# Endoplasmic reticulum stress in the lung:
## lessons from $\alpha_1$-antitrypsin deficiency

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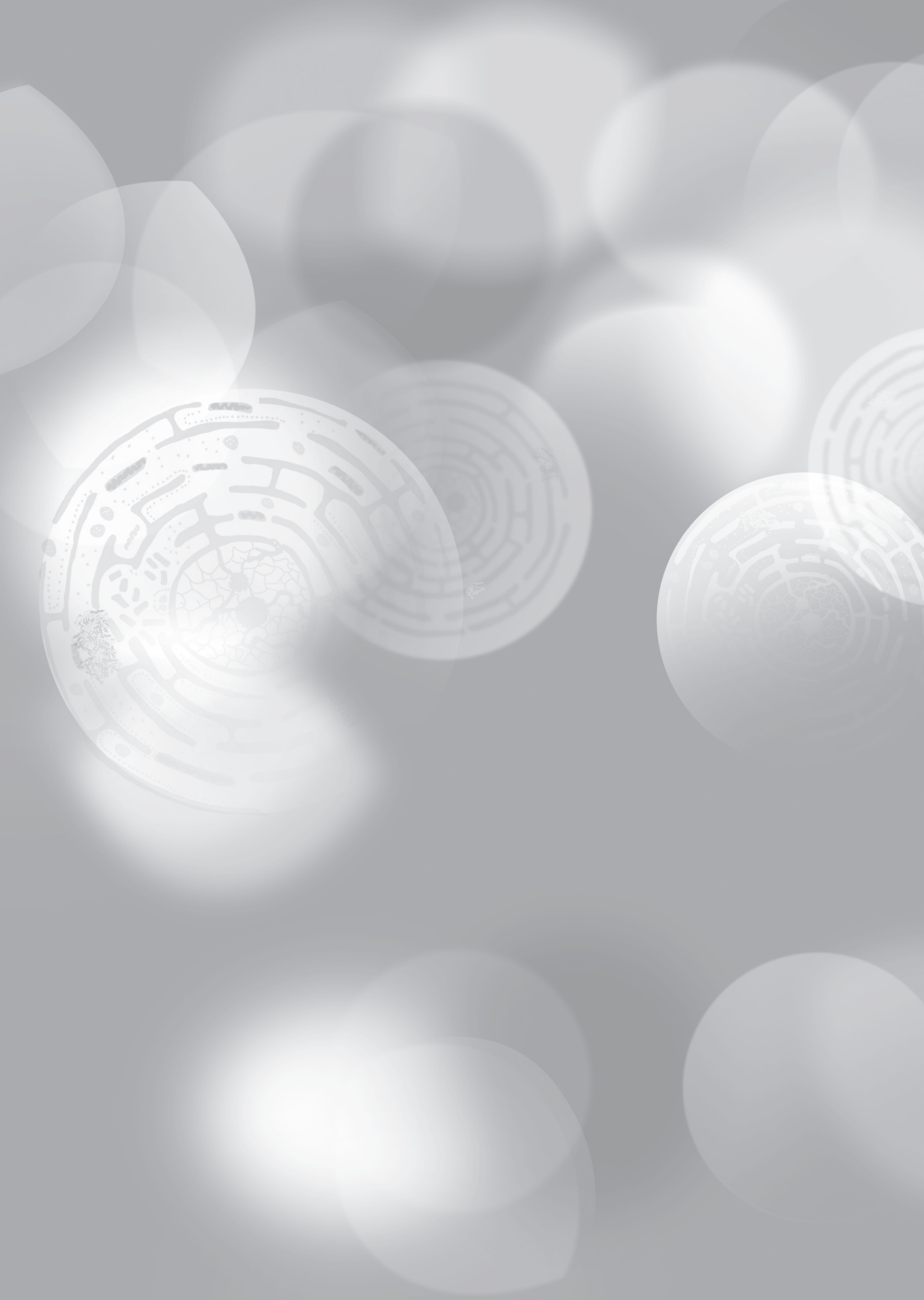


# Chapter 1

General introduction

## Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is one of the most important causes of morbidity and mortality worldwide. In 2008, COPD was already the fourth leading cause of death in the world, but the number of patients is still rising and the World Health Organization (WHO) predicts that COPD will become the third most common cause of mortality in 2030 (1). COPD is a systemic disease characterised by a progressive airflow limitation which is not fully reversible. Patients with COPD usually present with cough, sputum production, dyspnoea and impaired exercise tolerance. It is associated with a chronic and abnormal inflammatory response of the lung, primarily caused by exposure to cigarette smoke and/or other risk factors such as biomass cooking and occupational exposure to dust and chemical reagents (2-5). The diagnosis is traditionally confirmed by an airflow limitation defined as a decreased ratio of forced expiratory volume in 1 second ($FEV_1$) over forced vital capacity (FVC), and a reduction of the $FEV_1$ on spirometry that is not fully reversible by inhaling a bronchodilator (6).



## Alpha$_1$-antitrypsin deficiency as a risk factor of COPD

Alpha$_1$-antitrypsin deficiency is the most widely recognised genetic disorder causing COPD. It was first described in 1963 by Carl-Bertil Laurell and his fellow investigator Sten Erikkson, who discovered two emphysematous patients missing a normal $\alpha_1$-band on plasma protein electrophoresis (7). After this initial finding, over 100 variants of $\alpha_1$-antitrypsin have been reported and the characterisation of different genetic variants and their prevalence has been an important challenge. The different genetic mutations were classified by the proteinase inhibitor (Pi) nomenclature according to their electrophoretic mobility compared to the normal M variant (PiMM) and it became apparent that the majority of mutations in the *SERPINA1* gene, leading to $\alpha_1$-antitrypsin deficiency, were due to single amino acid substitutions (8). The most frequent and severe mutation in Northwest Europe and North America is the Z mutation (E342K), which originated in Scandinavia, and compromises over 95% of all $\alpha_1$-antitrypsin deficiency cases (8). Approximately 4% of the affected individuals are heterozygous (PiMZ) for this variant,

while roughly 1 in 1700 Northern Europeans are PiZZ homozygotes, which is as common as cystic fibrosis-associated CFTR mutation. Although the PiZZ homozygotes are extremely prone to develop disease (9), there is a high variability in clinical presentation. About 10% of the new-borns exhibit prolonged jaundice and juvenile cirrhosis, of which the majority clinically recover. In contrast, only 2-3% develop severe hepatitis and cirrhosis of the liver during childhood or adulthood requiring liver transplantation (10-12). In most homozygotes who smoke tobacco, the mutation is accompanied by a rapidly developing and progressive early-onset lung emphysema. Although the increased risk of developing COPD in PiMZ individuals remains controversial (13, 14), a meta-analysis revealed an odds-ratio of 2.31 (95% confidence interval 1.60-3.35; (15)). Historically, it has been suggested that severe lung and liver disease rarely coexists in the same patient (16, 17). However, Dawwas *et al*. (18) recently demonstrated that 63.2% of PiZZ individuals with clinical lung disease had a history or clinical findings of liver disease, and 17.5% had evidence of severe fibrosis or cirrhosis as confirmed by ultrasound or liver biopsy. The management of the lung condition of this deficiency with intravenous augmentation therapy of $\alpha_1$-antitrypsin is at present not fully accepted to be effective, and disease progression is ultimately a major reason for lung transplantation (19).



**Pathogenesis**

Alpha$_1$-antitrypsin deficiency is a so-called conformational disease, which develops as a result of a mutation that alters protein conformation, leading to protein misfolding, aggregation and depositions within cells or tissues. Besides $\alpha_1$-antitrypsin deficiency, other members of this group of disorders include other serine protease inhibitor (serpin) mutants (serpinopathies), Alzheimer's and Parkinson's disease, prion encephalopathies and amyloidosis (20). Alpha$_1$-antitrypsin, a member of the serpin superfamily, is the major serpin active within the lung, but is mainly synthesised by hepatocytes (21). In addition, small amounts are made by peripheral blood monocytes, alveolar macrophages and lung epithelial cells (22-24). One of the most important inhibitory functions of $\alpha_1$-antitrypsin is its irreversible inactivation of neutrophil elastase, thereby protecting lung tissue against

the destructive effects of neutrophil elastase released by degranulating neutrophils during inflammation. More recently, the anti-inflammatory properties of $\alpha_1$-antitrypsin have become apparent, including the inhibition of TNFα gene expression (25), inhibition of a disintegrin and metalloprotease (ADAM)17 activity in neutrophils and endothelial cells (26, 27), and the regulation of CD14 expression and cytokine release in monocytes (28, 29).



The native structure of $\alpha_1$-antitrypsin is composed of three β-sheets (A-C), nine α-helices (A-I) and an exposed mobile reactive centre loop (Figure 1A; (30, 31)). Upon docking with neutrophil elastase, the enzyme cleaves the P1-P1' peptide bond of the reactive centre loop, which can subsequently be inserted as an additional central strand (strand 5) into the β-sheet A (32). This results in stabilisation of the $\alpha_1$-antitrypsin, inactivation of the active site of the elastase and subsequent clearance of the serpin-enzyme complex from the circulation by various cell types, including hepatocytes (32). Although Z $\alpha_1$-antitrypsin is transcribed and translated at the same rate as normal M $\alpha_1$-antitrypsin, the mutation affects post-translational folding of the Z $\alpha_1$-antitrypsin polypeptide chain inside the endoplasmic reticulum (ER). This has been hypothesised to generate an unstable intermediate state, termed M*, in which β-sheet A is more exposed, allowing it to accept the reactive centre loop of another Z $\alpha_1$-antitrypsin molecule to form a dimer (Figure 1B; (33-35)). Subsequent extension would then form longer chains of loop-sheet polymers (36). Other possible molecular mechanisms of polymerisation described are the β-hairpin linkage model (37) and the more recently suggested triple-strand linkage model or C-terminal swap (38) (Figure 1C-D). While polymers do not only lack any inhibitory activity, their formation leads to accumulation within the ER of hepatocytes and subsequent degradation by the proteasome, resulting in a plasma deficiency (33).

Thus, the current concept is that the accumulation of intracellular polymers of $\alpha_1$-antitrypsin causes liver pathology through toxic gain-of-function, while early-onset emphysema is due, in large part, to loss-of-function resulting from a protease-antiprotease imbalance (39). However, after the discovery of polymers in broncho-alveolar lavage fluid and pulmonary tissue up to ten years following liver transplantation (40), it has been

proposed that these polymers can be produced locally within the lung. As these polymers are chemotactic for neutrophils *in vivo* and *in vitro*, such local formation might contribute to sustained inflammation in subjects with $\alpha_1$-antitrypsin deficiency (41).

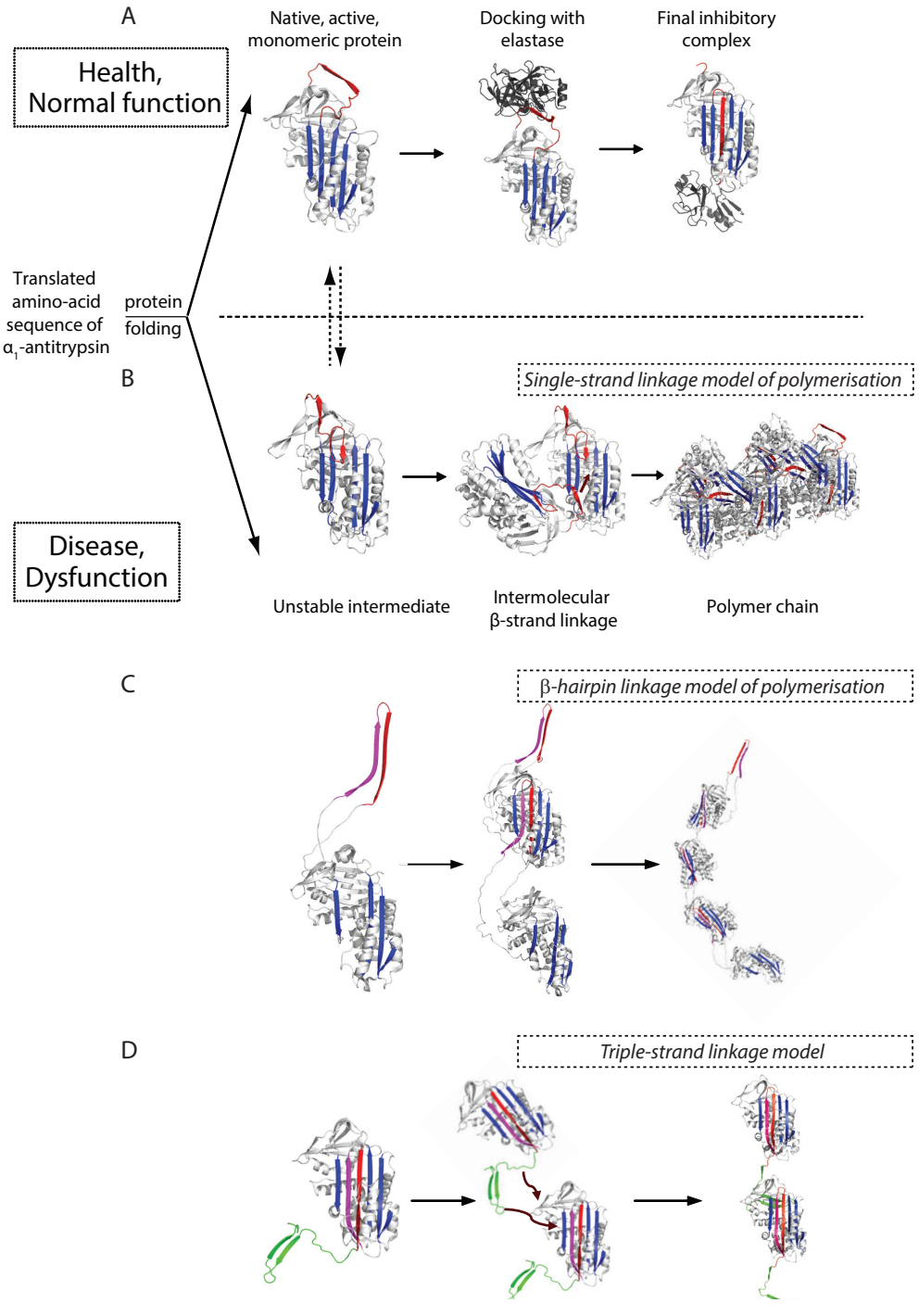
## ER stress

Within the ER, proteins destined for secretion and insertion into the cell-membrane are folded to their tertiary structure and undergo post-translational modifications, such as disulphide bond formations and glycosylation. The organelle's quality control system allows only properly folded proteins to exit the ER and traffic to their site of action (42). Delayed folding can be caused by mutant proteins, rapid surges in demand for protein secretion and metabolic disturbances such as hypoxia. The imbalance between protein load and the ER's capacity to handle newly synthesised proteins is termed "ER stress" (43-45). Moreover, toxins such as tunicamycin, an irreversible inhibitor of N-linked glycosylation, and thapsigargin, a sarcoendoplasmic reticulum calcium ATPase (SERCA)-pump inhibitor, lead to protein misfolding and thus chemically-induced ER stress. Since misfolded ER client proteins are prone to aggregation, they pose a cytotoxic risk to the cell. Accordingly, early in evolution eukaryotes developed an Unfolded Protein Response (UPR) to deal with this threat (44).



### The UPR

The UPR is activated by three distinct stress sensors: inositol-requiring enzyme 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). In the resting state, each sensor is inactive. One theory states that inactivity is maintained through the binding of a chaperone called glucose-regulated protein 78 (GRP78, also known as BiP) (46, 47). With the accumulation of unfolded or misfolded proteins inside the ER lumen, GRP78 dissociates from its kinase to bind these proteins. This sequestration of GRP78 is the central trigger for each kinase to become activated, thereby initiating distinct downstream pathways (Figure 2). More recently, it has been suggested that PERK and IRE1 may interact directly with misfolded


A
Native, active, monomeric protein
Docking with elastase
Final inhibitory complex
Health, Normal function
Translated amino-acid sequence of α1-antitrypsin
protein folding
B
Single-strand linkage model of polymerisation
Disease, Dysfunction
Unstable intermediate
Intermolecular β-strand linkage
Polymer chain
C
β-hairpin linkage model of polymerisation
D
Triple-strand linkage model

<<< **Figure 1. Folding of $\alpha_1$-antitrypsin and its misfolding in $\alpha_1$-antitrypsin deficiency.**

A. The reactive centre loop (red) of native $\alpha_1$-antitrypsin binds to neutrophil elastase (dark grey). This allows the conformational change leading to an additional strand into β-sheet A (blue) and the inactivation of neutrophil elastase. B-D. Three proposed models for Z $\alpha_1$-antitrypsin polymerisation. All mechanisms involve an unstable intermediate. In the single-strand linkage model (B), the reactive centre loop of one $\alpha_1$-antitrypsin molecule is inserted in between strands 3 and 5A of another molecule. The β-hairpin linkage model (C) proposes that both the reactive centre loop and strand 5A translocate into another $\alpha_1$-antitrypsin molecule. The newest model is the tirple-strand linkage model (D). The reactive centre loop self inserts and strands 1C, 5 and 6B translocate between the molecules. Reproduced with permission from B. Gooptu and D.A. Lomas (104).

proteins (48, 49).

Early during ER stress, the dimerisation and subsequent trans-autophosphorylation of PERK strongly activates this kinase (50, 51). It then phosphorylates eukaryotic translation initiation factor 2 on its alpha subunit (eIF2α) causing global inhibition of protein synthesis, and thereby reducing the load of newly synthesised proteins entering the ER (50). In addition, eIF2α phosphorylation promotes a gene expression programme known as the integrated stress response (ISR (52); see Chapter 2). This is mediated by the translation of specific mRNAs, notably activating transcription factor 4 (ATF4) (53). ATF4 triggers a signalling cascade leading to the induction of the transcription factor C/EBP homologous protein (CHOP), which in turn induces growth arrest and DNA damage-inducible protein 34 (GADD34, also known as PPP1R15A) (54). In complex with protein phosphatase 1 (PP1), GADD34 dephosphorylates eIF2α thus completing a negative feedback loop and enabling the recovery of general protein synthesis (55). Another target gene of CHOP encodes ERO1α, an ER oxidase that in conjunction with Protein Disulphide Isomerase (PDI) catalyses the generation of disulphide bonds in ER client proteins (54).

In parallel, the dissociation of GRP78 releases ATF6 to be transported from the ER to the Golgi via the specific coat protein complex II (COPII) vesicles (56, 57). Here, site 1 and site 2 proteases (S1P and S2P, respectively) cleave ATF6, liberating a cytosolic N-terminal

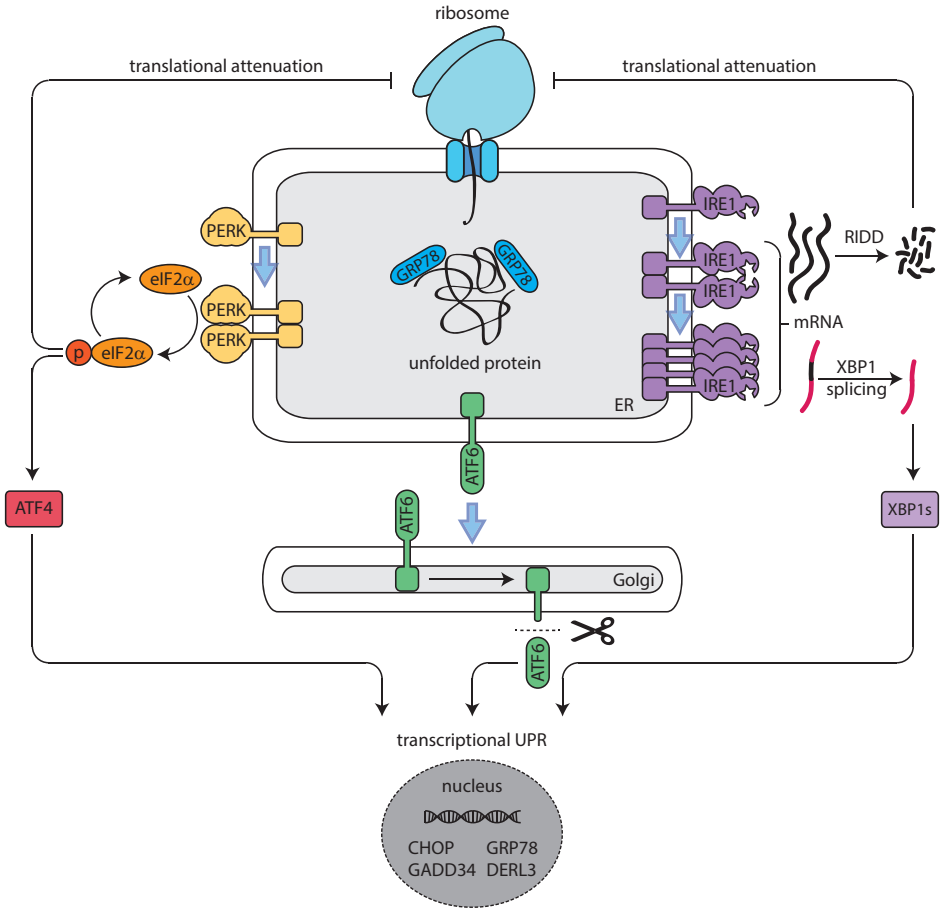



**Figure 2. The unfolded protein response (UPR).**

Three endoplasmic reticulum (ER) signal transducers sense the protein-folding state inside the ER. When unfolded or misfolded proteins accumulate in the ER lumen, PERK, ATF6 and IRE1 become activated through a loss of binding to GRP78 (also called BiP) and/or directly by interacting with the misfolded proteins. PERK activation leads to the phosphorylation of eIF2α and subsequent global translational attenuation. Controversially, it activates a pathway involving ATF4 and its downstream targets CHOP and GADD34, of which the latter is able to dephosphorylate eIF2α. The endoribonuclease activity of IRE1 results in the splicing of *XBP1* mRNA and reduces mRNA stability (a process called RIDD), thereby contributing to the inhibition of global mRNA translation. Activation of ATF6 results in its translocation to the Golgi and its cleavage to allow translocation into the nucleus and activation of UPR target genes. Reproduced with permission from J.E. Chambers, University of Cambridge (105).

fragment (ATF6c) that travels to the nucleus and binds ER stress response elements (ERSE) to initiate translation of UPR target genes, including many chaperons (58), to enhance protein folding (59).

Like PERK, IRE1 is activated via dimerisation and trans-autophosphorylation (60-62). Unlike PERK, however, this triggers the RNAase domain of IRE1 to initiate the unconventional splicing of the mRNA encoding *X-box binding protein-1* (*XBP1*) (63). *Spliced XBP1* mRNA encodes an active transcription factor that, together with ATF6c, induces expression of UPR target genes, such as the chaperones *GRP78* and *GRP94* (63). In addition, *spliced XBP1* induces components involved in the ER-associated degradation (ERAD) pathway (see below; (64)). Moreover, phosphorylated IRE1 facilitates the degradation of mRNAs encoding proteins destined to enter the ER, thus further diminishing the influx of new proteins into the ER; a process called Regulated IRE1-Dependent Decay (RIDD, (65)). Finally, activated IRE1α may recruit TRAF2, which, via activation of cJUN $NH_2$-terminal kinase (JNK), can lead to enhanced cytokine release and eventually cell death (66).



**ERAD and apoptosis**

When the UPR fails to restore protein homeostasis by reducing the influx of newly synthesised proteins into the ER and increasing the protein folding capacity, a third mechanism is evoked: the ERAD. This degradation pathway targets terminally unfolded or misfolded proteins for proteasomal degradation, relieving the ER of its burden (67).

Whereas induction of the UPR during acute ER stress is a necessity for cell survival, chronic ER stress and prolonged activation of the UPR is associated with increased cell death (54, 68-70). As mentioned above, IRE1 activation can trigger apoptosis via the TRAF2-JNK pathway. Urano *et al.* (66) showed that TRAF2 binds to IRE1α leading to the activation of JNK. Accordingly, *Ire1α*$^{-/-}$ fibroblasts are impaired in JNK signalling in response to ER stress. Because sustained JNK activity has been associated with apoptosis, this pathway may contribute to IRE1α-mediated cell death (71-73). Other possible links between IRE1 and ER stress-induced apoptosis may involve increased RIDD activity of essential proteins (74), and the interaction of IRE1 with pro-apoptotic proteins such as Bak and Bax (75).

Furthermore, prolonged high level of GADD34 expression have been shown to induce cell death, perhaps by promoting on-going protein secretion via the dephosphorylation of eIF2α, thereby overloading an already stressed ER (54). Of note, CHOP has often been reported as pro-apoptotic. However, this is misleading as CHOP does not directly induce cell-death target genes, but instead appears to promote apoptosis indirectly via its target genes, especially GADD34 and ERO1α, which increase protein synthesis and oxidation within the ER (54).



**ER overload response (EOR)**

Besides the activation of the well-characterised UPR, ER stress also induces the activation of the poorly understood ER overload response (EOR), also known as the ordered protein response (76). This response was initially observed in cells transfected with an expression vector for immunoglobulin μ chain, which are unable to exit the ER, leading to the activation of nuclear factor-kappa B (NF-κB) (77). Furthermore, several ER stress inducers selectively caused the induction of NF-κB or the activation of the UPR, suggesting that the activation of NF-κB required a distinct signal. Although NF-κB activation can follow inhibition of IκB translation by PERK (78), a UPR-independent signal for NF-κB activation appears to be the accumulation of ordered proteins causing distension of the ER (79). This accumulation leads to the efflux of $Ca^{2+}$, which, together with the production of reactive oxygen species (ROS), is an essential messenger signal for NF-κB activation (76).

## ER stress and inflammation in $\alpha_1$-antitrypsin deficiency

For many years, the protease-antiprotease imbalance in the lungs was thought to be the main explanation for early-onset emphysema in patients with Z $\alpha_1$-antitrypsin deficiency. However, over the years, $\alpha_1$-antitrypsin appeared to possess additional roles besides its antiprotease activity, which might be altered due to the Z mutation. Although the Z mutation causes a conformational change and an accumulation of $\alpha_1$-antitrypsin polymers in the ER of hepatocytes, to date these polymers have not been convincingly shown to activate the UPR directly (80, 81). However, recently it has been recognised that the intracellular polymerisation of Z $\alpha_1$-antitrypsin may prime cells to ER stress if exposed to a second insult (82, 83). For instance, Chinese hamster ovary (CHO) cells overexpressing Z $\alpha_1$-antitrypsin show impaired protein mobility within the ER due to distension of the ER by polymer accumulation (84). Importantly, this accumulation does not elicit activation of an ATF6-luciferase reporter or trigger the splicing of *XBP1* mRNA. However, when these cells encounter a "second hit", such as the chemical ER stress inducer tunicamycin or a more physiological glucose starvation, they show an exaggerated activation of the ATF6-luciferase reporter together with increased *XBP1 splicing* (84). It has been suggested that Z $\alpha_1$-antitrypsin expression in human embryonic kidney cells leads to the cleavage of caspase-4 and -7, resembling thapsigargin-induced apoptosis (85), but this appears to be cell-type specific since 16HBE cells expressing Z $\alpha_1$-antitrypsin show reduced levels of apoptosis and decreased caspase-3 activity, similar to M $\alpha_1$-antitrypsin expressing 16HBE cells (81). The enhanced sensitivity of Z $\alpha_1$-antitrypsin expressing cells to ER stress may contribute to lung disease through the induction of inflammation and cell death in pulmonary epithelial cells and macrophages following exposure to a secondary trigger, but to date there is limited evidence for this hypothesis within the lung.

The constitutive activation of NF-κB in lung epithelial cells expressing Z $\alpha_1$-antitrypsin has been thought to reflect protein polymer formation leading to increased cytokine release and inflammation (83, 86, 87). Carroll and colleagues previously showed increased IκBα degradation and increased cytokine production together with intracellular accumulation of $\alpha_1$-antitrypsin in ZZ monocytes (80). It is worth noting, however, that

the conformation of the retained $\alpha_1$-antitrypsin protein remained unclear and so the mechanism for the hyperinflammatory phenotype of Z $\alpha_1$-antitrypsin cells remains unknown.

**NF-κB**

The transcription factor NF-κB regulates many genes involved in inflammation and cell death, and therefore plays a key role in inflammation and immunity (88). It is the main regulator of numerous cytokines and chemokines such as interleukin (IL)-8 (89). NF-κB is a cytosolic protein consisting of the p50/p52 and p65 (or RelA) subunits and is inactive when in complex with inhibitor kappa-B alpha (IκBα; (90, 91)). The phosphorylation of this IκBα, which results in its degradation by the proteasome, reveals the nuclear localisation site of NF-κB (92, 93). Subsequently, NF-κB translocates into the nucleus, where it is able to bind to specific DNA sequences and regulate transcription (89, 94). A key regulatory step in the translocation of this transcription factor is activation of a high molecular weight IkappaB kinase (IKK) complex, which phosphorylates IκBα (95, 96). The IKK complex consists of three subunits, of which IKKα and IKKβ are catalytically active and IKKγ serves as a regulatory unit (97). Numerous pathways lead to the activation of NF-κB, with most exogenous stimuli acting via the classical IKK-IκB pathway. Examples important for innate immunity include cytokine receptors, such as tumor necrosis factor (TNF) receptor, and Toll-like receptors, the main class of pattern-recognition receptors (Figure 3). However, it is now generally acknowledged that NF-κB can also be activated via mitogen-activated protein kinase (MAPK) signalling cascades (98, 99).

**MAP kinases**

The MAPKs are a conserved group of serine/threonine kinases that mediate a wide range of cellular responses, including cell growth, differentiation, cell survival and the immune response (reviewed in (100)). There are six distinct groups of MAPK, but the most extensively studied MAPKs are the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the p38 MAPK and the c-Jun NH2-terminal kinases (JNK).

ERK1/2 was the first MAPK discovered and its upstream kinases and signalling cascades have been widely studied. Upon stimulation, Ras activates c-Raf, which will lead to phosphorylation of the mitogen-activated protein/extracellular signal-regulated kinase kinases (MEK) which, in turn, phosphorylate ERK1/2. The initiation of this pathway generally starts via the activation of tyrosine kinases receptors on the cell membrane. In the lung, the tyrosine kinase receptor epidermal growth factor (EGF) receptor (EGFR) plays an important role in epithelial cell proliferation, cytokine induction, and cell survival by activating ERK1/2 (101). EGF and EGF-like growth factors, of which heparin binding-EGF (HB-EGF), amphiregulin (AREG) and transforming growth factor alpha (TGFα) are the most important, are membrane-bound growth factors, which, upon cleavage by metalloproteases, as ADAMs, trigger EGFR signalling (102, 103).

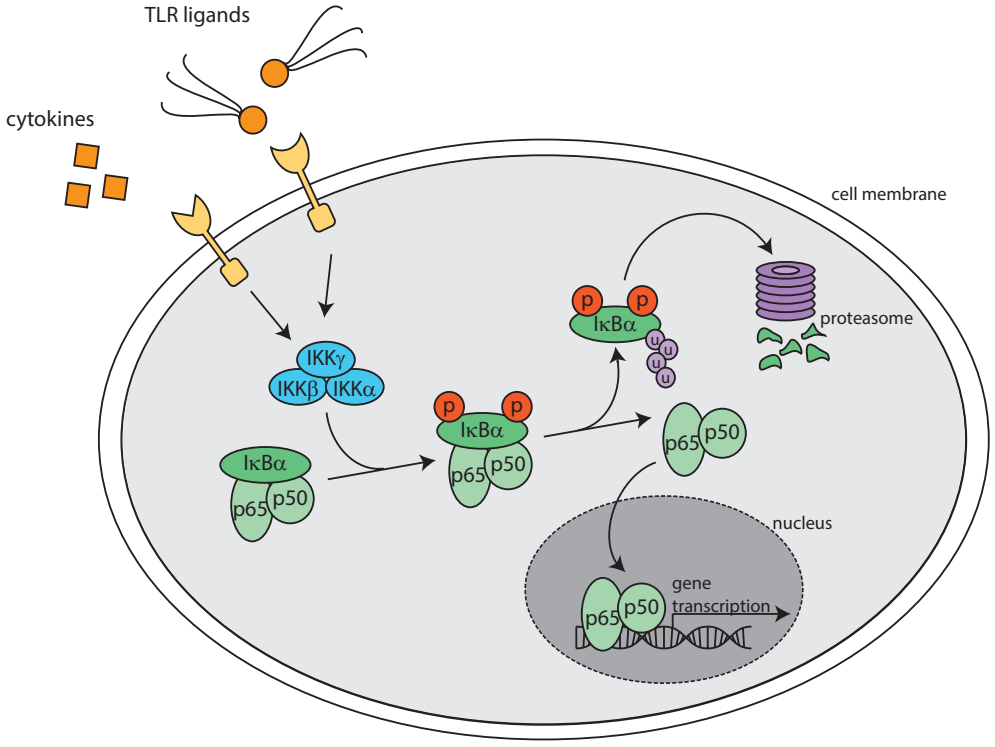



**Figure 3. Activation of the NF-κB pathway.**

A variety of triggers, such as cytokines and Toll-like receptor (TLR) ligands, bind to their receptor, resulting in the activation of the IκB kinase (IKK) complex. IKK phosphorylates the inhibitor of NF-κB (IκBα), thereby releasing IκBα from NF-κB and exposing the nuclear localisation site of NF-κB. IκBα is degraded by the proteasome and the activated NF-κB is then translocated into the nucleus where it activates its target genes.

## Outline / aim of the thesis

Altered physiological stress responses, including ER stress, may contribute to the development of lung diseases. The aberrant folding of Z $\alpha_1$-antitrypsin and its accumulation within the ER has led some to suggest that $\alpha_1$-antitrypsin deficiency might represent an ER stress-induced disease. However, as discussed above, there is much controversy about the extent to which Z $\alpha_1$-antitrypsin triggers the UPR. The studies described in this thesis focus on the induction of ER stress in bronchial epithelial cells and (monocyte-derived) macrophages and its contribution to inflammation in lung disease, with a specific focus on Z $\alpha_1$-antitrypsin deficiency.



ER stress, leading to the unfolded protein response (UPR), is a complex cellular process. As discussed in this introduction, activation of the PERK-arm of the UPR leads to phosphorylation of eIF2α and subsequent global translational repression. In addition to PERK, three other kinases can also initiate translational attenuation via the phosphorylation of eIF2α and its downstream targets. These pathways are collectively called the integrated stress response (ISR). This thesis starts in **Chapter 2** with a review on the importance of the ISR in lung diseases.

Another important UPR-arm, leading to the induction of ER chaperones to allow refolding, is the IRE1-pathway. In **Chapter 3,** we validated a new technique that allows rapid and reliable detection of one of the key features of this arm, namely the *splicing of XBP1* mRNA.

Bacteria and especially viruses, which have been more extensively studied than other microbes, can induce splicing of *XBP1* mRNA, phosphorylation of eIF2α and induction of GADD34, with a lack of CHOP induction. Recently, it has been proposed that this microbe-induced specific stress response induced by microbes, which mimics an ER stress response and an ER stress independent activation of the ISR, should be termed the "microbial stress response (MSR)". However, the precise description is not yet clearly defined and we therefore investigate the ER stress response and ISR induced by secreted virulence factors of *Pseudomonas aeruginosa* in **Chapter 4**.

Z $\alpha_1$-antitrypsin is a misfolded protein that polymerises and subsequently

accumulates in the ER. However, overexpression of the protein does not initiate the ER stress response, but rather primes the cell for a "second hit". Instead, this accumulation leads to an enhanced NF-κB response. In **Chapter 5**, we investigated what is causing the increased inflammatory (NF-κB) signalling in primary bronchial epithelial cells with an endogenous expression of Z $\alpha_1$-antitrypsin. We explored the possibility of polymer formation as an underlying mechanism and investigated whether endogenous Z $\alpha_1$-antitrypsin primes primary bronchial epithelial cells for a second hit, and thereby causing this augmented response.



Previous studies have shown that macrophages are also able to produce $\alpha_1$-antitrypsin. Since macrophages constitute a heterogeneous population, we first investigated in **Chapter 6** whether there is a difference in $\alpha_1$-antitrypsin production between the two main macrophage subtypes of healthy donors that can be generated *in vitro*. Subsequently, in **Chapter 7**, we assessed the $\alpha_1$-antitrypsin production by monocyte-derived macrophages of Z $\alpha_1$-antitrypsin patients and the cellular consequences of its expression. Finally, a summary and general discussion of these studies are presented in **Chapter 8**.

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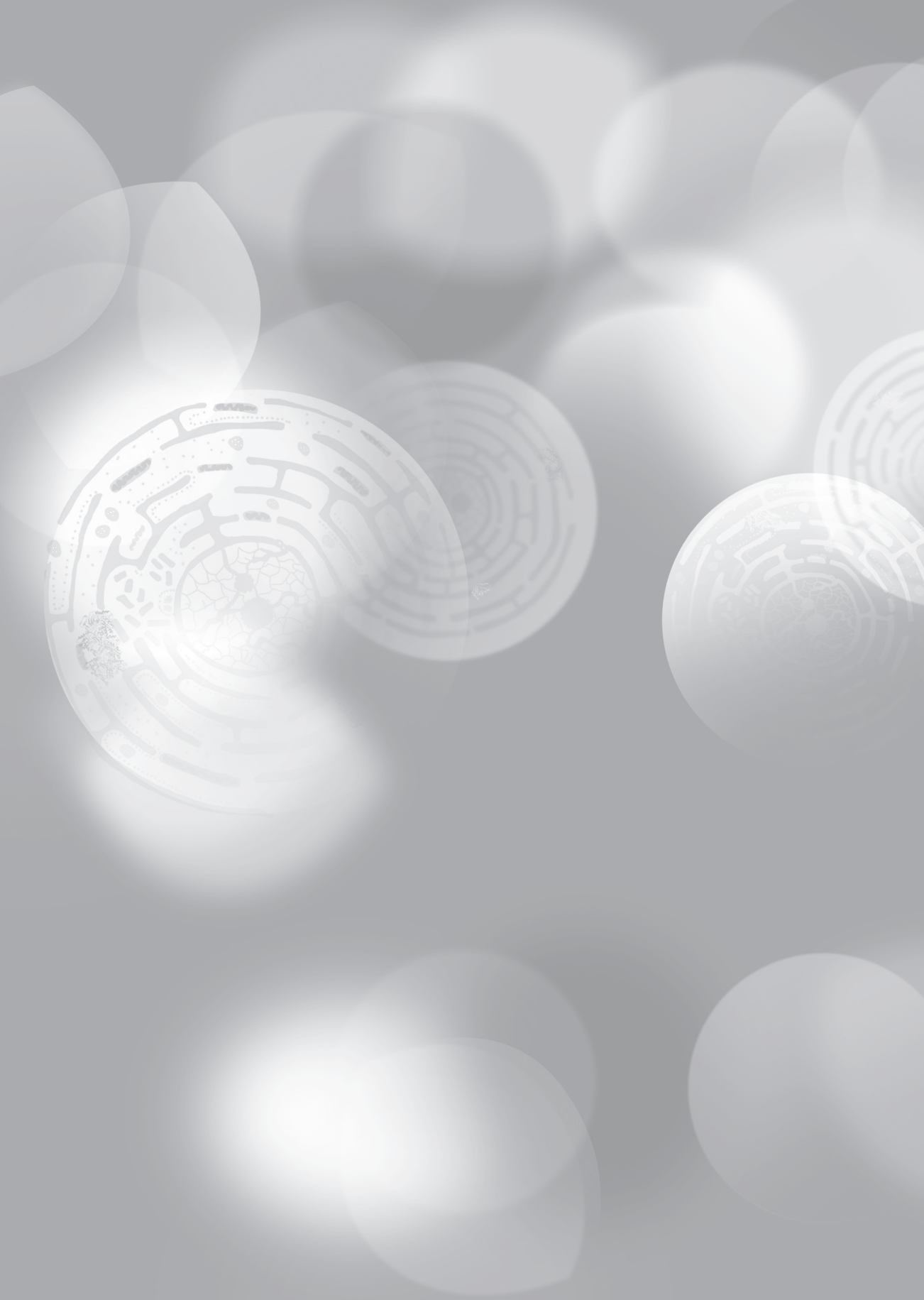

# Chapter 2

## The integrated stress response in lung disease

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## Abstract

**Lungs are repeatedly exposed to inhaled toxic insults, such as smoke, diesel exhaust and microbes, which elicit cellular stress responses. The phosphorylation of eIF2α by one of four stress-sensing kinases triggers a pathway called the integrated stress response (ISR) that helps protect cellular reserves of nutrients and prevents that accumulation of toxic proteins. In this review, we will discuss how activation of the ISR has been shown to play an important role in pulmonary pathology and how its study may help in the development of novel therapies for diverse conditions from hypoxia to cancer.**

## Introduction

Because of its direct contact with the outside world, the lung is continuously challenged by inhaled insults, including smoke, diesel exhaust and a multitude of microbes, all of which trigger various cellular stress pathways. Recent studies have underlined the critical role played by one of these stress pathways, which involves phosphorylating the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α). This single molecular event serves to integrate signalling from multiple stress-sensors and so has been termed the "integrated stress response" (ISR) (Figure 1). In this review, we will discuss the ISR with particular regard to its evolving role in pulmonary medicine and we highlight how this knowledge is enabling the development of novel therapies.



## The integrated stress response (ISR)

Activation of the ISR occurs when one of four homologous stress-sensing kinases is triggered. This family comprises General Control Nonrepressible-2 (GCN2), Heme-Regulated Inhibitor (HRI), Protein Kinase R (PKR) and PKR-like Endoplasmic Reticulum Kinase (PERK) (1). GCN2 evolved first and allows all eukaryotes, including yeast, to respond to amino acid starvation. As multicellular animals developed, this kinase gave rise to a family of proteins that could respond to other stresses. HRI was identified as a kinase responsive to iron-deficiency; however, over time, it has been shown to respond also to other stressful stimuli including damaging oxidation. PKR responds to the appearance of double-stranded RNA within the cell, which occurs most commonly during viral infections; and finally, PERK senses the efficiency of protein folding within the endoplasmic reticulum (ER), thus enabling cells to trigger the ISR during ER stress. This pathway induced by PERK activation is also one of the three arms of the unfolded protein response (UPR) to ER stress, which has been reviewed elsewhere (2).

When eIF2α is phosphorylated, protein synthesis is blocked, which serves a number of cytoprotective roles. During ER stress, it lowers the rate at which new proteins enter the ER thereby off-loading its overburdened chaperones; in conditions of amino acid starvation or iron limitation, it reduces the rate at which these nutrients are consumed

to make new proteins; and during viral infection, blocking protein synthesis can impede viral replication. Furthermore, the phosphorylation of eIF2α paradoxically promotes the translation of a subset of mRNAs, most notably that encoding activating transcription factor 4 (ATF4). Indeed, most canonical ISR target genes are transactivated by this transcription factor and act to adapt the cell to these stresses. For example, up-regulation of numerous transporters increases the rate of amino acid import, thus overcoming nutrient limitation, while also providing the substrates needed for the synthesis of the antioxidant glutathione (Figure 1). In addition, ATF4 induces expression of another transcription factor, C/EBP homologous protein (CHOP), which cooperates with ATF4 to induce the eIF2α phosphatase growth arrest and DNA damage-inducible protein 34 (GADD34; also known as PPP1R15A) (3). GADD34 completes a negative feedback loop and allows protein synthesis to recover by dephosphorylating eIF2α (4).



It is often claimed that CHOP is a pro-death transcription factor, but this is inaccurate. In some situations, the expression of CHOP promotes cell survival (5); however, in conditions of very prolonged stress, for example in models of chronic disease, the loss of CHOP is clearly protective. This protective effect of CHOP deletion reflects the toxic consequence of recovering protein synthesis (via GADD34) if the original stress has not adequately been ameliorated by the ISR. In addition, another target of CHOP is the ER oxidase ERO1α, which is up-regulated during the ISR to promote oxidative protein folding, but can itself contribute to cellular oxidative stress (Figure 1). Finally, CHOP contributes to the induction of pro-inflammatory genes, such as interleukin (IL)-8 (6).

## Activation of the ISR in lung pathology

### Inhaled toxins

Of the pulmonary insults recognised to trigger the ISR, perhaps the best known is oxidative stress, frequently caused by exposure to cigarette smoke. *In vitro* studies have shown that cigarette smoke extract induces apoptosis in a CHOP-dependent manner and this can be antagonised by antioxidants (7, 8). ER stress appears to be a major mediator of

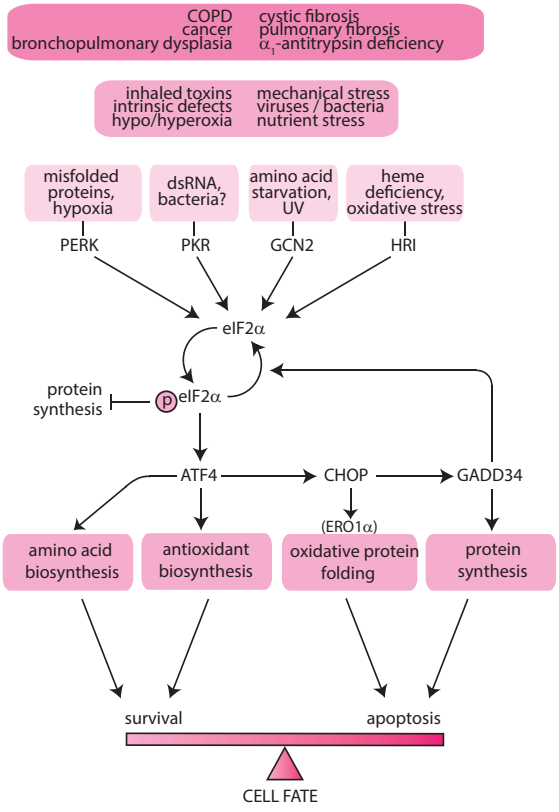


**Figure 1. The integrated stress response (ISR) in the lung.**

Phosphorylation of eIF2α by the stress-sensing kinases GCN2, PKR, PERK or HRI, leads to activation of the ISR by a wide range of insults including amino acid starvation, viral infection, protein misfolding and iron deficiency. This attenuates global protein synthesis via the inhibition of eIF2B, which normally functions to maintain the translation initiation factor eIF2 in its active GTP-bound state. Simultaneously, limiting levels of active (GTP-bound) eIF2 lead to the translation of the transcription factor ATF4. Targets of ATF4 include amino acid transporter and synthesases, which serve a cytoprotective role. Via induction of the transcription factor CHOP and the eIF2α phosphatase GADD34, ATF4 also promotes the recovery of protein synthesis and oxidative protein folding (through induction of the oxidase ERO1α). During severe or prolonged stress, the recovery of protein translation and protein oxidation can contribute to the failure of homeostasis and ultimately to cell death. COPD, chronic obstructive pulmonary disease.

the response to cigarette smoke extract because inhibition of the kinase PERK can block this induction of CHOP (7). Consistent with this, heightened levels of ER stress have been observed within the lungs of current smokers (9) and patients with COPD (10). It has recently been demonstrated that exposure even to the smoke of a single cigarette can impair oxidative protein folding in lungs of mice, probably through impaired function of the ER enzyme Protein Disulphide Isomerase (PDI) (11).

Recently, an airway gene expression signature associated with COPD was described and included 98 genes, many of which are targets of ATF4 (12). When ATF4 is overexpressed in epithelial cells *in vitro*, this COPD gene signature is recapitulated. The observation that many of the COPD-associated changes in gene expression in bronchial biopsies can be reversed by inhaled corticosteroid therapy suggests that treatments might be targeted to this mechanism in future (12).



When mice are exposed to inhaled environmental particulate pollution, they also show evidence of ISR activation due to ER stress (13), as do cultured human cells treated with diesel exhaust particles (14). Since the effects of particulate matter can be antagonised by antioxidants, these too most likely reflect oxidative stress impairing the function of the ER (13). In fact, the toxicity of particulates may be mediated by the ISR, since CHOP deficient cells are protected from particulate-induced apoptosis.

