Function of monocytes and monocyte-derived macrophages in α_1-antitrypsin deficiency

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ABSTRACT α_1-antitrypsin deficiency is the most widely recognised genetic disorder causing chronic obstructive pulmonary disease (COPD). Mutant Z α_1-antitrypsin expression has previously been linked to intracellular accumulation and polymerisation of this proteinase inhibitor. Subsequently, this has been described to underlie an exaggerated endoplasmic reticulum stress response and enhanced nuclear factor-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 granulocyte-macrophage or macrophage colony-stimulating factor to obtain pro- and anti-inflammatory macrophages (mφ-1 and mφ-2, respectively).

We first showed that, in contrast to monocytes, pre-stressed mφ-1 and mφ-2 from healthy blood donors display an enhanced endoplasmic reticulum stress response upon a lipopolysaccharide trigger (*XBP1* splicing, *CHOP*, *GADD34* and *GRP78* mRNA). However, this endoplasmic reticulum stress response did not differ between monocyte-derived macrophages and monocytes from ZZ patients compared to MM controls. Furthermore, these ZZ cells 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 endoplasmic reticulum stress and pro-inflammatory cytokine release is not altered.

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Introduction

α_1-antitrypsin is an important serine proteinase inhibitor that protects lung tissue from the destructive effects of serine proteases, such as neutrophil elastase, proteinase 3 and cathepsin G, which are released by degranulating neutrophils. Moreover, α_1-antitrypsin is thought to display anti-inflammatory activity including cytokine inhibition [1–3], inhibition of extracellular signal-regulated protein kinase (ERK)1/2 [4] and regulation of CD14 expression [5]. Although α_1-antitrypsin is primarily synthesised in the liver, previous studies 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 α_1-antitrypsin comprises >95% of the mutations leading to severe α_1-antitrypsin deficiency. Due to this mutation, the Z α_1-antitrypsin is not properly folded, which leads to the formation of polymers that accumulate as Periodic acid–Schiff -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, thus, early-onset lung emphysema [10]. Polymers of Z α_1-antitrypsin have been identified in lung lavage [11, 12] and have shown pro-inflammatory properties that may exacerbate inflammation and lung damage [11, 13–15], particularly in the cigarette smoking Z α_1-antitrypsin homozygote. Mulgrew *et al.* [15] showed that Z α_1-antitrypsin polymers could still be detected in lung lavage 10 years after liver transplantation, suggesting local secretion and polymerisation of Z α_1-antitrypsin within the lung. However, even after a decade, the source of these polymers remains unclear.

The ER is the site of secretory and membrane protein folding and its quality control systems ensure that only properly folded proteins exit the organelle for secretion or integration into the cell membrane. Accumulation of unfolded or misfolded proteins in the ER induces "ER stress", thereby activating intracellular signal transduction pathways collectively called the unfolded protein response (UPR) [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 protein degradation (ERAD) pathway to remove terminally misfolded proteins [17]. Furthermore, the UPR not only orchestrates ER homeostasis, it has also been shown to be involved in ER stress-induced nuclear factor (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-β in B-cells and macrophages, respectively [19, 20].

Misfolded monomeric Z α_1-antitrypsin is predominantly degraded by ERAD whilst polymers are cleared by autophagy [21, 22]. Interestingly, this does not activate the UPR within cells overexpressing Z α_1-antitrypsin [23–25]. However, it does prime cells to an exaggerated ER stress response upon a "second hit", probably due to the impaired protein mobility within the ER caused by α_1-antitrypsin polymers [25]. In addition to the enhanced sensitivity to ER stress, cells expressing Z α_1-antitrypsin also display an augmented NF-κB response with subsequent increase in cytokine secretion [4, 23, 24, 26]. Upon a second hit, such as exposure to lipopolysaccharide (LPS) or tumour necrosis factor (TNF)-α, this inflammatory response is further increased [4, 26].

Peripheral blood monocytes are the precursors for various subsets of lung macrophages, including alveolar macrophages, which are increased in chronic lung diseases such as chronic obstructive pulmonary disease (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 [29, 30]. Based largely on *in vitro* studies into development of human monocyte-derived macrophages, distinct macrophage subpopulations have been identified. For instance, human monocytes exposed to granulocyte-macrophage colony-stimulating factor will activate the classical pathway of macrophage differentiation, resulting in pro-inflammatory type I (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 type II (mφ-2) macrophages (also called alternatively activated macrophages) can be derived from human monocytes exposed to macrophage colony-stimulating factor, 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 [34].

Carroll *et al.* [26] previously showed intracellular accumulation of α_1-antitrypsin and subsequent activation of the UPR in monocytes from homozygous Z α_1-antitrypsin deficiency patients. Since we have previously shown differential α_1-antitrypsin production by different macrophage subsets [8], we set out to test the hypothesis that mφ-1 macrophages are able to produce Z α_1-antitrypsin polymers. Furthermore,

	PiZZ patients
TABLE 1 Patient characteristics	
Subjects	7
Age years	52 (43–57)
Male/female	4/3
FEV$_1$ %	50 (33–84)
FEV$_1$/FVC %	38 (28–65)
Smoking status	
Current smoker	0
Ex-smoker	2
Never-smoker	5

Data are presented as n or mean (range). FEV$_1$: forced expiratory volume in 1 s; FVC: forced vital capacity.

we hypothesised that this subset contributes to the enhanced inflammation due to the activation of the UPR and increased NF-κB activation.

