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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 α_1-band on plasma protein electrophoresis (7). After this initial finding, over 100 variants of α_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 α_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 α_1-antitrypsin deficiency cases (8). Approximately 4% of the affected individuals are heterozygous (PiMZ) for this variant,

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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 α_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 α_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 α_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 α_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 α_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 α_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 α_1-antitrypsin is transcribed and translated at the same rate as normal M α_1-antitrypsin, the mutation affects post-translational folding of the Z α_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 α_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 α_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
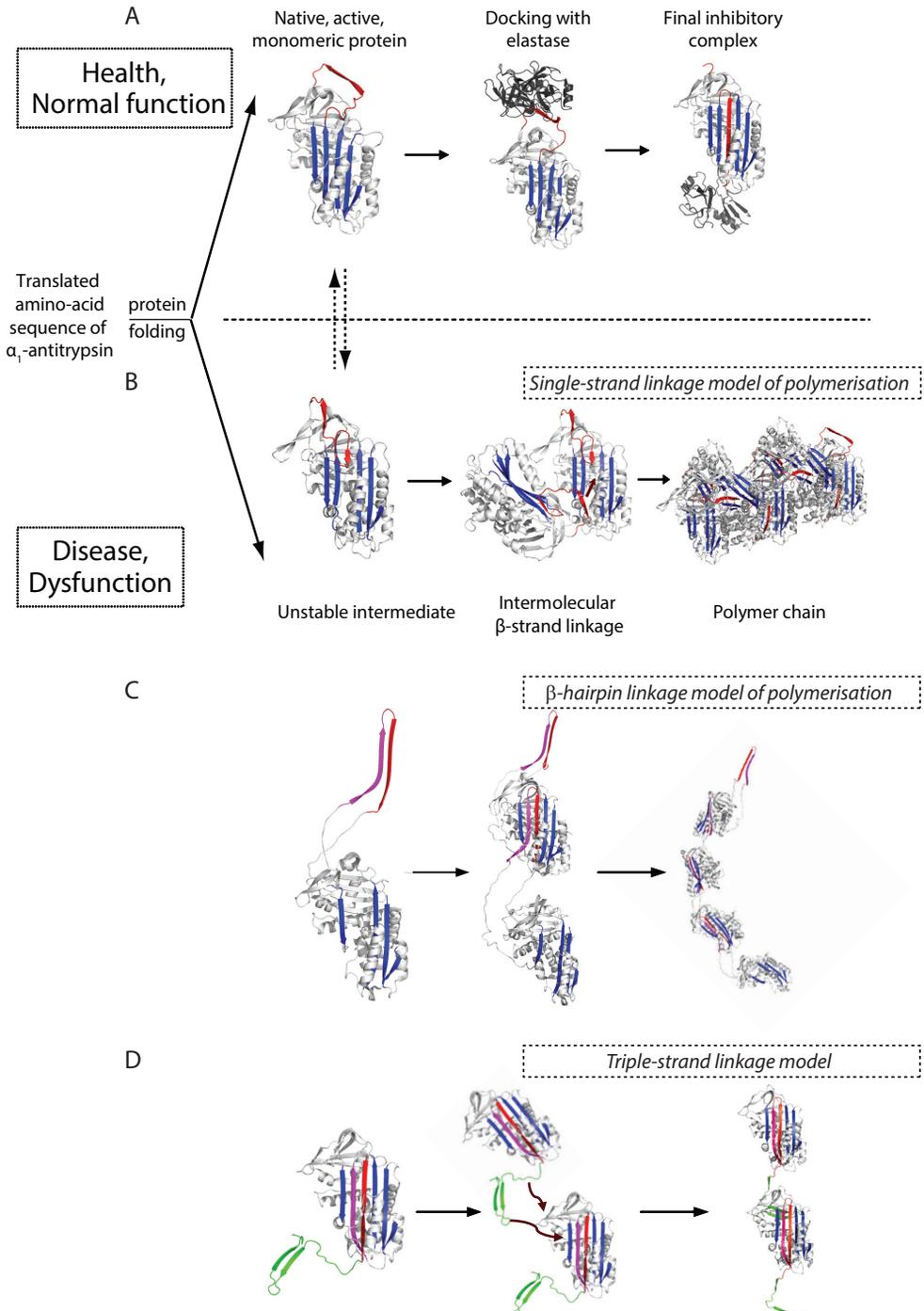
proposed that these polymers can be produced locally within the lung. As these polymers are chemotactic for neutrophils *in vivo* and *in vitro*, such local formation might contribute to sustained inflammation in subjects with α_1-antitrypsin deficiency (41).