It would be wrong to suggest that the ISR is purely toxic during pulmonary oxidative stress or that PERK is the sole kinase involved. For example, when mice are exposed to hyperoxia (95% $O_2$), phosphorylation of eIF2α does not involve ER-stress (5). Instead, PKR is activated by unknown mechanisms to induce ATF4 and CHOP. In this instance, CHOP is protective since *Chop*$^{-/-}$ mice reared in 95% $O_2$ develop more severe lung injury, and even modest hyperoxia (80% $O_2$) causes higher mortality in CHOP deficient animals (5). The mechanism for this protection is unclear but CHOP appears to prevent increased epithelial permeability. But the complex role of CHOP in hyperoxic lung injury requires more study because in newborn mice with hyperoxia-induced bronchopulmonary dysplasia CHOP appears to mediate pathological apoptosis (15).

**Intrinsic defects**

Point mutations of secreted proteins can cause ER stress and activate PERK. Surfactant protein C (SFTPC), which is normally secreted by type II pneumocytes, is mutated in some rare cases of familial interstitial lung disease (16). A splice-site mutation (c.460+1A>G) that causes deletion of *SFTPC* exon4 results in protein misfolding and familial interstitial pneumonia. The *Sftpc* null mouse has a non-lethal phenotype suggesting that the disease-associated mutations in man may be caused by toxic gain-of-function. Accordingly, when the Δexon4 mutant is expressed in cells it causes ER stress and phosphorylation of eIF2α (17). It remains to be tested, however, whether *CHOP* or *GADD34* genotype can alter the toxicity of the SFTPC mutants. Although currently only a correlation, it is intriguing that elevated levels of ATF4 and CHOP protein have been detected in lung homogenates and in type II cells from patients with idiopathic pulmonary fibrosis compared with controls (18). It is therefore tempting to speculate that ISR activation may be a final common pathway in many cases of interstitial lung disease.



Mutations of the *SERPINA1* gene, which encodes $\alpha_1$-antitrypsin, can lead to its accumulation within the ER, but surprisingly most of these mutants fail to trigger the UPR, although they do enhance the cell's susceptibility to ER stress (19). The most common of these, the so-called Z allele, results from a missense mutation (E342K) that destabilises the protein such that most is degraded while some polymerises within the ER resulting in serum deficiency and early onset emphysema in most homozygous individuals. Approximately 10% of homozygotes also develop clinically significant liver disease but the mechanism for this is not entirely clear. The increased sensitivity of hepatocytes to ER stress and therefore activation of the ISR by PERK may be involved, since mice made to express the Z variant are far more susceptible to developing hepatic fibrosis following bile duct ligation, which causes ER stress, than are animals expressing wild type $\alpha_1$-antitrypsin (20). In this model, Z expressing mice show an exaggerated ISR as evidenced by significantly higher induction of CHOP (20) and it is known that CHOP expression is essential for cholestasis-induced hepatic fibrosis (21). However, these effects are critically dependent on the level of Z $\alpha_1$-antitrypsin expressed by a cell. Primary bronchial epithelial cells,

which secrete detectable levels of Z $\alpha_1$-antitrypsin, fail to achieve concentrations of the mutant protein within their ER to allow protein polymerisation (22). As a result, in contrast to hepatocytes, airway epithelial cells show no augmentation of CHOP or GADD34 expression upon a second hit of mild ER stress (22). Each tissue should therefore be considered individually, since monocytes, another $\alpha_1$-antitrypsin-expressing cell, have been reported to display enhanced expression of ATF4 when purified from Z homozygous individuals (23). Whether these cells, unlike hepatocytes, experience constitutive activation of the ISR in Z individuals is not clear. But it is plausible that isolation of the cells may be sufficiently stressful to trigger the ISR, which in itself represents an interesting question for future research.



**Infection**

The lung is exposed daily to large numbers of inhaled infectious micro-organisms. These represent the third class of stimuli causing activation of the ISR. The canonical stimulus for the eIF2α kinase PKR is dsRNA, which reduces the rate of protein translation and so impedes viral replication (as reviewed in (24)). Because of the need to recover translation in order to synthesise inflammatory cytokines, there is also a requirement for GADD34 induction and translational recovery as demonstrated by the increased susceptibility of GADD34 deficient cells and mice to viral infection (25). But there is redundancy within the system: for example the coronavirus IBV (infectious bronchitis virus) can activate both PKR and PERK, leading to expression of ATF4 and CHOP (26). Surprisingly, both kinases appear to mediate the cytotoxicity of this infection via CHOP-dependent apoptosis. Curiously, although PKR serves an antiviral role in most instances, in IBV infection, eIF2α phosphorylation contributes to enhance viral replication.

Activation of Toll-like receptors (TLRs), specifically TLR3 by dsRNA and TLR4 by lipopolysaccharide (LPS), can also trigger phosphorylation of eIF2α (24). The mechanism for this is not entirely clear, but may plausibly reflects increased ER activity due to the need to secrete cytokines and antimicrobial peptides upon TLR activation. Interestingly, the ISR shows specific modulation by TLR signalling that goes beyond simple activation.

In macrophages, when phosphorylation of eIF2α is induced by other stimuli, for example ER stress, activation of TLR4 by LPS blocks induction of ATF4, CHOP and GADD34 (27). It is now becoming clear how this can be explained. Normally, when eIF2α is phosphorylated it binds to and inhibits eIF2B, an enzyme responsible for maintaining eIF2α in its active GTP-bound state. Indeed, binding of phospo-eIF2α to eIF2B is responsible both for the inhibition of translation seen during the ISR and for the up-regulation of ATF4 and CHOP. However, activated TLR4 stimulates eIF2B thus overcoming the effects of eIF2α phosphorylation (28). It has been proposed that this mechanism may enable cells selectively to inhibit the ISR during chronic ER stress caused by infection and thus avoid the toxic effects of inducing CHOP and its target GADD34.



But the phosphorylation of eIF2α and subsequent activation of the ISR can have negative consequences during chronic infection and so may represent a therapeutic target. In cystic fibrosis, most causative mutations of CFTR do not cause robust ER stress directly because these mutations lie within its cytosolic portion (29, 30). But loss of CFTR function leads to the generation of thickened airway mucus with reduced clearance, which is prone to chronic colonisation by bacteria such as *Pseudomonas aeruginosa*. This induces local ER stress and can be recapitulated in normal airway epithelia by application of CF mucus directly (30), and explains why primary CF epithelia recover from ER stress if allowed to grow *in vitro* in the absence of colonised mucus (29). It has been noted, however, that the ER stress seen in models of CF in response to *P. aeruginosa* is deficient in PERK-eIF2α signalling (31). This may contribute to the chronic inflammation seen in CF, since pharmacological induction of eIF2α phosphorylation with the drug salubrinal was found to lessen the inflammatory response of CF cells. The mechanism by which ER stress fails to activate the ISR in this context has not been studied in detail, but may involve the novel TLR-eIF2B pathway introduced above.

Where on-going translation is essential for efficient immune responses, for example in PKR-activated dendritic cells, a modified ISR is activated in which robust induction of GADD34 prevents significant translational attenuation (32). This and other observations, including those of the TLR-eIF2B pathway, have given rise to the concept of a

specific "microbial stress response", which shares many features of the canonical ISR, but with modifications such as less robust induction of CHOP (reviewed in (24)). However, the induction of components of the ISR, including CHOP, can differ substantially between models, perhaps because of cell type or the stimuli used, and thus many 'flavours' of the ISR may exist. For example, when dendritic cells are challenged either with ER stress or TLR agonists, efficient induction of CHOP is required for secretion of interleukin (IL-) 23 (33), which appears to contrast with the response elicited by PKR activation. It should also be noted that viruses and bacteria have evolved numerous mechanisms to escape the antimicrobial effects of the ISR. For example, respiratory syncytial virus (RSV) can sequester activated PKR and prevent phosphorylation of eIF2α (34).



**Nutrient stress**

Being the oldest in evolutionary terms, GCN2 is ubiquitous in eukaryotes. In terms of pulmonary pathology, it has been studied most in the context of tumour biology as it functions to match amino acid supplies with demand, which can be a limiting factor in cancer growth. The induction of ATF4 during nutrient stress promotes the induction of many amino acid transporters and synthetic enzymes in tumour cells, such as asparagine synthetase (35). As a result, inhibition of the GCN2-ATF4 pathway can reduce proliferation and cell survival (35). This accounts for the up-regulation of GCN2 (both activated and total) in lung cancer samples compared to healthy controls or the surrounding non-tumorous cells (35).

In addition to limiting the levels of nutrients, such as amino acids required for macromolecule biosynthesis, nutrient deprivation also profoundly affects the metabolism of a tumour. A consequence of this is a heightened level of ER stress that is seen in many hypoxic tumours (36, 37). It is therefore unsurprising that ER stress and the UPR have been implicated in the pathophysiology of solid tumours including human lung cancer (8). Evidence of ER stress is associated with more aggressive tumours and resistance to chemotherapy (36). As the mediator of eIF2α phosphorylation during ER stress, PERK has proved necessary for the growth of larger solid tumours (37) and contributes to resistance

to therapy (36). It ensures that protein synthesis remains at a level consistent with the supply of nutrients and energy, and through ISR-mediated activation of autophagy it also promotes the recycling of nutrients from old and damaged organelles (38). Under such conditions, the recovery of translation mediated by CHOP and GADD34 would be expected to be toxic. Indeed, loss of GADD34 in tumours may prevent apoptosis and promote cell survival in hypoxic conditions (39). Accordingly, GADD34 expression correlates with the degree of differentiation of malignant mesothelioma, with lower levels of expression being seen in the more aggressive sarcomatoid subtype (40). It is therefore tempting to speculate that GADD34 may be a tumour suppressor in ER stressed environments, but this has yet to be tested formally.



**Mechanical stress**

Finally, the pulmonary epithelium is not a static culture of cells as it is frequently treated in the laboratory. Instead, it is a rhythmically flexing and stretching structure. The forces placed upon it profoundly alter its biology, as the challenges of invasive ventilation in acute lung injury have shown. Marked mechanical stress has been shown to induce apoptosis in a PERK dependent manner (41). Surprisingly, other limbs of the UPR do not appear to be activated, even during prolonged mechanical stress. Moreover, the mechanism of apoptosis is independent of CHOP-GADD34. This is likely to be a fruitful area for research and there will be much to learn from other areas of biology, for example the bone where cyclical loading, rather than static loads, have been show to modulate protein synthesis via activation of PKR (42).

## Therapeutic options

It is clear that the ISR plays a crucial role in many aspects of pulmonary pathophysiology and the basic science of ISR signalling is sufficiently well understood that it could plausibly be manipulated for therapeutic gain. In many cases, the transient activation of the ISR plays a cytoprotective role. For that reason, agents that promote phosphorylation of eIF2α have been shown to be protective. The small molecule salubrinal

was identified as a compound that prevented the dephosphorylation of eIF2α during ER stress and during infection with herpes simplex virus (HSV) (43). Although it has been claimed to inhibit the eIF2α phosphatases directly, convincing evidence for this is lacking. Nevertheless, it can increase cellular levels of phosphorylated eIF2α with cytoprotective results. Amelioration by this drug of inflammatory signalling in *Pseudomonas*-exposed CF cells has already been mentioned (31), but salubrinal has also been shown to be protective in the monocrotaline model of pulmonary hypertension (44) and the toxicity of cigarette smoke extract (45). More recently, the drug guanabenz, used previously as an anti-hypertensive, has been found to inhibit GADD34 thus increasing phosphorylation of eIF2α and promoting cell survival during chronic ER stress (46). The hypotensive effects of this drug, which are unrelated to its effects on the ISR, limit its use in animals, but it is likely that future GADD34 inhibitors will be useful in situations where activation of the ISR would be beneficial.



In contrast, blockade of the cytoprotective effects of the ISR will be useful in chemotherapy. Potent small molecules have been developed, such as GSK2656157, that show high selectivity for PERK, which is especially important in malignancy, and in animal models have proved effective at inhibiting tumour growth through alterations in amino acid metabolism and angiogenesis (47).

## Conclusion and future directions

The progress from understanding the basic biology of the ISR to its role in pulmonary pathology has been dramatic and rapid. The breadth of noxious stimuli to which it responds may hamper efforts to identify which stress or stresses were the original insult in some diseases; however, the very fact that multiple kinases converge on a single substrate to regulate translation during disease offers many advantages for research and treatment. If selectivity is required, identification of the upstream kinase should offer this, while modulation of the downstream phosphatase(s) can generate a more broad effect. But many unknowns remain, which will make the ISR an exciting and fruitful area for respiratory research for many years to come.

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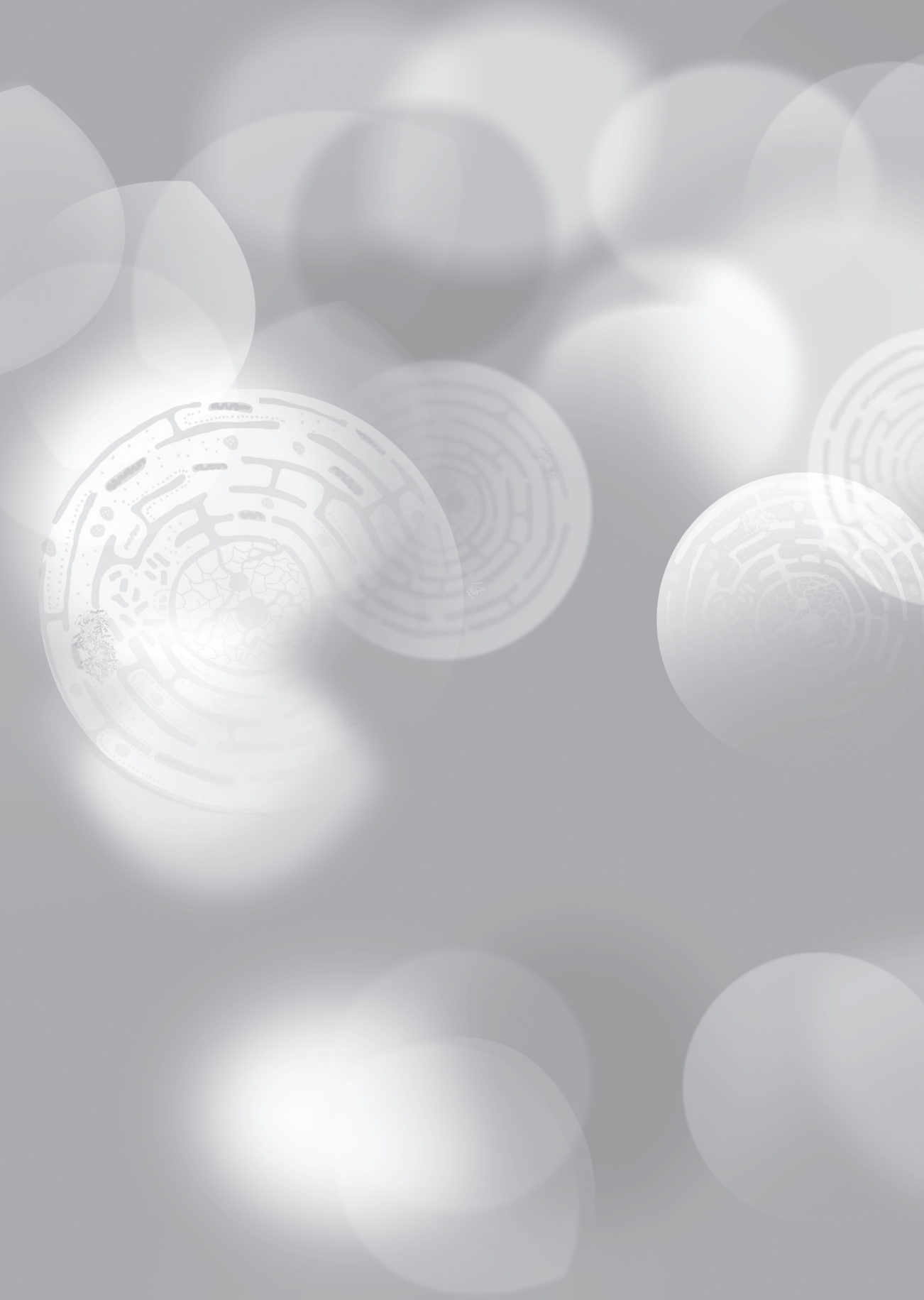

# Chapter 3

## A quantitative method for detection of *spliced X-box binding protein* (*XBP1*) mRNA as a measure of endoplasmic reticulum stress

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## Abstract

**Endoplasmic reticulum (ER) stress is increasingly recognised as an important mechanism in a wide range of diseases including cystic fibrosis, $\alpha_1$-antitrypsin deficiency, Parkinson's and Alzheimer's disease. Therefore, there is an increased need for reliable and quantitative markers for detection of ER stress in human tissues and cells. Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum can cause ER stress, which leads to the activation of the unfolded protein response (UPR). UPR signaling involves splicing of X-box binding protein-1 (*XBP1*) mRNA, which is frequently used as a marker for ER stress. In most studies, the splicing of the *XBP1* mRNA is visualised by gel electrophoresis which is laborious and difficult to quantify. In the present study we have developed and validated a quantitative RT-PCR method to detect the spliced form of *XBP1* mRNA.**

## Introduction

Endoplasmic reticulum (ER) stress induced by protein misfolding is an important mechanism in cellular stress in a variety of diseases. When protein folding in the ER is compromised, the unfolded proteins accumulate in the ER which leads to ER stress. ER stress triggers the unfolded protein response (UPR), a transcriptional induction pathway which is aimed at restoring normal ER functioning (1).

The UPR is mediated by three ER stress receptors: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein-1 (IRE1) and activating transcription factor-6 (ATF6). In the absence of ER stress, all three ER stress receptors are maintained in an inactive state through their association with the ER chaperone protein GRP78 (BiP). ER stress results in the dissociation of BiP from the three receptors, which subsequently leads to their activation (2). Dissociation of BiP from PERK leads to autophoshorylation and thereby activation of PERK and subsequent phosphorylation of translation initiation factor eIF2α, resulting in an inhibition of mRNA translation, and eventually in the translation of the transcription factor ATF4. Dissociation of BiP from ATF6 leads to translocation of ATF6 to the Golgi complex where it is cleaved by proteases into an active transcription factor. Active ATF6 moves to the nucleus and induces expression of genes with an ER stress response element (ERSE) in their promoter such as the ER chaperone BiP and the transcription factors C/EBP homologous protein (CHOP) and X-box binding protein-1 (XBP1). Dissociation of BiP from IRE1 leads to the activation of IRE1 which cleaves a 26-nucleotide intron from the *XBP1* mRNA. The *spliced XBP1* mRNA encodes a stable, active transcription factor that binds to the UPRE or ERSE sequence of many UPR target genes, leading to transcription of ER-chaperone proteins (2, 3).



The UPR can be induced experimentally by chemicals like thapsigargin and tunicamycin. Thapsigargin blocks the ER calcium ATPase pump, leading to depletion of ER calcium stores and tunicamycin blocks N-linked glycosylation of proteins. Both chemicals lead to high levels of stressors which are expected to rapidly activate all three components of the UPR (4).

An increasing number of studies have reported the involvement of ER stress in a variety of diseases, including cystic fibrosis, $\alpha_1$-antitrypsin deficiency, Parkinsons' and Alzheimers' disease. Therefore there is a growing demand for quantifiable markers to measure ER stress. The splicing of *XBP1* mRNA is considered to be an important marker for ER stress, however the quantification is difficult because the splicing is mainly visualised by gel electrophoresis after conventional RT-PCR. We have now developed a simple and quantitative method to measure spliced human *XBP1* by using quantitative real-time RT-PCR, and we show that the results obtained with this method correlate with data for *BiP* and *CHOP* mRNA.




**Figure 1. Location of the forward *XBP1spl* primer on the *spliced* and *unspliced XBP1* mRNA.**

## Results and discussion

Several publications described methods for monitoring ER stress and the UPR, and *spliced XBP1* mRNA is generally considered to be a relevant marker for ER stress. However, the semi-quantitative conventional RT-PCR method is currently used to assess the splicing of the *XBP1* mRNA. Only Hirota *et al*. (5) developed a quantitative real-time RT-PCR method for measuring *spliced XBP1*, but this method does involve an additional step with a restriction enzyme during the PCR reaction, which we consider to be more laborious and more complex. Both Samali *et al*. (6) and Zhao *et al*. (7) recently reviewed methods for monitoring ER stress, and recommended the analysis of *spliced XBP1* by conventional RT-PCR for detection of ER stress.



In our study we have designed primers that span the 26bp intron of the *XBP1* mRNA, in order to amplify only the *spliced XBP1* mRNA. Because of the similarities between the sequence of the *XBP1* mRNA just before this intron and the last part of the intron itself, only a very few options were possible to design a specific forward primer for the *spliced XBP1* mRNA (Figure 1). We tested the specificity of the *XBP1spl* primers by sequencing. The PCR product of the thapsigargin treated, as well as the DMSO treated cells, both matched the *spliced XBP1* mRNA and no *unspliced XBP1* mRNA was detected with the new primers. These results also indicate that in DMSO treated cells there is a low level of *spliced XBP1* mRNA present.

Next, we compared the semi-quantitative conventional RT-PCR method with the newly developed quantitative real-time RT-PCR method by conducting a time course experiment with thapsigargin and DMSO on primary bronchial epithelial cells. In both methods the *spliced XBP1* mRNA was mostly expressed after 6 hours, and its levels were found to be comparable between the two methods (Figure 2). Next, we used the *XBP1spl* primers to perform a quantitative RT-PCR in a dose-response experiment on primary bronchial epithelial cells, using various concentrations of thapsigargin as well as two concentrations of tunicamycin. In addition, *CHOP* and *BiP* mRNA were analysed and correlated with levels of *spliced XBP1* mRNA (Figure 3A and 3B). We found a significant and high correlation between *CHOP* and *spliced XBP1* mRNA ($r=0.962$, $p<0.001$) as well

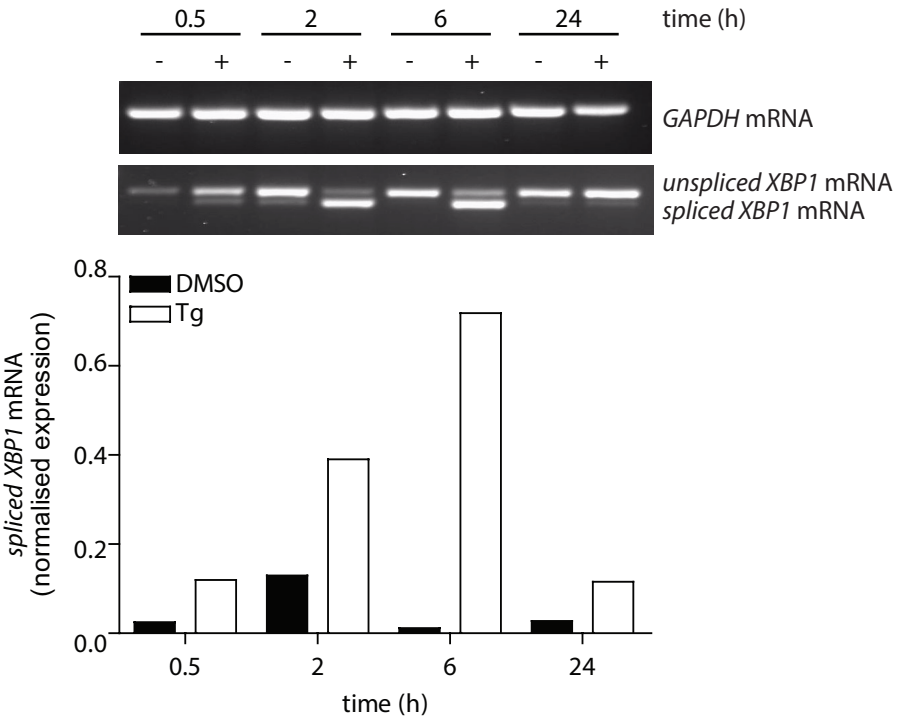




**Figure 2. Effect of thapsigargin exposure for various time periods on *spliced XBP1* expression in primary bronchial epithelial cells.**

The *spliced XBP1* mRNA was mostly expressed after 6 hours of stimulation with 50 nM thapsigargin, as shown by conventional RT-PCR (top) as well as by quantitative real-time RT-PCR (bottom).

as between *BiP* and *spliced XBP1* mRNA (r=0.884, p<0.001). Since both *CHOP* and *BiP* genes contain an ERSE region which is recognised by the XBP1 protein, a good correlation between the *spliced XBP1* and the more downstream ER stress markers *CHOP* and *BiP* was anticipated.

To evaluate whether the new *XBP1spl* primers could also be used in other cell lines, experiments were performed in two different epithelial cell lines, HK-2 (a proximal tubule epithelial cell line from normal adult human kidney) and A549 (a human lung carcinoma cell line with type II alveolar epithelial cell characteristics). We repeated the dose-response experiments on these cell lines and compared the results with those

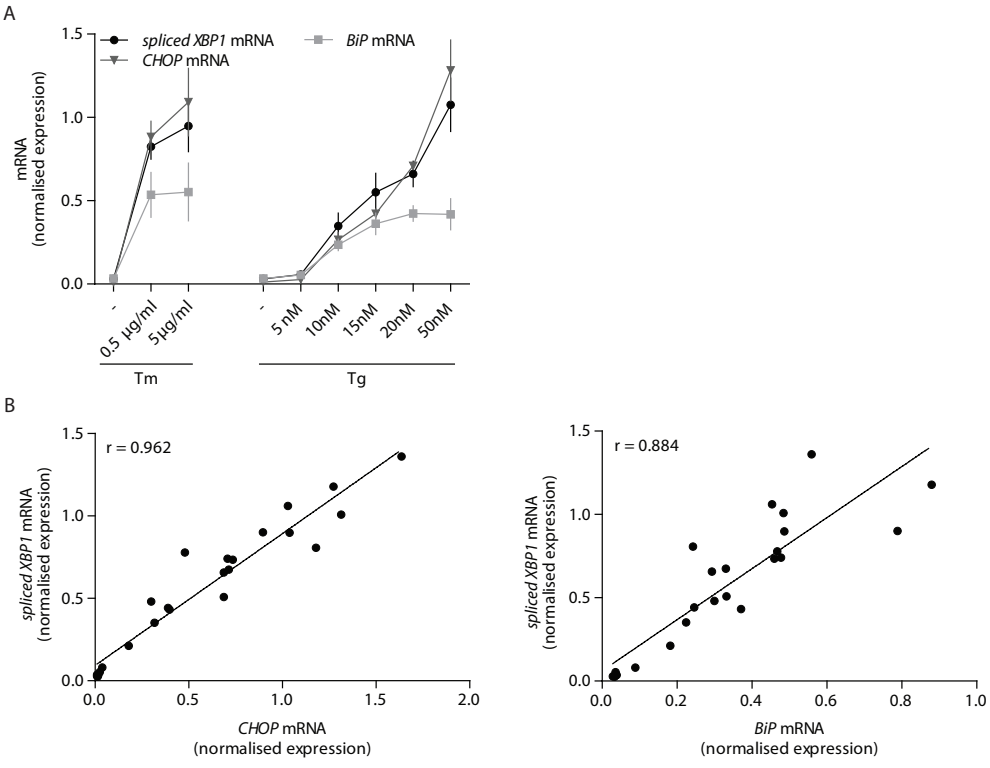


**Figure 3. Effect of thapsigargin and tunicamycin on markers of ER stress in primary bronchial epithelial cells.**

Primary bronchial epithelial cells were exposed to various concentrations of thapsigargin or tunicamycin for 6 hours, and next total RNA was isolated for quantitative RT-PCR-based detection of *spliced XBP1*, *CHOP* and *BiP* mRNA. The results showed a dose-dependent increase in *spliced XBP1* mRNA after exposure to both stimuli (A), that showed a significant correlation with levels of *CHOP* and *BiP* mRNA (B). Data are mean ± SEM using cells from 3 different donors (A), and data points represent the result of a single experiment (B).

previously obtained with the primary bronchial epithelial cells (Figure 4). We found a dose-dependent increase of *spliced XBP1* mRNA in both cell lines, similar to the results found in primary bronchial epithelial cells.

In conclusion, the novel quantitative real-time RT-PCR method described in this report is a reliable quantitative method to measure spliced human *XBP1* mRNA as a marker for ER stress.

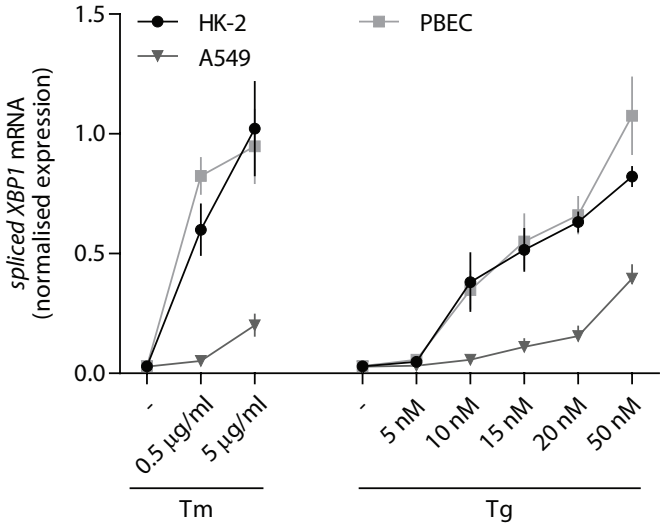


**Figure 4. Effect of thapsigargin and tunicamycin on spliced XBP1 mRNA in different cell lines.**

Primary bronchial epithelial cells, HK-2 and A549 cells were exposed to various concentrations of thapsigargin or tunicamycin, and next total RNA was isolated for quantitative real-time RT-PCR-based detection of spliced XBP1 mRNA. The results showed a dose-dependent increase in spliced XBP1 mRNA after exposure to both stimuli for all cell lines. Data are mean ± SEM using cells from 3 different donors (primary bronchial epithelial cells) or 3 separate experiments (HK-2, A549). Spliced XBP1 mRNA data for primary bronchial epithelial cells are the same as those shown in Figure 3A.

## Materials and methods

### Cell culture and stimulation

Primary bronchial epithelial cells were isolated from resected lung tissue obtained from patients undergoing surgery for lung cancer as described previously (8). Briefly, the cells were cultured in a 1:1 mixture of DMEM (Invitrogen, Carlbad, CA, USA) and BEGM (Clonetics, San Diego, CA, USA) , supplemented with 0.4% w/v BPE, 0.5 ng/ml EGF, 5 µg/ml insulin, 0.1 ng/ml retinoic acid, 10 µg/ml transferrin, 1 µM hydrocortisone, 6.5 ng/ml T3, 0.5 µg/ml epinephrine (all from Clonetics), 1.5 µg/ml BSA (Sigma-Aldrich, St Louis, MO, USA), 1mM Hepes (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Cambrex, East Rutherford, NJ, USA).



Immortalised human renal PTEC (HK-2, kindly provided by M. Ryan, University College Dublin, Dublin, Ireland) were grown in serum-free DMEM/HAM-F12 (Bio-Whittaker, Walkersville, MD) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Breda, The Netherlands), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), triiodothyronine (40 ng/ml), epidermal growth factor (10 ng/ml), and hydrocortisone (36 ng/ml, all purchased from Sigma).

Cells from the A549 human lung carcinoma cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were routinely cultured in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Cambrex, East Rutherford, NJ, USA) and 10 % (v/v) heat-inactivated FCS (Gibco) at 37°C in a 5 % CO2-humidified atmosphere.

ER stress was induced in cells by exposure to thapsigargin or tunicamycin (both Sigma). After reaching near-confluence, primary bronchial epithelial cells were exposed to thapsigargin (50 nM) for various time periods. For the dose-response experiment , primary bronchial epithelial cells from 3 different donors were stimulated with various concentrations of thapsigargin or tunicamycin for 6 hours. Dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) served as a solvent control for both thapsigargin and tunicamycin. The dose-response experiments were repeated on HK-2 cells and A549 cells

with 2 hours stimulation instead of 6 hours. This shorter duration of exposure in HK-2 and A549 cells was based on pilot experiments using these cell lines.

**Total RNA isolation and reverse-transcription**

After stimulation, the cells were washed twice with PBS and total mRNA was isolated using the RNeasy mini kit (Qiagen, Valencia CA, USA). DNase I amplification grade (Invitrogen) was used to remove genomic DNA. Total RNA concentration and purity were measured on a NanoDrop spectrophotometer (NanoDrop technologies, Wilmington USA). Next, cDNA synthesis was performed with M-MLV Reverse Transcriptase (Promega, Madison WI, USA).



**Semi-quantitative RT-PCR**

To amplify the *spliced* and *unspliced XBP1* mRNA, *XBP1* primers were used as described previously (3). PCR products were electrophoresed on 2.5% agarose gel. *GAPDH* (forward 5′ GGATGATGTTCTGGAGAGCC 3′, reverse 5′ CATCACCATCTTCCAGGAGC 3′) was used as a loading control. The size difference between the spliced and the unspliced *XBP1* is 26 nucleotides.

**Quantitative real-time RT-PCR**

Primers were designed to span the 26 base pair intron that is removed by IRE1 to obtain the *spliced XBP1* mRNA (*XBP1spl*; forward 5′ TGCTGAGTCCGCAGCAGGTG 3′ and reverse 5′ GCTGGCAGGCTCTGGGGAAG 3′). Also specific primers for *CHOP* mRNA (forward 5′ GCACCTCCCAGAGCCCTCACTCTCC 3′ and reverse 5′ GTCTACTCCAAGCCTTCCCCCTGCG 3′) and *BiP* mRNA (5) were used. Quantitative real time RT-PCR was carried out at 95°C for an initial 3 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 62°C for 15 seconds and extension at 72°C for 30 seconds using IQ SYBRGreen supermix (Bio-Rad, Hercules, CA, USA). Each assay was run on a Bio-Rad CFX Real-time PCR system in triplicates and arbitrary mRNA concentrations were calculated by the Bio-Rad software, using the relative standard curve method. Stable housekeeping genes were selected

using the Genorm software (9). Relative mRNA concentrations of *ATP5B* and *RPL13A* (GeNorm, Primerdesign, Southampton, UK) were used as reference genes to calculate the normalised expression of the *spliced XBP1* mRNA. The identity of the PCR products obtained with the *XBP1spl* primers was verified by DNA sequencing.

**Statistical analysis**

The results of the dose-response experiment were expressed as mean ± SEM. The correlation coefficient was determined by the use of Pearson's correlation statistics. The correlation coefficient was considered significant at p-values $< 0.05$.



## Acknowledgements

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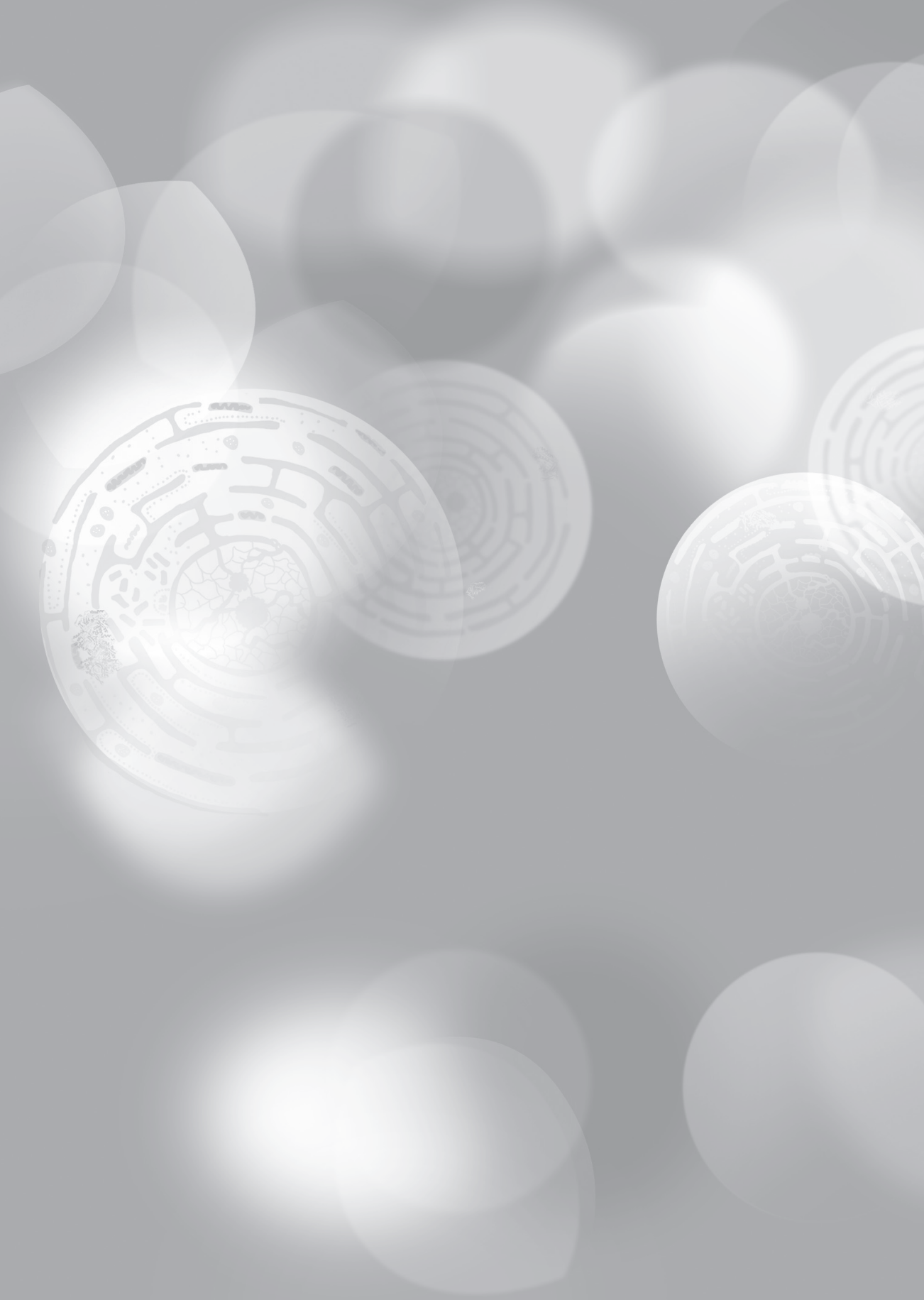

# Chapter 4

## Virulence factors of *Pseudomonas aeruginosa* induce both the unfolded protein and integrated stress responses in airway epithelial cells

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## Abstract

***Pseudomonas aeruginosa* infection can be disastrous in chronic lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease (COPD). Its toxic effects are largely mediated by secreted virulence factors including pyocyanin, elastase and alkaline protease (AprA). Efficient functioning of the endoplasmic reticulum (ER) is crucial for cell survival and appropriate immune responses, while an excess of unfolded proteins within the ER leads to "ER stress" and activation of the "unfolded protein response" (UPR). Bacterial infection and Toll-like receptor (TLR) activation trigger the UPR most likely due to the increased demand for protein folding of inflammatory mediators. In this study, we show that cell-free conditioned medium of the PAO1 strain of *P. aeruginosa,* containing secreted virulence factors, induces ER stress in primary bronchial epithelial cells as evidenced by splicing of *XBP1* mRNA, and induction of *CHOP*, *GRP78* and *GADD34* expression. Most aspects of the ER stress response were dependent on TAK1 and p38 MAPK, except for the induction of *GADD34* mRNA. Using various mutant strains and purified virulence factors, we identified pyocyanin and AprA as inducers of ER stress. However, the induction of *GADD34* was mediated by an ER stress-independent integrated stress response (ISR) mediated by the iron-sensing eIF2α kinase HRI. This increased *GADD34* expression protected against *Pseudomonas*-induced, iron-sensitive cell cytotoxicity. In summary, the virulence factors from *P. aeruginosa* induce ER stress in airway epithelial cells, and also trigger the ISR via activation of the iron-sensing kinase HRI, to improve cell survival of the host.**

## Introduction

The Gram-negative bacterium *P. aeruginosa* is an opportunistic pathogen that increases morbidity and mortality in chronic lung diseases, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD; GOLD stages III-IV) (1-3). *P. aeruginosa* often causes chronic infection due to its ease of developing antibiotic resistance and its ability to form biofilms in these patients. Furthermore, its survival in the host in the early stages of infection is supported by the secretion of toxins and virulence factors, including pyocyanin and its proteases elastase and alkaline protease (AprA) (reviewed in (4, 5)). Interestingly, their production appears to be lower in the later stages of infection (6, 7). Therefore, the specific role of these virulence factors in chronic infections is incompletely understood. Pyocyanin is a redox-active toxin that causes cellular senescence (8), ciliary dyskinesia (9), increased expression of interleukin (IL)-8 (10) and disruption of calcium homeostasis (11) in human lung epithelial cells. Pyocyanin inactivates $\alpha_1$-antitrypsin, contributing to the protease-antiprotease imbalance found in CF lungs (12), while *P. aeruginosa* elastase additionally cleaves many proteins of the extra-cellular matrix including collagen, fibrinogen and elastin, and cleaves opsonin receptors, thus contributing to the invasion of bacteria into the lung parenchyma (13). AprA is thought to modulate the host response and prevent bacterial clearance by degrading proteins of the host immune system, including TNFα and complement factors (14-16).



*P. aeruginosa* requires iron both for its respiration and for biofilm formation (17,18). Competition with the host is fierce and so *P. aeruginosa* has evolved specific strategies to obtain iron (19). It produces redox-active phenazine compounds to turn insoluble $Fe^{3+}$ to the more soluble $Fe^{2+}$, siderophores to scavenge iron and receptors for the uptake of iron-siderophore complexes, proteases to degrade host iron-binding proteins, and bacteriocins to eliminate competitors (reviewed in (19)). Moreover, iron availability regulates the production of virulence factors such as pyocyanin, AprA and exotoxin A (20) .

The endoplasmic reticulum (ER) functions to fold secretory and membrane proteins and its quality control systems ensure that only properly folded proteins exit

the organelle. Accumulation of incompletely folded proteins can impair ER homeostasis and induces "ER stress", which activates intracellular signal transduction pathways collectively called the "unfolded protein response" (UPR; Figure 1). This response restores ER homeostasis by reducing the influx of new proteins into the lumen of the ER and by enhancing the organelle's capacity to fold proteins; however, if the stress cannot be resolved then apoptotic cell death pathways are invoked (reviewed by Marciniak and Ron (21)).

Three distinct sensors detect ER stress: protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor-6 (ATF6) (21). Early during ER stress, the kinase PERK phosphorylates eukaryotic translation initiation factor 2 on its alpha subunit (eIF2α) causing the inhibition of protein synthesis and thus preventing the load on the ER from increasing further (22-24). In addition, this promotes the translation of specific mRNAs, for example that encoding the transcription factor ATF4 (25). One important target of ATF4 is the transcription factor called C/EBP homologous protein (CHOP), and both individually can trans-activate the *GADD34* gene (26). GADD34 is a phosphatase that selectively dephosphorylates eIF2α, completing a negative feedback loop and enabling the translation of other targets of the UPR (27). In parallel, IRE1 initiates the unconventional splicing of the mRNA encoding X-box binding protein-1 (XBP-1) (28). *Spliced XBP-1* mRNA encodes an active transcription factor that, in concert with ATF6, induces expression of UPR genes, such as the chaperones GRP78 (also known as BiP) and GRP94 (28-30).



The phosphorylation of eIF2α is a point at which the responses to several forms of stress are integrated (31, 32). During ER stress, PERK phosphorylates eIF2α, but eIF2α can also be phosphorylated by PKR responding to double-stranded RNA during viral infection (33, 34), by GCN2 during amino acid starvation (25, 35, 36), and by HRI during iron deficiency (reviewed in (31)). For this reason, the events initiated by eIF2α phosphorylation have been termed the "integrated stress response" (ISR; Figure 1 and (37)).

Abnormal function of the ER has been implicated in the pathogenesis of many diseases, including diabetes mellitus, atherosclerosis, Alzheimer's disease and cancer (21,

38). Remarkably, the ER also plays an important role during immune responses to infection and malignancy. For example, during bacterial infection, Toll-like receptor (TLR) activation triggers splicing of *XBP1* mRNA, possibly in response to the increased biosynthesis of secreted inflammatory mediators, increasing the capacity for protein secretion and thus contributing to an augmented inflammatory response (39-41). In addition, induction of GADD34 is required for cytokine expression during viral infection; however, in contrast to ER stress, pathogen-induced induction of GADD34 appears to be independent of CHOP (42, 43). Nevertheless, sustained activation of the UPR can impair the immune response by triggering cell death (26, 44).

Previously, it has been shown that infection of airway epithelia or *Caenorhabditis elegans* with *P. aeruginosa* can elicit an UPR (40, 45, 46). In worms, activation of the IRE1-XBP-1 branch of the UPR was dependent on p38 MAPK-signalling (40), but it is unknown if this signalling response is conserved in humans. Moreover, it is unclear whether living



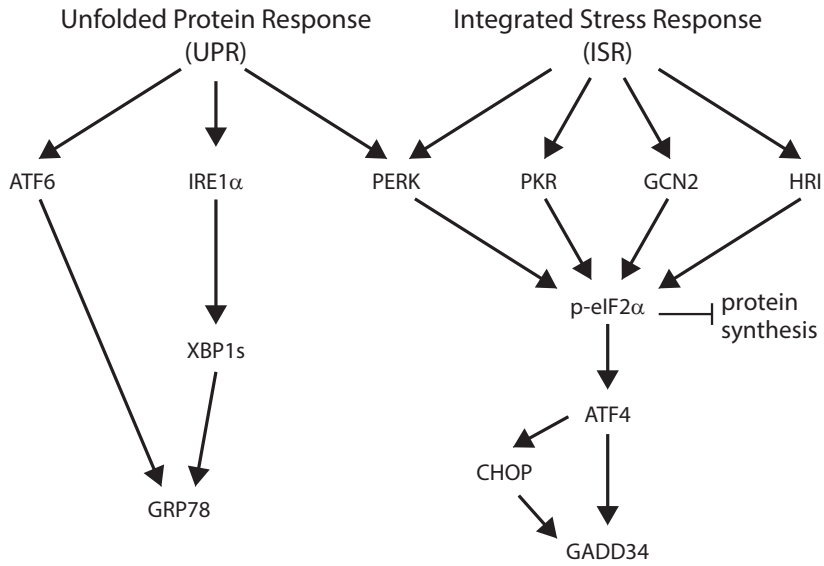



**Figure 1. Schematic overview of the unfolded protein response (UPR) and integrated stress response (ISR).**

bacteria are required for the induction of ER stress or if unidentified secreted factors are sufficient.

In the present study, we set out to test the hypothesis that virulence factors secreted by *P. aeruginosa* trigger the UPR in human cells via the p38 MAPK pathway. We found that p38 MAPK signalling was required for the response of human epithelial cultures to bacterial conditioned medium and that the secreted factors pyocyanin and AprA contribute to the induction of ER stress. Furthermore, we showed that induction of the ISR target *GADD34* is mediated by the iron-regulated kinase HRI and this induction protects the host against the toxic effects of *P. aeruginosa*.

## Results

### Conditioned medium of *P. aeruginosa* strain PAO1 (CM-PAO1) causes ER stress in primary bronchial epithelial cells

Infection with live *P. aeruginosa* has previously been shown to induce the UPR in mouse macrophages and human immortalized bronchial epithelial cells (41, 46). To identify whether *P. aeruginosa* could induce the UPR in primary bronchial epithelial cells and whether living bacteria were necessary for this, we stimulated primary bronchial epithelial cells with filter-sterilised conditioned medium (CM) from *P. aeruginosa* strain PAO1 (CM-PAO1), containing secreted virulence factors without living bacteria. Treatment with CM-PAO1 induced ER stress in a time- and dose-dependent manner, as evidenced by a 9.9-fold increase of splicing of *XBP1* mRNA ($p<0.001$), a 12.8-fold increase of *CHOP* mRNA ($p=0.02$) and a 16.2-fold increase of *GADD34* mRNA ($p<0.05$) after 8-12 hours (Figure 2A and 2B). This was accompanied by an increase in phosphorylation of eIF2α and protein expression of GADD34 and GRP78 protein (Figure 2C). This increase in phosphorylated eIF2α was accompanied by a decrease in global protein translation as assessed by puromycin incorporation into nascent proteins (Figure 2D) (47). In line with previous reports (48-50), CM-PAO1 gradually impaired epithelial integrity until the monolayer was completely disrupted after 24 hours. Although the epithelial layer was disrupted by CM-PAO1 (as reported by trans-epithelial resistance; Figure S1A), the cell membranes themselves remain intact as reported by exclusion of trypan blue stain (Figure S1B).



