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Endoplasmic reticulum stress in the lung : lessons from α1-antitrypsin deficiency

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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 α_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 α_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 α_1-antitrypsin secretion (7).

While hepatocytes are considered as the primary source of α_1-antitrypsin, human lung epithelial cells, monocytes and alveolar macrophages have also been shown to produce α_1-antitrypsin (7-10). Although these cells produce substantially lower amounts of α_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 α_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 α_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 α_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 α_1-antitrypsin.

Results

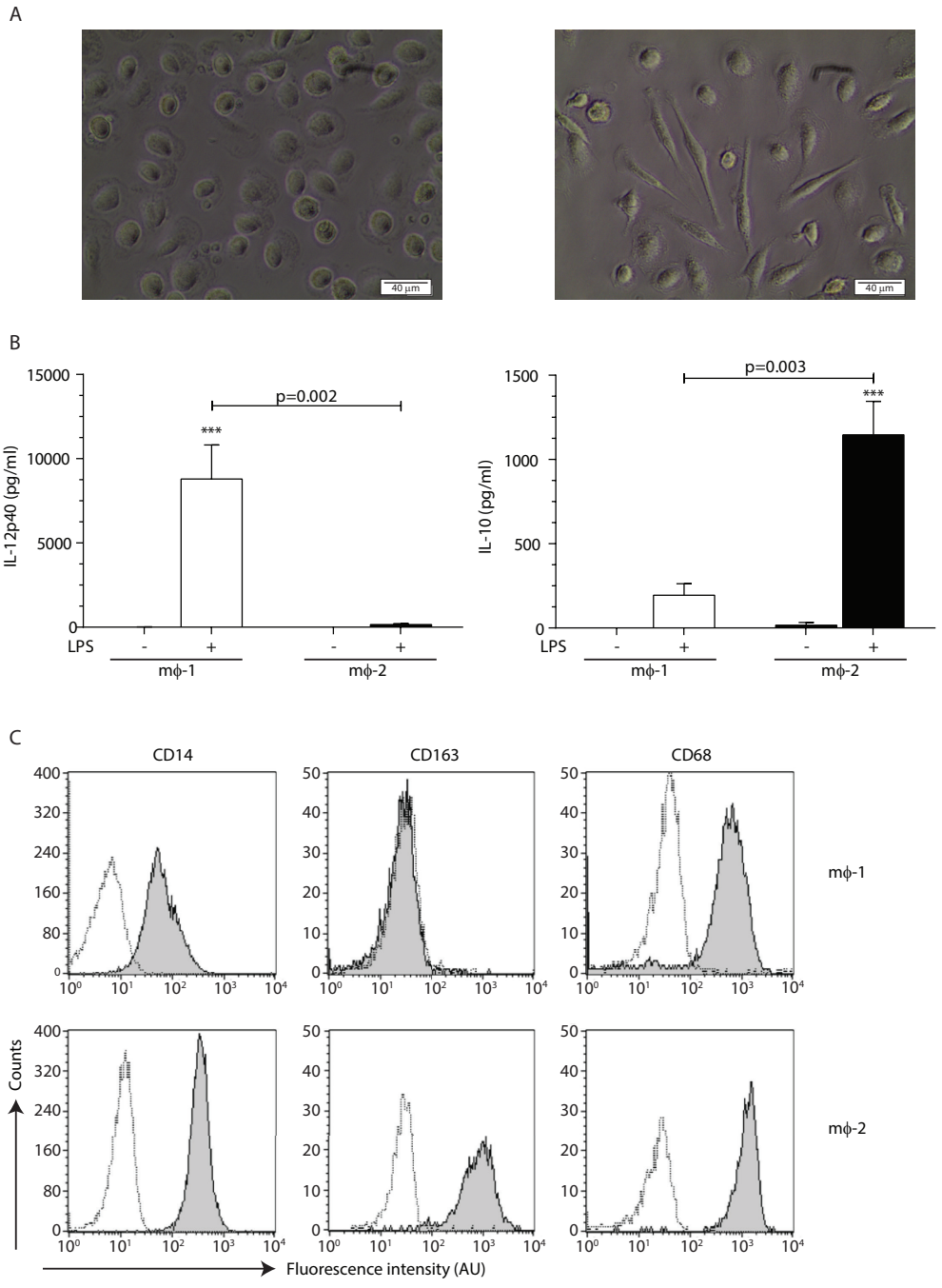
Differentiation of mφ-1 and mφ-2

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 α_1-antitrypsin than mφ-2

To evaluate whether the production of α_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 α_1-antitrypsin expression in lung epithelial cells and monocytes (7-9). Spontaneous release of α_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 α_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 α_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 α_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)

