml | Peprotech |
| GM-CSF | Mouse anti-human GM-CSF 2µg/ml | Biotinylated mouse anti-human IL-8 0.5µg/ml | 0.1% BSA in PBS-T (0,05% Tween20 in TBS), pH | TMB | 15,62-1000pg/ml | R&D System |

TMB- 3,3',5,5'-Tetramethylbenzidine; ABTS- 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

**Table 8 Composition and concentrations of reagents and antibodies used in ELISA**

**Establishing sample dilution factor**

To find the optimal sample dilution factor and to ensure that the analyte concentration was within the range of the standard curve linearity, 2-fold serial dilutions of selected samples were performed prior to analysis. The sample diultions fitting within the linear phase of the standard curve were used to determine the concentrations of the protein of interests. An example standard curve has been shown in figure 9.

## ELISA: Standard curve for IL-6



$y = 0.0009x$
$R^2 = 0.9763$

**Corrected plate readings** (y-axis)

**IL-6 Concentration (pg/ml)** (x-axis)
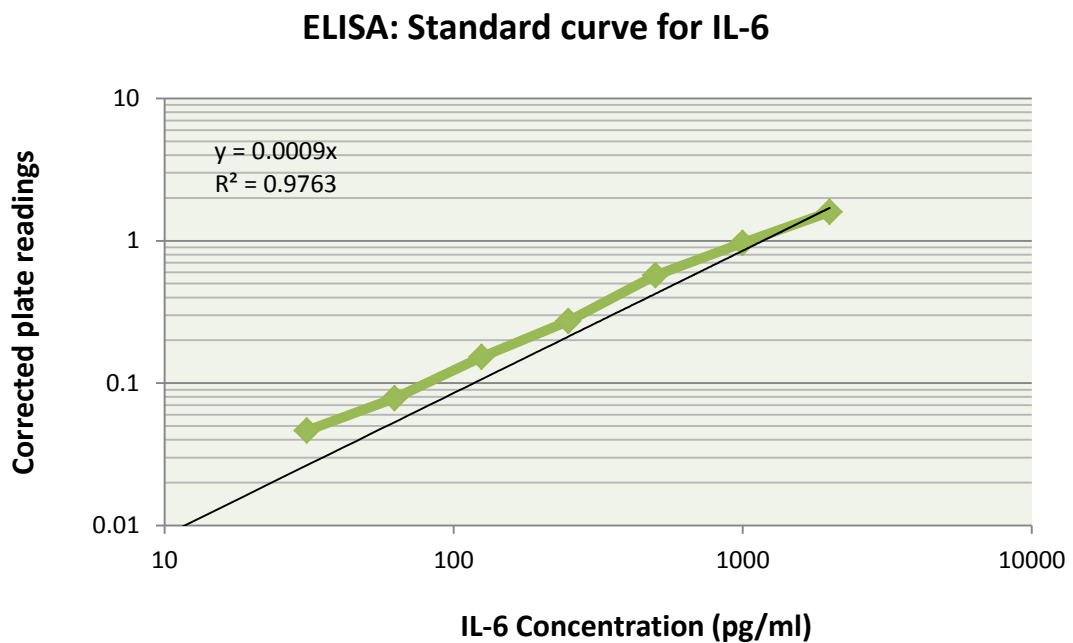
**Figure 9 An example of an ELISA standard curve.**

## 2.8 MSD electrochemiluminescence assay

IL-1α and IL-8 concentrations in BALs were measured using MSD electrochemiluminescence detection kits (Meso Scale Discovery) according to the manufactures instructions. Briefly, 25µl of sample or an appropriate human recombinant cytokine was loaded in technical duplicates into each well of a 96 plate and subjected to an overnight incubation at 4°C. Following the incubation the plates were washed 3 times with 1xPBS-T and 25µl of detection antibody was added to each well. After 2hrs incubation the plates were washed again and 150 of 2X read buffer was added to each well and the plates were read immediately on a SECTOR®

Imager 2400 plate leader (Meso Scale Discovery). The cytokine concentrations were calculated against a seven point standard curve ranging from 2500 to 0 pg/ml.

## 2.9 Immunocytofluorescence

Cells were seeded at $2x10^5$/ml in a 24 well plate and treated as indicated. The cells were fixed with 3% paraformaldehyde (Sigma) for 1hr at room temperature. To quench the autofluorescence the samples were incubated in 100mM glycine (Sigma) solution for 30mins. Cell membranes were permeabilized using 0.1% Triton X-100 solution (Sigma), blocked with 5% BSA (Sigma) in PBS-T and probed with 1:100 dilution of anti-human IL-6 (mouse monoclonal, R&D Systems) or fibronectin (mouse monoclonal, R&D Systems) antibody. Proteins were visualized using a corresponding secondary antibody conjugated with FITC or TRIC respectively (DAKO). The cell nuclei were stained with DAPI (Vectra Shield). Samples were analyzed using a Leica TCS-SP-2UV confocal microscope. To determine the background staining, 2°Ab controls were implemented in each experiment.

## 2.10 Immunohistofluorescence

Lung tissue sections were embedded in paraffin and sliced using a microtome. Prior to the staining the specimen were dewaxed in xylens for 5mins and fixed with 74% ethanol for 3mins. To reduce the surface tension the slides were boiled in 1xEDTA for 10mins. After cooling for 20mins at room temperature the samples were blocked with 1% BSA in PBS-T and incubated with an appropriate concentration of primary antibody overnight. Following the incubation the samples were washed 3 times with PBST and a fluorochrome-conjugated secondary antibody (DAKO) added for 2hrs. Cell nuclei were visualized with mounting solution containing DAPI (Vectashield). Samples were analyzed using a Leica TCS-SP-2UV confocal microscope. To determine the background staining, 2°Ab controls were implemented in each experiment.

## 2.11 RNA extraction

Total RNA from cells was isolated using Absolutely RNA MicroPrep (Stratagen). The extraction method is based on the ability of nucleic acids to bind to silicon matrix and uses chaotropic salt guanidine thiocyanate as a strong protein denaturant allowing to effectively lyse cells and prevent from RNA degradation by ribonucleases.

~5x105 cells were resuspended in 100μl lysis buffer containing 0.7μl β-Mercaptoethanol and homogenized by vortexing. Next, 100ul 75% ethanol was added and the mixture was vortexed for 5 seconds. The cell lyset was transferred into RNA-Binding Spin column seating within a 2-ml collection tube and span down for 1 min at 12000g.

In order to remove genomic DNA contamination, DNase treatment was performed. Prior to the procedure, the column was washed with 600μl 1XLow Salt Buffer and the matrix was dried by centrifugation for 2 mins at 12000g. Next, 30μl of DNase solution was added directly onto the matrix of the RNA-Binding Spin column and the samples were placed in a 37°C incubatior for 15 mins.

Following the DNase treatment, the matrix was washed once with 500μl of 1xHigh Salt Buffer containing guanidine thiocyanate  and twice with 500 and 300μl 1xLow Salt Buffer and, to dry the matrix, the samples were span down for 2mins at 12000g.

The RNA-Binding Spin column was transferred into a new 1.5ml collection tube and, in order to elute the RNA, 30ul of Elution Buffer was added directly onto the matrix. Following 2 mins incubation at room temperature, the column was span down for 1min at 12000g. The purified RNA solutions in Elution Buffer were stored at -80C.

The RNA concentrations were measured spectrophotometrically at the wavelength of 260nm using a NanoDrop instrument. The purity of the extracted RNA was assessed based on the A260/A280 ratio and its quality was verified by electrophoresis in 2% agarose gel in 1xTAE buffer (Figure 10).
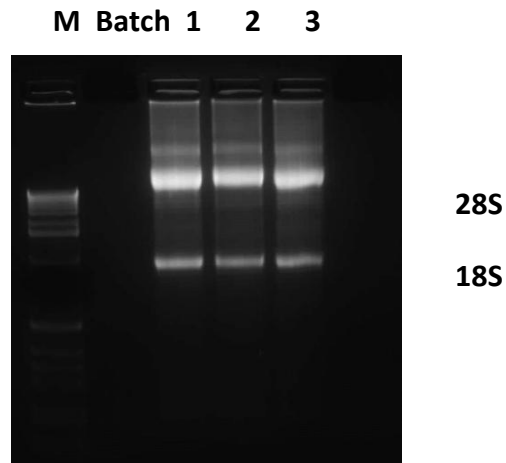
**Figure 10 An example of RNA quality check.** 20ng RNA was separated by electrophoresis in a 2% agarose gel containing ethidium bromide.

## 2.12 Reverse-transcription Polymerase Chain Reaction

Reverse transcription PCR was carried out with AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene) using 0.5-2ug of total RNA. In order to perform the first strand cDNA synthesis a 15.7 μl reaction mixture containing 12.7 μl of RNA and 3μl of random primers (0.1μg/μl) in RNase-free water was incubated for 5mins at 65°C in a thermocycler. Following the incubation the mixture was cooled down to room temperature for 10mins to allow the primers to anneal to RNA. Next, 2.0 μl of 10× AffinityScript RT Buffer, 0.8 μl of dNTP mix (25 mM each dNTP), 0.5 μl of RNase Block Ribonuclease Inhibitor (40 U/μl) and 1 μl of AffinityScript Multiple Temperature Reverse Transcriptase were added to the mixture to the total volume of 20μl. To extend the primers, the samples were incubated at 25°C for 10mins, after which the reaction temperature was increased for 60min to 55°C to allow cDNA synthesis. The reaction was terminated by incubating for 10mins at 70°C. cDNA samples were used immediately for PCR or stored at -20°C.

## 2.13 Polymerase Chain Reaction (PCR)

PCR was performed to verify the results of the first strand cDNA synthesis and to confirm the site-specificity of the primers designed to anneal fragments of the target cDNA for gene expression analysis by qRT-PCR. PCR reactions were carried out in 25µl volume. Following 1min initial denaturation step at 94°C, samples were subjected to PCR for 35 cycles as follows: 94°C denaturation (30s), 55-60°C annealing (30s) and 72°C elongation (1min). The final elongation step was performed at 72°C for 5mins. The products of the PCR reaction were analyzed by electrophoresis in 2% agarose gel with 0.5µg/ml ethidium bromide in 1xTAE buffer containing 40 mM Tris·HOAc and 2 mM EDTA (pH ~ 8.5).

Composition of the reaction mixture is as follows:

5µl 5x GoTaq Flexi Reaction Buffer,

2µl $MgCl_2$ (25mM)

0.5µl dNTPs (10mM)

0.5µl Forward Primer (10µM)

0.5µl Reverse Primer (10µM)

15.25µl $H_2O$

1µl cDNA (20ng)

0.25µl GoTaq Flexi DNA Polymerase (0.25U) (Promega).

### 2.14 XBP-1 splicing assay

1 µg total RNA was reverse-transcribed with AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent technologies) and 20ng of cDNA was amplified with JumpStart™ Taq polymerase using a pair of primers covering a 26 nucleotide intron (nucleotides 531-556), which is spliced out by IRE1 following ER-stress induction. DNA fragments were separated on 2% agarose gel.

### 2.15 Real-time PCR

Real-time PCR was performed using ABI PRISM 2000 instrument. GAPDH was used as the house-keeping gene. The reactions were performed in a 96 well plate format. Each well contained 6.5µl Sybr Green Master Mix (Sigma), 1µl forward and reverse primer mixture (10mM), 3.5µl $H_2O$ and 2µl cDNA (20ng). Each test was performed in duplicates with a no template control for each primer set. Results were normalized to the expression of the endogenous reference gene (GAPDH) and the relative gene expression was calculated using ΔΔCt method according to the following formula:

ΔΔCt= 2^(Ct of reference gene – Ct of gene of interest).

where Ct referes to a threshold cycle

### 2.16 Determination of protein concentration using BCA (bicinchoninic acid) protein assay

*Assay principle*

The BCA Protein Assay is based on reduction of $Cu^{2+}$ to $Cu^{1+}$ by protein in an alkaline environment. Cuprous cations ($Cu^{1+}$) are bound by bicinchoninic acid which results in forming a purple-blue product which exhibits a strong linear absorbance at 562nm correlating with protein concentrations. Total protein concentration is calculated using an 8-point (working range = 20-2000µg/ml) standard curve prepared by making serial dilutions of Bovine Serum Albumin (BSA). An exemplary standard curve is presented in figure 11.

## Protein (BSA) standard curve



$$y = 0.0009x + 0.0249$$
$$R^2 = 0.9983$$
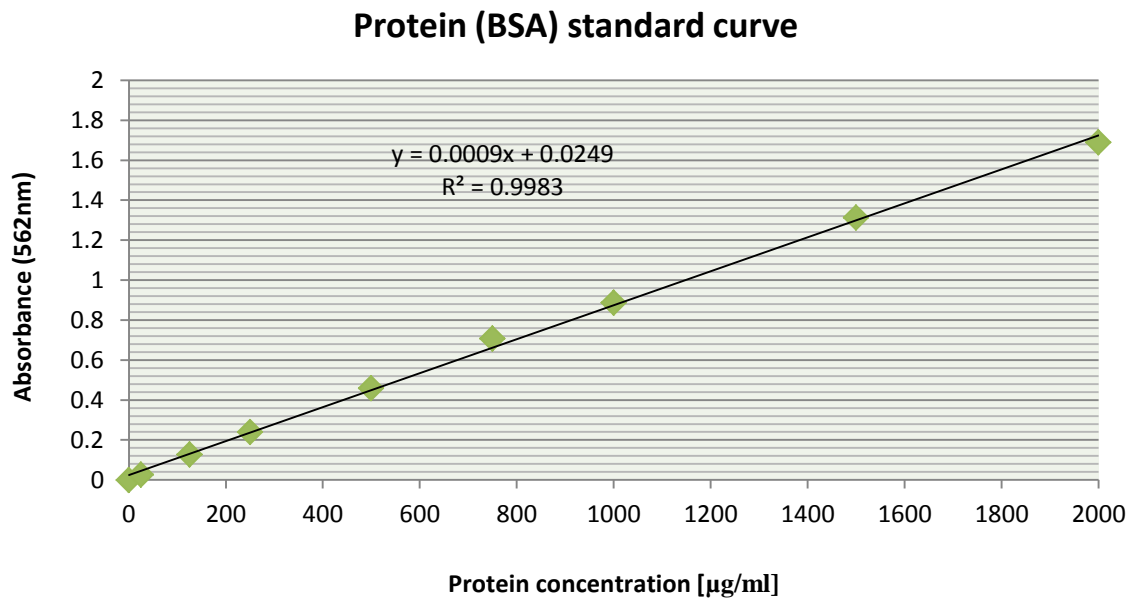
**Figure 11 An example of protein standard curve used to determine protein concentration with BCA assay.**

*Assay procedure*

25µL of each standard or unknown sample was pipetted in triplicates into a microplate well. Before loading, samples were diluted 1:16 with PBS. Next, 200µL of the working reagent prepared by mixing reagent A, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide, with reagent B, containing 4% cupric sulphate, in 50:1 ration was added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds. Subsequently, the plate was covered and incubated at 37°C for 30 minutes. To determine the protein concentration the absorbance was measured using a plate reader.

**2.17 Western bloting**

Protein was extracted from cells by sonication and protein concentration was assessed with BCA protein assay (Thermo Scientific) as described above. Aliquots containing equal amounts of protein were mixed at 1:6 ratio with 6 x sample loading buffer containing mercaptoethanol and the samples were boiled at 100°C for 5mins before loading on polyacrylamide gel containing SDS. Proteins were resolved using gradient 4-12% gels (Invitrogen) or 10% home-made acrylamide gels, prepared according to a recipe described in table 9.

| Separating gel mix | |
|---|---|
| **Component** | Volume |
| **30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide** | 3.75 ml |
| **1 M Tris·HCl, pH 8.9** | 2.813 ml |
| **H$_2$O** | 4.654 ml |
| **Add after degasing:** | |
| **10% (w/v) SDS** | 75 µl |
| **10% (w/v) ammonium persulfate** | 33.75 µl |
| **TEMED** | 7.5 µl |
| **Total** | 11.33 ml |
| **Stacking gel mix** | |
| **Component** | Volume |
| **30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide** | 0.50 ml |
| **1 M Tris·HCl, pH 6.8** | 0.938 ml |
| **H$_2$O** | 2.323 ml |
| **Add after degasing:** | |
| **10% (w/v) SDS** | 9.375 µl |
| **10% (w/v) ammonium persulfate** | 16.88 µl |
| **TEMED** | 5.625 µl |
| **Total** | 3.795 ml |

**Table 9 List of components used to prepare polyacylamide gels.**

Following SDS-PAGE electrophoresis in 1xTris-glycine buffer at 100V for 1.5hrs, protein was transferred overnight at 4°C in 1xTris-glycin buffer containing 20% methanol onto PVDF membrane using X-cell Blot Module (Invitrogen). The membranes were blocked with 5% BSA in 0.05% TBS-T for 1hr at RT, washed 3 times with 0.05% TBS-T and immunobloted overnight at 4°C with an appropriate primary antibody diluted in 5% BSA in 0.05% TBS-T. Following the incubation, the membranes were washed 3 times with 0.05% TBS-T and probed for 1hr with a corresponding secondary HRP-conjugated antibody. Next, the membranes were washed 3 times with 0.05% TBS-T buffer, after which peroxidase activity was detected using Pierce Chemiluminescent Substrate (Thermo-Scientific) and the protein was visualized with Gel-Doc system or using X-ray film. The content of buffers and reagents used for western blotting has been listed in table 10.

| GEL | NuPAGE Bis-Tris gradient (4-12%) gel (Invitrogen) | 10% SDS-PADE gel |
|---|---|---|
| **Sample loading buffer** | 4xLDL sample buffer + β-mercaptoethanol (2:1) | 6xSDS-PADE sample buffer<br>350 mM Tris·HCl, pH 6.8<br>30% (v/v) glycerol<br>10% (w/v) SDS<br>0.5 g/l bromophenol blue<br>2% (v/v) β-mercaptoethanol |
| **Running Buffet** | MES (0.1M 2-(N-morpholino) ethanesulfonic acid) | SDS-PAGE running buffer (0.192 M glycine, 0.0248 M Tris, 1 g/l SDS) |
| **Transfer Buffet** | Tris-glycine buffer with 20% methanol<br>(0.192 M glycine<br>0.0248 M Tris<br>20% v/v Met-OH) | Tris-glycine buffer with 20% methanol<br>(0.192 M glycine<br>0.0248 M Tris<br>20% v/v Met-OH) |
| **Wash buffer** | 0.05% TBS-T<br>50 mM Tris.HCl<br>150 mM NaCl<br>0.1% Tween 20<br>adjust pH to pH 7.4 with HCl | 0.05% TBS-T<br>50 mM Tris.HCl<br>150 mM NaCl<br>0.1% Tween 20<br>adjust pH to pH 7.4 with HCl |

**Table 10 List of buffers and reagents used for western blotting**

## 2.18 Statistical analysis

Statistical analysis was performed using one-way Anova followed by un-paired Student –t test (for normally distributed samples) or Kruskal-Wallis test followed by Mann U Whitney test (for not normally distributed samples). Correlation was analysed using Spearman's rank correlation.

# CHAPTER 3 ESTABLISHING AN IN-VITRO MODEL OF HUMAN BRONCHIAL EPITHELIAL CELL INJURY

## 3.1 Background

Innate immune signaling promotes inflammatory responses essential for tissue homeostasis, particularly in organs such as the lung, that are regularly challenged by environmental insults. Although bacterial and viral infections have been recognized as one of the main risk factors in chronic degenerative diseases, numerous studies demonstrated that chronic inflammation may also be induced by non-pathogen related-repetitive tissue injury which may persist even without ongoing infection. This phenomenon, termed 'sterile inflammation', is mediated by DAMPs (alarmins), that may be released by injured respiratory epithelium, posing the first line of defense against invading, exogenous agents. Chronic exposure to pathogen and damage associated danger signals often results in chronic inflammation and tissue fibrosis. Classically, myeloid cells, comprising monocytes, macrophages and neutrophils, are the main cellular triggers of innate immune signaling, via engagement of receptors recognizing DAMPs and PAMPs, However there is growing evidence of immune functions for tissue resident fibroblasts, whose numbers significantly increase in fibrotic organs [35].

Epithelial damage associated with alarmin release is a hallmark of many chronic degenerative diseases including chronic lung allograft rejection, liver cirrhosis or arthritis. In chronic lung allograft rejection, bronchial epithelial cell injury, necrosis, and ulcerations of mucosa are observed. This epithelial damage is accompanied by infiltration of leukocytes, neutrophils and macrophages and subsequent expansion of myofibroblasts causing tissue scarring and airway obliteration [13].

Although numerous alarmins have been identified so far, their potential involvement in the pathogenesis of chronic degenerative lung diseases remains unclear. The studies investigating the immunogenic potential of alarmins, that have been conducted to date, focused on the influence of these molecules on the functions of immunocytes. No studies, however, have been performed on the interactions between alarmins released by damaged human bronchial epithelial cells and fibroblasts.

The initial aim of this study was to establish an in vitro model of human bronchial epithelial cell damage and to identify alarmins released by these cells following injury that may be implicated in chronic fibrotic lung diseases by activating human lung fibroblasts. This objective has been met by establishing the influence of different injurious stimuli on human bronchial cell viability and alarmin release and investigating the changes in inflammatory and fibrotic phenotype of fibroblasts exposed to conditioned media from the injured epithelium.

Since alarmins were first described in 1994 [202], in order to investigate the role of danger signals in different in vitro and in vivo models of chronic degenerative diseases, several models of cell injury have been established.

Although, the pathophysiology of chronic degenerative diseases such as chronic allograft rejection has been studied for decades, the consensus as to the underlying cause of the progressive epithelial damage has still not been reached.

The main injurious stimuli used to induce cell death and alarmin release in vitro, include freeze-thaw stress, oxidative stress, osmotic stress and cell lysis by sonication.

Increased number of neutrophils, which is often observed in lung following transplantation, has drawn some researchers to the conclusion that another factor that may contribute to epithelial damage may be proteolytic enzymes such as elastase, that may be produced by activated neutrophils and whose main function is the digestion of protein components of invading pathogens [203].

Additionally, some research indicate that cellular damage in chronic inflammatory diseases, which are associated with very intensive production of proinflammatory factors, may be caused by stress in the endoplasmic reticulum (ER stress), which can be caused by accumulation of unfolded proteins. Increased production of proteins may occur in fibrotic and chronic inflammatory diseases which are characterized by intensive expression of proteins building the extracellular matrix, and cytokines and growth factors, which may be rapidly produced in large quantities [204].

A commonly used and well accepted animal model of pulmonary fibrosis and inflammation, uses bleomycin to induce epithelial damage, which initiates fibrotic and inflammatory changes in the lung.

Bleomycins are a family of compounds possessing antibiotic activity that are produced by the bacterium Streptomyces verticillis. Bleomycin A2 and B2 found application in anti-cancer therapy and are used in the treatment in diseases such as Hodgkin's lymphoma, squamous cell carcinomas and testicular cancer. One of the side effects of bleomycin therapy is pulmonary toxicity which may be manifested by progressive lung fibrosis. This property of bleomycin has been used in an animal model of pulmonary fibrosis. In vitro studies revealed that bleomycin may act as an intercalating agent and cause DNA damage leading to cell death. The bleomycin molecule consists of a bithiazole component which can intercalate into the DNA strand and pyrimidine and imidazole components which bind iron and oxygen and may form oxidants causing DNA damage. Moreover bleomycin may contribute to cell damage by inducing lipid peroxidation. Hence, the main mechanism of bleomycin induced cell damage is oxidative stress which causes deregulation of the intracellular homeostasis leading to necrotic cell death. This property of bleomycin is used to reduce tumor size in anti-cancer therapy [205] [206].

In patients and animals treated with bleomycin, damage to alveolar epithelium is observed. This epithelial damage initiates influx of neutrophil and may lead to chronic inflammation and fibrosis. Similar changes are observed in lungs of patients following transplantation. The studies performed to date suggest that the degeneration of the respiratory epithelium post transplantation may be mediated by several factors including allogenic injury and oxidative stress that may be induced for example by activated neutrophils which are a substantial source of reactive oxygen species and free radicals [207] [208].

The mechanisms of cell death induced by the aforementioned methods differ significantly and therefore the choice of the cell injury technique should be carefully considered when designing a study.

In lung transplant recipients, epithelial damage in the respiratory tract may be accentuated by environmental factors such as airborne particles, smog or cigarette

smoke. A recent study conducted on post-transplant patients, revealed that the level of air pollution may have a substantial influence on the allograft fate. The study demonstrated that post-transplant patients living in areas characterized by higher levels of air pollution were more likely to develop bronchiolities obliterans syndrome in comparison to allograft recipients from less polluted neighborhoods [209].

Considering all the points listed above, in the present study, in order to establish an in vitro model of human bronchial epithelial cells damage, oxidative stress, a proteolitic enzyme, elastase, cigarette smoke, ER stress and freeze-thaw stress, have been tested as potential injurious stimuli.

A flow chart showing the study experimental design is shown in figure 12.
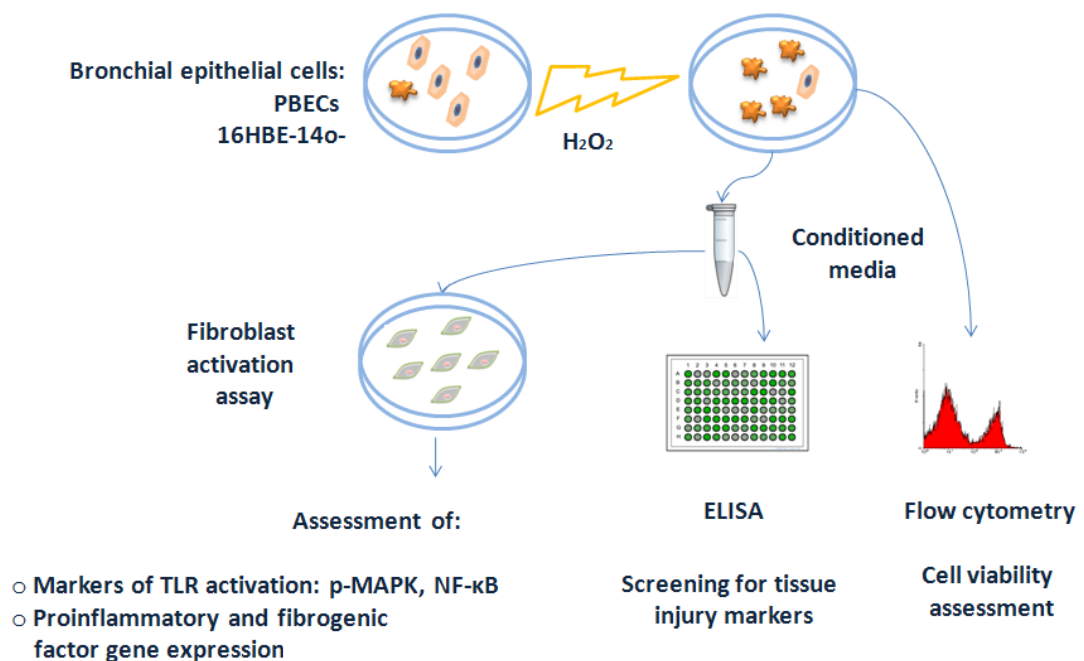


**Figure 12 A flow chart showing the study experimental design.**

Primary human bronchial epithelial cells (PBECs) were used in the project to establish an in vitro model of epithelial injury. Primary cells are considered the best in vitro model to predict in vivo responses, however, their lifespan is relatively short (4-5 passages) and their availability is limited. Therefore it was necessary to identify

a cell line whose response to injury is comparable to PBECs. Cell lines tested as potential candidates for that role included A549, BEAS-2B and 16HBE14o-.

To establish a lung epithelial cell injury model relevant to chronic degenerative lung diseases, PBECs cells and the aforementioned cell lines were exposed to the insults discussed above.

The influence of the treatments listed above on PBEC cell viability was assessed by flow cytometry or trypan blue staining. To establish if epithelial damage was associated with a release of danger signals, in the initial experiments, levels of marker alarmins: IL-1α, HMGB-1 and HSP-60 were measured using an ELISA. The choice of the marker alarmins was based on previous studies showing that these factors may be released by damaged cells and that they are upregulated during infection, which is one of the major causes of exacerbations in patients affected by fibrotic diseases [27]. Additionally, in subsequent experiments, IL-1α was used as a marker of injury to identify a cell line whose behavior in response to an injurious stimulus resembles PBECs. Once the appropriate cell line has been indentified, and a method of cell injury established, the model of an in vitro epithelial cell damage was verified by a measurement of other marker alarmins including HMGB-1 and HSP-60. IL-1α and HSP-60 protein concentrations were measured using commercially available sandwich ELISAs. In order to estimate HMGB-1 concentations in cell culture media, a direct home-made ELISA was developed.

**Hypotheses tested:**

1. Primary human bronchial epithelial cells (PBECs) and human lung epithelial cell lines (A549, 16HBE14o- and BES2B) damaged with an injurious stimulus such as oxidative stress, stress in the endoplasmic reticulum (ER stress), elastase or cigarette smoke release marker alarmins including IL-1α, HMGB-1 and HSP60.

2. Conditioned media from damaged human bronchial epithelial cells trigger gene expression of proinflammatory (eg IL-6) and fibrogenic (eg collagen type1) factors in human lung fibroblasts.

## 3.2 Development of direct ELISA for human HMGB-1 detection

To establish optimal conditions for HMGB-1 detection by direct ELISA, titrations of the primary antibody (monoclonal mouse anti-human HMGB-1, RnD systems), secondary antibody (polyclonal biotinylated donkey anti-mouse IgG, RnD systems) and HRP-streptavidin (Thermo Scientific – Pierce) were performed. 96 well ELISA plate was coated with hrHMGB1 resuspended in PBS at a range of concentrations from 300 to 0.41ng/ml. Following 24hrs incubation time, the wells were washed three times with 1XTBS-T and blocked with 5% BSA in PBS for 2hrs. Next, the primary antibody in 3% BSA-PBS at three different concentrations (0.5, 2, 5µg/ml) was added to the plate. After 2hrs incubation period the wells were washed 3 times with 1XTBS-T, after which the secondary antibody in 3% BSA-PBS was added at 2 different concentrations (1:1000, and 1:2000). The plate was incubated at RT for another 2hrs after which it was washed 3 times with 1XTBS-T and 100µl HRP-strepatavidin at 3 different concentrations (1:500, 1:1000 and 1:2000) was added to the adequate wells. After 20 mins, the plate was washed 3 times with 1XTBS-T and 100ul substrate solution (RnD systems) was added to each plate. The plate was incubated for another 20 mins in the dark, after which, to stop the reaction, 50µl of 2M $H_2SO_4$ was added to each well and the ODs were read at 450nm with ELISA plate reader. The combination of the reagent concentrations which gave the lower background and the strongest signal (Primary Ab - 2ug/ml; Secondary Ab – 1:2000; HRP-Streptavidin – 1:2000) were used in all the subsequent experiments. The ELISA detection limit was established according to the following equation: 2.5 x mean of background OD + mean of standard deviation. The analysis revealed that the assay detection limit was 10 ng/ml.

The tested concentrations of primary and secondary antibodies used to optimize HMGB-1 ELISA are listed in table 11. An example of an HMGB-1 ELISA standard curve is presented in figure 13.

| Reagent | Concentrations tested | Incubation time |
|---|---|---|
| Primary antibody - monoclonal mouse anti-human HMGB1, RnD systems | 0.5, 2, 5µg/ml | 2hrs |
| Secondary antibody - polyclonal biotinylated donkey anti-mouse IgG, RnD systems | 1:1000 1:2000 | 2hrs |
| HRP- streptavidin (Pierce) | 1:500 1:1000 1:2000 | 30mins |

**Table 11 Concentration of reagents used in the HMGB1 ELISA.**

## ELISA: Standard curve for HMGB1



$y = 0.0086x$
$R^2 = 0.9942$
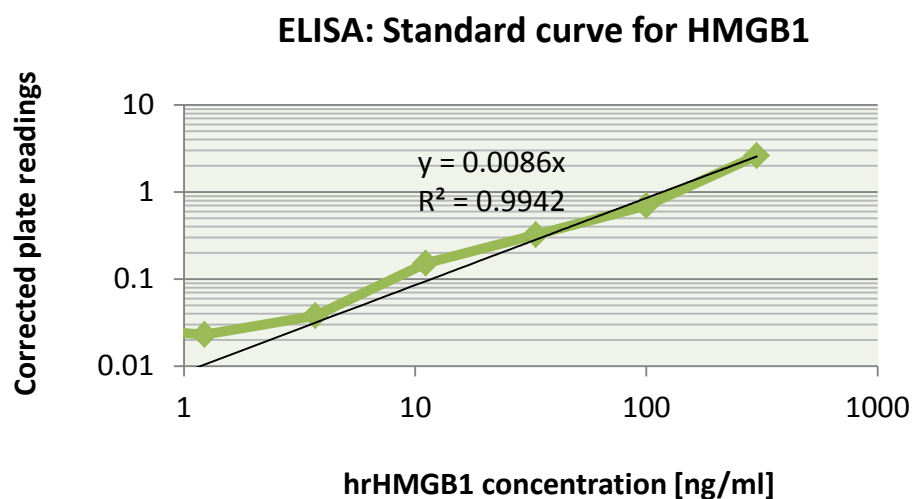
**Figure 13 Standard curve for HMGB1 detected using direct ELISA; 1°Ab concentration: 2µg/ml, 2°Ab conc. 1:2000, HRP-streptavidin conc. 1:2000**

**3.3 Influence of selected injurious stimuli on PBEC cell viability and alarmin release.**

To identify the most efficient and possibly physiologically relevant method to induce injury of human bronchial epithelial cells, PBEC cells obtained from bronchial brushings were used. The cells were cultured in SAGM media until the monolayers reached 80-90% confluency. Next, the cells were subjected to a treatment with

selected injurious stimuli and the cell viability was assessed by trypan blue staining or flow cytometry using PI. Moreover, to establish the influence of the tested injury methods on danger signal release, levels of marker alarmins were measured in conditioned media collected from the damaged PBEC cells.

## 3.4 Influence of oxidative stress on PBEC cell viability and marker alarmin (IL-1α, IL-1β, HMGB-1, HSP60) release.

To establish the optimal dose of hydrogen peroxide, sufficient to induce PBEC cell damage, the cells were treated with a range of $H_2O_2$ concentrations. The medium was replenished after 2hrs and the cell viability assessment and conditioned media collection were performed after another 24hrs. The results of the experiment revealed marked increase in IL-1α, HSP60 and HMGB-1 release by damaged PBEC cells in comparison to the untreated controls. Interestingly, increase in HMGB-1 release was associated with PBEC cell death, while elevated levels of IL-1α were detectable also in conditioned media from PBEC cells exposed to $H_2O_2$ doses, which did not have significant effect on the cell viability, suggesting that HMGB-1 may be considered a marker of cellular damage, while IL-1α, may function as an early danger signal, released by stressed cells prior to cell death. Since, according to some reposts, oxidative stress may trigger inflammasome activation, the levels of IL-1β, whose processing is caspase-1 and inflammasome dependant, were also measured in conditioned media from damaged cells. The ELISA analysis revealed that IL-1β was detectable in conditioned media following PBEC stimulation with $H_2O_2$, however its concentrations were approximately 5 times lower in comparison to IL-1α (Figure 14). Therefore, to establish a model of epithelial injury, which will allow to identify early danger signals that may be implicated in fibroblast activation, IL-1α was used as a marker alarmin in all the subsequent experiments performed to identify a suitable cell line and type of injury.
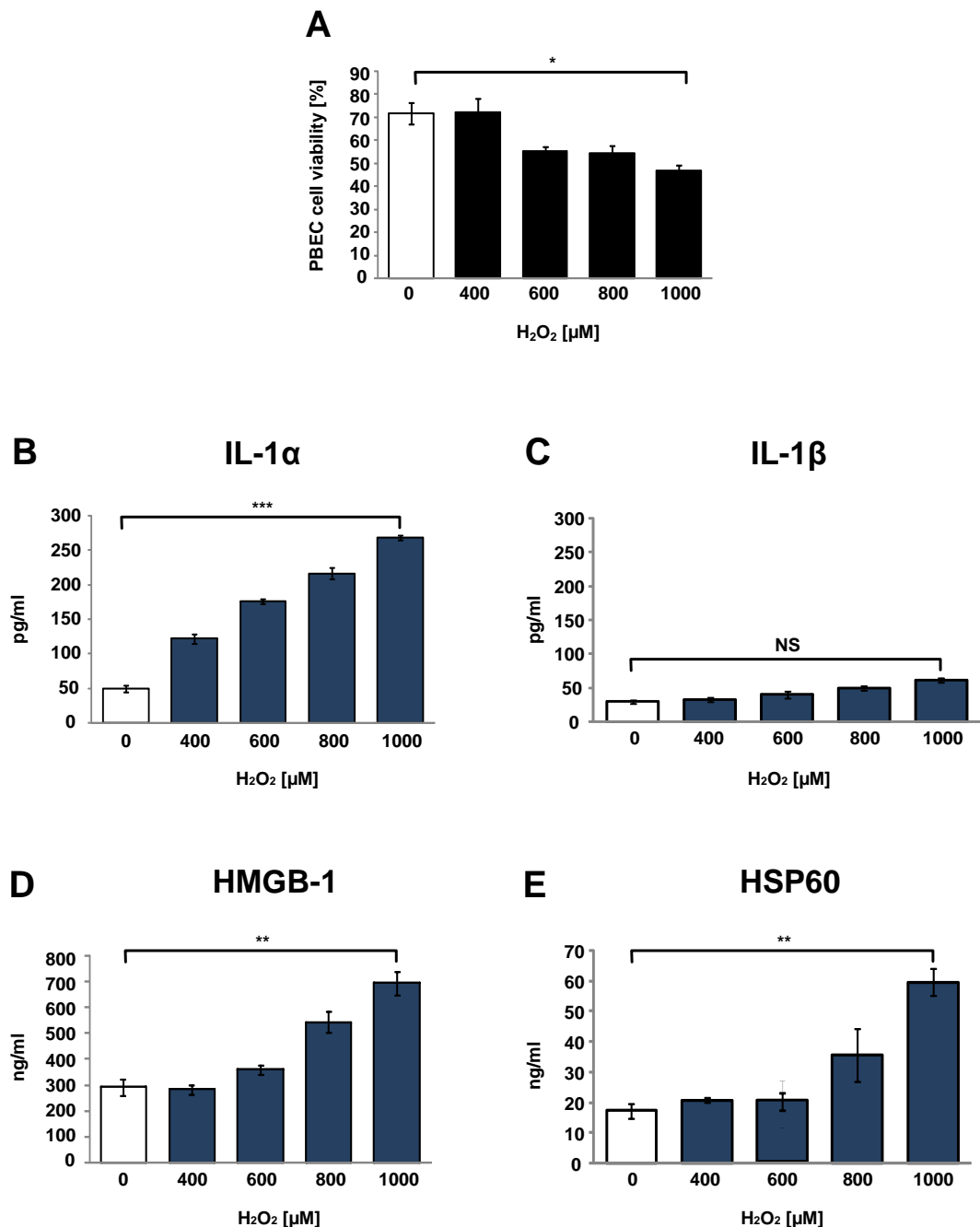
**Figure 14 Oxidative stress reduces primary human bronchial epithelial cell (PBEC) viability and induces alarmin release.** PBEC were treated with the indicated concentrations of $H_2O_2$ for 2hrs, after which the culture medium was replenished and the cells were subjected to a further incubation for 24hrs. Following the incubation, the cell viability was assessed by flow cytometry, using PI staining and the levels of IL-1α, IL-1β, HMGB-1 and HSP60 were analysed by ELISA. The ELISA analysis revealed that oxidative stress reduced PBEC cell viability (A) and induced IL-1α (B), HMGB-1 (D) and HSP60 (E) release. There was not significant difference in IL-1β (C) levels in conditioned media following $H_2O_2$ treatment and IL-1β levels were approximately 5 times lower in comparison to IL-1α. Data represent the mean±S.E.M from three independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**3.5 Influence of thapsigargin-induced ER stress on PBEC cell viability and IL-1α and HMGB-1 release.**

To induce ER stress, PBEC cells were exposed to 1, 10 or 50µM doses of thapsigargin (TG) for 2hrs, after which the culture media was replenished and the cells were re-incubated for an additional 24hrs and the cell viability and IL-1α and release were assessed. Additionally, in a control experiment, the levels of HMGB-1 in conditioned media from TG-treated PBEC cells were also measured. Induction of unfolded protein response was measured 2hrs post TG treatment using XBP-1 splicing assay.