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

Health,
Normal function

Native, active,
monomeric protein

Docking with
elastase

Final inhibitory
complex

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 α₁-antitrypsin and its misfolding in α₁-antitrypsin deficiency.**

A. The reactive centre loop (red) of native α₁-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 α₁-antitrypsin polymerisation. All mechanisms involve an unstable intermediate. In the single-strand linkage model (B), the reactive centre loop of one α₁-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 α₁-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
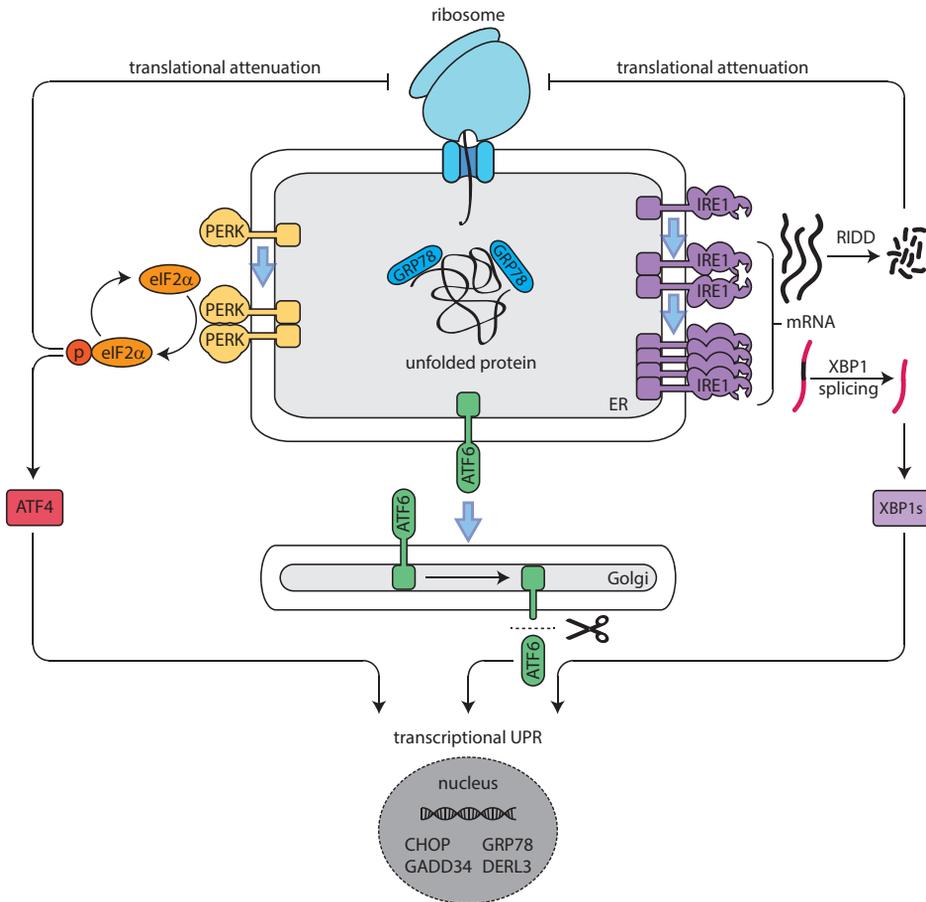
Figure 2. The unfolded protein response (UPR).