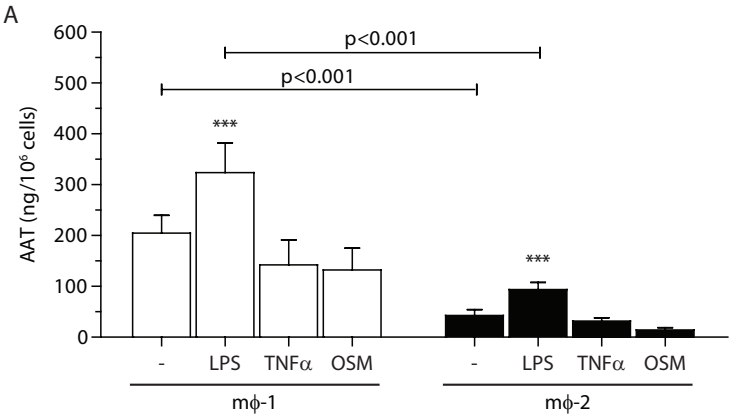
<<< **Figure 1. Differentiation of human monocytes into pro- (mφ-1) and anti-inflammatory (mφ-2) macrophages.**

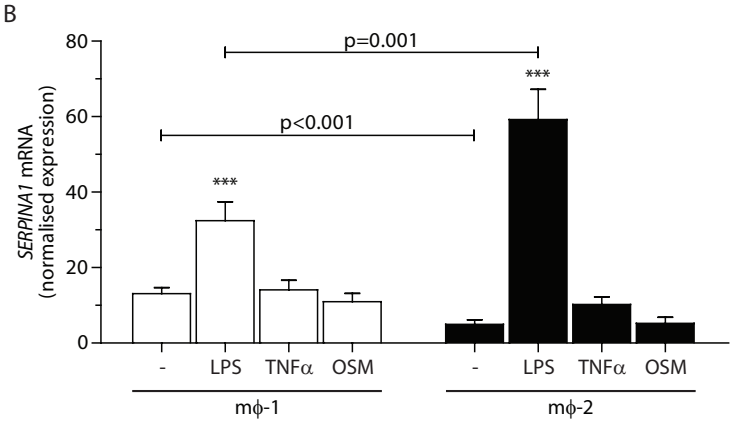
Human monocytes were cultured for 6 days in the presence of GM-CSF or M-CSF to obtain mφ-1 and mφ-2, respectively. A. After 6 days, mφ-1 (left) typically showed a 'fried-egg' morphology; mφ-2 (right) appeared as 'spindle-like' cells as visualised by phase contrast microscopy. Images are representative 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.

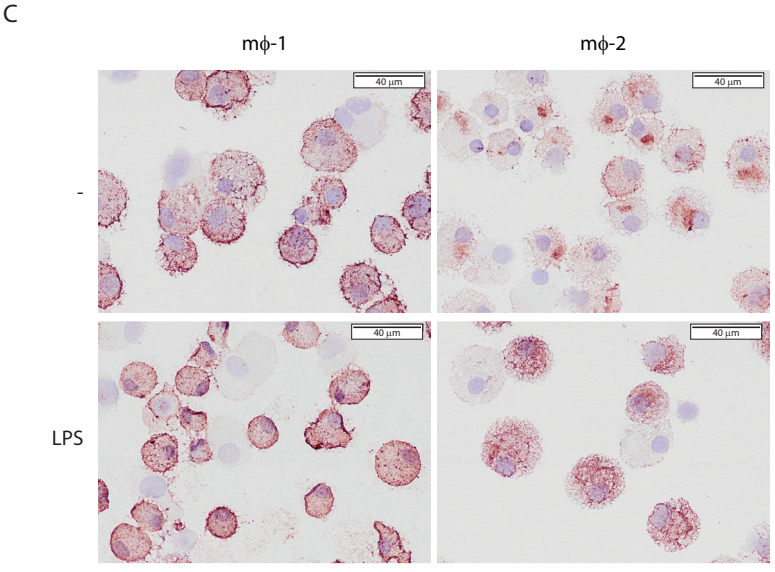
in the cell supernatant, cell-associated α_1-antitrypsin of mφ-1 and mφ-2 was examined by immunocytochemistry. Analysis showed that in mφ-1 compared to mφ-2, the staining intensity of cell-associated α_1-antitrypsin was markedly higher, and that mφ-1 cells showed a more membrane-associated pattern (Figure 2C). These findings suggest that the increased *SERPINA1* mRNA after LPS stimulation observed in mφ-1, is directly translated and secreted as α_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 α_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
***
***
-
LPS
TNFα
OSM
-
LPS
TNFα
OSM
mϕ-1
mϕ-2