### Induction of ER stress in human bronchial epithelial cells by *P. aeruginosa* is dependent on p38 MAPK

Infection of *C. elegans* with *P. aeruginosa* has been reported to cause splicing of *XBP1* mRNA in a p38 MAPK-dependent manner (40). To exclude the effects of donor variation and complex nutrient/growth factor requirement of primary cells, we therefore tested whether exposure of 16HBE cells, a SV-40 transformed bronchial epithelial cell line, to *P. aeruginosa* conditioned medium would trigger phosphorylation of p38 MAPK and

activate the UPR. We observed that CM-PAO1 caused prolonged phosphorylation of p38 MAPK in 16HBE cells up to 6 hours (Figure 3A). We reasoned that the activation of p38 MAPK after 15 minutes might represent the activation of TLR signalling, since stimulation of HEK-TLR2 or HEK-TLR4 cells (51) with CM-PAO1 demonstrated robust TLR2 and TLR4 activation (data not shown). The sustained activation was similar to that observed in C. elegans infected with *Pseudomonas* (40) (Figure 3A), which suggests the importance of p38 MAPK in the induction of the UPR. To examine if p38 MAPK signalling was required for the ER stress response, we pre-treated 16HBE cells with an inhibitor of p38 MAPK (SB203580) or an inhibitor of TAK1 (5Z-7-oxozeanol, also known as LL-Z1640-2), a kinase upstream of p38 MAPK. We then exposed cells to CM-PAO1, and observed that both compounds markedly reduced activation of p38 by CM-PAO1 (Figure 3B). In addition, both compounds reduced secretion of IL-8 in response to CM-PAO1 treatment (Figure 3C). Of note, these compounds strongly inhibited splicing of *XBP1* mRNA and abrogated the induction of *CHOP* and *GRP78* mRNA (Figure 3D). However, the induction of *GADD34* was insensitive to the inhibitors (Figure 3D) suggesting the involvement of an additional pathway independent of *CHOP*.



**Pyocyanin is able to induce ER stress**

To prepare *P. aeruginosa* conditioned medium, cultures were grown for 5 days (see Experimental procedures and (48)) to a high optical density at which quorum-sensing is activated in this strain, thus triggering the production of a variety of virulence factors among which the cytotoxic exoproduct pyocyanin. When pyocyanin levels in *P. aeruginosa* conditioned medium were measured, values up to 5.5 µg/ml (26 µM) were detected (Figure 4A), which were similar to values observed in sputum of CF patients colonised with *P. aeruginosa* (52). We therefore tested whether purified pyocyanin could induce ER stress in 16HBE cells. Treatment with purified pyocyanin caused dose-dependent splicing of *XBP1* mRNA, induction of *CHOP* and *GRP78* mRNAs and expression of GRP78 and GRP94 protein (Figure 4B-C), maximal at 10 µM (2.1 µg/ml). In contrast, *GADD34* mRNA continued to rise up to a maximum at ≥ 30 µM (6.3 µg/ml) pyocyanin (Figure 4B). Once again, this

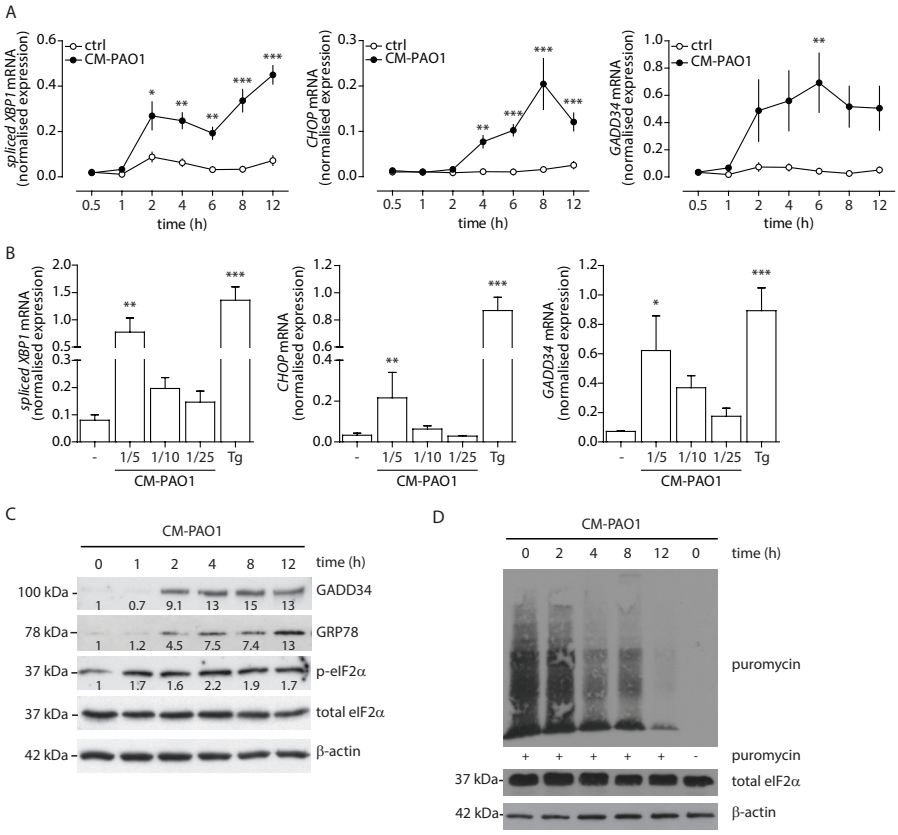



**Figure 2. *P. aeruginosa* secreted virulence factors induce ER stress in primary bronchial epithelial cells.**

A. Time-dependent induction of ER stress in primary bronchial epithelial cells, as assessed by *spliced XBP1*, *CHOP* and *GADD34* mRNA after treatment with CM-PAO1 (n=5; mean ± SEM). B. Dose-response of *spliced XBP1*, *CHOP* and *GADD34* mRNA in primary bronchial epithelial cells treated with CM-PAO1 for 12 hours (n=5; mean ± SEM). C. Time-dependent phosphorylation of eIF2α and synthesis of GADD34 and GRP78 (visualised with anti-KDEL antibody). Relative quantifications for each protein are shown within (representative of n=3). D. Time-dependent decrease of puromycin incorporation in nascent proteins. Total eIF2α and β-actin serve as loading controls. * p<0.05, ** p<0.01, ***p<0.001 versus control (ctrl) two-way repeated-measurements ANOVA (Bonferroni *post-hoc*) or untreated (-) with a one-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

suggested that induction of *GADD34* in this system might not simply reflect activation by ER stress. As expected, pyocyanin potently induced secretion of IL-8 by 16HBE cells (Figure 4D) (10).

Next, we wished to determine if pyocyanin was also an important mediator of the observed ER stress response by CM-PAO1. To this end, *P. aeruginosa* bacterial cultures were supplemented with iron to suppress pyocyanin production together with other iron-regulated factors (Figure 4A). The conditioned medium prepared in this manner was significantly less efficient at triggering the splicing of *XBP1* mRNA and at increasing expression of *GRP78* mRNA (Figure 4E). Surprisingly, *CHOP* mRNA was not significantly affected (Figure 4E), whereas *GADD34* mRNA induction was completely abrogated. These experiments provided only indirect support for the involvement of pyocyanin, since iron supplementation also affects production of other *P. aeruginosa* virulence factors and may also affect host cells. Since pyocyanin is a redox active toxin, we tested the effect of co-administration of the anti-oxidants N-acetylcysteine (10 mM) and glutathione reduced ethyl-ester (10 mM) for 24 hours. Both failed to ameliorate the ER stress response suggesting that pyocyanin caused ER dysfunction independent of causing oxidative

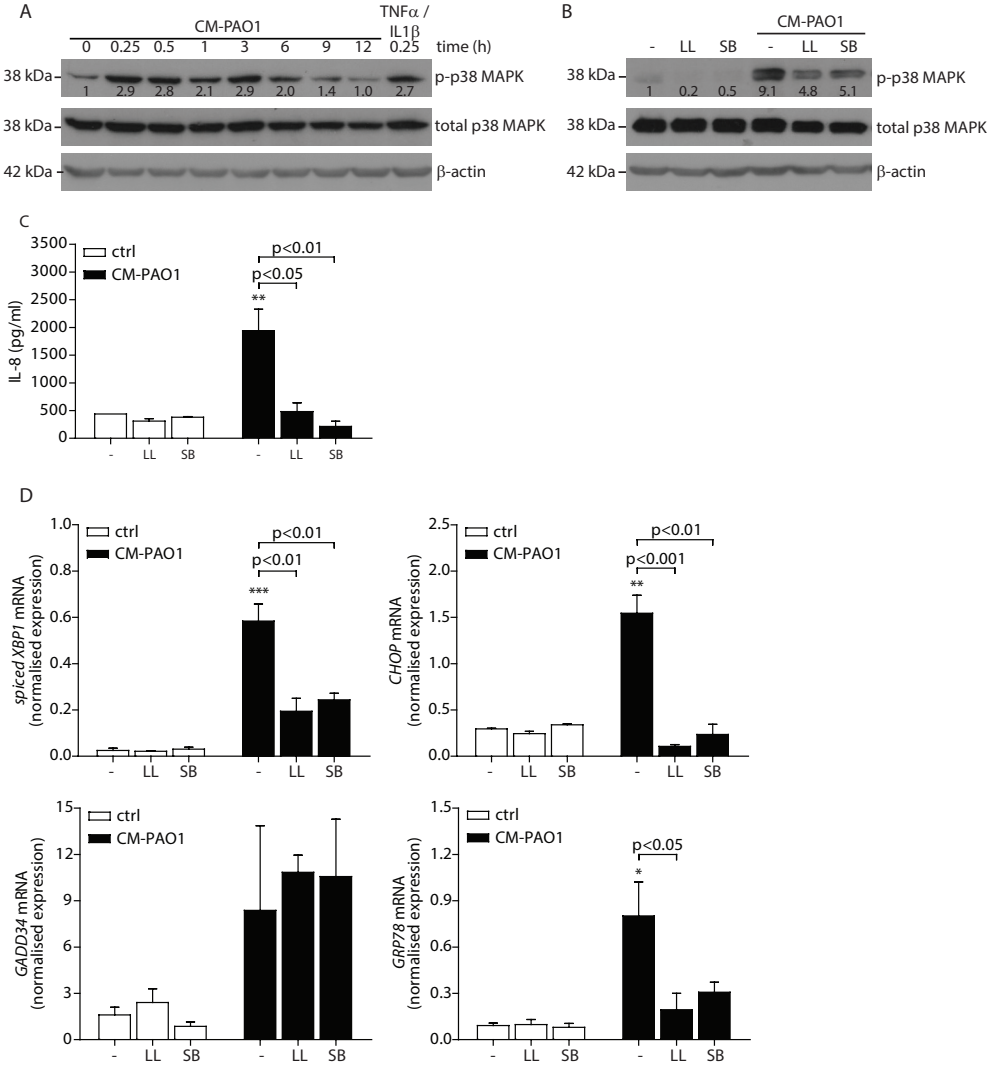


**Figure 3. Conditioned medium of *P. aeruginosa* induces ER stress via TAK1-p38 MAP kinase (MAPK).** >>>

A. Time-dependent phosphorylation of p38 MAPK in 16HBE after treatment with CM-PAO1. Total p38 MAPK and β-actin serve as loading controls. Numbers display the relative quantifications for phosphorylated p38 MAPK to total p38 MAPK (representative of n=3). B. Western blot of phosphorylated p38 MAPK from 16HBE after pre-treatment for 30 min with the TAK-1 inhibitor LL-Z1640-2 (LL) or p38 MAPK inhibitor SB203580 (SB), followed by CM-PAO1 stimulation for 6 hours. Total p38 MAPK and β-actin serve as loading controls. Numbers display the relative quantifications for phosphorylated p38 MAPK to total p38 MAPK (representative of n=3). C. IL-8 release of 16HBE cells treated as in B (n=3; mean ± SEM). D. Normalised mRNA levels of spliced *XBP1*, *CHOP*, *GADD34* and *GRP78* in 16HBE cells, treated as B (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus untreated (-) with a one-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

stress (53, 54) (data not shown).

Taken together, these observations suggested that conditioned medium of *P. aeruginosa* caused ER stress via multiple virulence factors, including pyocyanin. Furthermore, the induction of *GADD34* appeared to involve an additional pathway independent of *CHOP*.



**Identifying other factors mediating ER stress**

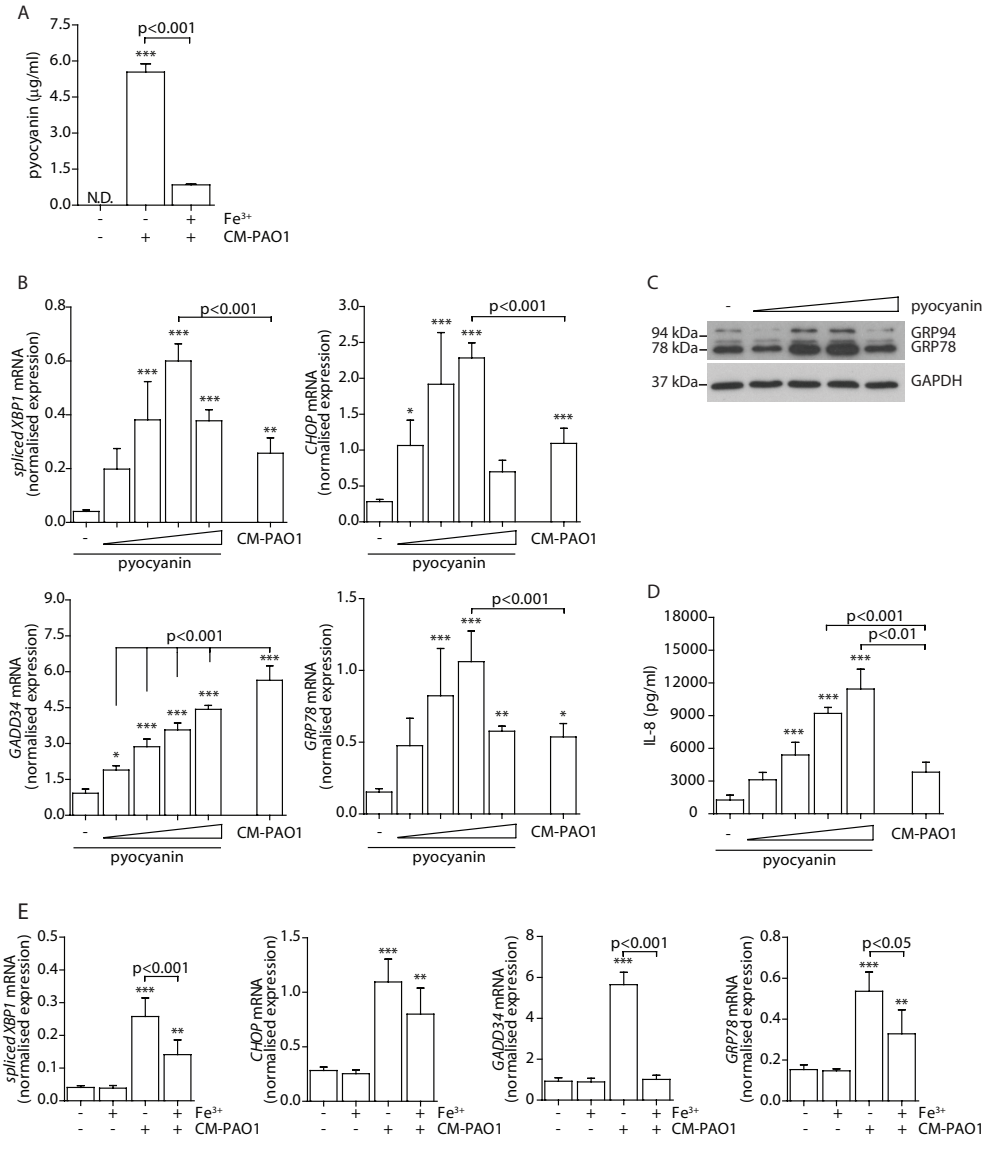
Having found evidence for the involvement of multiple virulence mechanisms in the induction of ER stress, we next attempted to determine their identities. The *P. aeruginosa* AB toxin exotoxin A is known to cause translational attenuation by catalysing the ADP-ribosylation of elongation factor 2 (EF2) (55). We investigated whether purified exotoxin A could also induce ER stress, but detected no increase in *spliced XBP1*, *CHOP*, *GADD34* or *GRP78* mRNA nor the phosphorylation of eIF2α (Figure S2A-B). Next, to explore more broadly the involvement of other potential virulence factors, we made use of strains of *P. aeruginosa* that lacked specific toxic products: PAN8, a *lasB aprE* double mutant, which is deficient in the production of elastase (56) and the secretion of AprA; PAN11, a *xcpR lasB* mutant, which is deficient in the production of elastase and the secretion of all other substrates of the type II protein secretion system but still produces AprA; and PAO25, a *leu arg* double mutant derivative of PAO1 and the direct parental strain of both mutants (table S1). CM-PAO25 did not differ from CM-PAO1 in the content of all toxins measured (Figure S3A-C). In spite of the aprE mutation, still traces of AprA were detected in the culture supernatant of the PAN8 strain (Figure 5A), presumably due to cell lysis during the 5 days growth period.



**Figure 4. Pyocyanin is able to cause ER stress.** >>>

A. Quantitation of pyocyanin in CM-PAO1. Iron ($Fe^{3+}$) was supplemented in the culture medium of the bacteria to inhibit virulence factor secretion (n=3; mean ± SEM). B. Normalised mRNA expression levels of *spliced XBP1*, *CHOP*, *GADD34* and *GRP78* after pyocyanin treatment (0-1-3-10-30 μM) (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. C. Western blot of GRP78/GRP94 and GAPDH (loading control) from 16HBE cell lysates treated as in B. (representative of n=3). D. IL-8 release of 16HBE cells treated as in B (n=3; mean ± SEM). E. *Spliced XBP1*, *CHOP*, *GADD34* and *GRP78* mRNA levels in 16HBE cells exposed to CM-PAO1 derived after growth in the presence or absence of iron ($Fe^{3+}$) (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ versus untreated (-) with a one-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

When 16HBE cells were incubated with CM-PAN8 (lacking elastase and AprA), induction of *spliced XBP1* and *GRP78* mRNA were completely abolished, and only minimal induction of *CHOP* mRNA remained (Figure 5B). The CM-PAN11 (containing AprA, but no elastase or other substrates of the type 2 secretion system) caused measurable splicing of *XBP1* mRNA and the induction of *CHOP* and *GRP78* mRNA, albeit significantly less than the conditioned medium from the parental *P. aeruginosa* PAO25 strain (Figure 5B). Of note,



stimulating 16HBE cells with purified elastase did not elicit an ER stress response within 24 hours (data not shown). On the other hand, incubation with 10 nM purified AprA induced the splicing of *XBP1* mRNA, and up-regulated *CHOP* and *GRP78* mRNA (Figure 5C). These experiments suggested that, in addition to pyocyanin, AprA also contributed to the induction of ER stress in 16HBE cells.

Remarkably, once again the induction of *GADD34* mRNA followed a distinct trend from the other markers of ER stress. Particularly a lack of AprA (in CM-PAN8) was correlated with an increased expression of *GADD34* (Figure 5B). Likewise, purified AprA did not induce *GADD34* mRNA (Figure 5C). This suggested that an unrelated mechanism regulated *GADD34* induction by CM-PAO1 and that this might be independent of ER stress.

**_GADD34_ is regulated via the integrated stress response (ISR) independent of PERK**



To examine the involvement of ER stress-dependent and -independent responses to CM-PAO1, we next made use of the specific inhibitor of IRE1, 4μ8C, which blocks splicing of *XBP1* mRNA during ER stress ((57) and Figure 6A). Of note, this compound not only attenuated the ER stress response elicited by CM-PAO1, but, interestingly, also attenuated the secretion of IL-8 by 16HBE in response to CM-PAO1 (Figure S4A).

During ER stress, the kinase PERK phosphorylates eIF2α, thereby activating the ISR. When *Perk*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were exposed to CM-PAO1, the induction of *Gadd34* mRNA was unaffected, while the response to the ER stress-inducing agent tunicamycin (Tm) was abrogated (Figure 6B). However, phosphorylation of eIF2α was required for the induction of *Gadd34* mRNA in response to CM-PAO1 as demonstrated by the failure of the conditioned medium to induce *Gadd34* mRNA in fibroblasts homozygous for the *eIF2α*$^{AA}$ mutation, which renders them insensitive to all eIF2α kinases (Figure 6C). Moreover, Atf4, a transcription factor translationally up-regulated upon phosphorylation of eIF2α, was essential for the induction *Gadd34* mRNA by CM-PAO1 (Figure 6D). As we have shown previously (26), Chop was only partially required for tunicamycin (ER stress)-induced expression of *Gadd34* mRNA (Figure S4B). The same was observed for CM-PAO1, although it did not reach statistical significance (Figure S4B).

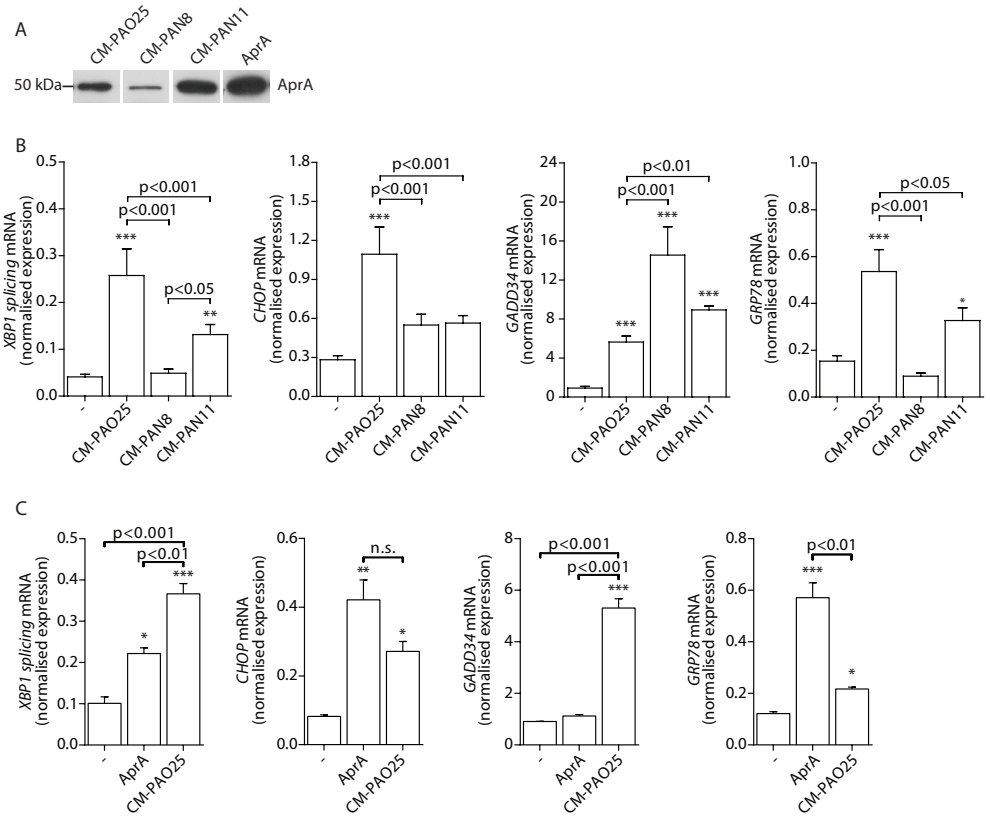
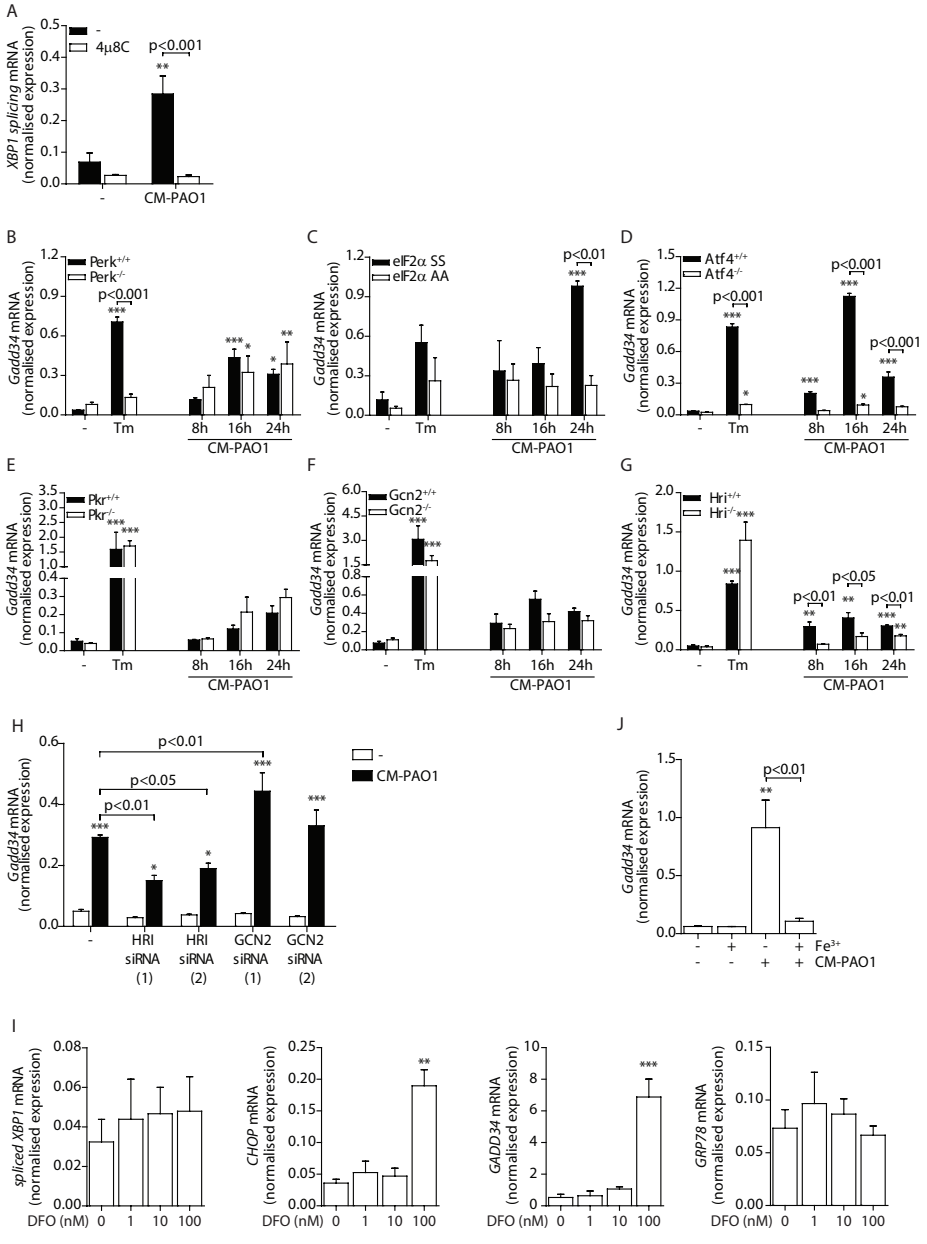



**Figure 5. The *P. aeruginosa* secreted virulence factors alkaline protease (AprA) and pyocyanin mediate ER stress.**

A. Western blot analysis of conditioned medium (CM) of strains PAO25, PAN8 and PAN11 for AprA (representative of n=2; complete blot is showed in Figure S1B). Equal volumes of the conditioned medium were loaded onto the gel. AprA displays 200 nM purified AprA and serves as a positive control. B. Normalised expression levels of *spliced XBP1*, *CHOP*, *GADD34* and *GRP78* mRNA in 16HBE cells after stimulation with CM-PAO25, CM-PAN8 or CM-PAN11 (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. C. Normalised expression values of *spliced XBP1*, *CHOP*, *GADD34* and *GRP78* mRNA in 16HBE cells after stimulation with 10 nM purified AprA. All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. * p<0.05, ** p<0.01, ***p<0.001 versus untreated (-) with a one-way repeated-measurements ANOVA (Bonferroni *post-hoc*).


A
XBP1 splicing mRNA (normalised expression)
-
4μ8C
p<0.001
CM-PAO1
B
Gadd34 mRNA (normalised expression)
Perk+/+
Perk-/-
p<0.001
Tm
8h
16h
24h
CM-PAO1
C
eIF2α SS
eIF2α AA
p<0.01
D
Atf4+/+
Atf4-/-
p<0.001
E
Pkr+/+
Pkr-/-
F
Gcn2+/+
Gcn2-/-
G
Hri+/+
Hri-/-
p<0.05
H
p<0.01
p<0.05
HRI siRNA (1)
HRI siRNA (2)
GCN2 siRNA (1)
GCN2 siRNA (2)
J
Fe3+
I
spliced XBP1 mRNA (normalised expression)
CHOP mRNA (normalised expression)
GADD34 mRNA (normalised expression)
GRP78 mRNA (normalised expression)
DFO (nM) 0 1 10 100

<<< **Figure 6. *GADD34* mRNA expression is regulated via the activation of the integrated stress response by *P. aeruginosa*.**

A. *Spliced XBP1* mRNA in 16HBE cells after treatment with CM-PAO1 in the presence of 30 μM 4μ8C, a selective inhibitor of the ER stress responsive kinase IRE1α (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. B-G. *Gadd34* mRNA normalised expression in *Perk*$^{-/-}$, *eIF2α AA*, *Atf4*$^{-/-}$, *Pkr*$^{-/-}$, *Gcn2*$^{-/-}$ and *Hri*$^{-/-}$ mouse embryonic fibroblasts (MEFs) exposed to CM-PAO1 for 8, 16 or 24 hours or tunicamycin (Tm) for 6 hours as a positive control (n=3; mean ± SEM). All values are normalised to the housekeeping genes *Actb* and *Sdha*. H. *GADD34* mRNA levels in HeLa cells upon exposure to CM-PAO1 after knock-down of GCN2 or HRI with siRNA (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. I. Normalised expression values of spliced *XBP1*, *CHOP*, *GADD34* and *GRP78* mRNA in 16HBE cells after stimulation with 1-100 nM deferoxamine (DFO). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. J. *Gadd34* mRNA levels in wild-type MEFs after repletion of the cell culture medium with iron ($Fe^{3+}$) when treated with CM-PAO1 (n=3; mean ± SEM). The first lane (- $Fe^{3+}$, - CM-PAO1) reflects medium control cells, without adding or depleting iron from the cell culture medium. All values are normalised to the housekeeping genes *Actb* and *Sdha*. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus untreated (-) with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).



Interestingly, murine fibroblasts stimulated with CM-PAO1 failed to splice *Xbp1* mRNA (Figure S4C), suggesting that activation of IRE1 by CM-PAO1 may be less important in this cell type than in human epithelial cells. However, reassuringly, ISR-dependent signalling in response to *P. aeruginosa* toxins was preserved in these cells and, once again, expression of *Chop* mRNA was regulated via eIF2α and Atf4. As had been observed for *Gadd34*, *Chop* induction was independent of PERK, suggesting that in MEFs treated with CM-PAO1, *Chop* was induced by a stimulus other than ER stress (Figure S4D-F).

We next examined which eIF2α kinase was responsible for activation of the ISR by CM-PAO1. To this end, we made use of *Pkr*$^{-/-}$, *Gcn2*$^{-/-}$ and *Hri*$^{-/-}$ MEFs (25,58,59) and

observed a significant deficit of CM-PAO1 induction of *Chop* and *Gadd34* mRNA in *Hri*$^{-/-}$ cells, suggesting the involvement of the iron-sensing kinase HRI (Figure 6E-G, and Figure S4G-I). Although we observed no significant effect on the induction of *Gadd34* mRNA in *Gcn2*$^{-/-}$ cells (Figure 6F), it has been suggested previously that GCN2 is involved in the stress response induced by *P. aeruginosa* in gut epithelial cells (60). We therefore went on to deplete either GCN2 or HRI in HeLa cells using two separate siRNA oligonucleotides for each gene and obtained similar results, supporting a role for HRI rather than GCN2 (Figure 6H and Figure S4J).

Since RPMI is an iron-poor medium, we reasoned that the CM-PAO1 would limit iron availability to epithelial cells (e.g. by siderophores; (61)), which might activate HRI through depletion of iron from the culture medium. We therefore first evaluated the effect of iron depletion of the epithelial cell culture medium using deferoxamine (DFO). DFO treatment resulted in a marked increase in the expression of the ISR and UPR related genes *CHOP* and *GADD34*, whereas *GRP78* and *spliced XBP1* were not affected (Figure 6I). This is line with selective activation of the ISR by iron depletion. We next confirmed the presence of the iron-chelating siderophore pyoverdine in the CM-PAO1 by the bright fluorescence of the medium upon exposure to UV light (data not shown). To test the possible involvement of iron depletion in CM-PAO1-mediated *Gadd34* induction, we supplemented the epithelial cell culture medium with iron, which indeed completely suppressed the induction of *Gadd34* mRNA (Figure 6J, and Figure S4K).



Taken together, these data demonstrate that CM-PAO1 induces splicing of *XBP1* mRNA (ER stress) in human bronchial epithelial cells, while induction of *GADD34* predominantly reflects an iron-dependent ISR mediated by the eIF2α kinase HRI.

**The role of *Gadd34* induction in cell survival**

During chronic ER stress in cell and animal models of disease, the induction of *GADD34* appears to mediate cellular toxicity (26, 44). In contrast, during the acute stress of SERCA pump inhibition by thapsigargin, *GADD34* has been shown to be protective (62). To test the role of ER stress-independent induction of *GADD34* by exposure to CM-PAO1,

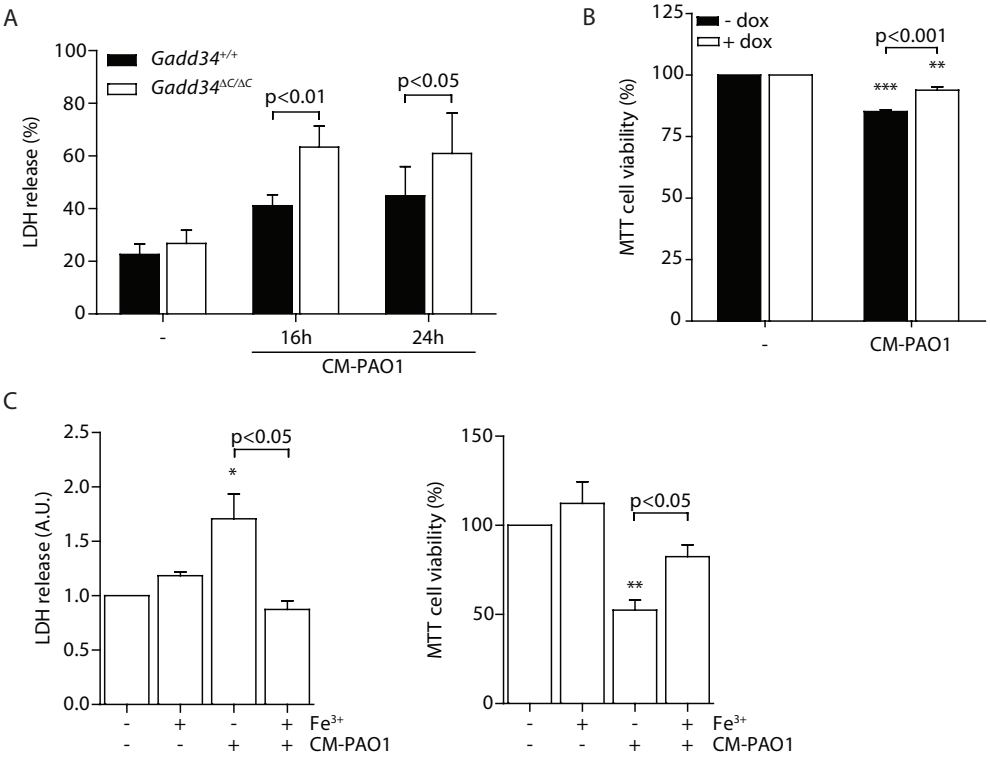



**Figure 7. Induction of *GADD34* protects against *P. aeruginosa* mediated cell cytotoxicity.**

A. LDH release of *Gadd34*$^{+/+}$ and *Gadd34*$^{\Delta C/\Delta C}$ MEFs after stimulation with CM-PAO1 for 16 and 24 hours (n=3; mean ± SEM). B. MTT cell viability of HeLa cells conditionally expressing *GADD34* (± dox) after treatment with CM-PAO1 (n=3; mean ± SEM). C. LDH release (left) and cell viability assessed with a MTT assay (right) of wild-type MEFs treated with CM-PAO1 after repleting the cell culture medium with iron ($Fe^{3+}$) (n=3; mean ± SEM). * p<0.05, ** p<0.01, *** p<0.001 versus untreated (-) with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

we made use of *Gadd34*$^{\Delta C/\Delta C}$ MEFs (62), which lack *GADD34* phosphatase activity. Cells expressing wild-type *Gadd34* were more resistant to the cytotoxic effects of CM-PAO1 compared with *Gadd34*$^{\Delta C/\Delta C}$ fibroblasts, as reported by the release of lactate dehydrogenase (LDH) (Figure 7A). To confirm these findings, we repeated these experiments in HeLa cells

expressing *GADD34* from a tetracycline-responsive promoter. The induction of GADD34 protein with doxycycline significantly increased cell viability on exposure to CM-PAO1 (Figure 7B). When the cell culture medium of wild-type cells was supplemented with iron, the release of LDH was prevented (Figure 7C, left panel). Iron supplementation was also observed to rescue the reduction of cell viability reported by MTT assay (Figure 7C, right panel). Taken together, these data suggest that the toxicity of CM-PAO1 is sensitive to iron and that HRI-mediated induction of *GADD34* is protective in this context. Supplementation with iron relieves both the cytotoxicity and the requirement for induction of *GADD34*.

## Discussion

It is known that a normal response to ER stress is required for an efficient innate immune response to bacterial infection (40), but whether live bacteria are required for this has been unclear. In this study, we have shown that secreted virulence factors of *P. aeruginosa* cause ER stress in primary bronchial epithelial cells and in different cell lines, and that this is mediated by TAK1 and phosphorylated p38 MAPK. In addition, we have identified *GADD34* induction via an ER-stress independent ISR. We have demonstrated pyocyanin to be one of the factors eliciting these responses, while AprA contributes to the activation of the UPR. In contrast, activation of the ISR with induction of *GADD34* mRNA is a response to reduced iron availability and serves a cytoprotective role during exposure to conditioned medium of *P. aeruginosa*.

In line with these observations, phosphorylation of p38 MAPK has previously been shown to be involved in the splicing of *XBP1* upon infection with *P. aeruginosa* (40, 46), although the involvement of TAK1 upstream of p38 MAPK and its essential involvement in the activation of *CHOP* and *GRP78* are novel findings. Interestingly, *GADD34*, classically a downstream target of *CHOP*, was regulated independently of the TAK1-p38 MAPK pathway. The induction of *GADD34* is only partially dependent on *CHOP* (Figure S2A and (26)), but it is absolutely reliant on phosphorylation of eIF2α and ATF4 (26). This is concordant with the recent description of a virus-induced "microbial stress response" mediated via the PKR/eIF2α/ATF4 pathway, which fails to induce *CHOP*, but potently induces *GADD34* (42, 43).



In contrast to the response of human airway epithelial cells, *P. aeruginosa* conditioned medium failed to cause splicing of *Xbp1* mRNA in murine fibroblasts, suggesting that ER stress may not be a conserved feature of the cellular response to this insult. This is unsurprising, as induction of ER stress is known to be highly cell-type dependent (41). In the absence of ER stress in the murine fibroblasts, the induction of *Chop* and *Gadd34* suggests that activation of the ISR by the secreted virulence factors may be a more conserved response. Of note, in human primary bronchial epithelial cells, the induction of *CHOP* seems primarily subordinate to an ER stress-induced ISR, rather

than the microbial stress response (Figure S7). Consequently, induction of *CHOP* was dependent on the TAK1-p38 MAPK pathway in those cells (Figure 2D) and its induction was only partially inhibited when bacterial cultures were supplemented with iron (Figure 3E), in contrast to MEFs where *Chop* induction was dependent on HRI (Figure S2I).

Recent evidence suggests that bacterial components may function as triggers for the UPR. Flagellin has been shown to induce an atypical ER stress response in CF bronchial epithelial cells during live infection (46), while N-(3-oxo-dodecanoyl) homoserine lactone (C12) has been observed to cause phosphorylation of eIF2α and activation of p38 MAPK (63). We have now shown that at least two secreted virulence factors, pyocyanin and AprA, also contribute to this ER stress response to *Pseudomonas*. More research has to be done to assess the involvement of (other) individual virulence factors.

High concentrations of pyocyanin also mediated an ER stress-independent, ISR-dependent induction of cytoprotective *GADD34* (Figure 3B). We were able to identify a crucial role for iron availability and for the iron-sensing kinase HRI in this response. Interestingly, AprA was not involved in the induction of this response but rather appeared to dampen it, since considerably higher *GADD34* expression was observed when conditioned medium of the aprE mutant PAN8 was used to stimulate the cells (Figure 4B). Among other possibilities, an explanation for this observation could be that AprA present in the conditioned medium of the wild-type strain partially degrades HRI, a possibility that warrants further investigations. The discovery of this ER stress-independent ISR may plausibly offer novel potential therapeutic targets.



It has been shown recently that *spliced XBP1* is required for C12-mediated apoptosis (63). Remarkably, exposure of cells to C12 does not itself trigger the splicing of *XBP1* mRNA suggesting that basal levels of *spliced XBP1* are both necessary and sufficient for this response. Moreover, the transcriptional activity of *spliced XBP1* does not appear to be required for this cell death, indicating that the spliced XBP1 protein may have additional, as yet unidentified, activities. C12 appears able to trigger the ISR in an ER stress-independent matter, although the mechanism for this remains to be determined. It would be interesting to determine if C12 can activate HRI.

Chronic elevation of GADD34 in ER stress can mediate cellular toxicity (26), but GADD34 has shown to be protective during the acute stress of SERCA pump inhibition with thapsigargin, which depletes the ER of calcium (62). As with thapsigargin, *P. aeruginosa* has been associated with altered ER calcium signalling (39, 45). It is therefore of interest that expression of *GADD34* reduced cell toxicity and increased cell survival upon iron deficiency caused by treatment with conditioned medium from *P. aeruginosa*. It has been shown that lungs of cystic fibrosis patients lack the ability to induce *GADD34* (46), which might plausibly lead to increased cytotoxicity or altered innate immunity due to *Pseudomonas* infection of the lungs of CF patients.

In summary, secreted virulence factors of the PAO1 strain of *P. aeruginosa,* including pyocyanin and AprA, are sufficient to elicit an ER stress response but the relative contribution of these virulence factors remains to be investigated. In contrast to these virulence factors, iron depletion causes an ER stress-independent ISR. The induction of *GADD34* via this response ameliorates the toxic effects of *P. aeruginosa* conditioned medium.

## Materials and Methods

### Bacterial strains and preparation of conditioned medium of *P. aeruginosa*

All strains used in this study are listed in Table S1. CM was prepared as described previously with slight modifications (48). Briefly, overnight bacterial cultures in Luria Broth were inoculated 1:50 into RPMI 1640 (Gibco, Life Technologies, Breda, the Netherlands) and incubated at 37°C shaking at 200 rpm. After 5 days, the cultures were centrifuged and supernatants were filter-sterilised through 0.22 µm pore-size filter (Whatman, Dassel, Germany) to obtain CM. Pyocyanin and AprA levels in CM were measured as described previously (64, 65).

### Cell culture



Primary bronchial epithelial cells were obtained from tumour-free resected lung tissue by enzymatic digestion as described previously (66). 16HBE cells (passage 4-15; kindly provided by Dr. D.C. Gruenert, University of California, San Francisco, CA, USA) were cultured in MEM (Invitrogen) supplemented with 1 mM HEPES (Invitrogen), 10% (v/v) heat-inactivated FCS (Bodinco, Alkmaar, the Netherlands), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from BioWhittaker). All MEFs were maintained as described previously (23, 26, 37, 67, 68). HEK-TLR2 and HEK-TLR4 (51) were a kind gift from M. Yazdanbakhsh (Leiden University Medical Center, the Netherlands). HeLa cells were transfected for 6 hours with two different ON-TARGETplus Human EIF2AK1 siRNA (GCACAAACUUCACGUUACU and GAUUAAGGGUGCAACUAAA) or EIF2AK4 siRNA (GGAAAUUGCUAGUUUGUCA and GACCAUCCCUAGUGACUUA) and knockdown was assessed 48 hours after transfection (Figure S5).

GADD34-N1-eGFP (kind gift form S. Shenolikar, Duke-NUS Graduate Medical School Singapore, Singapore) was excised with BglII and NotI and ligated into pTRE2-hyg plasmid (Clontech Laboratories, Mountain View, CA, USA) digested with BamHI and NotI. HeLa Tet-On advanced cells (Clontech Laboratories) were transfected with the pTRE2-hyg_ GADD34-eGFP plasmid and selected with 600 µM hygromycin to generate a stable cell

line conditionally expressing GADD34-GFP (Figure S6). Positive cell clones were visualised by GFP expression in response to 1 µg/ml of doxycycline. Once identified, expanded and characterised, these clones were maintained in DMEM (Sigma) supplemented with 10% FBS and antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin, 200 µg/ml G418 and 200 µM hygromycin). Expression of GADD34 was typically induced using 1 µg/ml doxycycline (Sigma) for 24 hours.

Cells were exposed at 80-90% confluence for 24 hours (unless stated otherwise) to CM-PAO1 (1 in 5 dilution, unless stated otherwise), pyocyanin (1-30 µM), ammonium iron (III) citrate (100 µM; $Fe^{3+}$), exotoxin A (1-10 ng/ml) and/or DFO (1-100 nM) as indicated (all from Sigma). Puromycin (10 µg/ml; Sigma) was added 30 minutes before harvesting. Thapsigargin (100 nM; Sigma), TNFα and IL-1β (both 20 ng/ml; Peprotech, Rocky Hill, NJ) were used as positive controls. The compounds SB203580 (10 nM; Sigma) and 5Z-7-oxozeanol (also called LL-Z1640-2; 100 nM; TebuBio, Heerhugowaard, the Netherlands) were added 30 minutes before stimulation for the inhibition of p38 MAPK and TAK1, respectively. The specific IRE1-inhibitor 4µ8C (30 µM) (57) was a kind gift from Prof. dr. D. Ron, University of Cambridge.



**Western Blot**

Cells were lysed in buffer H (10 mM HEPES, pH 7.9, 50 mM NaCl, 500 mM sucrose, 0.1 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM PMSF, 1X Complete™ protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany)) supplemented with phosphatase inhibitors (10 mM tetrasodium pyrophosphate, 17.5 mM β-glycerophosphate, and 100 mM NaF (25, 27)) for detection by antibodies directed against phospho-eIF2α (Cell Signaling Technology, Danvers, MA, USA), eIF2α (gift from Prof. D. Ron), KDEL (Enzo Life Sciences), GADD34 (ProteinTech, Chicago, IL, USA), puromycin (Millipore, Billerica, MA, USA), β-actin and GAPDH (CellSignalling), or in sample buffer (0.2 M Tris-HCl pH 6.8, 16% (v/v) glycerol, 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 0.003% (w/v) bromophenol blue) for detection by antibodies directed against phospho-p38 MAPK and total p38 MAPK (both CellSignalling). The proteins in the samples were separated using a 10%

SDS-PAGE gel and transferred onto a nitrocellulose membrane. After blocking with PBS containing 0.05% Tween-20 (v/v) and 5% skimmed-milk (w/v), the membrane was incubated overnight with the primary antibody (1:1000) in TBS with 0.05% Tween-20 (v/v) and 5% BSA (w/v) at 4°C. Next, the membrane was incubated with HRP-labelled anti-mouse or anti-rabbit antibody (Sigma) in blocking buffer for 1 hour and developed using ECL (ThermoScientific).