A relatively low dose of TG (1µM) resulted in slight increase of PBEC cell viability and no IL-1α release. 10µM TG induced IL-1α release, but did not have a significant effect on PBEC cell viability, while 50µM TG significantly reduced the number of viable cells and resulted in IL-1α and HMGB-1 release (figure 15).
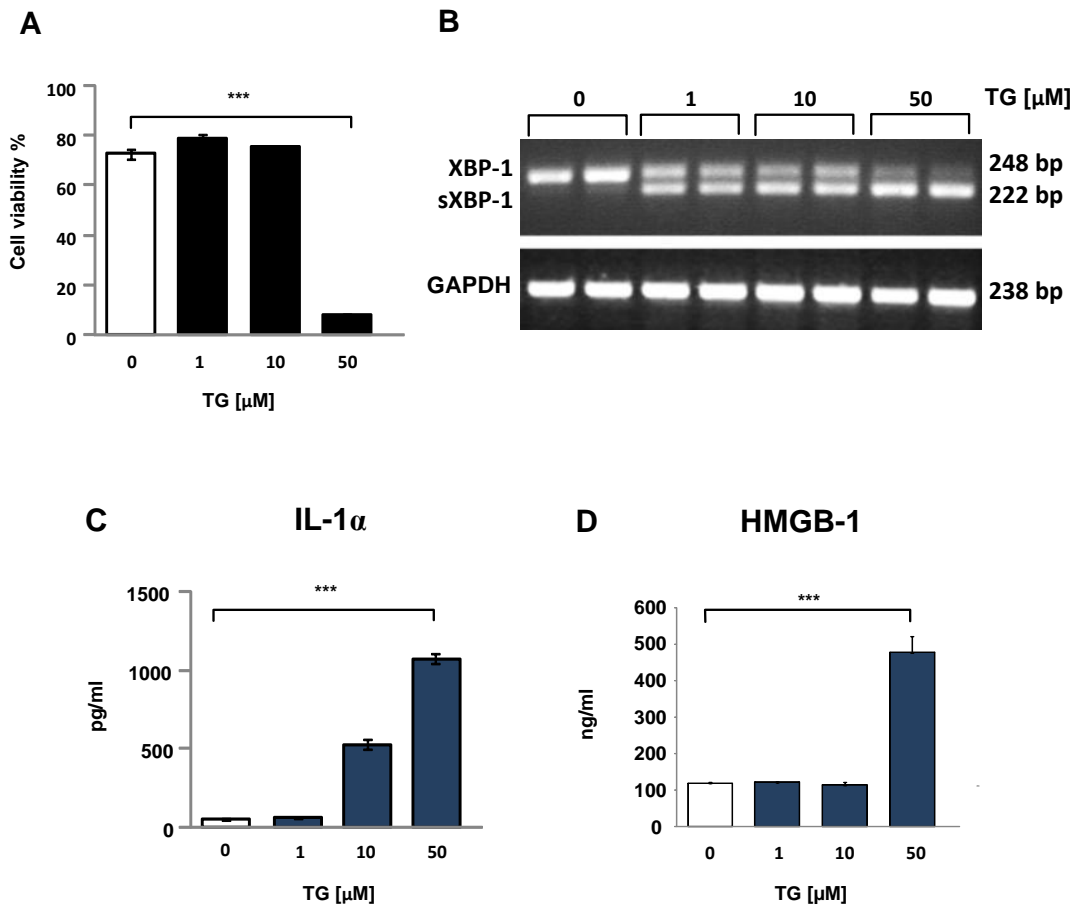
**Figure 15 Thapsigargin-induced stress in the endoplasmic reticulum (ER stress) reduces PBEC cell viability and induces IL-1α and HMGB-1 release.** Primary human bronchial epithelial cells (PBEC) were pulsed with the indicated concentrations of thapsigargin (TG) for 2hrs. Following the treatment, the culture medium was replenished and the cells were re-incubated for a further 24hrs. Next, the cell viability was assessed by trypan blue staining and IL-1α and HMGB-1 concentrations were measured with ELISA. Induction of ER stress was confirmed using XBP-1 splicing assay (B).Treatment with 50μM TG significantly reduced PBEC cell viability (A) and TG doses of 50μM induced IL-1α (C) and HMGB-1 (D) release. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, **p<0.01, ***p<0.001.

## 3.6 Influence of human neutrophil elastase treatment on PBEC cell viability and IL-1α release

80-90% confluent PBEC cell monolayers were cultured for 2hrs in culture media containing the indicated concentrations of human neutrophil elastase (Sigma), after which the culture media was replenished and the cells were re-incubated for an additional 24hrs and the cell viability and IL-1α release were assessed. The analysis revealed that the elastase treatment did not have a significant influence on PBEC cell viability or IL-1α release (figure 16).
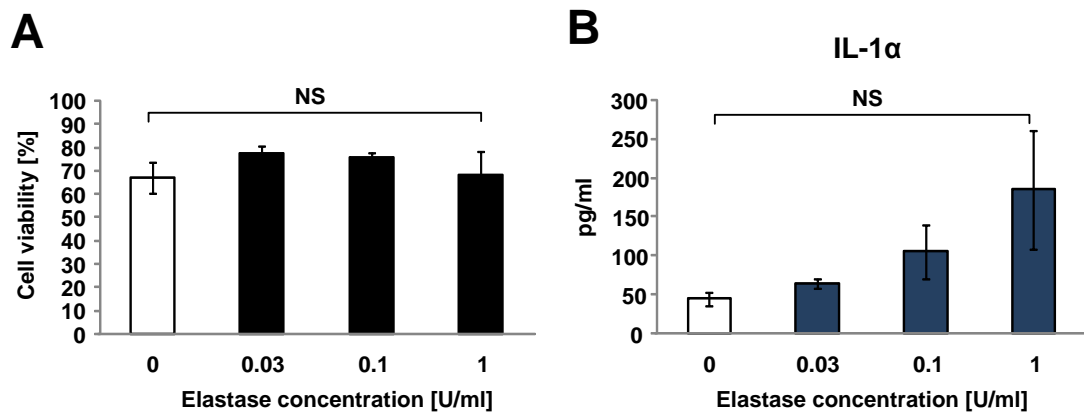


**Figure 16 Influence of elastase treatment on PBEC cell viability and IL-1α release**

PBEC cells were treated with the indicated concentrations of human neutrophil elastase for 2hrs. Following the treatment the medium was replenished and the cells were re-incubated for further 24hrs. Next, the cell viability was assessed using trypan blue staining and IL-1α protein concentration in conditioned media was measured using ELISA. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. No significant differences were detected between samples.

## 3.7 Influence of cigarette smoke extract on PBEC cell viability and IL-1α release

Cigarette smoke extract (CSE) was freshly prepared prior to the experiment by bubbling the mainstream of cigarette smoke directly into 20mls of the culture medium by constant vacuum. The CSE solution was filtrated through 0.2um filter and diluted down to the final concentrations of 25%, 12.5% and 6.25%. The PBEC cells were cultured in media containing the aforementioned concentrations of CSE for 2hrs, after which the culture media was replenished and the cells were re-incubated for an additional 24hrs. Following the incubation, the cells were harvested and subjected to cell viability assessment by FACS and the conditioned media were collected and IL-1α concentrations were measured by ELISA.

The analysis revealed that the CSE treatment did not have a significant influence on PBEC cell viability or IL-1α release (figure 17).
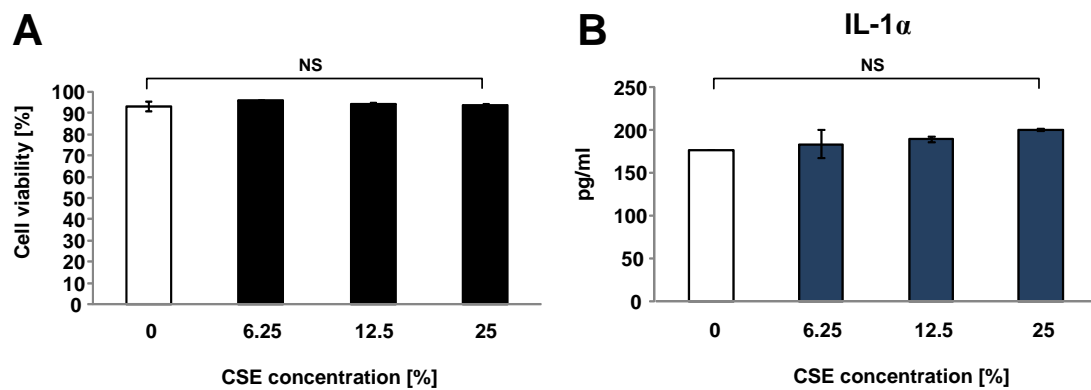


**Figure 17 Influence of cigarette smoke extract on PBEC cell viability and IL-1α release**

PBEC cells were treated with the indicated concentrations of cigarette smoke extract (CSE) for 2hrs. Following the tretamnet, the medium was replenished and the cells were re-incubated for further 24hrs. Next, the cell viability (A) was assessed using trypan blue staining and IL-1α protein concentration (B) in conditioned media was measured using ELISA. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. No significant differences were detected between samples.

## 3.8 Influence of freeze-thaw stress on PBEC cell viability and IL-1α and HMGB-1 release

To establish if alarmins may be released from PBEC cells damaged without an intervention of chemical substances, in a control experiment, PBECs cells were subjected to 3 freeze-thaw (F/T) cycles at -80°C/37°C. The cells were left to recover for 24hrs, after which the cell viability was assessed by flow cytometry and the conditioned media collected for ELISA. The FACS analysis did not reveal the presence of viable cells in a culture following treatment. As assessed by ELISA, freeze-thaw stress-induced cell damage was associated with increased release of IL-1α and HMGB-1 (figure 18).
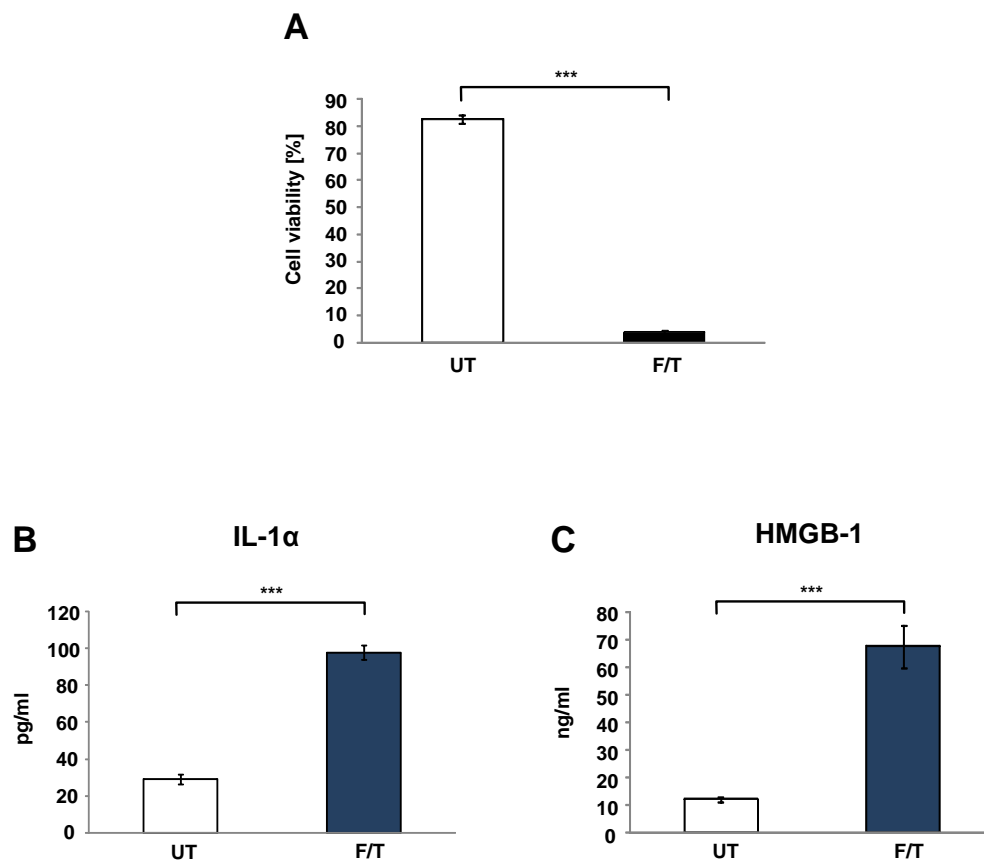
**Figure 18 Influence of freeze-thaw stress on PBEC cell viability and alarmin release**

PBEC cells were subjected to three cycles of freeze/thaw (F/T) at -80°C. Following the tretamnet the cells were re-incubated at 37°C for additional 24hrs, after which, the cell viability (A) was assessed using trypan blue staining and IL-1α (B) and HMGB-1 (C) protein concentration in conditioned media were measured using ELISA. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. * p<0.05, ** p<0.01, *** p<0.001.

**3.9 Identification of a human lung epithelial cell line that mimics behavior of PBEC cells in response to an injurious stimulus.**

Primary human bronchial epithelial cells are the most reliable in vitro model to investigate the behavior of respiratory epithelium in the context of chronic inflammatory lung diseases. However due to limited availability and relatively short lifespan (4-5 passage numbers) of PBEC cells, it was necessary to identify a human lung epithelial cell line that would mimic the behavior of PBEC cells in response to injurious stimuli. The cell lines appointed as the potential candidate to that role included: adenocarcinomic human alveolar basal epithelial cells (A549), normal human bronchial epithelial cells transformed with Adenovirus 12-SV40 virus (BEAS-2B) and the SV40-transformed human bronchial lung cells (16HBE14o-). To assess their suitability to serve as an alternative in vitro model of epithelial injury, the aforementioned cell lines were subjected to treatment with $H_2O_2$-induced oxidative stress, after which the cell viability and IL-1α release were assessed by trypan blue and ELISA respectively.

### 3.10 Influence of $H_2O_2$ on A549 cell viability and IL-1α release

In order to induce oxidative stress, A549 cells were treated with a range of $H_2O_2$ doses (up to 800μM) for two hours. Following the treatment, the culture medium was replenished and the cells were subjected to a further incubation at 37°C for 24hrs. Next, the conditioned media were collected and tested for IL-1α by ELISA and the cell viability was measured by Trypan blue staining. The analysis revealed that the $H_2O_2$ treatment reduced the A549 cell viability in a dose dependant manner, however it did not induce IL-1α release. Based on this observation, A549 cells were found unsuitable to be used as a cell line to establish an in vitro model of human lung epithelial injury (figure 19).
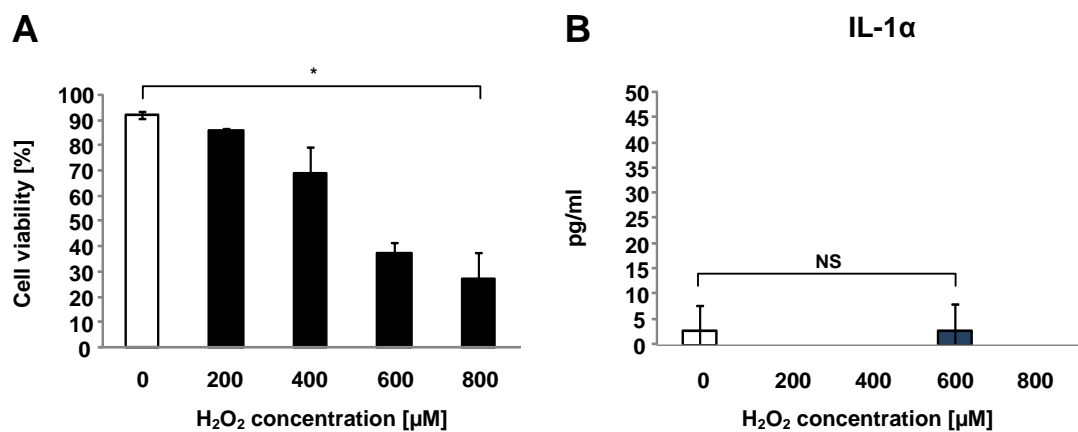


**Figure 19 Influence of $H_2O_2$ on A549 cell viability and IL-1α protein release**

A549 cells were treated with the indicated concentrations of $H_2O_2$ for 2hrs, after which culture medium was replenished and cells were re-inculated for additional 24hrs. Following the incubation the cell viability was assessed by FACS and IL-1α concentrations were measured by ELISA. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed unpaired Student t test. * <0.05, **<0.01, ***<0.001.

## 3.11 Influence of $H_2O_2$ on BEAS-2B cell viability and IL-1α release

BEAS-2B cells were treated with $H_2O_2$ doses ranging from 0 to 800μM for 24hrs, after which the cell viability was assessed by trypan blue staining and the conditioned media collected for ELISA. Oxidative stress reduced cell viability however it did not have significant impact on IL-1α release (Figure 20). These results show that the BEAS-2B cell line's response to oxidative stress does not resemble primary lung epithelial cells and therefore it would not serve as a reliable model in our study.
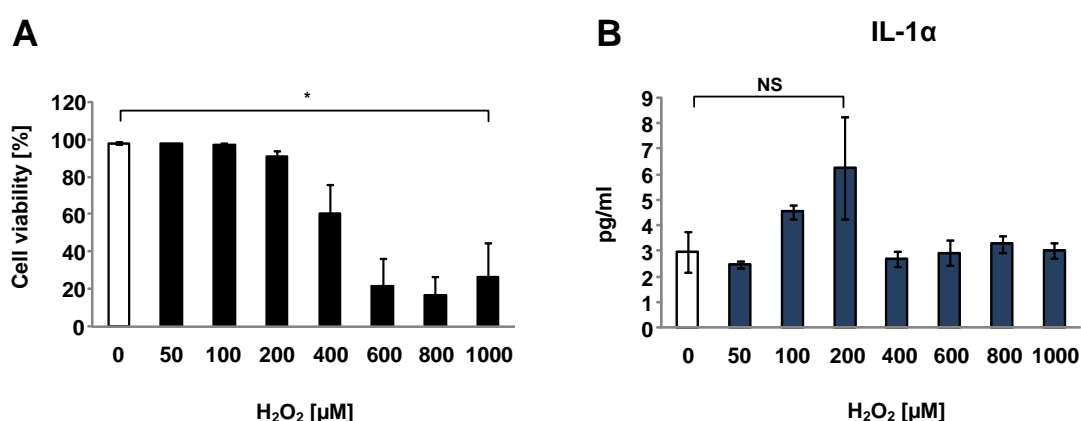


**Figure 20 Influence of $H_2O_2$ on BEAS-2B cell viability and IL-1α protein release**

BEAS-2B cells were treated with the indicated concentrations of $H_2O_2$ for 2hrs, after which culture medium was replenished and cells were re-inculated for an additional 24hrs. Following the incubation the cell viability was assessed by FACS and IL-1α concentrations were measured by ELISA. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student t test. * <0.05, **<0.01, ***<0.001.

## 3.12 Influence of $H_2O_2$ on 16HBE14o- cell viability and alarmin release.

16HBE14o- cells were treated with decreasing doses of $H_2O_2$ ranging from 400 to 12.5μM. Following 24hrs incubation the cell viability was assessed by trypan blue staining and the conditioned media collected for ELISA. $H_2O_2$-induced oxidative stress reduced the cell viability in a dose dependant manner and led to increased release of IL-1α, HSP60 and HMGB-1 (figure 21), suggesting that 16HBE14o- behavior in response to stress resembles PBEC responses and that it could be considered as a potential candidate to establish an in vitro model of human lung epithelium injury.

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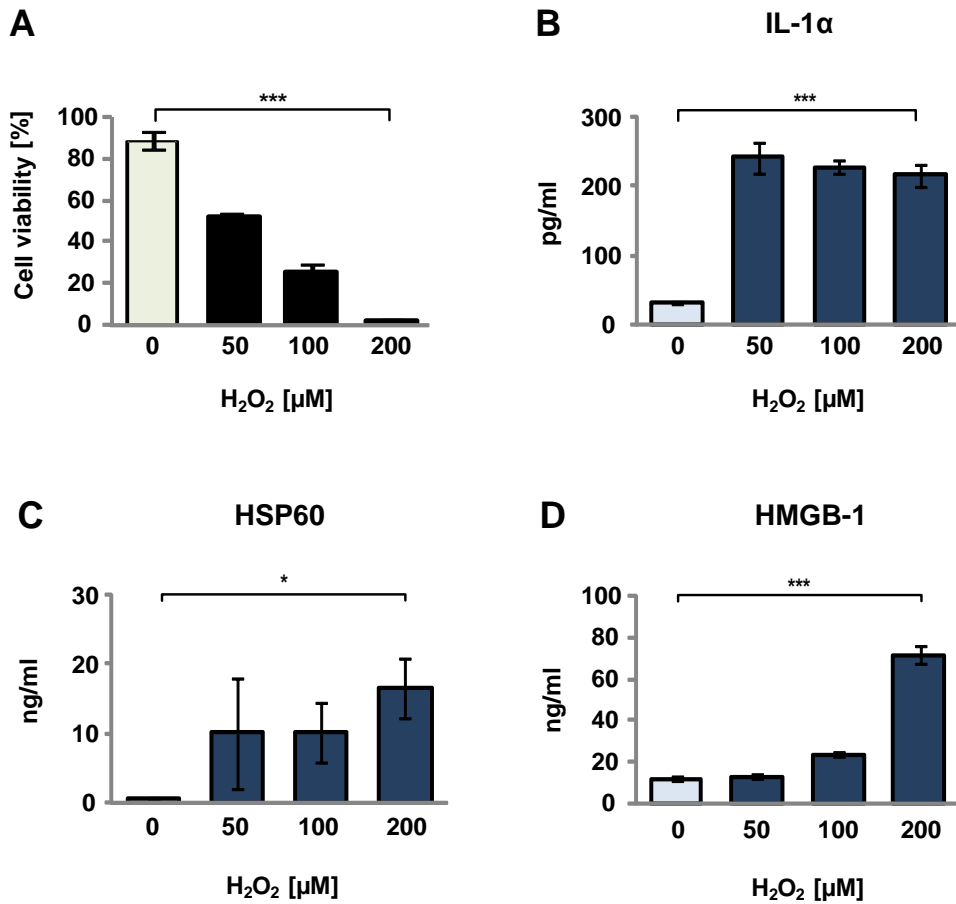
**Figure 21 Influence of $H_2O_2$ -induced oxidative stress 16HBE-14o- cell viability and marker alarmin release.**

Subconfluent (80%) 16HBE-14o- cell monolayers were treated with the indicated doses of $H_2O_2$ for 2hrs, after which the medium was replenished and the cells were re-incubated for additional 24hrs. Following incubation, cell viability was assessed by flow cytometry and IL-1α concentrations were measured with ELISA. $H_2O_2$ – induced oxidative stress reduced culture viability (n=3, $p<0.001$) (A) and upregulated release of alarmins: IL-1α (n=3, $p<0.001$) (B), HSP60 (n=3, $p<0.05$) (C) and HMGB1 (n=3, $p<0.01$) (D) (asterisks correspond to the statistical significance between untreated controls and cells treated with 200μM $H_2O_2$ dose which was used to damage 16HBE-14o- cells in all subsequent experiments). Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

## 3.13 Influence of freeze-thaw stress on 16HBE14o- cell viability and marker alarmin release.

Since secretion of some proinflammatory cytokines has previously been attributed to the activity of oxidative stress, we next decided to establish whether the release of the marker alarmins from 16HBE14o- cells can also be achieved using an injury not involving any chemical substances. For that, in a control experiment, 16HBE14o- cell monolayers in culture media were subjected to 3 freeze (-80ºC/10 min)- thaw (37ºC/20min) cycles. Next, the cells were re-incubated at 37°C for a further 24hrs, after which the cell viability was assessed by flow cytometry and the levels of marker alarmins in the conditioned media were measured by ELISA. The flow cytometric analysis revealed only the presence of cell debris in cultures subjected to freeze-thaw stress. The treatment resulted in increased release of IL-1α and HMGB-1 (Figure 22).

**A**

**B** IL-1α

**C** HMGB-1
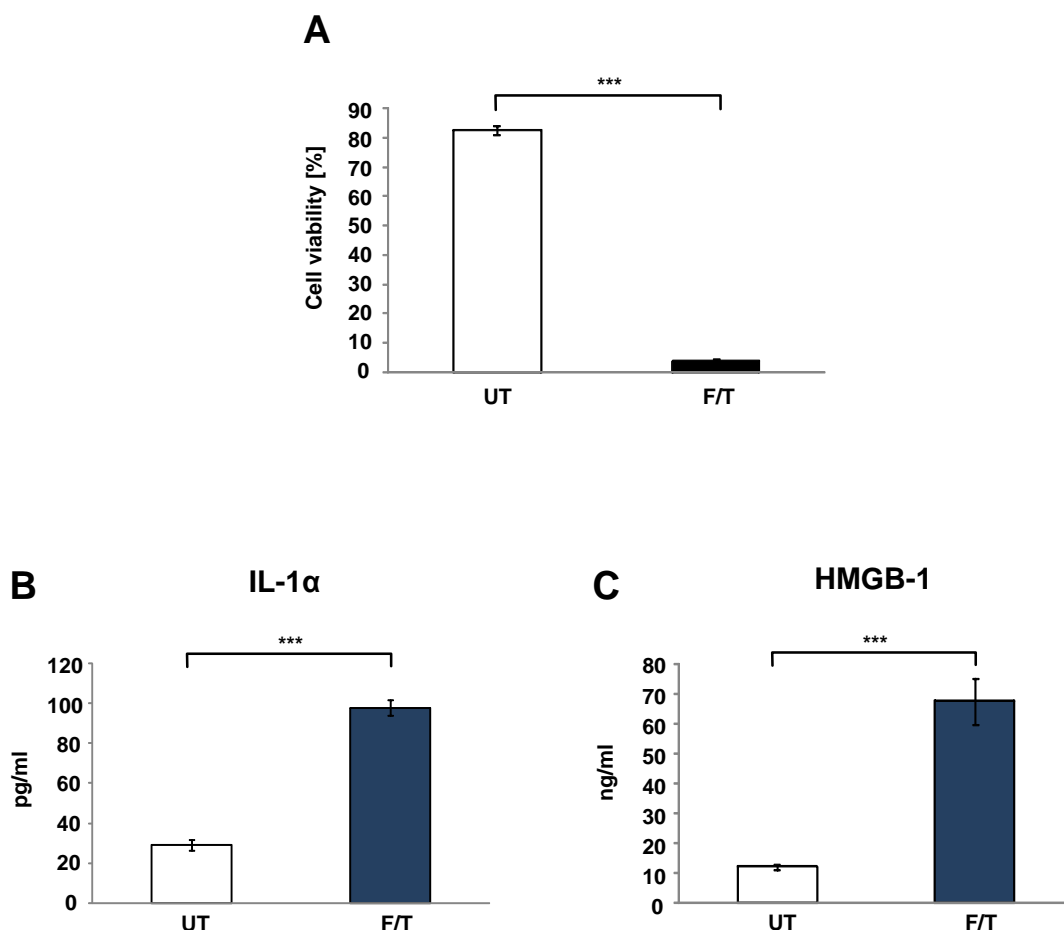
**Figure 22 Influence of freeze-thaw stress on 16HBE14o- cell viability and alarmin release**

16HBE14o- cells were subjected to three cycles of freeze/thaw (F/T) at -80°C. Following the treatment the cells were re-incubated at 37°C for additional 24hrs, after which, the cell viability (A) was assessed using trypan blue staining and IL-1α (B) and HMGB-1 (C) protein concentration in conditioned media were measured using ELISA. The freeze-thaw treatment significantly reduced the cell viability and induced IL-1α and HMGB-1 release (P<0.001 in all cases). Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, ** p<0.01, *** p<0.001.

## 3.14 Influence of conditioned media from damaged epithelial cells on fibroblast activation

### 3.14.1 Optimisation of qRT-PCR reactions

In order to investigate the influence of conditioned media from damaged epithelial cells on fibroblasts activation, a number of primers were designed to measure expression of various proinflammatory and fibrogenic genes by qRT-PCR. For each primer pair PCR reaction efficiency was assessed.

To establish the real-time PCR reaction efficiency, serial dilutions of cDNA were prepared and a series of PCR reactions were run using the same concentration of primers. Next, titration curves were prepared by plotting Ct values on Y axis against log values of cDNA concentration on X axis. The reaction efficiency was calculated according to the following equation: Efficiency=$10^{(-1/-slope)}-1$, where slope is represented by the 'a' value in equation describing the linearised titration curve (y=ax+b). Efficiencies of all the primers tested in the study, were between 80-100% (0.8-1). An example calculation of efficiency of PCR reaction using GAPDH primers is presented in figure 23.
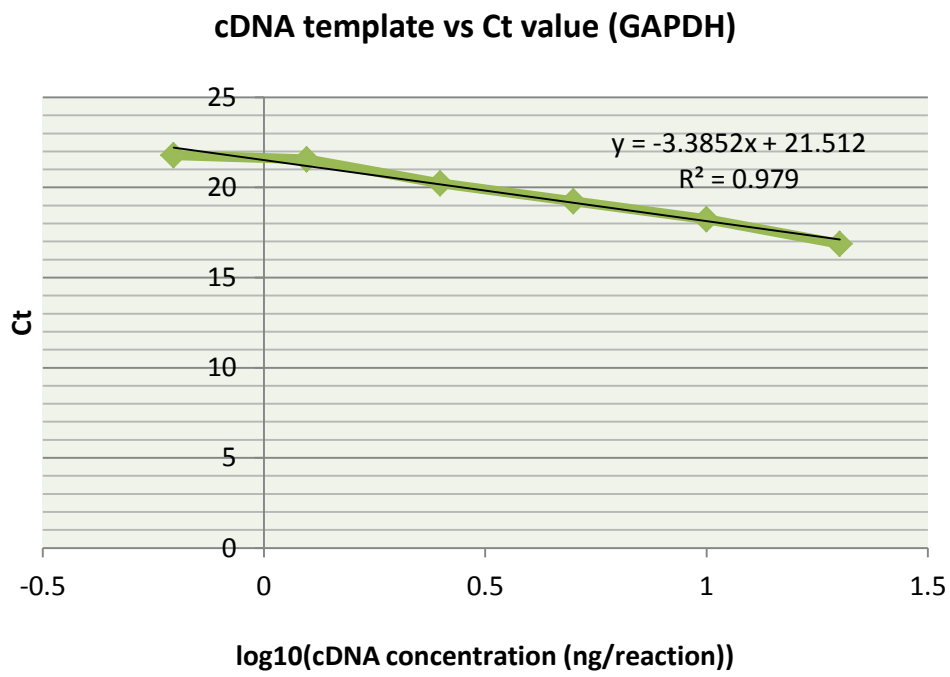
**Figure 23 An example of qRT-PCR standard curve**

Slope = -3,3852

Efficiency=10^(-1/slope)-1

Hence

Efficiency=0.97 or 97%

### 3.14.2 Melt curve analysis

Sybr Green based amplicon detection is based on the ability of the fluorescent dye to bind to double stranded DNA, meaning that apart from the target template it can also bind to primer dimers, PCR products from misannealed primers and contaminating DNA. Therefore to ensure that the desired template was detected, a dissociation (melting) curve was generated following each PCR reaction. Melt curve analysis allow to establish the point of inflection which can be defined as the melting temperature of the amplicon. Considering, that products of different sizes and different nucleotide content can be distinguished based on their melting temperature, melt curve analysis allows to detect any by-products binding Sybr Green. An exemplary melting curve for GAPDH has been presented in figure 24. A single peak shows that during the PCR reaction only one product was amplified. Primer dimmers or DNA contamination would appear as additional picks.

Dissociation curve
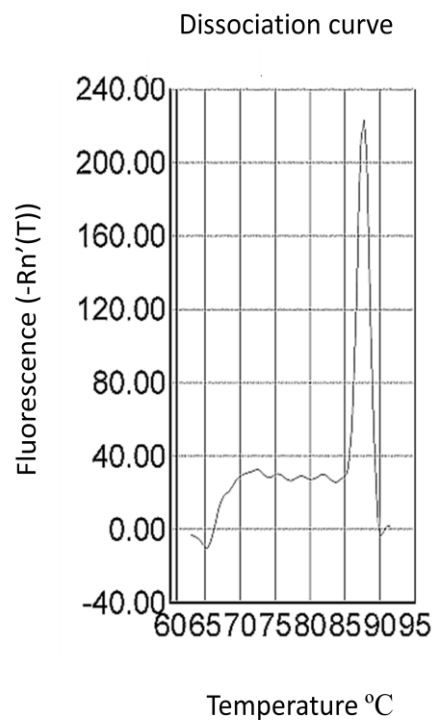


**Figure 24 An example of melting curve generated following qRT-PCR reaction**

Additionally, to assess primer specificity, the PCR products were resolved on a 2% agarose gel. The analysis revealed a presence of a single product on gel for all primer pairs, confirming, that a single PCR product is amplified (Figure 25).

117
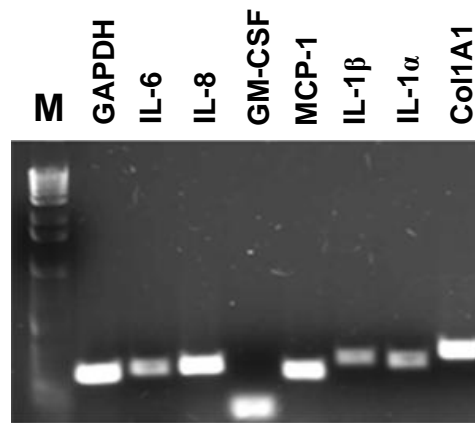
**Figure 25 Verification of primer site specificity by PCR.** 200ng RNA was converted into cDNA and following PCR reaction, 10µl of the amplification product was separated by electrophoresis in 2% agarose gel containing ethidium bromide.

## 3.15 Influence of conditioned media from damaged 16HBE14o- cells on proinflammatory cytokine and fibrogenic factor gene expression in MRC5 cells.

To establish if alarmins released from damaged lung epithelial cells may activate innate immune signalling in lung fibroblasts, MRC5 cells were cultured for 5hrs in fresh culture media or conditioned media collected from 16HBE-14o- cells that were untreated or damaged with $H_2O_2$-induced oxidative stress or thapsigargin-induced ER stress and gene expression of proiflammatory cytokines (IL-6, IL-8, MCP-1, GM-CSF, IL-1α, IL-1β) assessed by qRT-PCR. Fibroblast treatment with conditioned media from damaged epithelial cells led to an increase in expression of all of the measured genes, suggesting that alarmins released from damaged epithelial cells may trigger a proinflammatory phenotype in lung fibroblasts (figure 26 and 27).

Additionally, to assess if factors released from damaged cells may trigger fibrogenic responses in fibroblasts, MRC5 cells were treated under serum free conditions for 1, 2 or 3 days with conditioned media from 16HBE14o- cells damaged with $H_2O_2$. Following the treatment, the cells were harvested for RNA and gene expression of collagen 1A1 (Col1A1) was assessed by qRT-PCR. To confirm the responsiveness of fibroblasts to a fibrogenic stimulus, fibroblasts were treated for 2 days with 5ng/ml of TGFβ. The treatment with TGFβ resulted in marked upregulation of Col1A1 gene expression, however the conditioned media from damaged cells had no effect (figure 28).
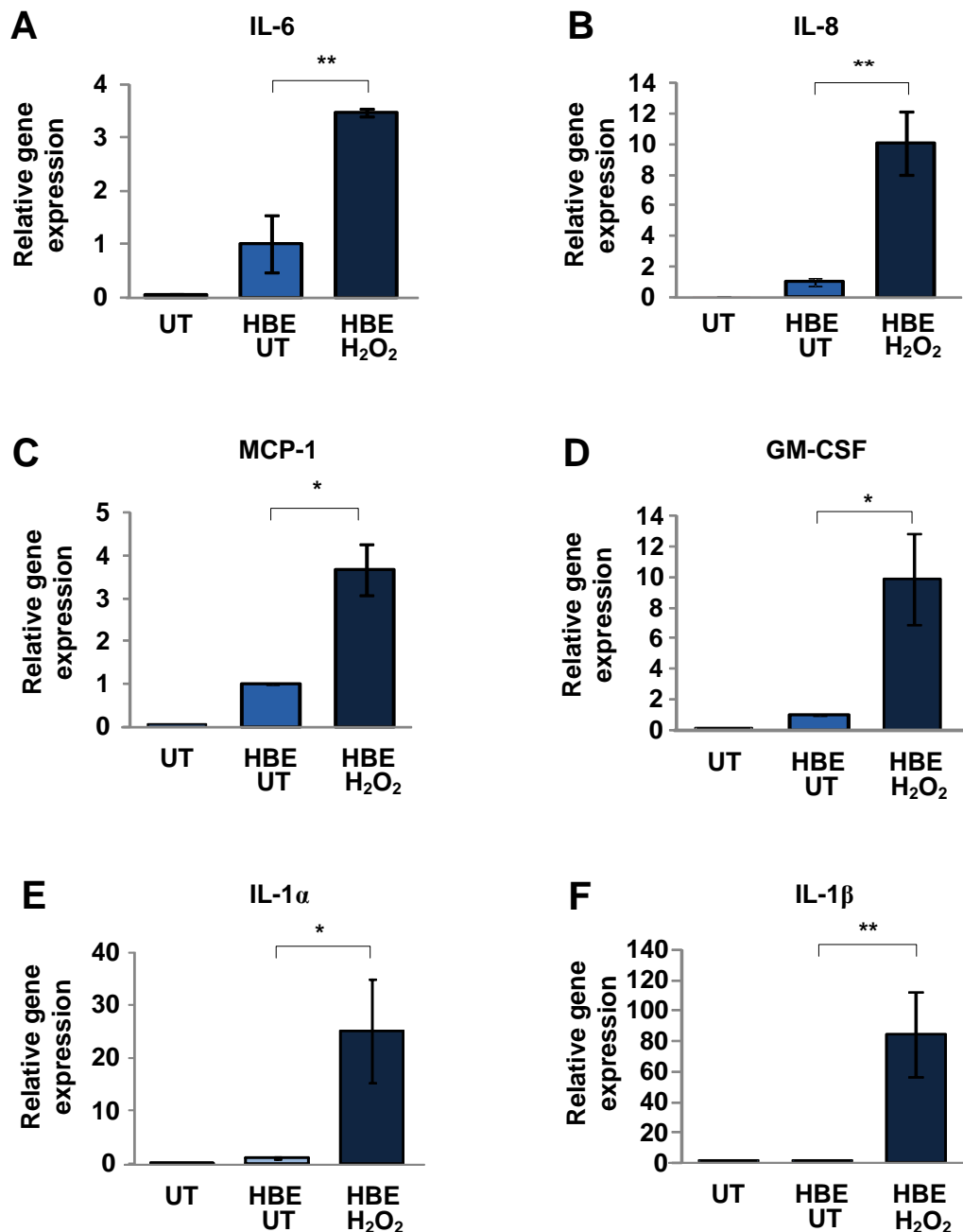
**Figure 26 Influence of conditioned media from 16HBE-14o- cells damaged with $H_2O_2$ (200μM) on proinflammatory cytokine gene expression in MRC5 cells.**

5hrs treatment of MRC5 cells with conditioned media from 16HBE14o- cells damaged with 200μM $H_2O_2$ (HBE $H_2O_2$) stimulated gene expression of IL-6 ($p < 0.01$) (A), IL-8 ($p < 0.01$) (B), MCP-1 ($p < 0.05$) (C), GM-CSF ($p < 0.05$) (D), IL-1α ($p < 0.05$) (E) and IL-1β (0.01) (F) in comparison to treatment with conditioned media from undamaged cells (HBE UT). Cytokine gene expression was assessed following 5hrs treatment by qRT-PCR using GAPDH as a reference gene. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
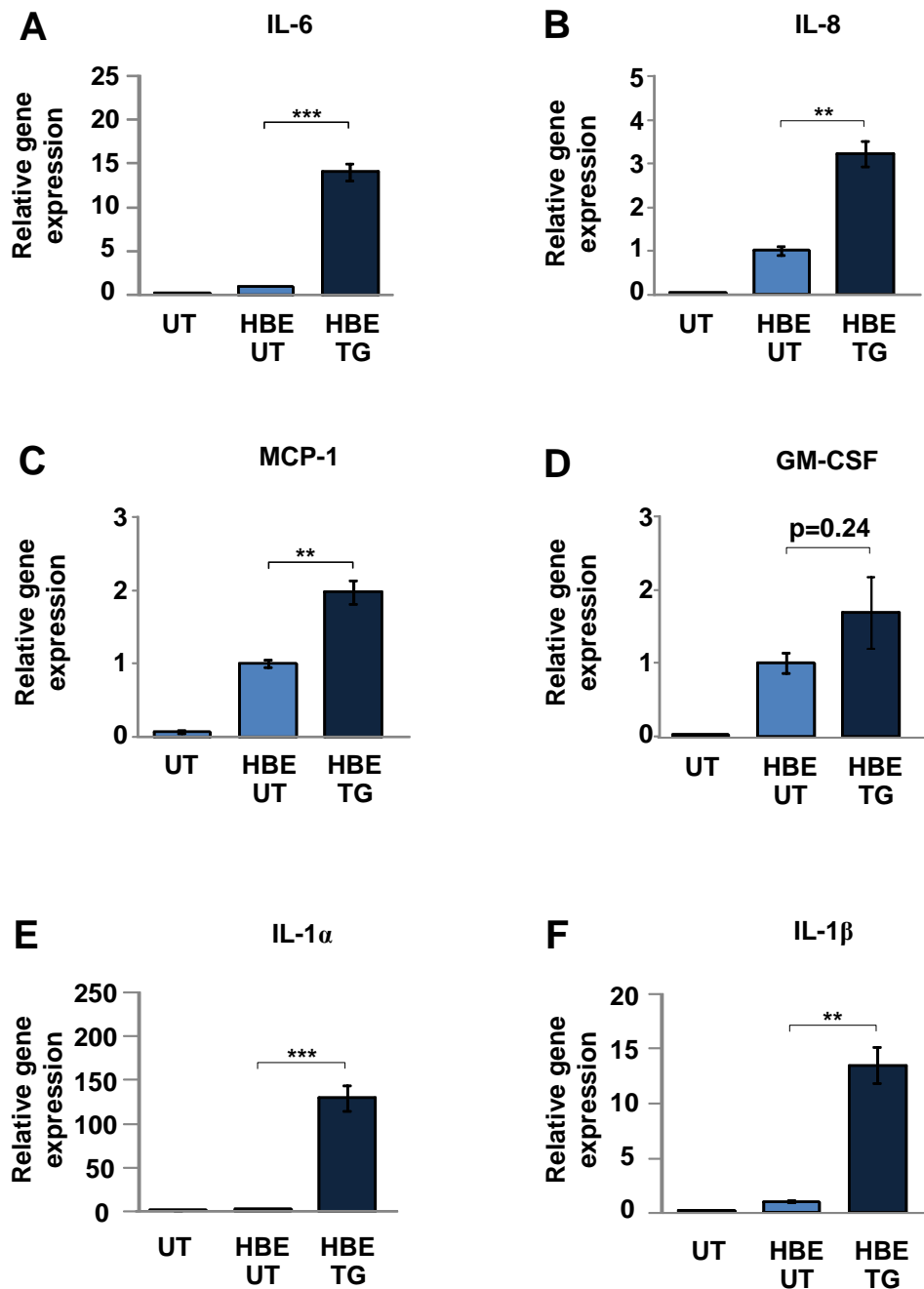
**Figure 27 Influence of conditioned media from 16HBE-14o- cells damaged with 50µM thapsigarging (TG) on proinflammatory cytokine gene expression in MRC5 cells.**

5hrs treatment of MRC5 cells with conditioned media from 16HBE14o- cells damaged with 200µM thapsigargin (TG) stimulated gene expression of IL-6 ($p<0.001$) (A), IL-8 ($p<0.01$) (B), MCP-1 ($p<0.01$) (C), GM-CSF ($p=0.24$) (D), IL-1α ($p<0.001$) (E) and IL-1β ($p<0.01$) (F) in comparison to treatment with conditioned media from undamaged cells (HBE UT). Cytokine gene expression was assessed following 5hrs treatment by qRT-PCR using GAPDH as a reference gene. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.
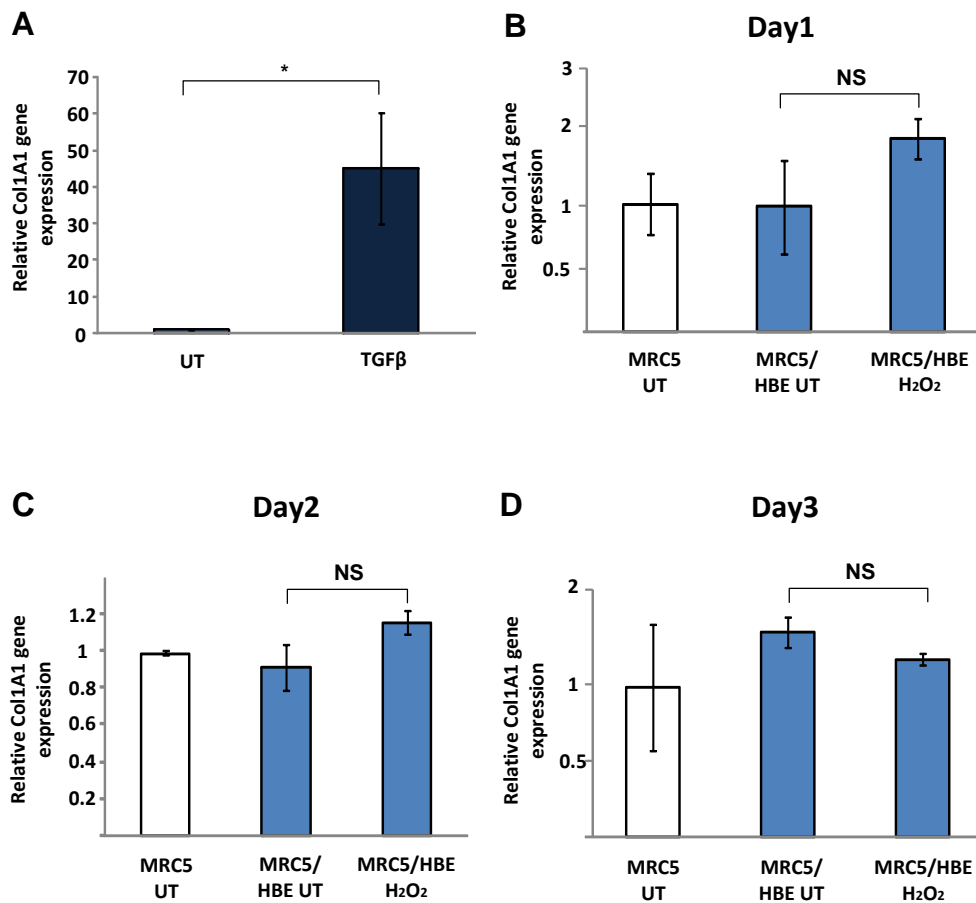
**Figure 28 Influence of conditioned media from damaged epithelial cells on collagen 1A1 gene expression.**

MRC5 cells were treated with 5ng/ml of TGFβ for 2 days, or conditioned media from untreated (HBE UT) or damaged with $H_2O_2$ (HBE $H_2O_2$) 16HBE14o- cells for 1,2 or 3 days, after which the cells were harvested for RNA and subject to qRT-PCR analysis for collagen1A1 (Col1A1) gene expression. The analysis revealed that, although TGFβ triggered expression of Col1A1 ($p<0.05$), conditioned media from damaged cells was with no effect. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

## 3.16 Discussion

The results of the aforedescribed experiments indicate that $H_2O_2$ induced-oxidative stress and thapsigargin (TG)-induced ER stress may be used to trigger damage of human bronchial epithelial cells resulting in the release of alarmins. The epithelial damage was induced by pulsing the cells with $H_2O_2$ or TG for a relatively short period of time (2hrs), which allowed for replenishing the media when cells were still viable. This approach allowed for excluding the injuring agents as factors inducing inflammatory responses in fibroblasts following administration of conditioned media from damaged epithelial cells.

