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

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Running title: Macrophage function in α_1-antitrypsin deficiency

Take home message

No evidence for polymer formation and excessive inflammatory responses in cultured mononuclear phagocytes from α_1-antitrypsin deficient patients

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 do display an enhanced ER stress response upon a LPS trigger (*XBP1* splicing, *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 ER stress and pro-inflammatory cytokine release is not altered.

Introduction

Alpha$_1$-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, α_1-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 α_1-antitrypsin is primarily synthesized 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 α_1-antitrypsin comprises more than 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 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 α_1-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 α_1-antitrypsin homozygote. In 2004, Mulgrew *et al.* [15] showed that Z α_1-antitrypsin polymers could still be detected in lung lavage ten 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) (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 synthesized 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 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 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 α_1-antitrypsin and subsequent activation of the UPR in monocytes from homozygous Z α_1-antitrypsin deficiency patients. Since we have shown previously 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, we hypothesized that this subset contributes to the enhanced inflammation due to the activation of the UPR, and due to an increased NF-κB activation.

Material and methods

Subjects

The ZZ α_1-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.

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. We observed no reduction in cell viability after 24 hours 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 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 RT-PCR (qPCR)

RNA was isolated using Maxwell RNA extraction (Promega, Madison, WI, USA) according to manufacturer's instructions. Quantitative 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.

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*, *GRP78* mRNA and the splicing of *XBP1* mRNA, in all cell types at both 4 hours and 24 hours (figure 1A-D). 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 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 *IκB* and *IL8* mRNA in monocytes at 4 hours, 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 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) and was not caused by a lack of *SERPINA1* mRNA, which encodes α_1-antitrypsin (Figure 3C). 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; Figure 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 (Figure 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 (Figure 3A), and did not significantly up-regulate the total α_1-antitrypsin production after LPS treatment. Interestingly, the intracellular α_1-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 and 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 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] 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 (Figure 4A and 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 of an enhanced α_1-antitrypsin production, these cells were stimulated with LPS. After 24 hours, mφ-2 from Z α_1-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 (Figure 4C). In fact, these levels appeared to be lower in ZZ macrophages compared to MM macrophages (Figure 4C).

Production of Z α_1-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 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. 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 and confirming the appropriate differentiation of the macrophage-subsets [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 *IκB* and *cFos* mRNA and the release of IL-8 (figure 5D). *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 α_1-antitrypsin polymers in the lung lavage of a ZZ α_1-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 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, 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 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 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 [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 α_1-antitrypsin within the ER of unknown conformation, and an exaggerated ER stress response. We also detected the intracellular retention of Z α_1-antitrypsin (figure 3A-B), since the ratio of α_1-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 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, 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 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 [48], 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, 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, 49]. 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 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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Figure legends

Figure 1. ER stress response upon LPS treatment in MM monocytes and MM monocyte-derived macrophages experiencing ER stress. A. Monocytes (mono), and macrophages type I (mφ-1) and type II (mφ-2) were pre-incubated with thapsigargin (Tg) for 1 hour followed by LPS treatment for 4 hours (left) or 24 hours (right) as indicated. The splicing of *XBP1* mRNA was measured with quantitative RT-PCR, normalised to *ATP5B* and *ACTB* mRNA. B-D. Cells were treated as in A and *CHOP*, *GADD34* and *GRP78* mRNA was measured, respectively. 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*).

Figure 2. Inflammatory response upon LPS treatment in MM monocytes and MM monocyte-derived macrophages experiencing ER stress. A. Cells were treated as in figure 1 and *IkB*, *cFos* and *IL8* mRNA was measured. B. Pre-incubated cells with thapsigargin (Tg; 1 hour) were subjected to 24 hours LPS treatment and IL-10, IL-12p40 and IL-8 cytokine release were measured in cell supernatant by ELISA. All mRNA 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*).

Figure 3. Alpha$_1$-antitrypsin production by monocyte-derived macrophages from ZZ patients and MM controls. A. Total α_1-antitrypsin and α_1-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 α_1-antitrypsin and α_1-antitrypsin polymer levels in whole cell lysates. C. Cells were treated as in A. and *SERPINA1* mRNA was measured. All mRNA 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*).

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. All mRNA 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*).

Figure 5. No increased inflammatory response in monocytes and monocyte-derived macrophages from ZZ patients compared to MM controls. A. IL-8, IL-10 and IL-12p40 release of macrophages type I (mφ-1) and type II (mφ-2) after 24 hours LPS treatment measured by ELISA. B. mRNA levels of *IkB* and *cFos* in mφ-1 and mφ-2 treated as in A. C. Representative western blot of the activation of the MAP kinases ERK1/2 of whole cell lysates from mφ-1 and mφ-2 treated as in A. Densitometry of n=4. D. IL-8, IL-10 and IL-12p40 release of monocytes treated as in A. All mRNA 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*).