**Quantitative reverse-transcriptase polymerase chain reaction (qPCR)**

Total RNA was isolated using Qiagen RNeasy mini kit (Qiagen/Westburg, Leusden, the Netherlands). Quantitative reverse-transcriptase polymerase chain reaction (qPCR) was performed as described previously (69) using the primer pairs as defined in Table S2. Relative mRNA concentrations of *RPL13A* and *ATP5B* (GeNorm, PrimerDesign Ltd., Southampton, UK) were used as housekeeping genes for human genes and *Actb* (β-actin) and *Sdha* for mouse genes to calculate normalized expression.



**ELISA**

IL-8 (Sanquin, Amsterdam, the Netherlands) were measured using commercially available ELISA kit according to manufacturer's instructions.

**Cytotoxicity assays**

LDH release was measured with a LDH-cytotoxicity colorimetric assay kit following manufacturer's instructions (Biovision, Milpitas, CA, USA). Thiazolyl blue tetrazolium bromide (MTT; Sigma) was dissolved in a 5 mg/ml stock concentration in sterile water and cells were incubated with a 1:10 dilution for 2 hours at 37°C. Next, the water-insoluble formazan formed from MTT is viable cells was dissolved in isopropanol for 10 min before the absorbance was read at 570 nm wavelength.

**Electric Cell-sensing Impedance Sensing**

Epithelial barrier function was measured using ECIS (Applied Biophysics, Troy, NY,

USA) as described previously (70). Resistance was measured at 1000 Hz and cells were stimulated with CM-PAO1 when the resistance was stable.

### Statistical analysis

Results are expressed as mean ± SEM. Data were analysed using one- or two-way analysis of variance (ANOVA) and corrected with the Bonferroni *post-hoc* test. Differences with p-values < 0.05 were considered to be statistically significant.

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## Supplementary data

Table S1. Bacterial strains

| Strain | Characteristics | Ref. |
|---|---|---|
| PAO1 | Wild-type | ATCC; BAA-47 |
| PAO25 | PAO1 *leu arg* | (71) |
| PAN8 | PAO25 *lasB::km$^R$ aprE::ΩHg* | (56) |
| PAN11 | PAO25 *xcpR-54 lasB::km$^R$* | (56) |

Table S2. qPCR primers

| Name | Forward primer / Reverse primer | Melting temp (°C) | Ref. |
|---|---|---|---|
| | | | |
| HUMAN | | | |
| *CHOP* | 5′ GCACCTCCCAGAGCCCTCACTCTCC 3′<br>5′ GTCTACTCCAAGCCTTCCCCCTGCG 3′ | 62 | (69) |
| *GADD34* | 5′ ATGTATGGTGAGCGAGAGGC 3′<br>5′ GCAGTGTCCTTATCAGAAGGC 3′ | 62 | (72) |
| *GRP78* | 5′ CGAGGAGGAGGACAAGAAGG 3′<br>5′ CACCTTGAACGGCAAGAACT 3′ | 62 | (73) |
| *XBP1spl* | 5′TGCTGAGTCCGCAGCAGGTG 3′<br>5′ GCTGGCAGGCTCTGGGGAAG 3′ | 62 | (69) |
| | | | |
| MOUSE | | | |
| *Actb* | 5′TCCTGGCCTCACTGTCCA 3′<br>5′ GTCCGCCTAGAAGCACTTGC 3′ | 59 | (74) |
| *Chop* | 5′ GGAGCTGGAAGCCTGGTATGA G 3′<br>5′ GCAGGGTCAAGAGTAGTGAAGG 3′ | 59 | (57) |
| *Gadd34* | 5′ CCCGAGATTCCTCTAAAAGC 3′<br>5′ CCAGACAGCAAGGAAATGG 3′ | 59 | (75) |
| *Sdha* | 5′TTGCTACTGGGGGCTACGGGC 3′<br>5′TGACCATGGCTGTGCCGTCC 3′ | 59 | - |
| *Xbp1s* | 5′ CTGAGTCCGAATCAGGTGCAG 3′<br>5′ GTCCATGGGAAGATGTTCTGG 3′ | 59 | (76) |

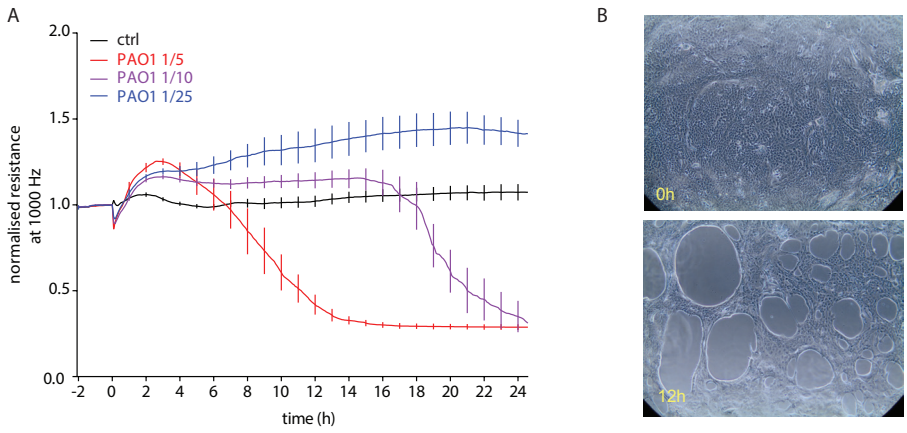



**Figure S1. Conditioned medium of strain PAO1 causes disruption of the epithelial barrier function.** A. Time- and dose-dependent decrease in epithelial resistance measured by ECIS®. Primary bronchial epithelial cells were cultured on golden electrodes a described previously and epithelial resistance was measured every 5 minutes at 1000 Hz. Values are displayed as a relative number of the resistance at time point 0 (n=3; mean ± SEM). B. Trypan blue staining of primary bronchial epithelial cells incubated for 12 hours with CM-PAO1.

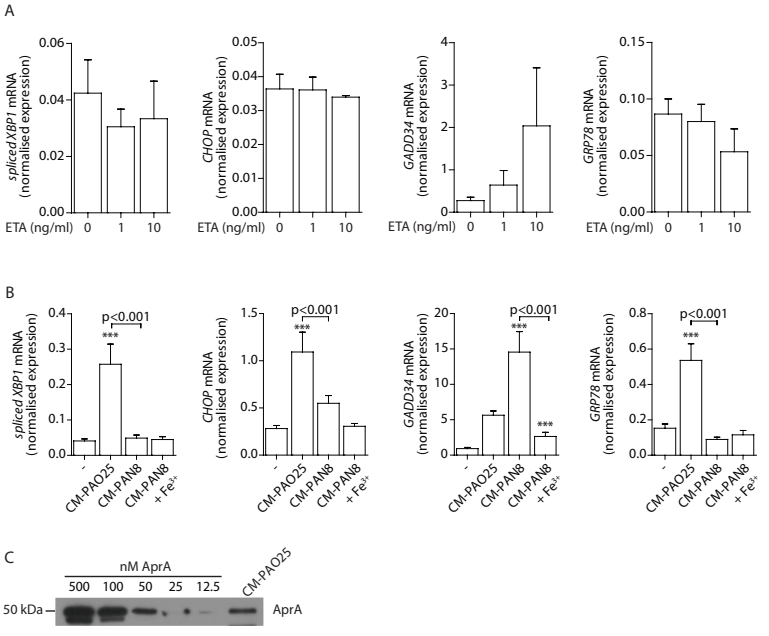





**Figure S2. Exotoxin A does not elicit an ER stress response in 16HBE cells.**

A. Normalised expression levels of *spliced XBP1*, *CHOP*, *GADD34* and *GRP78* mRNA in 16HBE cells after stimulation with 0, 1 or 10 ng/ml *P. aeruginosa* exotoxin A (ETA) (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. B. Normalised expression levels of *spliced XBP1*, *CHOP*, *GADD34* and *GRP78* mRNA in 16HBE cells after stimulation with in 16HBE cells after stimulation with CM-PAO25, CM-PAN8 or CM-PAN8+$Fe^{3+}$ (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. C. AprA western blot of a standard curve of purified AprA. Undiluted CM-PAO25 is used to semi-quantify AprA content within. * p<0.05, ** p<0.01, *** p<0.001 versus untreated (-) with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

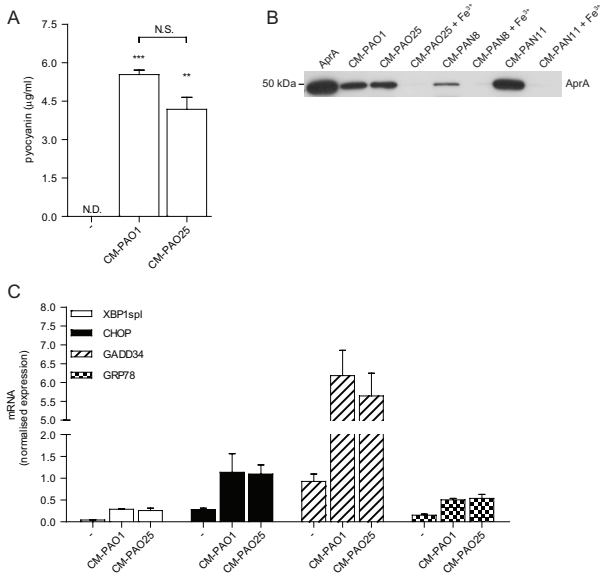





**Figure S3. Conditioned medium of strain PAO1 and PAO25 are comparable in inducing ER stress.** A. Quantitation of pyocyanin in CM-PAO1 and CM-PAO25 (n=3; mean ± SEM). B. Western blot for AprA levels present in CM-PAO1, -PAO25, -PAO25 cultured in the presence of iron (PAO25 + $Fe^{3+}$), -PAN8 and -PAN11 (representative of n=3). C. *Spliced XBP1*, *CHOP, GADD34* and *GRP78* mRNA levels in 16HBE cells treated with CM-PAO1 or CM-PAO25 (n=3; mean ± SEM). * p<0.05, ** p<0.01, *** p<0.001 versus untreated (-) with a one-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

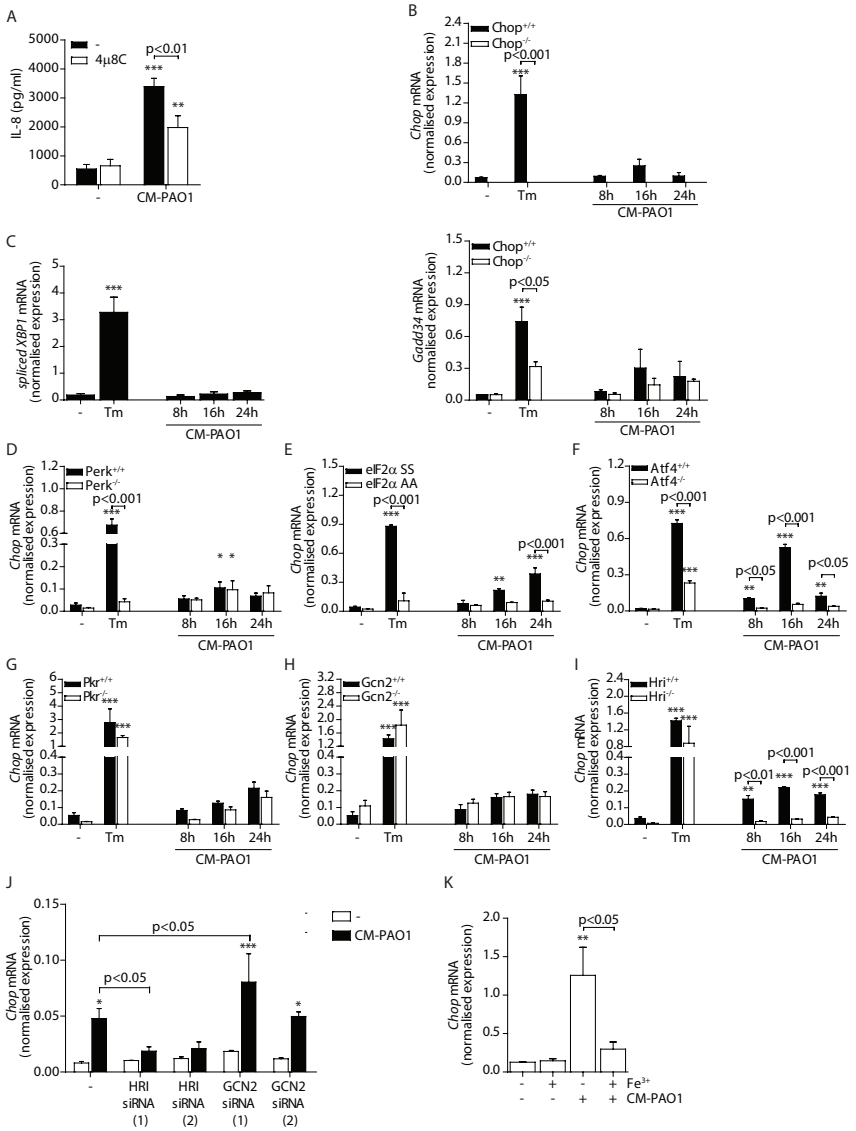

A
IL-8 (pg/ml)
-
4μ8C
p<0.01
CM-PAO1
B
Chop mRNA (normalised expression)
Chop+/+
Chop-/-
p<0.001
Tm
8h
16h
24h
CM-PAO1
C
spliced XBP1 mRNA (normalised expression)
Gadd34 mRNA (normalised expression)
p<0.05
D
Perk+/+
Perk-/-
E
eIF2α SS
eIF2α AA
F
Atf4+/+
Atf4-/-
G
Pkr+/+
Pkr-/-
H
Gcn2+/+
Gcn2-/-
I
Hri+/+
Hri-/-
J
HRI siRNA (1)
HRI siRNA (2)
GCN2 siRNA (1)
GCN2 siRNA (2)
K
Fe3+

**Figure S4. *CHOP* can be regulated via the ER stress independent ISR.**

<<< A. IL-8 release of 16HBE cells after treatment with CM-PAO1 in the presence of 30 μM 4μ8C, a selective inhibitor of the ER stress responsive kinase IRE1 (n=3; mean ± SEM). B. *Chop* and *Gadd34* mRNA induction in *Chop*$^{+/+}$ or *Chop*$^{-/-}$ MEFs exposed to CM-PAO1 for 8, 16 or 24 hours or tunicamycin (Tm) for 6 hours as a positive control (n=3; mean ± SEM). All values are normalised to the housekeeping genes *Actb* and *Sdha*. C. Splicing of *XBP1* mRNA in wild-type MEFs after treatment as in B. (n=3; mean ± SEM). All values are normalised to the housekeeping genes *Actb* and *Sdha*. D-I. *Chop* mRNA normalised expression in *Perk*$^{-/-}$, *eIF2α AA*, *Atf4*$^{-/-}$, *Pkr*$^{-/-}$, *Gcn2*$^{-/-}$ and *Hri*$^{-/-}$ mouse embryonic fibroblasts (MEFs) treated as in A. (n=3; mean ± SEM). All values are normalised to the housekeeping genes *Actb* and *Sdha*. J. *CHOP* mRNA levels in HeLa cells upon exposure to CM-PAO1 after knock-down of HRI or GCN2 with siRNA (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. K. *Gadd34* mRNA levels in wild-type MEFs after repletion of the cell culture medium with iron ($Fe^{3+}$) when treated with CM-PAO1. The first lane (- $Fe^{3+}$, - CM-PAO1) reflects medium control cells, without adding or depleting iron from the cell culture medium (n=3; mean ± SEM). All values are normalised to the housekeeping genes *Actb* and *Sdha*. * p<0.05, ** p<0.01, *** p<0.001 versus untreated (-) with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

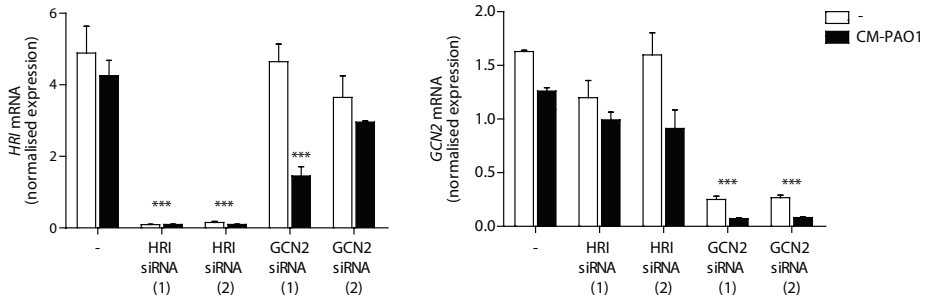



**Figure S5. *HRI* and *GCN2* knock down in epithelial cells.**

*HRI* and *GCN2* expression in HeLa cells after transfection with two different siRNA for each gene. (n=3; mean ± SEM). All values are normalised to the housekeeping genes *RPL13A* and *ATP5B*. * p<0.05, ** p<0.01, *** p<0.001 versus untreated (-) with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).



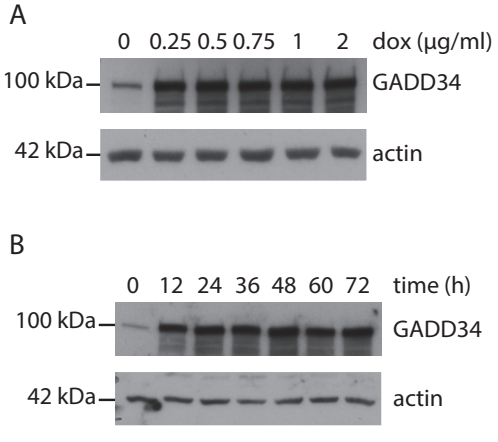



**Figure S6. Expression of GFP-tagged *GADD34* in HeLa Tet-ON cells .**

A. Response of HeLa cells incubated for 24 hours with a range of doxycycline concentrations (n=3). B. Time-dependent induction of GFP-tagged GADD34 in HeLa cells treated with 0.5 µg/ml doxycycline (n=3).

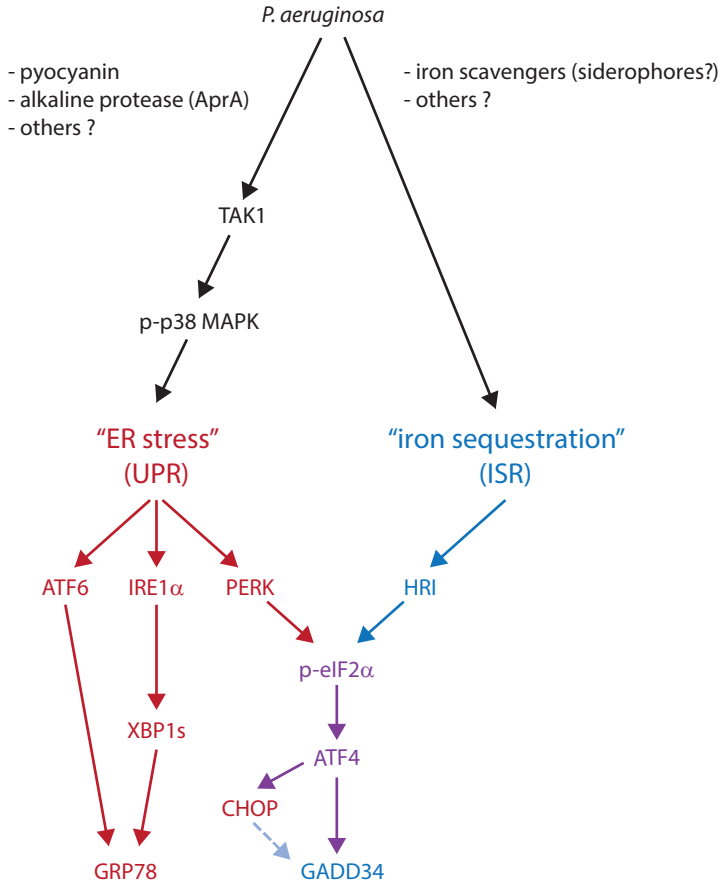
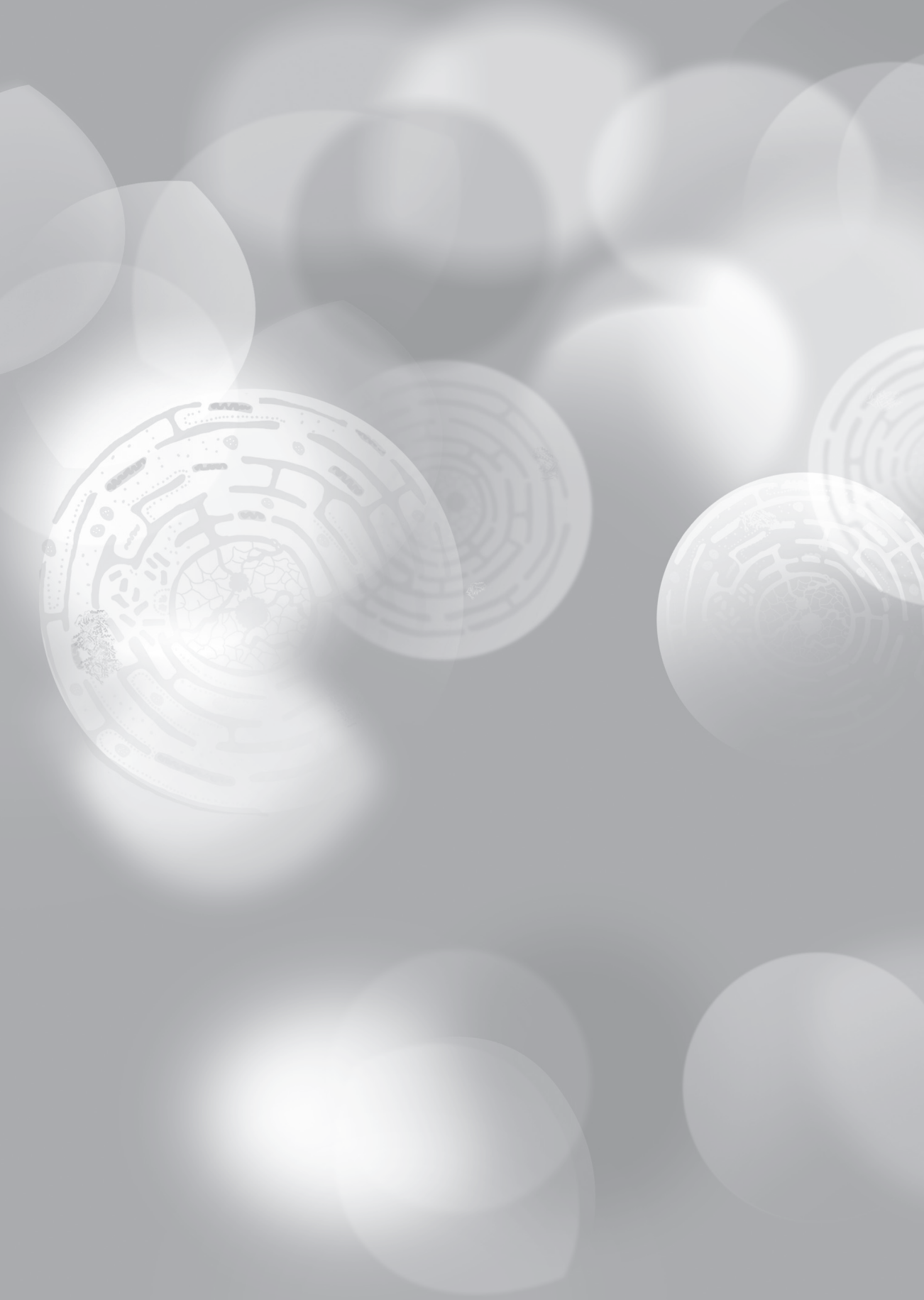





**Figure S7. Schematic overview.**

Secreted virulence factors of *P. aeruginosa* induce both the UPR and the ISR. UPR induction is dependent on the TAK1-p38 MAPK pathway, whereas the induction of the ISR is mediated via iron deficiency. In human bronchial epithelial cells, the UPR causes *XBP1* splicing, and the induction of GRP78 and CHOP (all in red). Iron deficiency, most likely in part caused by sequestration of iron by secreted siderophores, leads to activation of GADD34 via the ER stress independent kinase HRI (in blue). The common pathway is displayed in purple. In our model, it seems unlikely that CHOP influences GADD34. It is yet unknown whether cells distinguish between the phosphorylation of eIF2α by different kinases, and thereby influence specific induction of downstream targets.



# Chapter 5

## Increased ERK signalling promotes inflammatory signalling in primary airway epithelial cells expressing Z $\alpha_1$-antitrypsin

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[*] Joint senior authors

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## Abstract

**Overexpression of Z $\alpha_1$-antitrypsin is known to induce polymer formation, prime the cells for ER stress and initiate NF-κB signalling. However, whether endogenous expression in primary bronchial epithelial cells has similar consequences remains unclear. Moreover, the mechanism of NF-κB activation has not yet been elucidated. Here we report excessive NF-κB signalling in resting primary bronchial epithelial cells from ZZ patients compared to wild-type (MM) controls, and this appears to be mediated by MEK, EGFR and ADAM17 activity. Moreover, we show that rather than being a response to protein polymers, NF-κB signalling in airway-derived cells represents a loss of anti-inflammatory signalling by M $\alpha_1$-antitrypsin. Treatment of ZZ primary bronchial epithelial cells with purified plasma M $\alpha_1$-antitrypsin attenuates this inflammatory response, opening up new therapeutic options to modulate airway inflammation in the lung.**

## Introduction

Alpha$_1$-antitrypsin is a 52-kDa serine protease inhibitor (or serpin), primarily produced by hepatocytes, but also secreted locally by lung epithelial cells and alveolar macrophages (1, 2). Its known function is to inhibit a number of serine proteases, including neutrophil elastase and proteinase 3, thereby preventing excessive degradation of the extracellular matrix. It has also been reported to exhibit anti-inflammatory properties, including the inhibition of TNFα gene expression (3), inhibition of a disintegrin and metalloprotease (ADAM)17 activity in neutrophils and endothelial cells (4, 5) and the regulation of CD14 expression and cytokine release in monocytes (6, 7).

The Z mutation (E342K) of $\alpha_1$-antitrypsin causes subtle misfolding of the protein that permits polymer formation and accumulation within the endoplasmic reticulum (ER) of hepatocytes or degradation by the proteasome leading to deficiency of the secreted protein (8, 9). This causes hepatic cirrhosis through toxic gain-of-function within the liver, most likely due to the retention of polymers, and early-onset lung emphysema, due in large part to the loss of protease inhibition (10). The discovery of polymers in broncho-alveolar lavage fluid and pulmonary tissue (11, 12), the pro-inflammatory nature of such extracellular polymers (11, 13) and their identification many years after liver transplantation (14) led to the proposal that pulmonary pathology could be induced by polymer-induced toxic gain-of-function with inflammation as an additional mechanism (15).



Secreted proteins are first folded within the ER where quality control systems ensure that only properly folded proteins exit the organelle (16). Accumulation of unfolded or misfolded proteins within the ER induces "ER stress", thereby activating intracellular signal transduction pathways, collectively called the unfolded protein response (UPR) (16). This complex cellular response evolved to restore ER homeostasis by reducing the load of newly synthesised protein while increasing the complement of molecular chaperones, which enhance ER protein folding capacity, and increasing the efficiency of misfolded protein degradation (ERAD) (17, 18). We have shown previously that mutant Z $\alpha_1$-antitrypsin is degraded predominantly by ERAD (19). Remarkably, the accumulation of polymers of Z $\alpha_1$-antitrypsin within the ER of hepatocytes does not activate the UPR but

instead increases the cell's sensitivity to ER-stress upon a 'second hit' due to impaired ER luminal protein mobility (20-22).

The transcription factor nuclear factor kappa B (NF-κB) regulates many genes involved in inflammation and cell death, including numerous cytokines and chemokines e.g. interleukin (IL)-8 (23). Phosphorylation of NF-κB is classically mediated through the phosphorylation of inhibitor kappa-B alpha (IκBα); however, NF-κB can also be activated via mitogen-activated protein kinase (MAPK) signalling cascades (24, 25).

Epidermal growth factor (EGF) and related mitogens such as heparin binding-EGF (HB-EGF), amphiregulin (AREG) and transforming growth factor (TGF)α are synthesised as membrane-bound proteins that upon cleavage by metalloproteases (MPs), including ADAMs, bind to and activate the EGF receptor (EGFR) (reviewed in (26)). Transactivation of the EGFR can also occur via activation of ADAMs by G-protein coupled receptor signalling (reviewed in (27)). Within the lung, EGFR activation can induce epithelial cell proliferation by activating ERK1/2. This is mediated by Ras activation of c-Raf, causing phosphorylation of the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK), which in turn phosphorylates ERK1/2 (28).

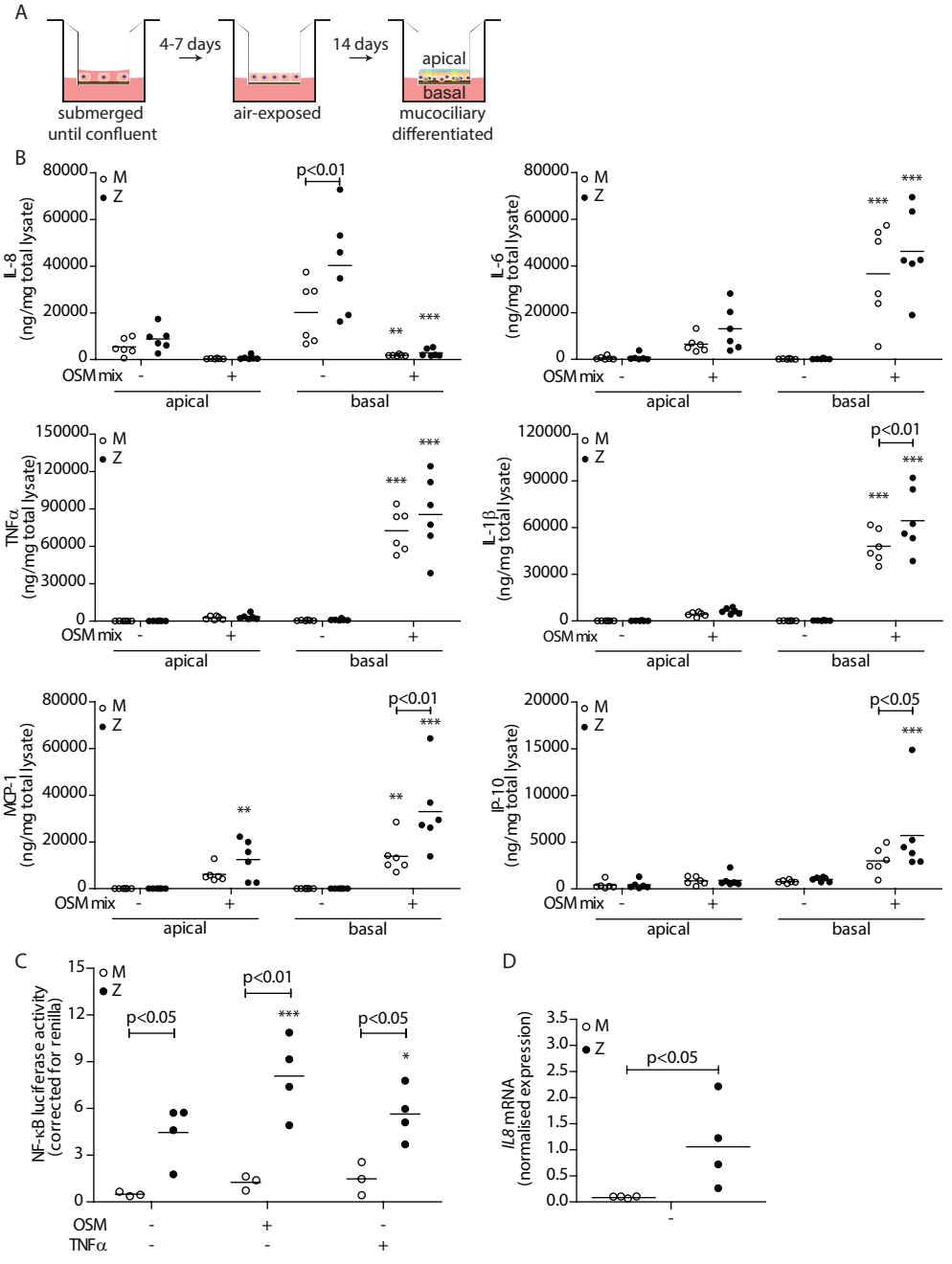


Mutants of members of the serpin superfamily, including $\alpha_1$-antitrypsin, have been shown to activate NF-κB signalling, postulated to be a response to the formation of protein polymers within the ER (20, 21, 29). However, this appears to be independent of their ability to prime cells for ER stress (29). Whether the local expression of Z $\alpha_1$-antitrypsin by airway epithelial cells *in vivo* leads to the formation of protein polymers and to the activation of the NF-κB pathway remains unclear. We report here the detection of NF-κB activation in primary bronchial epithelial cells isolated from patients homozygous for the Z mutation (ZZ) and demonstrate this to be mediated by increased ADAM17-dependent EGFR-MEK-ERK signalling in the absence of either detectable polymer formation or ER stress response. Instead, the activation of the EGFR in this setting represents a loss of M $\alpha_1$-antitrypsin phenotype.

## Results

### Z $\alpha_1$-antitrypsin activates NF-κB in lung epithelial cells

It is well-recognised that overexpression of Z $\alpha_1$-antitrypsin activates the NF-κB response leading to pro-inflammatory cytokine release (20, 21, 30). We therefore asked whether expression of Z $\alpha_1$-antitrypsin regulated by its endogenous promoter in airway epithelial cells could also activate this pathway. Primary bronchial epithelial cells were differentiated into mucin producing, ciliated epithelial cell layers (Figure 1A) and a multiplex ELISA (Meso Scale Discovery®) of apical washings (air exposed) and basal (liquid exposed) conditioned medium for IL-8, IL-6, TNFα, IL-1β, MCP-1 and IP-10 was performed (Figure 1B). This revealed that resting ZZ differentiated primary bronchial epithelial cells secreted more IL-8 basally when compared to MM cells ($p<0.01$). After combined stimulation with oncostatin M (OSM), TNFα and IL-1β (OSM-mix), ZZ differentiated primary bronchial epithelial cells showed significantly higher release of MCP-1 ($p<0.01$), IP-10 ($p<0.05$) and IL-1β ($p<0.01$) compared to MM controls. The reduced secretion of IL-8 most likely reflects the known inhibitory effect of OSM on IL-1β-induced IL-8 release (31). To determine whether this enhanced release of cytokines was mediated by increased NF-κB signalling, submerged cultures of patient-derived ZZ primary bronchial epithelial cells were induced to express increased levels of $\alpha_1$-antitrypsin by treatment with OSM and NF-κB activity was assayed using a luciferase reporter system (Figure 1C) (29). Low levels of basal NF-κB luciferase activity were detected in MM primary bronchial epithelial cells, whereas ZZ cells showed significantly higher activity at baseline ($p<0.05$ compared to MM; Figure 1C). When $\alpha_1$-antitrypsin production was increased by treatment with OSM, the NF-κB activity in ZZ primary bronchial epithelial cells increased significantly ($p<0.001$) and the difference between MM and ZZ cells further increased ($p<0.01$). Stimulation with TNFα, a cytokine known to induce NF-κB activation, showed the same difference between MM and ZZ cells ($p<0.05$). The same effect was seen in Tet-On A549 cells overexpressing either M or Z $\alpha_1$-antitrypsin (Figure S1). To test if the baseline difference in NF-κB activity was related to the transfection of the reporter constructs, we measured transcription


A
4-7 days
14 days
apical
basal
submerged
until confluent
air-exposed
mucociliary
differentiated
B
IL-8 (ng/mg total lysate)
IL-6 (ng/mg total lysate)
TNFα (ng/mg total lysate)
IL-1β (ng/mg total lysate)
MCP-1 (ng/mg total lysate)
IP-10 (ng/mg total lysate)
M
Z
p<0.01
p<0.05
OSM mix
apical
basal
C
NF-κB luciferase activity (corrected for renilla)
OSM
TNFα
D
IL8 mRNA (normalised expression)

<<< **Figure 1. Z $\alpha_1$-antitrypsin expression enhances NF-κB signalling in lung epithelial cells.**

A. Schematic diagram of culturing primary bronchial epithelial cells at an air (apical) - liquid (basal) interface. Once differentiated, the epithelium is a pseudo-stratified cell layer composed of ciliated cells, goblet cells and basal cells. B. Meso scale discovery® of apically and basally secreted cyto- and chemokines (IL-8, IL-6, TNFα, IL-1β, MCP-1 and IP-10). Cells were treated ± oncostatin M (100ng/ml; OSM) + TNFα/IL-1β (both 20 ng/ml; OSM mix) for 48 hours before harvesting apical washes and basal medium (mean, n=6). C. NF-κB luciferase activity of undifferentiated MM and ZZ cells. Submerged cells were cultured for 24 hours, then transfected with luciferase reporters for 6 hours and left 16 hours ± OSM or TNFα (20 ng/ml) as indicated. NF-κB reporter activity is corrected for Renilla (mean, n=3-4). D. Basal *IL8* mRNA expression levels of undifferentiated primary bronchial epithelial cells measured by qPCR (mean, n=4). *p<0.05, **p<0.01, ***p<0.001 versus – with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

of the NF-κB-dependent chemokine IL-8, which confirmed basal levels of inflammatory signalling were higher in ZZ primary bronchial epithelial cells compared with controls ($p<0.05$; Figure 1D).



**Z $\alpha_1$-antitrypsin does not form detectable polymers nor causes ER stress in lung epithelial cells**

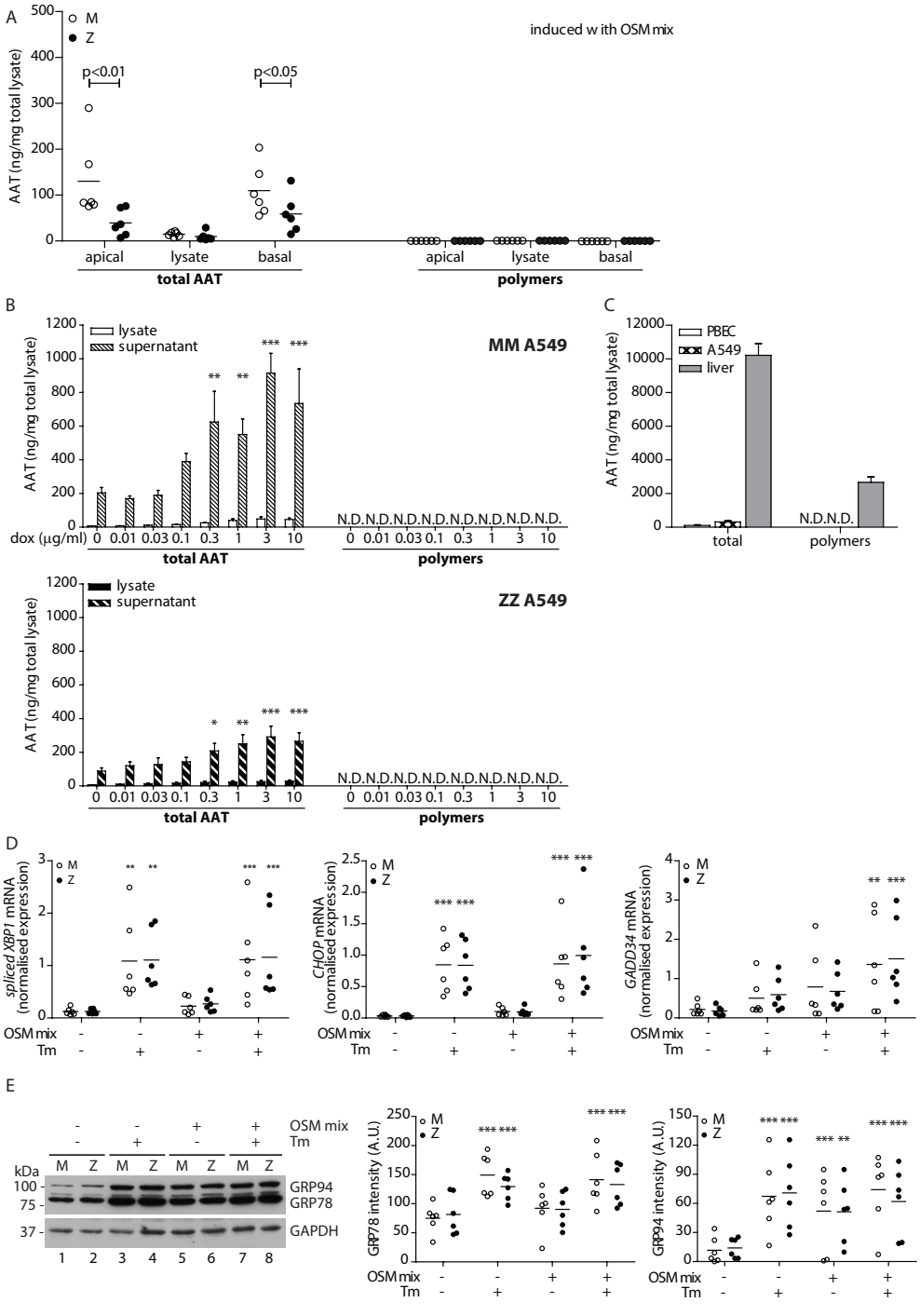
NF-κB activation by mutant serpins has previously been associated with the accumulation of protein polymers within the ER (20-22, 29). This accumulation has also been shown to exaggerate ER stress upon a second hit (20, 22). To verify whether this mechanism was responsible for the enhanced NF-κB signalling in fully differentiated ZZ primary bronchial epithelial cells, we first measured total secreted and intracellular $\alpha_1$-antitrypsin. Resting primary bronchial epithelial cells produced unquantifiable amounts of $\alpha_1$-antitrypsin, but after stimulation with OSM-mix for 48 hours, $\alpha_1$-antitrypsin was detectable in both the apical washes and basal supernatant (Figure 2A). Similar results were obtained after exclusion of the current smokers, indicating that the differences

in smoking status of the patients between MM and ZZ patients from which cells were obtained did not explain the differences in production of $\alpha_1$-antitrypsin (data not shown). Using the 2C1 monoclonal antibody that specifically detects naturally occurring polymers of Z $\alpha_1$-antitrypsin (32), we found no evidence of polymer formation (Figure 2A). Accordingly, we could detect no differences in ER protein mobility (Figure S2), which we have previously shown occurs in cells containing ER luminal polymers of $\alpha_1$-antitrypsin (22). To determine if the absence of polymer formation was a feature of lung epithelial cells, we generated stable transfected A549 lung carcinoma cell lines that conditionally expressed either M or Z $\alpha_1$-antitrypsin under the control of a Tet-On responsive promoter. As expected, M $\alpha_1$-antitrypsin-expressing A549 cells secreted five-times more $\alpha_1$-antitrypsin than did Z $\alpha_1$-antitrypsin-expressing A549 cells (Figure 2B). Again, we were unable to detect protein polymers in either the supernatant or cellular lysates (Figure 2B). Since polymer formation is dependent upon $\alpha_1$-antitrypsin concentration (8), we



**Figure 2. Polymer formation and an (exaggerated) ER stress response are not causing the augmented NF-κB response.** >>>

A. Total $\alpha_1$-antitrypsin (AAT) and $\alpha_1$-antitrypsin polymer production of fully differentiated primary bronchial epithelial cells stimulated with OSM mix for 48 hours. Note lack of polymer signal with the 2C1 antibody (mean, n=6). B. Total $\alpha_1$-antitrypsin and $\alpha_1$-antitrypsin polymer production of the overexpressing Tet-On A549 cells after inducing for 48 hours with doxycycline (dox; mean ± SEM, n=3). C. Total $\alpha_1$-antitrypsin levels produced by ZZ lung epithelial cells compared to ZZ liver homogenate (n=3 from one individual). D. Quantitative RT-PCR of fully differentiated primary bronchial epithelial cells treated ± OSM mix for 48 hours. Four hours before harvesting, cells were stimulated with tunicamycin (Tm; 1 µg/ml). *Spliced XBP1*, *CHOP* and *GADD34* mRNA levels are displayed normalised to the housekeeping genes *RPL13A* and *ATP5B* (mean, n=6). E. Western blot for GRP94 and GRP78 using anti-KDEL antibody. Cells were treated as in D, but stimulated for 16 hours with tunicamycin (mean, n=6). N.D. not detectable. *p<0.05, **p<0.01, ***p<0.001 versus – or 0 with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).


A
induced w ith OSM mix
AAT (ng/mg total lysate)
p<0.01
p<0.05
apical
lysate
basal
total AAT
polymers
B
lysate
supernatant
MM A549
ZZ A549
dox (µg/ml)
N.D.
C
PBEC
A549
liver
total
D
spliced XBP1 mRNA (normalised expression)
CHOP mRNA (normalised expression)
GADD34 mRNA (normalised expression)
OSM mix
Tm
E
kDa
GRP94
GRP78
GAPDH
GRP78 intensity (A.U.)
GRP94 intensity (A.U.)

compared the relative levels of $\alpha_1$-antitrypsin in tissue from an explanted cirrhotic ZZ liver (Figure 2C) with those in cultured airway epithelial cells. This revealed a 100-fold higher level of $\alpha_1$-antitrypsin in hepatic tissue and significant polymer accumulation (Figure 2C). While polymerisation of $\alpha_1$-antitrypsin *in vitro* is highly dependent upon protein concentration, the concentration-dependence of polymerisation within the crowded environment of the endoplasmic reticulum *in vivo* is not known. Therefore, to determine the critical concentration for the polymerisation of Z $\alpha_1$-antitrypsin in living cells, we induced the expression of Z $\alpha_1$-antitrypsin in Tet-On stable CHO stable cells (22) and measured both total $\alpha_1$-antitrypsin and polymer levels (Figure S3A). This revealed that levels of 300 ng $\alpha_1$-antitrypsin per mg total lysate protein are necessary before intracellular polymers can be detected in these cells (Figure S3A). To test this finding in lung epithelial-derived cells, we induced expression of Z $\alpha_1$-antitrypsin in Tet-On A549 cells with doxycycline and augmented the protein level by inhibiting endoplasmic reticulum associated degradation (ERAD) with lactacystin, a selective proteasome inhibitor. This increased the concentration of intracellular Z $\alpha_1$-antitrypsin above 300 ng $\alpha_1$-antitrypsin per mg total lysate, whereupon polymers were detected (Figure S3B). It therefore seems likely that the low levels of $\alpha_1$-antitrypsin produced by airway epithelia are insufficient to generate detectable polymers.



To define whether Z $\alpha_1$-antitrypsin expressed in epithelial cells alters the ER stress response, we induced expression of $\alpha_1$-antitrypsin in fully differentiated primary bronchial epithelial cells with OSM-mix in the presence or absence of the ER stress-inducing toxin tunicamycin. We detected no differences in the basal or OSM-mix-stimulated levels of *spliced XBP1*, *CHOP*, and *GADD34* mRNA between MM and ZZ primary bronchial epithelial cells (Figure 2D). As expected, tunicamycin increased the level of these transcripts; however, there was not an exaggerated ER stress response in ZZ epithelial cells (Figure 2D). Similarly, we were unable to detect differences in ER stress by western blot for the KDEL-positive chaperones, GRP94 and GRP78, in ZZ and MM cells (Figure 2E).