Numerous studies demonstrated that oxidative stress, which may be defined as an imbalance between the amounts of oxidant and anti-oxidant agents, persists in many chronic inflammatory lung diseases such as COPD [210], BOS [211] and cystic fibrosis (CF) [212]. Currently it is believed that oxidative stress in the lung is induced by increased levels of reactive oxygen species that may be generated by activated macrophages and neutrophils. Activated inflammatory cells produce free oxygen radicals (O2.) which may be converted to $H_2O_2$ by an enzyme - superoxide dismutase. In a secondary reaction, hydroxyl radicals can be formed in the presence of Fe2+ cations in a reaction not requiring enzymatic catalysis [213].

ROS may contribute to cellular damage by oxidizing membrane phospholipids resulting in formation of numerous molecules such as lipid hydroperoxide leading to membrane disruption, followed by DNA damage and cell death. Additionally, they may react with certain amino acids such as methionine, tyrosine and cysteine in proteins, altering their functions [214].

Previous studies suggest that ROS may induce inflammation in two ways: by activating redox-sensitive transcription factors including NF-κB and activator protein-1 (AP-1) leading to production of proinflammatory cytokines and by causing cellular damage, resulting in the release of IL-8 and inflammation-mediating danger signals [215]. Moreover, in order to maintain homeostasis in tissue, redox-sensitive transcription factors also induce expression of anti-oxidant factors such as c-glutamylcysteine synthetase involved in glutathione production.

Glutathione, which is one of the main anti-oxidant agents, poses a thiol group in its cysteine residue which is able to reduce free radicals by donating a free electron. It has been shown that glutathione contributes to tissue red-ox homeostasis by neutralization of free radicals and reactive oxygen species and maintaining antioxidants such as vitamins E and C in their reduced – active state [216]. Additionally, other studies demonstrated that it is also involved in regulation of nitric oxide cycle [217], apoptosis [218], mitochondrial respiration [219] and remodelling of the extracellular matrix [220] .

Decreased levels of GSH have been observed in BALs of COPD [221] and BOS [222] patients while increased levels of GSH have been reported in asthmatics [223]. GSH have been shown to be required to protect lung against cigarette smoke and airborne particle- induced lung injury [224].

Interestingly this study demonstrated that there were marked differences in terms of the level of resistance to oxidative stress between a human bronchial epithelial cell line 16HBE14o- and primary human bronchial epithelial cells, which have been derived from brushings obtained from post-transplant patients. One of the limitations of this study was the restricted availability of PBEC cells from healthy individuals, and therefore it is difficult to speculate if the extreme resistance of PBEC cells to $H_2O_2$ was due to the fact that they originated from patients who have been subject to immunosuppressive therapy required to prevent allograft rejection or if PBECs are naturally equipped in mechanisms allowing them to cope with oxidative stress which persists in the lung due to relatively high oxygen pressure.

The comparison of resistance to oxidative stress and glutathione levels between PBECs derived from healthy individuals and patients affected by chronic inflammatory and fibrotic lung diseases could not be performed in this study due to limited supply of normal PBEC cells, however it could be a subject of a follow up study in the future.

Resistance to oxidative stress and production of glutathione remains under control of numerous factors of endogenous and exogenous origin.

Factors able to downregulate glutathione production include Glucocorticoids (eg. dexamethasone) [225], TGFβ [226] and Cyclic adenosine monophosphate (cAMP) [227].

Production of glutathione and c-glutamylcysteine synthetase may be triggered by numerous factors including oxidants such as hydrogen peroxide, cigarette smoke, ozone, nitric oxide, hyperoxia, heat shock, cytokines and growth factors including TNFα and IL-1, radiation, some metals such as selenium, iron, cadmium and mercury. Many of these substances can be inhaled with air and therefore airway epithelium is most prone to their influence [228].

It has been shown for instance that inhaled ozone, a strong oxidizing agent which may cause lipid peroxidation in cellular membranes leading to epithelial damage, may also increase neutrophil accumulation in the lung and decrease lung function [229].

Airborne oxidants originating from air pollution have also been recognized as factors stimulating production of proinflammatory cytokines from airway epithelial cells [230].

Oxidants, as well as some proinflammatory cytokines such as TNFα induce production of ROS by mitochondria. Proinflammatory activity of ROS involved activation of NF-κB which requires phosphorylation and ubiquitination followed by proteolitic degradation of IκB, which functions as NF-κB inhibitor. ROS are also able to activate a MAP-kinase JNK leading to phosphorylation of c-Jun and AP-1 activation. Both AP-1 and NF-κB function as transcription factors modulating expression of numerous genes involved in maintaining red-ox homeostasis including c-glutamylcysteine synthetase (c-GCS), manganese superoxide dismutase (MnSOD), haemoxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS) [231].

Although the direct influence of oxidative stress on activation of proinflammatory signaling pathways has not been investigated in this study, our data suggest that it may lead to release of alarmins, which may initiate a very potent inflammatory response. Interestingly, a dose response experiment using different concentrations of $H_2O_2$, revealed that oxidative stress may induce IL-1α release from cells which are

still viable. Interestingly, HMGB-1 release was detected only when the cells were damaged, suggesting that IL-1α may serve as an early danger signal released prior to cell death, whereas HMGB-1 could be treated as a marker of epithelial damage.

One of main sources of free radicals causing oxidative stress, a human lung is exposed to, is cigarette smoke and much research has been done on its influence on the activation of the innate immune system. Cigarette smoke contains numerous free radicals and oxidants such as nitrogen dioxide and sulphulr dioxide and remains the underlying cause of many chronic diseases including COPD [232]. In our study, cigarette smoke extract did not induce epithelial damage or alarmin release, which may be due to a relatively short time of treatment. Previous studies, using longer time of treatment, reported a link between CSE and epithelial death [233]. Such model, however, would not be applicable in this study, where in order to distinguish between the influence of injurious stimulus and released alarmins on fibroblast functions, the damaging factor had to be removed before epithelial damage occurred.

Recent studies revealed that oxidative stress may also contribute to epithelial damage by inducing stress in the endoplasmic reticulum (ER stress). Gao *et al.,* demonstrated that $H_2O_2$ may trigger unfolded protein response by upregulating glucose regulated protein 78 (GRP 78) and protein ubiquitination [234].

My study demonstrated that ER stress induced by thapsigargin (TG) may result in epithelial damage and alarmin release.

The relationship between ER stress and chronic inflammation has been thoroughly investigated, and it is clear that some proinflammatory cytokines, such as IL-1β may cause ER stress and UPR. It is also known that ER stress may trigger proinflammatory responses in cells by activating the JNK pathway [235].

ER stress has been linked to several chronic conditions, including obesity driven inflammation [236], cystic fibrosis [237], cardiovascular disease [238] and COPD [239].

Although the activation of UPR was demonstrated in several chronic inflammatory diseases, it has not been clear, how ER stress may trigger inflammatory responses.

My study demonstrated that cells exposed to TG released the proinflammatory alarmin IL-1α, even when the cell viability was not affected, which supports our other observations that IL-1α may function as an early danger signal, released viable cells exposed to a stress stimulus.

Additionally my study demonstrated that fibroblasts treated with conditioned media from epithelial cells damaged with oxidative stress or ER stress show a proinflammatory potential manifested by increased expression of numerous proinflammatory cytokines, chemokines and growth factors, which have been recognized to play important functions in the pathophysiology of many chronic degenerative diseases. The proinflmmatory potential of fibroblasts is best pronounced by the amounts of IL-8 and IL-6 they are able to release upon stimulation, and which are comparable to levels generated by LPS-activated macrophages (data not shown). The functions and physiological importance of selected factors expressed by human lung fibroblasts, including IL-6, IL-8, GM-CSF and MCP-1 has been discussed in the introduction.

**CHAPTER 4 PATTERN RECOGNITION RECEPTOR GENE EXPRESSION PROFILING IN HUMAN LUNG FIBROBLASTS AND THEIR RESPONSIVENESS TO SELECTED PRR LIGANDS**

**4.1 Background**

Until quite recently fibroblasts have been regarded as cells whose role is limited to production of the ECM components. Recent reports however, suggest that fibroblasts are a substantial source of many proinflammatory and growth factors and that they may also function as mediators of inflammation. Comparative studies on fibroblasts from healthy individuals and patients with chronic arthritis demonstrated that the cells from the two test groups showed different cytokine expression patterns suggesting that fibroblasts are able to gain a new phenotype in chronically injured organs and that they may function as mediators of inflammation [240]. The molecular basis for the persistent phenotype of fibroblasts in chronically inflamed tissues remain unclear, however recent studies on RelB knock-out mice indicate that NF-κB signaling may play a crucial role in perpetuating the inflammatory response in chronic inflammatory diseases. Fibroblasts from endotoxin challenged RelB deficient mice secreted a wide array of inflammatory cytokines which caused accumulation of immune cells within hours. The proinflammatory fibroblast phenotype was attenuated following transfection with WT RelB, suggesting a direct link between the NF-κB pathway and fibroblast activation [241]. Moreover, Smith *et al.* showed that fibroblasts cultured in vitro in contact, via CD40, with hematopoetic cells secreted elevated levels of IL-6, IL-8, Cyclooxygenase-2 and the polysaccharide hyaluronan, which resembles interactions between immunocytes and antigen presenting cells [242]. Furthermore, RelB upregulation is associated with dendritic cell maturation, which emphasizes the similarity of the mechanism of activation between antigen presenting cells and fibroblasts [243]. Fibroblasts express a range of pattern recognition receptors (PRRs), including certain Toll-like receptors (TLR), Receptor for advanced glycation endproducts (RAGE) and NOD-like receptors (NLR), which suggests that they may respond to both exogenous and endogenous danger patterns. However the pattern of PRRs expression in different fibroblast populations has not yet been fully described. Therefore to better understand the role of fibroblasts in innate immune

signaling and fibrosis it is necessary to unravel via which molecules and receptors, activation of fibrogenic cells in different tissues is triggered.

Despite several reports investigating the function of selected TLRs in human lung fibroblasts [244] [245], detailed PRR gene expression profile in these cells has not been described before. Previous reports state that normal human lung fibroblasts - MRC5 cells express TLR3 and TLR9 [246] [247], which may indicate that they may play a role in mediating innate immune responses to viral and bacterial PAMPs.

Therefore to establish which receptors are involved in alarmin-mediated fibroblast activation and to identify alarmins responsible for triggering the proinflammatory phenotype in fibroblasts following treatment with conditioned media from damaged epithelial cells, we performed PRR gene expression profiling in normal primary human lung fibroblasts.

**Hypotheses tested:**

1. Primary human lung fibroblasts exhibit a unique and distinct from macrophages profile of Pattern Recognition Receptor gene expression, characterized by a high expression of IL-1R and TLR3 and are responsive to IL-1 and a TLR3 ligand, Poly I:C.

2. Nucleic acids, which may activate TLR3 and may be released from damaged cells, trigger a proinflammatory phenotype in lung fibroblasts treated with conditioned media from injured epithelial cells.

3. IL-1α is the key epithelial alarmin triggering innate immune responses in human lung fibroblasts.

4. Human lung fibroblasts show increased expression of proinflammatory cytokines (IL-6 and IL-8) in response to the epithelial alarmin IL-1α and a TLR3 ligand, Poly I:C and these two factors may act synergistically to increase expression of proinflammatory factors in fibroblasts.

5. Inflammation induced by IL-1α and Poly I:C in fibroblasts may be reduced by a steroid drug, dexamethasone, and chemical inhibitors targeting selected kinases within the IL-1R signaling pathway (IKK2i and TAK1i).

## 4.2 Pattern Recognition Receptor gene expression in primary human lung fibroblasts

The results described in chapter 3 indicate that some factors, potentially alarmins, that are released by damaged epithelial cells may trigger a proinflammatory phenotype in fibroblasts. The influence of conditioned media from damaged cells on expression of selected fibrogenic genes was also investigated, however despite a pronounced responsiveness of fibroblasts to TGFβ in terms of collagen gene upregulation, these cells failed to respond to conditioned media from damaged cells in the same way.

To establish which alarmins released from damaged lung epithelial cells may be responsible for innate immune signalling activation in lung fibroblasts, human primary lung fibroblast were subjected to PRR gene expression profiling by qRT-PCR. The analysis revealed that the cells expressed relatively high levels of TLR3 and TLR9 and very high levels of IL-1R. TLR3 and IL-1R expression was confirmed on the protein level by western blotting. Additionally IL-1R expression was detected by immunofluorescence using an antibody specific to human IL-1R (figure 29).

**A  PRR gene expression in primary human lung fibroblasts**
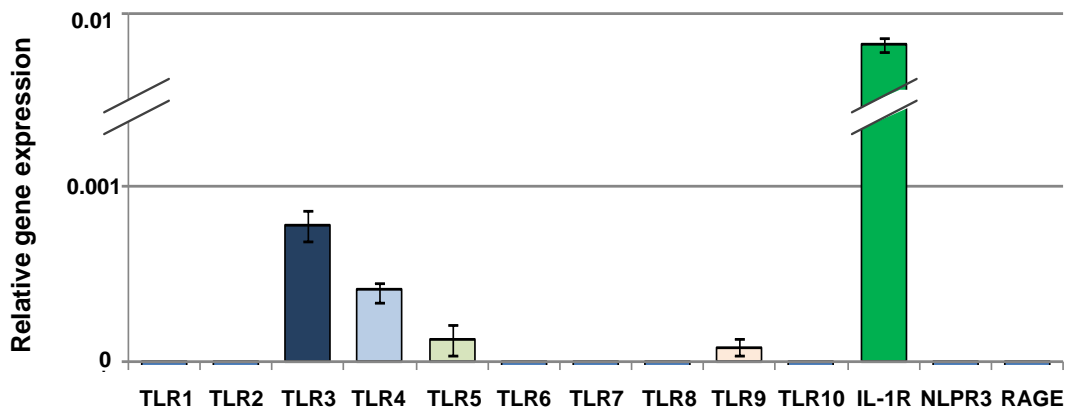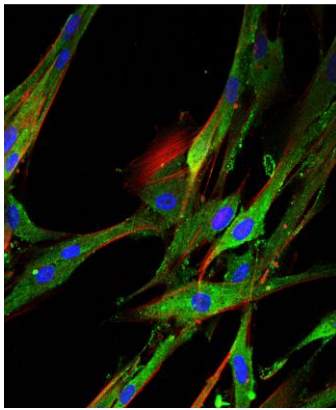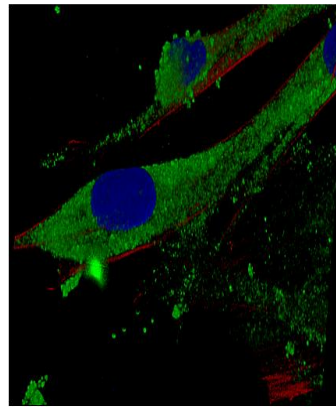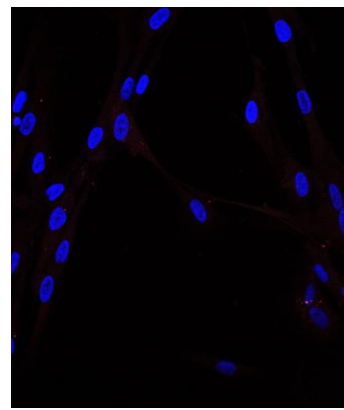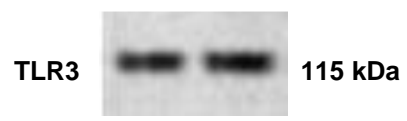
**Figure 29 Pattern Recognition Receptor (PRR) gene expression in primary human lung fibroblasts**

PRR gene expression profiling performed by qRT-PCR in unstimulated primary human lung fibroblasts isolated from 3 donor lungs revealed high levels of IL-1R and TLR3 and low levels of TLR2, TLR4, RAGE and NLPR3 transcripts (A). Data represent the mean±S.E.M, n=3. IL-1R expression was confirmed by immunocytofluorescence: IL-1R protein expression in primary human lung fibroblasts visualized using confocal incroscopy in 2D (B) and 3D (C) format; D - 2°antibody control. IL-1R (E) and TLR3 (F) expression was also confirmed on a protein level by western blotting.

## 4.3 Pattern Recognition Receptor gene expression in human lung fibroblast cell line MRC5

To confirm the results obtained using primary human lung fibroblast, a human lung fibroblast cell line – MRC5 - has also been used in the study. Therefore, to establish if MRC5 is a suitable cell line to study innate immune responses of human lung fibroblasts, the cell line has also been subjected to PRR gene expression profiling by qRT-PCR. The analysis confirmed that MRC5 cells, like PHLF, show very high expression of IL-1R and relatively high expression of TLR3. The levels of TLR2, characteristic to macrophages were very low and gene expression for TLR4 was below the qRT-PCR detection level. These results indicate that MRC5 cells show similar pattern of TLR expression as MRC5 cells, confirming that this cell line may serve as a reliable model to study immune responses in human lung fibroblasts (Figure 30).
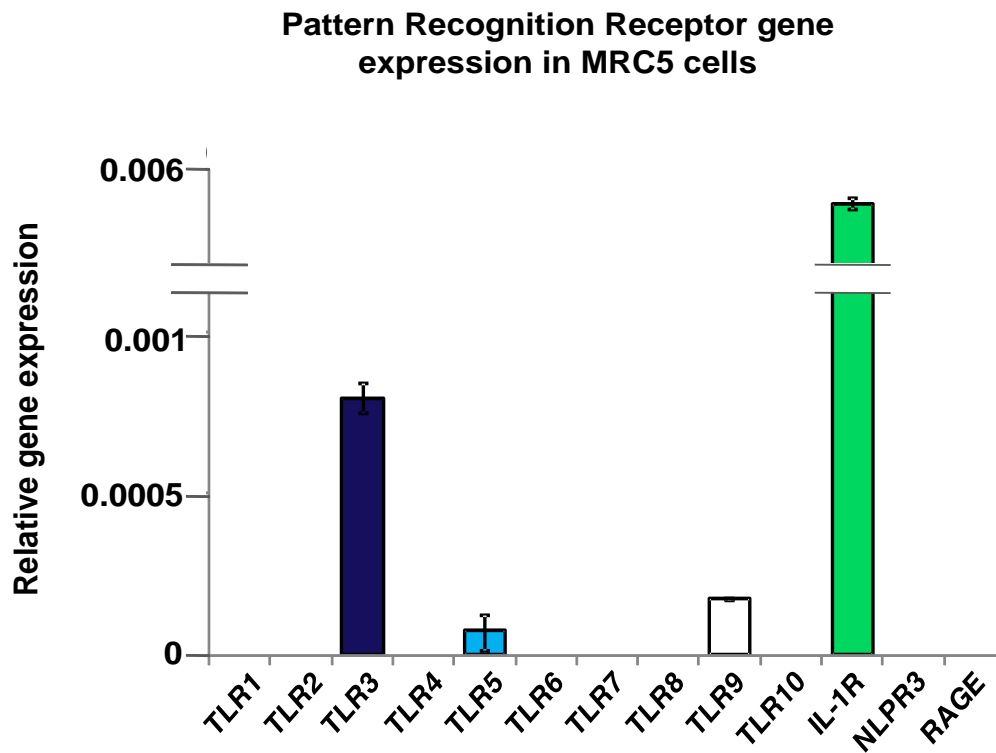


**Figure 30 Pattern Recognition Receptor gene expression in MRC5 cells.**

PRR gene expression measured by qRT-PCR revealed high expression levels of TLR3 and IL-1R in untreated MRC5 cells. GAPDH was used as a reference gene. Data represent the mean±S.E.M from 3 biological replicates.

**4.4 Pattern Recognition Receptor gene expression in activated THP-1 cells.**

To confirm the specificity of primers used to asses PRR gene expression, PRR expression was also measured in THP1 cells differentiated with 20ng/ml PMA. The qRT-PCR analysis revealed that the cells predominantly expressed TLR2 and TLR4 – a pattern characteristic for mature macrophages. The expression of TLR3 and TLR9 was relatively low in THP-1 cells, suggesting that the role of these cells, in contrast to fibroblasts, is focused on mediating innate immune responses to bacterial rather than viral PAMPs (figure 31).
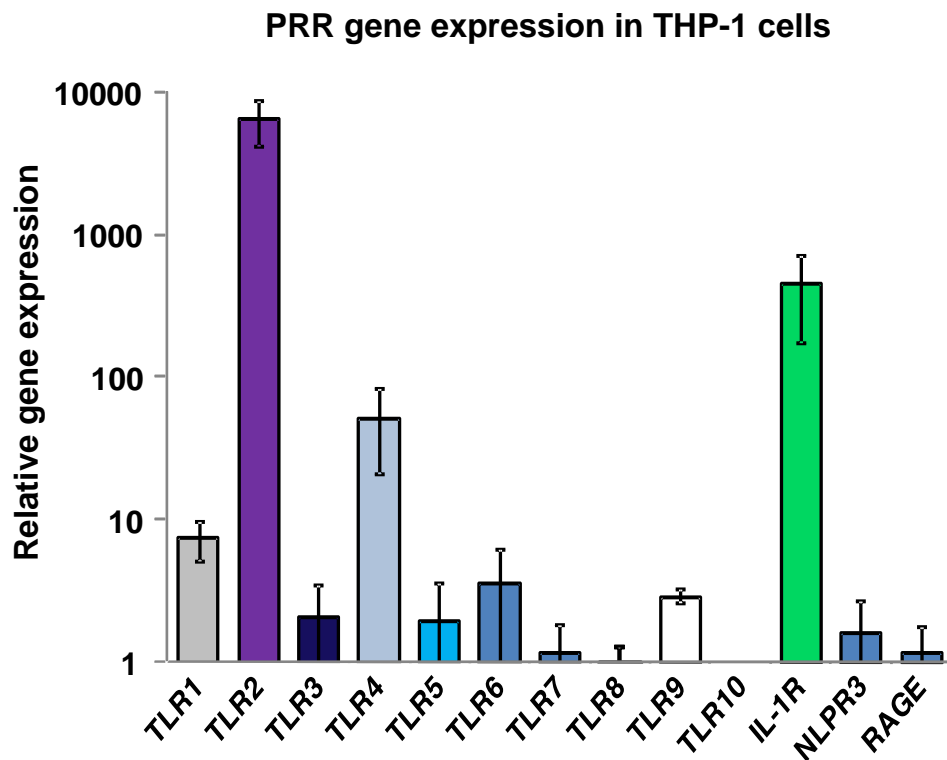


**Figure 31 Pattern recognition receptor (PRR) gene expression in THP-1 cells stimulated with PMA.**

THP-1 cells were treated for 25hrs with 20ng/ml of PMA, after which they were harvested for RNA and subject to qRT-PCR analysis. The analysis revealed that THP-1 cells express predominantly TLR2 and TLR4 – a pattern characteristic of activated macrophages. Note, to clarify the differences in expression levels between different PRRs in THP1 cells the data is expressed as logarithm of ΔΔCT values multiplied by a factor of 10000. Data represent the mean±S.E.M from 3 biological replicates.

## 4.5 Influence of selected PRR ligands on IL-6 gene expression in primary human lung fibroblasts

To confirm the observations described above and to investigate the responsiveness of fibroblasts to danger signals, primary human lung fibroblasts were treated for 6hrs with selected endogenous and exogenous PRR ligands including IL-1α (IL-1R ligand), Poly I:C – a synthetic analog of double stranded viral RNA (TLR3 ligand), LPS (TLR2 and TLR4 ligand) and HMGB-1 (TLR2, TLR4 and RAGE ligands). Following the treatment, the cells were harvested for RNA and subjected to qRT-PCR using IL-6 gene expression as a marker of innate immune signaling activation. The qRT-PCR analysis revealed that IL-1α was the most potent inducer of IL-6 gene expression in these cells, resulting in a substantial, 300-fold increase in expression of the target gene. Poly I:C also significantly upregulated IL-6 expression, while LPS and HMGB-1 had an insignificant or no effect on innate immune signaling activation in the human lung fibroblasts (Figure 32).
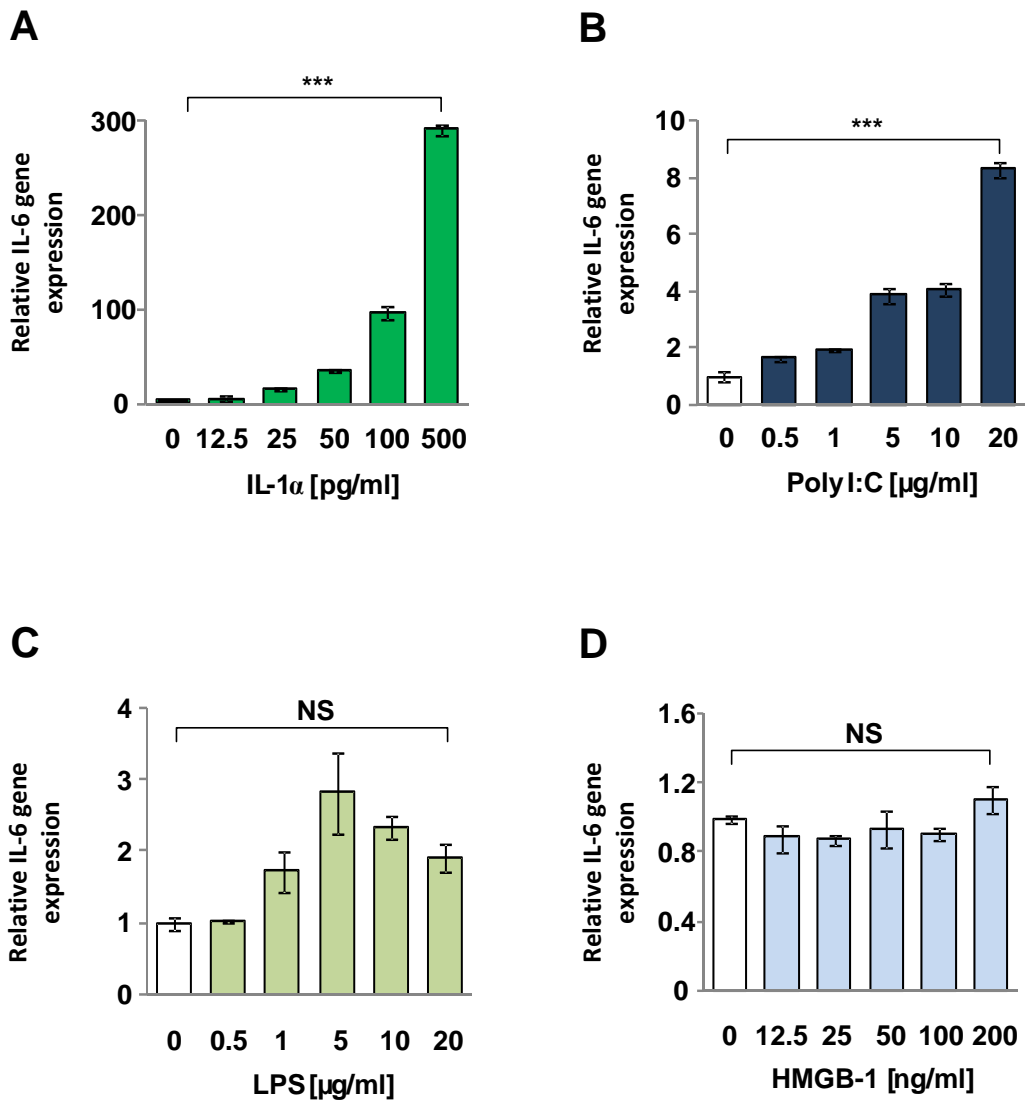
**Figure 32 Influence of selected Pattern Recognition Receptor ligands on IL-6 gene expression in primary human lung fibroblasts**

Primary human lung fibroblasts were challenged for 5hrs with selected PRR ligands including IL-1α (A) and Poly I:C (B), LPS (C) and HMGB-1 (D), after which the cells were harvested for RNA and subjected to qRT-PCR analysis to assess IL-6 gene expression. The analysis revealed that IL-1α is the most potent activator of IL-6 gene expression in primary human lung fibroblasts. Data represent the mean±S.E.M. from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, **p<0.01, ***p<0.001.

## 4.6 Influence of DAMP antagonists and benzonase on innate immune signaling activation in human lung fibroblasts treated with conditioned media from damaged human bronchial epithelial cells

Strong MRC5 cell responsiveness to Poly I:C suggests that TLR3 ligands may be involved in lung fibroblast activation. Previous reports indicate that one of the danger signals released from damaged cells that may activate innate immue system is endogenous RNA. To investigate whether TLR3 signaling in MRC5 cells may be triggered by endogenous RNA released by damaged epithelial cells, MRC5 cells were treated with the supernatants from 16HBE14o- cells injured with 200μM $H_2O_2$, that had been preincubated for 3hrs with or without 200U/ml nuclease (Benzonase, Sigma). Following the treatment, MRC5 cells were harvested for RNA and qRT-PCR analysis of IL-6 gene expression was performed. The analysis revealed that nuclease treatment did not have any influence on IL-6 gene expression when compared to the control (Figure 33). In a control experiment, MRC5 cells were treated with media containing 10ug/ml P I:C that had been preincubated for 3hrs with or without nuclease. The qRT-PCR analysis revealed that Benzonase treatment drastically reduced IL-6 gene expression in Poly I:C stimulated MRC5 cells, showing that the nuclease digestion was successful (Figure 34). To assess a deirect effect of Benzaonase on fibroblasts activation, an additional control could be performed where fibroblasts would be treated with Benzonase alone.
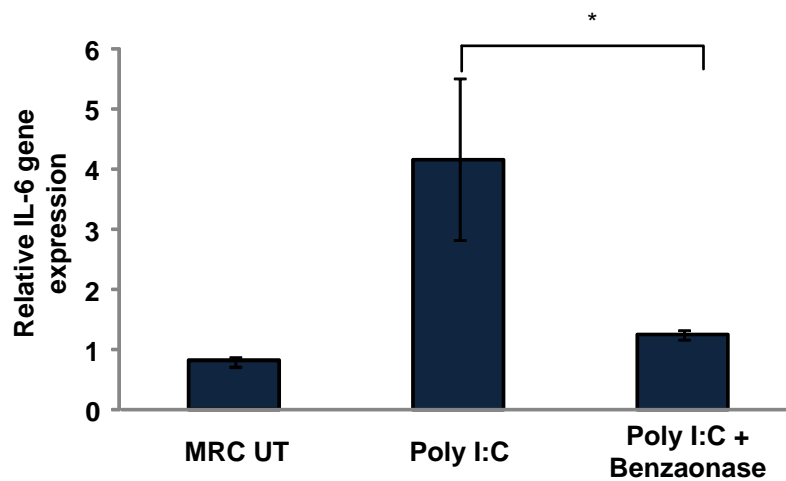
**Figure 33 Validation of the efficacy of a ribonuclease, benzonase, in a functional assay using Poly I:C as an analog of double stranded RNA and IL-6 gene expression as a marker of innate immune signaling activation.**

10µg/ml of Poly I:C was treated with bensonase for 1hr. Following the treatment, the solution of Poly I:C and the rybonuclease was added to MRC5 cell cultures for 5hrs. RT-PCR analysis revealed that bensonase efficiently inactivated Poly I:C and blocked its ability to upregulate IL-6 gene expression. Data represent the mean±S.E.M from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, **p<0.01, ***p<0.001.
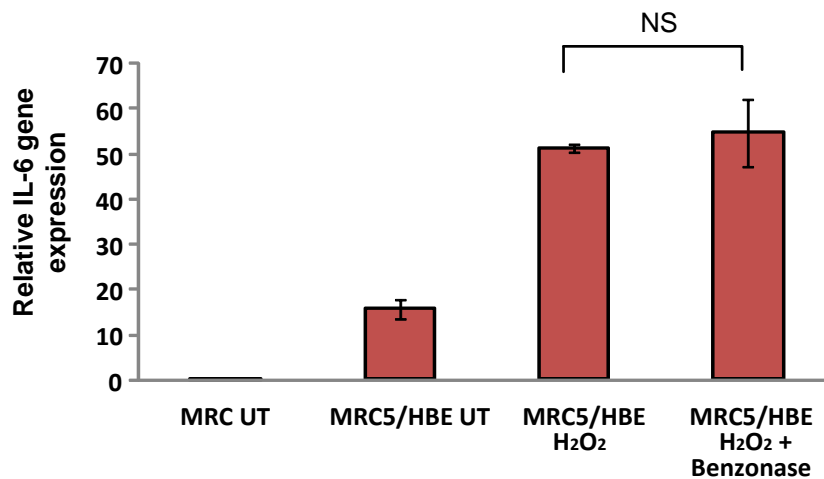


**Figure 34 Influence of a Benzonase on IL-6 gene expression in MRC5 cells treated with conditioned media from damaged 16HBE14o- cells.**

Conditioned media from damaged 16HBE14o- cells were treated with Bensonase for 1hr. Following the treatment, the conditioned media were added to MRC5 cell cultures for 5hrs. RT-PCR analysis revealed that Bensonase had no effect on IL-6 gene expression that was induced by conditioned media from damaged epithelial cells. Data represent the mean±S.E.M from 3 independent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**4.7 Influence of IL-1 antagonists on gene expression of selected proinflammatory cytokines in MRC5 cells treated with conditioned media from damaged epithelial cells**

Since IL-1R was the highest expressed PRR on human lung fibroblasts and IL-1α was a powerful inducer of fibroblast IL-6 gene expression I next determined the requirement of IL-1α/IL-1R signaling for stimulation of an inflammatory phenotype in fibroblasts by ROS-damaged epithelia.  MRC5 cells were treated for 5hrs with conditioned media collected form damaged 16HBE-14o- cells in the presence or absence of IL-1α blocking antibody (IL-1α BAb - 4μg/ml) or IL-1R antagonist (IL-1Ra – 500ng/ml). To confirm IL-1α BAb specificity as a negative control a neutralizing antibody against IL-1β (IL-1β BAb - 4μg/ml) was used. IL-1β BAb also served as an isotype control (both IL-1α BAb and IL-1β BAb are IgG isotype, have been raised in mouse and used at the same concentration of 4μg/ml).

The optimal doses and efficacy of IL-1Ra and IL-1α and IL-1β neutralizing antibodies were established in a functional assay using IL-6 gene expression as a marker of innate immune signaling activation in human lung fibroblasts (figure 35). In parallel, a cell viability study was performed to assess the possible toxic effect of the blocking agents. The analysis revealed that no decrease in fibroblast viability was observed following treatment with the indicated doses of IL-1 antagonists (data not shown).