C
mϕ-1
mϕ-2
-
LPS
40 µm
40 µm
40 µm
40 µm

<<< **Figure 2. Effects of LPS, tumor necrosis factor alpha (TNFα) and oncostatin M (OSM) on α_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 α_1-antitrypsin to visualise cell-associated/intracellular α_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 α_1-antitrypsin after LPS exposure for 48 hour was higher in mφ-2 compared to mφ-1 (Figure 3A), the absolute levels of secreted α_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 α_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 α_1-antitrypsin secretion was dependent on de novo mRNA synthesis and/or de novo protein synthesis, cells were treated with actinomycin D or

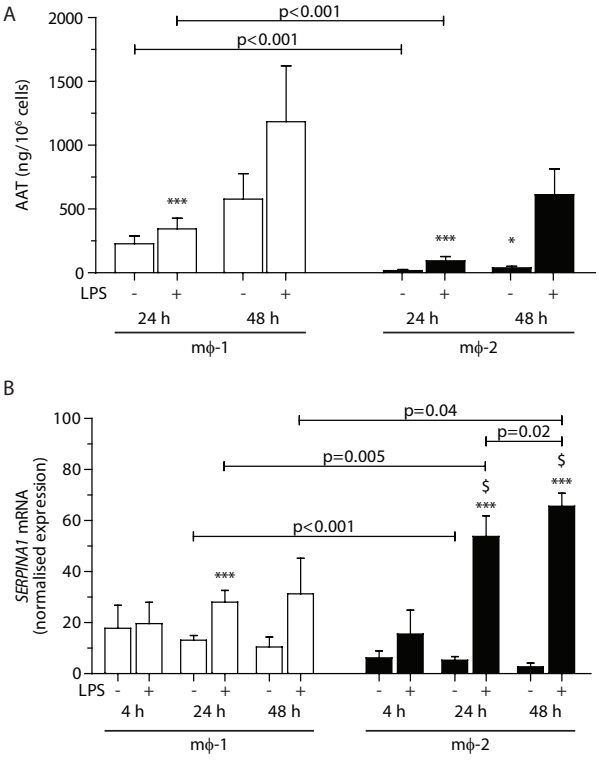


Figure 3. Kinetics of α_1-antitrypsin expression and secretion.

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 α_1-antitrypsin protein in LPS treated cells (data not shown). Similarly, blocking the de novo protein synthesis by cycloheximide showed reduced secreted amounts of α_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 α_1-antitrypsin in mφ-1 and mφ-2.

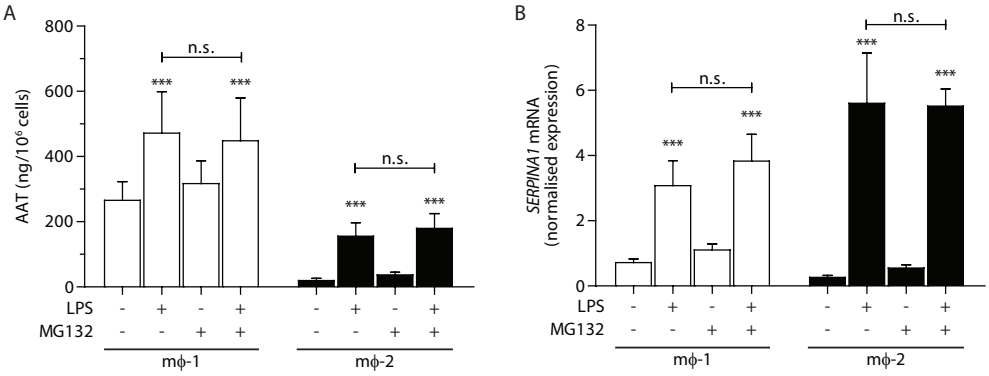


Figure 4. Effects of the proteasome inhibitor MG132 on α_1-antitrypsin (AAT) production of mφ-1 and mφ-2.