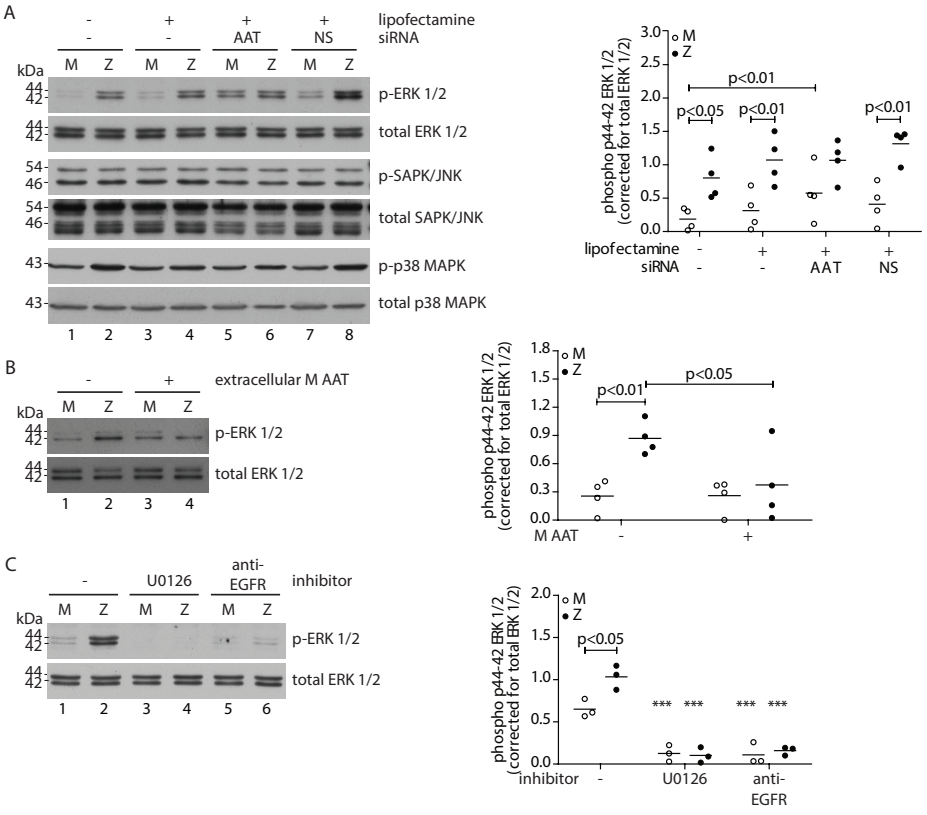





**Figure 3. Increased NF-κB response in ZZ primary bronchial epithelial cells is dependent on the ERK/MEK/EGFR pathway.**

A. Representative western blot of the activation of the MAP kinases ERK1/2, JNK and p38 MAPK of whole cell lysates from undifferentiated primary bronchial epithelial cells knocked-out for $\alpha_1$-antitrypsin (AAT) with siRNA. Cells were cultured overnight, transfected for 24 hours and left 48 hours before harvesting. Neuroserpin (NS) siRNA served as a control. Densitometry of four independent experiments in duplicate (mean, n=4). B. ZZ primary bronchial epithelial cells treated for 24 hours with 1 mg/ml purified plasma M $\alpha_1$-antitrypsin normalised ERK1/2 levels. Densitometry of four independent experiments in duplicate (mean, n=4). C. ZZ primary bronchial epithelial cells treated with 10 μM U0126 (a specific MEK inhibitor) for 8 hours or 2 μg/ml anti-EGFR blocking antibody for 24 hours. Densitometry of three independent experiments in duplicate (mean, n=3). *p<0.05, **p<0.01, ***p<0.001 versus – or 0 with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

**Loss of M $\alpha_1$-antitrypsin leads to increased activation of ERK, which is dependent on MEK and EGFR**

In order to understand the mechanism of inflammatory signalling in ZZ epithelial cells, we next evaluated activation of the NF-κB pathway components IKKβ, IκBα and p65. To evaluate MAPK signalling, we also measured JNK, p38 MAPK and ERK1/2. This revealed a significant difference only in the activation of ERK ($p<0.05$; Figure 3A and S4). Interestingly, depletion of $\alpha_1$-antitrypsin by siRNA caused phosphorylation of ERK1/2 in MM primary bronchial epithelial cells, but did not alter the phosphorylation of ERK1/2 in ZZ cells (Figure 3A). This effect was specific for ablation of $\alpha_1$-antitrypsin, since silencing a nonspecific serpin, neuroserpin (NS), did not increase phosphorylation of ERK1/2 in MM cells. This suggested that it was the lack of M $\alpha_1$-antitrypsin, rather than the presence of Z $\alpha_1$-antitrypsin, that might be responsible for the phosphorylation of ERK1/2 in ZZ primary bronchial epithelial cells. To test this, we treated ZZ primary bronchial epithelial cells with plasma purified M $\alpha_1$-antitrypsin and observed a suppression of ERK1/2 phosphorylation ($p<0.05$; Figure 3B). We wished to determine whether this loss of function phenotype reflected a lack of anti-inflammatory activity in Z $\alpha_1$-antitrypsin or if cells secreted insufficient Z $\alpha_1$-antitrypsin. Since concentration of plasma purified Z $\alpha_1$-antitrypsin to a degree required for this experiment would result in its polymerisation, we instead transiently transfected HeLa cells with either M or Z $\alpha_1$-antitrypsin or empty vector as control. After transfection, the cells produced high levels of $\alpha_1$-antitrypsin, with ZZ cells producing approximately 20% of the amount that MM cells produced (444 ng/mg $\alpha_1$-antitrypsin in the total lysate versus 1995 ng/mg $\alpha_1$-antitrypsin in the total lysate respectively; Figure S5A). Although 14% (33 ng/mg total lysate) of the extracellular Z $\alpha_1$-antitrypsin formed polymers (not shown), the protein was able to inhibit phosphorylation of ERK1/2 to a similar degree as M $\alpha_1$-antitrypsin (Figure S5B). This result is consistent with a model in which ZZ primary bronchial epithelial cells secrete insufficient $\alpha_1$-antitrypsin to inhibit ERK1/2 phosphorylation, rather than Z $\alpha_1$-antitrypsin lacking the anti-inflammatory activity per se.

To determine the mechanism of ERK1/2 phosphorylation in ZZ primary bronchial

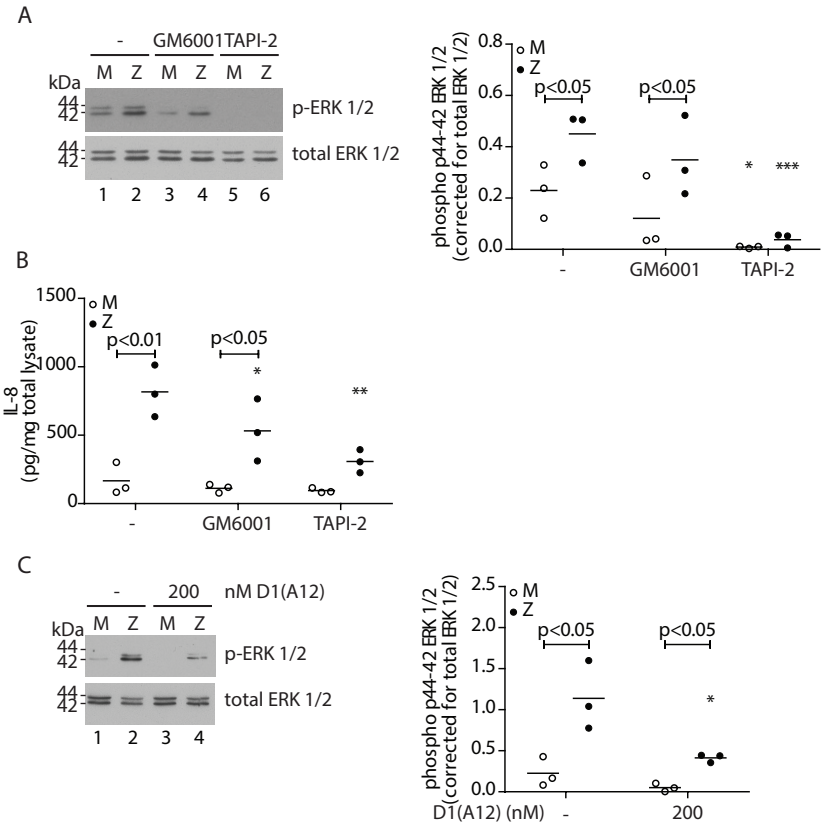
epithelial cells, we treated cells with U0126, a specific inhibitor of MEK, or an anti-EGFR blocking monoclonal antibody. Both reagents abrogated the phosphorylation of ERK indicating that the EGFR-MEK signalling pathway was involved and was activated by the ligand-binding site of EGFR (Figure 3C). Although under some circumstances the activation of ERK1/2 can lead to epithelial cell proliferation, we did not observe differences in the rate of MM and ZZ cell proliferation (Figure S6A). Attempts to assess the effect of ERK1/2 inhibition in each cell type were hampered by toxicity, but it did not appear that modulation of ERK1/2 activation affected proliferation of MM or ZZ cells differentially. Treatment of ZZ cells with purified M $\alpha_1$-antitrypsin did not appear to affect the rate of proliferation (Figure S6B).

**ZZ primary bronchial epithelial cells generate higher levels of ADAM17-dependent EGFR ligands**

We reasoned that this increased EGFR signalling might reflect increased cleavage of membrane-tethered ligands by MMPs and/or ADAMs. Therefore, we incubated MM and ZZ primary bronchial epithelial cells with GM6001, a broad-spectrum MP-inhibitor, or TAPI-2, a broad-spectrum MP inhibitor with enhanced ADAM17 inhibitory activity. TAPI-2 completely blocked the phosphorylation of ERK1/2 in ZZ cells ($p<0.001$), while GM6001 failed to affect phosphorylation (Figure 4A). Furthermore, only treatment with TAPI-2 reduced secretion of IL-8 from ZZ cells ($p<0.01$) (Figure 4B). Since this suggested the involvement of ADAM17, we next tested the effect of a specific ADAM17 blocking antibody, D1(A12) (33). At 200 nM, a concentration known to block the activity of ADAM17 (34), we observed a substantial decrease in phosphorylation of ERK1/2 ($p<0.05$; Figure 4C).



A previous report suggested that M $\alpha_1$-antitrypsin can directly inhibit ADAM17 derived from neutrophils (4), and so we attempted to reproduce this observation. We first performed an *in vitro* ADAM17 activity assay by incubating 1 nM ADAM17 with 0-30 μM purified plasma M $\alpha_1$-antitrypsin, but were unable to detect any inhibition of ADAM17 (Figure 4D). When using 10 μM β-mercaptoethanol in PBS or PBS alone, two potent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<<< **Figure 4. M $\alpha_1$-antitrypsin protects primary bronchial epithelial cells against constitutively activated ERK1/2 in an ADAM17-dependent fashion.**

A. Western blot for phosphorylated ERK1/2 of whole cell lysates from primary bronchial epithelial cells treated with 25 μM GM6001 (MP inhibitor) or 25 μM TAPI-2 (MMP and ADAM17 inhibitor) for 24 hours. Densitometry of three independent experiments in duplicate (mean, n=3). B. IL-8 secretion in supernatant of primary bronchial epithelial cells treated as in A (mean, n=3). C. Treatment with D1(A12), a specific ADAM17 blocking antibody, inhibits phosphorylation of ERK1/2 in ZZ cells after 6 hours. (–) represents a control IgG. Densitometry of three independent experiments in duplicate (mean, n=3). D. ADAM17 (1 nM) was incubated with 7.5 or 30 μM plasma M $\alpha_1$-antitrypsin. ADAM17 activity was assayed against a fluorogenic substrate. β-mercaptoethanol (10 μM) in PBS was used as a positive control for inhibition. (–) represents baseline ADAM17 activity against the fluorogenic substrate. E. Silver stain of a Bis-Tris non-reducing PAGE revealed no binding of ADAM17 to plasma M $\alpha_1$-antitrypsin incubated in a 1:1 molar ratio for 1 hour at 37°C. *p<0.05, **p<0.01, ***p<0.001 versus – with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).



ADAM17 inhibitors, we were able to inhibit its activity, confirming the functionality of our assay (Figure 4D). Moreover, we were unable to detect the formation of a complex between ADAM17 and either form of $\alpha_1$-antitrypsin (Figure 4E). Although it remains controversial as to whether cytoplasmic phosphorylation of ADAM17 plays an important regulatory role (35), we also tested for phosphorylation of ADAM17 in MM and ZZ cells grown with or without supplementary M $\alpha_1$-antitrypsin (Figure S7). We detected no differences between these conditions.

To explore this pathway further, we next measured whether ZZ primary bronchial epithelial cells generated more EGFR ligands than controls. We detected significantly higher levels of mRNA encoding HB-EGF and TGFα in ZZ cells compared with MM controls ($p<0.05$ and $p=0.05$; Figure 5A). When the EGFR was blocked using a monoclonal antibody to prevent ligand binding to its receptor, significantly more release of TGFα and AREG was

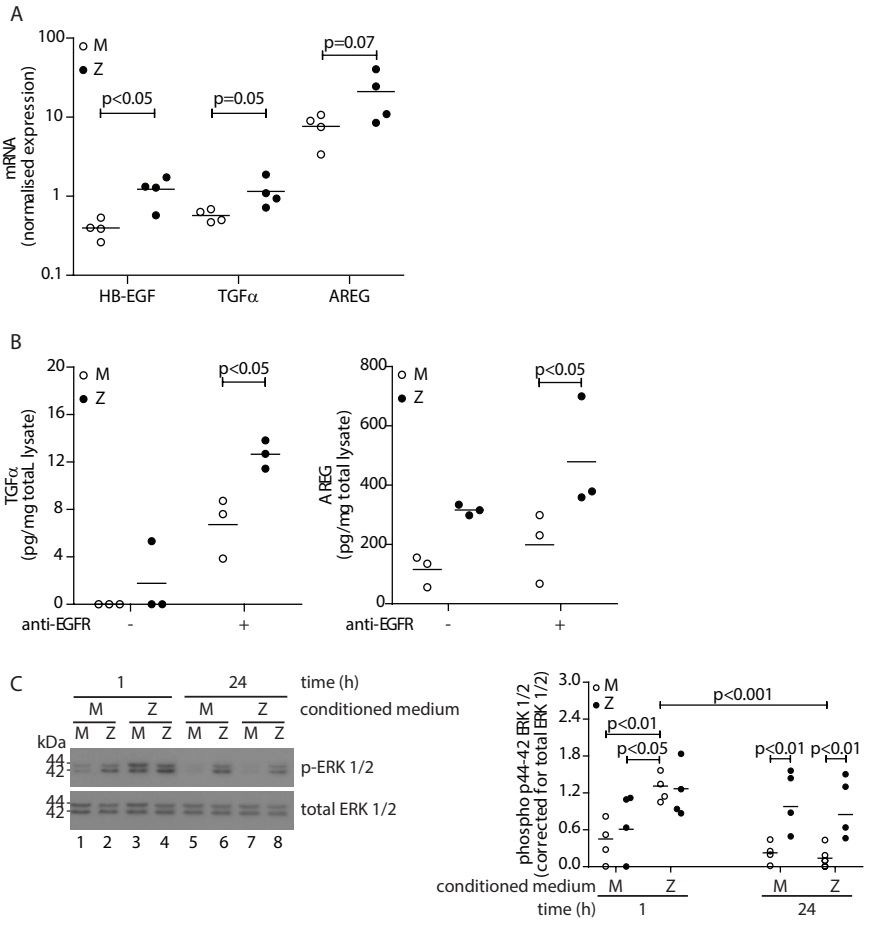




**Figure 5. EGFR ligands are enriched in ZZ primary bronchial epithelial cells.**

A. Basal expression levels of the EGFR ligands HB-EGF, TGFα and amphiregulin (AREG) in undifferentiated primary bronchial epithelial cells, measured by qPCR. EGF was not quantifiable (mean, n=4). B. Increased TGFα and AREG in the cell supernatant of ZZ primary bronchial epithelial cells after blockade of the EGFR, quantified by ELISA. HB-EGF was undetectable (mean, n=3). C. Conditioned medium from ZZ primary bronchial epithelial cell culture given to MM primary bronchial epithelial cells and vice versa. Phosphorylation of ERK1/2 was measured 1 and 24 hours after media exchange. Densitometry of four independent experiments in duplicate (mean, n=4). *p<0.05, **p<0.01, ***p<0.001 versus – with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

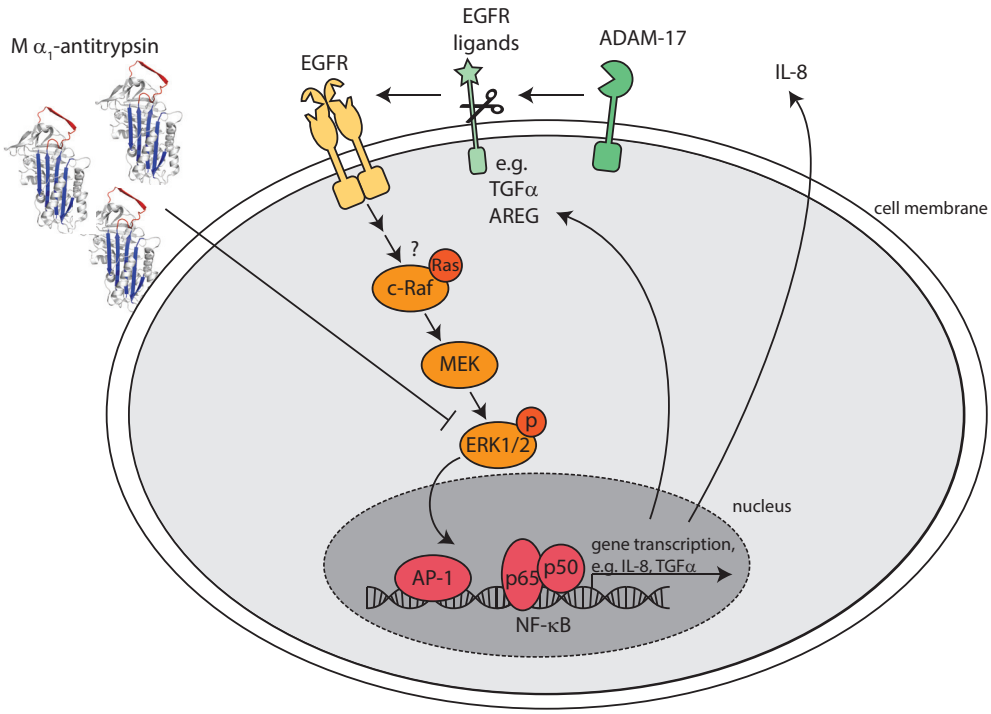




**Figure 6. ZZ primary bronchial epithelial cells show an enhanced inflammatory response dependent of the ERK/EGFR/ADAM17 pathway.**

Impaired expression of $\alpha_1$-antitrypsin in primary bronchial epithelial cells leads to increased phosphorylation of ERK1/2, which is dependent on MEK, EGFR and ADAM17. M $\alpha_1$-antitrypsin modulates this inflammatory response via a yet undefined mechanism.

detected in ZZ cells compared with MM cells ($p<0.05$; Figure 5B). Consistent with these observations, when we treated MM primary bronchial epithelial cells with conditioned medium derived from ZZ cultures, we observed a significant increase in phosphorylation of ERK1/2 after 1 hour ($p<0.01$), which returned to baseline after 24 hours ($p<0.001$; Figure 5C).

Taken together, these data indicate that the increased NF-κB signalling in ZZ primary bronchial epithelial cells is caused by phosphorylation of ERK1/2. This is due

to increased availability of ADAM17-dependent EGFR ligands leading to activation of the EGFR and signalling via MEK (Figure 6). Surprisingly, we were unable to detect the formation of polymers of $\alpha_1$-antitrypsin in ZZ primary bronchial epithelial cells or A549 lung adenocarcinoma cells overexpressing the protein, which may reflect the low levels of $\alpha_1$-antitrypsin expression of which these cells are capable.

## Discussion

For many years, it was thought that an imbalance between protease and antiprotease activity was solely responsible for the accelerated onset of emphysema in patients homozygous for the Z allele of $\alpha_1$-antitrypsin (reviewed in (36)). However, $\alpha_1$-antitrypsin has been found to possess additional roles to its antiprotease activity, including a range of anti-inflammatory properties (3-5), and the Z alleles can increase inflammatory NF-κB signalling when overexpressed pulmonary epithelial cells (20, 21, 30). The results of our current study using primary bronchial epithelial cells in which $\alpha_1$-antitrypsin is expressed under the control of its endogenous promoter, confirm and extend these findings. We found that at the low levels of $\alpha_1$-antitrypsin expression that occur in these cells, clinically relevant polymer formation is unlikely to occur. Sufficient $\alpha_1$-antitrypsin is generated by wild-type cells to suppress ERK1/2 and NF-κB signalling, but this anti-inflammatory effect appears to be lost in ZZ epithelia, suggesting a novel mechanism for airway pathology in $\alpha_1$-antitrypsin deficiency. Surprisingly, while ADAM17 is required to generate secreted EGFR ligands mediating inflammatory signals in these ZZ cells, we were unable to detect a direct inhibition of ADAM17 by M $\alpha_1$-antitrypsin.



Many heterologous overexpression systems have reported the presence of Z $\alpha_1$-antitrypsin polymers both intracellular and in conditioned medium (20, 21). Unexpectedly, but importantly, we were unable to detect 2C1-positive polymers in primary bronchial epithelial cultures. Although the 2C1 monoclonal antibody is highly specific for polymers, it is formally possible that it is less sensitive than other anti-polymer antibodies, for example ATZ11. However, in our hands both have similar avidity towards Z polymers, but the polyclonal antibody ATZ11 is less specific for polymers, detecting both Z monomers as well as Z polymers (32). Even if very low levels of Z $\alpha_1$-antitrypsin polymer is made within bronchial epithelial cells, it is unlikely to affect cellular function as we were unable to detect impaired ER protein mobility nor altered ER stress responsiveness as we have done previously for polymer-expressing cells (22).

We were unable to identify all components required for ERK1/2 activation in ZZ primary bronchial epithelial cells. It is likely that there exists a significant level of

redundancy in this system as we were able to detect multiple EGFR ligands to be increased in the conditioned medium of ZZ cultures. In our system, $\alpha_1$-antitrypsin did not directly inhibit ADAM17 as has been reported for neutrophils (4). Interestingly, it has recently been speculated that endocytosed $\alpha_1$-antitrypsin may modulate ADAM17 in endothelial cells (5). If intracellular $\alpha_1$-antitrypsin can indeed modulate these pathways, multiple potential mechanisms may explain this effect. ADAM-dependent transactivation of the EGFR by activation of G-protein coupled receptors can also occur (reviewed in (27)). For example, IL-8 can induce EGFR phosphorylation in epithelial cells via its receptors CXCR1 and CXCR2 (28, 37), while binding of $\alpha_1$-antitrypsin to IL-8 has been reported to prevent activation of CXCR1 (4). Although there are contradictory reports concerning the expression of CXCR1 and CXCR2 on bronchial epithelial cells (38, 39), we found that IL-8 release is increased in ZZ primary bronchial epithelial cells, which potentially might provide an autocrine inflammatory signal in the absence of sufficient $\alpha_1$-antitrypsin. Alternatively, CCL20 (also known as MIP-3α) and its receptor CCR6 have been shown to activate ADAM17 causing transactivation of EGFR (40, 41).



Taken together, our experiments have demonstrated that airway epithelial cells have anti-inflammatory activity due to the local synthesis of M $\alpha_1$-antitrypsin from its endogenous promoter and that this effect is lacking in ZZ homozygous epithelial cells because of a lack of $\alpha_1$-antitrypsin secretion. This raises the possibility that $\alpha_1$-antitrypsin augmentation may have unanticipated effects within the airway. It also raises the potential that targeted anti-EGFR therapy might have anti-inflammatory effects within the lung.

## Materials and methods

### Reagents and antibodies

OSM (100 ng/ml) was purchased from R&D systems (R&D systems, Minneapolis, MN, USA), and TNFα and IL-1β (both 10 ng/ml) from Peprotech (Peprotech, Rocky Hill, NJ, USA). Tunicamycin (1 µg/ml) was bought from Sigma (Sigma-Aldrich, St.Louis, MO, USA), U0126 (10 nM) from Promega (Promega, Madison, WI, USA), monoclonal (Ab-3) anti-EGFR antibody (2 µg/ml), GM6001 (25 µM) and TAPI-2 (25 µM) all from Calbiochem (Calbiochem, Darmstadt, Germany) and D1(A12) (200 nM) (33). Purified plasma M $\alpha_1$-antitrypsin (1 mg/ml) was derived as described previously (42). All antibodies for immunoblotting were from Cell Signaling (Cell Signaling Technology, Danvers, MA, USA), except secondary HRP-labeled antibodies (Sigma) and GRP94 and GRP78 was detected using an anti-KDEL monoclonal antibody (StressGen).

### Cell cultures



Primary bronchial epithelial cells were cultured submerged in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and bronchial epithelial growth medium (BEGM; Clonectics, San Diego, CA, USA) with BEGM SingleQuot supplements and growth factors (Clonetics), 1.5 µg/ml bovine serum albumin (BSA; Sigma-Aldrich), 1 mM HEPES (Invitrogen, Life Technologies, Breda, the Netherlands) and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma), shortened as full medium, at 37°C, 5% $CO_2$ (43). Starvation medium consist of full medium except BSA and the SingleQuot supplements EGF and BPE.

We carried out siRNA-mediated knockdown of $\alpha_1$-antitrypsin using a SMARTpool of ONTARGETplus $\alpha_1$-antitrypsin siRNA (Dharmacon, Lafayette, CO, USA) or SERPIN1 siRNA (Dharmacon) as a mock control. In general, 10 nM siRNA and 1 µl RNAiMAX (Invitrogen) was used according to manufacturer's descriptions and $\alpha_1$-antitrypsin expression, measured with qPCR and ELISA, was knocked down >90% after 72 hours by this method (Figure S8).

Primary bronchial epithelial cells were differentiated as described previously (44). Briefly, cells were cultured submerged until confluence on semi-permeable Transwell membranes (Corning Costar, Cambridge, MA, USA) in B/D medium with addition of retinoic acid (end-concentration 15 ng/ml; Sigma), and subsequently cultured air-exposed for 14 days to allow mucociliary differentiation.

A549 cells were obtained from American Type Culture Collection (ATCC) and stably transfected with the pTetON vector (Clontech) to obtain Tet-On A549 cells. ELISA confirmed that production of endogenous $\alpha_1$-antitrypsin protein was below the level of detection in these cells (data not shown). These were stably transfected with pTRE2-hyg plasmid encoding M or Z $\alpha_1$-antitrypsin as described previously (42). Cells were maintained in DMEM with 10% (v/v) Tetracycline-free Fetal Bovine Serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml geneticin and 400 µg/ml hygromycin B (selective antibiotics from Invitrogen) at 37°C, 5% $CO_2$. Expression of $\alpha_1$-antitrypsin was usually induced using 2 µg/ml doxycycline (Sigma) for 48 hours. HeLa cells were obtained from ATCC and transient transfected with pcDNA3.1 encoding M or Z $\alpha_1$-antitrypsin (or empty vector) as shown previously (19).



**Patient groups**

PiZZ $\alpha_1$-antitrypsin deficient patients were recruited in the Leiden University Medical Center (LUMC; Leiden, the Netherlands). ZZ primary bronchial epithelial cells were acquired by bronchial biopsy, with approval from the Medical Ethical Committee of the LUMC. Briefly, bronchial biopsies were washed with PBS, divided in 2mm pieces and placed into a fibronectin/collagen-coated 24-well plate. Twice daily, the explants were fed with 20 µl B/D medium until they became adherent (maximum of three days). Then, primary bronchial epithelial cells were expanded submerged with 500 µl B/D medium, replaced triweekly. When confluent, cells were frozen down in liquid nitrogen until further use. MM primary bronchial epithelial cells were obtained from tumour-free resected lung tissue as described previously (43) and matched to ZZ primary bronchial epithelial cells according to sex, GOLD-stage (0-III) and smoking status (non-, ex-, or current smoker)

(Table S1). In each case, care was taken to ensure that cells were sourced only from the 2nd-3rd branches of the bronchial tree in order that results from each were directly comparable.

**Measurement of total $\alpha_1$-antitrypsin, polymerised $\alpha_1$-antitrypsin and cytokine release**

Total and polymerised $\alpha_1$-antitrypsin were measured in whole cell lysate (intracellular) and supernatant (secreted) by ELISA as described previously (32). Cytokine release of fully differentiated cells was measured using a 4-plex Meso Scale Discovery (MSD) kit (IL-6, IL-8, TNFα and IL-1β) and singleplex kits (MCP-1 and IP-10; Meso Scale Discovery, Rockville, MD, USA). EGFR-ligands were measured using commercial available ELISA's following manufacturer's protocol (R&D systems). IL-8 release in supernatant of submerged primary bronchial epithelial cultures was quantified using an IL-8 ELISA kit (Sanquin, Amsterdam, the Netherlands).

**Protein mobility assay**



Submerged cultured primary bronchial epithelial cells were grown overnight on coated 35mm glass bottom petri dishes (MatTek Corporation, Ashland, MA, USA) and transiently transfected with an ER-GFP plasmid (45). Live cells were imaged on an LSM510 confocal microscope (DuoScan; Carl Zeiss Inc., Thornwood, NY, USA) at 37°C as defined in (22). Briefly, ER-GFP was visualised with a x63/1.4NA oil objective at 488nm laser and fluorescence recovery after photobleaching (FRAP) experiments were performed. Fluorescence recovery curves were obtained by transforming fluorescence intensities into a percentage scale in which the pre-bleach time point represents 100% of fluorescence intensity.

**Luciferase activity assays**

Transfection with luciferase-reporter plasmids was typically performed in 6-well plates with 1 µg of either p(5x)ATF6-luc (Firefly) or pELAM1-luc (Firefly) and 50 ng of pRL-TK (Renilla) as a transfection efficacy control (29). Cells were transfected for 6 hours with 2 µl Lipofectamine LTX (Invitrogen) in serum- and antibiotic free OptiMEM according to

manufacturer's instructions and lysed the next day using the recommended protocol of the Dual-Luciferase Reporter Assay (Promega).

**Western blotting**

Cells were lysed in 50 µl buffer H (10 mM HEPES, pH 7.9, 50 mM NaCl, 500 mM sucrose, 0.1 mM EDTA and 0.5%, (v/v) Triton X-100, 1 mM PMSF, 1X Complete™ protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) supplemented with phosphatase inhibitors (10 mM tetrasodium pyrophosphate, 17.5 mM β-glycerophosphate, and 100 mM NaF; (46, 47)). Samples were run on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. After blocking with PBS/0.05% Tween-20 (v/v)/5% skim-milk (w/v), the membrane was incubated with the primary antibody (1:1000) in TBS/0.05% Tween-20 (v/v)/5% BSA (w/v) overnight at 4°C. The HRP-labeled antibody was incubated for 1 hour in blocking buffer and developed with ECL (ThermoScientific).



**Quantitative RT-PCR**

Total RNA was isolated and normalised mRNA levels were calculated using *RPL13A* and *ATP5B* as housekeeping genes (48). Primers used are described in Table S2. IQ SYBRGreen supermix (Bio-rad , Herculus, CA, USA) was used for amplification of the cDNA.

**ADAM17 activity and binding assay**

ADAM17 activity assay was performed as descried earlier (49). In brief, 1 nM purified recombinant ADAM17 was incubated with or without 0-30 µM purified plasma M $\alpha_1$-antitrypsin and assayed for ADAM17 activity using the fluorogenic substrate MOCAc-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-$NH_2$ (R&D systems). Purified plasma M $\alpha_1$-antitrypsin (0.5 µg) was incubated for 1 hour at 37°C in a 1:1 molar ratio with ADAM17, ran on a Native Page Bis Tris (3 – 12% w/v; Invitrogen) gel and visualised by Silver stain. Both ADAM17 and purified plasma M $\alpha_1$-antitrypsin were incubated in 50mM Tris-HCl (pH 7.4), 100mM NaCl, 10 mM CaCl.

**Statistical analysis**

Results from primary bronchial epithelial cells are expressed as single patients (each dot is the average of one patient in duplicate). Results from Tet-On A549 are shown as mean ± SEM. Data were analysed using two-way repeated-measures analysis of variance (ANOVA) or Student t-test as appropriate. Differences with p-values < 0.05 were considered to be statistically significant.

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## Supplementary data

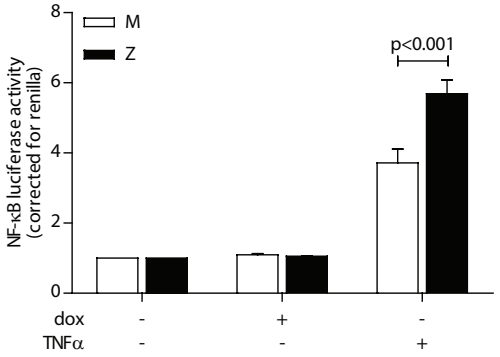



**Figure S1. Tet-On A549 cells overexpressing Z $\alpha_1$-antitrypsin show enhanced NF-κB signalling upon an inflammatory stimulus.**

Cells were treated as in Figure 1C, only cells were induced with doxycycline (dox) instead of OSM (mean ± SEM, n=3).



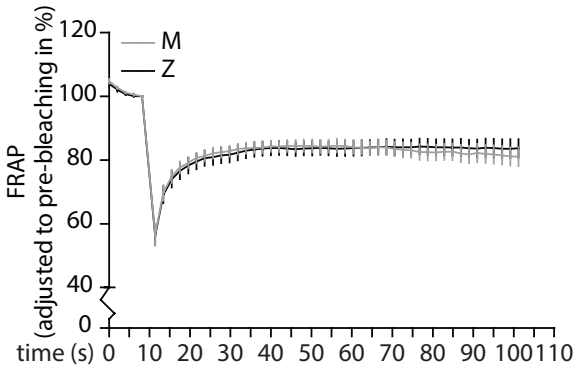



**Figure S2. Z $\alpha_1$-antitrypsin does not alter ER protein mobility.**

Primary bronchial epithelial cells were transiently transfected with ER-GFP and fluorescence recovery was measured by fluorescence recovery after photo-bleaching (FRAP). Recovery is displayed relative to pre-bleach fluorescence intensity (mean ± SEM, n=9-10).

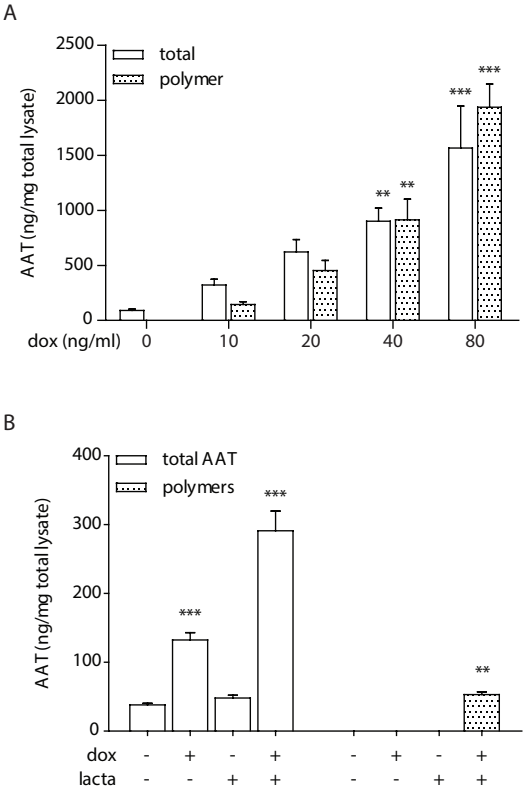



**Figure S3. Critical concentration for polymer formation within cells.**

A. Dose-response doxycycline on a CHO stable cell line overexpressing Z $\alpha_1$-antitrypsin under the control of a Tet-On responsive promotor. Intracellular total and polymeric Z $\alpha_1$-antitrypsin levels are measured by ELISA. B. Z $\alpha_1$-antitrypsin-expressing A549 cells induced with doxycycline and stimulated in the presence of lactacystin. Intracellular total and polymeric Z $\alpha_1$-antitrypsin levels are measured by ELISA.

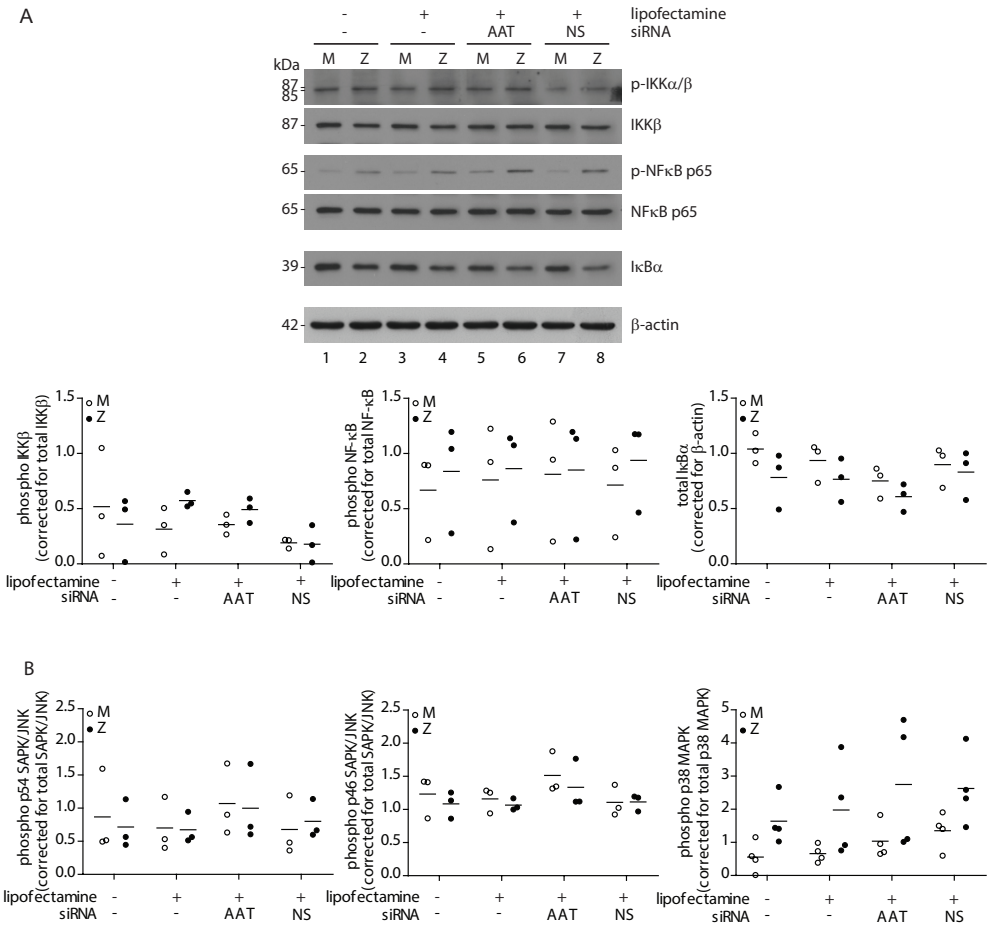
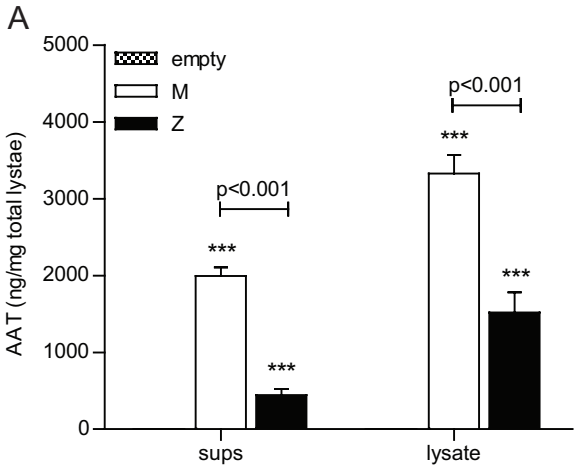





**Figure S4. Increased NF-κB response in ZZ primary bronchial epithelial cells is caused by increased phosphorylated ERK.**

A. Representative western blots of the activation of the NF-κB proteins IKKβ and NF-κB p65 and degradation of total IκBα of whole cell lysates from undifferentiated primary bronchial epithelial cells knocked-down for $\alpha_1$-antitrypsin by siRNA (see Figure 3A). Densitometry was done on four independent experiments in duplicate (mean, n=3). B. Densitometry of the Western blots shown in Figure 3A (mean, n=3-4).



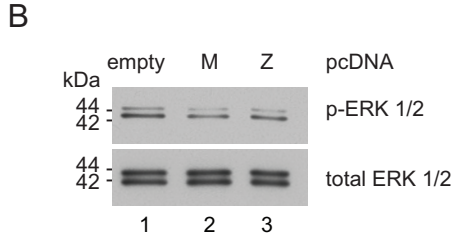






**Figure S5. Overexpression of Z $\alpha_1$-antitrypsin downregulates ERK1/2 phosphorylation.**

A. Alpha$_1$-antitrypsin (AAT) production after transfection of HeLa cells with pcDNA3.1 containing M or Z $\alpha_1$-antitrypsin (or empty vector as control). B. Representative western blot of phospho ERK1/2 in HeLa cells after transfection with M or Z $\alpha_1$-antitrypsin. High amounts of Z $\alpha_1$-antitrypsin are able to inhibit phospho ERK1/2 at the same rate as M $\alpha_1$-antitrypsin does.

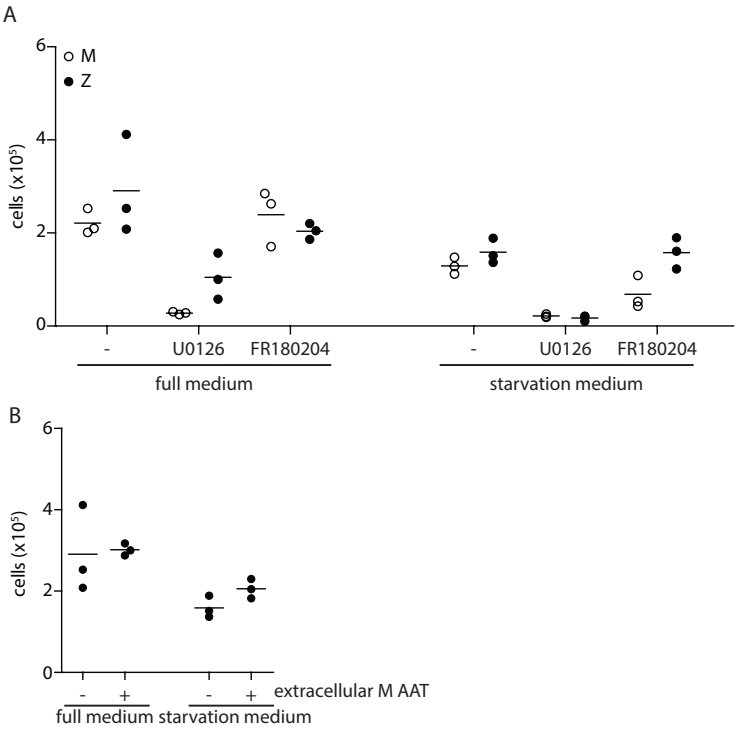





**Figure S6. Increased ERK1/2 phosphorylation in ZZ cells does not enhance proliferation.**

A. Cells were seeded at $3{\cdot}10^4$ cells/well and cultured for 48 hours as indicated. Starvation medium is full medium except BSA and the SingleQuot supplements EGF and BPE to minimise the effects of exogenous growth factors. FR180204, a specific ERK inhibitor did not change proliferation rates. U0126, a specific MEK inhibitor, was toxic to both MM and ZZ cells. B. ZZ cells were seeded at $3{\cdot}10^4$ cells/well and cultured for 48 hours in the presence of 1 mg/ml M $\alpha_1$-antitrypsin.

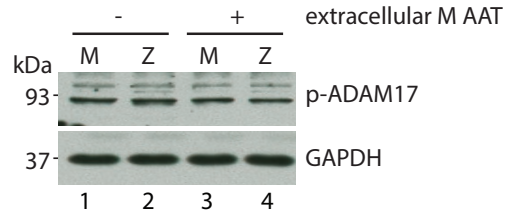



**Figure S7. ZZ cells do not display increased phosphorylation of ADAM17.**

ZZ primary bronchial epithelial cells were treated for 24 hours with 1 mg/ml purified plasma M $\alpha_1$-antitrypsin. Representative western blot of phosphorylated ADAM17 (Thr735) (n=3).

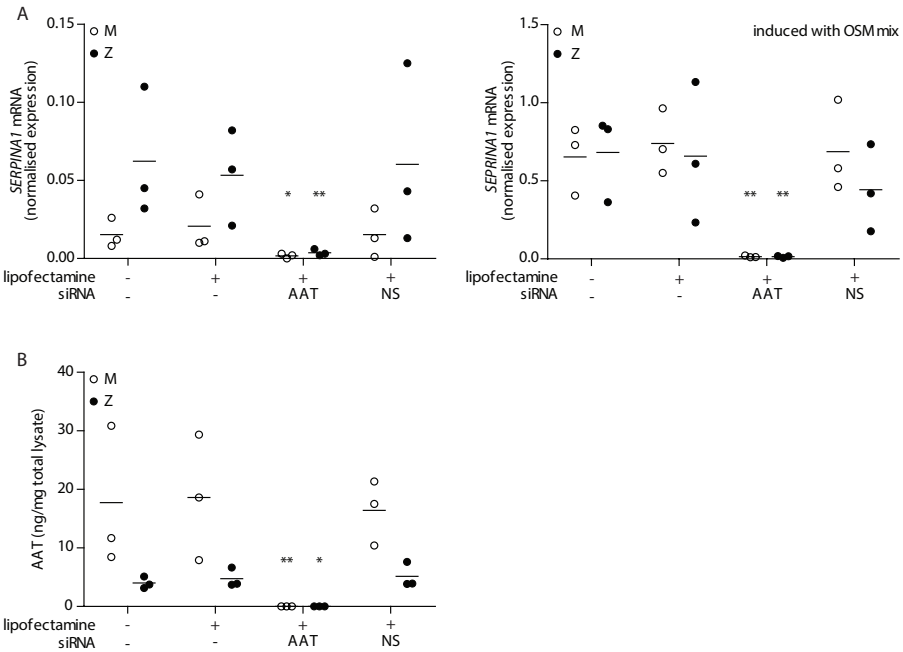



**Figure S8. Confirmation of $\alpha_1$-antitrypsin knock-down by siRNA in primary bronchial epithelial cells.**

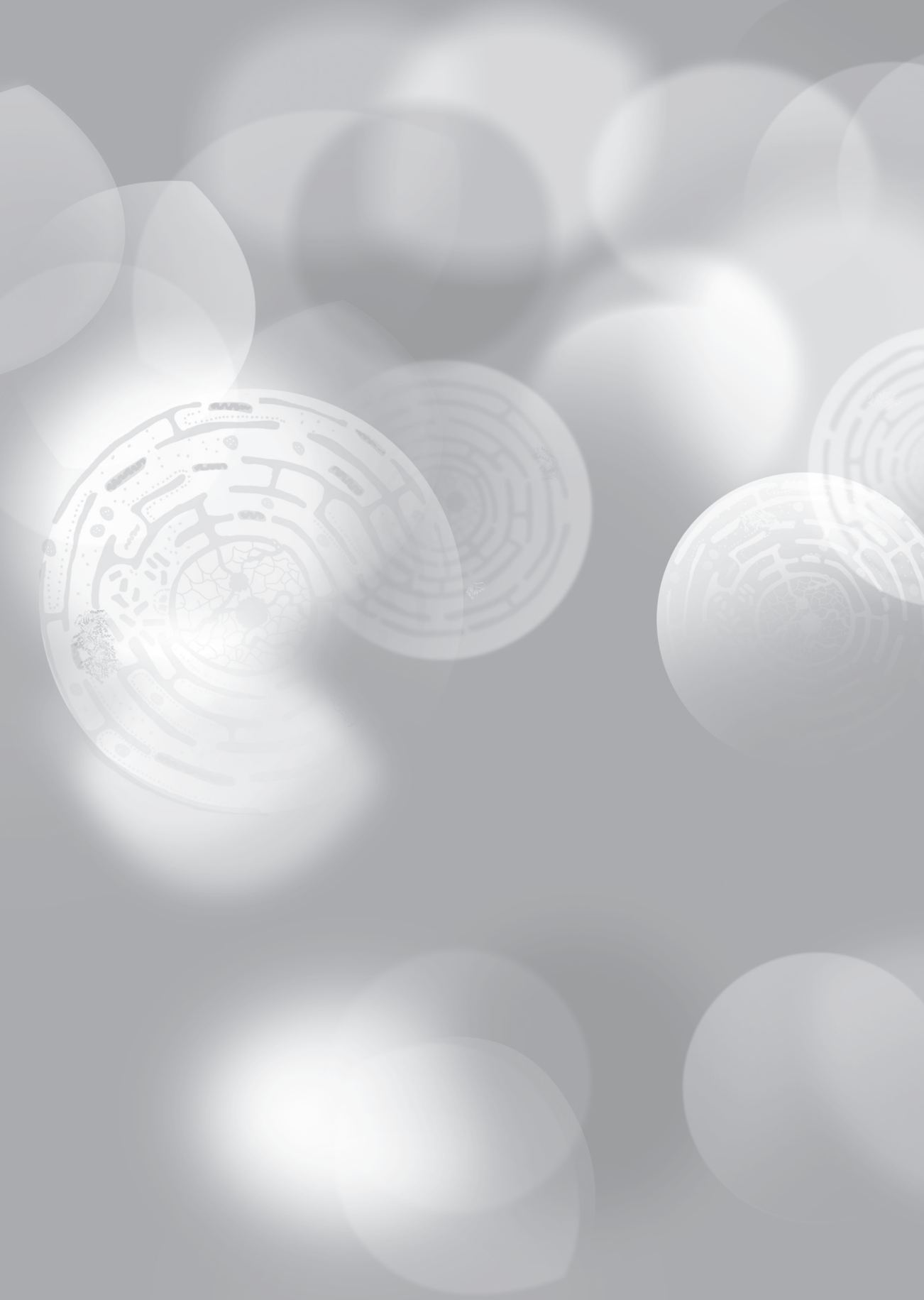
A. *SERPINA1* mRNA knock-down was >90% efficient (left panel) and after $\alpha_1$-antitrypsin up-regulation with OSM mix >98% efficient (right panel). Neuroserpin (NS) siRNA served as a control (mean, n=3). B. $\alpha_1$-antitrypsin was undetectable in cell supernatant after knock-down of $\alpha_1$-antitrypsin, measured by ELISA (mean, n=3).