The qRT-PCR analysis revealed that IL-1α NAb and IL-1Ra significantly reduced IL-6 gene expression in MRC5 cells challenged with media from damaged epithelial cells while IL-1β NAb had no effect (figure 36).
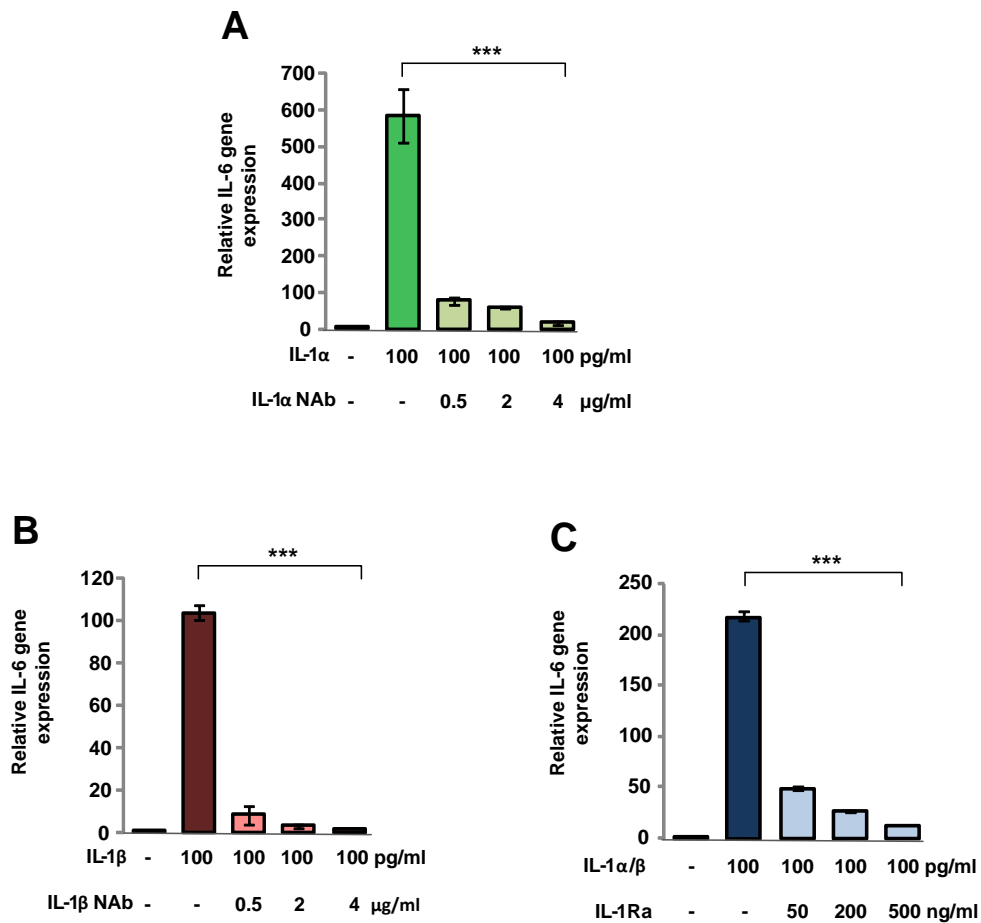
**Figure 35 Validation of the efficacy of the IL-1α neutralizing antibody (IL-1α NAb), IL-1β neutralizing antibody (IL-1β NAb) and IL-1 receptor antagonist (IL-1Ra).**

The ability of IL-1α NAb, IL-1β NAb and IL-1Ra to inhibit IL-1 biological activity was assessed in a bioassay using IL-6 gene expression as a marker of IL-1R activation. IL-1α and IL-1β (100 pg/ml) were incubated for 1hr at 37°C in the presence of the corresponding monoclonal neutralizing antibodies at various concentrations. Following incubation, primary human lung fibroblasts were treated for 5hrs with IL-1α **(A)** or IL-1β **(B)** in the presence or absence of the neutralizing antibodies and subjected to qRT-PCR analysis to measure IL-6 gene expression. Primary human lung fibroblasts were treated with various concentrations of IL-1Ra for 1hr after which 100pg/ml of IL-1 was added to the culture. Following 5 hrs incubation cells were harvested for total RNA and subjected to qRT-PCR analysis to measure IL-6 gene expression **(C)**. All the antagonists tested reduced IL-6 gene expression in a dose dependant manner (n=3, p<0.001). The following concentrations of IL-1 antagonists: IL-1α NAb 4µg/ml, IL-1β NAb 4µg/ml, IL-1Ra 500ng/ml were used in all the subsequent experiments. Data represent the mean±S.E.M from 3 biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, **p<0.01, ***p<0.001.
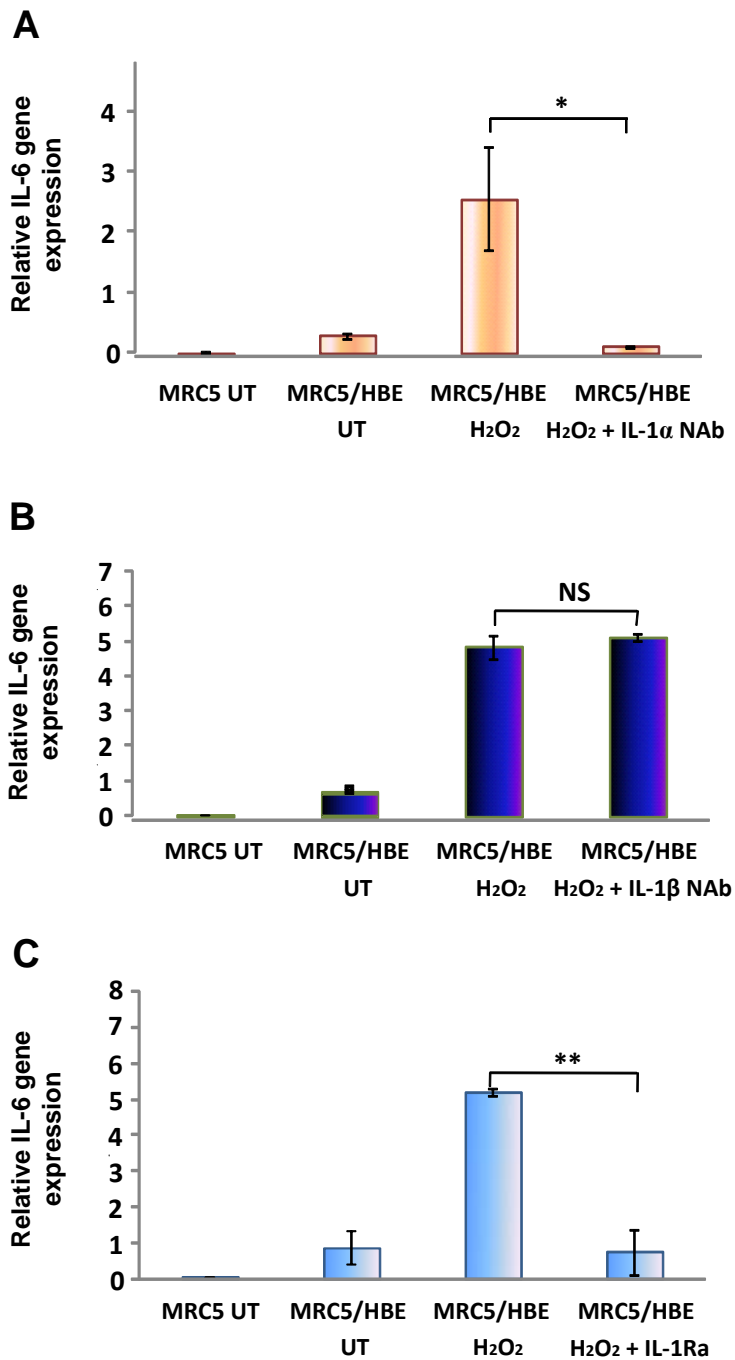
**Figure 36 Influence of IL-1 antagonists on IL-6 expression in MRC5 cells treated with conditioned media from 16HBE14o- cells damaged with 200μM $H_2O_2$.**

MRC5 cells were treated for 5hrs with conditioned media from 16HBE14o- cells damaged with oxidative stress in the presence or absence of IL-1α (A) or IL-1β (B) neutralizing antibodies (BAb) or IL-1R antagonist (IL-1Ra) (C). Treatment with conditioned media from damaged cells significantly induced IL-6 gene expression in MRC5 cells. This effect was significantly reduced by IL-1α BAb and IL-1Ra but not IL-1β Bab. Data represent the mean±S.E.M from 3 indepenent experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**4.8 Influence of IL-1 antagonists on gene and protein expression of selected proinflammatory cytokines in primary human lung fibroblasts treated with conditioned media from PBEC cells damaged with oxidative stress.**

To confirm the reproducibility of the aforedescribed results, an analogous experiment was performed using human primary bronchial epithelial cells derived from 5 patients and human primary lung fibroblasts isolated from 3 different patients. The qRT-PCR analysis showed that the fibroblast treatment with conditioned media collected from PBEC cells damaged with 1mM $H_2O_2$ led to upregulation of prinflammatory cytokine gene expression: IL-6, IL-8, MCP-1, GM-CSF, IL-1α and IL-1β and that this induction was significantly reduced by IL-1α NAb and IL-1Ra but not by IL-1β Nab.

To confirm the results of the qRT-PCR analysis described above on the protein level, primary human lung fibroblasts were treated for 5hrs with conditioned media from damaged epithelial cells in the presence or absence of IL-1α or IL-1β neutralizing antibody or IL-1Ra. Following the treatment the conditioned medium was replaced with fresh culture medium and the cells were incubated at 37°C for an additional 24hrs. After the incubation, the conditioned media from fibroblasts were collected and subjected to ELISA for IL-6, IL-8, MCP-1 and GM-CSF. The ELISA analysis confirmed that IL-1α NAb and IL-1Ra but not IL-1β NAb significantly reduced IL-6, IL-8, MCP-1 and GM-CSF protein release from lung fibroblasts pre-treated with conditioned media from damaged epithelial cells IL-1α and IL-1β were below the detection level (figures 37-42).

**Figure 37 Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on IL-6 gene expression (A) and IL-6 protein secretion (B) by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with $H_2O_2$.**

Primary human lung fibroblasts (PF) were treated for 5hrs with conditioned media from damaged PBECs (PBECs $H_2O_2$) that had been preincubated for 1hrs at 37°C in the presence or absence of IL-1α (4µg/ml) or IL-1β (4µg/ml) neutralizing antibody. To inhibit IL-1R signalling, PF were pretreated for 1hrs with 500ng/ml of IL-1Ra after which the culture media was replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. Following 5hrs treatment samples were collected for RNA or the conditioned medium was replaced with fresh culture medium and the cells incubated at 37°C for additional 24hrs. Subsequently, the conditioned media from the fibroblasts was collected and subjected to ELISA. IL-1α NAb and IL-1Ra significantly reduced induction of (A) IL-6 gene expression (90%, p<0.001 and 91% p<0.01 resp., data represented as S.E.M. from 5 individual experiments performed in biological triplicates). Additionally, conditioned media from ROS-damaged epithelial cells induced fibroblast secretion of (G) IL-6 (25ng/ml, data represented as S.E.M. from 3 individal experiments performed in biological duplicates) and that effect was significantly reduced by IL-1α Nab and IL-1Ra but not IL-1β Nab. Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**Figure 38 Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on IL-8 gene expression (A) and IL-8 protein secretion (B) by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with H$_2$O$_2$.**

Primary human lung fibroblasts (PF) were treated for 5hrs with conditioned media from damaged PBECs (PBECs H$_2$O$_2$) that had been preincubated for 1hrs at 37°C in the presence or absence of IL-1α (4μg/ml) or IL-1β (4μg/ml) neutralizing antibody. To inhibit IL-1R signalling, PF were pretreated for 1hrs with 500ng/ml of IL-1Ra after which the culture media was replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. Following 5hrs treatment samples were collected for RNA or the conditioned medium was replaced with fresh culture medium and the cells incubated at 37°C for additional 24hrs. Subsequently, the conditioned media from the fibroblasts was collected and subjected to ELISA. IL-1α NAb and IL-1Ra significantly reduced induction of (A) IL-8 gene expression (93%, p<0.01 and 95%, n<0.01 resp., data represented as S.E.M. from 5 individual experiments performed in biological triplicates). Additionally, conditioned media from ROS-damaged epithelial cells induced fibroblast secretion of (G) IL-8 (1μg/ml, data represented as S.E.M. from 3 individal experiments performed in biological duplicates) and that effect was significantly reduced by IL-1α Nab and IL-1Ra but not IL-1β Nab. Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**A** GM-CSF

| | | | | | | |
|---|---|---|---|---|---|---|
| PBECs UT | - | + | - | - | - | - |
| PBECs H$_2$O$_2$ | - | - | + | + | + | + |
| IL-1α NAb | - | - | - | + | - | - |
| IL-1β NAb | - | - | - | - | + | - |
| IL-1Ra | - | - | - | - | - | + |

**B** GM-CSF

| | | | | | | |
|---|---|---|---|---|---|---|
| PBECs UT | - | + | - | - | - | - |
| PBECs H$_2$O$_2$ | - | - | + | + | + | + |
| IL-1α NAb | - | - | - | + | - | - |
| IL-1β NAb | - | - | - | - | + | - |
| IL-1Ra | - | - | - | - | - | + |

**Figure 39 Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on GM-CSF gene expression (A) and GM-CSF protein secretion (B) by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with H$_2$O$_2$.**

Primary human lung fibroblasts (PF) were treated for 5hrs with conditioned media from damaged PBECs (PBECs H$_2$O$_2$) that had been preincubated for 1hrs at 37°C in the presence or absence of IL-1α (4μg/ml) or IL-1β (4μg/ml) neutralizing antibody. To inhibit IL-1R signalling, PF were pretreated for 1hrs with 500ng/ml of IL-1Ra after which the culture media was replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. Following 5hrs treatment samples were collected for RNA or the conditioned medium was replaced with fresh culture medium and the cells incubated at 37°C for additional 24hrs. Subsequently, the conditioned media from the fibroblasts was collected and subjected to ELISA. IL-1α NAb and IL-1Ra significantly reduced induction of (A) GM-CSF gene expression (95%, p<0.001 and 94%, p<0.001 resp., data represented as S.E.M. from 5 individual experiments performed in biological triplicates). Additionally, conditioned media from ROS-damaged epithelial cells induced fibroblast secretion of (G) GM-CSF (0.7ng/ml, data represented as S.E.M. from 3 individal experiments performed in biological duplicates) and that effect was significantly reduced by IL-1α Nab and IL-1Ra but not IL-1β Nab. Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**A** MCP-1

| | | | | | |
|---|---|---|---|---|---|
| PBECs UT | - | + | - | - | - | - |
| PBECs H$_2$O$_2$ | - | - | + | + | + | + |
| IL-1α NAb | - | - | - | + | - | - |
| IL-1β NAb | - | - | - | - | + | - |
| IL-1Ra | - | - | - | - | - | + |

**B** MCP-1

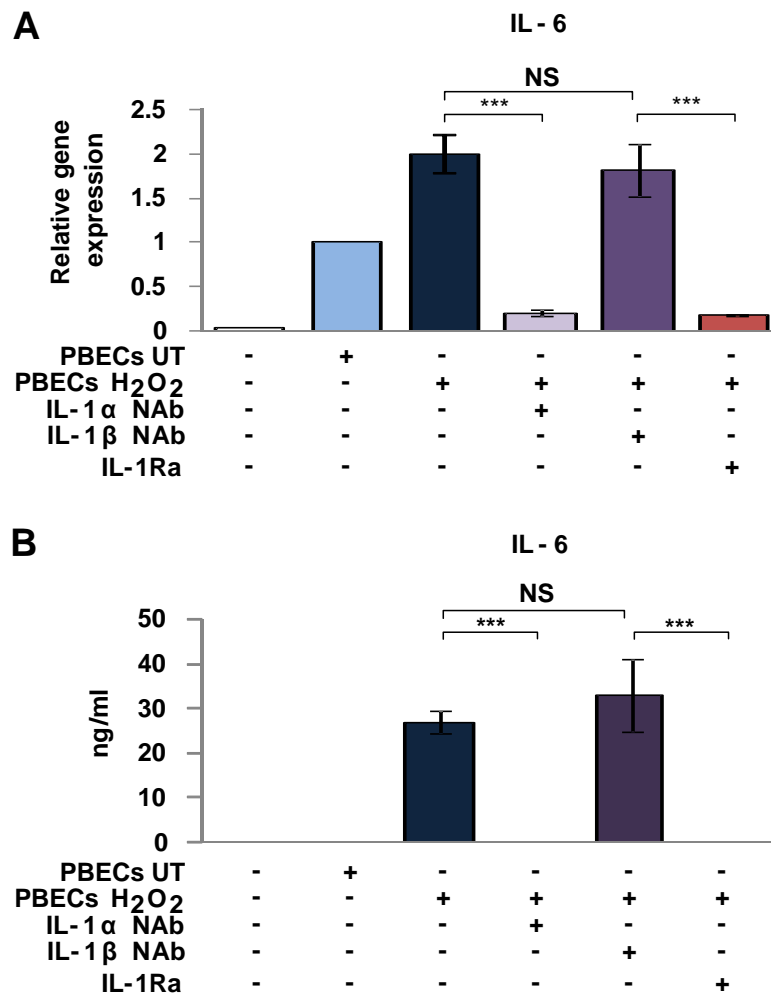| | | | | | |
|---|---|---|---|---|---|
| PBECs UT | - | + | - | - | - | - |
| PBECs H$_2$O$_2$ | - | - | + | + | + | + |
| IL-1α NAb | - | - | - | + | - | - |
| IL-1β NAb | - | - | - | - | + | - |
| IL-1Ra | - | - | - | - | - | + |

**Figure 40 Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on MCP-1 gene expression (A) and MCP-1 protein secretion (B) by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with H$_2$O$_2$.**

Primary human lung fibroblasts (PF) were treated for 5hrs with conditioned media from damaged PBECs (PBECs H$_2$O$_2$) that had been preincubated for 1hrs at 37°C in the presence or absence of IL-1α (4μg/ml) or IL-1β (4μg/ml) neutralizing antibody. To inhibit IL-1R signalling, PF were pretreated for 1hrs with 500ng/ml of IL-1Ra after which the culture media was replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. Following 5hrs treatment samples were collected for RNA or the conditioned medium was replaced with fresh culture medium and the cells incubated at 37°C for additional 24hrs. Subsequently, the conditioned media from the fibroblasts was collected and subjected to ELISA. IL-1α NAb and IL-1Ra significantly reduced induction of (A) MCP-1 gene expression (92%, p<0.001 and 93%, p<0.001 resp., data represented as S.E.M. from 5 individual experiments performed in biological triplicates). Additionally, conditioned media from ROS-damaged epithelial cells induced fibroblast secretion of (G) MCP-1 (20ng/ml, data represented as S.E.M. from 3 individal experiments performed in biological duplicates) and that effect was significantly reduced by IL-1α Nab and IL-1Ra but not IL-1β Nab. Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01, ***p<0.001.
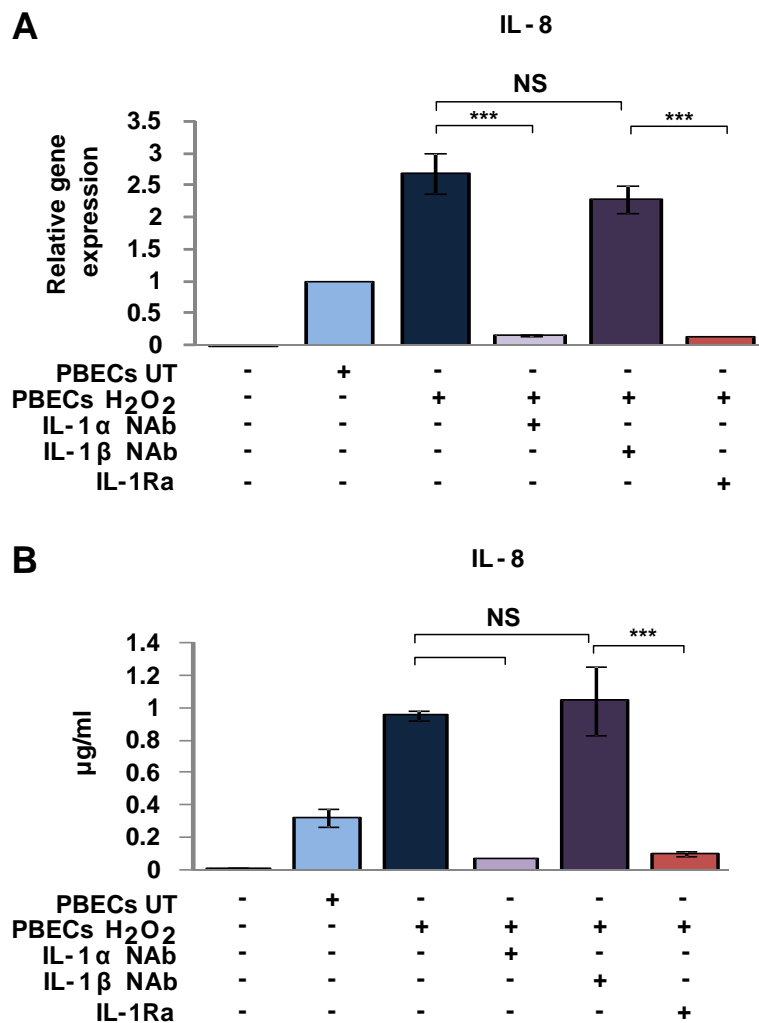
**IL-1α**

| | | | | | | |
|---|---|---|---|---|---|---|
| PBECs UT | - | + | - | - | - | - |
| PBECs H$_2$O$_2$ | - | - | + | + | + | + |
| IL-1α NAb | - | - | - | + | - | - |
| IL-1β NAb | - | - | - | - | + | - |
| IL-1Ra | - | - | - | - | - | + |

**Figure 41 Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on IL-1α gene expression by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with H$_2$O$_2$.**

Primary human lung fibroblasts (PF) were treated for 5hrs with conditioned media from damaged PBECs (PBECs H$_2$O$_2$) that had been preincubated for 1hrs at 37°C in the presence or absence of IL-1α (4μg/ml) or IL-1β (4μg/ml) neutralizing antibody. To inhibit IL-1R signalling, PF were pretreated for 1hrs with 500ng/ml of IL-1Ra after which the culture media was replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. Following 5hrs treatment samples were collected for RNA. IL-1α NAb and IL-1Ra significantly reduced induction of IL-1α (35%, $p<0.05$ and 42% $p<0.05$ resp., data represented as S.E.M. from 5 individual experiments performed in biological triplicates). Statistical analysis was performed using Student's t test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.
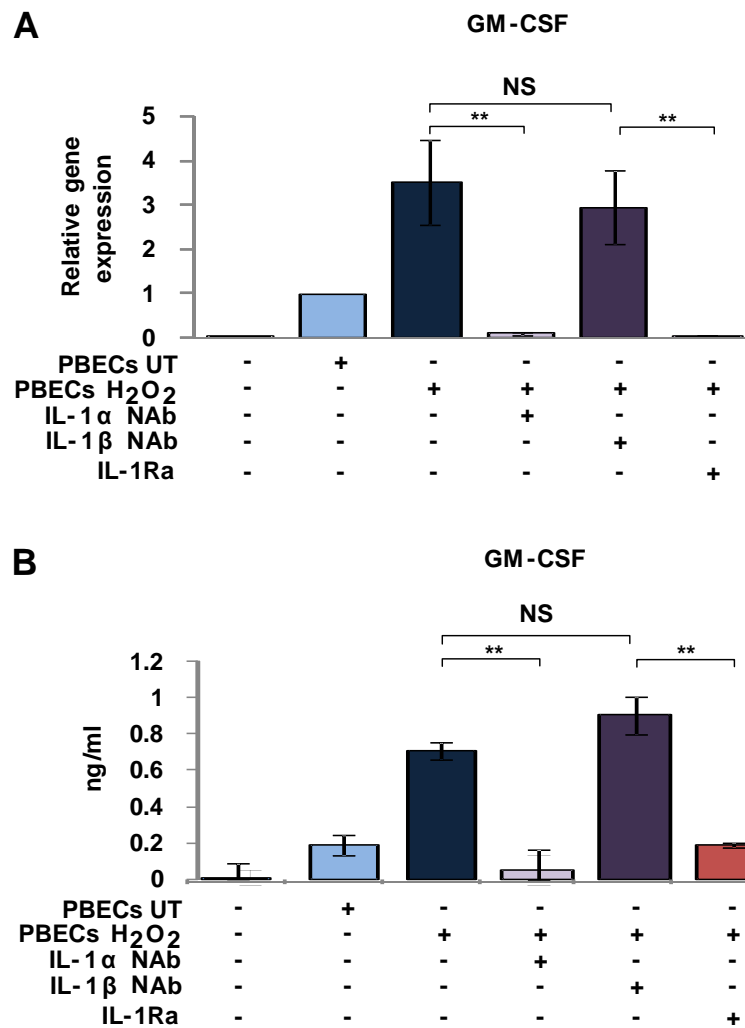
# IL-1β



**Figure 42 Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on IL-1β gene expression by primary human lung fibroblasts stimulated with conditioned media from PBEC cells damaged with $H_2O_2$.**

Primary human lung fibroblasts (PF) were treated for 5hrs with conditioned media from damaged PBECs (PBECs $H_2O_2$) that had been preincubated for 1hrs at 37°C in the presence or absence of IL-1α (4µg/ml) or IL-1β (4µg/ml) neutralizing antibody. To inhibit IL-1R signalling, PF were pretreated for 1hrs with 500ng/ml of IL-1Ra after which the culture media was replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. Following 5hrs treatment samples were collected for RNA. IL-1α NAb and IL-1Ra significantly reduced induction of IL-1β (77%, p<0.001 and 79%, p<0.001 resp., data represented as S.E.M. from 5 individual experiments performed in biological triplicates). Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**4.9 Influence of IL-1 antagonists on gene expression of selected proinflammatory cytokines in primary human lung fibroblasts treated with conditioned media from 16HBE14o- cells damaged with thapsigargin-induced stress in the endoplasmic reticulum (ER stress).**

To confirm the reproducibility of the results described before using a different model of epithelial injury, an analogous experiment was performed using conditioned media from 16HBE14o- damaged with 50µM thapsigargin and human primary lung fibroblasts. The qRT-PCR analysis showed that the fibroblast treatment with conditioned media collected from 16HBE14o- cells damaged with ER stress led to upregulation of proinflammatory and fibrogenic cytokine gene expression: IL-6, IL-8, MCP-1, GM-CSF, IL-1α and IL-1β, which was significantly reduced by IL-1α NAb and IL-1Ra but not by IL-1β Nab (Figure 43).
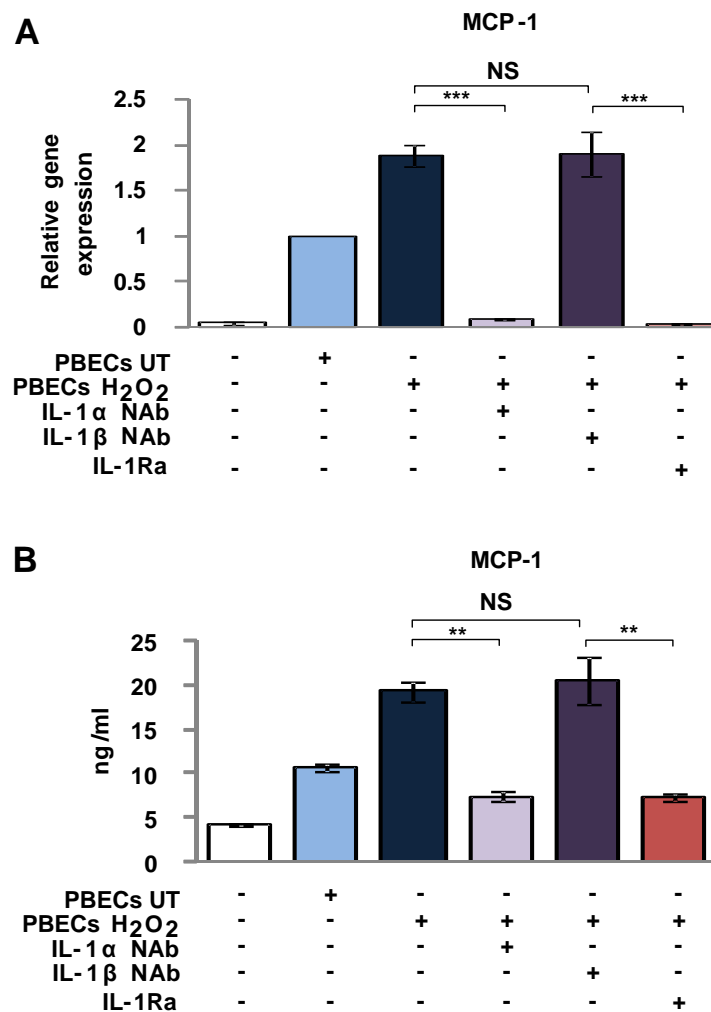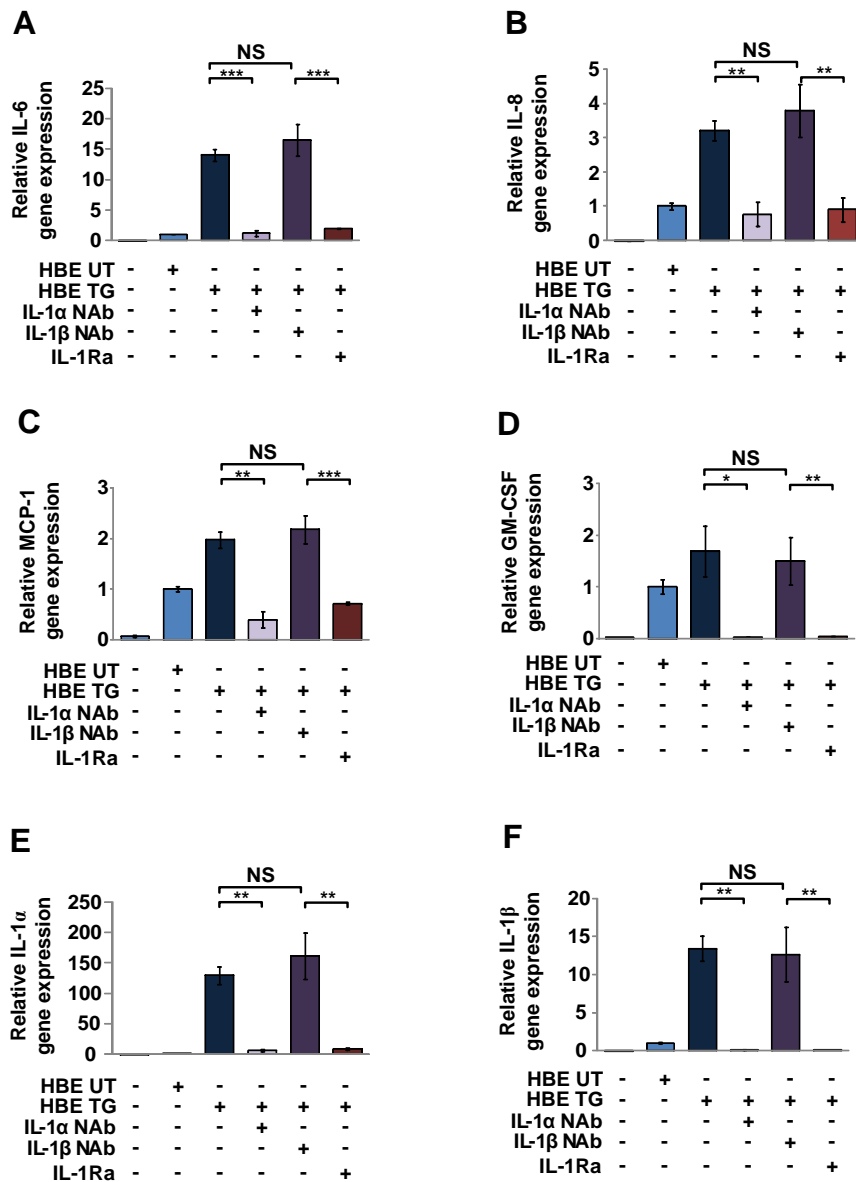
**Figure 43 Influence of IL-1α (IL-1α NAb) and IL-1β (IL-1β NAb) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on proinflammatory cytokine gene expression by primary human lung fibroblasts stimulated with conditioned media from 16HBE14o- cells (HBE) damaged with 50µM thapsigargin (TG)**.

Primary human lung fibroblasts (PF) were treated for 5hrs with conditioned media from 16HBE14o- damaged with 50µM thapsigargin (HBE TG) that had been preincubated for 1hrs at 37°C in the presence or absence of IL-1α (4µg/ml) or IL-1β (4µg/ml) neutralizing antibody. To inhibit IL-1R signalling, PHLF were pretreated for 1hrs with 500ng/ml of IL-1Ra after which the culture media was replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. Following 5hrs treatment samples were collected for RNA. IL-1α NAb and IL-1Ra significantly reduced induction of IL-6, IL-8, MCP-1, GM-CSF, IL-1α and IL-1β induced following treatment with conditioned media from damaged epithelial cells. Data represent the mean±S.E.M, from 3 individual experiments performed on two biological replicates. Statistical analysis was performed using unpaired Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**4.10 Influence of IL-1 antagonists on phosphorylation of selected PRR signaling molecules in primary human lung fibroblasts treated with conditioned media from damaged epithelial cells**

To investigate the influence of alarmins released from damaged epithelial cells on TLR signalling activation, primary human lung fibroblasts were treated for 30mins with conditioned media from untreated or damaged epithelial cells and phosphorylation of downstream TLR signalling molecules (ERK, JNK, p38, p65, IKK) analyzed by western blotting. The analysis revealed that the treatment with conditioned media from damaged cells led to MAP-kinase and NF-κB activation in comparison to control. Both IL-1α neutralizing antibody and IL-1Ra diminished phosphorylation of the kinases in response to conditioned media from 16HBE-14o-cells while IL-1β neutralizing antibody had no effect (Figure 44).
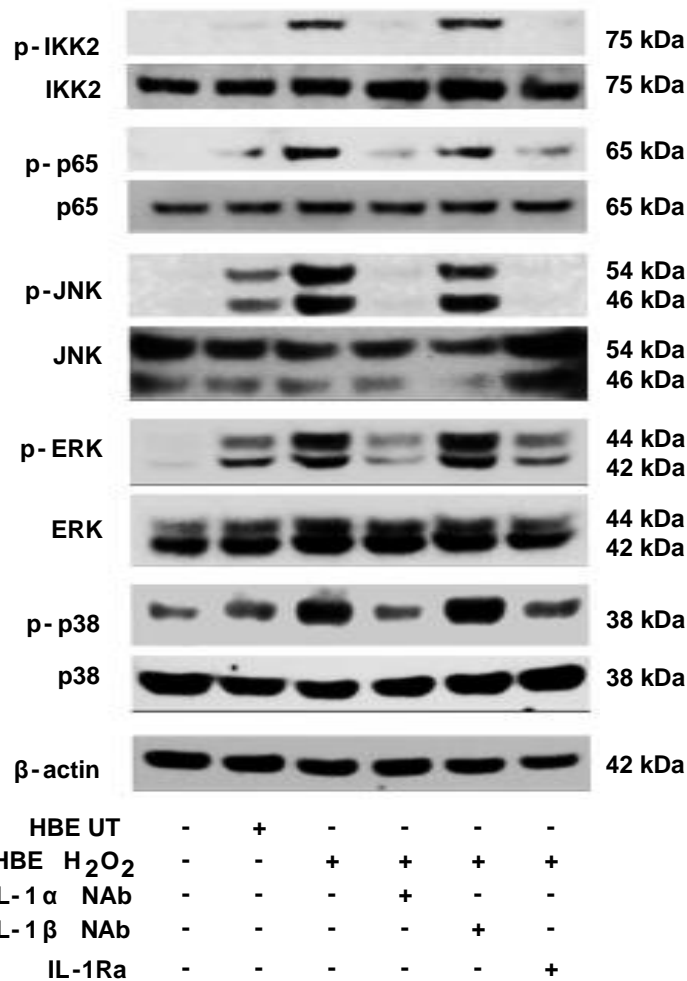
| | p-IKK2 | | 75 kDa |
| | IKK2 | | 75 kDa |
| | p-p65 | | 65 kDa |
| | p65 | | 65 kDa |
| | p-JNK | | 54 kDa / 46 kDa |
| | JNK | | 54 kDa / 46 kDa |
| | p-ERK | | 44 kDa / 42 kDa |
| | ERK | | 44 kDa / 42 kDa |
| | p-p38 | | 38 kDa |
| | p38 | | 38 kDa |
| | β-actin | | 42 kDa |

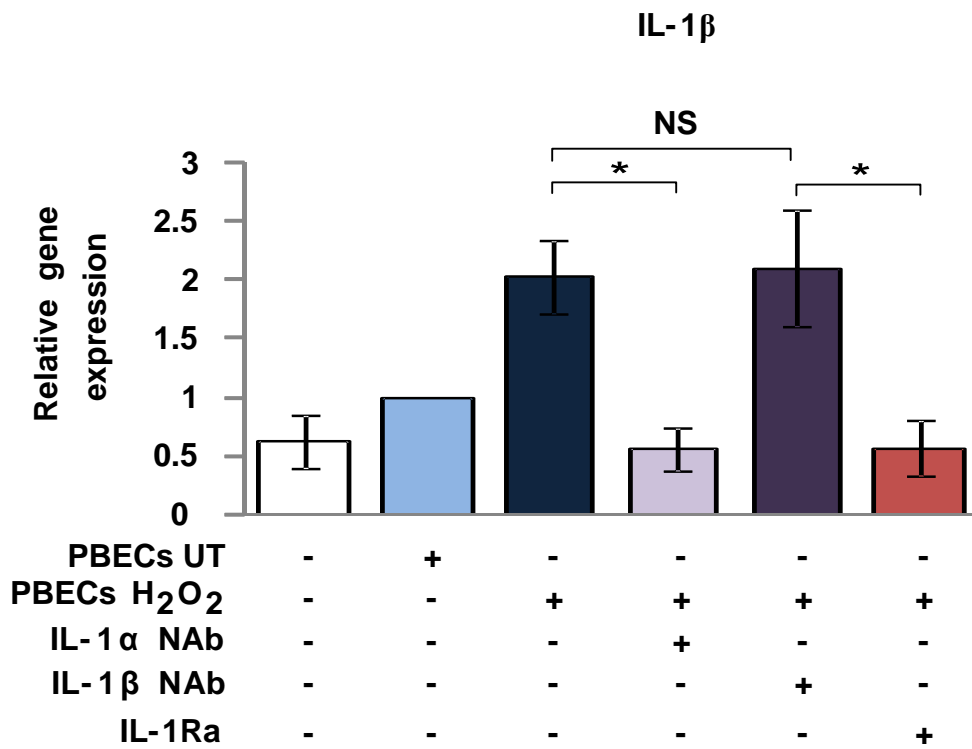| | | | | | | |
|---|---|---|---|---|---|---|
| HBE UT | - | + | - | - | - | - |
| HBE H₂O₂ | - | - | + | + | + | + |
| IL-1α NAb | - | - | - | + | - | - |
| IL-1β NAb | - | - | - | - | + | - |
| IL-1Ra | - | - | - | - | - | + |

**Figure 44 Influence of IL-1α (IL-1α Nab) and IL-1β (IL-1β Nab) neutralizing antibodies and IL-1 receptor antagonist (IL-1Ra) on phosphorylation of downstream TLR signaling molecules in primary human lung fibroblasts induced following treatment with conditioned media from H₂O₂ damaged 16HBE-14o- cells (HBE).**

Primary human lung fibroblasts (PF) were treated for 30mins with conditioned media from damaged 16-HBE14o- cells (HBE H₂O₂) that had been preincubated for 1hrs at 37°C in the presence or absence of IL-1α (4μg/ml) or IL-1β (4μg/ml) neutralizing antibody. To inhibit IL-1R signalling, PF were pretreated for 1hrs with 500ng/ml of IL-1Ra after which the culture media was replaced with conditioned media from damaged epithelial cells containing the same concentration of IL-1Ra. 30mins treatment with conditioned media from damaged epithelial cells increased phosphorylation of downstream TLR signaling molecules including p-65, IKK2, JNK, ERK and p38 in primary human lung fibroblasts in comparison to treatment with media from undamaged cells. This effect was strongly reduced by IL-1α NAb and IL-1Ra but not IL-1β NAb. Protein phoshorylation was assessed by western blotting. β-actin was used as loading control (n=3).

## 4.11 Synergy between IL-1α and Poly I:C in stimulating proinflammatory cytokine expression in human lung fibroblasts

As described above, both IL-1α and Poly I:C may stimulate expression of IL-6 in primary human lung fibroblasts. Therefore it may be hypothesized that there exist a crosstalk between IL-1R and TLR3 and that their ligands they may act synergistically to upregulate expression of proinflammatory cytokines. To test this hypothesis, human primary lung fibroblast were treated with 10μg/ml of Poly I:C or 500pg/ml of IL-1α or a combination of these two factors for 5 or 24hrs. Following the treatment the cells were harvested for RNA and subjected to qRT-PCR to assess IL-6 and IL-8 gene expression. The analysis revealed that Poly I:C significantly accentuated IL-1α induced IL-6 and IL-8 gene expression and that the synergistic effect was more pronounced after 24hrs (figure 45).