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 α_1-antitrypsin as an intermediate phenotype

To investigate whether all monocyte-derived cell-lineages are able to produce α_1-antitrypsin, immature monocyte-derived dendritic cells (iDC) were stimulated with LPS for 24 hours. Immature DC released 65 ng/10^6 cells α_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 α_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 α_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

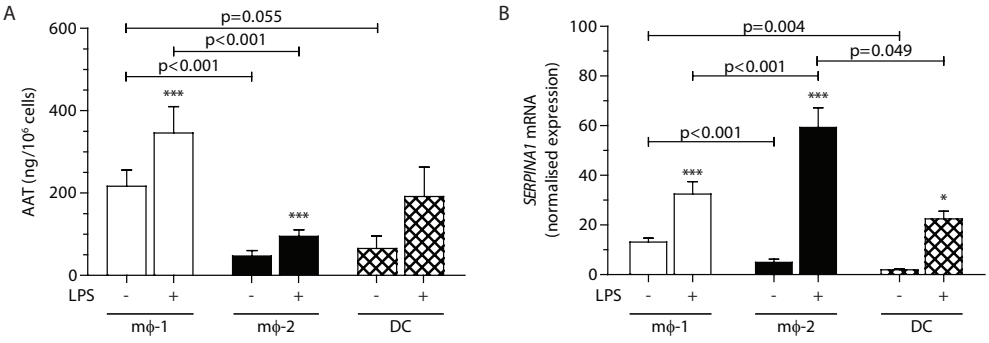


Figure 5. Effects of LPS on α_1-antitrypsin production of dendritic cells (DC) compared to mφ-1 and mφ-2.

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).

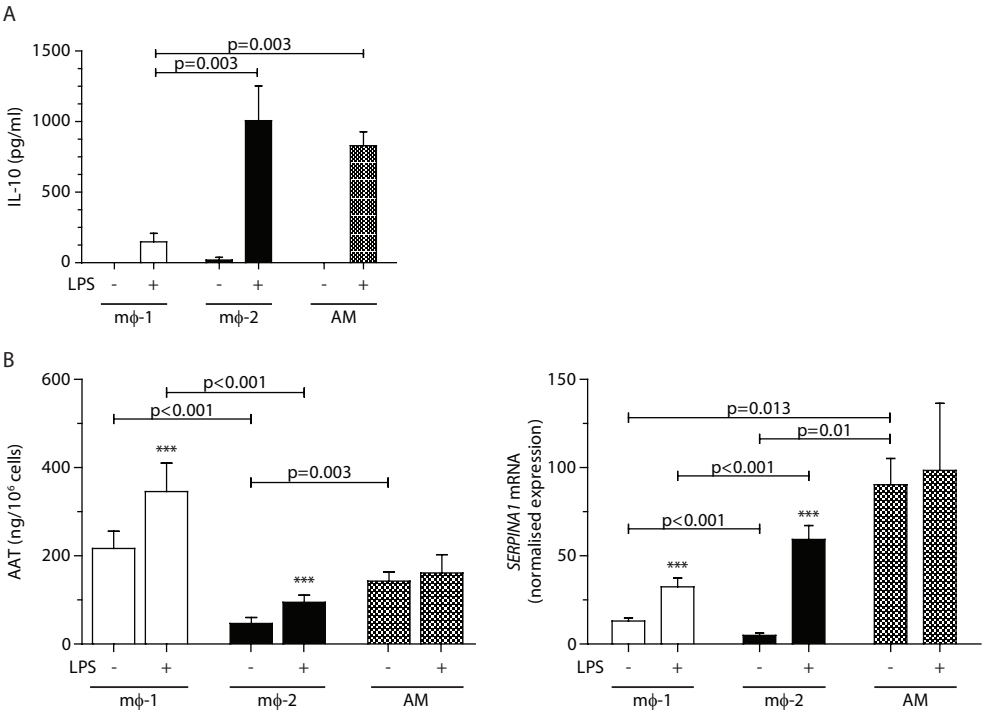
of α_1-antitrypsin by alveolar macrophages, we stimulated alveolar macrophages for 24 hours in the presence of LPS. Characterisation of these cells by measuring IL-12p40/IL-23p40 and IL-10 secretion and evaluating cell surface markers showed that these alveolar macrophages produce high amounts of IL-10 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 α_1-antitrypsin, which was not further increased by LPS stimulation (162 ng/10^6 cells). These α_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 α_1-antitrypsin was comparable with mφ-1 (Figure 6C), suggesting that produced α_1-antitrypsin remains cell-associated.