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 α$_1$-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 α$_1$-antitrypsin deficiency. However, over the years, α$_1$-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 α$_1$-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 α$_1$-antitrypsin may prime cells to ER stress if exposed to a second insult (82, 83). For instance, Chinese hamster ovary (CHO) cells overexpressing Z α$_1$-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 α$_1$-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 α$_1$-antitrypsin show reduced levels of apoptosis and decreased caspase-3 activity, similar to M α$_1$-antitrypsin expressing 16HBE cells (81). The enhanced sensitivity of Z α$_1$-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 α$_1$-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 α$_1$-antitrypsin in ZZ monocytes (80). It is worth noting, however, that

the conformation of the retained α_1-antitrypsin protein remained unclear and so the mechanism for the hyperinflammatory phenotype of Z α_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).
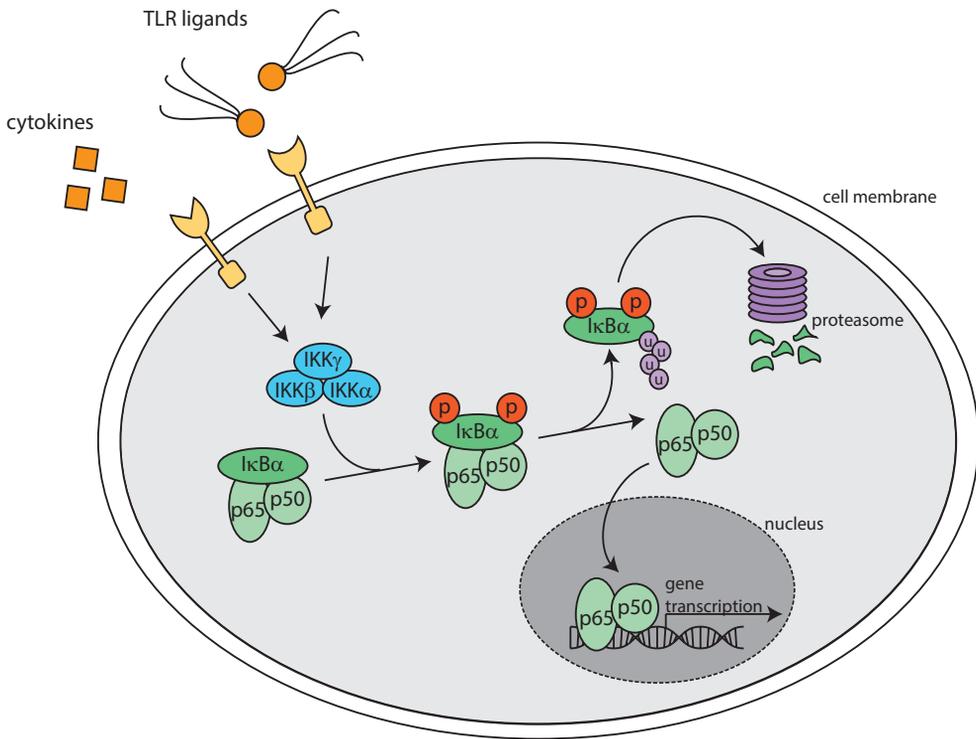
Figure 3. Activation of the NF-κB pathway.

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 α_1-antitrypsin and its accumulation within the ER has led some to suggest that α_1-antitrypsin deficiency might represent an ER stress-induced disease. However, as discussed above, there is much controversy about the extent to which Z α_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 α_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 α_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 α_1-antitrypsin. We explored the possibility of polymer formation as an underlying mechanism and investigated whether endogenous Z α_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 α_1-antitrypsin. Since macrophages constitute a heterogeneous population, we first investigated in **Chapter 6** whether there is a difference in α_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 α_1-antitrypsin production by monocyte-derived macrophages of Z α_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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