Table S1. Patient characteristics.

| | PiMM controls | PiZZ patients |
|---|---|---|
| age (mean, range) | 61 (51-83) | 52 (43-57) |
| sex (M/F) | 3/3 | 3/3 |
| GOLD-stage (0/I/II/III) | 2/2/1/1 | 3/1/1/1 |
| smoking status (current/never/ex) | 2/0/4 | 0/1/5 |

Table S2. qPCR primers.

| Name | Forward primer / Reverse primer | Melting temp. (°C) | Ref. |
|---|---|---|---|
| *AREG* | 5′ GGTGGTGCTGTCGCTCTTG 3′<br>5′ AGGTGTCATTGAGGTCCAATCC 3′ | 62 | - |
| *CHOP* | 5′ GCACCTCCCAGAGCCCTCACTCTCC 3′<br>5′ GTCTACTCCAAGCCTTCCCCCTGCG 3′ | 62 | (48) |
| *EGF* | 5′TGCAGAGGGATACGCCCTAA 3′<br>5′ CAAGAGTACAGCCATGATTCCAAA 3′ | 62 | - |
| *GADD34* | 5′ ATGTATGGTGAGCGAGAGGC 3′<br>5′ GCAGTGTCCTTATCAGAAGGC 3′ | 62 | (50) |
| *HB-EGF* | 5′TGGACCTTTTGAGAGTCACTT TATCC 3′<br>5′ CGTGCTCCTCCTTGTTTGGT 3′ | 62 | - |
| *IL8* | 5′ CTGGACCCCAAGGAAAAC 3′<br>5′TGGCAACCCTACAACAGAC 3′ | 60 | - |
| *SERPINA1* | 5′ AAGGCAAATGGGAGAGACCC 3′<br>5′ AAGAAGATGGCGGTGGCAT 3 | 60 | (51) |
| *TGFα* | 5′ AGGTCCGAAAACACTGTGAGT 3′<br>5′ AGCAAGCGGTTCTTCCCTTC 3′ | 62 | - |
| *XBP1spl* | 5′ TGCTGAGTCCGCAGCAGGTG 3′<br>5′ GCTGGCAGGCTCTGGGGAAG 3′ | 62 | (48) |



# Chapter 6

## Alpha$_1$-antitrypsin production by pro- and anti-inflammatory macrophages and dendritic cells

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## Abstract

**Alpha$_1$-antitrypsin acts as an important neutrophil elastase inhibitor in the lung. Although the hepatocyte is considered as the primary source of α$_1$-antitrypsin, local production by monocytes, macrophages and epithelial cells may contribute to the formation of an anti-elastase screen. Since monocytes can differentiate into a heterogeneous population of macrophages with subpopulations ranging from pro-inflammatory properties (mφ-1) to anti-inflammatory properties (mφ-2) and into dendritic cells (DC), we studied whether lipopolysaccharide (LPS), tumor necrosis factor alpha (TNFα) and oncostatin M enhance α$_1$-antitrypsin production differentially in cultured mφ-1, mφ-2 and DC. Monocytes from healthy blood donors were cultured for 7 days in the presence of GM-CSF, M-CSF, or GM-CSF and IL-4 to obtain mφ-1, mφ-2 and immature(i)DC, respectively. Next, cells were stimulated with LPS, TNFα or oncostatin M and synthesis of α$_1$-antitrypsin was assessed by quantitative RT-PCR, immunocytochemistry and ELISA. Spontaneous release of α$_1$-antitrypsin was higher in mφ-1 than in mφ-2 and iDC and only LPS significantly increased α$_1$-antitrypsin production in mφ-1, mφ-2 and DC, whereas TNFα and oncostatin M did not affect α$_1$-antitrypsin secretion. The secretion levels of the related protease inhibitors α$_1$-antichymotrypsin and secretory leucocyte proteinase inhibitor (SLPI) were below the limits of detection by ELISA. In contrast to the protein data, analysis by quantitative RT-PCR showed that 24 hours LPS exposure caused a maximal 2.1-fold *SERPINA1* mRNA increase in mφ-1, a 21-fold increase in mφ-2 and 11-fold increase in DC. These data suggest that cellular differentiation is a regulator of local α$_1$-antitrypsin production.**

## Introduction

Alpha$_1$-antitrypsin, a member of the serine protease inhibitor (SERPIN) superfamily, is not only a major inhibitor of the neutrophil-derived serine proteases neutrophil elastase, cathepsin G and proteinase 3, but also complexes with trypsin, chymotrypsin and thrombin. In inflammatory lung disease, one of the most important inhibitory functions of $\alpha_1$-antitrypsin is the irreversible binding and inactivation of neutrophil elastase, thereby protecting lung tissue against the destructive effects of neutrophil elastase released by degranulating neutrophils during inflammation (1). Alpha$_1$-antitrypsin deficiency is the major identified genetic risk factor for chronic obstructive pulmonary disease (COPD) and is characterised by early-onset pulmonary emphysema, which is partially explained by a protease-antiprotease imbalance (reviewed by Stockley (2)). In addition, many other airway diseases, including bronchiectasis, cystic fibrosis and certain phenotypes of asthma, are associated with neutrophilic inflammation and the number of neutrophils present in the lung is correlated to the disease severity (3-5). Therefore, neutrophil elastase and $\alpha_1$-antitrypsin are also implicated in the pathogenesis of these diseases.

Alpha$_1$-antitrypsin is primarily synthesised in hepatocytes and its secretion is enhanced during an inflammatory response. This increase is mainly mediated by pro-inflammatory cytokines like interleukin (IL)-6, IL-1β and tumor necrosis factor alpha (TNFα) (6). Members of the IL-6 family, including oncostatin M, have also been shown to induce $\alpha_1$-antitrypsin secretion (7).



While hepatocytes are considered as the primary source of $\alpha_1$-antitrypsin, human lung epithelial cells, monocytes and alveolar macrophages have also been shown to produce $\alpha_1$-antitrypsin (7-10). Although these cells produce substantially lower amounts of $\alpha_1$-antitrypsin compared to hepatocytes, its production is also augmented by cytokines such as IL-6, oncostatin M, TNFα and IL-1β, and thereby these cells may contribute to the formation of an anti-elastase shield in the lung during inflammation. In addition, monocytes showed an increase in $\alpha_1$-antitrypsin secretion after lipopolysaccharide (LPS) exposure, indicating the importance of the regulation of the anti-protease screen as a defense during infection, particularly in the microenvironment of lung inflammatory cells

(9, 11).

Macrophages constitute a heterogeneous population with subpopulations displaying pro-inflammatory properties and those with repair-inducing and anti-inflammatory properties (reviewed by Gordon and Taylor (12)). Previous studies have already shown the heterogeneity of these different subsets *in vivo* (reviewed by Mosser and Edwards (13)). *In vitro* studies has shown that macrophages can be polarized into type I (mφ-1) or type II (mφ-2) macrophages in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF), respectively (14). Pro-inflammatory mφ-1, or classically activated macrophages, are characterised by production of pro-inflammatory cytokines like IL-6, IL-12p40 and IL-23p40 and promotion of T-helper 1 response (14). In contrast, anti-inflammatory mφ-2, or so-called alternatively activated macrophages, are characterised by production of IL-10 in the absence of pro-inflammatory cytokines, promotion of T regulatory responses and ingestion of early apoptotic cells (15, 16). Alveolar macrophages have been shown to be immunosuppressive with poor antigen-presenting capacities (17, 18), and thus display characteristics of mφ-2. However, recent studies have suggested a role for (chronic) inflammation causing phenotype switching of alveolar macrophages and monocyte-derived macrophages *in vivo* and *in vitro* (19-21).



Monocytes and macrophages play a key role in the early defense in the lung and, more importantly, these cell types are involved in the pathogenesis of COPD (20). Furthermore, dendritic cells (DC), the third *in vitro* monocyte-derived cell type, may also contribute to the formation of the anti-elastase screen in the lung. However, the regulation of $\alpha_1$-antitrypsin production by mφ-1, mφ-2 and DC has not yet been studied. Therefore, we hypothesised that LPS and pro-inflammatory cytokines like TNFα and oncostatin M can induce $\alpha_1$-antitrypsin production by mφ-1, mφ-2 and DC. To this end, we used monocyte-derived mφ-1 and mφ-2 macrophages and dendritic cells, as well as alveolar macrophages to characterise the production of $\alpha_1$-antitrypsin.

## Results

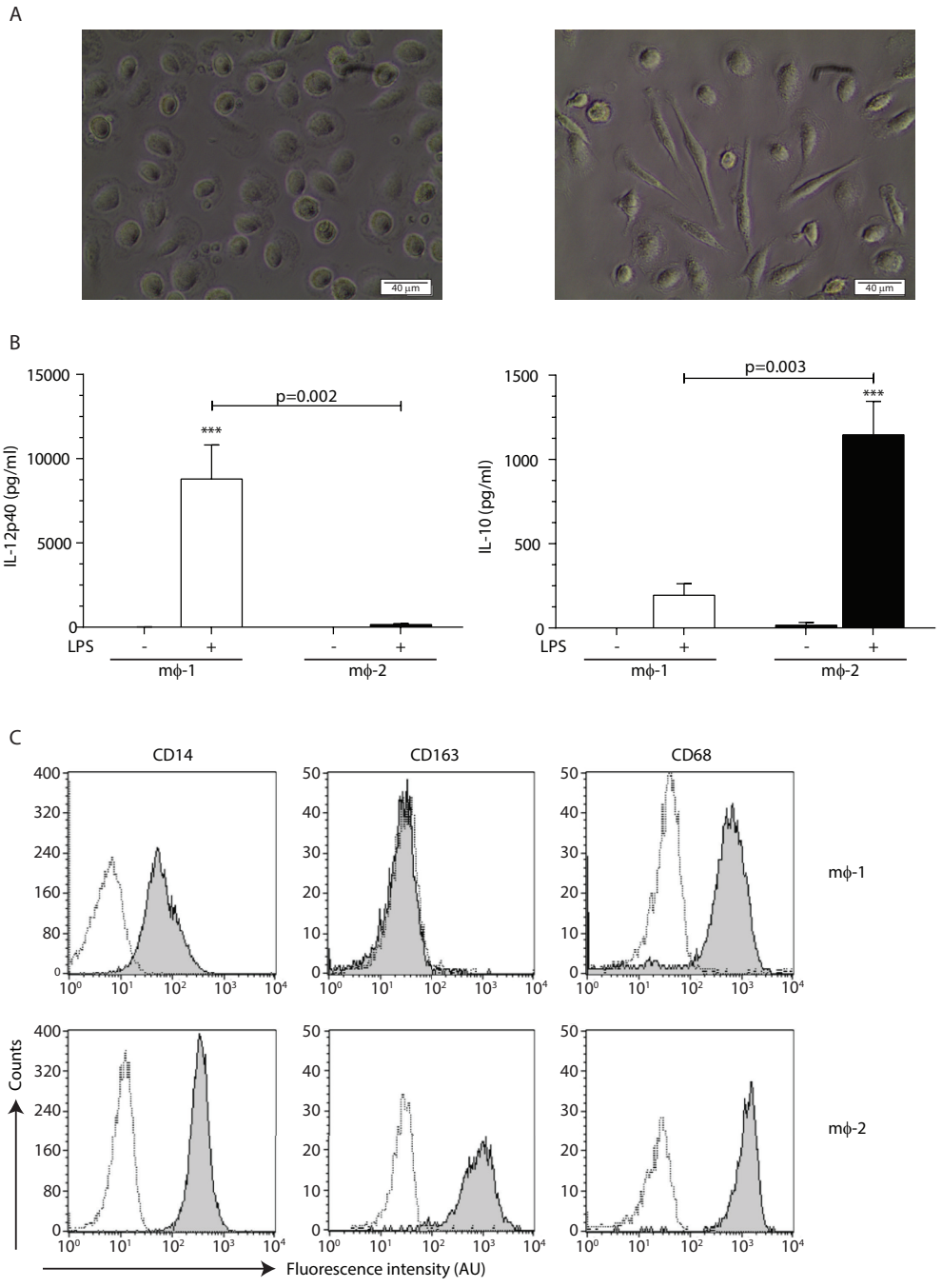
### Differentiation of mφ-1 and mφ-2

Differentiation of human blood monocytes into pro- (mφ-1) and anti-inflammatory (mφ-2) macrophages was assessed by morphological characteristics, by measuring IL-12p40/IL-23p40 and IL-10 secretion and by evaluating cell surface markers. After 6 days, morphological distinct subsets were observed (Figure 1A). After stimulation with LPS (100 ng/ml) for 24 hours, mφ-1 produced significantly more IL-12p40 than mφ-2, whereas mφ-2 produced significantly more IL-10 than mφ-1 (Figure 1B). In line with previous studies, mφ-2 expressed CD163 and high levels of CD14, whereas mφ-1 were $CD14^{low}$ and showed no detectable expression of CD163 (Figure 1C) (22, 23). Both cell types were positive for the intracellular cell marker CD68, confirming that these cells were classical macrophages.

### Mφ-1 produces more $\alpha_1$-antitrypsin than mφ-2

To evaluate whether the production of $\alpha_1$-antitrypsin differed between mφ-1 and mφ-2, monocyte-derived macrophages were stimulated with LPS, TNFα or oncostatin M, known to be inducers of $\alpha_1$-antitrypsin expression in lung epithelial cells and monocytes (7-9). Spontaneous release of $\alpha_1$-antitrypsin after 24 hours was higher in mφ-1 than mφ-2 (204 ng/$10^6$ cells vs. 42 ng/$10^6$ cells; $p < 0.001$, Figure 2A). LPS significantly enhanced $\alpha_1$-antitrypsin secretion from both mφ-1 and mφ-2 and levels were significantly higher in mφ-1 than mφ-2 (323 ng/$10^6$ cells vs. 93 ng/$10^6$ cells; $p = 0.003$). TNFα and oncostatin M did not affect $\alpha_1$-antitrypsin secretion (Figure 2A), whereas measurement of enhanced IL-8 in the cell supernatant by ELISA confirmed the activation of the macrophages by these cytokines (data not shown). Interestingly, after 24 hours LPS exposure, the normalised expression of *SERPINA1* mRNA, the gene encoding $\alpha_1$-antitrypsin, was significantly lower in mφ-1 than mφ-2 (Figure 2B). In line with the protein data, neither TNFα nor oncostatin M showed an effect at mRNA level.

To explain the differences in *SERPINA1* mRNA levels and measured protein levels


A
40 µm
40 µm
B
IL-12p40 (pg/ml)
15000
10000
5000
0
p=0.002
***
LPS
-
+
-
+
mϕ-1
mϕ-2
IL-10 (pg/ml)
1500
1000
500
0
p=0.003
***
C
CD14
CD163
CD68
mϕ-1
mϕ-2
Counts
Fluorescence intensity (AU)

<<< **Figure 1. Differentiation of human monocytes into pro- (mφ-1) and anti-inflammatory (mφ-2) macrophages.**

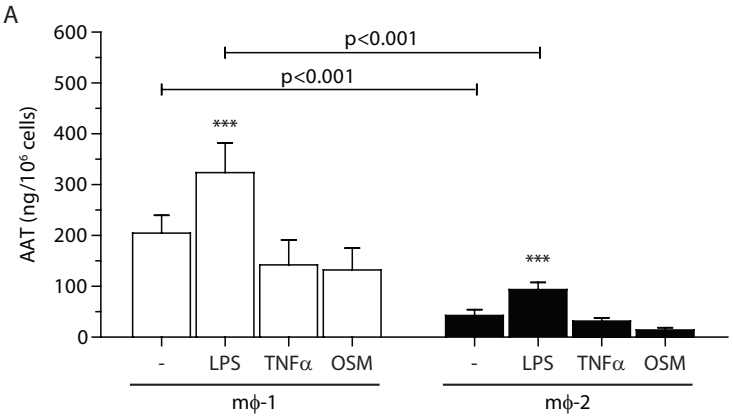
Human monocytes were cultured for 6 days in the presence of GM-CSF or M-CSF to obtain mφ-1 and mφ-2, respectively. A. After 6 days, mφ-1 (left) typically showed a 'fried-egg' morphology; mφ-2 (right) appeared as 'spindle-like' cells as visualised by phase contrast microscopy. Images are representative of at least five independent donors. B. After stimulation with LPS for 24 hours, IL-12p40/IL-23p40 and IL-10 concentrations in cell supernatant were measured by ELISA (n=10; different donors). C. Cell surface expression (closed histograms) of CD14, CD163 and CD68 on mφ-1 and mφ-2 was determined by flow cytometry. Open histograms represent matched isotype controls. Data are representative of at least three independent experiments using separate donors. These results confirm appropriate differentiation towards mφ-1 and mφ-2 cells.

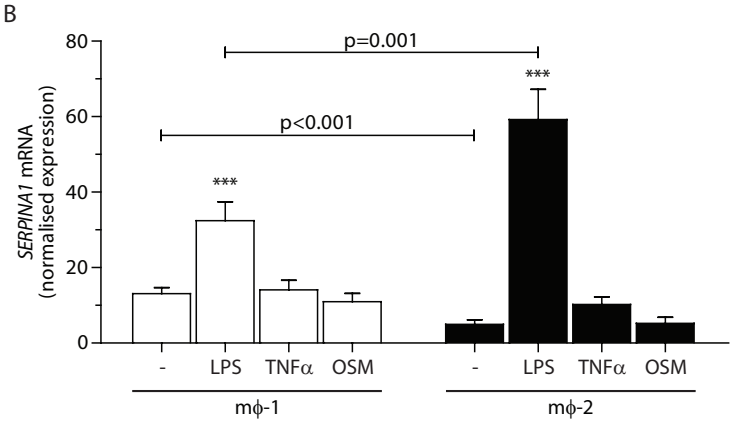
in the cell supernatant, cell-associated $\alpha_1$-antitrypsin of mφ-1 and mφ-2 was examined by immunocytochemistry. Analysis showed that in mφ-1 compared to mφ-2, the staining intensity of cell-associated $\alpha_1$-antitrypsin was markedly higher, and that mφ-1 cells showed a more membrane-associated pattern (Figure 2C). These findings suggest that the increased *SERPINA1* mRNA after LPS stimulation observed in mφ-1, is directly translated and secreted as $\alpha_1$-antitrypsin protein. However, the enhanced *SERPINA1* mRNA in mφ-2 after LPS exposure could not be detected to the same extent at protein level. Levels of secreted $\alpha_1$-antichymotrypsin, another member of the serpin family, and SLPI were below the limits of detection of the ELISA.



**Alpha$_1$-antitrypsin is secreted in a time-dependent way**

To verify the apparent discrepancy between the effect of LPS exposure on *SERPINA1* mRNA and protein levels (both cell-associated and secreted), we stimulated both mφ-1 and mφ-2 with 100 ng/ml LPS for 4, 24 and 48 hours. In mφ-2, an LPS-induced increase in *SERPINA1* mRNA was already detected by quantitative RT-PCR after 4 hours,


A
AAT (ng/10^6 cells)
600
500
400
300
200
100
0
p<0.001
p<0.001
***
***
-
LPS
TNFα
OSM
-
LPS
TNFα
OSM
mϕ-1
mϕ-2


B
SERPINA1 mRNA
(normalised expression)
80
60
40
20
0
p=0.001
p<0.001
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-
LPS
TNFα
OSM
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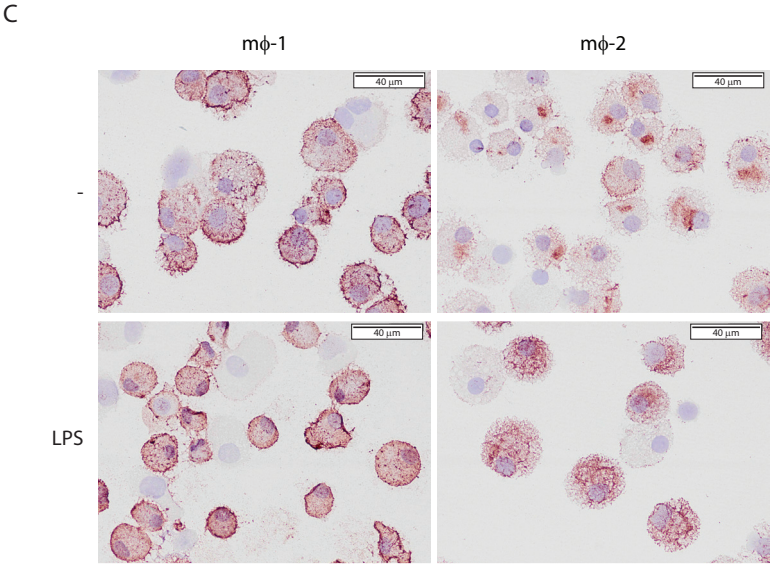
LPS
TNFα
OSM
mϕ-1
mϕ-2




C
mϕ-1
mϕ-2
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LPS
40 µm
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<<< **Figure 2. Effects of LPS, tumor necrosis factor alpha (TNFα) and oncostatin M (OSM) on $\alpha_1$-antitrypsin synthesis and secretion of mφ-1 and mφ-2.**

Mφ-1 (open bars) and mφ-2 (closed bars) were stimulated with LPS (100 ng/ml), TNFα (10 ng/ml) or oncostatin M (100 ng/ml) for 24 hours. Cell supernatants were collected, total RNA was isolated and cytospin preparations were made. A. Alpha$_1$-antitrypsin (AAT) protein levels in cell supernatant measured by ELISA (*** $p<0.001$ versus control; n=5). B. *SERPINA1*, *ATP5B* and *ACTB* mRNA concentrations were determined by quantitative RT-PCR (qPCR), with SERPINA1 normalised to *ATP5B* and *ACTB* as assessed using GeNorm software (*** $p<0.001$ versus control; n=6). C. $1{\cdot}10^5$ cells of each sample were used for cytospin preparation. Slides were stained with monoclonal antibody $\alpha_1$-antitrypsin to visualise cell-associated/intracellular $\alpha_1$-antitrypsin. Representative photomicrographs are shown.

whereas a significant difference in *SERPINA1* mRNA induction upon LPS exposure in mφ-1 was only seen after 24 hours (Figure 3B). The highest fold increase in *SERPINA1* mRNA was seen after 48 hours in both mφ-1 and mφ-2 (4-fold vs. 62-fold increase compared to control-treated mφ-1 or mφ-2, respectively). Although mφ-2 showed the highest fold increase at mRNA level and although the relative increase in secreted $\alpha_1$-antitrypsin after LPS exposure for 48 hour was higher in mφ-2 compared to mφ-1 (Figure 3A), the absolute levels of secreted $\alpha_1$-antitrypsin were still higher in mφ-1 than mφ-2 (Figure 3A), indicating that the different kinetics could only explain a part of the inconsistency in mRNA and protein levels.



To further explore the discrepancy, cells were incubated with LPS in the presence or absence of MG132, a proteasome inhibitor which reduces the ubiquitin-proteasomal degradation. After 24 h, MG132 did not influence the *SERPINA1* mRNA nor the $\alpha_1$-antitrypsin secretion in both mφ-1 and mφ-2 (Figure 4A and 4B), indicating that differences in mRNA and protein could not be explained by an increased degradation by the proteasome. To ensure that the increase of $\alpha_1$-antitrypsin secretion was dependent on de novo mRNA synthesis and/or de novo protein synthesis, cells were treated with actinomycin D or

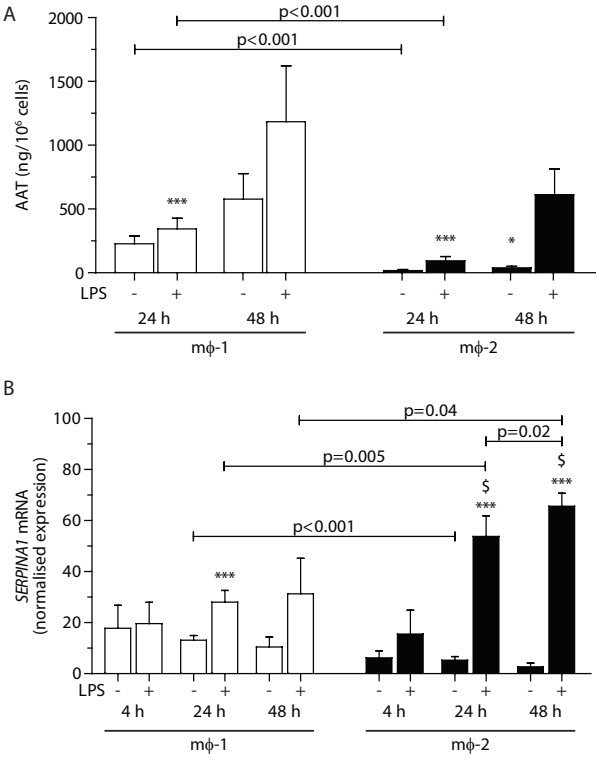





**Figure 3. Kinetics of $\alpha_1$-antitrypsin expression and secretion.**

Mφ-1 (open bars) and mφ-2 (closed bars) were stimulated with 100 ng/ml LPS and both cell supernatant and RNA were harvested at different time points as indicated. A. Alpha$_1$-antitrypsin secretion in cell supernatants were measured by ELISA (* $p<0.05$, *** $p<0.001$ versus control at 24 hours; n=4) and B. *SERPINA1* mRNA production was determined using quantitative RT-PCR. All samples were normalised to *ATP5B* and *ACTB* (*** $p<0.001$ versus control at 24 hours; $ $p<0.05$ versus LPS-stimulated mφ-2 at 4 hours; n=4).

cycloheximide, respectively. Actinomycin D fully inhibited the increase in both *SERPINA1* mRNA and $\alpha_1$-antitrypsin protein in LPS treated cells (data not shown). Similarly, blocking the de novo protein synthesis by cycloheximide showed reduced secreted amounts of $\alpha_1$-antitrypsin by both mφ-1 and mφ-2, demonstrating that de novo mRNA synthesis and protein synthesis are required for an up-regulation of $\alpha_1$-antitrypsin in mφ-1 and mφ-2.

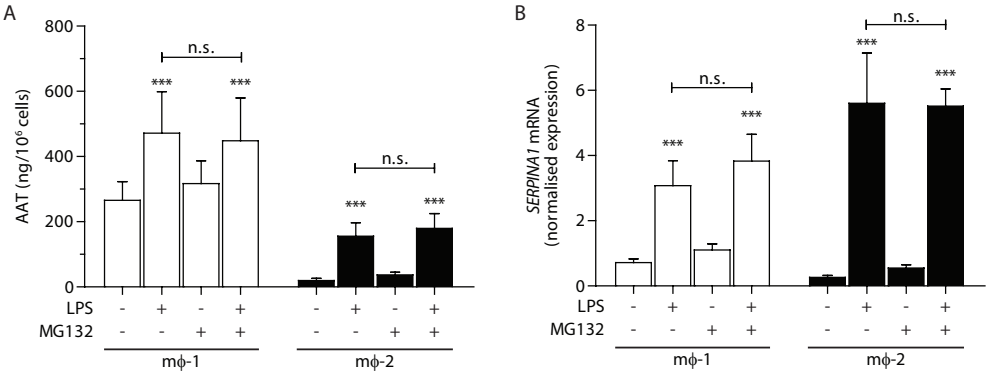



**Figure 4. Effects of the proteasome inhibitor MG132 on $\alpha_1$-antitrypsin (AAT) production of mφ-1 and mφ-2.**

Mφ-1 (open bars) and mφ-2 (closed bars) were stimulated for 24 hours with 100 ng/ml LPS and/or MG132 as indicated. A. Alpha$_1$-antitrypsin secretion in cell supernatants were measured by ELISA (*** $p<0.001$ versus control, n.s. not significant; n=6) and B. *SERPINA1* mRNA production was determined using qPCR and were normalised to *ATP5B* and *ACTB* (*** $p<0.001$ versus control, n.s. not significant; n=4).

### DC produce $\alpha_1$-antitrypsin as an intermediate phenotype

To investigate whether all monocyte-derived cell-lineages are able to produce $\alpha_1$-antitrypsin, immature monocyte-derived dendritic cells (iDC) were stimulated with LPS for 24 hours. Immature DC released 65 ng/$10^6$ cells $\alpha_1$-antitrypsin compared to 204 ng/$10^6$ cells and 42 ng/$10^6$ cells for mφ-1 and mφ-2, respectively (Figure 5A). Amounts of $\alpha_1$-antitrypsin secreted by mature LPS-exposed DC (mDC) were elevated (190 ng/$10^6$ cells) compared to control treated iDC. At mRNA level, mDC showed a 12-fold increase of *SERPINA1* after 24 hours compared to control treated iDC (Figure 5B). These results suggest an intermediate phenotype of DC concerning the production of $\alpha_1$-antitrypsin. Levels of ACT and SLPI were below the limits of detection.



### Alpha$_1$-antitrypsin production by alveolar macrophages

Alveolar macrophages are known to be very heterogeneous. To determine which *in vitro* subset of monocyte-derived macrophages best represents the production

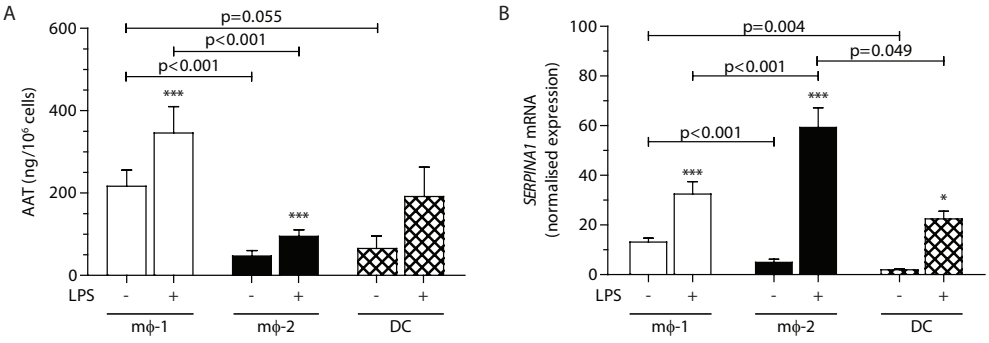



**Figure 5. Effects of LPS on $\alpha_1$-antitrypsin production of dendritic cells (DC) compared to mφ-1 and mφ-2.**

Human monocytes were cultured for 6 days in the presence of GM-CSF, M-CSF or GM-CSF + IL-4 to allow appropriate differentiation of mφ-1 (open bars), mφ-2 (closed bars) and DC (diamond bars), respectively. After 6 days, cells were stimulated with 100 ng/ml LPS and cell supernatant and RNA were isolated. A. Alpha$_1$-antitrypsin (AAT) secretion in cell supernatants were measured by ELISA (*** p<0.001 versus control; n=4) and B. *SERPINA1* mRNA production was determined using quantitative RT-PCR. All samples were normalised to *ATP5B* and *ACTB* (* p<0.05, *** p<0.001 versus control; n=4).



of $\alpha_1$-antitrypsin by alveolar macrophages, we stimulated alveolar macrophages for 24 hours in the presence of LPS. Characterisation of these cells by measuring IL-12p40/IL-23p40 and IL-10 secretion and evaluating cell surface markers showed that these alveolar macrophages produce high amounts of IL-10 in the complete absence of IL-12p40/IL-23p40 (Figure 6A) and were CD163$^{high}$ using immunocytochemistry (data not shown), both typical features of mφ-2. Alveolar macrophages produced 143 ng/10$^6$ cells of $\alpha_1$-antitrypsin, which was not further increased by LPS stimulation (162 ng/10$^6$ cells). These $\alpha_1$-antitrypsin levels are comparable to those produced by mφ-2 after LPS stimulation (Figure 6B) and were confirmed by quantifying the *SERPINA1* mRNA levels (Figure 6B). Interestingly, cell-associated $\alpha_1$-antitrypsin was comparable with mφ-1 (Figure 6C), suggesting that produced $\alpha_1$-antitrypsin remains cell-associated.

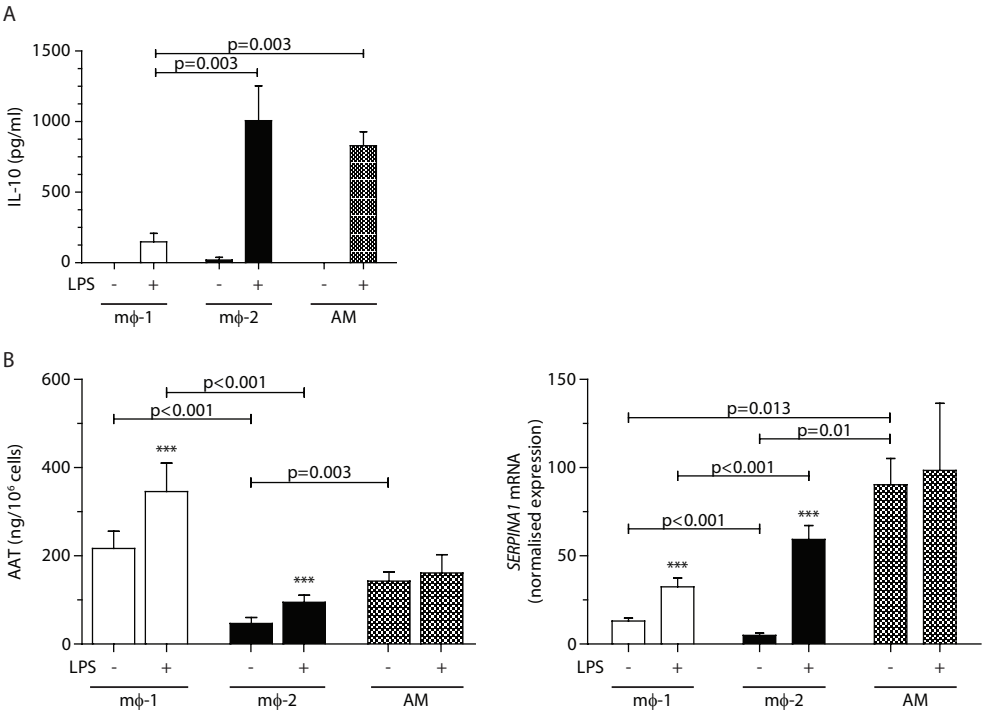
## Discussion

In the present study, we demonstrated that differentiated monocyte-derived macrophages, monocyte-derived DC and alveolar macrophages produce $\alpha_1$-antitrypsin, although to different extent. Only LPS could significantly induce *SERPINA1* mRNA synthesis and $\alpha_1$-antitrypsin secretion in the monocyte-derived cell types, suggesting that in addition to its pro-inflammatory activities, LPS also contributes to the prevention of lung tissue destruction by proteases during inflammation via the up-regulation of $\alpha_1$-antitrypsin.

Many lung diseases, including $\alpha_1$-antitrypsin deficiency, cystic fibrosis and neutrophilic asthma, are characterised by a neutrophil-dominated inflammation, where a protease-antiprotease imbalance can result in lung injury. It is interesting that both mφ-1 and mφ-2 did not increase $\alpha_1$-antitrypsin production following TNFα or oncostatin M treatment, whereas IL-8 secretion was increased by these cytokines. Previous studies with human bronchial epithelial cells already showed that both TNFα and oncostatin M can increase $\alpha_1$-antitrypsin production by these cells (8, 10, 24), and that both LPS and TNFα could up-regulate $\alpha_1$-antitrypsin in monocytes (9). In contrast, Perlmutter *et al.* (25) showed earlier the inability of monocytes to release $\alpha_1$-antitrypsin following exposure to TNFα. Together with our results, these studies indicate the complexity of the regulation of the antiprotease shield by macrophages in the microenvironment of the lung. In addition, to the best of our knowledge, we are the first to describe the capacity of DC to synthesise and secrete $\alpha_1$-antitrypsin. The role of DC-produced $\alpha_1$-antitrypsin in the regulation of the protease-antiprotease imbalance should be further investigated.



Although $\alpha_1$-antitrypsin is the major antiprotease present in the lung, $\alpha_1$-antichymotrypsin and SLPI can contribute to the protection of the alveolar tissue against neutrophil elastase, cathepsin G and proteinase-3. In contrast to our expectation, we were unable to detect $\alpha_1$-antichymotrypsin or SLPI produced by mφ-1, mφ-2 or DC with ELISA, although it has been reported that alveolar macrophages produce $\alpha_1$-antichymotrypsin and SLPI in response to LPS (26, 27). Moreover, a recent study showed that bone marrow derived DC from mice could produce SLPI in response to LPS (28). This discrepancy may


A
IL-10 (pg/ml)
1500
1000
500
0
p=0.003
p=0.003
LPS - + - + - +
mϕ-1
mϕ-2
AM
B
AAT (ng/10^6 cells)
600
400
200
0
p<0.001
p<0.001
***
p=0.003
***
SERPINA1 mRNA (normalised expression)
150
100
50
0
p=0.013
p=0.01
p<0.001
p<0.001
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LPS - + - + - +
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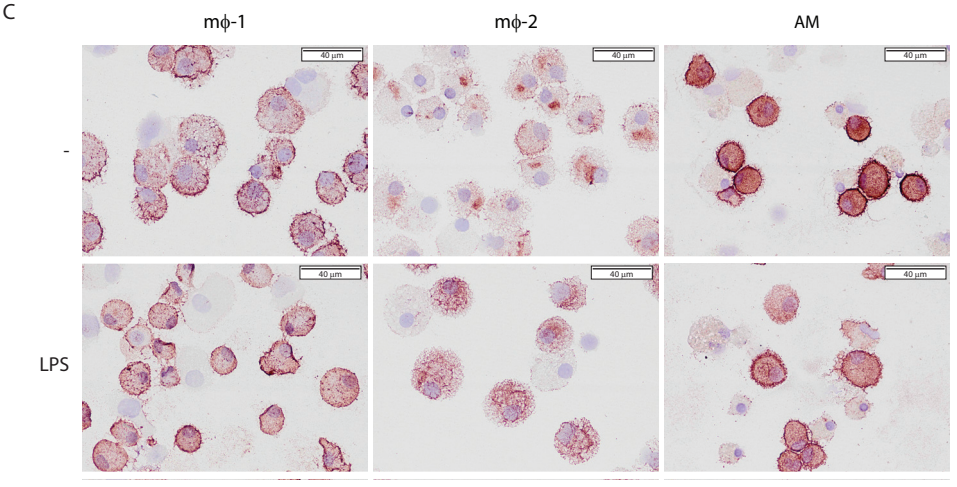
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mϕ-1
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<<< **Figure 6. Effects of LPS on $\alpha_1$-antitrypsin production of alveolar macrophages (AM) compared to mφ-1 and mφ-2.**

Alveolar macrophages were obtained from 4 patients susceptible of sarcoidosis by bronchoalveolar lavage and cultured for 24 hours in the presence of LPS as indicated. Mφ-1 and mφ-2 were obtained for blood monocytes of healthy blood donors. A. Alpha$_1$-antitrypsin (AAT) protein levels in cell supernatant of mφ-1 (open bars), mφ-2 (closed bars) and alveolar macrophages (checker board bars) were measured by ELISA (*** $p<0.001$ versus control; n=4). B. *SERPINA1*, *ATP5B* and *ACTB* mRNA concentrations were determined by quantitative RT-PCR, with *SERPINA1* normalised to *ATP5B* and *ACTB* as assessed using GeNorm software (*** $p<0.001$ versus control; n=4). C. $1{\cdot}10^5$ cells of each sample were used for cytospin preparation. Slides were stained with a monoclonal mouse antibody against $\alpha_1$-antitrypsin to visualise cell-associated/intracellular $\alpha_1$-antitrypsin. Images are representative for at least two different donors. Photomicrographs of mφ-1 and mφ-2 are identical to those shown in Figure 2C, and shown for comparison.

be explained by the different roles of DC in mice and human.

For decades, human alveolar macrophages have been known to express *SERPINA1* mRNA (29) and to increase the *SERPINA1* mRNA levels when exposed to LPS (30). However, to our knowledge this is the first time that $\alpha_1$-antitrypsin production is explored in alveolar macrophages, mφ-1 and mφ-2. In contrast to our findings in mφ-1 and mφ-2, we did not find an increase in $\alpha_1$-antitrypsin induction by LPS in alveolar macrophages. This finding could be explained by the fact that we used alveolar macrophages obtained from subjects during the diagnostic work-up of sarcoidosis. Wikén *et al*. (31) showed that there is no evidence of altered alveolar macrophages polarisation in patients with sarcoidosis, although others reported increased IP-10 and CCL-20 production by alveolar macrophages from sarcoidosis patients (32, 33). The phenotype of alveolar macrophages appears to be influenced by the unique environment in the lung (34), and the plasticity of alveolar macrophages may have affected their ability to produce $\alpha_1$-antitrypsin. At present, it is unclear whether the inability of LPS to increase $\alpha_1$-antitrypsin secretion in alveolar macrophages and the fact that these cells produce high amounts of both $\alpha_1$-antitrypsin

and IL-10 (which is in contrast to our findings in monocytes-derived macrophages), is related to differences between alveolar macrophages and *in vitro* generated monocytes-derived macrophages or to a disease specific phenomenon.

Functionally, mφ-1 and mφ-2 are distinct subsets concerning the cytokine production and T cell response, and evidently regarding the $\alpha_1$-antitrypsin production: mφ-1 release more $\alpha_1$-antitrypsin spontaneously and after stimulation with LPS than mφ-2, whereas *SERPINA1* mRNA levels are significantly higher in mφ-2 after LPS exposure. Recently, mφ-1 have been shown to degrade more rapidly IκBα and consequently more rapidly activate NF-κB pathway than mφ-2 following LPS treatment (21), providing a possible explanation for the observed differences in kinetics. However, these differences could only partially explain the observed differences in $\alpha_1$-antitrypsin secretion. Experiments inhibiting the proteasome did not provide any insights regarding the discrepancies, excluding the possibility of enhanced intracellular degradation of $\alpha_1$-antitrypsin. Blocking the de novo mRNA synthesis and de novo protein synthesis did not reveal an underlying mechanism concerning the transcriptional regulation and stability of *SERPINA1* mRNA. Therefore, we suggest a translational block for $\alpha_1$-antitrypsin in mφ-2 as a possible mechanism.



Several of our findings showed that mφ-2 *in vitro* resemble alveolar macrophages, which is supported by the data of other studies (17, 18). However, alveolar macrophages constitute a heterogeneous population and have been shown to be able to switch their phenotype during (chronic) inflammation and smoking (19, 20, 35). Our data indicate that $\alpha_1$-antitrypsin production of all monocyte-derived cell lineages may contribute to the restriction of neutrophil-mediated tissue injury.

In conclusion, this study provides evidence that both mφ-1 and mφ-2 are able to produce $\alpha_1$-antitrypsin, though in different amounts, which is partially explained by the high spontaneous release by the mφ-1. Moreover, also the third monocyte-derived cell lineage, namely DC, is capable to release $\alpha_1$-antitrypsin and therefore may contribute to the anti-elastase screen in the lung.

## Materials and methods

### Isolation and culture of monocyte-derived cells

Monocytes were isolated from buffy coats of healthy blood donors (Sanquin Blood Bank, Leiden, The Netherlands) using magnetic-labeled anti-CD14 beads (Myltenyi Biotec, Auburn, CA, USA) per manufacturer's instructions. Next, cells were cultured for 6 days in medium (RPMI 1640, Invitrogen, Breda Life Technologies, The Netherlands) containing 10% fetal calf serum (FCS, Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all Bio Whittaker, Walkersville, MD, USA) at 37°C in 5% $CO_2$ atmosphere in the presence of GM-CSF (5 ng/ml; Invitrogen) or M-CSF (50 ng/ml; R&D systems, Minneapolis, MN, USA) to obtain mφ-1 and mφ-2 macrophages, respectively (22). At day 6, cells were stimulated with LPS (100 ng/ml; LPS from *Pseudomonas aeruginosa*, Sigma-Aldrich, St. Louis, MO, USA), human TNFα (10 ng/ml; Peprotech, Rocky Hill, NJ, USA), oncostatin M (100 ng/ml; R&D Systems) and MG132 (10 nM; Sigma) for 4, 24 or 48 hours as indicated, at 37°C in 5% $CO_2$. Actinomycin D (1 µg/ml) and cycloheximide (10 µg/ml) were both purchased from Sigma.

Immature DC were generated by culturing CD14-isolated monocytes for 6 days in the presence of 5 ng/ml GM-CSF and 10 ng/ml IL-4 (14). Appropriate differentiation was ensured by determining the cell surface markers CD1a and CD83 and measuring the amounts of IL-12p40/IL-23p40 and IL-10 in the supernatant after LPS stimulation for 24 hours.



### Isolation and culture of alveolar macrophages

Alveolar macrophages were obtained from left-over material of a bronchoalveolar lavage (BAL) that was obtained as part of the diagnostic procedure for the diagnosis of sarcoidosis, and the patients were not on current treatment. Culture of alveolar macrophages was performed as described previously (36). Briefly, the collected BAL fluid was centrifuged, washed twice in PBS and finally cells were resuspended in RPMI culture medium with supplements as described above. Cells were allowed to adhere by incubation

for 1 hour at 37°C in 5% $CO_2$ atmosphere. The non-adherent cells were removed by three washes with medium and the remaining cells were stimulated as indicated. The adherent population of cells was > 95% pure macrophages, as assessed using CD68 flow cytometry.

**Flow cytometry**

Cell surface markers were assessed by standard flow cytometry using a FACSCalibur cytometer (Becton and Dickinson, La Jolla, CA, USA) and CellQuest Pro software. APC-labeled anti-human CD14 and PE-labeled anti-human CD163 were purchased from BD Biosciences/Pharmingen (Temse, Belgium). Anti-human CD68 (FITC-labeled; eBioscience, Vienna, Austria) was used for intracellular staining and appropriate IgG antibodies were used as isotype-control. Cells were incubated with the antibodies for 30 minutes on ice in PBS containing 0.5% BSA (w/v) and 0.2% sodium-azide (w/v) (both from Sigma). After fixation, intracellular staining was performed in PBS containing 1% saponine (Sigma) and 5% FCS.