Materials and methods

Subjects

The ZZ α_1-antitrypsin deficiency patients were stable without any sign of an exacerbation. 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, were nonsmokers and all had a MM genotype as confirmed by reverse transcriptase (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).

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 h and stimulated with 100 ng·mL^{-1} *Pseudomonas aeruginosa* LPS (Sigma-Aldrich) for 4 h or 24 h as indicated. We observed no reduction in cell viability after 24 h of thapsigargin treatment compared to control-treated cells by trypan blue exclusion (data not shown).

ELISA

Total and polymerised α_1-antitrypsin were measured in cell supernatant by ELISA as described previously [36] and expressed per mg of whole cell lysate to correct for differences in cell number and/or lysate. Intracellular levels were determined using whole cell lysate. Limit of detection for polymers was 3.0 ng·mg^{-1} total lysate. 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% weight/volume acrylamide SDS-PAGE gel. Proteins were detected with specific primary antibodies to phosphorylated-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 RT-PCR

RNA was isolated using Maxwell RNA extraction (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitative PCR was performed as described previously [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 ANOVA and Bonferroni *post hoc* analysis. Differences were considered statistically significant with a p-value <0.05.

Primer name	Forward primer	Reverse primer	Melting temperature °C	[Ref.]
CHOP	5′ GCA CCT CCC AGA GCC CTC ACT CTC C 3′	5′ GTC TAC TCC AAG CCT TCC CCC TGC G 3′	62	[37]
GADD34	5′ ATG TAT GGT GAG CGA GAG GC 3′	5′ GCA GTG TCC TTA TCA GAA GGC 3′	62	[38]
IL-8	5′ CTG GAC CCC AAG GAA AAC 3′	5′ TGG CAA CCC TAC AAC AGA C 3′	60	
SERPINA1	5′ AAG GCA AAT GGG AGA GAC CC 3′	5′ AAG AAG ATG GCG GTG GCA T 3′	60	[8]
XBP1s	5′ TGC TGA GTC CGC AGC AGG TG 3′	5′ GCT GGC AGG CTC TGG GGA AG 3′	62	[37]

IL: interleukin.