Since IL-6 may also be accumulated in intracellular globules, a immunohistochemical analysis was performed to establish if the IL-1α and Poly I:C may also have an effect on the levels of intracellular IL-6. The analysis showed that both IL-1α and Poly I:C treatment caused accumulation of IL-6 within intracellular granules in fibroblasts and that the stimulation with the combination of the two factors significantly enhanced the amount of the stored cytokine and increased the number of cells positively stained for IL-6 (figure 46).
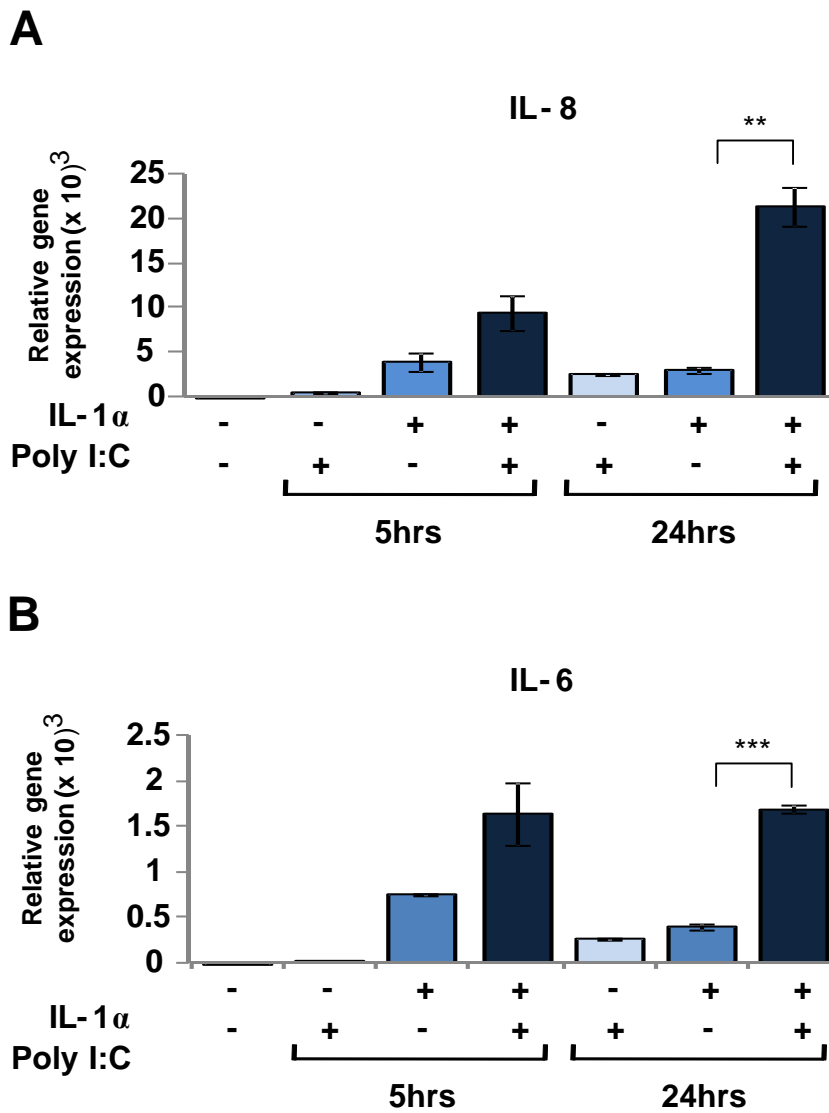
**Figure 45 Influence of IL-1α and Poly I:C on selected proinflammatory cytokine gene expression in primary human lung fibroblats in the course of time.**

Primary human lung fibroblasts were treated with IL-1α, Poly I:C or a combination of these two factors for 5 or 24hrs. Following the treatment RNA was collected and qRT-PCR analysis was performed to assess gene expression of IL-8 (A) and IL-6 (B). GAPDH was used as a reference gene. Data represent the mean±S.E.M. from 3 individual experiments performed on two biological replicates. Statistical analysis was performed using Student's t test.  *p<0.05, **p<0.01, ***p<0.001.
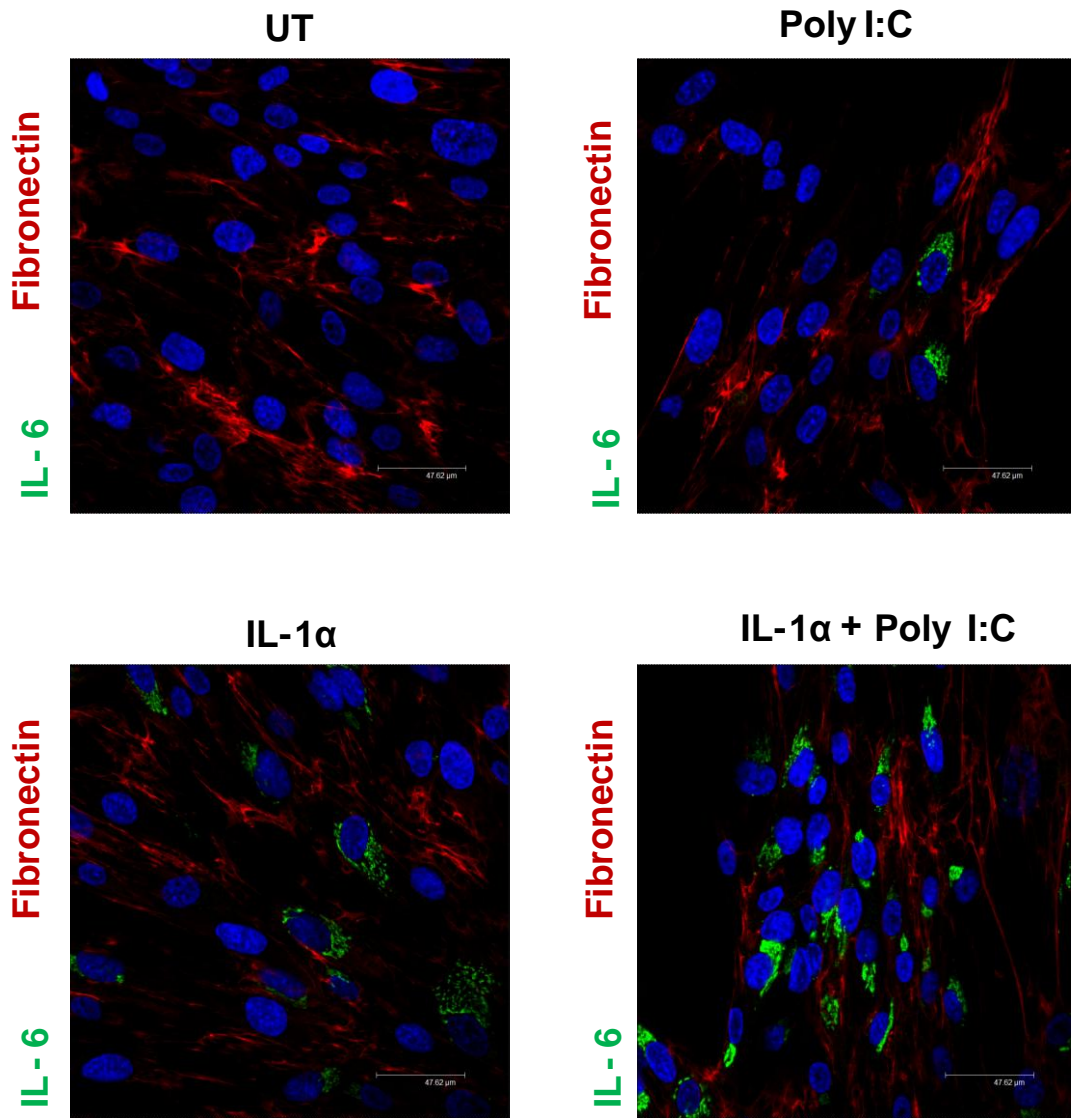
**Figure 46 Influence of IL-1α and Poly I:C on of IL-6 protein acumulation in primary human lung fibroblasts.**

Primary human lung fibroblasts were treated for 24hrs with IL-1α (100pg/ml), Poly I:C (10µg/ml) or a combination of these two factors. Following treatment the cells were subjected to immunofluorescent staining to visualize intracellular IL-6 protein (n=3). Red – fibronectin, green – IL-6, blue – DAPI.

**4.12 Influence of inhibitors of IL-1R signaling molecules on IL-α and Poly I:C induced inflammation.**

To investigate signaling pathways via which IL-1α and Poly I:C-induced inflammation is mediated, several chemical agents and commercially available drugs including chemical inhibitors of two major kinases mediating IL-1R and TLR3 signaling: TAK1 and TAK1, a steroid drug, dexamethasone and a macrolide antibiotic, azithromycin, were tested.

TAK1 inhibitor ((5Z)-7-Oxozeaenol) is a resorcyclic lactone of fungal origin that forms a covalent complex with TAK1 and inhibites both the kinase and the ATPase activity of TAK1. (5Z)-7-Oxozeaenol inhibits IL-1-induced activation of NF-κB (IC50 = 83 nM) and JNK/p38.

IKK2i (5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide) is an ureidocarboxamido thiophene compound that acts as a potent, ATP-competitive inhibitor of IKK-2 (IC50 = 18 nM) with selectivity over IKK-1, JNK and p38 MAPK. (Information obtained from the supplier (Calbiochem) datasheet).

Human primary lung fibroblast were exposed to IKK2i (0.078-10μM), TAK1i (0.0078-1μM) for 1hrs, after which the cells were treated with IL-1α or Poly I:C or a combination of these two factors for 24hrs. Following the treatment conditioned media from fibroblast cultures were collected for ELISA. The analysis revealed that IKK2i and TAK1i significantly reduced IL-6 nad IL-8 protein release form fibroblasts treated with IL-1α and Poly I:C (figures 44-47). Additionally, to confirm these results on the RNA level, an analogous experiment was performed using 1μM IKK2i or TAK1i and gene expression of IL-8 and IL-6 was measured by qRT-PCR. The analysis revealed that the treatment resulted in marked downregulation of IL-6 and IL-8 gene expression induced by IL-1α and Poly I:C. These results were further confirmed on the protein level by ELISA (figures 47-54). Neither of the tested IKK2i or TAKi doses reduced fibroblast cell viability (data not shown).
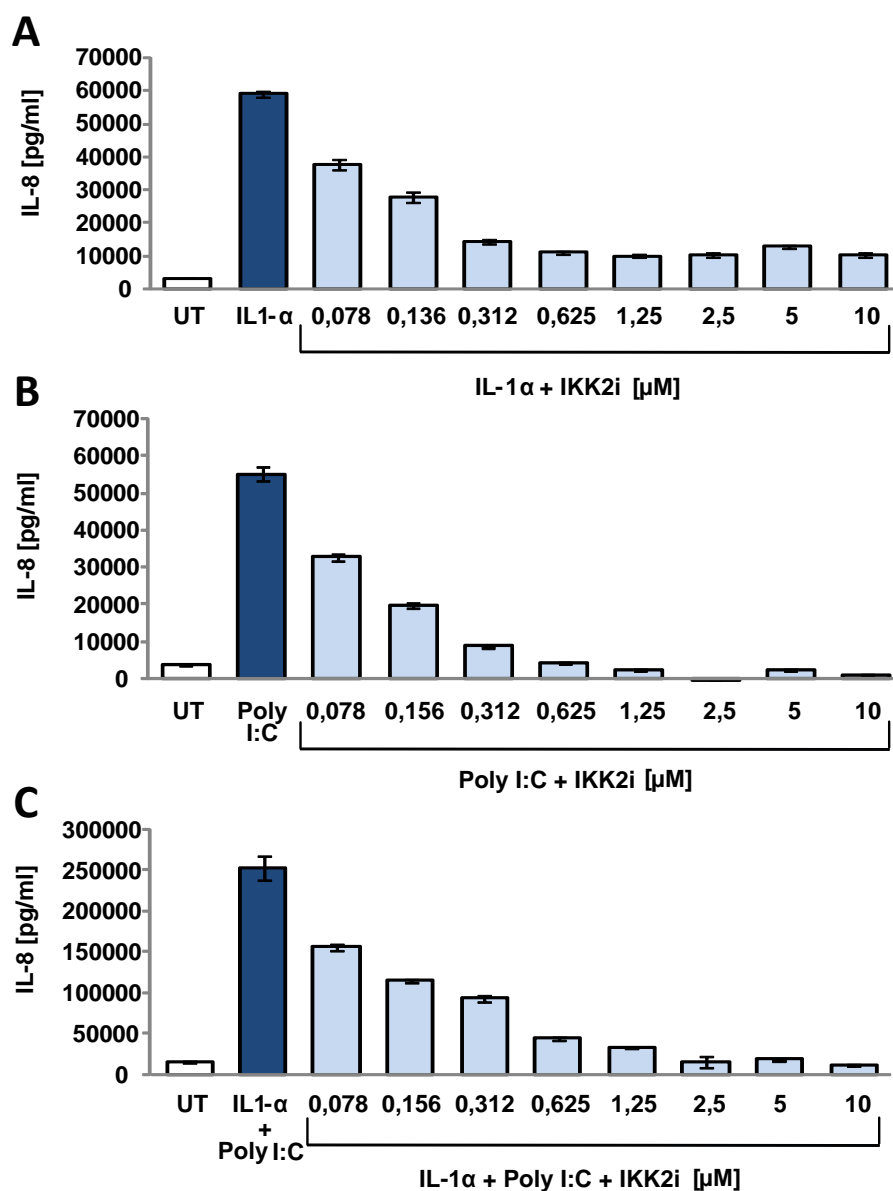
**Figure 47 Influence of IKK2i on IL-1α and Poly I:C induced IL-8 protein secretion by primary human lung fibroblasts.**

To establish the optimal dose of IKK2i required to inhibit IL-1α and Poly I:C- induced cytokine production in primary human lung fibroblasts the indicated IKK2i concentrations were tested in an assay using IL-8 protein secretion as markers of innate immune signaling activation. Primary human lung fibroblasts were treated with the indicated concentrations of IKK2i for 1hr after which 100pg/ml of IL-1α (A) or 10μg/ml of Poly I:C (B) alone or in combination were added to the culture (C). Following 24 hrs incubation conditioned media were collected and subjected to ELISA analysis    IL-8. IKK2i doses above 0.625μM were sufficient to inhibit IL-8 secretion by >90% in response to IL-1α and Poly I:C. Consequently a 1μM IKK2i dose was used in subsequent experiments assessing the influence of IKK2i on IL-1α and Poly I:C-induced IL-8 gene expression. Data represented as S.E.M. from 3 biological replicates.
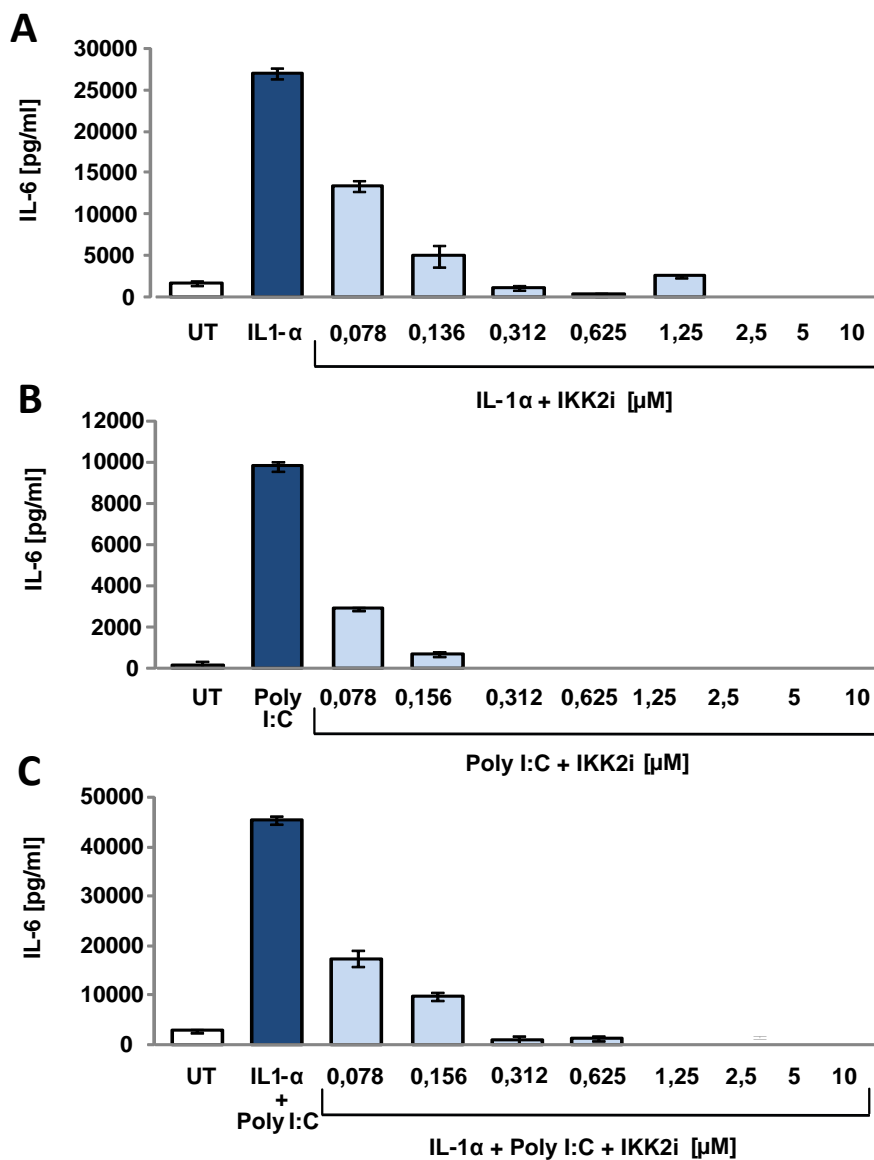
**Figure 48 Influence of IKK2i on IL-1α and Poly I:C induced IL-6 protein secretion by primary human lung fibroblasts.**

To establish the optimal dose of IKK2i required to inhibit IL-1α and Poly I:C- induced cytokine production in primary human lung fibroblasts the indicated IKK2i concentrations were tested in an assay using IL-6 protein secretion as markers of innate immune signaling activation. Primary human lung fibroblasts were treated with the indicated concentrations of IKK2i for 1hr after which 100pg/ml of IL-1α **(A)** or 10μg/ml of Poly I:C **(B)** alone or in combination were added to the culture **(C)**. Following 24 hrs incubation conditioned media were collected and subjected to ELISA analysis IL-6. IKK2i doses above 0.625μM were sufficient to inhibit IL-6 secretion by >90% in response to IL-1α and Poly I:C. Consequently a 1μM IKK2i dose was used in subsequent experiments assessing the influence of IKK2i on IL-1α and Poly I:C-induced IL-6 gene expression. Data represented as S.E.M. from 3 biological replicates.
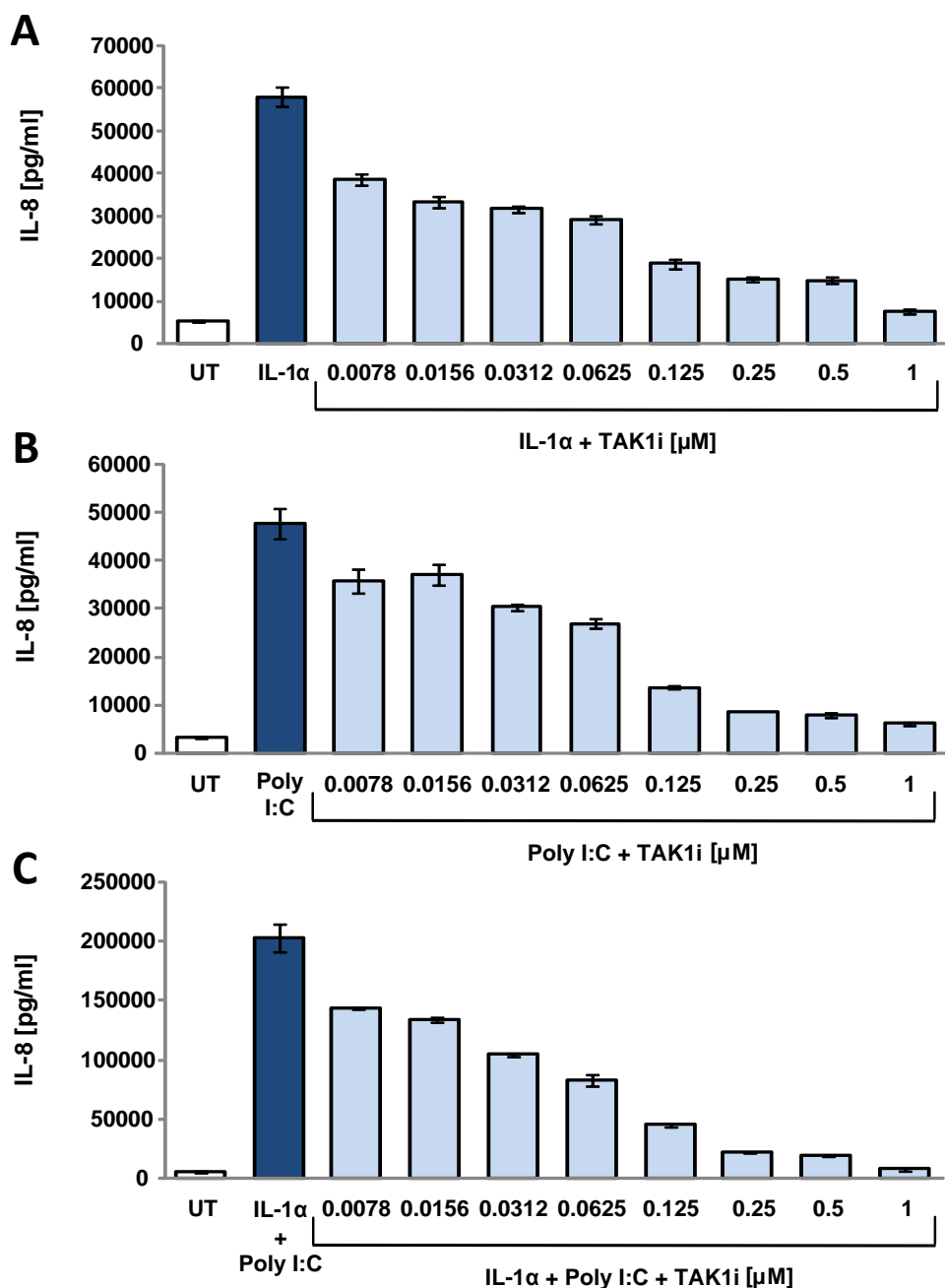
**Figure 49 Influence of TAK1i on IL-1α and Poly I:C induced IL-8 protein secretion by primary human lung fibroblasts**

Optimal TAK1i dose to inhibit IL-1α and Poly I:C- induced cytokine production by primary human lung fibroblasts was established analogically as described in SF.6 legend. The analysis revealed that a 1µM dose of TAK1i was sufficient to inhibit IL-8 protein secretion by >90% in response to IL-1α and Poly I:C. Consequently this dose was used in the subsequent experiments assessing the influence of TAKi on IL-1α and Poly I:C-induced IL-8 gene expression. Data represented as S.E.M. from 3 biological replicates.
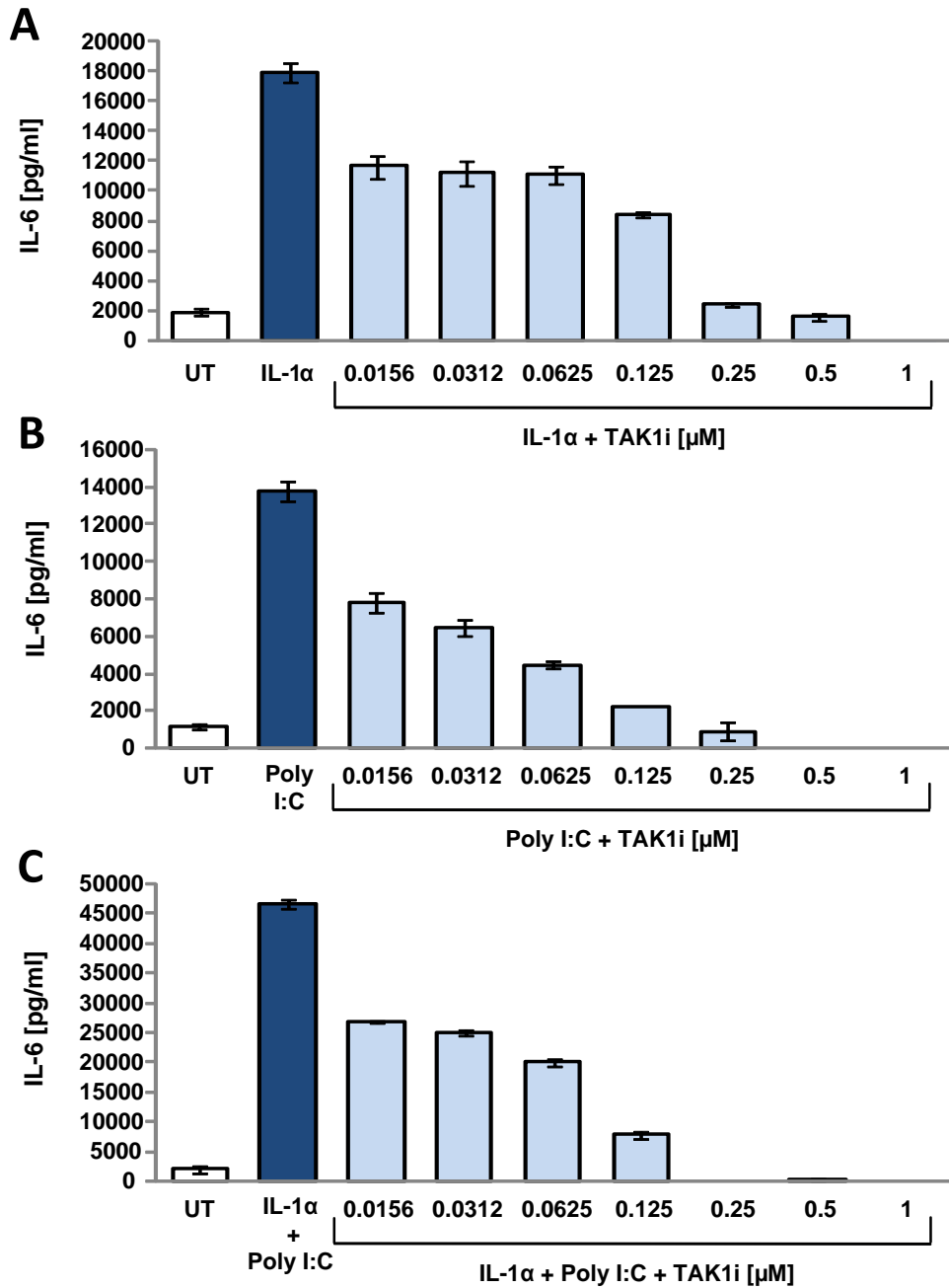
**Figure 50 Influence of TAK1i on IL-1α and Poly I:C induced IL-6 protein secretion by primary human lung fibroblasts**

Optimal TAK1i dose to inhibit IL-1α and Poly I:C- induced cytokine production by primary human lung fibroblasts was established analogically as described in SF.6 legend. The analysis revealed that a 1µM dose of TAK1i was sufficient to inhibit IL-6 protein secretion by >90% in response to IL-1α and Poly I:C. Consequently this dose was used in the subsequent experiments assessing the influence of TAKi on IL-1α and Poly I:C-induced IL-6 gene expression. Data represented as S.E.M. from 3 biological replicates.

**A**



**B**
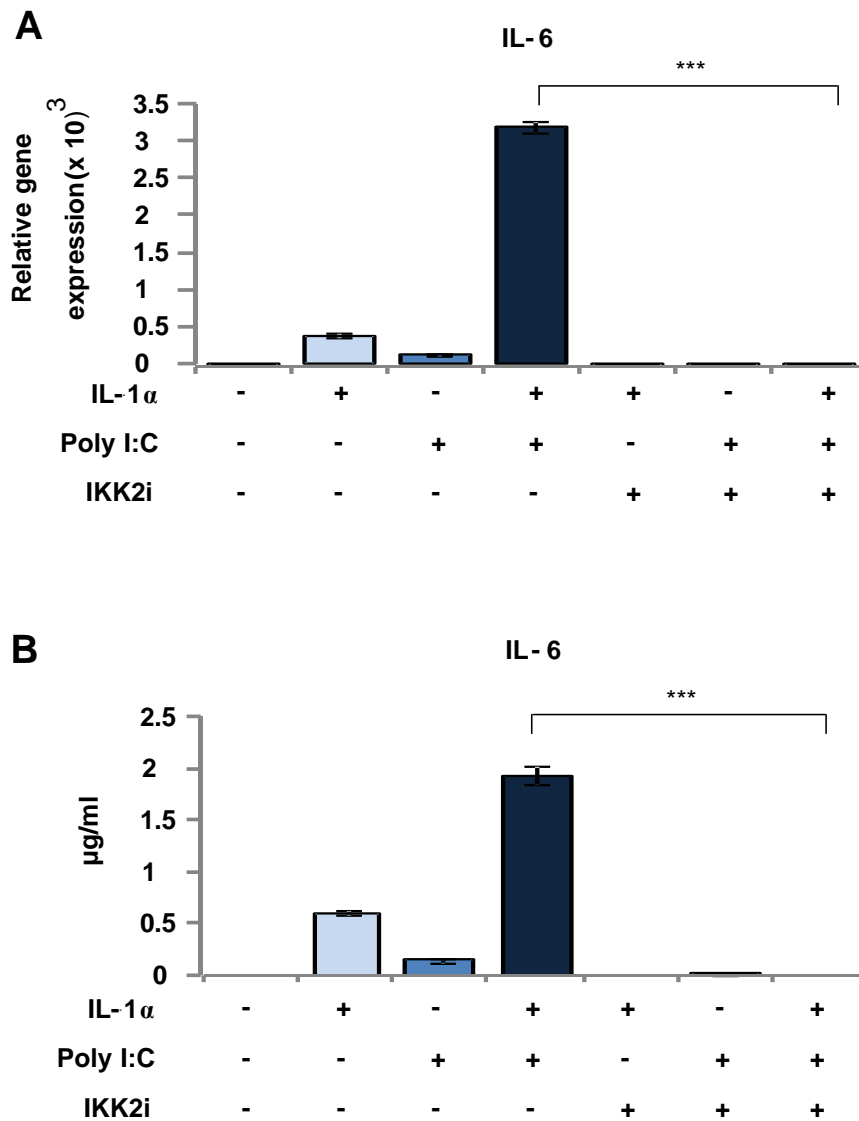


**Figure 51 Influence of IKK2 inhibitor (IKK2i) on IL-6 gene expression and protein release from primary human lung fibroblasts in response to IL-1α and Poly I:C.**

24hrs co-treatment with IL-1α (100pg/ml) and Poly I:C (10µg/ml) markedly enhanced gene expression of IL-6 (A) and protein release (B) in primary human lung fibroblast in comparison to treatment with IL-1α or Poly I:C alone. 1hr pretreatment with IKK2i (1µM) inhibitor significantly reduced IL-6 gene expression and protein release by primary human lung fibroblasts in response to treatment with IL-1α and Poly I:C individually or in combination. Relative gene expression was measured by qRT-PCR using GAPDH as a housekeeping gene. IL-6 protein concentrations were analyzed by ELISA. Data represent S.E.M. from 3 individual experiments performed on two biological replicates. Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01, ***p<0.001.
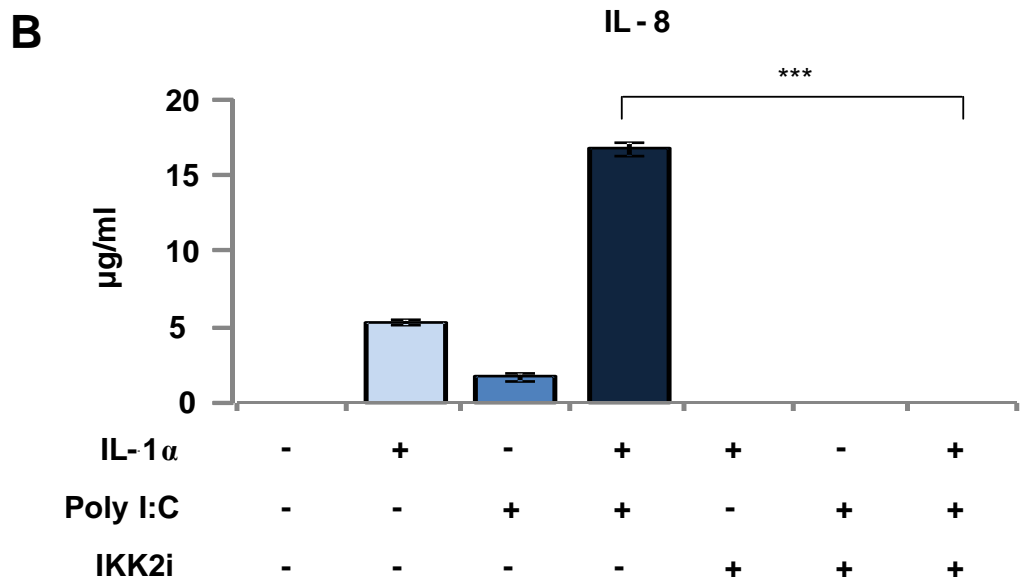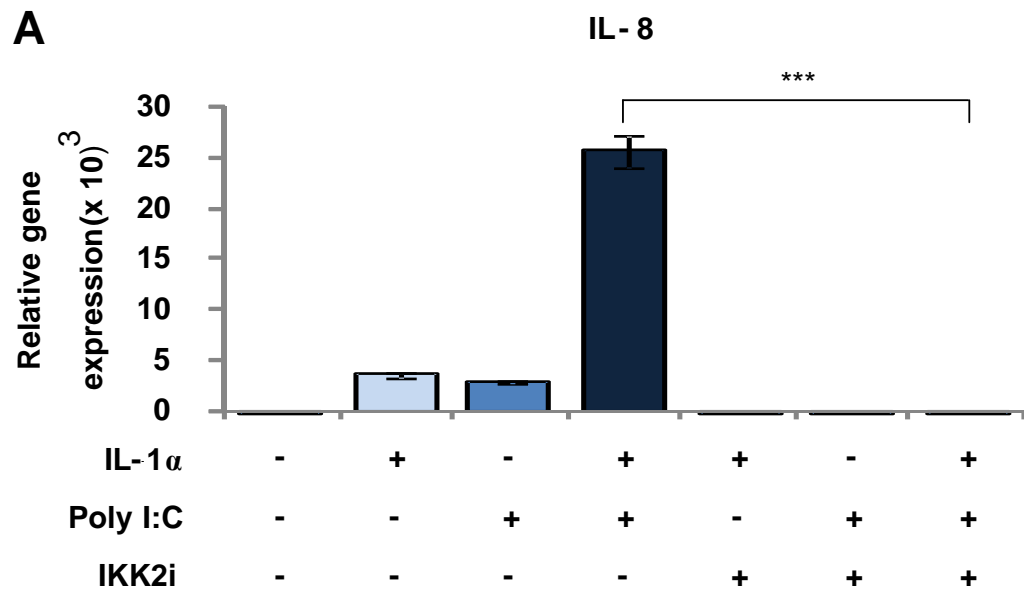
**Figure 52 Influence of IKK2 inhibitor (IKK2i) on IL-8 gene expression and protein release from primary human lung fibroblasts in response to IL-1α and Poly I:C.**

24hrs co-treatment with IL-1α (100pg/ml) and Poly I:C (10µg/ml) markedly enhanced gene expression of IL-8 **(A)** and protein release **(B)** in primary human lung fibroblast in comparison to treatment with IL-1α or Poly I:C alone. 1hr pretreatment with IKK2i (1µM) inhibitor significantly reduced IL-8 gene expression and protein release by primary human lung fibroblasts in response to treatment with IL-1α and Poly I:C individually or in combination (n=3, p<0.001 in all cases). Relative gene expression was measured by qRT-PCR using GAPDH as a housekeeping gene. IL-8 protein concentrations were analyzed by ELISA. Data represent S.E.M. from 3 individual experiments performed on two biological replicates. Statistical analysis was performed using Student's t test.  *p<0.05, **p<0.01, ***p<0.001.

**A**

IL - 6



| IL-1α | - | + | - | + | + | - | + |
| Poly I:C | - | - | + | + | - | + | + |
| TAK1i | - | - | - | - | + | + | + |

**B**

IL- 6



| IL-1α | - | + | - | + | + | - | + |
| Poly I:C | - | - | + | + | - | + | + |
| TAK1i | - | - | - | - | + | + | + |

**Figure 53 Influence of TAK1 inhibitor (TAK1i) on IL-6 gene expression and protein release from primary human lung fibroblasts in response to IL-1α and Poly I:C.**

24hrs co-treatment with IL-1α (100pg/ml) and Poly I:C (10μg/ml) markedly enhanced gene expression of IL-6 **(A)** and protein release **(B)** in primary human lung fibroblast in comparison to treatment with IL-1α or Poly I:C alone. 1hr pretreatment with TAK1 (1μM) inhibitor significantly reduced IL-6 gene expression and protein release by primary human lung fibroblasts in response to treatment with IL-1α and Poly I:C individually or in combination (n=3, p<0.001 in all cases). Relative gene expression was measured by qRT-PCR using GAPDH as a housekeeping gene. IL-6 protein concentrations were analyzed by ELISA. Data represent S.E.M. from 3 individual experiments performed on two biological replicates. Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01, ***p<0.001.

**A** IL- 8

**B** IL- 8

**Figure 54 Influence of TAK1 inhibitor (TAK1i) on IL-8 gene expression and protein release from primary human lung fibroblasts in response to IL-1α and Poly I:C.**

24hrs co-treatment with IL-1α (100pg/ml) and Poly I:C (10μg/ml) markedly enhanced gene expression of IL-8 **(A)** and protein release **(B)** in primary human lung fibroblast in comparison to treatment with IL-1α or Poly I:C alone. 1hr pretreatment with TAK1 (1μM) inhibitor significantly reduced IL-8 gene expression and protein release by primary human lung fibroblasts in response to treatment with IL-1α and Poly I:C individually or in combination (n=3, p<0.001 in all cases). Relative gene expression was measured by qRT-PCR using GAPDH as a housekeeping gene. IL-8 protein concentrations were analyzed by ELISA. Statistical analysis was performed using unpaired Student's t test.  *p<0.05, **p<0.01, ***p<0.001.

## 4.13 Influence of glucocorticoids and macrolide antibiotics on inflammation induced by IL-1α and Poly I:C

Currently patients affected by chronic post-transplant lung rejection are subject to strict anti-inflammatory and immunosuppressive therapy, which involves drugs such as macrolide antibiotics including azithromycin and glucocorticois.

In order to establish the influence of the aforementioned drugs on inflammation induced by IL-1α and Poly I:C, human primary lung fibroblasts were exposed to dexamethasone (0.1325-5µM) for 1hrs or azithromycin (0.078-5µM) for 24hrs, after which the cells were treated with IL-1α or Poly I:C or a combination of these two factors for 24hrs.

Following the treatment the conditioned media were collected and subjected to ELISA form IL-8 and IL-6.