**Immunocytochemistry**



Expression of $\alpha_1$-antitrypsin by mφ-1, mφ-2 and alveolar macrophages in cytospin preparations was demonstrated by immunocytochemistry. Cells in cytospin preparations were fixed with 4% (w/v) formaldehyde for 30 minutes at room temperature. Next, cells were incubated with PBS/0.3% Triton X-100 for 30 minutes for permeabilisation and stained with mouse monoclonal IgG1 anti-$\alpha_1$-antitrypsin (1:500, Abcam, Cambridge, UK) or control mouse IgG1 as a negative control (DAKO, Glostrup, Denmark) at room temperature for 1 hour. As a secondary antibody, the horseradish peroxidase conjugated anti-mouse Envision system (DAKO) was used, with NovaRED (Vector, Burlingame,CA) as the chromagen. The sections were counterstained with Mayer's haematoxylin (Klinipath, Duiven, The Netherlands).

**Enzyme-linked immunosorbent assay (ELISA)**

Macrophage differentiation was verified by assessing the secretion of IL-12p40/IL-23p40 (R&D systems; sensitivity: 62.5 pg/ml) and IL-10 (Sanquin, Amsterdam, The Netherlands; sensitivity: 4.096 pg/ml) by sandwich ELISA according to manufacturer's description. Specific ELISA's for $\alpha_1$-antitrypsin and $\alpha_1$-antichymotrypsin were purchased from Kordia (Leiden, The Netherlands; sensitivity: 0.342 ng/ml) and Immunology Consultants Laboratory, Inc. (ICL, Newberg, OR, USA; sensitivity: 3.125 ng/ml), respectively. Levels of SLPI in the cell supernatant were quantified as described previously (37).

**Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR)**

Total RNA was isolated using Qiagen RNeasy mini kit (Qiagen/Westburg, Leusden, The Netherlands) and cDNA was synthesised in equal amounts per experiment. Quantitative RT-PCR was performed using the following primer pair: *SERPINA1* (gene encoding $\alpha_1$-antitrypsin) sense-primer 5' AAGGCAAATGGGAGAGACCC 3' and anti-sense 5' AAGAAGATGGCGGTGGCAT 3'. Quantitative RT-PCR was performed on the iCycler PCR machine and MyiQ real-time PCR detection system using iQ SYBR Green Supermix (all from Bio-Rad, Hercules, USA) for 40 cycles at 60°C. The levels of the reference genes *ACTB* and *ATP5B* were used for normalisation, and their stability was determined by using GeNorm software (PrimerDesign Ltd., Southampton, UK).



**Statistical analysis**

Results were expressed as mean ± S.E.M. Mixed model analysis was performed to explore the overall effect. If the mixed model analysis was significant, we performed the paired t-test or one-way ANOVA as indicated using SPSS Statistics 17.0. Differences at p-values $< 0.05$ were considered as significant.

**Acknowledgements**

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of Medical Statistics (Leiden University Medical Centre, the Netherlands) for their help in the statistical analysis. This work was supported in part by a grant from the Netherlands Asthma Foundation (3.2.08.032).

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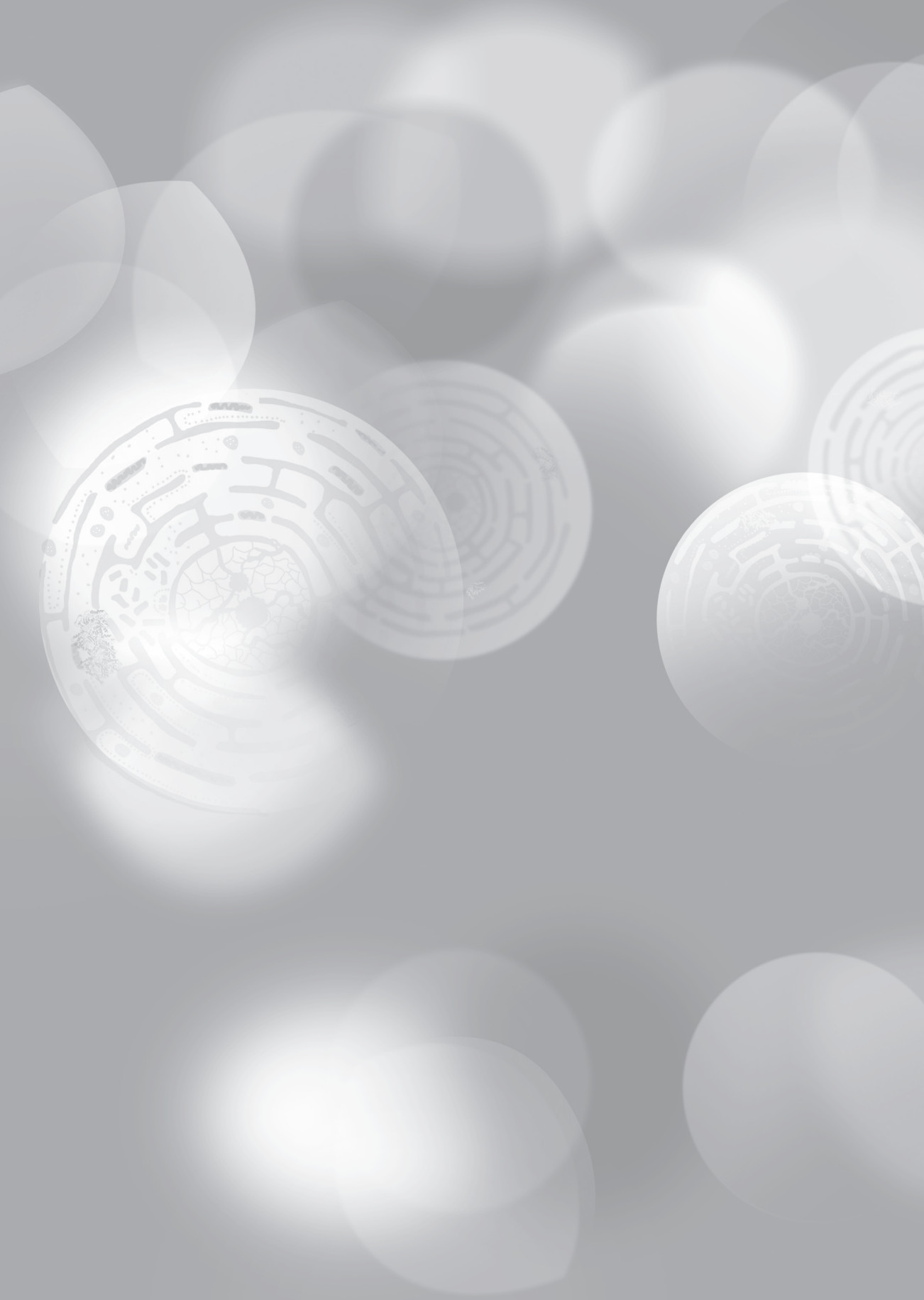

# Chapter 7

## Function of monocytes and monocyte-derived macrophages in $\alpha_1$-antitrypsin deficiency

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*Submitted*

## Abstract

**Alpha$_1$-antitrypsin deficiency is the most widely recognised genetic disorder causing COPD. Mutant Z $\alpha_1$-antitrypsin expression has previous been linked to intracellular accumulation and polymerisation of this proteinase inhibitor. Subsequently, this has been described to underlie an exaggerated endoplasmic reticulum (ER) stress response and enhanced NF-κB signalling. However, whether monocyte-derived macrophages display the same features remains unknown. Monocytes from homozygous PiZZ $\alpha_1$-antitrypsin deficiency patients and PiMM controls were cultured for 6 days in the presence of GM-CSF or M-CSF to obtain pro- and anti-inflammatory macrophages (mφ-1 and mφ-2, respectively). We first show that in contrast to monocytes, pre-stressed mφ-1 and mφ-2 from healthy blood donors display an enhanced ER stress response upon a LPS trigger (*spliced XBP1*, *CHOP*, *GADD34* and *GRP78* mRNA). However, this ER stress response did not differ between monocyte-derived macrophages and monocytes from ZZ patients compared to MM controls. Furthermore, these ZZ cells also do not secrete higher cytokine levels, and $\alpha_1$-antitrypsin polymers were not detectable by ELISA. These data suggest that monocyte-derived macrophages are not the local source of Z $\alpha_1$-antitrypsin polymers found in the lung and that the ER stress response and pro-inflammatory cytokine release is not altered.**

## Introduction

Alpha$_1$-antitrypsin is an important serine proteinase inhibitor (serpin) that protects lung tissue from the destructive effects of serine proteases such as neutrophil elastase, proteinase 3 and cathepsin G that are released by degranulating neutrophils. Moreover, $\alpha_1$-antitrypsin is thought to display anti-inflammatory activity including cytokine inhibition (1-3), inhibition of ERK1/2 (4) and regulation of CD14 expression (5). Although $\alpha_1$-antitrypsin is primarily synthesised in the liver, we and others have shown that it can also be produced locally by lung epithelial cells, alveolar macrophages and dendritic cells (4, 6-8).

The Z mutation (E342K) of $\alpha_1$-antitrypsin comprises more than 95% of the mutations leading to severe $\alpha_1$-antitrypsin deficiency. Due to this mutation, the Z $\alpha_1$-antitrypsin is not properly folded, which leads to the formation of polymers that accumulate as PAS positive inclusions within the endoplasmic reticulum (ER) of hepatocytes (9). This toxic gain-of-function within the liver causes hepatic cirrhosis and the concomitant plasma deficiency causes a protease-antiprotease imbalance within the lung and hence early-onset lung emphysema (10). Polymers of Z $\alpha_1$-antitrypsin were identified in lung lavage (11, 12) and shown to have pro-inflammatory properties that may exacerbate inflammation and lung damage (11, 13-15), particularly in the cigarette smoking Z $\alpha_1$-antitrypsin homozygote. In 2004, Mulgrew *et al.* (15) showed that Z $\alpha_1$-antitrypsin polymers could still be detected in lung lavage ten years after liver transplantation, suggesting local secretion and polymerisation of Z $\alpha_1$-antitrypsin within the lung. However, even after a decade, the source of these polymers remains unclear.



The ER is the site of secretory and membrane protein folding and its quality control systems ensure that only properly folded proteins exit the organelle for secretion or integration into the cell membrane. Accumulation of unfolded or misfolded proteins in the ER induces "ER stress", thereby activating intracellular signal transduction pathways collectively called the unfolded protein response (UPR) (reviewed by Marciniak and Ron (16)). The aim of this complex cellular response is to maintain ER homeostasis initially by reducing the influx of newly synthesised proteins into the ER lumen and subsequently by

enhancing the protein-folding capacity of the ER. Cells also increase expression of proteins of the ER associated degradation (ERAD) pathway to remove terminally misfolded proteins (17). Furthermore, the UPR not only orchestrates ER homeostasis, it has also be shown to be involved in ER stress-induced NF-κB activation (18). For example, X-box binding protein 1 (XBP1), a key modulator of the UPR, has been shown to control the production of interleukin (IL)-6 and interferon (IFN)-β in B cells and macrophages, respectively (19, 20).

Misfolded monomeric Z $\alpha_1$-antitrypsin is predominantly degraded by ERAD whilst polymers are cleared by autophagy (21, 22). Interestingly, this does not activate the UPR within cells overexpressing Z $\alpha_1$-antitrypsin (23-25). However, it does prime cells to an exaggerated ER stress response upon a "second hit", probably due to the impaired protein mobility within the ER caused by $\alpha_1$-antitrypsin polymers (25). In addition to the enhanced sensitivity to ER stress, cells expressing Z $\alpha_1$-antitrypsin also display an augmented NF-κB response with subsequent increase in cytokine secretion (4, 23, 24, 26). Upon a second hit, such as exposure to lipopolysaccharide (LPS) or tumour necrosis factor (TNF)α, this inflammatory response is further increased (4, 26).

Peripheral blood monocytes are the precursors for various subsets of lung macrophages, including alveolar macrophages, which are increased in chronic lung diseases such as COPD (27) and are associated with the pathogenesis and disease severity of this condition (28). In the healthy lung, alveolar macrophages have been shown to be immunosuppressive with poor antigen-presenting capacities, but different macrophage phenotypes can develop when monocytes are exposed to different (micro-)environmental signals (reviewed in (29, 30)). Based largely on *in vitro* studies into development of human monocytes-derived macrophages, distinct macrophage subpopulations have been identified. For instance, human monocytes exposed to GM-CSF will activate the classical pathway of macrophage differentiation, resulting in pro-inflammatory mφ-1 macrophages releasing pro-inflammatory cytokines and promoting a T-helper 1 response (31). On the other side of the spectrum, the anti-inflammatory mφ-2 macrophages (also called alternatively activated macrophages), can be derived from human monocytes exposed to M-CSF, and are characterised by the production of IL-10, the induction of T

regulatory cells and the phagocytosis of apoptotic cells (32, 33). However, recent studies have shown altered alveolar macrophage polarisation with an "intermediate phenotype" and impaired phagocytosis in COPD patients (reviewed in (34)).

Carroll *et al*. (26) previously showed intracellular accumulation of $\alpha_1$-antitrypsin and subsequent activation of the UPR in monocytes from homozygous Z $\alpha_1$-antitrypsin deficiency patients. Since we have shown previously differential $\alpha_1$-antitrypsin production by different macrophage subsets (8), we set out to test the hypothesis that mφ-1 macrophages are able to produce Z $\alpha_1$-antitrypsin polymers. Furthermore, we hypothesised that this subset contributes to the enhanced inflammation due to the activation of the UPR, and due to an increased NF-κB activation.

## Results

### Monocytes and monocyte-derived macrophages experiencing ER stress display an exaggerated response upon LPS

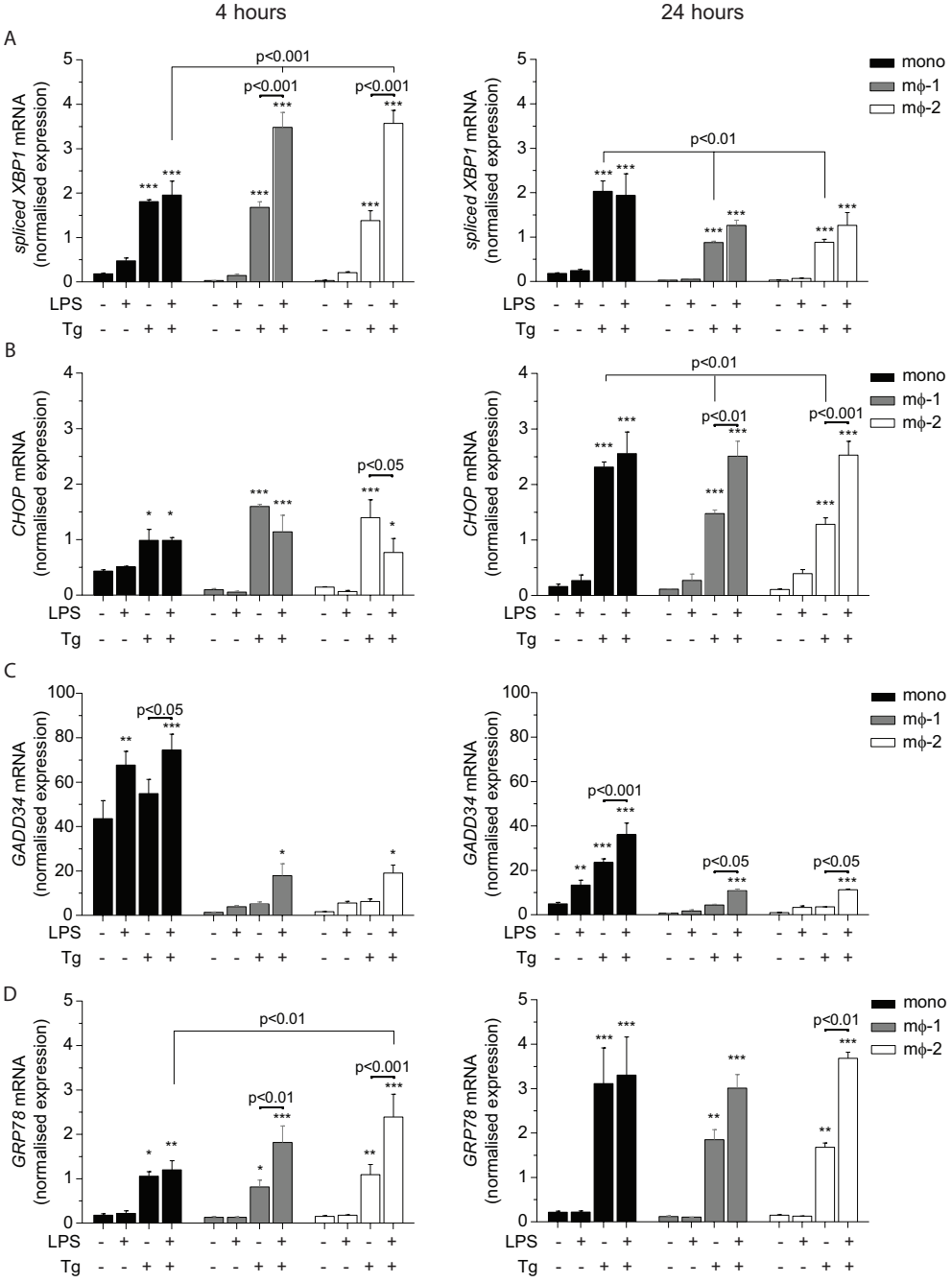
Thapsigargin inhibits the sarcoendoplasmic reticulum calcium ATPase, thereby releasing the $Ca^{2+}$ stores from the ER and inducing the UPR and activation of NF-κB (38, 39). To confirm that low-grade ER stress can lead to an exaggerated UPR upon a second hit in monocytes and monocytes-derived macrophages, we pre-treated these cells isolated from MM donors with thapsigargin for 1h, and subsequent stimulation with LPS for 4 or 24 hours. As expected, thapsigargin significantly increased *CHOP*, *GADD34*, *GRP78* mRNA and the splicing of *XBP1* mRNA in all cell types at both 4 hours and 24 hours (Figure 1A-D). This response was slower in monocytes compared to both mφ-1 and mφ-2, since the levels of *CHOP* and *spliced XBP1* mRNA were significantly lower at 4 hours, and significantly higher at 24 hours ($p<0.01$; Figure 1A-B). LPS induced considerably higher levels of all four UPR genes in mφ-1 and mφ-2 at either 4 hours (for *spliced XBP1* and *GRP78* mRNA) or 24 hours (for *CHOP* and *GADD34* mRNA).

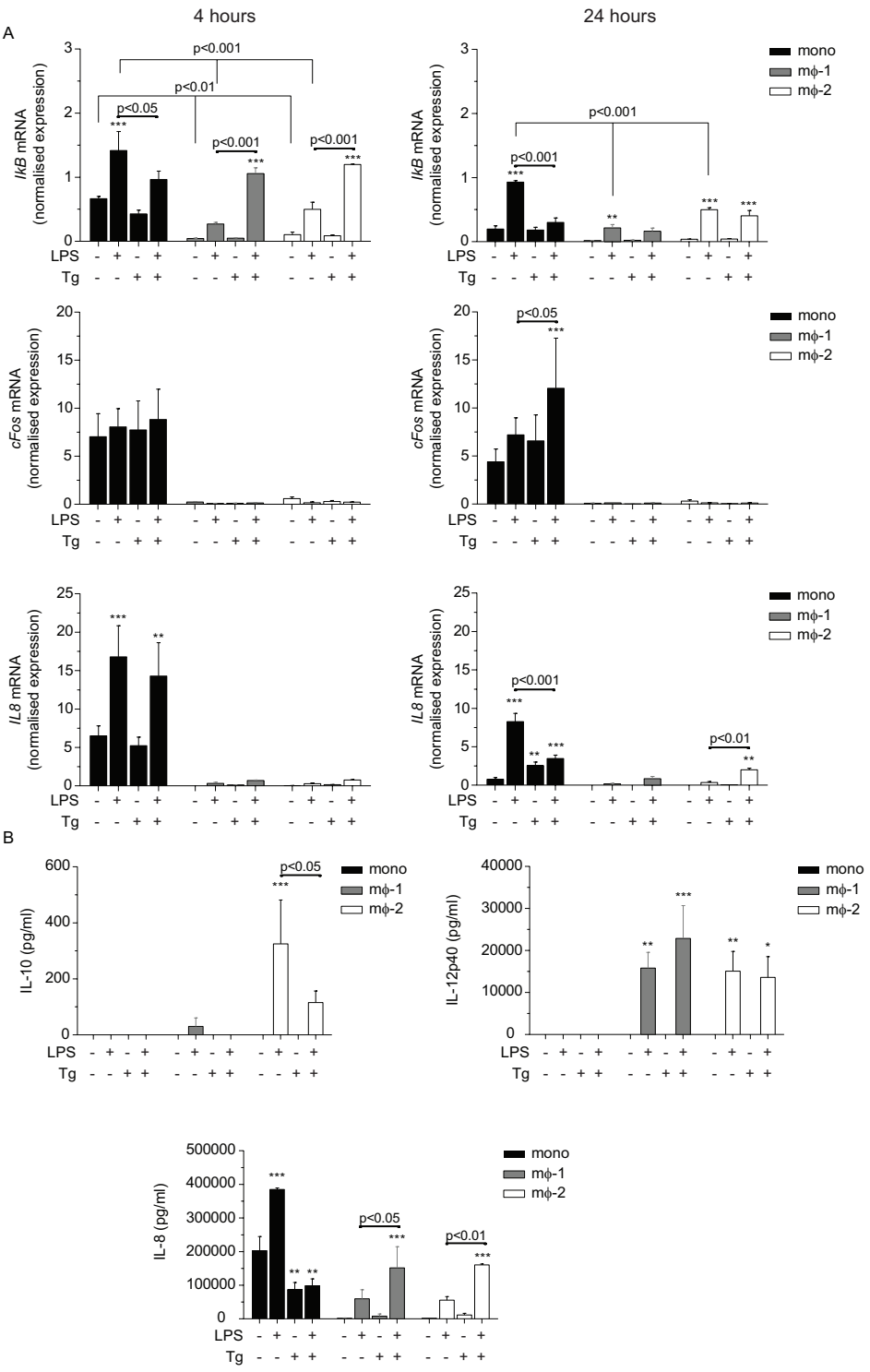
Next, we verified whether this increased ER stress response was accompanied by an increase in NF-κB response. Basal levels of *IκB*, *cFos* and *IL8* mRNA were significantly higher in monocytes compared to both mφ-1 and mφ-2 (Figure 2A). LPS significantly increased all three parameters in monocytes at 4 hours, but not in mφ-1 or mφ-2.



**Figure 1. ER stress response upon LPS treatment in MM monocytes and MM monocyte-derived macrophages experiencing ER stress.** >>>

A. Monocytes (mono), and macrophages type I (mφ-1) and type II (mφ-2) were pre-incubated with thapsigargin (Tg) for 1 hour followed by LPS treatment for 4 hours (left) or 24 hours (right) as indicated. The splicing of *XBP1* mRNA was measured with quantitative RT-PCR, normalised to *ATP5B* and *ACTB* mRNA. B-D. Cells were treated as in A and *CHOP, GADD34* and *GRP78* mRNA was measured, respectively. *p<0.05, **p<0.01, ***p<0.001 versus untreated with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).


4 hours
24 hours
A
B
C
D
spliced XBP1 mRNA (normalised expression)
CHOP mRNA (normalised expression)
GADD34 mRNA (normalised expression)
GRP78 mRNA (normalised expression)
mono
mϕ-1
mϕ-2
LPS
Tg
p<0.001
p<0.01
p<0.05


A
4 hours
24 hours
mono
mϕ-1
mϕ-2
IκB mRNA (normalised expression)
cFos mRNA (normalised expression)
IL8 mRNA (normalised expression)
p<0.001
p<0.01
p<0.05
LPS
Tg
B
IL-10 (pg/ml)
IL-12p40 (pg/ml)
IL-8 (pg/ml)

<<< **Figure 2. Inflammatory response upon LPS treatment in MM monocytes and MM monocyte-derived macrophages experiencing ER stress.**

A. Cells were treated as in Figure 1 and *IkB*, *cFos* and *IL8* mRNA was measured. B. Pre-incubated cells with thapsigargin (Tg; 1 hour) were subjected to 24 hours LPS treatment and IL-10, IL-12p40 and IL-8 cytokine release were measured in cell supernatant by ELISA. *p<0.05, **p<0.01, ***p<0.001 versus untreated with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

Remarkably, mφ-1 and mφ-2 experiencing ER stress did show enhanced *IκB* mRNA levels after 4 hours of LPS treatment (p<0.001), whereas in monocytes this level actually decreased (p<0.05; Figure 2A). After 24 hours no differences were observed anymore.

To conclude, these data demonstrate that monocyte-derived macrophages display an exaggerated ER stress response and NF-κB response upon a second hit when experiencing ER stress, a phenomenon not observed in monocytes.

**Monocytes and monocyte-derived macrophages from ZZ patients lack the production of detectable polymers**

It has been known for a long time that monocytes (40) and (monocyte-derived) macrophages produce $\alpha_1$-antitrypsin (8, 41). However, it remains unknown whether macrophages from Z $\alpha_1$-antitrypsin patients (ZZ cells) are a source of Z $\alpha_1$-antitrypsin polymers found in the lung and experience an exaggerated ER stress response. Therefore, we first confirmed our previous findings (8) that pro-inflammatory mφ-1 macrophages secrete significantly more $\alpha_1$-antitrypsin compared to anti-inflammatory mφ-2 macrophages in both MM and ZZ cells (p<0.001; Figure 3A). As expected, the levels of $\alpha_1$-antitrypsin in the cell supernatant of MM cells were up to five times higher compared to the supernatant of ZZ cells. This could only in part be explained by the intracellular retention of Z $\alpha_1$-antitrypsin (Figure 3B). The production of $\alpha_1$-antitrypsin in both mφ-1 and mφ-2 was increased after 24 hours LPS treatment (p<0.05 and p<0.01, respectively;

Figure 3A-B). When we used the 2C1 monoclonal antibody to specifically detect naturally occurring $\alpha_1$-antitrypsin polymers, we were unable to detect Z $\alpha_1$-antitrypsin polymers in any cell type (Figure 3A-B), whereas liver homogenate from a cirrhotic ZZ liver revealed accumulation of Z polymers (data not shown). To verify whether this was due to their differentiation, we evaluated the total $\alpha_1$-antitrypsin and polymer production of monocytes from the same donors. Unstimulated monocytes released equal amounts of total $\alpha_1$-antitrypsin measured in the cell supernatant compared to mφ-2 (Figure 3A), and did not significantly up-regulate the total $\alpha_1$-antitrypsin production after LPS treatment. Interestingly, the intracellular $\alpha_1$-antitrypsin levels were significantly higher in both MM as ZZ monocytes compared to pro- or anti-inflammatory macrophages (Figure 3B). However, the polymer levels were undetectable in both the cell supernatant and whole cell lysate of ZZ monocytes (Figure 3A-B).

**No evidence for the activation of the unfolded protein response in ZZ monocytes and monocyte-derived macrophages**

It has been shown that the overexpression of Z $\alpha_1$-antitrypsin to levels that cause its polymerisation leads to an exaggerated ER stress response upon a second hit (24, 25), whereas the presence of monomeric Z $\alpha_1$-antitrypsin alone does not trigger the UPR in primary bronchial epithelial cells (4). Carroll *et al*. (26) showed a slightly enhanced UPR in resting ZZ monocytes in the presence of intracellular accumulated Z $\alpha_1$-antitrypsin. However, the conformation of this retained Z $\alpha_1$-antitrypsin remained unclear. Therefore, to examine whether our ZZ monocytes and ZZ monocyte-derived macrophages experience increased ER stress, we investigated the expression of several UPR target genes; *CHOP*, *GADD34* and *GRP78* and the splicing of *XBP1* mRNA. In resting cells, there was no evidence of an increased ER stress response in ZZ cells compared to MM cells (Figure 4A-B). In addition, beside basal *GADD34* mRNA levels, which were elevated in monocytes, there was no significant difference in the basal expression of most UPR genes between monocytes, mφ-1 and mφ-2, indicating that the differentiation of monocytes into macrophages does not alter the stress status (Figure 1A-D and Figure 4A-B). Next, to investigate the influence

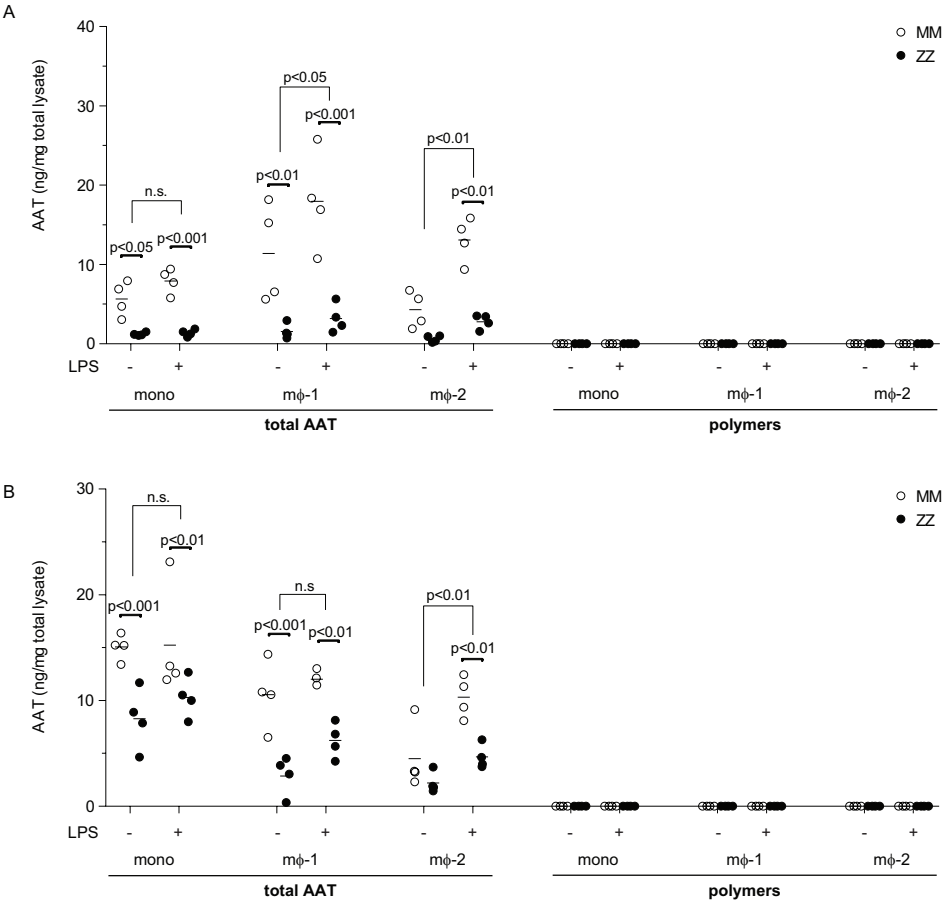





**Figure 3. Alpha$_1$-antitrypsin production by monocyte-derived macrophages from ZZ patients and MM controls.**

A. Total $\alpha_1$-antitrypsin (AAT) and $\alpha_1$-antitrypsin polymer production measured in cell supernatant of monocytes (mono), and macrophages type I (mφ-1) and type II (mφ-2) after 24 hours LPS treatment. B. As in A. Total $\alpha_1$-antitrypsin and $\alpha_1$-antitrypsin polymer levels in whole cell lysates. *p<0.05, **p<0.01, ***p<0.001 versus untreated with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

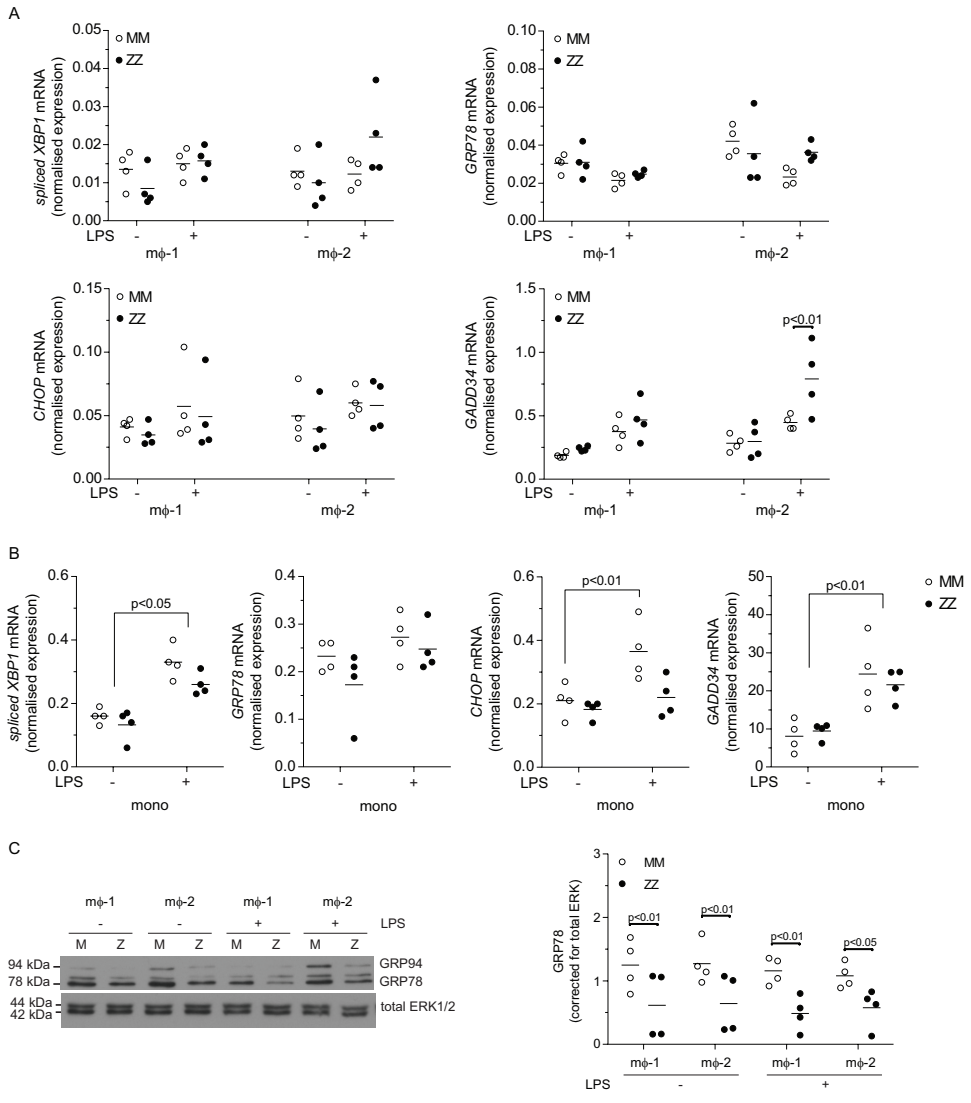



**Figure 4. No exaggerated ER stress response in monocytes and monocyte-derived macrophages from ZZ patients compared to MM controls.** A. mRNA levels in macrophages type I (mφ-1) and type II (mφ-2) of the ER stress genes *spliced XBP1*, *CHOP*, *GADD34* and *GRP78* after 24 hours LPS treatment measured by quantitative RT-PCR. B. Monocytes were treated and subjected to analysis as in A. C. Representative western blot for GRP94 and GRP78 using anti-KDEL antibody. Monocyte-derived macrophages were treated as in A. Densitometry of n=4. *p<0.05, **p<0.01, ***p<0.001 versus untreated with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

of an enhanced $\alpha_1$-antitrypsin production, these cells were stimulated with LPS. After 24 hours, mφ-2 from Z $\alpha_1$-antitrypsin patients showed a significant increase in *GADD34* mRNA (Figure 4A). However, this difference could not be detected in monocytes (Figure 4B). In line with previous research (42, 43), LPS significantly increased *CHOP* and *GADD34* mRNA levels and the splicing of *XBP1* mRNA.

When we assessed GRP78 protein levels by western blot, we were unable to detect its increase in mφ-2 from ZZ patients (Figure 4C). In fact, these levels appeared to be lower in ZZ macrophages compared to MM macrophages (Figure 4C).

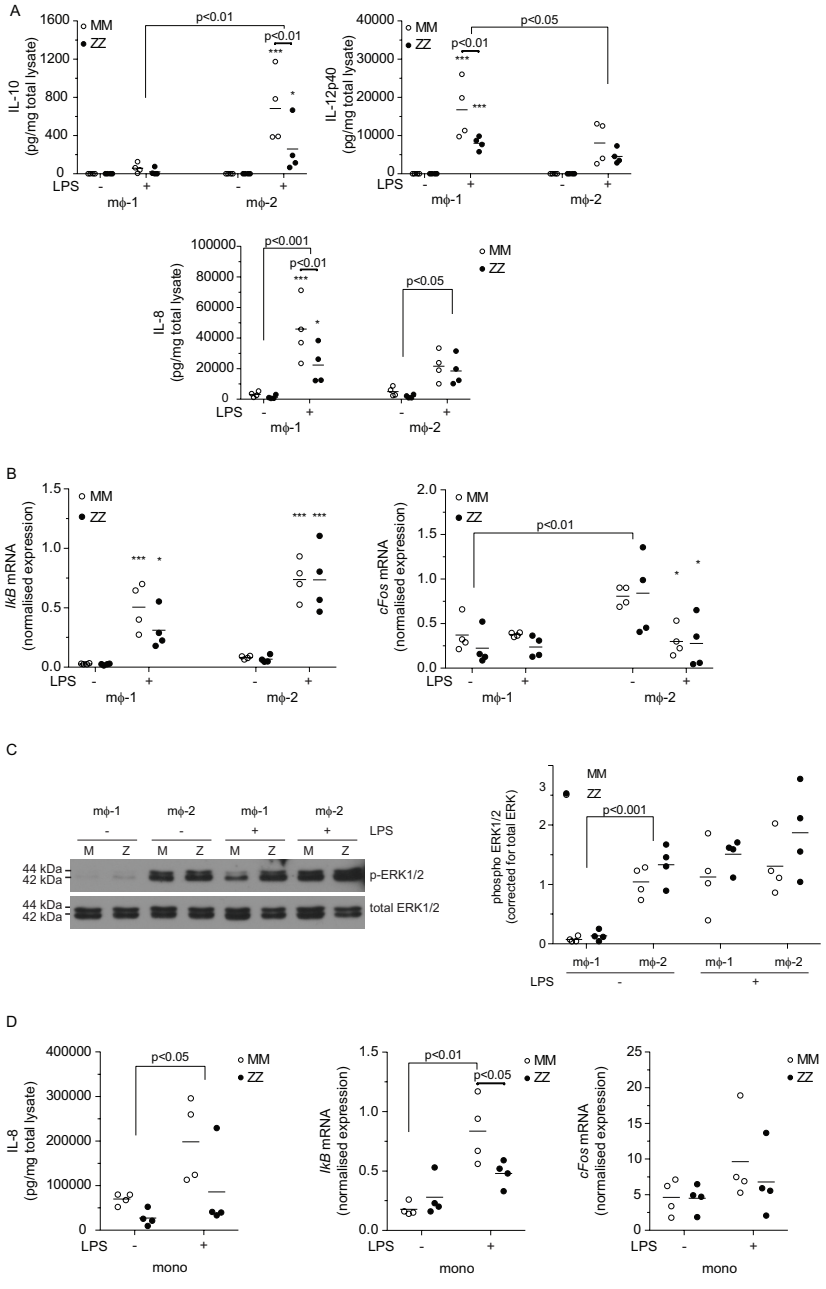
**Production of Z $\alpha_1$-antitrypsin in monocyte-derived macrophages does not alter NF-κB signalling**

We and others (4, 23, 44) have shown that the presence of monomeric Z $\alpha_1$-antitrypsin is associated with an enhanced NF-κB response in epithelial cells, even in the absence of polymers and an exaggerated ER stress response. However, so far the data for monocytes are inconsistent (26, 45) and data for macrophages are lacking. Therefore, we first measured the release of IL-12p40, IL-10 and IL-8 with or without LPS treatment. As shown previously (31, 46), mφ-1 produced more IL-12p40 compared to mφ-2 after 24 hours of LPS, whereas mφ-2 produced more IL-10 (Figure 5A). There was no difference in their IL-8 release. However, in contrast to our expectations, MM macrophages produced enhanced levels of all three cytokines compared with ZZ macrophages after 24 hours LPS ($p<0.01$; Figure 5A). There were no significant differences observed for all cytokines between resting MM and ZZ cells.



To verify whether this difference in cytokine release was caused by an increased NF-κB or ERK1/2 signalling, we measured *IκB* and *cFos* mRNA (Figure 5B) and phosphorylation of ERK1/2 (Figure 5C). We were unable to detect any difference, either basally or after 24 hours of LPS, in *IκB* and *cFos* mRNA or phosphorylation of ERK1/2 between MM and ZZ macrophages. However, mφ-2 showed higher levels of ERK1/2 phosphorylation in resting cells compared to mφ-1 (Figure 5C).

Once again, to ensure that the differentiation of monocytes into macrophages


A
IL-10 (pg/mg total lysate)
IL-12p40 (pg/mg total lysate)
IL-8 (pg/mg total lysate)
MM
ZZ
p<0.01
p<0.05
p<0.001
LPS
mϕ-1
mϕ-2
B
IκB mRNA (normalised expression)
cFos mRNA (normalised expression)
C
mϕ-1
mϕ-2
LPS
M
Z
44 kDa
42 kDa
p-ERK1/2
total ERK1/2
phospho ERK1/2 (corrected for total ERK)
D
mono

<<< **Figure 5. No increased inflammatory response in monocytes and monocyte-derived macrophages from ZZ patients compared to MM controls.** A. IL-8, IL-10 and IL-12p40 release of macrophages type I (mφ-1) and type II (mφ-2) after 24 hours LPS treatment measured by ELISA. B. mRNA levels of *IkB* and *cFos* in mφ-1 and mφ-2 treated as in A. C. Representative western blot of the activation of the MAP kinases ERK1/2 of whole cell lysates from mφ-1 and mφ-2 treated as in A. Densitometry of n=4. D. IL-8, IL-10 and IL-12p40 release of monocytes treated as in A. *p<0.05, **p<0.01, ***p<0.001 versus untreated with a two-way repeated-measurements ANOVA (Bonferroni *post-hoc*).

did not influence our results, we determined *IκB* and *cFos* mRNA and the release of IL-8 (Figure 5D). *IκB* mRNA was higher in MM monocytes compared to ZZ monocytes. Although not significant, this was also observed for IL-8 in the cell supernatant (Figure 5D). These results indicate that the differentiation of monocytes into macrophages does not alter the behaviour of either MM or ZZ cells concerning the parameters measured.

## Discussion

After the discovery of Z $\alpha_1$-antitrypsin polymers in the lung lavage of a ZZ $\alpha_1$-antitrypsin deficiency patient who underwent a liver transplantation ten years before (15), the search for the responsible cell type emerged. We have shown recently that primary bronchial epithelial cells of ZZ $\alpha_1$-antitrypsin deficiency patients are unlikely to be the source of polymers (4). In this study, polymers were also not detectable in both monocytes and monocyte-derived macrophages from ZZ patients. Furthermore, we show that these cells do not show an exaggerated ER stress nor an increased NF-κB response to a second trigger such as LPS.

Interestingly, we have recently shown that resting ZZ $\alpha_1$-antitrypsin primary bronchial epithelial cells display increased NF-κB activation even in the absence of detectable polymers and without an exaggerated ER stress response (4). This enhanced NF-κB response in these cells was explained by the inability to produce significant amounts of Z $\alpha_1$-antitrypsin by these cells to inhibit ERK1/2 phosphorylation via the epidermal growth factor receptor (EGFR) (4). Monocytes are reported to lack substantial EGFR expression (47), which may explain why we were unable to detect this increased NF-κB response in ZZ monocytes and monocyte-derived macrophages. This is in line with Aldonyte *et al*. (45), who showed lower TNFα release by ZZ monocytes. On the other hand, Carroll *et al*. (26) performed a similar study comparing monocytes isolated from peripheral blood from MM and ZZ individuals, where they did find a difference in the release of IL-6, IL-8 and IL-10. We cannot exclude that differences in handling and isolation of monocytes between our study and that of Carroll *et al*. explains the different results. The increase in cytokine release found by Carroll *et al*. was accompanied by the accumulation of Z $\alpha_1$-antitrypsin within the ER of unknown conformation, and an exaggerated ER stress response. We also detected the intracellular retention of Z $\alpha_1$-antitrypsin (Figure 3A-B), since the ratio of $\alpha_1$-antitrypsin in the whole cell lysate and the supernatant was higher in ZZ (up to 3.5 to 1) compared to MM cells (up to 1 to 1). It needs to be noted that these ratios may not be accurate especially for ZZ cells, since the $\alpha_1$-antitrypsin levels measured were close to the limit of detection of the ELISA. Although we obtained preliminary evidence for increased

retention of $\alpha_1$-antitrypsin in ZZ cells, this does not fully explain the difference in secreted $\alpha_1$-antitrypsin between MM and ZZ cells. This conclusion is based on the observation that the total amount of $\alpha_1$-antitrypsin produced (sum of whole cell lysates and supernatant) is lower in ZZ than MM cells (data not shown). It would be interesting to investigate whether the remaining difference between MM and ZZ cells can be explained by degradation of Z $\alpha_1$-antitrypsin via ERAD by treating these cells with a proteasome inhibitor.

Previously, we have determined the critical Z concentration at which Z $\alpha_1$-antitrypsin is likely to form polymers, namely 300 ng/mg total lysate protein (4). In this study, monocytes and monocyte-derived macrophages did not reach this concentration (maximum of 30 ng/mg total lysate protein by MM cells), which could explain why we were unable to detect 2C1-positive polymers intracellularly or in their cell supernatant. It is noteworthy that this critical Z concentration has been established in different epithelial cell lines. Currently, we are unable to exclude the possibility that this concentration might be lower in mononuclear cell lineages.

To our knowledge, this is the first study directly comparing monocytes and monocyte-derived macrophages of ZZ patients and MM controls in response to ER stress and a secondary trigger like LPS. In our opinion, it is important that we have compared these subsets, since it not only excludes the possibility that our findings in the monocyte-derived macrophages could have been explained by alterations in their behaviour whilst differentiating, it also reveals unknown differences between these subsets in the expression of inflammatory markers like *cFos* and *IL8* mRNA and secretion of IL-8, IL-10 and IL-12p40. The cellular mechanisms behind these differences and their biological significance are important issues to be addressed, but beyond the scope of this study.



Although monocyte-derived macrophages are in general a good model to study macrophage behaviour, we and others have shown previously that these cellular subsets *in vitro* might not always represents alveolar macrophages *in vivo* (8, 48). Therefore, theoretically it is still possible that alveolar macrophages are a source of polymers within the lung *in vivo*, although the levels secreted by MM alveolar macrophages are comparable with monocytes and monocyte-derived macrophages *in vitro* (8, 41). However, it needs

to be noted that marked differences exist in the characteristics of alveolar macrophages between patients with and without COPD (reviewed in (34)). For example, alveolar macrophages from COPD patients have been shown to be unable to efficiently ingest microorganisms and apoptotic cells. Interestingly, this inability of COPD cells is already present in monocyte-derived macrophages obtained from peripheral blood of COPD patients. This not only validates the use of these cells *in vitro*, it also illustrates the complexity of defining the ultimate alveolar macrophage phenotype. Future studies with alveolar macrophages obtained from broncho-alveolar lavage of Z $\alpha_1$-antitrypsin deficiency patients will help to better understand the role of macrophages in Z $\alpha_1$-antitrypsin deficiency *in vivo*.

Taken together, this study extends our understanding of the current view of Z $\alpha_1$-antitrypsin polymerisation, exaggerated ER stress response and NF-κB signalling by all cell types expressing Z $\alpha_1$-antitrypsin. However, more research needs to be done to completely understand the underlying mechanisms for these phenomena.