Results

Monocytes and monocyte-derived macrophages experiencing ER stress display an exaggerated response on 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 [39, 40]. 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 1 h, and subsequent stimulation with LPS for 4 h or 24 h. As expected, thapsigargin significantly increased CHOP, GRP78 mRNA and the splicing of XBP1 mRNA, in all cell types at both 4 h and 24 h (fig. 1). In contrast, GADD34 mRNA levels remained unchanged. 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 h, and significantly higher at 24 h (p<0.01) (fig. 1a–d). LPS induced considerably higher levels of all four UPR genes in mφ-1 and mφ-2 at either 4 h (for spliced XBP1 and GRP78 mRNA) or 24 h (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 (fig. 2a–f). LPS significantly increased IκB and IL8 mRNA in monocytes at 4 h, but not in mφ-1 or mφ-2. Remarkably, mφ-1 and mφ-2 experiencing ER stress did show enhanced IκB mRNA levels after 4 h of LPS treatment (p<0.001), whereas in monocytes this level actually decreased (p<0.05) (fig. 2a–f). After 24 h 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 [41] and (monocyte-derived) macrophages produce α_1-antitrypsin [8, 42]. 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) (fig. 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, in part, only be explained by the intracellular retention of Z α_1-antitrypsin (fig. 3b) and was not caused by a lack of SERPINA1 mRNA, which encodes α_1-antitrypsin (fig. 3c). The production of α_1-antitrypsin in both mφ-1 and mφ-2 was increased after 24 h LPS treatment (p<0.05 and p<0.01, respectively) (fig. 3a and b). When we used the 2C1 monoclonal antibody to specifically detect naturally occurring α_1-antitrypsin polymers, we were unable to detect Z α_1-antitrypsin polymers in any cell type (fig. 3a and 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 α_1-antitrypsin and polymer production of monocytes from the same donors. Unstimulated monocytes released equal amounts of total α_1-antitrypsin measured in the cell supernatant compared to mφ-2 (fig. 3a), and did not significantly upregulate the total α_1-antitrypsin production after LPS treatment. Interestingly, the intracellular α_1-antitrypsin levels were significantly higher in both MM and ZZ monocytes compared to pro- or anti-inflammatory macrophages (fig. 3b). However, the polymer levels were undetectable in both the cell supernatant and whole cell lysate of ZZ monocytes (fig. 3a and b).
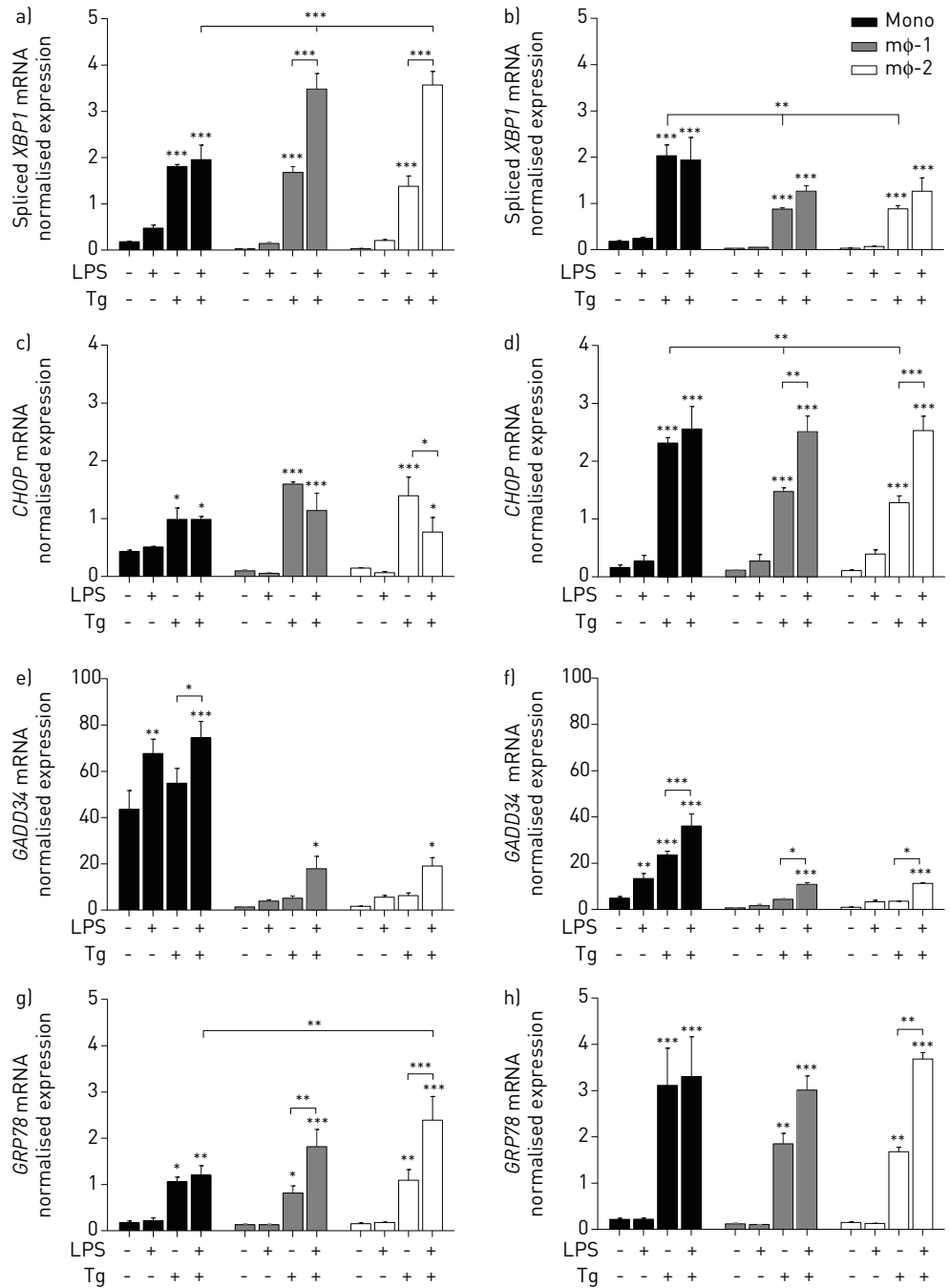
FIGURE 1 Endoplasmic reticulum stress response after lipopolysaccharide (LPS) treatment for 4 h (a, c, e, g) or 24 h (b, d, f, h) in MM monocytes and MM monocyte-derived macrophages experiencing endoplasmic reticulum stress. a, b) Monocytes (Mono), and macrophages type I (mφ-1) and type II (mφ-2) were pre-incubated with thapsigargin (Tg) for 1 h followed by LPS treatment as indicated. The splicing of *XBP1* mRNA was measured with quantitative reverse transcriptase-PCR, normalised to *ATP5B* and *ACTB* mRNA. c–h) Cells were treated as before and c, d) *CHOP*, e, f) *GADD34* and g, h) *GRP78* mRNA were measured. All values are normalised to the housekeeping genes *ACTB* and *ATP5B*. *: p<0.05, **: p<0.01, ***: p<0.001 *versus* untreated with a two-way repeated-measurements ANOVA (Bonferroni *post hoc*).