The analysis revealed that dexamethasone significantly reduced the production of IL-6 and IL-8 in response to IL-1α and Poly I:C, while azithromycin was effective in dumping down inflammtion induced by Poly I:C but not IL-1α (figures 55-58).
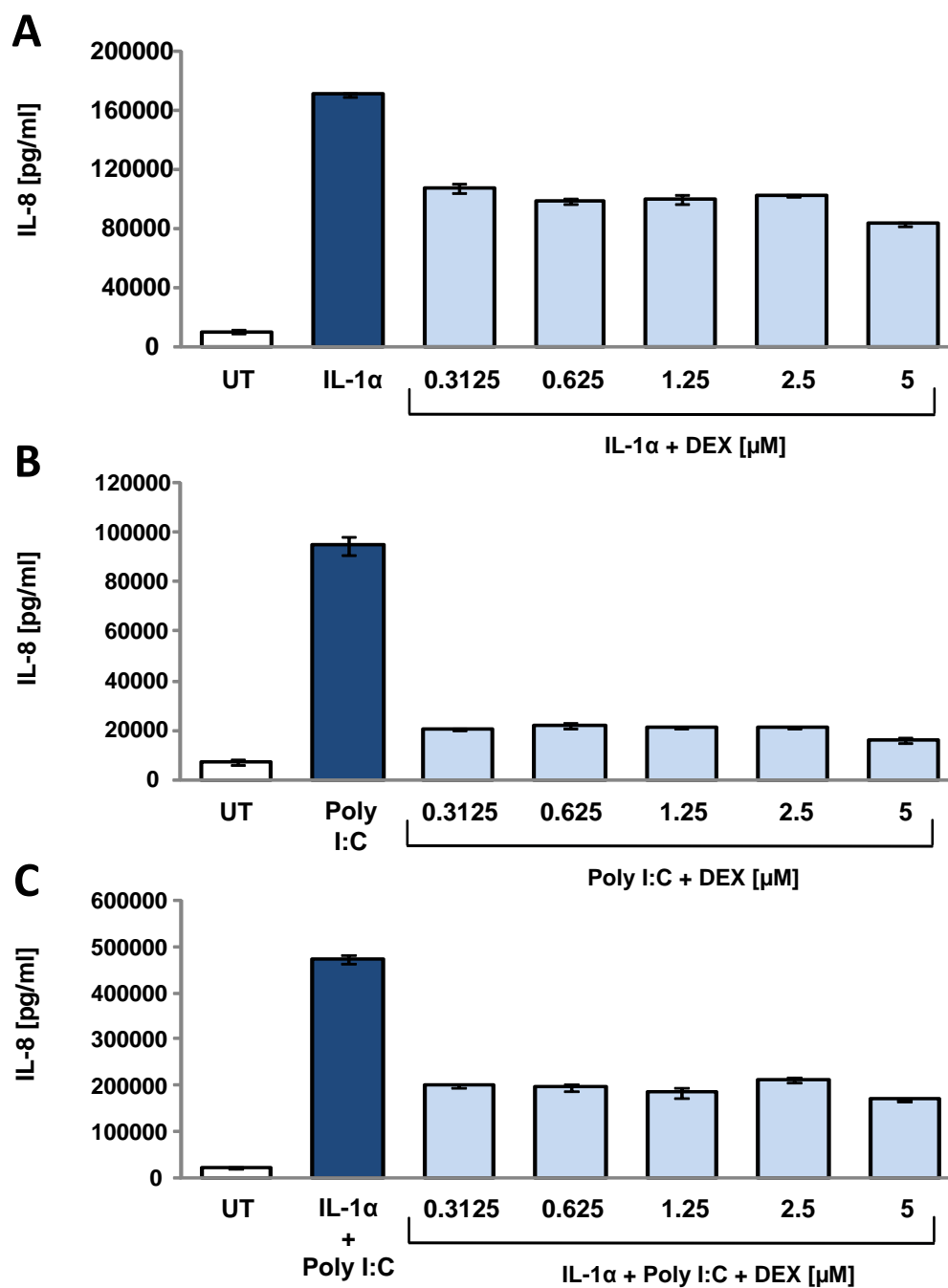
**Figure 55 Influence of Dexamethasone (DEX) on IL-1α and Poly I:C induced IL-8 protein secretion by primary human lung fibroblasts**

Primary human lung fibroblasts were treated with the indicated concentrations of Dexamethasone (DEX) for 1hr after which 100pg/ml of IL-1α (A) or 10pg/ml of Poly I:C (B) alone or in combination were added (C) to the culture and the cells were incubated at 37°C for the further 24hrs. Following the incubation conditioned media were collected and subjected to ELISA analysis for IL-8. All the tested DEX doses significantly reduced IL-1α and Poly I:C induced IL-8 secretion. Data represented as S.E.M. from 3 biological replicates.
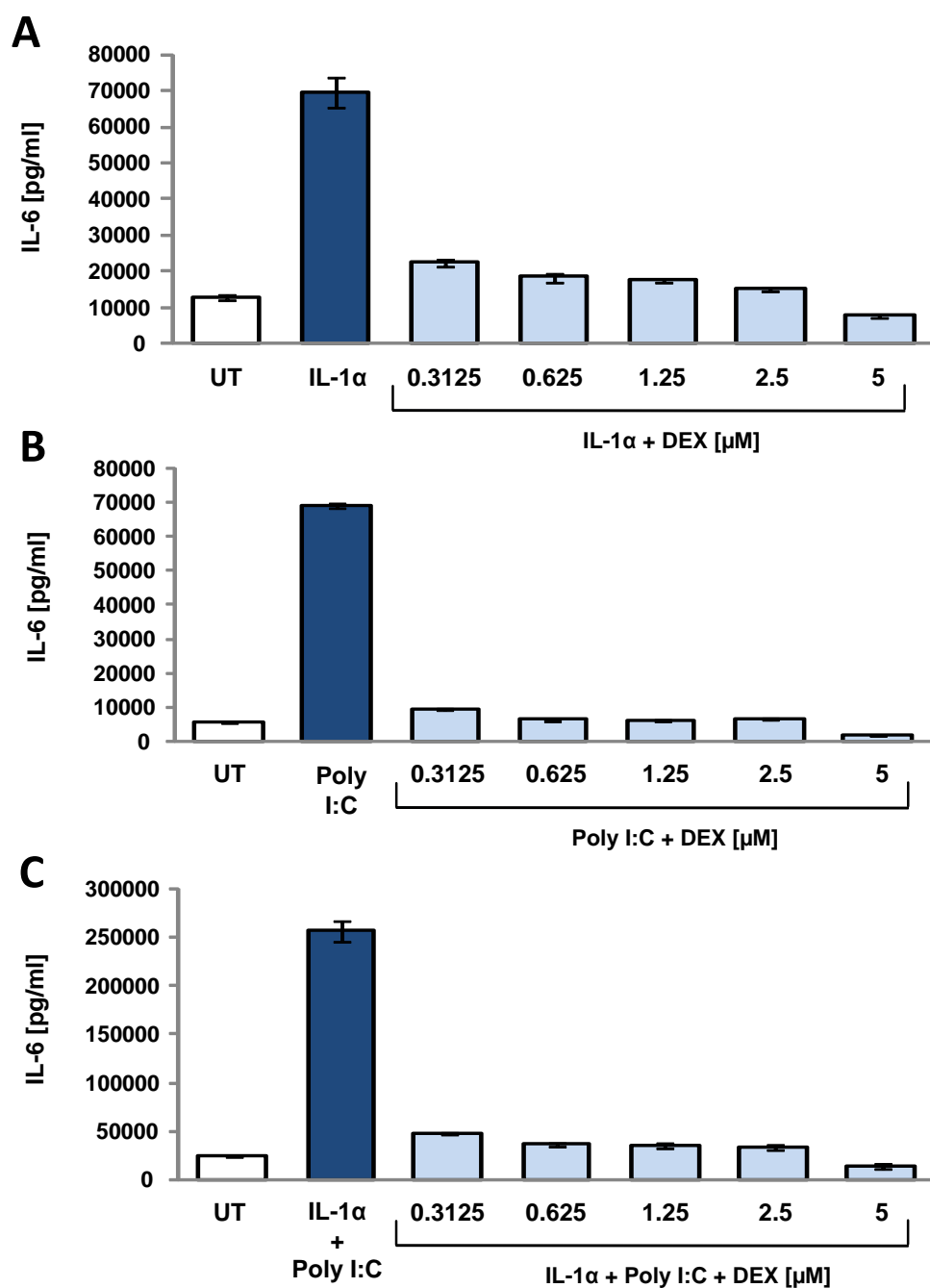
**Figure 56 Influence of Dexamethasone (DEX) on IL-1α and Poly I:C induced IL-6 protein secretion by primary human lung fibroblasts**

Primary human lung fibroblasts were treated with the indicated concentrations of Dexamethasone (DEX) for 1hr after which 100pg/ml of IL-1α (A) or 10pg/ml of Poly I:C (B) alone or in combination were added (C) to the culture and the cells were incubated at 37°C for the further 24hrs. Following the incubation conditioned media were collected and subjected to ELISA analysis for IL-6. All the tested DEX doses significantly reduced IL-1α and Poly I:C induced IL-6 secretion. Data represented as S.E.M. from 3 biological replicates.
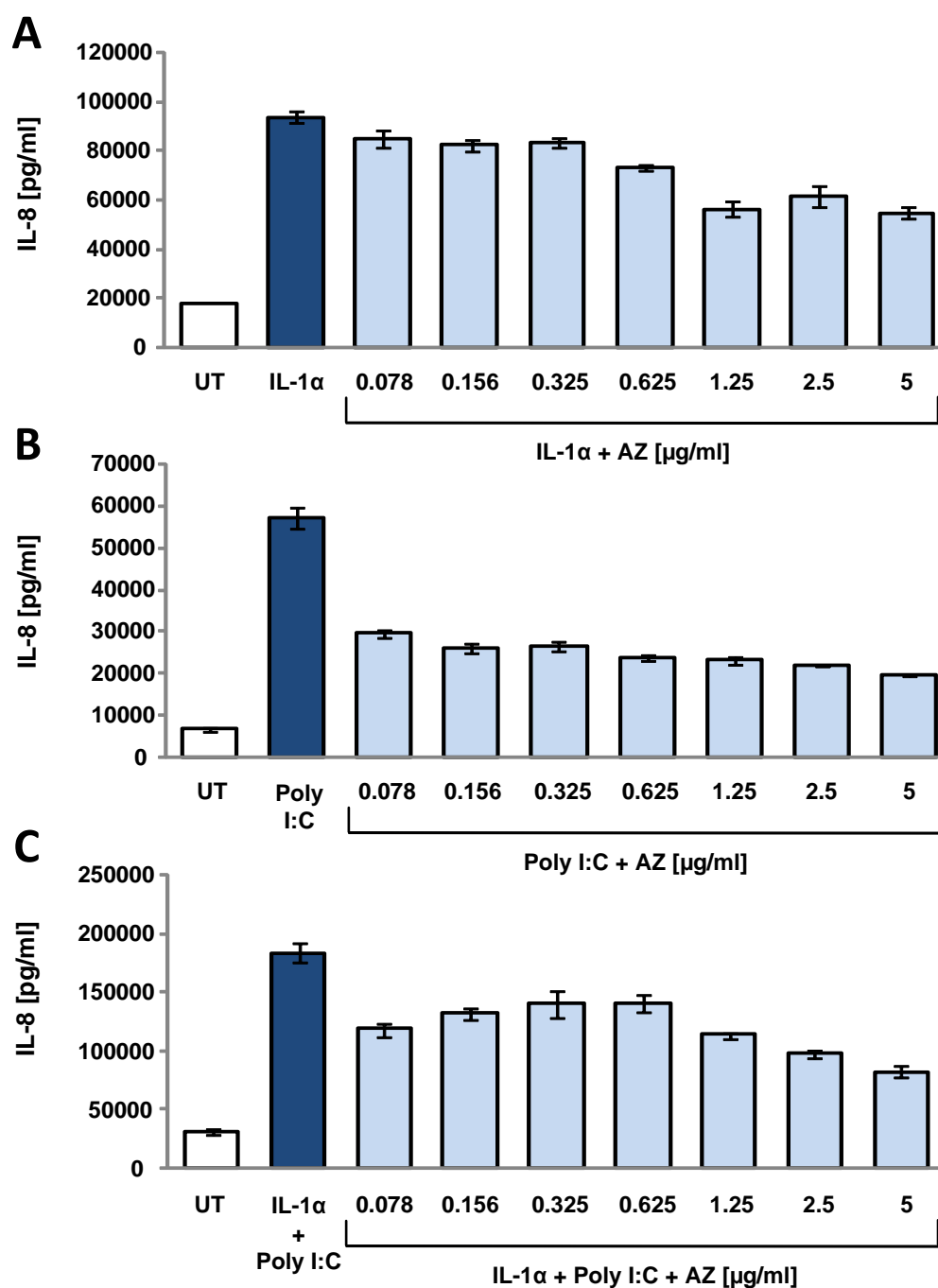
**Figure 57 Influence of Azithromycin (AZ) on IL-1α and Poly I:C induced IL-8 protein secretion by primary human lung fibroblasts**

Primary human lung fibroblasts were treated with the indicated concentrations of Azithromycin (AZ) for 24hr after which 100pg/ml of IL-1α (A) or 10pg/ml of Poly I:C (B) alone or in combination (C) were added to the culture and the cells incubated at 37°C for the further 24hrs. Following the incubation conditioned media were collected and subjected to ELISA analysis for IL-8. AZ partially reduced Poly I:C induced (B) IL-8 protein secretion but had no significant effect on the productions of IL-8 induced by IL-1α (A). Data represented as S.E.M. from 3 biological replicates.
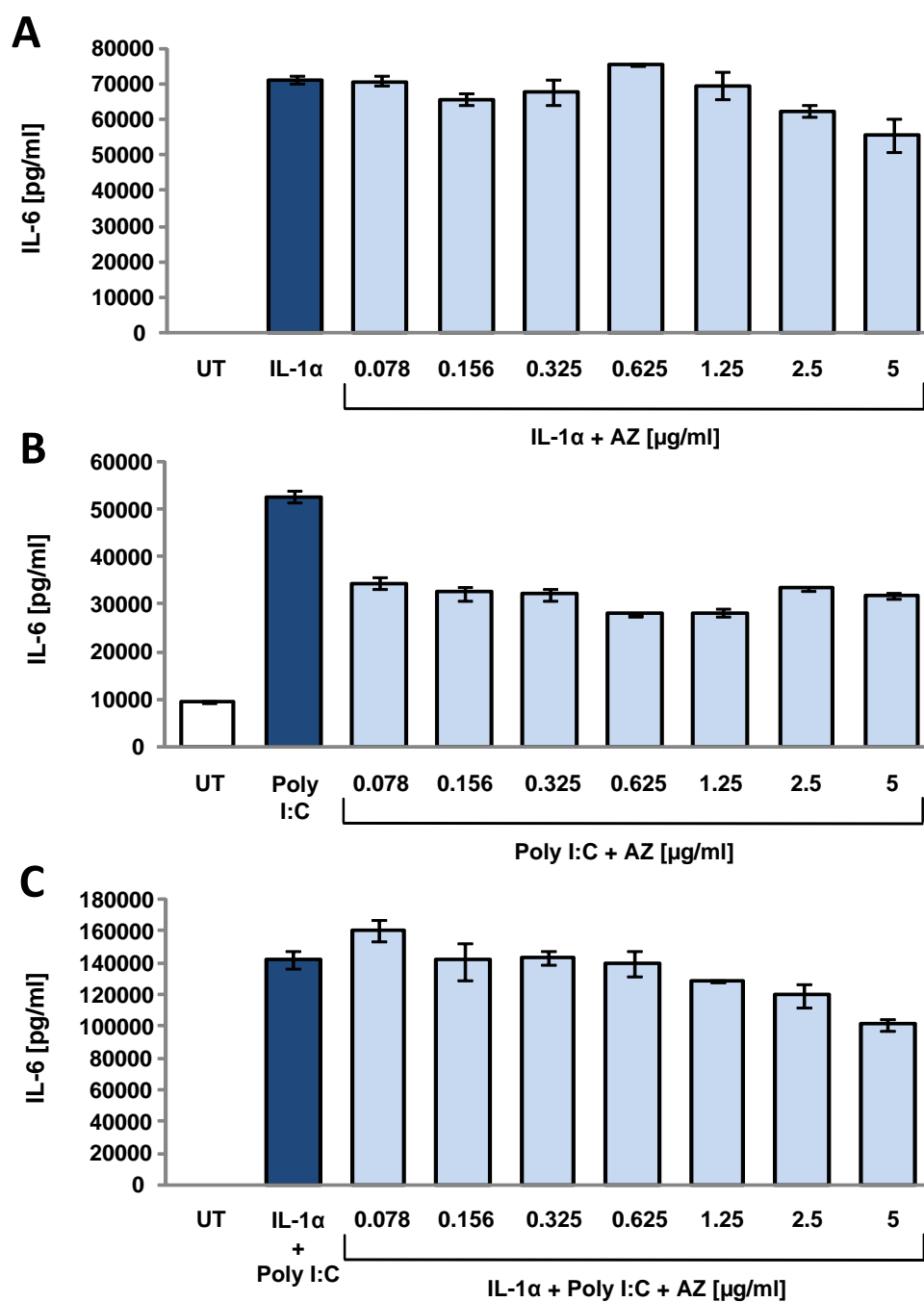
**Figure 58 Influence of Azithromycin (AZ) on IL-1α and Poly I:C induced IL-6 protein secretion by primary human lung fibroblasts**

Primary human lung fibroblasts were treated with the indicated concentrations of Azithromycin (AZ) for 24hr after which 100pg/ml of IL-1α (A) or 10pg/ml of Poly I:C (B) alone or in combination (C) were added to the culture and the cells incubated at 37°C for the further 24hrs. Following the incubation conditioned media were collected and subjected to ELISA analysis for IL-6. AZ partially reduced Poly I:C induced (B) IL-8 (n=3, p<0.01) protein secretion but had no significant effect on the productions of IL-6 induced by IL-1α (A). Data represented as S.E.M. from 3 biological replicates.

## 4.14 Influence of IL-1α on IL-8 protein secretion in primary human alveolar macrophages and PBEC cells

The main cellular key players involved in mediating innate immune responses in the lung include immune cells such as alveolar and interstitial macrophages and structural cells including alveolar and bronchial epithelial cells and interstitial fibroblasts. Therefore to establish how these cells may contribute to inflammatory responses mediated by IL-1α, primary human bronchial epithelial cells and human primary alveolar macrophages were subjected to a 24hrs treatment with a range of IL-1α doses, after which the conditioned media were collected for IL-8 ELISA. As a positive control TNFα or a lysate from Pseudomonas auriginosa were used to stimulate PBEC and alveolar macrophages respectively.

The analysis revealed that, the IL-1α treatment stimulated release of IL-8 from PBEC cells, however not from macrophages. To evaluate this result, macrophages were further stimulated with a range of IL-1β doses, however this treatment did not have a significant impact on their activation either (figure 59 and 60). Alveolar macrophages used in the experiment described above were isolated by centrifugation from BALs of 4 anonymous lung transplant recipients.
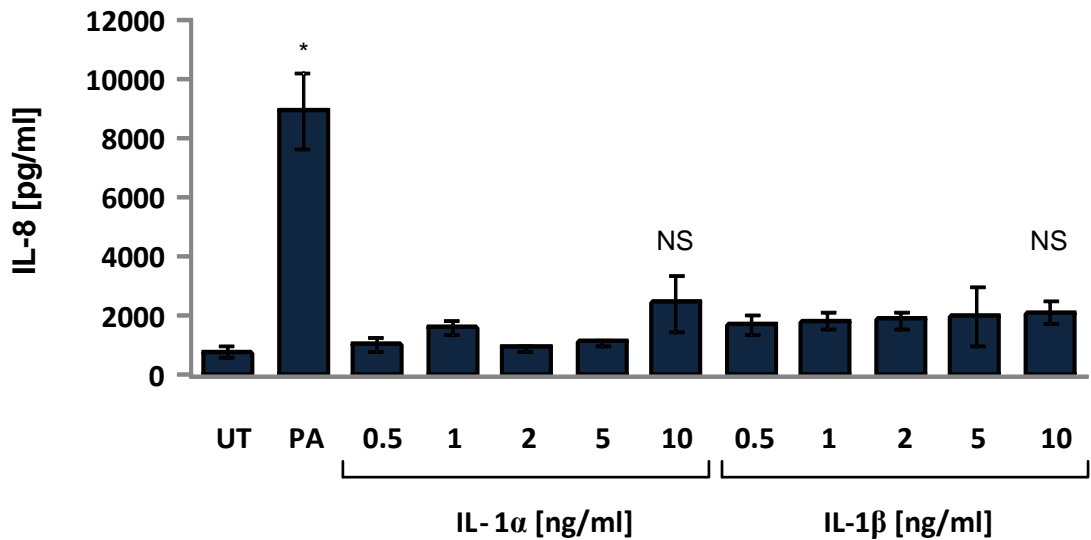
**Figure 59 Influence of IL-1α and IL-1β on IL-8 protein secretion by primary human alveolar macrophages.**

Primary human alveolar macrophages were challenged for 24hrs with the indicated doses of IL-1α and IL-1β, after which the conditioned media were collected and subject to ELISA for IL-8. As a positive control 25μl of a lysate from Pseudomonas aeruginosa (PA) was used. Data represent the mean±S.E.M. from 4 individual experiments performed on macrophages derived from 4 post-transplant patients. Statistical analysis was performed using Student's t t test.
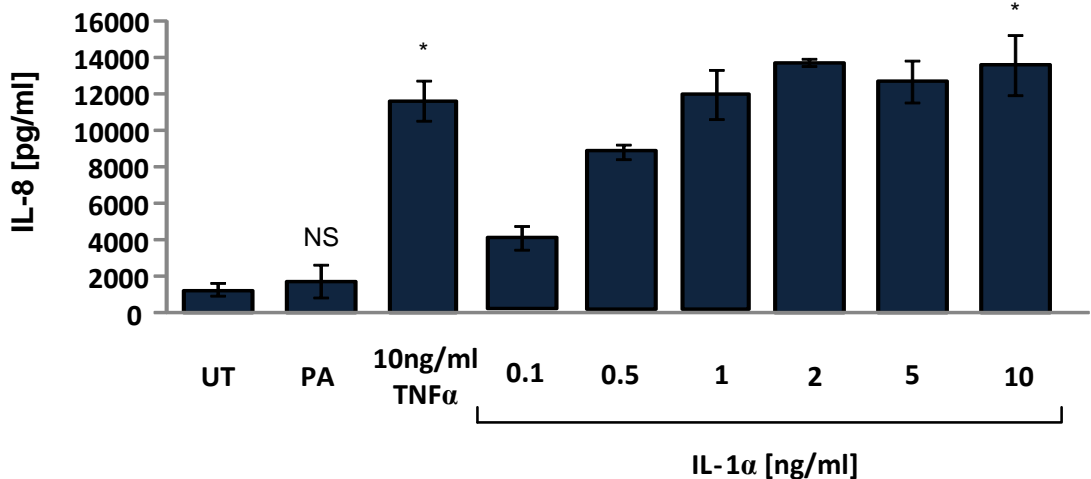


**Figure 60 Influence of IL-1α on IL-8 protein secretion by primary human bronchial epithelial cells (PBEC).**

Primary human bronchial epithelial cells (PBEC) were challenged for 24hrs with the indicated doses of IL-1α, TNFα or 25μl of a lysate from Pseudomonas aeruginosa (PA), after which the conditioned media were collected and subject to ELISA for IL-8. Data represent the mean±S.E.M. from 3 individual experiments performed on two biological replicates. Statistical analysis was performed using Student's t test.

**4.15 Discussion**

**4.15.1 Influence of IL-1α on fibroblast activation**

Fibroblasts represent a heterogeneous population of structural cells whose number significantly increases in fibrotic tissue where they may exceed 50% of the total cell population. Quiescent fibroblasts in normal tissues show low proliferation rate, limited contractibility and low expression levels of structural proteins including α-SMA and fibronectin. In fibrotic tissues and some tumors fibroblasts become activated, proliferate faster, gain the ability to migrate and express significantly higher levels of fibrogenic proteins. The overgrowth of fibrotic tissue is usually accompanied by progressive degeneration of the epithelial lining [248].

For decades, fibroblasts were regarded as cells whose role was limited to the production of the extracellular matrix. Therefore the majority of studies published to date focused on identifying factors that may stimulate fibroblasts proliferation and expression of fibrogenic factors in fibrotic tissues.

In many fibrotic diseases, tissue fibrosis is accompanied by chronic inflammation, however the role of fibroblasts in this process has not been investigated before.

Numerous studies investigating fibroblast biology suggest that fibroblasts are not a homogenous cell population, and that even fibroblasts residing in the same tissue may have different origin and behavior [249].

Although previous studies suggest that alarmins released from damaged cells may contribute to fibrosis, we did not find a link between IL-1α or conditioned media from injured epithelial cells and activation of pro-fibrotic genes in human lung fibroblasts. Fibroblast treatment with media from damaged cells failed to upregulate collagen gene expression. The literature lacks information on the influence of IL-1α on fibrotic factor expression, suggesting that there is no a direct link between IL-1α and the overgrowth of fibrotic tissue.

However previous reposts support our findings regarding the proinflammatory potential of IL-1α and the role of fibroblasts as mediators of chronic inflammation.

A study performed by Kawaguchi *et al.* demonstrated that endogenous IL-1α released by systemic sclerosis (SS) fibroblasts may stimulate production of IL-6 and PDGF-A in an autocrine manner [250]. Moreover the study demonstrated that blocking IL-6 using a specific monoclonal antibody reduced expression of procollagen I in SS fibroblasts. Additionally, Kawaguchi et all showed that fibroblasts from SS tissues produce increased levels of IL-1α when compared to fibroblasts from healthy individuals.

This observation is in compliance with our results, demonstrating that IL-1α may upregulate expression of its own transcripts in fibroblasts, which may exacerbate inflammation in tissues with ongoing epithelial injury.

Endogenous, cell bound IL-1α has also been recognized as the main factor responsible for upregulation of IL-6 and IL-8 in senescent fibroblasts [251]. Moreover senescent fibroblasts showed increased levels of IL-1α mRNA and intracellular protein. The number of senescent cells increases with age. Senescent fibroblasts are also present in some tumors and their presence is associated with increased production of proinflammatory cytokines.

The role of IL-1α as the main factor stimulating production of proinflammatory cytokines in the tumor microenvironment was demonstrated in a study performed using cancer-associated fibroblasts (CAFs). The study performed by Tjomsland *et al.* has shown that coculture of pancreatic ductal adenocarcinoma (PDAC) with CAFs leads to IL-1α dependant upregulation of proinflammatory factor (IL-1α, IL-6, CXCL8, VEGF-A, CCL20, and COX-2. CAFs) gene expression in fibroblasts, suggesting that anti-IL-1α therapies could be implemented in pancreatic cancer treatment [252].

The significance of IL-1α in tumor development was also confirmed in a study performed by *Xie et al.* demonstrating that fibroblasts promote squamous cell carcinoma cell matrigel invasive ability via a cathepsin K and IL-1α dependant manner [253].

IL-1α was also shown to promote a proinflammatory phenotype characterized by upregulation of IL-6, IL-8 and IL-1β gene expression in rheumatoid fibroblast-like

synovial cell line [254]. This process was demonstrated to be NF-κB and androgen receptor (AR) dependant and could be blocked by dihydrotestosterone.

Although the relationship between primary human lung fibroblasts and IL-1α released from damaged bronchial epithelial cells has not been studied before, there are reports indicating that IL-1α may contribute to fibroblast mediated inflammation in injured tissues. A study performed by Turner *et al.* confirms that IL-1α is capable of upregulating expression of IL-6, TNFα and IL-1β and increasing phosphorylation of ERK, JNK, p38 in cardiac myofibroblasts [255]. These results are in agreement with my observations, however primary human lung fibroblasts expressed insignificant levels of TNFα even after stimulation with IL-1α (data not shown).

Inflammation mediated by myofibroblasts, which are the main cell type building the heart structure, is of great importance in conditioned such as myocardial infarction, which is associated with death of myocardial fibroblasts [256].

The results described above are in compliance with our observations, demonstrating that fibroblasts may be a substantial source of numerous proinflammatory cytokines including IL-6, IL-8, MCP-1 and GM-CSF.

The current study revealed that upon stimulation with picogram concentrations of IL-1α, fibroblasts release nanogram to microgram amounts of IL-6 and IL-8, which resemblance the behavior of LPS-stimulated macrophages.

Additionally, my observation, that primary human alveolar macrophages do not secrete increased amounts of IL-8 in response to IL-1α, suggest that fibroblasts may play the key role in mediating inflammation in response to IL-1α released from injured epithelium.

### 4.15.2 Crosstalk between IL1α and TLR3

The current study demonstrated that a synthetic TLR3 ligand may synergize with IL-1α, resulting with marked upregulation of proinflammatory cytokines including IL-6 and IL-8, which may suggest that viral infections may exacerbate inflammation induced following epithelial injury.

The synergistic effect between IL-1α and Poly I:C and the crosstalk between TLR3 and IL-1R have not been described before. Previous studies however indicate that there exists a cooperation between TLR3 and IL-1R adaptor molecules: TRIF and MYD88. Ouyang *et al.* suggest that the synergistic effect between MYD88 dependant TLR4 and TLR3 signaling upon stimulation with LPS and TLR3 ligands: Poly I:C or CpG-DNA, that is manifested by enhanced expression of IL-12 and IL-6 may be mediated by IRF-5 which may directly interact with MyD88 to regulate expression of proinflammatory cytokines [257]. Moreover the study showed that the treatment with ligands of MYD88 and TRIF dependant TLRs results in a synergistic induction of IκBα – a regulator of NF-κB pathway. The results obtained in our study suggest that the synergy between IL-1α and Poly I:C which also signal via MYD88 and TRIF respectively may be NF-κB dependant and mediated by IKK2.

The current study demonstrated that the inflammation induced by IL-1α and Poly I:C could be effectively blocked with IKK2i, TAKi and a steroid drug dexamethasone, which functions as an inhibitor of NF-κB signaling pathway.

The role of IKK2 in TLR3 signaling finds confirmation is a study performed by Orita et all, which has shown that Poly I:C induced expression of adhesion molecules VCAM-1 and ICAM-1 in human corneal fibroblasts can be attenuated by IKK2i and a selective inhibitor of PI3Ks (LY294002) [258].

The involvement of TAK1 in the mediation of TLR3 signaling has been confirmed in a study by Bhattacharyya *et al.,* demonstrating that Poly I:C stimulates phosphorylation of TAK1 in mouse peritoneal macrophages, and this effect can be substantially attenuated by pretreatment with dexamethasone [259].

Additionally, Yao et all, has shown that both TAK1 and IKK2 are phosphorylated following IL-1R activation, and that this process is proceeded by IRAK1 phosphorylation and ubiquitin-dependant degradation, suggesting that IKK2 and TAK1 also participate in IL-1 induced signal transduction [260].

The role of IKK2 in IL-1R signaling also finds confirmation in studies by Salmerón et al [261] and Schwabe et al [262].

Although the crosstalk between IL-1R and TLR3 has not been investigated before, the studies exemplified above confirm our observations that signals triggered by both IL-1α and Poly I:C may be transduced by IKK2 and TAK1, and that it can be blocked using glucocorticoid drugs.

**4.15.3 Role of viral infections in chronic post-transplant lung rejection**

My study demonstrated that Poly I:C an analog of viral dsRNA ma activate innate immune responses in primary human lung fibroblasts.

Viral infections have been recognized as an important factor contributing to the pathogenesis of many chronic inflammatory diseases.

Following infection viruses replicate inside cells and therefore TLRs sensing viral PAMPs are located intracellularly. Two main TLRs capable to recognize viral danger signals include TLR3, sensing double stranded viral RNA and TLR7, able to bind single stranded viral RNA. Both TLR3 [263] and TLR7 [264] are located cytosolically in the endosomal membrane.

Another two receptors implicated in the recognition of double stranded viral RNA include RIG-I (retinoic-acid-inducible protein I/Ddx58) and MDA5 (melanoma-differentiation-associated gene 5/Ifih1). Although, both of these receptors are able to bind double stranded RNA, a study performed by Kato et al, demonstrated that there are differential roles of RIG-I and MDA5 in the recognition of RNA viruses. Using RIG-I-/- and MDA5-/- mice, Kato and al, have shown that RIG-I is required for the interferon production following infection with paramyxoviruses, influenza virus and Japanese encephalitis virus, while MAD5 is essential for picornavirus detection. Moreover the study revealed that RIG-I and MAD5 deficiency in mice was associated with increased susceptibility to viral infections in comparison to the control (WT) group [265].

These results suggest that intacellular receptors able to recognize double stranded RNA are essential for effective clearance of viral pathogens. One of the main mechanisms involved in counteracting viral infections in production of type I interferons, which inhibit cell division. Activation of TLR3 [266], RIG-I [267] and MAD5 [268] leads to increased expression of type I interferons, which may inhibit

cell proliferation as well as many other proinflammatory cytokines including IL-8 and IL-6.

Upregulation of type I interferons and proinflammatory cytokines is essential for viral pathogen clearance during acute infection. However unresolved or repetitive infections may lead to chronic inflammation causing tissue injury and progressive degeneration of the respiratory epithelium.

Viral infections have been associated with the pathogenesis of several chronic lung diseases including BOS [173], IPF [269] and lung cancer [270].

A retrospective study on 259 adult lung transplant recipients performed over a 5 year period, demonstrated that patients with community-acquired respiratory viral (CARV) infections were more likely to develop BOS and BOS-related death [173].

These observations were confirmed in another study performed by Billings et al, which demonstrated that patients with the lower respiratory tract infection with CARV showed predisposition to high-grade BOS development. Moreover the study has shown that patients BOS patients were predisposed to CARV infections, which may be due to immunosuppressive therapy that the lung transplant recipients are subjected to [271].

These results found confirmation in an animal study performed by Kuo et al, who have shown that infections with Sendai virus (SdV) can lead to enhanced tracheal allograft rejection in mouse and that this effect could be prevented by pretransplant immunization against SdV infection [272].

The results described above are in agreement with our observations that Poly I:C, a synthetic analog of double stranded viral RNA, accentuated fibroblast-mediated production of proinflammatory cytokines induced by IL-1α, suggesting that viral infection may exacerbate inflammation triggered by epithelial damage. This observation may explain why lung transplant recipients are more susceptible to viral infections.

Apart from enhancing inflammatory responses, viral infections may contribute to fibrosis, which is characteristic for many degenerative diseases including BOS and IPF.

The relationship between viral infections and pulmonary fibrosis was confirmed in a study conducted by Qiao et al, revealing that mice infected with H5N1virus have developed interstitial and intra-alveolar fibrosis, thickened and collapsed alveoli, had elevated hydroxyproline levels and significantly increased dry lung-to-body weight ratio compared to the control group [273].

Although viral infections have been recognized as an important risk factor increasing the likelihood of complications following lung transplantation, coming in contact with viral pathogens is not a prerequisite for BOS development.

Despite impaired immune system, whose functions in post-transplant patients are substantially limited by the immunosupresive therapy, viral infections are often effectively combated and cleared in BOS patients. There is however a vast spectrum of viruses which developed sophisticated mechanisms which allow them to effectively multiply in the immunosupressed microenvironment.

One of the mechanisms allowing selected viruses for more effective succession is the encoding of the nonstructural proteins NS1 and NS2, which regulate cellular RNA transport, splicing and translation and acts as interferon inhibitors. Genes encoding NS1 and NS2 proteins have been found in RNA of viruses such as influenza virus H5N1 [274] and human respiratory syncytial virus (RSV), that has been implicated as an important risk factor in BOS [275].

Considering that type I interferons by suppressing viral replication, provide the main mechanism of defense against viruses, NS1 and NS2-dependant inhibition of interferon activity may have serious implications in patients whose functions of the immune system has been compromised by the immunosupresive therapy.

Although the exact mechanism of NS1 and NS2-dependant inhibition of interferon function is unclear, several lines of evidence suggest that it may be executed by the supression of Stat2 expression, which acts as a signaling molecule within Interferon-α/β receptor (IFNAR) signaling pathway [275].

Another virus that often affects lung transplant recipients is cytomegalovirus (CMV), however its role in BOS pathogenesis is controversial [276].

Studies performed by Paraskeva et al [172] and Danziger-Isakov et al [277], suggest that there is an association between CMV infection and BOS development.

**4.15.4 Steroid and macrolide resistance in chronic lung allograft rejection**

Current immunosuppressive drugs used in lung transplant recipients include calcineurin inhibitors such as cyclosporine or tacrolimus and corticosteroids such as prednisolone and therapies in bronchiolitis obliterans syndrome include macrolide antibiotics, mainly azithromycin [183]. Our study revealed that inflammation induced by IL-1α in fibroblasts can be partially blocked by the corticosteroid drug, dexamethasone, but not with azithromycin. Interestingly, both drugs were able to partially inhibit production of proinflammatory cytokines triggered by Poly I:C.

Glucocorticoid drugs, including dexamtethasone, act via a cytoplasmic, glucocorticoid receptor (GR) [278]. Upon ligand binding, GR is translocated from cytoplasm to nucleus, where it functions as a transcription factor activating a vast array of anti-inflammatory genes including lipocortin I and p11/calpactin binding protein, downregulating release of arachidonic acid [279], secretory leucocyte protease inhibitor (SLPI) [280], and the decoy IL-1 type II receptor [281].

Steroids are effective anti-inflammatory drugs, however a large number of patients affected by chronic inflammatory diseases develop steroid resistance [282]. Glucocorticoid resistance has been recognized as a major obstacle in the treatment of a vast array of diseases including COPD [283], acute respiratory distress syndrome [284], asthma [285], rheumatoid arthritis [286], and inflammatory bowel disease [287]. Extensive studies identified several potential mechanisms responsible for this process, with the most probable being: activation of (MAP) kinase pathways by certain proinflammatory cytokines [288], upregulation of macrophage migration inhibitory factor (MIF) [289], increased activation of the transcription factor activator protein 1 (AP-1) [290], reduced histone deacetylase-2 (HDAC2) expression [291], and increased P-glycoprotein-mediated drug efflux [292].

Patients who developed steroid resistance may be subject to alternative anti-inflammatory therapies using inhibitors of calcineurin [293], phosphodiesterase 4 [294] or NFκB [295], although these drugs are associated with severe side effects.

My study demonstrated that inflammation induced by IL-1α can be effectively reduced using glucocorticoids. Therefore, anti-IL-1α therapies using IL-1Ra or monoclonal anti-IL-1α antibodies could be considered as potential treatment to reduce inflammation in patients who do not respond to glucocorticoids. IL-1Ra has been clinically tested and its use is not associated with major side effects, which makes it a promising candidate for an anti-inflammatory drug to treat long-lasting conditions [296].

Additionally, the current study demonstrated that inflammation induced by a suboptimal dose of 100pg/ml of IL-1α could not be effectively reduced by azithromycin, which is commonly used to treat chronic post-transplant lung rejection. It is estimated that azithromycin is effective in approximately 50% of post-transplant patients who develop BOS. The exact mechanism of action of this macrolide antibiotic is still to be unraveled, however according to some studies azithromycin reduces inflammation by inhibiting production of proinflammatory cytokines. Interestingly, azithromycin was shown to be effective also in BOS patients who remained infection free, suggesting that its mechanism of action is not narrowed down to anti-microbial properties. Although azithromycin was demonstrated to be beneficial for a significant number of patients who developed chronic post-transplant lung rejection, many subjects do not respond to macrolides, which is a significant obstacle in the treatment of BOS.

Many bacteria commonly found in post-transplant patients, including Streptococci spp., eg. Streptococcus pneumoniae, Staphylococcus aureus and nontuberculous mycobacteria may develop resistance to macrolides, which partially explains the basis of azithromycin resistance. [297].The results described in the current study suggest that anti-IL-1α compounds could be considered as a potential anti-inflammatory therapy for post-transplant patients who are unresponsive to azithromycin.

## 4.15.5 PRR expression and responsivenes to Damage and Pathogen Associated Molecular Patterns of cells participating in mediating innate immunity in the lung

The pattern of PRR expression is cell and tissue specific. High expression of the majority of PRR has been found in alveolar macrophages and neutrophils, which are responsible mainly for mediating inflammatory responses to bacterial danger signals. Interestingly, my study revealed that primary human alveolar macrophages, in contrary to primary human lung fibroblasts and bronchial epithelial cells, do not respond to IL-1α or IL-1β, suggesting that the inflammatory responses to IL-1R ligands are mediated by structural rather than immune cells. However, one of the limitations of the study was the fact that the only available source of primary human alveolar macrophages were BALs from post-transplant patients, and therefore it cannot be rulled out that macrophages derived from airways of healthy subjects could respond to IL-1 treatment in a different way.

The primary macrophages used in the study responded positively to PA treatment, which indicates that these cells exhibit functional TLR2 and TLR4 signaling.

TLR2 is able to recognize a vast array of bacterial wall components including peptidoglycan and lipoarabinomannan, which is a glycolipid derived from Mycobacterium tuberculosis [298], while TLR4 ligands LPS (a cell wall component of gramm-negative bacteria) [299], respiratory syncytial virus protein F [300], and Taxol, a plant derived product, possessing anti-mitotic properties that is currently used in anti-cancer therapy [301]. Another TLR that is able to recognize bacterial danger signals is TLR5, which may bind flagellin, a structural protein present in the flagellum of almost all flagellated bacteria including Helicobacter pylori and Esterichia coli [302]. TLRs expressed in the lung are also able to recognize endogenous danger signals including β-defensins (recognized by TLR4) [303], HSP60 (a TLR2 ligand) [304] and fibronectin fragments (TLR4 ligands) [305] that are generated in response to tissue injury.

Although alveolar macrophages has been considered to be the key mediators of innate immune responses in the lung, increasing evidence, including the present study, suggests that also structural cells may play an important role in this process.

Type II human alveolar epithelial cells were found to express TLR2 mRNA transcripts and protein, suggesting that the respiratory epithelium may be involved in mediating infalammatory responses to mycobacterial PAMPs such as peptidoglycan, lipoteichoic acid, lipoproteins, and lipoarabinomannan [306]. Moreover TLR2 and TLR4 were demonstrated to be involved in pathogen clearance following infection with Mycobacterium tuberculosis, the causative agent of the majority of tuberculosis cases [307].

Innate immune responses to pathogen derived danger signals may also be mediated by bronchial epithelial cells that showed increased levels of β-defensins and IL-8 following treatment with E. coli derived LPS [308]. A detailed information on the TLR expression pattern in primary human bronchial epithelial cells has been provided by study performed by Mayer et al, who report that these cells express functional TLR1-6 and TLR9. Moreover the authors have demonstrated that PBEC cells respond much stronger to Gram positive than Gram negative bacteria, which may be due to the lack of expression of CD36, which functions of a co-receptor supporting TLR2 signaling [309].

Additionally, my study demonstrated that primary human bronchial epithelial cells are responsive to IL-1α, suggesting that damage to epithelium may trigger the inflammatory responses in adjacent epithelial cells in a paracrine manner.

Bronchial and alveolar epithelial cells pose the first line of defense against invading pathogens and microparticles in the lung. However in many chronic inflammatory lung diseases the respiratory epithelium undergoes a progressive degenerative process and becomes replaced by intensively proliferating myofibroblasts which may take over the role of the innate immune response mediators.

# CHAPTER 5 IL-1α AND OTHER PROINFLAMMATORY CYTOKINE PROTEIN PROFILING IN LUNG ALLOGRAFT RECIPIENTS

## 5.1 Background

Chronic lung allograft dysfunction produces a significant loss of function of the transplanted lung and is believed to represent the process of chronic rejection and accounts for the limited long term survival after lung transplant [13]. When the process affects the small and medium sized airways causing airflow limitation it is referred to as bronchiolitis obliterans syndrome (BOS). BOS is commonly a progressive condition and is often accompanied by repeated or chronic lower respiratory tract infection. Histologically, BOS is characterized by progressive loss of bronchial epithelium, neutrophil influx, chronic inflammation and fibroproliferation causing small airway obliteration. Interestingly, small airway scarring may also occur in non-transplant patients suggesting that allogenic reaction is not a prerequisite for epithelial degeneration [310]. Numerous studies suggest that specific viral or bacterial infections significantly increase the risk of developing BOS both in post-transplant and non-transplant patients [310, 311]. Continuous tissue injury that may be caused by repeated or unresolved infection which may lead to release of alarmins, activation of innate immunity and attraction of ROS-producing neutrophils that drive the vicious cycle of chronic inflammation [95].