## Material and methods

### Subjects

The ZZ $\alpha_1$-antitrypsin deficiency patients were stable without any sign of an exacerbation. Patient characteristics of these patients can be found in Table 1. Control MM subjects were asymptomatic without evidence of any disease or a (family) history of respiratory disease and/or allergy. They were aged-matched to the ZZ patients, non-smokers and all had a MM genotype as confirmed by reverse transcription polymerase chain reaction (RT-PCR; (35)). Individuals gave written informed consent to take part in this study, as approved by the Medical Ethical Committee of Leiden University Medical Centre, Leiden, the Netherlands.

Table 1. Patient characteristics

| | PiZZ patients (n=7) |
|---|---|
| age (mean, range) | 52 (43-57) |
| sex (M/F) | 3/3 |
| FEV1 (mean, range in %) | 50 (33-84) |
| FEV1/FVC (mean, range in %) | 38 (28-65) |
| Smoking status (current/ex/never) | 0/1/5 |



### Cell culture

Monocytes were isolated from fresh blood and differentiated into mφ-1 or mφ-2 as described previously (8) or used directly as monocytes. Monocytes and monocyte-derived macrophages were pre-incubated with 100 nM thapsigargin (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour and stimulated with 100 ng/ml *Pseudomonas aeruginosa* LPS (Sigma) for 4 or 24 hours as indicated.

**ELISA**

Total and polymerised $\alpha_1$-antitrypsin were measured in cell supernatant by ELISA as described previously (36). Intracellular levels were determined using whole cell lysate. Limit of detection for polymers was 3.0 ng/mg total lysate. Interleukin (IL)-8, IL-10 and IL12p40 was measured as described previously (4, 8).

**Western blot analysis**

Western blot analysis was performed as described previously (4). Briefly, samples were separated on a 10% w/v acrylamide SDS-PAGE gel. Proteins were detected with specific primary antibodies to phospho-ERK1/2, total ERK1/2, and GAPDH (all Cell Signaling Technology, Beverly, MA, USA). GRP78 and GRP94 were visualised by using a monoclonal antibody against the KDEL-sequence (Enzo Life Sciences, Raamsdonksveer, the Netherlands). Although monocytes were seeded in the same density as monocyte-derived macrophages, the protein content of monocytes was too low to perform western blot analysis.

**Quantitative real-time polymerase chain reaction (RT-PCR)**

RNA was isolated using Maxwell RNA extraction (Promega, Madison, WI, USA) according to manufacturer's instructions. Quantitative RT-PCR was performed as described (37) with the primer pairs as described in Table 2.



**Statistical analysis**

Results are expressed as individual donors (each dot is one donor), unless otherwise stated. Data were analysed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) and compared with two-way repeated measurements analysis of variance (ANOVA) and Bonferroni *post-hoc* analysis. Differences were considered statistically significant with p-values $< 0.05$.

**Acknowledgement**

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Table 2. qPCR primers

| Name | Forward primer/ Reverse primer | Melting temp. (°C) | Ref. |
|---|---|---|---|
| *CHOP* | 5' GCACCTCCCAGAGCCCTCACTCTCC 3'<br>5' GTCTACTCCAAGCCTTCCCCCTGCG 3' | 62 | (37) |
| *GADD34* | 5' ATGTATGGTGAGCGAGAGGC 3'<br>5' GCAGTGTCCTTATCAGAAGGC 3' | 62 | (51) |
| *IL8* | 5' CTGGACCCCAAGGAAAAC 3'<br>5' TGGCAACCCTACAACAGA C 3' | 60 | - |
| *XBP1spl* | 5' TGCTGAGTCCGCAGCAGGTG 3'<br>5' GCTGGCAGGCTCTGGGGAAG 3' | 62 | (37) |

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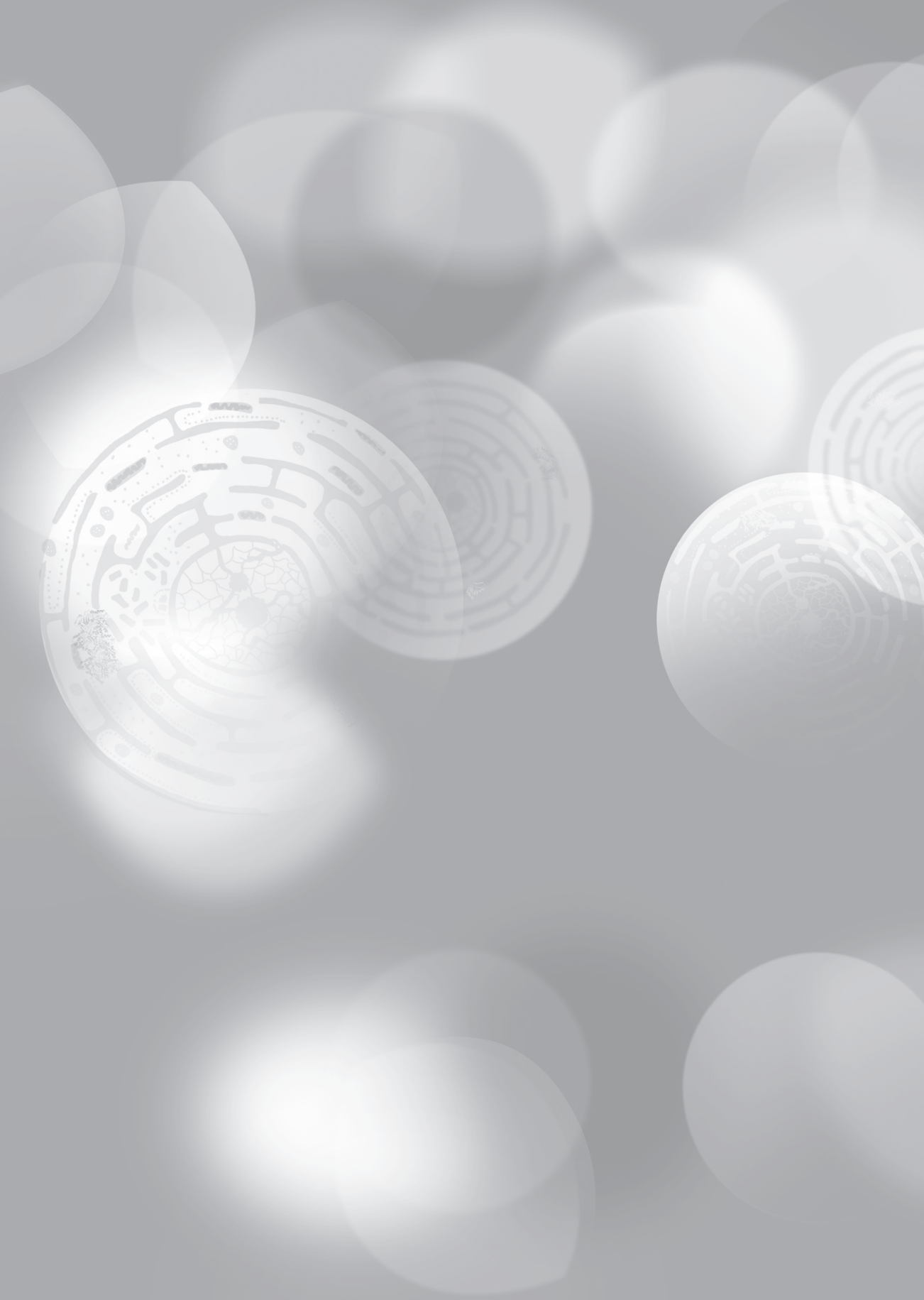

# Chapter 8

**General discussion**

## Summary and general discussion

### Main findings

Stress responses have been shown to play a central role in health and disease in the lung. For instance, acute activation of the integrated stress response (ISR; chapter 2, Figure 1) upon viral infection reduces viral replication and enhances appropriate cytokine release including that of interferons. On the other hand, chronic activation of the integrated stress response, for example by cigarette smoke exposure, may lead to enhanced cell death (reviewed in chapter 2). Until last year, the importance of both the ISR as well as the unfolded protein response (UPR; chapter 1, Figure 2) in bacterial infection received little attention. We examined how virulence factors of *Pseudomonas aeruginosa* elicit both stress responses in bronchial epithelial cells (chapter 4). The induction of endoplasmic reticulum (ER) stress, which was mediated via the TAK1-p38 MAPK pathway, was caused by secreted virulence factors of *P. aeruginosa*. Pyocyanin and alkaline protease were the main contributors to the observed effect. To evaluate the ER stress response and subsequent UPR, we made use of our newly developed primers presented in chapter 3. In this way, we were able to monitor the splicing of *XBP1* mRNA in a reliable and quantitative way. The induction of *GADD34* was caused by the ability of the *Pseudomonas* supernatant to sequester iron leading to iron deficiency for the epithelial cells *in vitro*, which was mediated exclusively by the eIF2α kinase HRI and the ISR. This induction of *GADD34* expression appeared to be cytoprotective for bronchial epithelial cells exposed to conditioned medium of *P. aeruginosa*.



Whereas the study of the ISR remained a relatively small field, research in the role of the ER stress response and UPR in the pathophysiology of lung diseases expanded massively in the past five years. In particular conformational diseases, such as Alzheimer's and Parkinson's diseases, amyloidosis and Z $\alpha_1$-antitrypsin deficiency, gained much attention due to the structural (conformational) change of a mutant protein leading to intracellular polymerisation and eventually disease (1). A subclass of conformational diseases that result from a point mutation in a protein member of the serpin superfamily

of serine proteinase inhibitors, like Z $\alpha_1$-antitrypsin deficiency, are also referred to as serpinopathies. Central questions in the study of serpinopathies are whether protein polymerisation causes ER stress and related cell death (toxic gain of function); how these serpins are related to increased inflammation found in cells expressing mutants (loss of function); and how ER stress and inflammation contribute to the pathophysiology of the associated diseases. In case of Z $\alpha_1$-antitrypsin deficiency, much of the answers to these questions were derived from liver samples or overexpressing models. These studies showed that overexpression of Z $\alpha_1$-antitrypsin leads to accumulation and polymerisation inside the ER, but does not elicit the UPR (2). Remarkably, polymers do prime cells to generate an exaggerated ER stress response upon a so-called second hit (3, 4). Furthermore, polymer formation activates caspases-4 (and -12) and enhances the NF-κB response (2). After the discovery of polymers in bronchoalveolar lavage fluid and lung tissue (5, 6), even 10 years after liver transplantation (7), it was hypothesised that these polymers and the accompanying cellular consequences would contribute massively to the pathophysiology of the lung disease. And so the search for the responsible cell type emerged. Therefore, we investigated whether two of the major sources of $\alpha_1$-antitrypsin in the lung, bronchial epithelial cells (chapter 5) and macrophages (chapter 7), were the cells responsible for the production of these polymers. In chapter 5 we determined the "critical Z $\alpha_1$-antitrypsin concentration"; the minimal concentration of $\alpha_1$-antitrypsin producted by a cell in order to be able to form polymers. We showed that both primary bronchial epithelial cells (chapter 5) and monocyte-derived-macrophages (chapter 7) are able to secrete detectable amounts of $\alpha_1$-antitrypsin, however, they do not reach the concentration threshold to form polymers. Furthermore, monomeric Z $\alpha_1$-antitrypsin does not elicit an exaggerated ER stress response upon a second hit in these cells. Importantly, even in the absence of these polymers, primary bronchial epithelial cells do exhibit an enhanced NF-κB response, dependent of the EGF receptor. This might also explain the absence of this exaggerated response in monocyte-derived macrophages (chapter 7). Since macrophages constitute a heterogeneous population, we studied the two extremes of a continuum of macrophage phenotypes, the pro- (mφ-1) and anti-inflammatory (mφ-2) macrophages, and examined

differences in its $\alpha_1$-antitrypsin production (chapter 6 and 7).

Based on my main findings, this discussion is subdivided into three main topics. First, I will describe macrophage heterogeneity in COPD and its possible implications for $\alpha_1$-antitrypsin deficiency. Then, I will discuss my experiences with measuring the ER stress response, thereafter I address potential therapeutic options for ER stress associated diseases and $\alpha_1$-antitrypsin deficiency.

**Macrophage heterogeneity**

Macrophage diversity enables these cells to possess a wide variety of functions in the innate immune response, such as phagocytosis, the production of effector molecules and antigen presentation to T cells (8). However, the broad range of different macrophage phenotypes is not only explained by their anatomical site, but is also a result of the local environment within one anatomical compartment (8, 9). Furthermore, once differentiated into a subset, a macrophage is still able to adapt to changing conditions, altering its functions, which is termed macrophage plasticity. Although monocytes can be differentiated into distinct macrophage subtypes *in vitro*, the plasticity and dependence on the local milieu are features that make it difficult to mimic *in vivo* monocyte-into-macrophage differentiation (9). Therefore, results derived from two polarised subtypes *in vitro*, the mφ-1 and mφ-2, might not always represent the phenotype of e.g. alveolar macrophages *in vivo*. This was demonstrated in chapter 6, where mφ-1 macrophages produce approximately 4-fold more $\alpha_1$-antitrypsin compared to the mφ-2 macrophages *in vitro*, and both increase production after LPS treatment. However, alveolar macrophages, which display more a mφ-2 phenotype according to their IL-10 production and complete lack of IL-12p40 secretion (chapter 6 and (10)), produce intermediate amounts of $\alpha_1$-antitrypsin *in vitro*, which is unresponsive to LPS stimulation. However, it needs to be noted that marked differences exist in the characteristics of alveolar macrophages between patients with and without COPD (11), illustrating the complexity of defining the ultimate alveolar macrophage phenotype.



Nevertheless, access to locally differentiated alveolar macrophages can be

limited, for instance when it is unethical or too dangerous to perform bronchoscopy. Interestingly, some altered characteristics found in alveolar macrophages can also be detected in monocyte-derived macrophages and even in monocytes. Prieto *et al*. (12) showed that circulating monocytes of COPD patients exhibit decreased phagocytic capacity to *Escherichia coli*, and a similar defect was seen in alveolar macrophages (13). More recently, different studies showed the same phagocytic impairment of the more relevant pathogen *Haemophilus influenzae* by both monocyte-derived macrophages as alveolar macrophages of patients with COPD (13-15). The same impairment was found in the ability to phagocyte apoptotic cells (16, 17); however, the phagocytosis of latex beads did not seem to be altered (15, 17). Often smoking has been proposed to be the underlying cause, since healthy controls are better able to phagocytose apoptotic cells compared to smokers and the same holds for ex-smokers with COPD compared to smoking COPD patients (16). Furthermore, macrophages isolated from induced sputum of smoking COPD patients were shown to have predominantly a mφ-1 phenotype (18), whereas in healthy lungs it would resemble a mφ-2 macrophage and thus would contain an increased ability to phagocytose compared to mφ-1. In line, smoking cessation in COPD has been associated with a phenotype shift back towards the mφ-2 phenotype (19). On the other hand, the same reduced phagocytic capacity in monocytes and monocyte-derived macrophages of COPD patients suggests an inherent defect rather than a consequence of local differentiation or environmental exposure of macrophages in the lung.



Since $\alpha_1$-antitrypsin is known to exhibit also anti-inflammatory properties ((20-22) and chapter 5), a genetic defect could provide additional insights in altered macrophage functions in chronic lung diseases. Indeed, $\alpha_1$-antitrypsin deficiency is associated with rapidly growing mycobacterial infections, and monocyte-derived macrophages treated with $\alpha_1$-antitrypsin appeared to be less prone to infection with *Mycobacterium abscessus* (23). However, whether this is due to a reduced phagocytic capacity needs to be investigated. Furthermore, to my knowledge, it is not known whether the phenotype-shift of alveolar macrophages found in COPD is also found in $\alpha_1$-antitrypsin deficiency.

Interestingly, it has been shown that *in vitro* and *in vivo* treatment with azithromycin improves phagocytic function in alveolar macrophages from COPD patients. Therefore, it would be highly relevant to explore the effect of such treatment on alveolar macrophage function in $\alpha_1$-antitrypsin deficiency, and to study the clinical benefit of such treatment.

**Monitoring ER stress**

The ER stress field developed rapidly with many investigators becoming interested in the UPR. Therefore, many experimental methods have been described to study the UPR. However, some of them are very laborious and others extremely difficult because of the lack of (proper) antibodies or low endogenous expression of certain proteins. Here, I will discuss my experience with different tools to measure and interpret the activation of each arm of the UPR.

PERK is an eIF2α kinase mediating the activation of one of the three branches of the UPR, and is known to have a poor endogenous expression in cells, which makes detection of its phosphorylation (as well as that of IRE1α) hard to measure. Moreover, PERK becomes highly phosphorylated upon activation, so its mobility is altered on SDS-PAGE resulting in an activation-dependent mobility shift (24). When specific questions require the detection of PERK activation, it is important to perform immunoprecipitation of PERK from cell lysates before immunoblotting (24). However, this method is very difficult and both time- and material-consuming. Once PERK is studied in the context of the ISR, and the involvement of this kinase in the activation of the ISR, then the use of knock-out MEFs or silencing RNA interference (RNAi) is advised. Alternatively, one can use downstream markers to evaluate the activation status of this pathway in general. There are good antibodies available to detect the phosphorylation of eIF2α (see Chapter 4), however, it is recommended to treat the cells with care and to replenish the media with pre-warmed (37°C) media one hour before treatment. Moreover, stimulation should be started when the cells are only 50% confluent and the use of an acute stressor as a positive control, such as thapsigargin, is advised to detect a notable change. For the detection of the nuclear proteins ATF4 and CHOP (25, 26), large quantities of nuclear extracts are needed, and

therefore, in my opinion, too expensive to use in primary cell cultures. Additionally, until recently, no appropriate antibodies were available for GADD34. Instead, CHOP, as well as GADD34, can be measured quantitatively at mRNA levels by quantitative RT-PCR (qPCR). Taken together, in most cases, phosphorylation of eIF2α, together with *CHOP* and *GADD34* mRNA levels are sufficient to verify the activation of this pathway. However, detection of these downstream mediators does not allow the identification of PERK or another kinase as being responsible for eIF2α phosphorylation.

Like PERK, phophorylation of IRE1 (the starting point for activation of another branch of the UPR) is difficult to measure, but fortunately detection of its most important downstream target, the splicing of *XBP1* mRNA, is more straight forward. Until 2006, the splicing of *XBP1* mRNA was mainly assessed by detection of PCR fragments following RT-PCR and gel electrophoresis (27). By this method, it was not only difficult to quantify the *spliced XBP1*, it was also very laborious and challenging to obtain a conclusive result. In 2006, Hirota *et al*. (28) introduced a new method by which the spliced form could be quantified by cleavage of the RT-PCR product of double stranded (unspliced) *XBP1* with the restriction enzyme PstI. This method requires the addition of the restriction enzyme after 2-4 cycles of annealing-elongating during the qPCR, and is therefore complex and prone to errors. In our search for reliable and simple methods to screen for the involvement of the UPR, we developed a specific primer for the quantitative detection of *spliced XBP1* by qPCR (Chapter 3). *Spliced XBP1* is highly transcriptionally active and regulates the transcription of UPR target genes via direct binding to the UPR element (UPRE) and ER stress response element (ERSE) when in complex with NF-Y (27). ATF6 is the sensor of the third branch of the UPR, and this transcription factor binds to and acts via the ERSE as well (29). Therefore, separation of the UPR target genes belonging to *spliced XBP1* specifically is difficult. Using *Xbp1*$^{-/-}$ and *Atf6*$^{-/-}$ MEFs treated with tunicamycin, Lee *et al*. (30) identified ERdj4, a heat shock protein 40 family member that interacts with ERAD (31) and chaperone p58$^{IPK}$ as specific downstream targets of *spliced XBP1*.



Interestingly, the same group did not identify a specific marker for ATF6. However, both Kaufman's lab (32) and Mori's lab (33, 34) performed transcriptional profile analysis

and microarray analyses on *ATF6α*$^{-/-}$ MEFs. Both groups, independently, pointed towards Derlin-3, a functional component of the ERAD, as a sole downstream target. It is worth mentioning that in both screens *p58*$^{IPK}$ was significantly reduced in *ATF6α*$^{-/-}$ treated with tunicamycin, which suggests that p58$^{IPK}$ may not be specific for detection of *spliced XBP1*. Due to this shared binding site, namely the ERSE, the opposite might be true, with so-called ATF6 specific genes also being responsive to *spliced XBP1*. For instance, bronchial epithelial cells transfected with the commonly used specific ATF6 luciferase reporter plasmid (p5xATF6-luc), showed a 15-fold increase in luciferase activity when treated with tunicamycin for 6 hours. Remarkably, in my hands, this increase was totally abolished when the cells were co-stimulated with 4μ8C (35), a specific IRE1 inhibitor (unpublished data).

A rather general, but important indicator of ER stress is the detection of the KDEL sequence (Lys-Asp-Glu-Leu). This sequence keeps soluble ER resident proteins, such as Glucose Regulated Proteins 78 and 94 (GRP78; also referred to as BiP and GRP94, respectively) and Protein Disulphide Isomerase (PDI) retained inside the ER. Especially the chaperone GRP78 is a key regulator in ER stress, since the activation of the UPR is dependent on its release from the three ER stress transducers. Activation of the three branches of the UPR eventually lead to the induction of UPR target genes, amongst which GRP78. Therefore, GRP78 up regulation is indicative for an ER undergoing stress.

## Therapeutic approaches

### ER stress mediated diseases

As reviewed in **Chapter 2**, there is increasing evidence of the involvement of the ISR in various chronic lung diseases and lung cancer, which has mainly been associated with an increased PERK activity. Chronic ER stress is in most cases detrimental for the cell, leading to cell death and worsening of the disease, whereas in cancer, increased PERK signalling favours tumour progression and cancer cell survival (36, 37). For that reason, the inhibition of the PERK-arm is currently receiving much attention and significant advances in therapeutical approaches to block this kinase have been made over the last years. However, ER stress-mediated cell death is not only induced via PERK, but can also be triggered via the IRE1α-JNK signalling cascade. Therefore, inhibition of this arm might be relevant as well. In 2012, the laboratorium of David Ron (35) used high-throughput screening to identify the aforementioned 4μ8C as a potent inhibitor of IRE1. Interestingly, this compound not only inhibits the ER stress kinase IRE1α, but is also predicted to be able to inhibit the second isoform, IRE1β (38, 39). The expression of this second IRE1 was initially reported to be restricted to the epithelial cells in the gastrointestinal tract (40). It has long been suggested that this kinase regulates ER homeostasis of goblet cells via an interaction with mucin production. Furthermore, it was assumed that this kinase might also be expressed in lung goblet cells to regulate mucin expression. However, no real evidence existed until very recently, when Tsuru *et al*. (39) showed that *IRE1β*$^{-/-}$ mice showed increased *mucin 2* (*MUC2*) mRNA stability and accumulation of MUC2 protein in the ER of intestinal goblet cells. Four months later, Martino *et al*. (41) presented the same findings for airway goblet cells. In line with this finding, I found that fully differentiated primary bronchial epithelial cells treated with 4μ8C showed reduced expression *MUC5AC* and *MUC2* mRNA and decreased secretion of mucin 5AC (unpublished data). Therefore, it is a potential therapeutic target for patients with ER stress-associated lung diseases and mucus overproduction, like COPD and cystic fibrosis.

Also during chronic infections, ER stress and activation of the UPR are important

causes of cell death. For *P. aeruginosa*, *spliced XBP1* has recently been shown to be crucial in N-(3-oxo-dodecanoyl) homoserine lactone (C12)-mediated apoptosis (42). Blocking IRE1 might be relevant to counteract this effect. In contrast, in chapter 4, we have shown that *GADD34* serves a cytoprotective role in human bronchial epithelial cells and mouse fibroblasts upon exposure to secreted virulence factors of *P. aeruginosa*. Inhibition of *GADD34* would in this case be detrimental. We have not investigated the presence and contribution of C12 in our conditioned medium from *P. aeruginosa* to the observed effects, which should be done in the future as this might be relevant to further reduce the cytotoxicity of *P. aeruginosa* in bronchial epithelial cells. Another approach to treat *P. aeruginosa* infection that needs to be considered is the inhibition of individual virulence factors to reduce the interference with the physiological ISR and ER stress response of the host. However, we were unable to ascribe the observed effect to a single virulence factor, making this approach more complex. Future studies will give us more insights in the contribution of each virulence factor, possibly opening up new therapeutic targets.

Although the development of many ER stress inhibitors, such as the PERK inhibitor (43) and the IRE1α inhibitor 4μ8C (35), are still in the early stages, it has demonstrated the importance of the right balance in ISR activation, and further development of inhibitors might lead to therapeutic options in the different lung diseases. Recently, encouraging results were obtained using a PERK inhibitor that inhibited neurodegeneration in prion-infected mice (44). However, one should bear in mind that only the stressed cells need targeting, since a physiological and functional ISR is crucial to maintain healthy tissue.



**Alpha$_1$-antitrypsin deficiency**

The discovery of the association between mutants of $\alpha_1$-antitrypsin and early-onset lung emphysema made the protease-antiprotease imbalance hypothesis the dominant model for the development of the disease. However, restoring this imbalance with intravenous $\alpha_1$-antitrypsin augmentation therapy did not cure the disease nor had its expected effects on disease progression (45). For this reason, $\alpha_1$-antitrypsin augmentation therapy remains unsupported for the treatment of $\alpha_1$-antitrypsin deficiency in several

countries, amongst which the Netherlands and the United Kingdom.

**Treating the defect**

The group of David Lomas at the University of Cambridge (now relocated to University College London) made major contributions in the development of novel strategies to treat $\alpha_1$-antitrypsin deficiency. They showed that small molecules that block surface cavities of $\alpha_1$-antitrypsin could inhibit polymerisation *in vitro* and *in vivo* (46). However, this treatment also inactivated $\alpha_1$-antitrypsin as a neutrophil elastase inhibitor (46). Moreover, the effective concentrations were expected to be too toxic to use in patients. Due to the enormous potential of this approach as a treatment, future research must certainly provide us new small molecules that are less toxic.

The polymers found in the lung are thought to arise from local production, as they have been reported to be still present in bronchoalveolar lavage fluid ten years after liver transplantation (7). Since we showed in chapter 5 and 7 that both cultured primary bronchial epithelial cells and monocyte-derived macrophages from ZZ patients do not produce these polymers, the origin of these polymers is still unknown. The main question to be answered, in my opinion, is whether only one specific type of cells is responsible for the polymer formation or whether $\alpha_1$-antitrypsin secreted locally by various cells is retained in the interstitium and epithelial lining fluid, and that at these sites the critical Z $\alpha_1$-antitrypsin concentrations is reached that is required for extracellular polymer formation. Another explanation could be that the extracellular milieu favours polymer formation, and thus lower secreted concentrations might allow polymerisation. Once we know the answer, we might be able to use the small molecules also as a potential lung therapy.



A second approach for developing a curative treatment for $\alpha_1$-antitrypsin deficiency is the use of genetic corrections and induced pluripotent stem cells (iPS). Rashid *et al*. (47) presented the derivation of iPS from fibroblasts of skin biopsies of $\alpha_1$-antitrypsin deficiency patients. Genetic correction of the point mutation underlying the Z mutation (E342K) restored $\alpha_1$-antitrypsin production and its proper activity in iPS-

derived liver-like cells (48). The challenge remains to differentiate these corrected liver cells to fully mature hepatocytes and to restore the mutations that resulted from the iPS development. Another approach was used by the Birmingham group, who showed that $\alpha_1$-antitrypsin secretion from monocytes carrying the Z mutation can be corrected with small DNA fragments encoding M $\alpha_1$-antitrypsin (49). Whether this approach can form the basis for development of new treatments for $\alpha_1$-antitrypsin deficiency is unclear at present.

**Blocking the MEK-EGFR pathway**

Based on the studies described in this thesis (chapter 5 and 7), we could argue that bronchial epithelial cells are the main contributors to the local inflammation in the lungs of $\alpha_1$-antitrypsin deficiency patients. Even in the absence of polymers, they exhibit an increased inflammatory response as demonstrated by increased ERK1/2 signalling and subsequent cytokine release. Interestingly, we were able to reduce these effects by treating the cells with extracellular M $\alpha_1$-antitrypsin. This might implicate that inhaled $\alpha_1$-antitrypsin augmentation therapy could diminish local inflammation through an effect on for example neutrophil influx and survival, and local macrophage differentiation. In my opinion, the primary outcome of a first clinical proof-of-concept study using inhaled $\alpha_1$-antitrypsin should therefore be the measurement of inflammatory markers, such as IL-8, in bronchoalveolar lavage and/or sputum. Subsequent more long-term studies would be needed to explore the effect on lung function (including $FEV_1$, VC, CO diffusion and single-breath nitrogen test) and alveolar pathology as assessed by densitometric CT scans. Secondary outcomes could comprise bacterial colonisation and exacerbation rates. However, I would like to stress that these read-outs are very complex and not solely dependent on increased inflammation, and therefore possibly not conclusive. Furthermore, the increased ERK1/2 phosphorylation was dependent on the 'classical' MEK-EGFR pathway, which is shared with non-small cell lung carcinoma and certain breast cancers (50, 51). Major therapeutic advances have already been made in the cancer field, and the use of existing MEK inhibitors, EGFR monoclonal antibodies or tyrosine kinase

inhibitors should also be considered as a treatment for $\alpha_1$-antitrypsin deficiency related emphysema. Further research has to be done to confirm these therapeutic options as actual treatment in $\alpha_1$-antitrypsin deficiency. However, it needs to be noted that a safety and efficacy trial with an EGFR inhibitor in COPD patients did not show convincing evidence for an effect on mucin stores and furthermore was associated with adverse effects (52).

## Future directions

This thesis started with the initial question about the role of polymers and its relation to ER stress in the development of Z $\alpha_1$-antitrypsin deficiency related emphysema. When the years progressed, it became evident that polymers might not be produced locally by lung cells and that monomeric Z $\alpha_1$-antitrypsin does not prime cells for an exaggerated ER stress response upon a second hit. In contrast, we can conclude that there is a prominent role for ER stress in other lung related diseases and bacterial infections. Two important questions have been raised by these studies, but still remain to be answered in full:

1) Stress responses during bacterial infection:

- What is the role of the each virulence factor in the induction of ER stress and the ER stress-independent ISR by *P. aeruginosa* and can we use that for future treatment?
- Are these mechanisms specific for virulence factors of *P. aeruginosa*, or are they applicable to other (Gram negative) respiratory pathogens as well?



2) Alpha$_1$-antitrypsin deficiency:

- Is there local production of Z $\alpha_1$-antitrypsin polymers in the lung?
- Would aerosolised $\alpha_1$-antitrypsin be suitable as a therapy to reduce lung inflammation?

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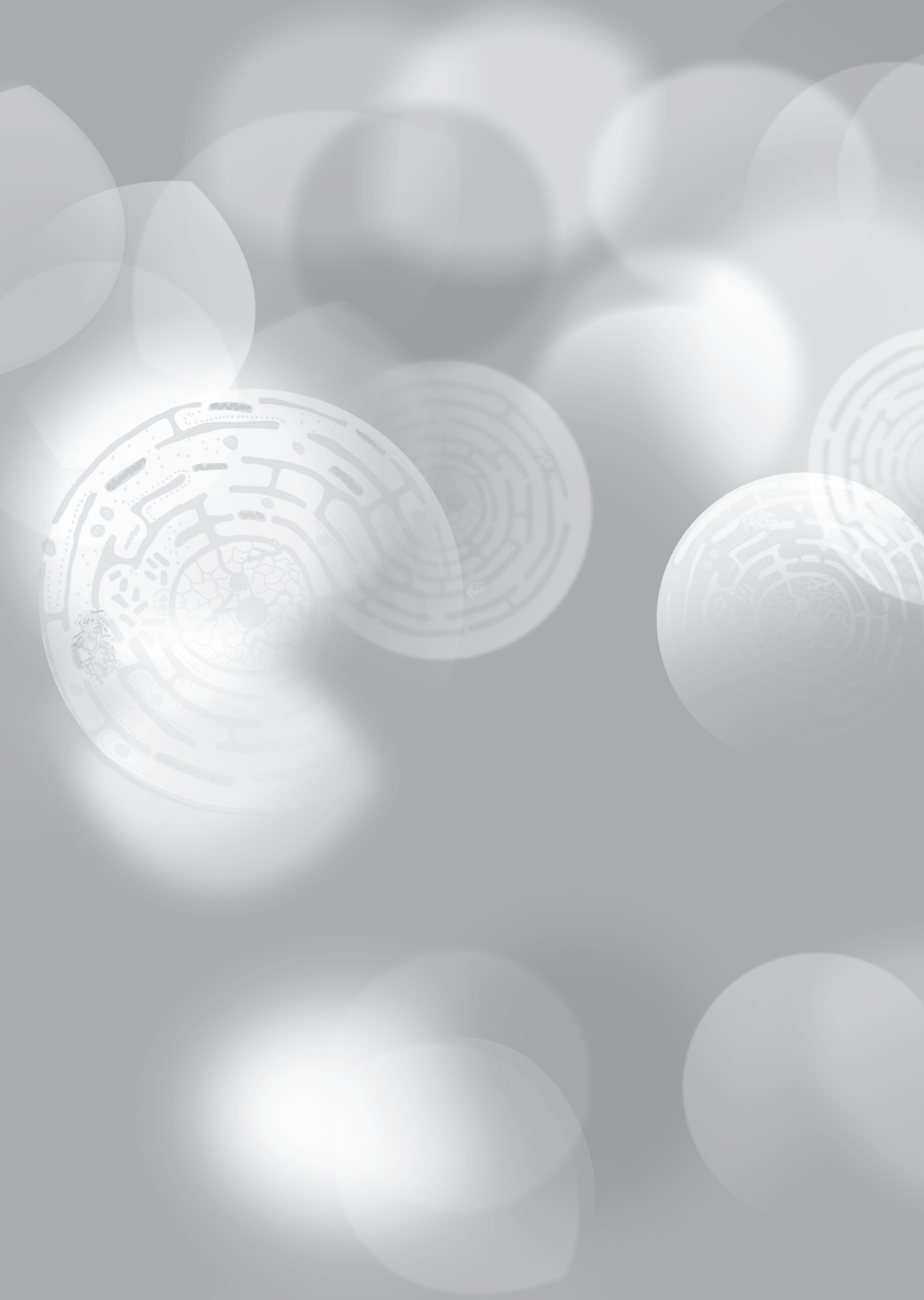

# Addendum

**Nederlandse samenvatting**

**Publications**

**Curriculum Vitae**

**Dankwoord (acknowledgements)**

## Nederlandse samenvatting

Chronic obstructive pulmonary disease (chronisch obstructieve longziekte; afgekort COPD), een verzamelnaam voor longemfyseem en chronische bronchitis, is een irreversibele chronische longziekte die voornamelijk wordt veroorzaakt door roken en mogelijk ook door luchtvervuiling. Door het roken worden de longblaasjes definitief afgebroken (longemfyseem) en raken de luchtwegen ontstoken waarbij vaak slijm wordt gevormd (chronische bronchitis). Behalve roken kunnen ook erfelijke afwijkingen lijden tot de ontwikkeling van longemfyseem. De bekendste en meest belangrijke genetische risicofactor is een tekort aan $\alpha_1$-antitrypsine ($\alpha_1$-antitrypsine deficiëntie), waarbij mensen door een mutatie een erfelijk tekort hebben aan het eiwit $\alpha_1$-antitrypsine. In de gezonde long is $\alpha_1$-antitrypsine een remmer van eiwit-afbrekende enzymen die vrijkomen bij ontsteking (proteasen), en draagt op die manier bij aan het beschermen van de long tegen weefselschade die kan ontstaan tijdens een ontstekingsproces. Alfa$_1$-antitrypsine wordt vooral in de lever aangemaakt en bereikt de long via de bloedbaan, maar wordt ook lokaal in de long door macrofagen en epitheelcellen geproduceerd. De concentratie $\alpha_1$-antitrypsine in de long echter wordt in hoge mate bepaald door productie in de lever. Bij pasgeborenen kan een mutatie in $\alpha_1$-antitrypsine leiden tot ernstige leverontstekingen (hepatitis) en geelzucht, en bij volwassenen, en met name rokers, tot een zeer sterk verhoogde kans op de ontwikkeling van longemfyseem vanaf het 35$^e$ levensjaar.

De productie van eiwitten in een cel begint in de nucleus (kern) met het aflezen van DNA: de transcriptie. Het ontstane messenger-RNA (mRNA) verplaatst naar het cytoplasma, waar het door de ribosomen vertaald wordt naar een aminozuurketting (polypeptide): de translatie. Dit polypeptide wordt in het endoplasmatisch reticulum (ER) gevouwen in zijn tertiaire structuur: de posttranslationele modificatie. Alleen correct gevouwen eiwitten zijn biologisch actief en worden uitgescheiden door het ER. Dus naast het vouwen van eiwitten, vervult het ER ook een functie in de kwaliteitscontrole van gevormde eiwitten. Het vouwen van eiwitten kan door verschillende zaken verstoord worden, met als gevolg een ophoping van verkeerd gevouwen eiwitten in het ER, wat ER stress oplevert. De complexe fysiologische cellulaire reactie van het ER heeft als doel

de homeostase in een cel te herstellen door enerzijds het aanbod van nieuw te vouwen eiwitten te reduceren (translationele repressie) en anderzijds de capaciteit van het vouwen te verhogen. Bij langdurige ER stress geeft deze respons aanleiding tot een verhoogde ontstekingsreactie en geprogrammeerde celdood. Naast ER stress kunnen drie andere oorzaken een translationele repressie geven, te weten ijzergebrek, virus infecties en aminozuurtekorten; dit wordt gezamenlijk de Integrated Stress Reponse (ISR) genoemd.

Veruit de meest voorkomende mutatie die leidt tot $\alpha_1$-antitrypsine deficiëntie is de zogenaamde Z mutatie. Bij deze mutatie wordt het Z $\alpha_1$-antitrypsine nog wel gemaakt in de lever, maar kan het eiwit zich niet goed vouwen in het ER. Dit leidt niet alleen tot ophoping van het Z $\alpha_1$-antitrypsine in het ER, maar ook tot samenklontering van de opgehoopte eiwitten (polymeren) en een beperkte uitscheiding in de bloedbaan. Door het ontstane tekort aan $\alpha_1$-antitrypsine wordt de long lokaal onvoldoende beschermd tegen de proteasen die vrijkomen bij de door o.a. roken ontstane ontsteking; daarnaast dragen de polymeren van Z $\alpha_1$-antitrypsine lokaal in de long ook zelf bij aan de ontsteking doordat ze ontstekingscellen aantrekken (chemotaxie).

Het proces van polymeervorming en de vraag of deze polymeren ook ER stress veroorzaken is uitgebreid onderzocht in levercellen. Interessant hierbij is dat er in deze cellen geen ER stress optreedt, ondanks de ophoping van fout-gevouwen Z $\alpha_1$-antitrypsine in het ER. Echter, de cellen die polymeren bevatten zijn wel extra gevoelig voor ER stress veroorzaakt door processen van buitenaf, met als gevolg dat deze cellen meer ontstekingsmediatoren uitscheiden en sneller dood gaan. In de afgelopen jaren rees de vraag of dit proces zich niet alleen in levercellen maar ook lokaal in de macrofagen en epitheelcellen van de long afspeelt, waardoor deze cellen ook zelf zouden kunnen bijdragen aan het ontstekingsproces in de long. Die vraag staat in dit proefschrift centraal. Daarnaast worden in dit proefschrift andere (extracellulaire) oorzaken van de ER stress respons bestudeerd.

In **hoofdstuk 1** wordt een algemene inleiding over COPD en $\alpha_1$-antitrypsine deficiëntie, de ER stress respons en cellulaire signaalverwerking bij ontstekingsprocessen gegeven. De ER stress respons is een complexe respons, die overlap heeft met andere stress responsen, zoals de eerder genoemde Integrated Stress Response (ISR). In de laatste jaren is steeds meer bekend geworden over de rol van de ISR in het ontstaan en het beloop van verschillende soorten longziekten, waaronder $\alpha_1$-antitrypsine deficiëntie. In **hoofdstuk 2** wordt deze kennis in detail besproken.

In **hoofdstuk 3** wordt een nieuwe techniek beschreven voor het nauwkeurig en kwantitatief meten van een centrale pijler in de ER stress respons, namelijk de splitsing van het mRNA dat de informatie bevat voor het X-box binding protein-1 (XBP-1). Deze techniek wordt vervolgens ook gebruikt in het onderzoek dat staat beschreven in **hoofdstuk 4, 5** en **7**.

Behalve intrinsieke factoren, zoals gen mutaties, kunnen ook extrinsieke factoren leiden tot de ophoping van eiwitten in het ER. Het is bijvoorbeeld bekend dat virussen voor een overmatige eiwitproductie in een cel zorgen, zodat niet alle eiwitten verwerkt kunnen worden en deze ophopen in het ER en ER stress geven. Tot voor kort was niet bekend of bacteriën eenzelfde fenomeen konden veroorzaken. In **hoofdstuk 4** staat onderzoek centraal naar de ER stress respons en de ISR in luchtwegepitheelcellen die werden blootgesteld aan virulentie factoren van de bacterie *Pseudomonas aeruginosa*. Hieruit bleek dat de inductie van de ISR, en dan met name productie van GADD34 via de ijzer gereguleerde receptor HRI, cruciaal was voor de overleving van in het laboratorium gekweekte luchtwegepitheelcellen.

De gevolgen van Z $\alpha_1$-antitrypsine en de polymeren in levercellen is uitgebreid bestudeerd en het is al langere tijd bekend dat deze polymeren ook aanwezig zijn in de longen, en in één patiënt zelfs tien jaar na een levertransplantatie. Lokale productie van deze polymeren en de cellulaire consequenties voor deze cellen zouden in grote mate kunnen bijdragen aan de ontwikkeling van longemfyseem, en mogelijk ook in het onderhouden van de ziekte. In **hoofdstuk 5** en **7** werden longepitheelcellen en macrofagen, de twee grootste bronnen van lokale $\alpha_1$-antitrypsine productie in de long, onderzocht

op het produceren van polymeren. In **hoofdstuk 5** werd de "kritieke Z $\alpha_1$-antitrypsine concentratie" (de drempel) vastgesteld waarbij cellen overgaan tot het produceren van de polymeren. Uit deze drempelconcentratie bleek dat zowel luchtwegepitheelcellen (**hoofdstuk 5**) als macrofagen (**hoofdstuk 7**) niet voldoende Z $\alpha_1$-antitrypsine produceren om polymeervorming mogelijk te maken. Uit beide hoofdstukken bleek ook dat bij de afwezigheid van deze polymeren geen overgevoeligheid bestaat voor ER stress. Aan de andere kant produceren de longepitheelcellen van Z $\alpha_1$-antitrypsine deficiënte patiënten wel overmatig veel ontstekingsmediatoren (**hoofdstuk 5**), en dragen daarmee dus bij aan de verhoogde ontstekingsreactie in de long (hyperinflammatie). Macrofagen vertonen deze "hyperinflammatoire" respons niet (**hoofdstuk 7**), waarschijnlijk omdat deze cellen de EGF receptor (de receptor verantwoordelijk voor de ontstekingsreactie in de epitheelcellen) nauwelijks tot expressie brengen. Daarnaast werden in **hoofdstuk 6** en **7** verschillen in $\alpha_1$-antitrypsine productie door verschillende soorten macrofagen onderzocht. Daarin werd gevonden dat bij zowel gezonde mensen als bij $\alpha_1$-antitrypsine deficiënte patiënten de ontstekingsbevorderende macrofagen veel meer $\alpha_1$-antitrypsine maken dan de ontstekingsremmende macrofagen.

Deze resultaten geven ons meer inzicht in het ziekteproces bij patiënten met type ZZ $\alpha_1$-antitrypsine deficiëntie met longemfyseem. Naar aanleiding van de uitkomsten van **hoofdstuk 5** zou bijvoorbeeld een therapie met EGF receptor blokkers of geïnhaleerd $\alpha_1$-antitrypsine tot de mogelijkheden behoren om zo de ontstekingsreactie lokaal in de long te onderdrukken. Hier moet echter meer onderzoek voor gedaan worden om daarover duidelijkheid te krijgen.

## Publications

van 't Wout EFA, Hiemstra PS, Marciniak SJ (2014) The integrated stress response in lung diseases, *Am J Respir Cell Mol Biol*., in press

van 't Wout EFA, Dickens JA, van Schadewijk A, Haq I, Kwok HF, Ordóñez A, Murphy G, Stolk J, Lomas, DA, Hiemstra PS, Marciniak SJ (2014) Increased ERK signalling promotes inflammatory signalling in primary airway epithelium expressing Z $\alpha_1$-antitrypsin, *Hum Mol Gen*. 23:929-41

Thomas SE, Malzer E, Ordóñez A, Dalton LE, van 't Wout, EFA, Liniker E, Crowther DC, Lomas DA, Marciniak SJ (2013) p53 and translation attenuation regulate distinct cell cycle checkpoints during endoplasmic reticulum (ER) stress, *J Biol Chem*. 288:7606-17

van 't Wout EFA, van Schadewijk A, Savage NDL, Stolk J, Hiemstra PS (2012) Production of $\alpha_1$-antitrypsin by pro- and anti-inflammatory macrophages and dendritic cells, *Am J Respir Cell Mol Biol*. 46(5):607-13.

van Schadewijk A, van 't Wout EFA, Stolk J, Hiemstra PS (2012) A quantitative method for detection of spliced X-box binding protein-1 (XBP1) mRNA as a measure of endoplasmic reticulum (ER) stress*, Cell Stress Chaperones*. 17(2):275-9

van 't Wout EFA, van Schadewijk A, van Boxtel R, Dalton LE, Clarke HJ, Tommassen J, Marciniak SJ, Hiemstra PS (2014) Virulence factors of *Pseudomonas aeruginosa* induce both the unfolded protein and integrated stress responses, submitted

van 't Wout EFA, van Schadewijk A, Lomas DA, Stolk J, Marciniak SJ, Hiemstra PS (2014) Monocytes and monocyte-derived macrophages function in $\alpha_1$-antitrypsin deficiency, submitted

Ninaber MK, Hamersma WBGJ, Schouffoer AA, van 't Wout EFA, Stolk J (2014) The global peripheral chemoreflex drive in patients with systemic sclerosis: a rebreathing and exercise study, submitted

## Curriculum Vitae

Emily Fiona Ariëlle van 't Wout was born on 20 April 1987 in Moerkapelle, the Netherlands. After graduating cum laude for her pre-university education at the Erasmus College, Zoetermeer, the Netherlands, she started studying Medicine in 2005 at the University of Leiden, the Netherlands. In addition to her study, she conducted a research project on the chemoreflex responsiveness in sclerodermia at the Department of Pulmonology under supervision of Dr. Jan Stolk and Maarten K. Ninaber. In March 2009, she started her scientific project as a medical student on nicotine-acetylcholine receptors in COPD and $\alpha_1$-antitrypsin deficiency at the Department of Pulmonology with Prof. dr. Pieter S. Hiemstra and Dr. Jan Stolk. After the completion of her doctorate in September 2009, she started her PhD project in the same department, of which the results are described in this thesis. As part of her PhD, she conducted a three months traineeship in the laboratory of Prof. dr. David A. Lomas and Dr. Stefan J. Marciniak at the Cambridge Institute for Medical Research (University of Cambridge, United Kingdom). She subsequently received the European Alpha$_1$-antitrypsin Laurell's Training Award which allowed her to finish the last year of her PhD at the University of Cambridge, United Kingdom. In January 2014 she started her clinical rotations to obtain her MD degree.

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Lab Longziekten, bedankt voor de gezellige tijd. Marianne en Renate, ik zal nooit zo goed worden als jullie in het kweken van cellen, maar ik heb genoten van jullie privélessen in mijn beginperiode! Annemarie, heel erg bedankt voor het begeleiden van mijn eerste babystapjes in de wondere wereld van het lab. Ik vond het fijn dat ik jouw eerste student mocht zijn (en ook gelijk de laatste, al weet ik nou niet of dat een heel goed teken is) en al je ondersteuning tijdens mijn werk als promovenda en daarna.

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