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Function of monocytes and monocyte-derived macrophages in α₁-antitrypsin deficiency

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Abstract

Alpha_1-antitrypsin deficiency is the most widely recognised genetic disorder causing COPD. Mutant Z α_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 α_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 α_1-antitrypsin polymers were not detectable by ELISA. These data suggest that monocyte-derived macrophages are not the local source of Z α_1-antitrypsin polymers found in the lung and that the ER stress response and pro-inflammatory cytokine release is not altered.
Introduction

Alpha₁-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, α₁-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 α₁-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 α₁-antitrypsin comprises more than 95% of the mutations leading to severe α₁-antitrypsin deficiency. Due to this mutation, the Z α₁-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 α₁-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 α₁-antitrypsin homozygote. In 2004, Mulgrew et al. (15) showed that Z α₁-antitrypsin polymers could still be detected in lung lavage ten years after liver transplantation, suggesting local secretion and polymerisation of Z α₁-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 α₁-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 α₁-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 α₁-antitrypsin polymers (25). In addition to the enhanced sensitivity to ER stress, cells expressing Z α₁-antitrypsin also display an augmented NF-kB 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 α1-antitrypsin and subsequent activation of the UPR in monocytes from homozygous Z α1-antitrypsin deficiency patients. Since we have shown previously differential α1-antitrypsin production by different macrophage subsets (8), we set out to test the hypothesis that mφ-1 macrophages are able to produce Z α1-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.
Function of macrophage subsets in α₁-antitrypsin deficiency

- **A** spliced XBP1 mRNA (normalised expression)
  - 4 hours
  - 24 hours

- **B** spliced CHOP mRNA (normalised expression)
  - 4 hours
  - 24 hours

- **C** spliced GADD34 mRNA (normalised expression)
  - 4 hours
  - 24 hours

- **D** spliced GRP78 mRNA (normalised expression)
  - 4 hours
  - 24 hours
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 \( α_1 \)-antitrypsin (8, 41). However, it remains unknown whether macrophages from \( Z \) \( α_1 \)-antitrypsin patients (ZZ cells) are a source of \( Z \) \( α_1 \)-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 \( α_1 \)-antitrypsin compared to anti-inflammatory mφ-2 macrophages in both MM and ZZ cells (\( p<0.001 \); Figure 3A). As expected, the levels of \( α_1 \)-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 \( Z \) \( α_1 \)-antitrypsin (Figure 3B). The production of \( α_1 \)-antitrypsin in both mφ-1 and mφ-2 was increased after 24 hours LPS treatment (\( p<0.05 \) and \( p<0.01 \), respectively;
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Figure 3A-B). When we used the 2C1 monoclonal antibody to specifically detect naturally occurring α₁-antitrypsin polymers, we were unable to detect Z α₁-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 α₁-antitrypsin and polymer production of monocytes from the same donors. Unstimulated monocytes released equal amounts of total α₁-antitrypsin measured in the cell supernatant compared to mφ-2 (Figure 3A), and did not significantly up-regulate the total α₁-antitrypsin production after LPS treatment. Interestingly, the intracellular α₁-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 α₁-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 α₁-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 α₁-antitrypsin. However, the conformation of this retained Z α₁-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. Alpha₁-antitrypsin production by monocyte-derived macrophages from ZZ patients and MM controls.

A. Total α₁-antitrypsin (AAT) and α₁-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 α₁-antitrypsin and α₁-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 α₁-antitrypsin production, these cells were stimulated with LPS. After 24 hours, mφ-2 from Z α₁-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 α₁-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 α₁-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
did not influence our results, we determined \(\text{IκB}\) and \(\text{cFos}\) mRNA and the release of IL-8 (Figure 5D). \(\text{IκB}\) 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 α₁-antitrypsin polymers in the lung lavage of a ZZ α₁-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 α₁-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 α₁-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 α₁-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 α₁-antitrypsin within the ER of unknown conformation, and an exaggerated ER stress response. We also detected the intracellular retention of Z α₁-antitrypsin (Figure 3A-B), since the ratio of α₁-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 α₁-antitrypsin levels measured were close to the limit of detection of the ELISA. Although we obtained preliminary evidence for increased
retention of α₁-antitrypsin in ZZ cells, this does not fully explain the difference in secreted α₁-antitrypsin between MM and ZZ cells. This conclusion is based on the observation that the total amount of α₁-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 α₁-antitrypsin via ERAD by treating these cells with a proteasome inhibitor.

Previously, we have determined the critical Z concentration at which Z α₁-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 α₁-antitrypsin deficiency patients will help to better understand the role of macrophages in Z α₁-antitrypsin deficiency in vivo.

Taken together, this study extends our understanding of the current view of Z α₁-antitrypsin polymerisation, exaggerated ER stress response and NF-κB signalling by all cell types expressing Z α₁-antitrypsin. However, more research needs to be done to completely understand the underlying mechanisms for these phenomena.
Material and methods

Subjects

The ZZ α₁-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

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<td>sex (M/F)</td>
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<td>FEV1 (mean, range in %)</td>
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<td>FEV1/FVC (mean, range in %)</td>
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<td>Smoking status (current/ex/never)</td>
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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 α1-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

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