Chronic inflammation and progressive fibroproliferation are the two characteristic features of BOS [312]. The relevance of IL-1α in BOS has not been evaluated before and therefore to address the clinical relevance of this alarmin in BOS, IL-1α concentrations were measured in BALs of 52 post-transplant patients patients who developed BOS or remained stable. Additionally, a correlation between IL-1α levels, IL-8 and neutrophil percentage was assessed.

The levels of IL-1α and other selected peoinflammatory cytokines were also measured in BALs of post-transplant who developed different phenotypes of chronic post-transplant lung rejection.

**Hypotheses tested:**

1. Increased IL-1α levels in BALs of lung transplant recipients are associated with development of BOS.

2. IL-1α levels positively correlate with IL-8 concentrations and neutrophil percentage in BALs of post-transplant patients.

3. Neutrophilic phenotypes of chronic post-transplant lung rejection (ARAD - Azithromycin reversible allograft dysfunction and PAN - persistent airway neutrophilia), are characterized by increased IL-1α levels in BALs.

## 5.2 Characteristics of patients participating in the study

Fifty two lung transplant recipients within 3 years of transplantation were identified from a cohort under follow up at the Institute of Transplantation, Freeman Hospital, Newcastle (table 5). These included 26 who developed chronic lung allograft dysfunction as BOS (16 Male and 10 Female) and 26 who remained stable and BOS-free (21 Male and 5 Female). All recipients had undergone surveillance bronchoscopic evaluation at 1, 3, 6 and 12 months post transplant and again at the time BOS was suspected or diagnosed. A standardized bronchoalveolar lavage (BAL) comprising 180mls of sterile normal saline was performed from a subsegment of the right middle or lower lobe or left lower lobe. 119 BAL samples were collected from the group who developed BOS and 94 BAL samples were collected from the stable group.

**BOS group**

| | |
|---|---|
| **No. of Patients** | n=26 |
| **Median Age** | 46 years |
| **Age range** | 23 - 63 years |
| **Sex distribution** | 10 Female : 16 Male |
| **Underlying condition** | COPD (n=9), Cystic Fibrosis (n=7), Fibrotic Lung Disease (n=5), alpha-1 anti- trypsin deficiency (n=2), Asthma (n=1), Hystiocytosis X (n=1), Lymphangioleiomyomatosis (n=1) |
| **Mean BAL samples** | 5 / patient |
| **Type of Transplant** | 17 Bilateral Lung : 9 Single Lung |
| **Organisms cultured from BAL** | Pseudomonas Aeruginosa (n=19), Candida Albicans (n=13), Aspergillus fumigatus (n=6), Proteus Mirabilis (n=4), Stenotrophomonas maltophilia (n=4), Staph Aureus (n=2), Acinetobacter baumannii (n=1), Enterobacter cloacae (n=1), Haemophilus influenza (n=1), Serratia (n=1), Klebsiella pneumonia (n=1) |

**non-BOS group**

| | |
|---|---|
| **No. of Patients** | n=26 |
| **Median Age** | 45 years |
| **Age range** | 19 - 64 years |
| **Sex distribution** | 5 Female : 21 Male |
| **Underlying condition** | Cystic Fibrosis (12), Fibrotic Lung Disease (8), COPD (2), alpha-1 anti-trypsin deficiency (2), Primary Pulmonary Hypertension (1), Bronchiectasis (1) |
| **Mean BAL samples** | 3.62 / patient |
| **Type of Transplant** | 18 Bilateral Lung, 7 Single Lung and 1 Heart-Lung |
| **Organisms cultured from BAL** | Pseudomonas Aeruginosa (14), Candida Albicans (18), Proteus Mirabilis (), Aspergillus fumigatus (7), MRSA (7), Staph Aureus (4), E. Coli (2), Serratia (1), Burkholderia cepacia complex (1), Exophiala sp (1). |

**Table 12 Demographics of lung transplant recipients participating in the study**

**5.3 Levels of IL-1α in BALs of post-transplant patients in lead up to BOS diagnosis**

To evaluate the clinical relevance of the investigated alarmins as mediators of inflammation in human degenerative lung disease, IL-1α and HMGB-1 levels were measured in bronchoalveolar lavage (BAL) fluid collected longitudinally from 26 lung transplant recipients in follow up to BOS diagnosis. An MSD and ELISA analysis revealed that in patients, HMGB-1 levels did not change (data not shown) in the lead up to BOS whereas levels of IL-1α were significantly higher upon BOS diagnosis (4.71 (1.12-14.67) pg/ml) than when measured at least 3 months before the disease was recognized (2.01 (0-14.67) pg/ml) (Figure 61).

One of the risk factors, significantly increasing the risk of developing BOS post transplantation are bacterial, viral and fungal infections. Invading pathogens not only contribute to chronic inflammation, but may also cause tissue damage. Therefore to assess the effect of pathogen-induced airway injury on IL-1α concentration, the influence of bacterial infections on inflammatory parameters was examined in 26 lung transplant recipients who developed BOS and an age matched control group of 26 who remained free from BOS. The MSD analysis revealed that lung transplant recipients with positive BAL bacterial cultures had higher BAL concentrations of IL-1α (BOS 4.38 (0.64-40.11); non-BOS 3.36 (0-63) pg/ml) compared to patients whose BAL cultures were negative for bacteria (IL-1α: BOS 2.41 (0-19.08); non-BOS 1.42 (0-17.41) pg/ml) (figure 61). The same pattern was also observed for IL-8 (figure 63).

The expression of IL-1α protein in human bronchial and alveolar epithelium was confirmed using immunohistofluorescence (figure 62). This observation confirms that injured respiratory epithelium may be one of the potential sources of IL-1α in BAL.
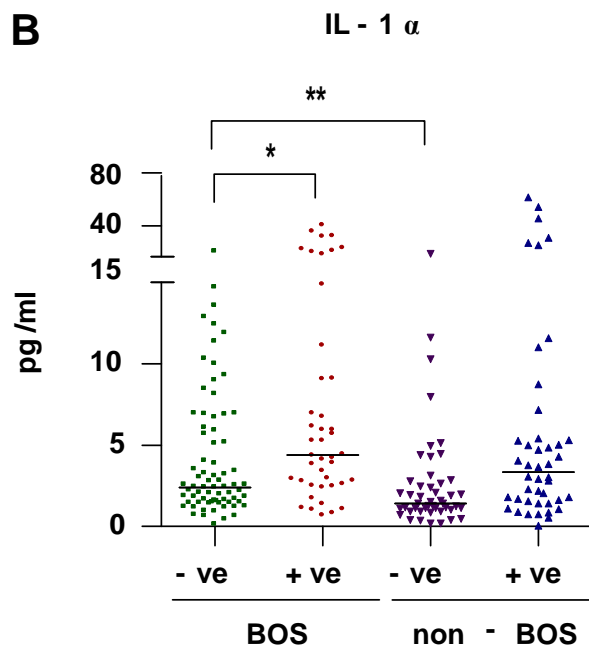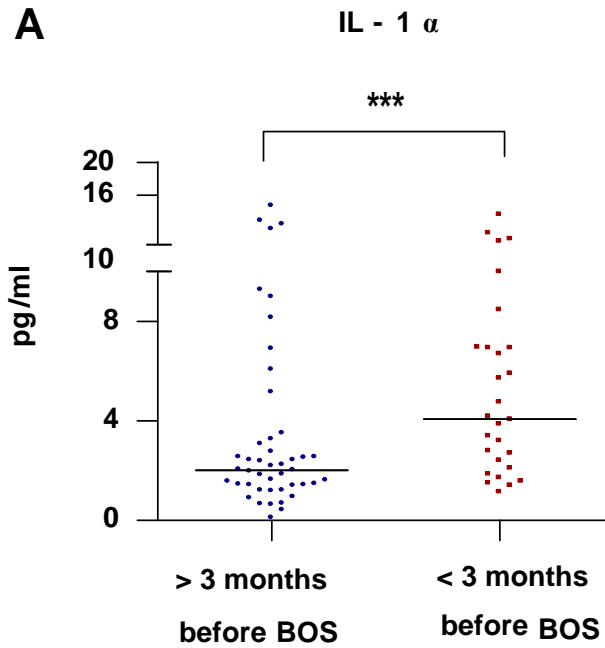
**Figure 61 IL-1α levels in BALs of post-transplant patients in lead up to BOS diagnosis.**

IL-1α (A) concentrations in BALs of lung transplant recipients who develop BOS: Group 1: BAL samples taken >3 months before BOS diagnosis (>3 months before BOS), group 2: BAL samples taken in the 3 months before and after BOS diagnosis (<3 months before BOS). IL-1α (B) concentrations in culture positive (+ve) and culture negative (-ve) BAL from post-lung transplant recipients who went on to develop BOS (BOS) or remained stable (non-BOS). IL-1α concentrations were measured using MSD assay. Data was analyzed using Mann-Whitney U test and is presented as median. *p<0.05, **p<0.01, ***p<0.001.

**A**

**B**

**C**
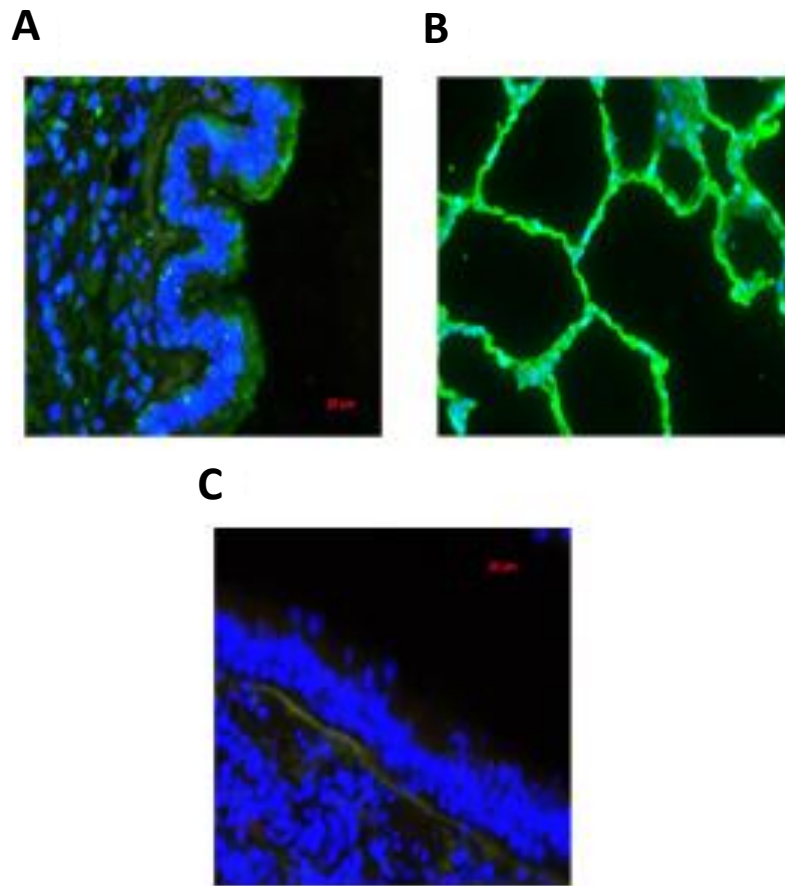
Figure 62 IL-1α protein expression in human (A) bronchial and (B) alveolar epithelium visualized with immunohistofluorescence using anti-human IL-1α antibody. (C) Secondary antibody control (staining performed in the presence of the secondary antibody conjugated with FITC but in the absence of the primary antibody). Magnification: x20.
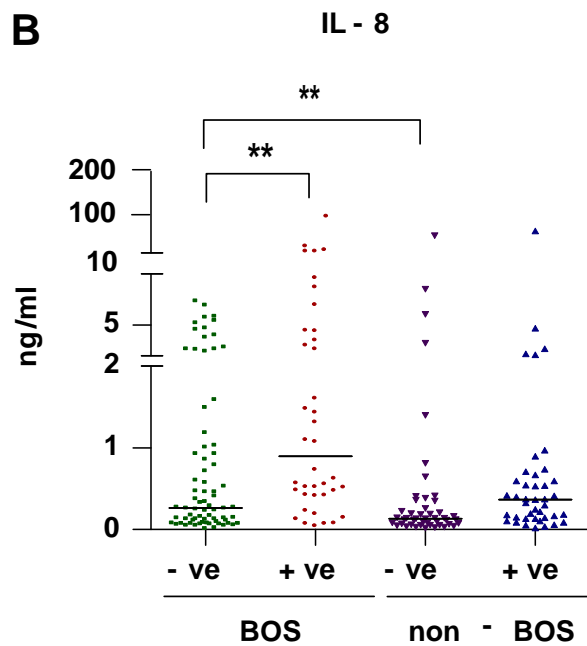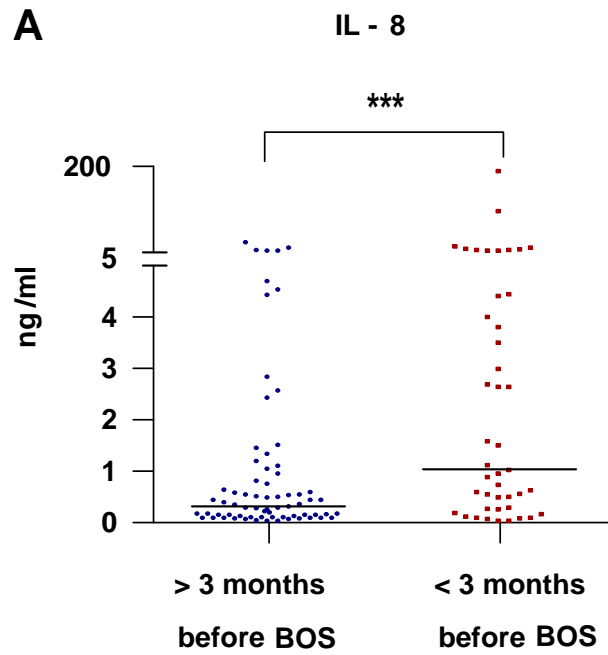
**Figure 63 IL-8 levels in BALs of post-transplant patients in lead up to BOS diagnosis.**

IL-8 (A) concentrations in BALs of lung transplant recipients who develop BOS: Group 1: BAL samples taken >3 months before BOS diagnosis (>3 months before BOS), group 2: BAL samples taken in the 3 months before and after BOS diagnosis (<3 months before BOS). IL-8 (B) concentrations in culture positive (+ve) and culture negative (-ve) BAL from post-lung transplant recipients who went on to develop BOS (BOS) or remained stable (non-BOS). Data was analyzed using Mann-Whitney U test and is presented as median.  *p<0.05, **p<0.01, ***p<0.001.

## 5.4 Correlation between IL-1α, IL-8 concentrations and neutrophil percentage in BALs of post-transplant patients who developed BOS

One of characteristic clinical features of BOS is increased number of neutrophils in BAL. Neutrohils may occur as an aftermath of infection or tissue damage and their main role is to fight invading pathogens and to help restore tissue architecture. Unresolved inflammation may result in accumulation of neutrophils in the lung, which may lead to further epithelial damage. The main chemotactic cytokine promoting neutrophil migration is IL-8.

Therefore to establish if there is a relationship between the levels of IL-1α, IL-8 and neutrophil percentage in BAL of post-transplant patients, a correlation analysis was performed using Spearman'rank correlation coefficient. The analysis revealed that there was a positive and statistically significant correlation between IL-1α concentrations and neutrophil percentage (figure 64) and IL-1α and IL-8 levels (figure 65).
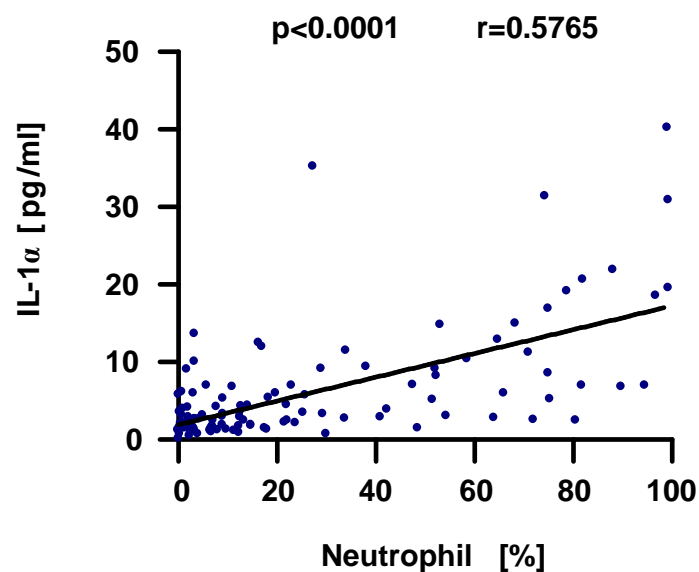


**Figure 64 Correlation between IL-1α concentrations and neutrophil percentage in BALs of post-transplant patients who developed  BOS.**

Correlation between IL-1α concentrations in BALs of BOS patients and percentage of neutrophils over total cell number. Correlation was tested using Spearman rank correlation coefficient.
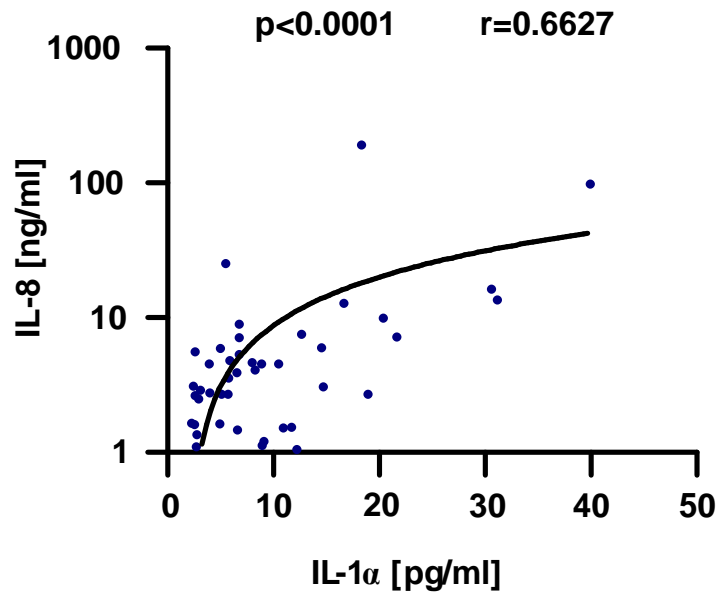
**Figure 65 Correlation between IL-1α and IL-8 concentrations in BALs of post-transplant patients who developed BOS.**

Correlation between IL-1α and IL-8 concentrations in BALs of BOS patients was tested using Spearman rank correlation coefficient. Note, Y axis is in logarithmic scale.

**5.5 Correlation between IL-1α levels and selected proinflammatory cytokine protein concentrations in BALs of post-transplant patients**

The in vitro experiments conducted during the study demonstrated that IL-1α released from damaged epithelial cells is able to significantly increase expression of numerous proinflammatory cytokines including IL-8, IL-6, MCP-1 and GM-CSF. All these cytokines may be produced by activated fibroblasts. IL-8 is a potent chemokine, attracting neutrophils to the site of injury, which may explain a positive correlation between IL-1α concentrations and neutrophil percentage described in above. MCP-1 and GM-CSF function as activations and cheoattractants for macrophages which are a powerful source of proinflammatory cytokines such as TNFα and IL-1β. Therefore it may be hypothesized that a positive correlation could also be observed in BALs between IL-1α and TNFα and IL-1β even though they are directly not produced by fibroblasts.

To test this hypothesis, the levels of IL-1α and selected proinflammatory cytokines including IL-8, IL-6, TNFα and IL-1β were measured in BALs of two individual cohorts of post-transplant patients (Newcastle study n=26, Leuven study n=70) and a correlation analysis using Spearman'rank correlation coefficient was performed. The analysis revealed that here was a positive and statistically significant correlation between IL-1α concentrations and the levels of all the measured cytokines in both patient cohorts (figure 66 and 67).
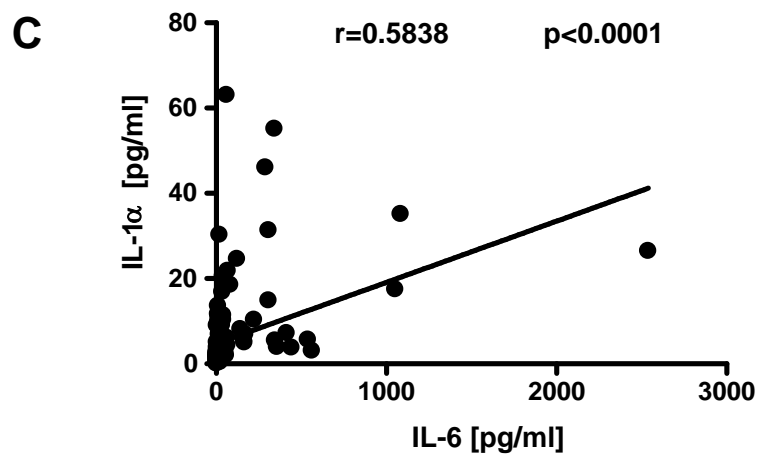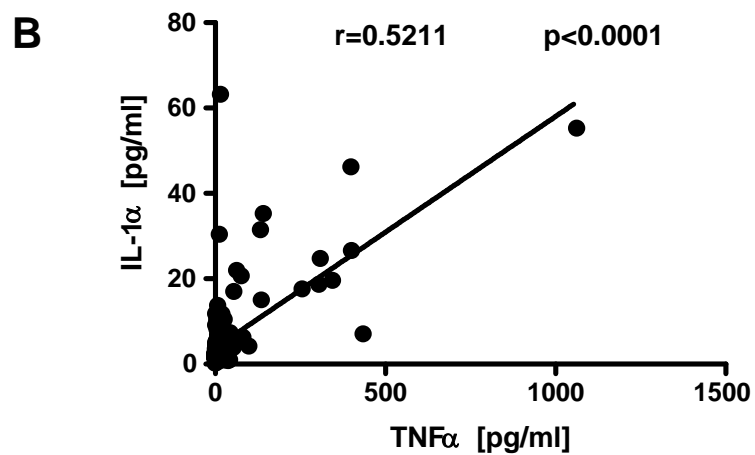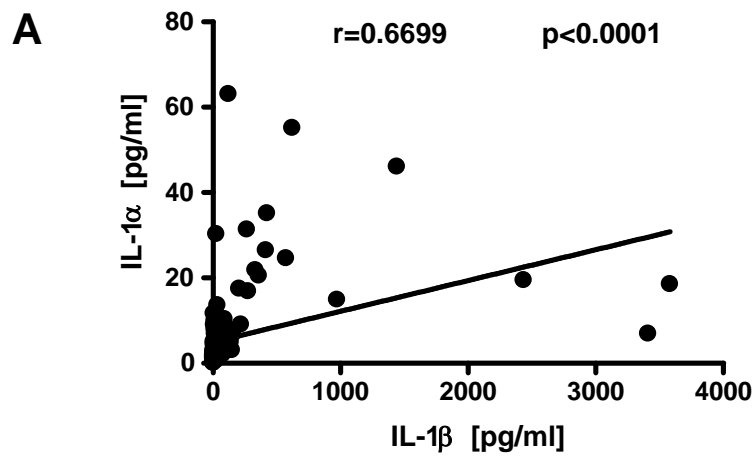
**Figure 66 Correlation between IL-1α levels and selected proinflammatory cytokine protein concentrations in BALs of post-transplant patients (Newcastle study).**

Concentrations of selected IL-1α, IL-1β, IL-6, TNFα were measured in BALs of post-transplant patients using MSD. Correlations were tested using Spearman rank correlation coefficient.
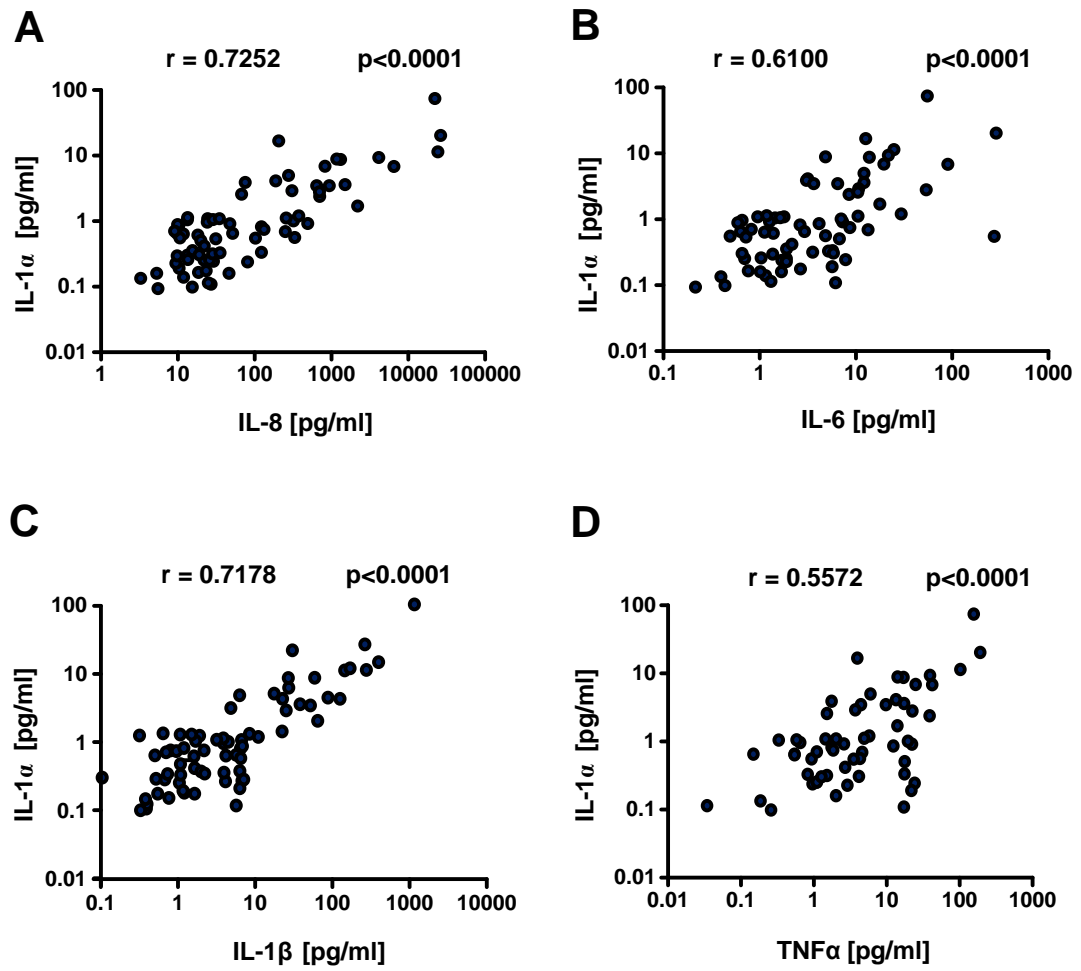
**Figure 67 Correlation between IL-1α levels and selected proinflammatory cytokine protein concentrations in BALs of post-transplant patients (Leuven study).**

Concentrations of selected proinflammatory cytokines were measured in BALs of post-transplant patients using MSD assay. Correlations were tested using Spearman rank correlation coefficient.

## 5.6 IL-1α and other proinflammatory cytokine protein profiles in different phenotypes of chronic lung allograft rejection

Recently it has been suggested that chronic post-transplant lung rejection does not have a uniform pathophysiology, but in fact it can be divided into several different phenotypes depending on the magnitude of fibrotic changes, inflammation, deformations in tissue architecture and responsiveness to commonly used therapies including macrolide treatment [313] [314] [315]. Taking under consideration these parameters, the following BOS phenotypes have been proposed: ARAD (Azithromycin reversible allograft dysfunction), PAN (persistent airway neutrophilia), fBOS (fibroproliferative BOS) and RAS (restrictive allograft dysfunction). The ARAD and PAN are characterized by relatively high percentage of neutrophils in BAL and high levels of inflammation but very moderate level of fibrosis. fBOS patients show lower levels of BAL neutrophils and inflammation but very severe fibrosis causing airway obliteration. The RAS phenotype is characterized by relatively low levels of inflammation and limited presence of fibrotic lesions in the lung parenchyma, yet very substantial decline in lung function (FEV$_1$). The proposed phenotypic dichotomy of chronic lung allograft dysfunction is presented in figure 68.
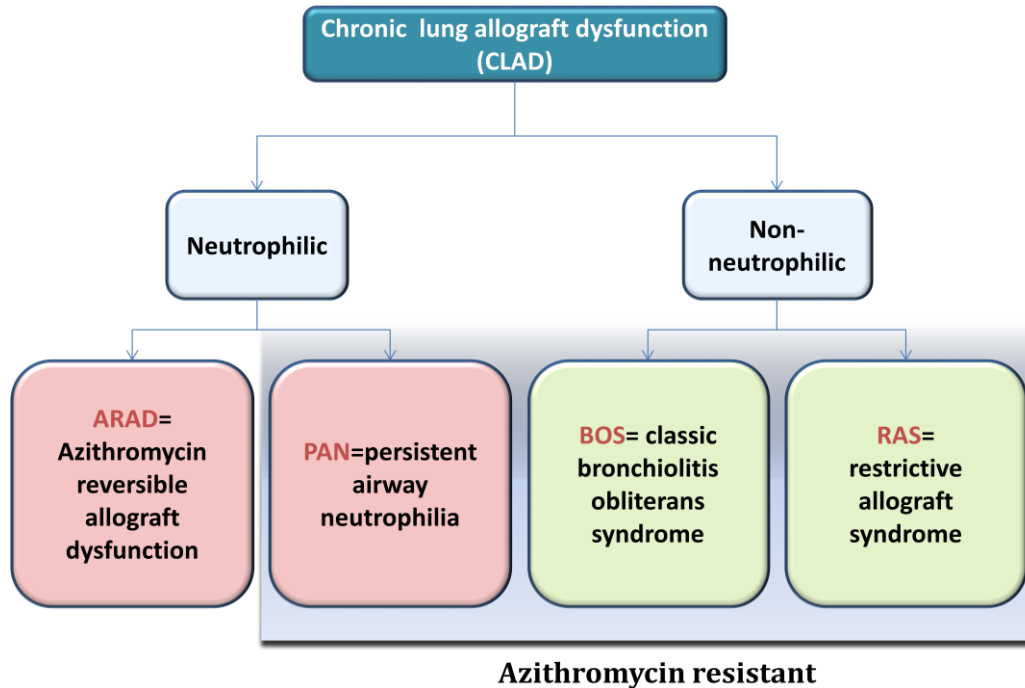
**Figure 68 Phenotypic dichotomy of chronic lung allograft dysfunction**

Cytokine and alarmin profile of different chronic lung allograft dysfunction (CLAD) phenotypes have not been determined before, and therefore to address this issue levels of IL-1α, TNFα, IL-1β, IL-6 and IL-8 were measured with MSD in BALs of 50 post-transplant patients who has been classified according to the criteria described above.

The analysis revealed significant differences in the levels of the measured factors between the groups. The highest levels of IL-1α, IL-1β, TNFα, IL-8 and IL-6 were detected in the PAN group, while the concentrations of this cytokines in the fBOS group were comparable the control group (post-transplant patients who did not develop chronic lung allograft dysfunction). Increased levels of IL-6 were also observed in the RAS group, however this increase was not associated with upregulation of IL-1α, IL-1β or TNFα, suggesting that an alternative mechanism may be responsible for promoting IL-6 expression in this group (figure 69).
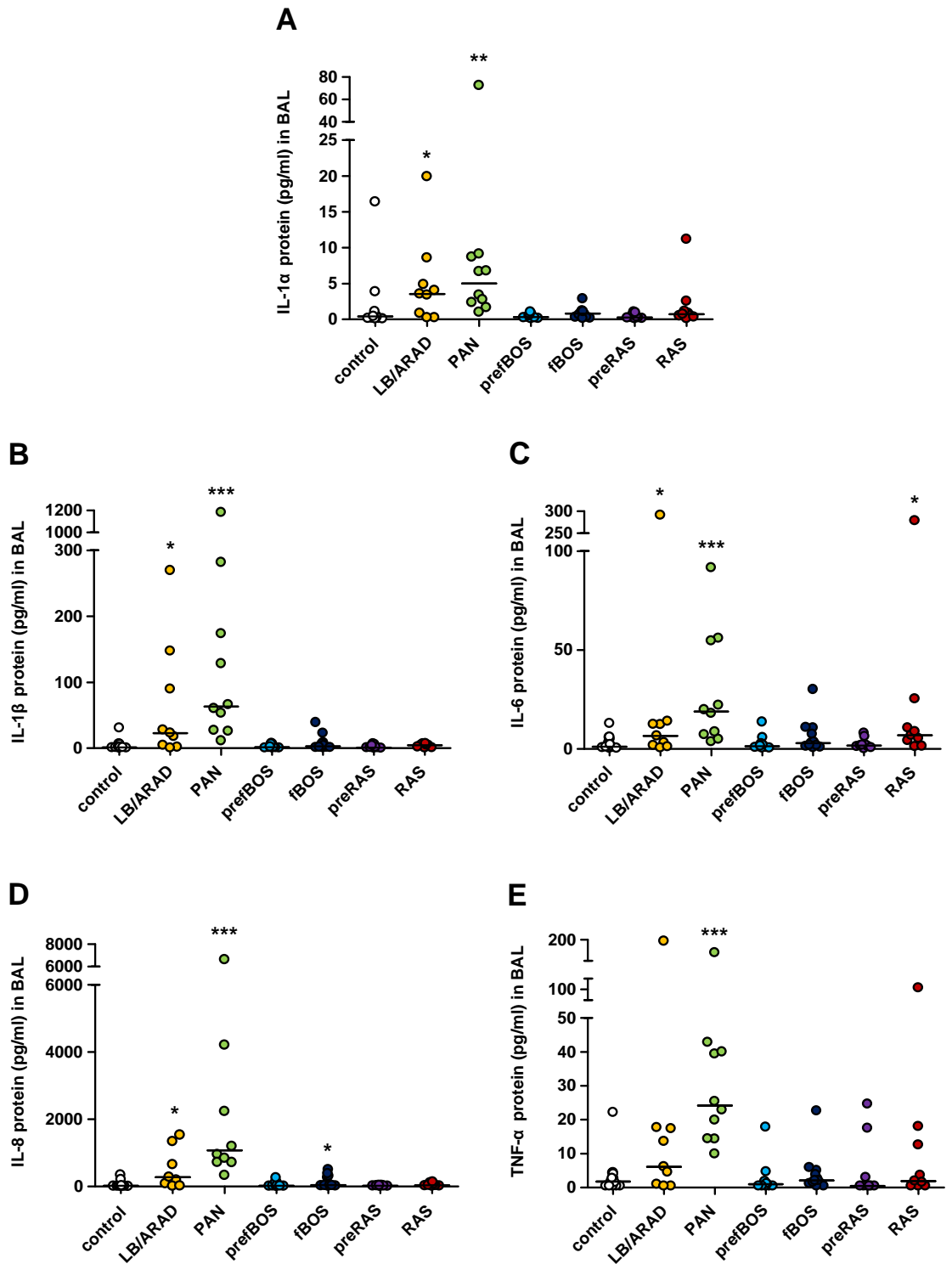
**Figure 69 Proinflammatory cytokine protein concentrations in BALs of lung transplant recipients who developed different BOS phenotypes.**

ARAD (Azithromycin reversible allograft dysfunction), PAN (persistent airway neutrophilia), fBOS (fibroproliferative BOS) and RAS (restrictive allograft dysfunction). Data was analyzed using Mann-Whitney U test and is presented as median.

## 5.7 Influence of BALs from lung transplant recipients who developed distinctive phenotypes of chronic lung allograft rejection on PBEC cell viability

One of the characteristics of chronic lung allograft dysfunction is progressive damage to bronchial epithelium. However this issue has not been studied in the context of different BOS phenotypes before. Therefore, to establish if any association exists between epithelial damage and different BOS phenotypes, primary bronchial epithelial cells were subjected to a 24hrs treatment with BALs (1:1 BAL to medium) collected from the 70 post-transplant patients who has been classified as ARAD, PAN, fBOS and RAS. Following the treatment, PBEC cell viability was assessed using XTT toxicity assay.

The analysis revealed there was a significant decrease in viability of PBEC cells cultured in the presence of BALs collected from PAN patients (figure 70).
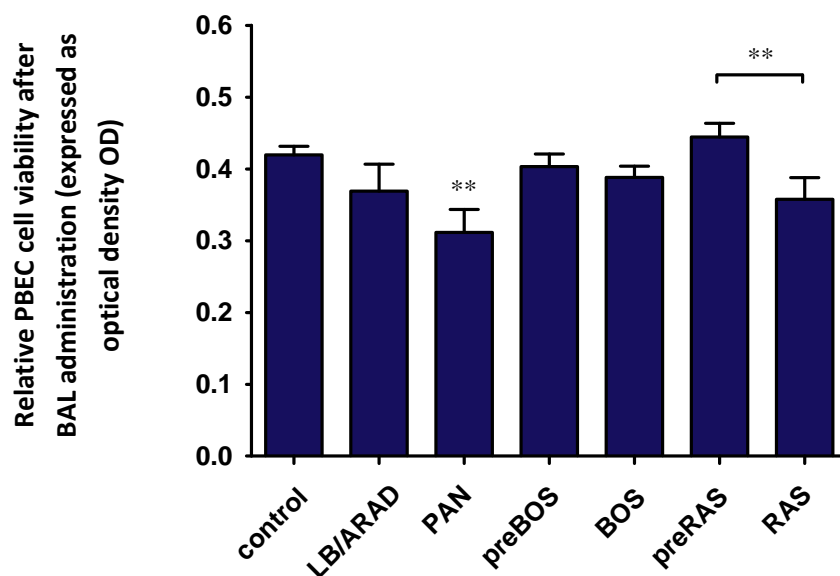


**Figure 70 Primary human bronchial epithelial cell (PBEC) viability in reponse to BALs from lung transplant recipients who developed different BOS phenotypes**

PBEC cells were treated with 1:1 culture medium/BALs from lung transplant recipients who developed different BOS phenotypes for 24hrs, after which the cell viability was assessed by XTT assay. Data was analyzed using Mann-Whitney U test and is presented as mean ± S.M.E.

**5.8 Correlation between IL-1α and other proinflammatory cytokine concentrations in BALs of post-transplant patients and PBEC cell viability in response to these BALs**

To further evaluate if there is any association between PBEC cell death and concentration of IL-1α and other proinflammatory cytokines present in BALs used to treat PBEC cells, a Spearman's rank correlation coefficient was calculated. The analysis revealed that there was a negative correlation between IL-1α, IL-1β, IL-6, IL-8 and TNFα levels in BALs and PBEC cell viability, suggesting that increased inflammation may be associated with epithelial damage (figure 71).
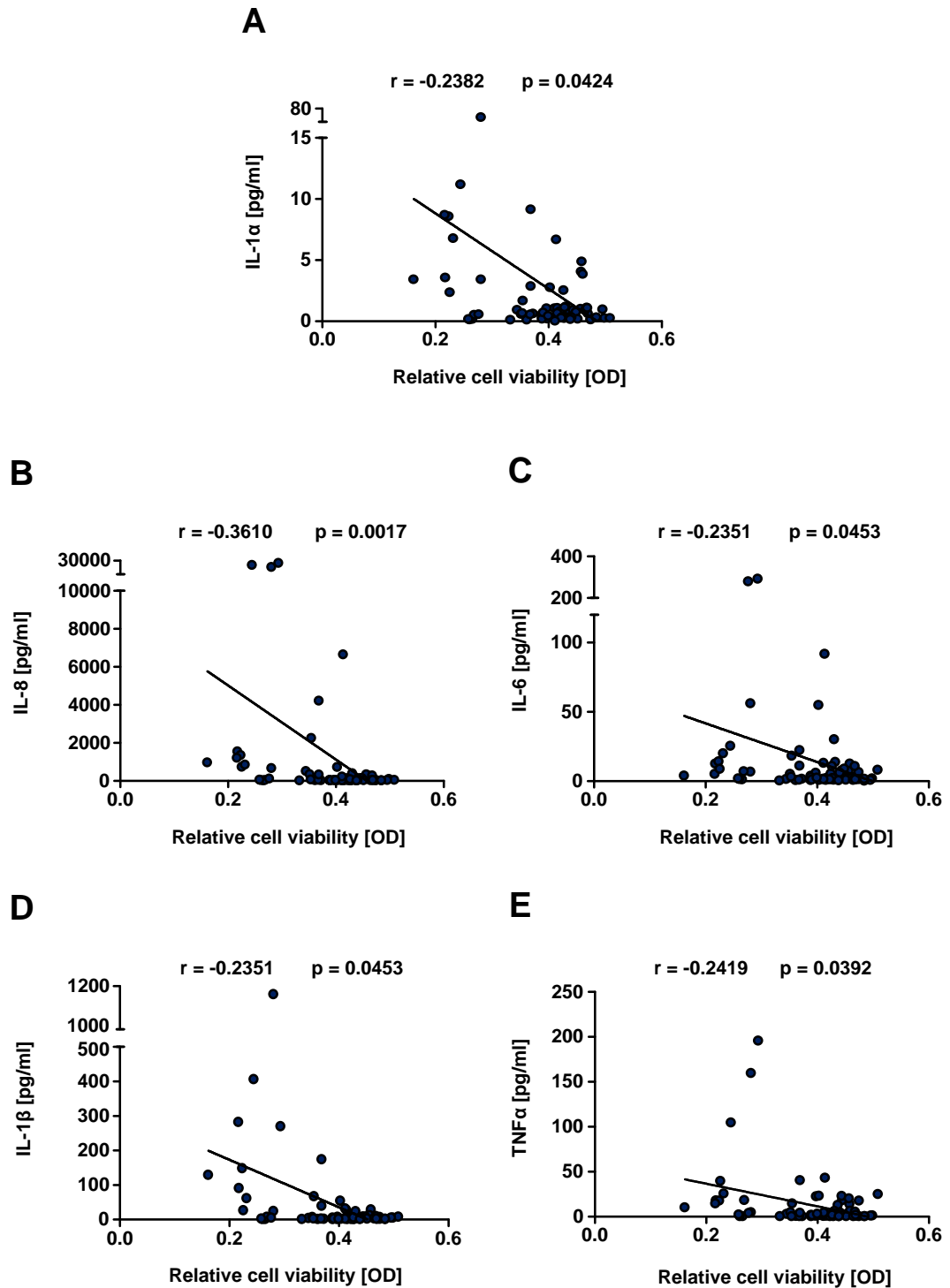
**Figure 71 Correlation between PBEC cell viability and the levels of proinflammatory cytokines in BALs of lung transplant recipients**

PBEC cells were treated with 1:1 culture medium/BALs from lung transplant recipients who developed different BOS phenotypes for 24hrs, after which the cell viability was assessed by XTT assay. The correlation between relative cell viability expressed as OD and cytokine concentrations in BALs was assessed using Spearman rank correlation coefficient.