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 α_1-antitrypsin to levels that cause its polymerisation leads to an exaggerated ER stress response upon a second hit [24, 25], whereas the presence of monomeric Z α_1-antitrypsin alone does not trigger the UPR in primary bronchial epithelial cells [4]. CARROLL *et al.* [26]
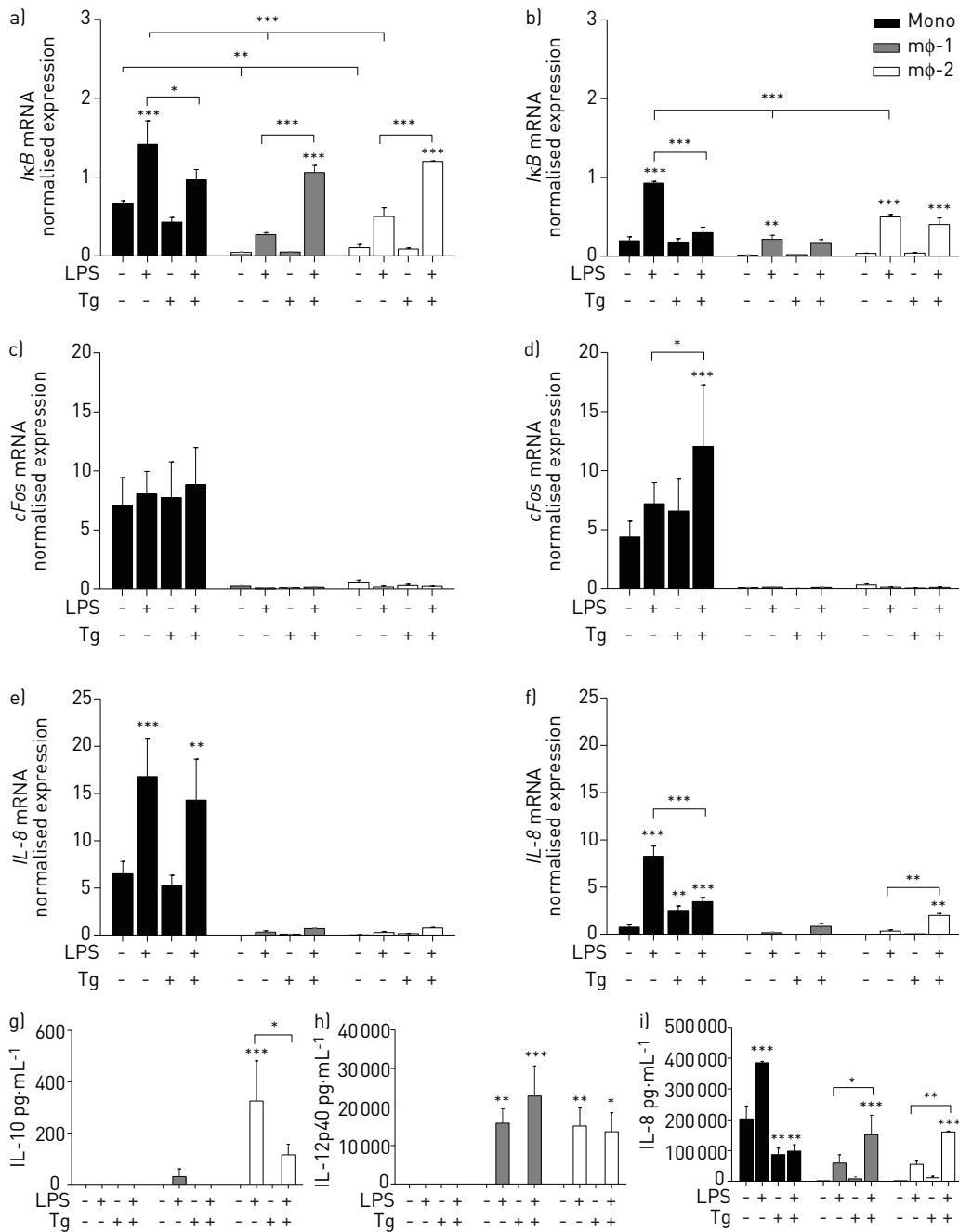
FIGURE 2 Inflammatory response after a, c, e) 4 h or b, d, f) 24 h lipopolysaccharide (LPS) treatment in MM monocytes and MM monocyte-derived macrophages experiencing endoplasmic reticulum stress. a–f) Cells were treated as in figure 1 and *IkB*, *cFos* and *IL-8* mRNA were measured. g–i) Pre-incubated cells with thapsigargin (Tg; 1 h) were subjected to 24 h LPS treatment and g) interleukin (IL)-10, h) IL-12p40 and i) IL-8 cytokine release were measured in cell supernatant by ELISA. All mRNA values are normalised to the housekeeping genes *ACTB* and *ATP5B*. Mono: monocytes; mφ-1: macrophages type I; mφ-2: macrophages type II *: p<0.05, **: p<0.01, ***: p<0.001 *versus* untreated with a two-way repeated-measurements ANOVA (Bonferroni *post hoc*).