Discussion

In the present study, we demonstrated that differentiated monocyte-derived macrophages, monocyte-derived DC and alveolar macrophages produce α_1-antitrypsin, although to different extent. Only LPS could significantly induce *SERPINA1* mRNA synthesis and α_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 α_1-antitrypsin.

Many lung diseases, including α_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 α_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 α_1-antitrypsin production by these cells (8, 10, 24), and that both LPS and TNFα could up-regulate α_1-antitrypsin in monocytes (9). In contrast, Perlmutter *et al.* (25) showed earlier the inability of monocytes to release α_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 α_1-antitrypsin. The role of DC-produced α_1-antitrypsin in the regulation of the protease-antiprotease imbalance should be further investigated.

Although α_1-antitrypsin is the major antiprotease present in the lung, α_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 α_1-antichymotrypsin or SLPI produced by mφ-1, mφ-2 or DC with ELISA, although it has been reported that alveolar macrophages produce α_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
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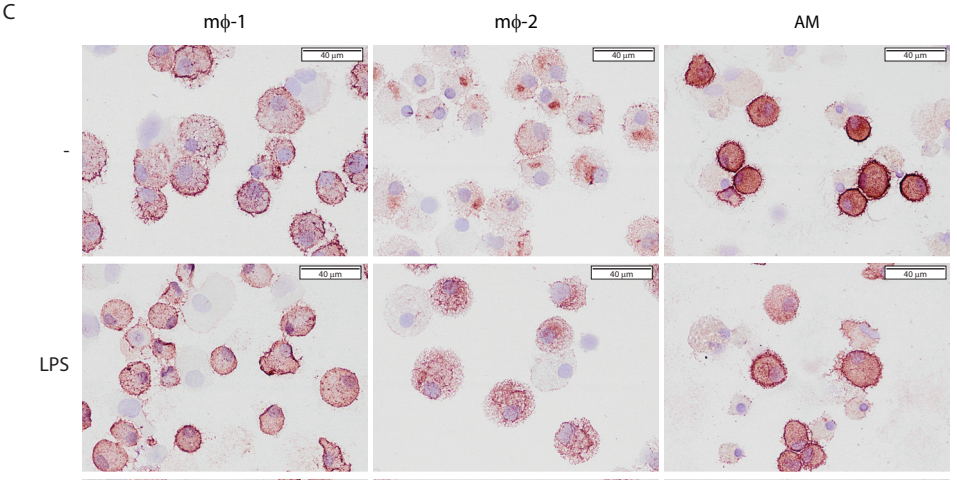
p=0.003
p=0.003
LPS - + - + - +
mϕ-1
mϕ-2
AM
B
AAT (ng/10^6 cells)
600
400
200
0
p<0.001
p<0.001
***
p=0.003
***
SERPINA1 mRNA (normalised expression)
150
100
50
0
p=0.013
p=0.01
p<0.001
p<0.001
***
***
LPS - + - + - +
mϕ-1
mϕ-2
AM


C
mϕ-1
mϕ-2
AM
-
LPS
40 µm

<<< **Figure 6. Effects of LPS on α_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 α_1-antitrypsin to visualise cell-associated/intracellular α_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 α_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 α_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 α_1-antitrypsin. At present, it is unclear whether the inability of LPS to increase α_1-antitrypsin secretion in alveolar macrophages and the fact that these cells produce high amounts of both α_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 α_1-antitrypsin production: mφ-1 release more α_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 α_1-antitrypsin secretion. Experiments inhibiting the proteasome did not provide any insights regarding the discrepancies, excluding the possibility of enhanced intracellular degradation of α_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 α_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 α_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 α_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 α_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 α_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-α_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 α_1-antitrypsin and α_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 α_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.

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