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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 *XBPI* 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 α1-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 α₁-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 α₁-antitrypsin deficiency, much of the answers to these questions were derived from liver samples or overexpressing models. These studies showed that overexpression of Z α₁-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 α₁-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 α₁-antitrypsin concentration”; the minimal concentration of α₁-antitrypsin produced 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 α₁-antitrypsin, however, they do not reach the concentration threshold to form polymers. Furthermore, monomeric Z α₁-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 α₁-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 α₁-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 α₁-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 α₁-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 α₁-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 α₁-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, α₁-antitrypsin deficiency is associated with rapidly growing mycobacterial infections, and monocyte-derived macrophages treated with α₁-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 α₁-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 α₁-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, phosphorylation 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 straightforward. 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 p58IPK 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 $ATF6^{α/−}$ 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 $p58^{1PK}$ was significantly reduced in $ATF6^{α/−}$ treated with tunicamycin, which suggests that $p58^{1PK}$ 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<sup>−/−</sup> 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 \textit{P. aeruginosa}, spliced \textit{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 \textit{GADD34} serves a cytoprotective role in human bronchial epithelial cells and mouse fibroblasts upon exposure to secreted virulence factors of \textit{P. aeruginosa}. Inhibition of \textit{GADD34} would in this case be detrimental. We have not investigated the presence and contribution of C12 in our conditioned medium from \textit{P. aeruginosa} to the observed effects, which should be done in the future as this might be relevant to further reduce the cytotoxicity of \textit{P. aeruginosa} in bronchial epithelial cells. Another approach to treat \textit{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 IRE1\(\alpha\) inhibitor 4\(\mu\)8C (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.

\textbf{Alpha\textsubscript{1}-antitrypsin deficiency}

The discovery of the association between mutants of \(\alpha\textsubscript{1}\)-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 \(\alpha\textsubscript{1}\)-antitrypsin augmentation therapy did not cure the disease nor had its expected effects on disease progression (45). For this reason, \(\alpha\textsubscript{1}\)-antitrypsin augmentation therapy remains unsupported for the treatment of \(\alpha\textsubscript{1}\)-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 α₁-antitrypsin deficiency. They showed that small molecules that block surface cavities of α₁-antitrypsin could inhibit polymerisation *in vitro* and *in vivo* (46). However, this treatment also inactivated α₁-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 α₁-antitrypsin secreted locally by various cells is retained in the interstitium and epithelial lining fluid, and that at these sites the critical Z α₁-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 α₁-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 α₁-antitrypsin deficiency patients. Genetic correction of the point mutation underlying the Z mutation (E342K) restored α₁-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 α₁-antitrypsin secretion from monocytes carrying the Z mutation can be corrected with small DNA fragments encoding M α₁-antitrypsin (49). Whether this approach can form the basis for development of new treatments for α₁-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 α₁-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 α₁-antitrypsin. This might implicate that inhaled α₁-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 α₁-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 FEV₁, 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 α1-antitrypsin deficiency related emphysema. Further research has to be done to confirm these therapeutic options as actual treatment in α1-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 α1-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 α1-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 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) Alpha1-antitrypsin deficiency:
   - Is there local production of Z α1-antitrypsin polymers in the lung?
   - Would aerosolised α1-antitrypsin be suitable as a therapy to reduce lung inflammation?
References