showed a slightly enhanced UPR in resting ZZ monocytes in the presence of intracellular accumulated Z α_1-antitrypsin. However, the conformation of this retained Z α_1-antitrypsin remained unclear. Therefore, to examine whether our ZZ monocytes and ZZ monocyte-derived macrophages experience increased ER stress, we investigated the expression of several UPR target genes; CHOP, GADD34 and GRP78 and the splicing of XBP1 mRNA. In resting cells, there was no evidence of an increased ER stress response in ZZ cells compared to MM cells (fig. 4a–h). 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
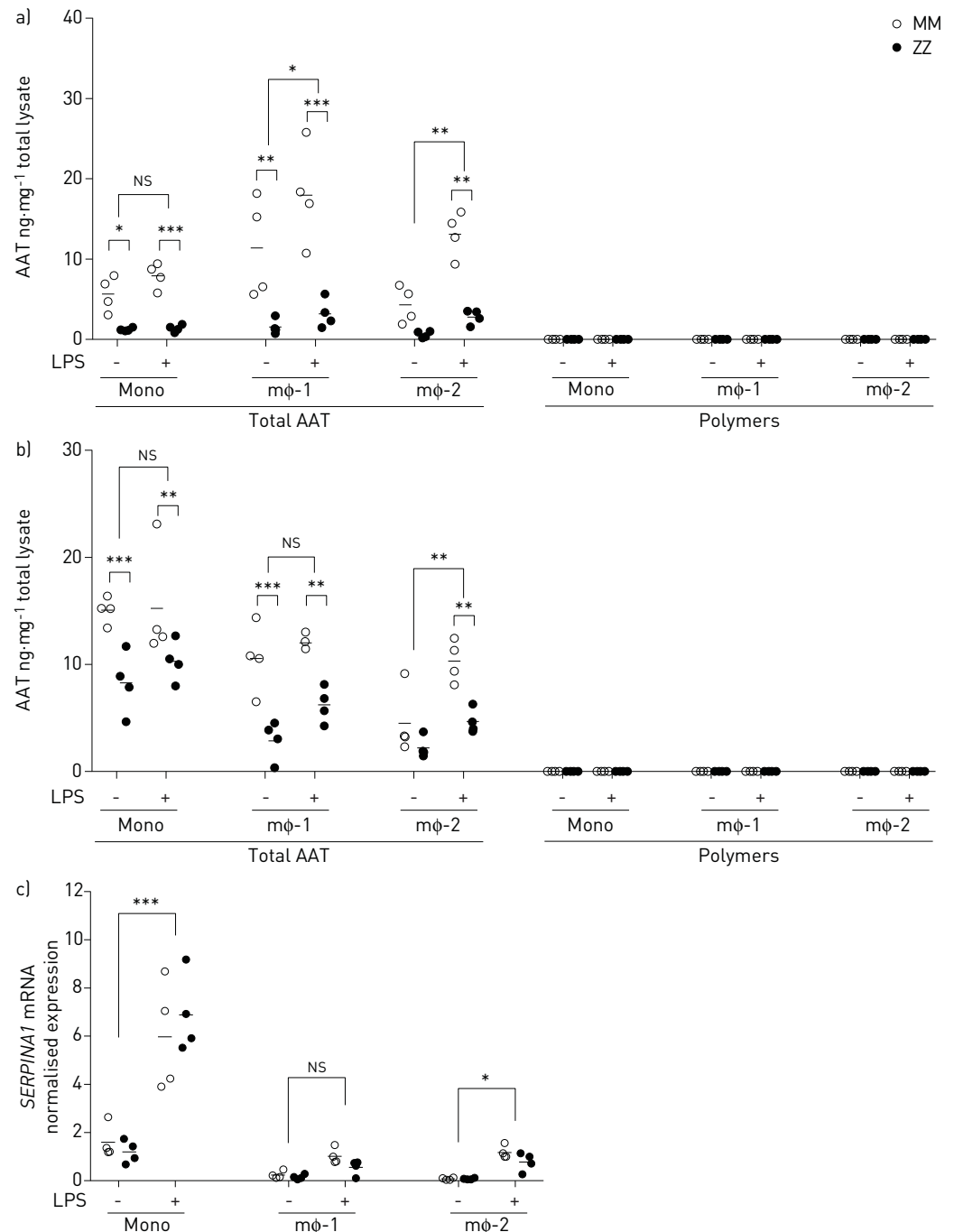
FIGURE 3 α_1-antitrypsin (AAT) production by monocyte-derived macrophages from ZZ patients and MM controls. a) Total AAT and AAT polymer production measured in cell supernatant of monocytes (Mono), and macrophages type I (mφ-1) and type II (mφ-2) after 24 h of lipopolysaccharide (LPS) treatment. b) Total AAT and AAT polymer levels in whole cell lysates measured in cell supernatant of monocytes, and mφ-1 and mφ-2 after 24 h of LPS treatment. c) Cells were treated as in (a) and *SERPINA1* mRNA was measured. All mRNA values are normalised to the housekeeping genes *ACTB* and *ATP5B*. NS: nonsignificant; *: p<0.05, **: p<0.01, ***: p<0.001 *versus* untreated with a two-way repeated-measurements ANOVA (Bonferroni *post hoc*).

between monocytes, mφ-1 and mφ-2, indicating that the differentiation of monocytes into macrophages does not alter the stress status (figs 1 and 4a–h). Next, to investigate the influence of an enhanced α_1-antitrypsin production these cells were stimulated with LPS. After 24 h, mφ-2 from Z α_1-antitrypsin patients showed a significant increase in GADD34 mRNA (fig. 4a–d). However, this difference could not be detected in monocytes (fig. 4e–h). In line with previous research [43, 44], 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 (fig. 4i and j). In fact, these levels appeared to be lower in ZZ macrophages compared to MM macrophages (fig. 4i and j).