**5.9 Discussion**

**5.9.1 Role of IL-1α in the pathogenesis of chronic inflammatory lung diseases**

Previous and the current study demonstrated that IL-1α and IL-1β are potent inducers of a vast plethora of proinflammatory cytokines including IL-6, IL-8, GM-CSF, MCP-1 and TNFα, which modulate numerous processes including T cell maturation and polarization, macrophage and neutrophil chemotaxis and wound healing. IL-1α and IL-1β may also upregulate the expression of their own genes, exacerbating the inflammatory responses.

All these cytokines are upregulated in many chronic inflammatory diseases including chronic post-transplant lung rejection and COPD [162] [135]. Macrophage and neutrophil influx is a hallmark of many injury- and pathogen-induces chronic diseases, and prolonged presence of these cells in the lung may cause the degeneration of the respiratory epithelium.

The role of IL-1β in the pathogenesis of chronic inflammatory lung diseases has been relatively widely described. However the implication of IL-1α in this process has not yet been insightfully investigated, and our study for the first time demonstrated that there is a relationship between the progression of bronchilitis obliterans syndrome post lung transplantation and IL-1α concentrations in BALs.

Although, the relevance of IL-1α has not been studied in the context of BOS before, there are reports linking IL-1α to neutrophilia in the COPD model of cigarette smoke induced inflammation in mice. Using IL-1α-/-, IL-1β-/- and IL-1R-/- mice, Botelho et all demonstrated that mice deficient in IL-1α and IL-1R showed decreased neutrophil influx to the lung following exposure to cigarette smoke. Interestingly, this process was independent of IL-1β and caspase-1 activation, suggesting that IL-1α is the main inducer of neutrophil influx post exposition to cigarette smoke [316].

These results were confirmed by Pauwels et al, who demonstrated that cigarette smoke-induced inflammation in mouse is IL-1α dependant. Interestingly, in this study also IL-1β deficiency resulted in decreased production of proinflammatory factors, however this process was independent of inflammasome activation [317].

Both IL-1α but not IL-1β may stimulate secretion of IL-8 from cells such as fibroblasts, epithelial and endothelial cells. The fact that in the two above described models of lung injury neutrophilia was IL-1α and not IL-1β dependant, may be explained by the different mechanisms underlying the release of these two cytokines.

My study demonstrated that IL-1α and not IL-1β is predominantly released by damaged normal human bronchial epithelial cells. Using a sensitive MSD analysis we established that detectable levels of IL-1α and IL-1β were present in BALs of lung transplant recipients who developed BOS (the current study and [179]). We also showed that although IL-1α upregulated IL-1β mRNA expression in fibroblasts it did not cause IL-1β protein release (data not shown).

Previous studies have shown that one of the main sources of IL-1β in inflamed tissues are macrophages [318] which tend to colonize chronically inflamed organs, and whose number in the lung tissue and BALs is significantly higher in COPD [319] and BOS [320] patients compared to healthy controls, which may indicate that IL-1β levels in the lung increase following macrophage influx. IL-1α release form damaged cells may therefore precede IL-1β secretion from macrophages invading the injured organ afterwards, which may explain why IL-1α and not IL-1β deficiency is sufficient to reduce neutrophil influx following tissue injury.

Moreover, my study demonstrated that IL-1α may stimulate secretion of GM-CSF and MCP-1 from fibroblasts. These two cytokines function as macrophage chemokines and activating factors respectively, suggesting that IL-1α may also be involved in mediating macrophage influx to the lung.

Although I did not investigate the relationship between IL-1α and macrophage percentage in BALs of post-transplant patients, previous reports suggest that there is a positive correlation between these two factors in the lung [321].

Additionally a study performed by Fitzgerald *et al.,* confirmed my observation that fibroblasts may function as a source of GM-CSF, which can mediate macrophage migration. In this study, fibroblasts gained the ability to produce GM-CSF following

exposure to TNFα and IL-1β or conditioned media from activated macrophages, which are a powerful source of these two cytokines [322].

These results are in compliance with my observations, that fibroblasts may produce proinflammatory cytokines in response to TNFα, suggesting that they may also function as important mediators of inflammation at the later stages of disease, when the production of proinflammatory factors is mediated mainly by macrophages, colonizing the inflamed lung.

One of the underlying causes of chronic inflammatory lung diseases is unresolved bacterial or viral infection. Pathogen-induced inflammation is mediated mainly by immunocytes with macrophages being the key players in this process. One of the cytokines released by macrophages activated by PAMPs is IL-1β, which similarly to IL-1α is a powerful inducer of proinflmmatory factor expression. The role of IL-1β in pathogen clearance during lung infection has been confirmed in a study performed by Kafka et all, who demonstrated that mice deficient in IL-1β show higher mortality rate following Streptococcus pneumoniae infection in comparison to WT controls or IL-1α-/- mice [323]. This observation indicates that IL-1β and not IL-1α plays a pivotal role in bacteria clearance during infection. Since IL-1β is strongly upregulated in chronic inflammatory lung diseases such as COPD and BOS, it has previously been suggested that anti-IL-1β therapies could be considered as a treatment in this diseases. However, the known role of IL-1β in the process of pathogen clearance, raised a question of the potential hazardous implications, that anti-IL-1β therapies may have in patients affected by bacterial infections.

The study conducted by Kafka et al, suggests that blocking IL-1α does not significantly affect the ability of the pulmonary immune system to fight infection, indicating that anti-IL-1α therapies could be used to dampen down inflammation induced by epithelial injury in patients suffering from bacterial infections.

Safe and effective inflammation management in post-transplant patients is a matter of great importance. Until quite recently, one of the most popular anti-inflammatory strategies post-transplantation was corticosteroid therapy. However this kind of treatment is associated with many side effects and a large percentage of patients suffering from chronic inflammation develop steroid resistance. Currently,

patients affected by chronic post-transplant lung rejection are subjected to macrolide antibiotic treatment, however this therapy is effective only in approx. 50% of cases [324]. Anti-IL-1α treatment has not been tested on BOS patients yet, however our and previous studies, suggest that this kind of treatment could allow to manage inflammation induced following epithelial injury, relatively safely, with minimal side effects and without the risk of uncontrolled infection.

The role of IL-1 was also investigated in the context of viral respiratory infection in mouse induced by influenza virus. Using IL-1R-/- mice, Schmitz et al demonstrated that IL-1 is required for neutrophil and CD4+ cell influx following influenza infection, however it does not have a major impact on virus titrate. The study however did not address the individual role of IL-1α in this process [325].

My study revelaed that there is an association between increased levels of IL-1α in BALs and bacterial infections. One of the limitations of the current study is its retrospective character and the lack of data on viral infections in the examined cohort of patients. To address these issues, a re-designed prospective study could be performed in the future.


**5.9.2 Role of bacterial infections in chronic post-transplant lung rejection**

Bacterial infections have been recognized as an important risk factor of many chronic degenerative diseases, including chronic post-transplant lung rejection. The immunosuppressive drug regime, that lung transplant recipients must adhere to, makes this group of patients highly prone to both viral and bacterial infections.

My study demonstrated that bacterial infections in lung transplant recipients who developed BOS were associated with significantly higher levels of proinflammatory cytokines including IL-8, when compared to post-transplant patients who remained stable, which may be due to significantly higher levels of IL-1α in BALs of BOS patients who were culture positive.

The association between high levels of IL-1α and bacterial colonization, finds explanation in several studies demonstrating that bacterial PAMPs such as LPS may

stimulate expression of IL-1α [326] [327]. Moreover bacterial pathogens invading lung may cause damage to the bronchial and alveolar epithelium, inducing IL-1α release from injured epithelial cells. Unresolved bacterial infections, which are a common complication in patients subjected to a strict immunosuppressive regime, may therefore exacerbate inflammation by triggering production of proinflammatory cytokines directly via interactions between PAMPs and corresponding TLRs and indirectly via upregulation of IL-1α expression and the induction of its release by injuring the epithelial layer.

Bacterial pneumonia is a frequent complication following lung transplantation. Recent studies demonstrated that bacterial microbes were isolated in up to 80% of lung transplant recipients [328]. A multicenter prospective study performed within the period of 2 years on a cohort of 236 lung transplant recipients (LT), revealed that the incidence of bacterial infections in this group was 72 episodes per 100 LT/year. Pseudomonas aeruginosa was isolated in 24.6%, Acinetobacter baumannii and Staphylococcus aureus each in 14%, Escherichia coli, Klebsiella pneumoniae, and Stenotrophomonas maltophilia each in 5.3%, Pseudomonas putida, Serratia marcescens, and Burkholderia cepacia each in 1.8%, and Mycobycterial infections were found in 5.3% of cases [328].

Pseudomonas aeruginosa is the most frequently isolated bacteria from lung following transplantation. Several studies demonstrated that lung transplant recipients with cystic fibrosis show higher susceptibility to infection caused by this bacteria, which may spread from extrapulmonary reservoirs of the recipient [329] [174].

Moreover, two independent studies [174] [330] demonstrated that the frequency of lung colonization with Pseudomonas aeruginosa can be associated with BOS.

Increased incidence of BOS has also been reported in lung transplant recipients affected by infections with Burkholderia cepacia [331], Chlamydia pneumonia [332] and Mycobacterium tuberculosis [333].

Apart from pulmonary infections, another common complication affecting lung transplant recipients is bacteremia, defined as a presence of bacteria in blood.

A prospective, multicentre study performed between 2000-2004, revealed that bacteremia was documented in 56 of lung transplant recipients and that multiple antibiotic resistance was registered in 48% of the isolates. The most frequent isolates included P. aeruginosa (14/56), S. aureus (9/56) B. cepacia (5/56), Enterococcus faecalis (5/56), Staphylococcus epidermidis (4/56), and Klebsiella pneumoniae (4/56) [334].

Complications following lung transplantation have also been attributed to fungal infection, which are estimated to affect 15 to 35% lung allograft recipients. It has been established that 80% of all fungal infections post transplantation is caused by Aspergillus spp. and Candida spp. [335].

A study by Saïd-Sadier demonstrated that inflammatory responses to conidia and hyphae of Aspergillus fumigates may be NLRP3 and inflmmasome dependant and are manifested by caspase-1 activation and increased secretion of IL-1β by monocytes [336].

This data is in compliance our recent onservations showing that bacterial infections are associated with upregulation of proinflammatory cytokines including IL-1α, TNFα, IL-1β and IL-8 in BALs of post-transplant patients who developed BOS (the current study and [179]).

**CHAPTER 6 SUMMARY DISCUSSION**

**6.1 Fibroblasts as mediators of immune responses**

Although fibroblasts are historically considered as cells whose main role is the production of the components of the extracellular matrix and tissue contraction during the wound healing process, emerging evidence suggests that fibroblasts may also function as important mediators of immune responses. Our study revealed that primary human lung fibroblasts express high levels of IL-1R and TLR3, suggesting that they may mediate immunity induced by IL-1α, IL-1β as well as by viral double stranded RNA and endogenous RNA, which may also be released from injured cell [337].

Primary human lung fibroblasts expressed also low levels of transcripts for TLR2 and TLR4, however they did not respond to LPS or HMGB-1, which distinguishes them form macrophages, which very strongly respond to TLR2 and TLR4 ligands.

Human lung fibroblasts responsiveness to IL-1 and a synthetic TLR3 ligand – Poly I:C, suggest that these cells may play a pivotal role as inflammation mediators during tissue injury and viral but potentially not bacterial infections. Progressive tissue degeneration is characteristic for many chronic diseases including chronic post-transplant lung rejection. Viral infections have been recognized as one of potential risk factors, increasing the probability of developing BOS post transplantation. Considering that fibroblasts become one of the most abundant cell type is fibrotic tissues, it may be therefore hypothesized that they play an important role in mediating chronic inflammation in BOS patients affected by viral infections.

Fibroblasts are also a source of type I interferons, which inhibit proliferation of virus-infected cells. This fibroblasts characteristic not only is of significant physiological importance, but it also found a practical implication, as fibroblasts induced with riboinosinic-ribocytidylic acid are used to produce human interferon for clinical use [338].

The TLR gene expression profile in primary human lung fibroblasts have not been described in literature before. There are however reports investigating PRR gene expression in human gingival, synovial and skin fibroblasts.

According to the study performed by Uehara et al, human gingival fibroblasts express mRNA for TLR1-9 and respond to selected PAMPs including FSL-1 (a synthetic lipoprotein derived from Mycoplasma salivarium functioning as TLR2/TLR6 ligand), Poly I:C (TLR3 agonist), lipid A (a component of LPS, activating TLR2 and TLR4), ssPolyU (TLR8 agonist) and CpG DNA (TLR9 ligand), suggesting that these cells may play an important function in promoting inflammatory responses to viral and bacterial danger signals [339].

The skin and synovial fibroblasts were found to express transcripts for TLR1-6, with TLR2 and TLR3 being significantly higher in the synovial fibroblasts. Moreover both skin and synovial fibroblasts responded positively to bacterial lipopeptide, poly (I:C), lipopolysaccharide and flagellin by producing increased amounts of MMP1, MMP3, MMP9 and MMP13, suggesting these cells may participate in tissue remodeling following viral and bacterial infections [340].

Moreover, synovial fibroblasts were found to produce a vast array of proinflammatory cytokines including granulocyte chemotactic protein (GCP)-2, RANTES, monocyte chemoattractant protein (MCP)-2, IL-8, growth-related oncogene-2, macrophage-inflammatory protein 1alpha, MCP-1, EXODUS, and CXCL-16. GCP-2 and RANTES in response to bacterial the TLR-2 ligand bacterial peptidoglycan, suggesting that similarly to human lung fibroblasts, the synovial fibroblasts also may function as mediators of inflammation [341].

Interestingly, in my study, unlike synovial fibroblasts, neither MRC5 cells nor primary fibroblasts isolated from human lung tissues responded to TLR2 agonist treatment, indicating that there are distinctive differences between fibroblasts originating from different tissues.

The role of human and lung fibroblast in mediating inflammation has recently been described in an elegant study performed by Kitamura et al, who demonstrated that mouse and human lung fibroblasts may regulate dendritic cell trafficking, airway inflammation, and fibrosis via integrin αvβ8–mediated activation of TGF-β.

The study revealed that conditional deletion of lung fibroblast αvβ8 led to inhibition of airway inflammation, and a reduction in innate and adaptive immune responses and fibrosis induced by adenovirus and IL-1β.

Moreover the study demonstrated that human lung fibroblasts derived from COPD lung show increased responsiveness to IL-1β in terms of αvβ8-dependent TGF-β activation, collagen expression, and proinflammatory gene expression when compared with normal lung fibroblasts, suggesting that the microenvironment of tissue exposed to injurious stimuli may modulate fibroblast behavior [342].

Additionally, Saalbach *et al.,* demonstrated that human dermal fibroblasts may interact with dendritic cells (DC) in a β2 integrins -Thy-1 (CD90)/ ICAM-1 dependant manner, leading to upregulation of CD83, CD86, CD80, and HLA-DR in DC. The study also demonstrated that fibroblast-induced DC may modulate T cells activation via induction of CD25 expression [343].

The modulation of T cell responses by fibroblasts may be also performed via stimulation of IL-23 protein secretion by DC, which is required for Th17 cell differentiation [344].

All the studies cited above and the results described in this report suggest that fibroblasts may play pivotal role in regulating innate and adaptive immune responses, during tissue injury and infection.

**6.2 IL-1α as mediator of innate immune responses and its role in chronic inflammatory diseases and wound healing**

IL-1, initially named as LAF (lymphocyte-activating factor) was discovered in 1972 by Gery *et al.* [345]. However it was not until 1985, that it was recognized that IL-1 consists of two different proteins, currently known as IL-1α and IL-1β [346]. The majority of studies, published since then, focused on the biology of IL-1β. One of the reasons behind this, was that the concentrations of IL-1β during inflammation induced by injury or infection, are significantly higher compared to IL-1α levels.

The functions and origin of IL-1β have been thoroughly investigated and now it is clear that activated macrophages are one of the main sources of this protein during

inflammation. IL-1β, alike other members of IL-1 family, lacks a signal peptide and cannot be conventionally secreted to the extracellular space. IL-1β is expressed as a pro-peptide, which requires a proteolitic cleavage by caspase-1. One of the characteristic properties of macrophages is constitutive expression of caspase-1, meaning that these cells are able to secret active IL-1β without inflammasome activation. Macrophage stimulation with LPS is sufficient to promote these cells to IL-1β secretion. However, the same stimulus will not induce IL-1β release from epithelial cells or fibroblasts.

Little however is known about the potential sources of IL-1α in tissue and the role of this IL-1 family member in the pathogenesis of chronic inflammatory lung diseases.

The clinical relevance of IL-1α in BOS has not been investigated before. In the current study we demonstrated, that elevated levels of IL-1α and IL-1β can be detected in BALs of post-transplant patients who developed chronic post-transplant lung rejection. The concentrations of IL-1α were significantly lower than IL-1β (data not shown), however, after correction for the BAL dilution factor, they were sufficient to trigger expression of proinflammatory cytokines in fibroblasts.

Moreover, I demonstrated that high IL-1α levels were associated with decreased PBEC cell viability following BAL administration, suggesting that there is a correlation between epithelial injury and IL-1α release.

Since epithelial degeneration is characteristic for many chronic inflammatory diseases, many studies have been performed in order to identify alarmins responsible for mediating inflammatory responses under these conditions [347]. These studies however, mainly aimed at identification of DAMPs released from immune cells such as macrophages or neutrophils, based on assumption that the danger signals originate from immunocytes undergoing apoptosis following pathogen/cell debris engulfment.

However, emerging evidence suggests that alarmins may also originate from damaged epithelium. Using primary bronchial epithelial cells (PBECs) and a human bronchial epithelial cell line (16HBE14o-), we demonstrated that damaged epithelial cells may be a source of alarmins including HMGB-1 and IL-1α. These results were

confirmed using three different types of injury, including H2O2- induced oxidative stress, freeze/thaw damage and thapsigarging-induced ER stress. Oxidative stress and ER stress may module gene expression and change the metabolic state of cells. Freeze/thaw damage however, results in a very rapid, necrotic cell injury and therefore may serve as a reliable control, confirming that the alarmin release is a result of cell death, rather than a consequence of the cell exposition to a chemical stimulus.

Additionally, the observation, that epithelial cells release IL-1α following injury induced by thapsigarging, which functions as an ER stress inducer and translation inhibitor, suggests that the mechanism of IL-1α release from damaged cells is translation-independent.

One of the characteristic features of alarmins is that they are stored in intracellular compartments and can be rapidly released following cell injury. Using an immunofluorescent staining technique, we demonstrated that substantial amounts of intracellular IL-1α protein are present in the cytoplasm of bronchial and alveolar epithelium of histological sections of normal human lung. This observation, suggests that IL-1α may be rapidly released from damaged respiratory epithelium, which poses the first line of defense against the invading pathogens and exogenous chemicals and therefore is prone to injury.

The observations described above are in compliance with previous studied demonstrating that IL-1α may function as an alarmin and can be rapidly released from damaged cells [182]. Moreover it has been demonstrated that the precursor form of IL-1α may act as a transcription factor, which can be translocated to the nucleus of macrophages upon stimulation with LPS and promote transcription of proinflammatory genes including IL-8 and IL-6 [348].

The role of IL-1α in the pathogenesis of chronic inflammatory lung diseases has been highlighted in several previous studies. For indtance, Yazdi *et al.*, demonstrated that nano-TiO provoked lung inflammation was substantially suppressed in IL-1R or IL-1α deficient mice compared to the wild type.

Nanoparticles may activate innate immune responses via two distinct mechanisms. Namely, they can activated inflammasome via specific receptors such as Nod-like receptors, leading to IL-1β processing and release, and they can cause cellular damage, leading to spontaneous IL-1α release [349].

Although, the mechanism of IL-1β processing has been relatively widely described, the exact way in which IL-1α enters the extracellular space remains unclear.

This issue has recently been addressed in a study performed by Gross el all, who suggests that IL-1α release from murine BMDC (bone marrow derived dendritic cells) cells may be both inflammasome-dependanat and independent. The authors propose that the deciding factor in this case is the type of stimulus. According to the study, caspase-1 independent IL-1α processing can be induces by stimulus such as Ca2+ influx, whereas caspase-1 dependant IL-1α release can be induced by NLRP3, NLRP1 or AIM2 ligands. Moreover, Ca2+ was shown to be involved in calpain-like protein-dependant IL-1α cleavage [350].

Caspase-1 is constitutively active in macrophages, which are able to secret IL-1β without inflammasome activation. In our study, however PMA-primed THP-1 cells failed to release IL-1α following LPS stimulation (data not shown). These observations and the results described in the aforementioned study [350], suggest, that the cell ability to release IL-1α and IL-1β may depend on the cell type and its metabolic state.

Numerous PAMPs and DAMPs, including IL-1α, may upregulate IL-1α and IL-1β gene expression. Therefore it may be hypothesized, that during infection or repetitive tissue injury, the potential of cells to release IL-1α may increase. However, the fact that significant amounts of IL-1α but not IL-1β can be rapidly released from epithelial cells not subjected to any pre-stimulation with proinflammatory factors, suggests that IL-1α is the initial danger signal initiating inflammation.

Although my study was performed in a context of chronic inflammatory/degenerative lung diseases, it is worth highlighting that the rapid IL-1α release following epithelial injury may also serve very important physiological

function. One of the physiological processed where IL-1α may play a significant role is wound healing.

Damage to the epithelial barrier initiates a cascade of events leading to macrophage and neutrophil recruitment, which by clearing the dead cell debris enable the subsequent regeneration of the damaged epithelium. Inflammation occurs rapidly post injury, and IL-1α which is stored inside cells may be released as the prime danger signal, initiating proinflammatory responses required for effective wound healing. Our study demonstrated that apart from fibroblasts, another cell type able to respond to IL-1α are epithelial cells, suggesting that paracrine interactions between damaged and undamaged epithelium may also significantly contribute to inflammation following injury.

In conclusion, the data obtained in this study suggests, that IL-1α is the initial danger signal released from injured epithelial cells, which may activate innate immune responses in lung fibroblasts, which by producing substantial amounts of chemokines and cytokines such as IL-8 and GM-CSF may potentially attract neutrophils and macrophages to the site of injury. Repetitive insult to the epithelium may therefore result in chronic inflammation mediated by IL-1α, which can additionaly be accentuated by viral PAMPs. Hence anti-IL-1α therapies could be considered as a novel therapeutic strategy to target chronic inflammatory lung diseases. A proposed sequence of events following epithelial injury in the lung has been shown in figure 72.
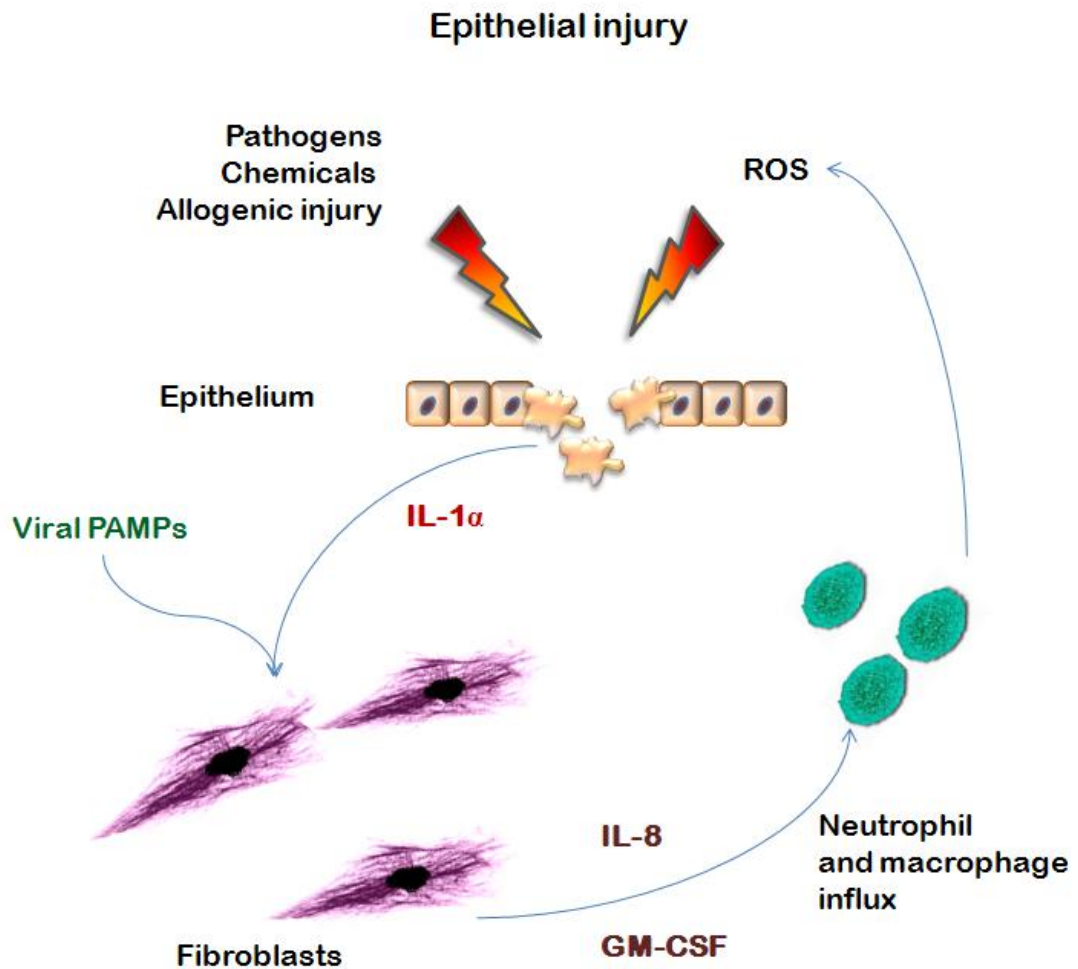
**Figure 72 Model of injury induced inflammation in the lung**

Diagram showing a proposed sequence of events following epithelial injury in the lung. Chemical, pathogenic or allogenic insult to epithelium causes release of alarmins including IL-1α which activates fibroblasts to release IL-8 attracting neutrophils to the site of injury. ROS producing neutrophils cause further damage to epithelium perpetuating the vicious circle of IL-1α-induced chronic inflammation.

## 6.3 Summary of main achievements

Repetitive damage to the airway or alveolar epithelium, associated with inflammation and tissue remodeling are common features of many chronic respiratory diseases. Chronic inflammation, characterized by intensive protein production may also cause ER stress and an unfolded protein response, which may lead to programmed cell death. Additionally, the lung epithelium is the first point of contact for inhaled oxidants/free radicals and these species are normally neutralized by antioxidants present in epithelial lining fluid. Defining how oxidant and ER-stress-induced epithelial cell damage stimulates inflammation and identifying the molecular mediators that cross-talk between damaged and dying epithelial cells and the immune system are important goals necessary to improve our knowledge of the pathophysiological events that underlie chronic pulmonary disease.

My work described here, makes the case for a new paradigm, that damaged bronchial epithelium can induce sterile innate immune signaling in lung fibroblasts resulting in a phenotypic switch towards a highly proinflammatory state.

In this study I was able to show that a single alarmin IL-1α, is both necessary and sufficient for the epithelial damage-induced phenotypic switch of lung fibroblasts to their inflammatory state. Blockade of IL-1α signaling completely inhibited the induction of proinflammatory cytokines by damaged epithelial cells as well as the intracellular signaling events namely IKK2/NF-κB activation that transduce IL-1R triggered signals to the transcriptional machinery of the fibroblast. I also demonstrated that there is a positive correlation between elevated levels of IL-1α, IL-8 and neutrophils in the chronic inflammatory lung disease, BOS after lung transplantation.

Additionally, my observation that TLR3 is the predominant TLR expressed in lung fibroblasts, and its ligation acts in synergy with IL-1α, raises the intriguing possibility that the degree to which fibroblasts impact on lung inflammation may be influenced by respiratory viral infections. The combined effects of IL-1α and TLR3 ligation on IL-8 and IL-6 expression were suppressed by DEX and inhibitors of IKK2 and its

213

upstream kinase TAK1. In contrast, the neomacrolide, Azithromycin, which has been shown to have anti-inflammatory properties and improve lung function in some patients with BOS (24) did not have major anti—inflammatory effect on IL-8 and IL-6 expression in fibroblasts in response to IL-1α. This may be an important observation as more than 50% of patients who develop BOS fail to respond to the anti-inflammatory actions of Azithromycin.

One of the main advantages of my study was the confirmation of the role of the epithelial alarmin IL-1α in triggering a proinflammatory phenotype in fibroblasts using primary human lung fibroblasts from a normal lung and two in vitro models of injury of human bronchial epithelial cells (oxidative stress and ER stress). However, all in vitro models have their limitations and one of the main drawbacks of this study was a limited access to PBEC cells from normal lung (the cells used in the study came from bronchial brushings obtained from stable post-transplant patients). Also, the behaviour of PBEC cells from diseased lung has not been investigated in this project.

The objective of my project was to identify alarmins that are released from injured bronchial epithelium and trigger sterile inflammation and fibroblast activation in the lung. However, normal lung is not sterile. Lungs are constantly exposed to airborne pathogens which are 'kept at bay' by the immune system. Therefore it may be assumed that all primary bronchial cells, unless obtained from a foetal tissue, have been primed with microbial and viral PAMPs. From that perspective the physiological relevance of the two models of epithelial injury used in the study can be questioned and therefore it would be beneficial to confirm the main results using a model of epithelial injury induced by viral or bacterial infection.

Additionally, from the clinical point of view it would be advantageous to measure the levels of IL-1α as well as to establish the ratio of total and pro-IL-1β in BALs of patients with BOS. My study revealed that the development of BOS was associated with increased concentrations of both IL-1α and total IL-1β (data not shown), however the levels of active IL-1β in BALs of these patients have not been established, and therefore it remains unclear which one of the aforementioned IL-1 family members may be implicated in the pathogenesis of BOS.

Interestingly my study revealed that IL-1α released from damaged epithelial cells triggers gene expression of both IL-1α and IL-1β which was not followed by IL-1α or IL-1β protein release. IL-1α also induced gene expression of another alarmin, IL-33 (data not shown) and resulted in accumulation of IL-6 proteins in intacellular granules. These properties of IL-1α and the fact that it is released not only from damaged but also still viable but stressed cells, suggests that this alarmin may function as the initial danger signal priming and preparing the adjacent cells to rapid innate immune response to agents which may potentially damage the bronchial epithelium. To confirm this hypothesis, another experiment could be performed where release of different alarmins would be measured from cells that were first primed with IL-1α and then damaged with different injurious stimuli.

Taken together, these observations build a case for investigating the therapeutic potential of biologics that attenuate IL-1α/IL-1R signaling in these chronic lung diseases which are associated with significant morbidity and premature mortality.

**6.4 Possible future work**

1. Confirm the role of fibroblasts and IL-1α as mediators of inflammation in vivo using an animal model of pulmonary fibrosis.

The current study resulted in identification of IL-1α as the key epithelial alarmin responsible for triggering a proinflammatory phenotype in primary human lung fibroblasts, however it did not provide an evidence that fibroblasts are important contributors to proinflmmatory responses in the microenvironment of injured tissue. To determine whether IL-1α-induced inflammation is mainly controlled by hemopoietic or nonhemopoietic cells in vivo, bone marrow (BM) chimeric mice between IL-1R and IL-1R-/- and IL-1a and IL-1a-/- mice could be generated and subjected to bleomycin-induced lung injury. This experiment could be next repeated using a more physiologically relevant model of virus-induced lung injury. Additionally, to distinguish between the role of epithelial cells and fibroblasts Cre-loxP-based mouse model for conditional knockdown of IL-1R in collagen-expressing cells could be used.

2. Confirm the role of IL-1α in activating innate immune responses in primary human lung fibroblasts using a model of rhinovirus (RV)-induced epithelial injury.

In the current study, in vitro models of oxidative stress and ER stress-induced-cell injury models were used to evaluate the role of epithelium-derived IL-1α in the activation of primary human lung fibroblasts. Both oxidative stress and ER stress have been associated with chronic inflammation, however the in vitro models using relatively high doses of $H_2O_2$ and thapsigargin are difficult to translate into an in vivo situation. Another factor that largerly contributes to exacerbations in chronic inflammatory lung diseases are viral infections, and therefore to evaluate the results obtained in the current study, the key experiments could be repeated using an in vitro model of epithelial injury induced by rhinovirus (RV), which has been recognized as a risk factor in BOS.

3. Investigate the interplay between damaged epithelial cells, fibroblasts and T cells.

The characteristic features of chronic post-transplant lung rejection include damage to the respiratory epithelium, fibroproliferation and influx of inflammatory cells including macrophages, neutrophils and T cells. Interaction between these type of cells and cytokines released by them, determine the microenvironment of the injured tissue. In a fibrotic tissue the balance between the number of these cellular components and their activation state becomes distorted. Evaluating how the interations between different immune and non-immune cellular components affect their phenotypes might increase the current understading of the relationship between fibrogenesis and activation of the innate and adaptive immune system.

4. Analyze the profile of cytokines expressed by human primary lung fibroblasts upon stimulation with IL-1α by MSD multiplex assay or a PCR microarray.

The current study revealed that IL-1α released by damaged human bronchial epithelial cells triggers a pro-inflammatory phenotype in fibroblasts characterized by increased gene expression of marker cytokines including IL-8, IL-6, MCP-1, GM-CSF, IL-1α and IL-1β. To fully unravel the proinflammatory potential of primary human lung fibroblasts, a profile of cytokines released by these cells upon stimulation with IL-1α could be evaluated by MSD multiplex assay and a PCR microarray.

5. Compare cytokine expression profile in normal primary human lung fibroblasts and fibroblasts derived from diseased tissue (derived from patients with COPD or BOS).

The experiments described in the current study were performed using normal human lung fibroblasts (MRC5) or primary human lung fibroblasts derived from normal/healthy tissues. To evelaute if there are phenotypic differences between normal fibroblasts and fibroblasts diseased lung, cytokine expression profile in normal fibroblasts and fibroblasts derived derived from patients with COPD or BOS could be evaluated using a PCR microarray or MSD multiplex assay.

6. Perform a prospective study to investigate the association between IL-1α concentrations in BALs, development of BOS and the incidence of bacterial and viral infections in post-transplant patients.

One of the limitations of the current study is its retrospective character. The time of BAL sampling was driven by clinical need and was not per pre-defined protocol which resulted in the difference in the number of samples collected from BOS and stable patients. Another limitation of this study is the lack of data on viral infections in the examined cohort of patients. To address these issues, a re-designed prospective study could be performed in the future.

## 6.5 Publications and presentations

**Published articles:**

*TNFα From Classically Activated Macrophages Accentuates Epithelial to Mesenchymal Transition in Obliterative Bronchiolitis.* (2013). Am J Transplant. 2013 Mar;13(3):621-33. 2013 Jan 17. Borthwick LA, Corris PA, Mahida R, Walker A, Gardner A, **Suwara M**, Johnson GE, Moisey EJ, Brodlie M, Ward C, Perry JD, Soyza AD, Mann DA, Fisher AJ.

*IL-1α released from damaged epithelial cells is sufficient and essential to trigger inflammatory responses in human lung fibroblasts.* (2013) Mucosal Immunol. 2013 Oct 30. doi: 10.1038/mi.2013.87. **Suwara MI**, Green NJ, Borthwick LA, Mann J, Mayer-Barber KD, Barron L, Corris PA, Farrow SN, Wynn TA, Fisher AJ, Mann DA.

**Manuscripts in preparation:**

*The Epithelial Alarmin Interleukin 1 alpha (IL-1α) is associated with development of Bronchiolitis Obliterans Syndrome.*

**MI Suwara**, LA Borthwick, NJ Green, R Mahida, KD Mayer-Barber, A Gardner, J Mann, TA Wynn, PA Corris, SN Farrow, DA Mann and AJ Fisher.

*Mechanistic differences between phenotypes of chronic lung allograft dysfunction after lung transplantation.*

**MI Suwara**, BM Vanaudenaerde, SE Verleden, R Vos, NJ Green, LA Borthwick, C Ward, DE Van Raemdonck, DA Mann, PA Corris, GM Verleden, AJ Fisher.

**Presentations:**

**2013 European Respiratory Society Conference, Barcelona**

*Crosstalk between lung fibroblasts and T-lymphocytes: implications for anti-viral responses in chronic lung disease (poster presentation)*

**Monika I Suwara**, Bart Vanaudenaerde, Nicola J Green, Elizabeth Moisey, Lee A Borthwick, Derek A Mann, Andrew J Fisher

**2013 British Association of Lung Research, Summer Conference**

*Transforming growth factor beta (TGFβ) and interleukin-1α (IL-1α) drive opposing functional phenotypes in primary human lung fibroblasts (PHLF) (poster presentation)*

Benjamin Emmerson, **Monika I Suwara**, Derek A Mann, Andrew A Fisher

**2013 American Thoracic Society Conference, Philadelphia**

*Interleukin-1 alpha released from airway epithelium in response to endoplasmic reticulum stress promotes induction of a pro-inflammatory lung fibroblast phenotype (poster presentation)*

Nicola J Green**, Monika I Suwara,** Derek A Mann and Andrew J Fisher

**2012 European Respiratory Society Conference, Vienna**

*Mechanistical differences within Bronchiolitis Obliterans Syndrome phenotypes after lung transplantation (oral presentation)*

**Monika I Suwara**, Bart M Vanaudenaerde, Stijn E Verleden, Lee A Borthwick, Robin Vos, Nicola J Green, Chris Ward, Dirk E Van Raemdonck, Derek A Mann, Paul A Corris, Geert M Verleden, Andrew J Fisher

**2012 Thorax: British Thoracic Society Winter Meeting, London, UK**

*Interleukin-33 in Chronic Lung Disease (discussion poster presentation)*

Nicola J Green, **Monika I Suwara**, Derek A Mann and Andrew J Fisher

**2012 European Respiratory Society Conference, Vienna**

*Interleukin-33 release from Airway Epithelium (discussion poster presentation)*

Nicola J Green, **Monika I Suwara,** Lee A Borthwick, Derek A Mann and Andrew J Fisher


**2011 The International Society for Lung and Heart Transplantation Conference, San Diego, USA**

*The epithelial alarmin, Interleukin 1α (IL-1α), is a potential fibrogenic factor in bronchiolitis obliterans syndrome (BOS) (poster presentation)*

**Monika I Suwara**, Rahul Mahida, Lee A Borthwick, Jelena Mann, Paul Corris, Stuart N Farrow, Andrew J Fisher, Derek A Mann


**2011 Lung Science Conference, European Respiratory Society, Estoril, Portugal**

*IL-1 α is the key epithelial alarmin which promotes proinflammatory phenotype in lung fibroblasts (poster presentation)*

**Monika I Suwara**, Lee A Borthwick, Jelena Mann, Stuart N Farrow, Andrew J Fisher, Derek A Mann


**2011 Thorax: Winter Meeting of the British Thoracic Society, Westminster, UK**

*Alarmins in bronchiolitis obliterans syndrome after lung transplantation (oral presentation)*

Rahul Mahida, **Monika I Suwara**, Gail Johnson, Derek A Mann, Paul A Corris, Lee A Borthwick, Andrew J Fisher


**2011 British Association of Lung Research, Summer Conference**

*The Role of IL-1 in Driving Epithelial to Mesenchymal Transition in Lung Epithelial Cells (poster presentation)*

Nicola J Green, **Monika I Suwara**, A Gardner, Lee A Borthwick and Andrew J Fisher

**2010 Thorax: British Thoracic Society Winter Meeting, London, UK**

*IL-1 α is the key epithelial alarmin which promotes fibroblast activation (oral presentation)*

**Monika I Suwara**, Lee A Borthwick, Jelena Mann, Stuart N Farrow, Andrew J Fisher, Derek A Mann

**2010 Institute of Cellular Medicine Research Day, Newcastle University, UK**

*Alarmins released by damaged human lung epithelia provoke fibroblast activation (poster presentation)*

**Monika I Suwara**, Lee Borthwick, Jelena Mann, Stuart Farrow, Andrew Fisher, Derek Mann

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