Production of Z α_1-antitrypsin in monocyte-derived macrophages does not alter NF-κB signalling
Previous studies have shown that the presence of monomeric Z α_1-antitrypsin is associated with an enhanced NF-κB response in epithelial cells, even in the absence of polymers and an exaggerated ER stress response [4, 23, 45]. However, at present, the data for monocytes are inconsistent [26, 46] 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 and confirming the appropriate differentiation of the macrophage-subsets [31, 47], mφ-1 produced more IL-12p40 compared to mφ-2 after 24 h of LPS, whereas mφ-2 produced more IL-10 (fig. 5a–c). 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 h LPS (p<0.01) (fig. 5a–c). 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 (fig. 5d and e) and phosphorylation of ERK1/2 (fig. 5f and g). We were unable to detect any difference, either basally or after 24 h 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 (fig. 5f and g).

Once again, to ensure that the differentiation of monocytes into macrophages did not influence our results, we determined IκB and cFos mRNA and the release of IL-8 (fig. 5h and i). 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 (fig. 5h and i). 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 α_1-antitrypsin polymers in the lung lavage of a ZZ α_1-antitrypsin deficiency patient who underwent a liver transplantation 10 years earlier [15], the search for the responsible cell type emerged. It has recently been shown that primary bronchial epithelial cells of ZZ α_1-antitrypsin deficiency patients are unlikely to be the source of polymers [4]. In this study, polymers were also not detectable in both monocytes and monocyte-derived macrophages from ZZ patients. Furthermore, it was shown that these cells do not show an exaggerated ER stress or an increased NF-κB response to a second trigger such as LPS.

Interestingly, we have recently shown that resting ZZ α_1-antitrypsin primary bronchial epithelial cells display increased NF-κB activation even in the absence of detectable polymers and without an exaggerated ER stress response [4]. This enhanced NF-κB response in these cells was explained by the inability of these to produce significant amounts of Z α_1-antitrypsin by these cells to inhibit ERK1/2 phosphorylation *via* the epidermal growth factor receptor (EGFR) [4]. Monocytes are reported to lack substantial EGFR expression [48], 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 the findings from ALDONYTE *et al.* [46], who showed lower TNF-α release by ZZ monocytes. However, 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.* [26] explains the different results. The increase in cytokine release found by CARROLL *et al.* [26] was accompanied by the accumulation of Z α_1-antitrypsin within the ER of unknown conformation, and an exaggerated ER stress response.

We also detected the intracellular retention of Z a1-antitrypsin (fig. 3a and b), since the ratio of a$_1$-antitrypsin measured in the whole cell lysate *versus* the supernatant was higher in ZZ (ratio of 3.5:1) compared to MM cells (ratio 1:1). It needs to be noted that these ratios may not be accurate especially for ZZ cells, since the α_1-antitrypsin levels measured were close to the limit of detection of the ELISA. Although we obtained preliminary evidence for increased retention of α_1-antitrypsin in ZZ cells, this does not fully explain the difference in secreted α_1-antitrypsin between MM and ZZ cells. This conclusion is based on the observation that the total amount of α_1-antitrypsin produced (sum of whole cell lysates and supernatant) is lower in ZZ than MM cells (data not shown). It would be interesting to investigate whether the remaining difference between MM and ZZ cells can be explained by degradation of Z α_1-antitrypsin *via* ERAD by treating these cells with a proteasome inhibitor.

Previously, we have determined the critical Z concentration at which Z α_1-antitrypsin is likely to form polymers, *i.e.* 300 ng·mg^{-1} total lysate protein [4]. In this study, monocytes and monocyte-derived
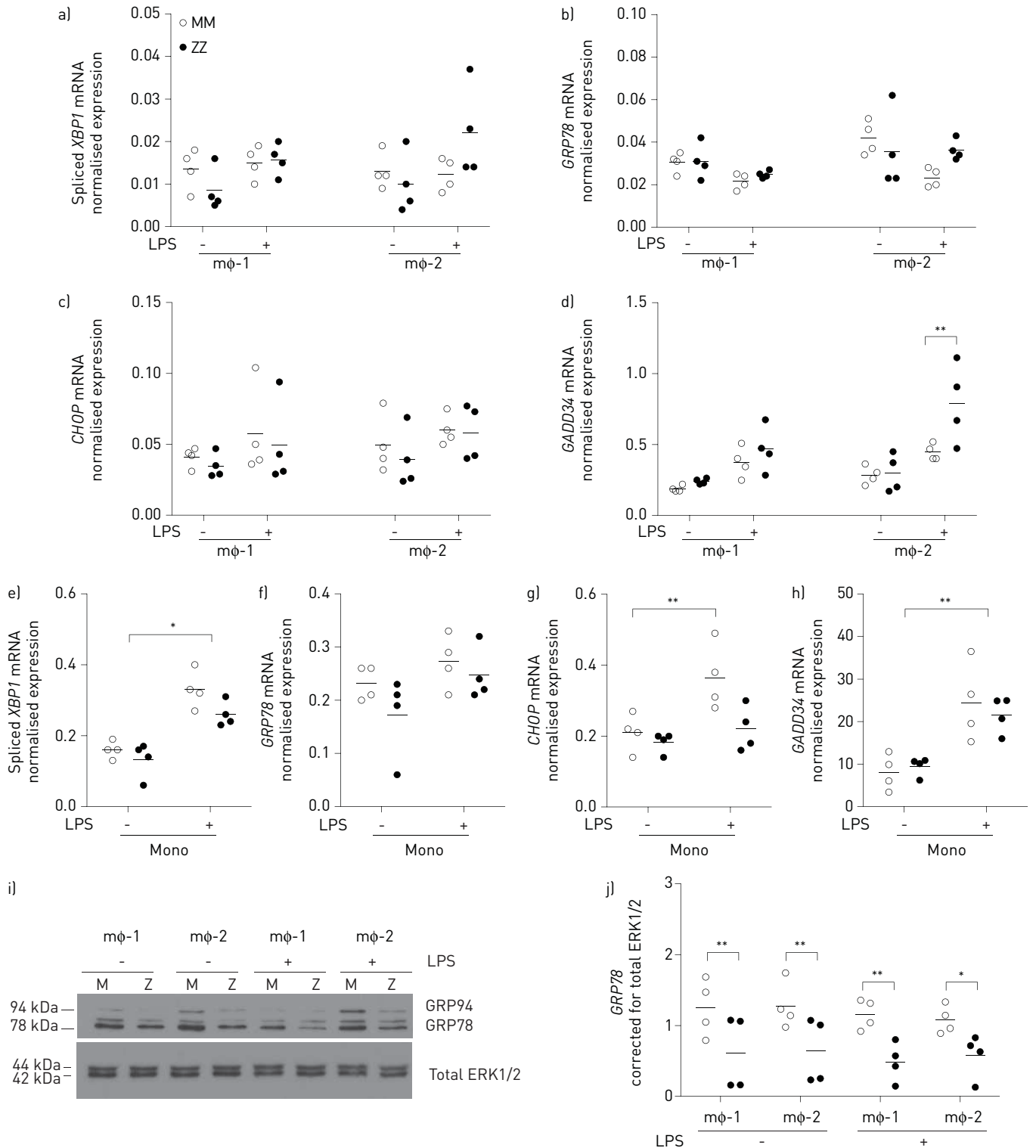
FIGURE 4 No exaggerated endoplasmic reticulum stress response in monocytes and monocyte-derived macrophages from ZZ patients compared to MM controls. a–d) mRNA levels in macrophages type I (mφ-1) and type II (mφ-2) of the endoplasmic reticulum stress genes a) spliced *XBP1*, b) *GRP78*, c) *CHOP* and d) *GADD34* after 24 h of lipopolysaccharide (LPS) treatment measured by quantitative reverse transcriptase-PCR. e–h) Monocytes (Mono) were treated and subjected to analysis as in (a–d). i, j) Representative western blot for GRP94 and GRP78 using anti-KDEL antibody. Monocyte-derived macrophages were treated as in (a–d). n=4 for densitometry. All mRNA values are normalised to the housekeeping genes *ACTB* and *ATP5B*. ERK: extracellular signal-regulated protein kinase. *: p<0.05, **: p<0.01 *versus* untreated with a two-way repeated-measurements ANOVA (Bonferroni *post hoc*).
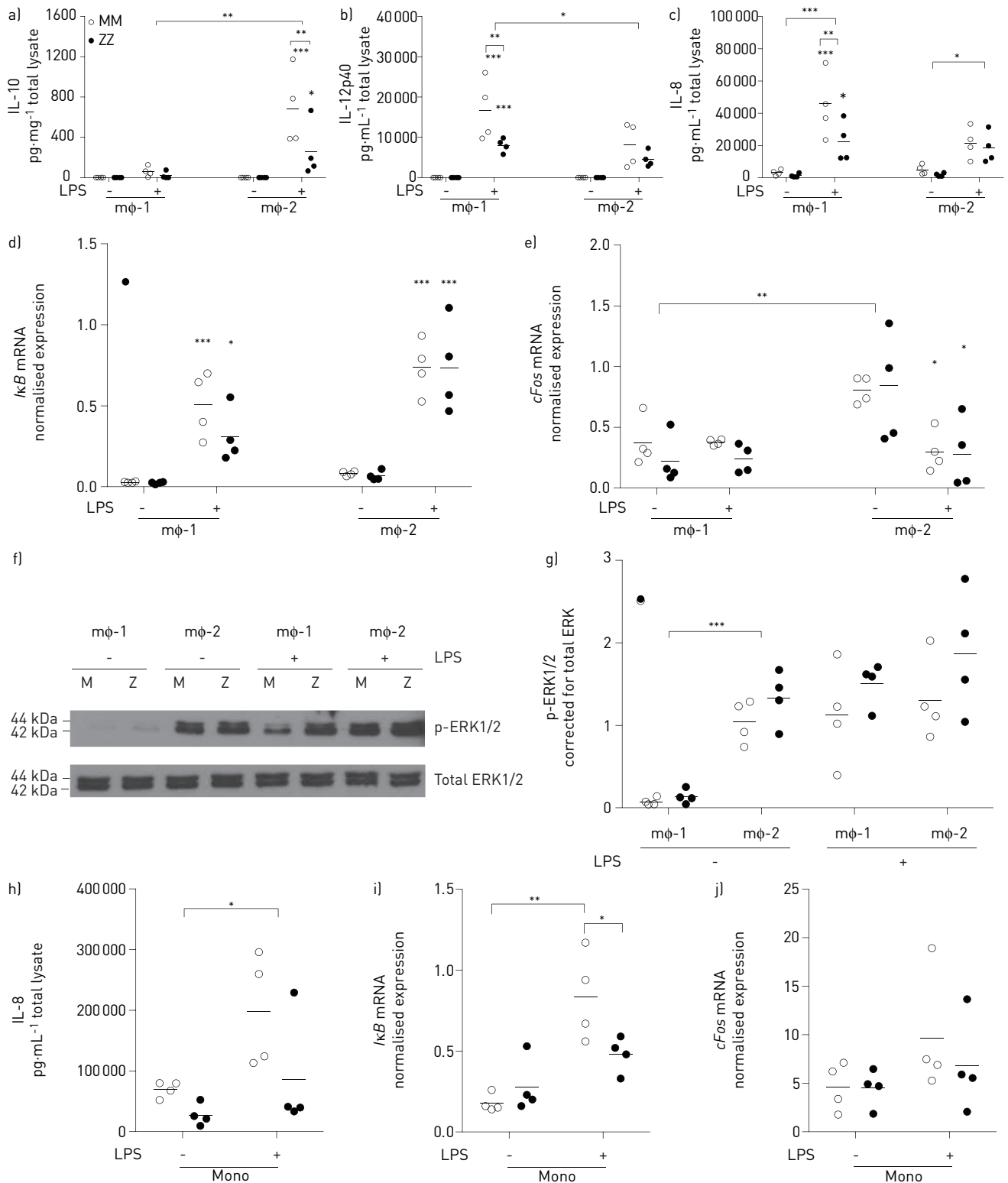
FIGURE 5 No increased inflammatory response in monocytes and monocyte-derived macrophages from ZZ patients compared to MM controls. a) Interleukin (IL)-10, b) IL-12p40 and c) IL-8 release of macrophages type I (mφ-1) and type II (mφ-2) after 24 h of lipopolysaccharide (LPS) treatment measured by ELISA. mRNA levels of d) *IkB* and e) *cFos* in mφ-1 and mφ-2 treated as in (a–c). f, g) Representative western blot of the activation of the mitogen-activated protein kinase extracellular signal-regulated protein kinase (ERK)1/2 of whole cell lysates from mφ-1 and mφ-2 treated as in (a–c). n=4 for densitometry. h) IL-8, i) *IkB* mRNA and j) *cFos* mRNA release of monocytes (Mono) treated as in (a–c). All mRNA values are normalised to the housekeeping genes *ACTB* and *ATP5B*. p: phosphorylated. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ *versus* untreated with a two-way repeated-measurements ANOVA (Bonferroni *post hoc*).

macrophages did not reach this concentration (maximum of 30 ng·mg^{-1} total lysate protein by MM cells), which could explain why we were unable to detect 2C1-positive polymers intracellular 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. We considered the possibility that the 2C1 monoclonal antibody used in the present study is less sensitive than the polyclonal ATZ11 antibody used by others [49], which could explain our inability to detect Z polymers. However, when we evaluated the binding characteristics of both antibodies, we found that both antibodies bind equally well to Z polymers, but the polyclonal antibody ATZ11 also bound to Z monomers [36]. Even if very low levels of Z α1-antitrypsin polymer are made within monocytes, it is unlikely to affect cellular function as we were unable to detect altered ER stress responsiveness as we have done previously for polymer-expressing cells [25].

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, but it also reveals unknown differences between these subsets in the expression of inflammatory markers like *cFos* and *IL8* mRNA, and the 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, previous studies have shown that these cellular subsets *in vitro* might not always represents alveolar macrophages *in vivo* [8, 50]. 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, 42]. However, it needs to be noted that marked differences exist in the characteristics of alveolar macrophages between patients with and without COPD 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* but also illustrates the complexity of defining the ultimate alveolar macrophage phenotype. Future studies with alveolar macrophages obtained from bronchoalveolar lavage of Z α$_1$-antitrypsin deficiency patients will help to better understand the role of macrophages in Z α$_1$-antitrypsin deficiency *in vivo*.

Taken together, this study extends our understanding of the current view of Z α$_1$-antitrypsin polymerisation, exaggerated ER stress response and NF-κB signalling by all cell types expressing Z α$_1$-antitrypsin. However, more research needs to be done to completely understand the underlying mechanisms for these phenomena.

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