Table 1. Patient characteristics

	PiZZ patients (n=7)
age (mean, range)	52 (43-57)
sex (M/F)	3/3
FEV1 (mean, range in %)	50 (33-84)
FEV1/FVC (mean, range in %)	38 (28-65)
Smoking status (current/ex/never)	0/2/5

Table 2. qPCR primers

Name	Forward primer	Reverse primer	Melting temp. (°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	[50]
IL8	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]

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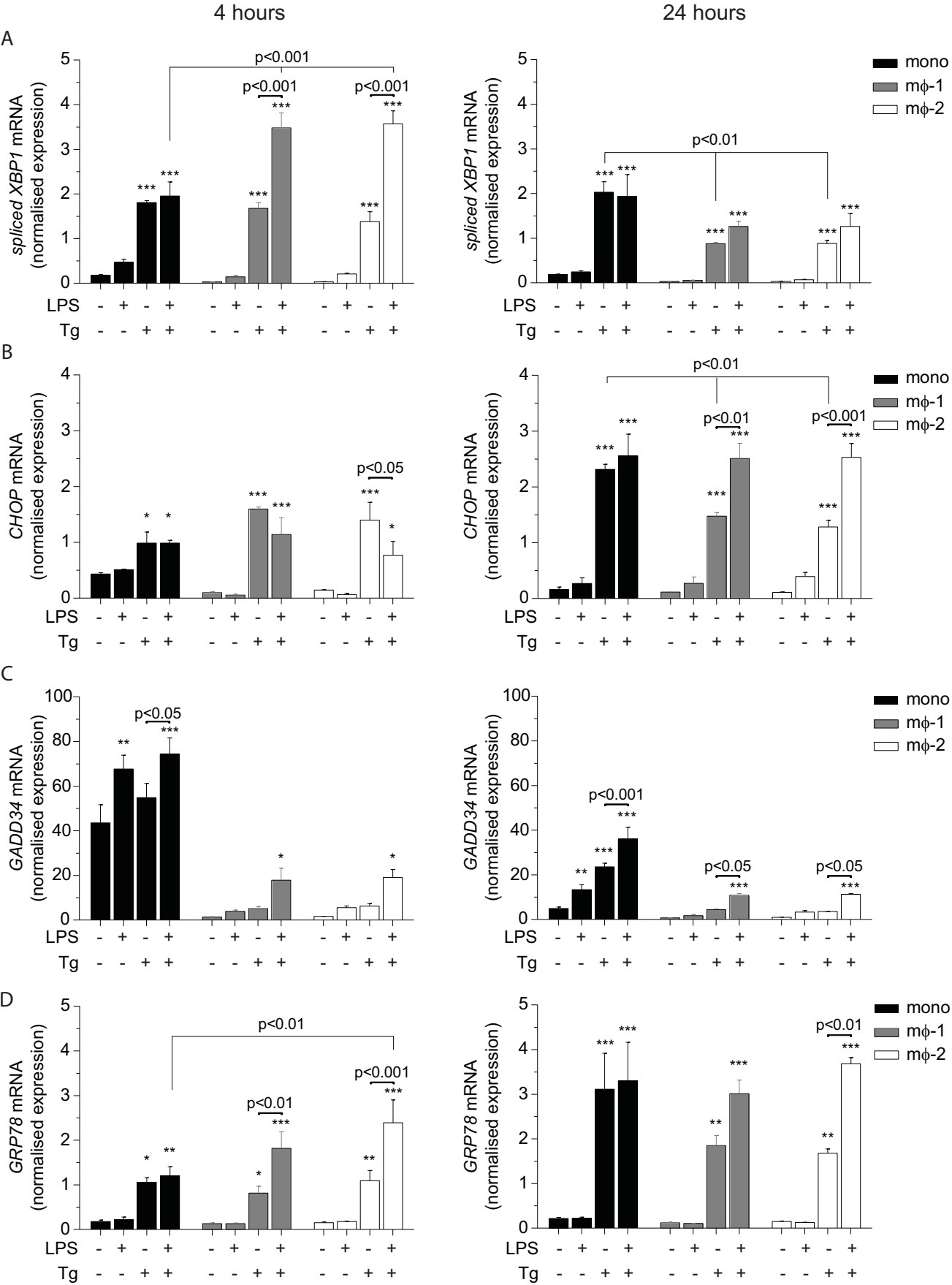
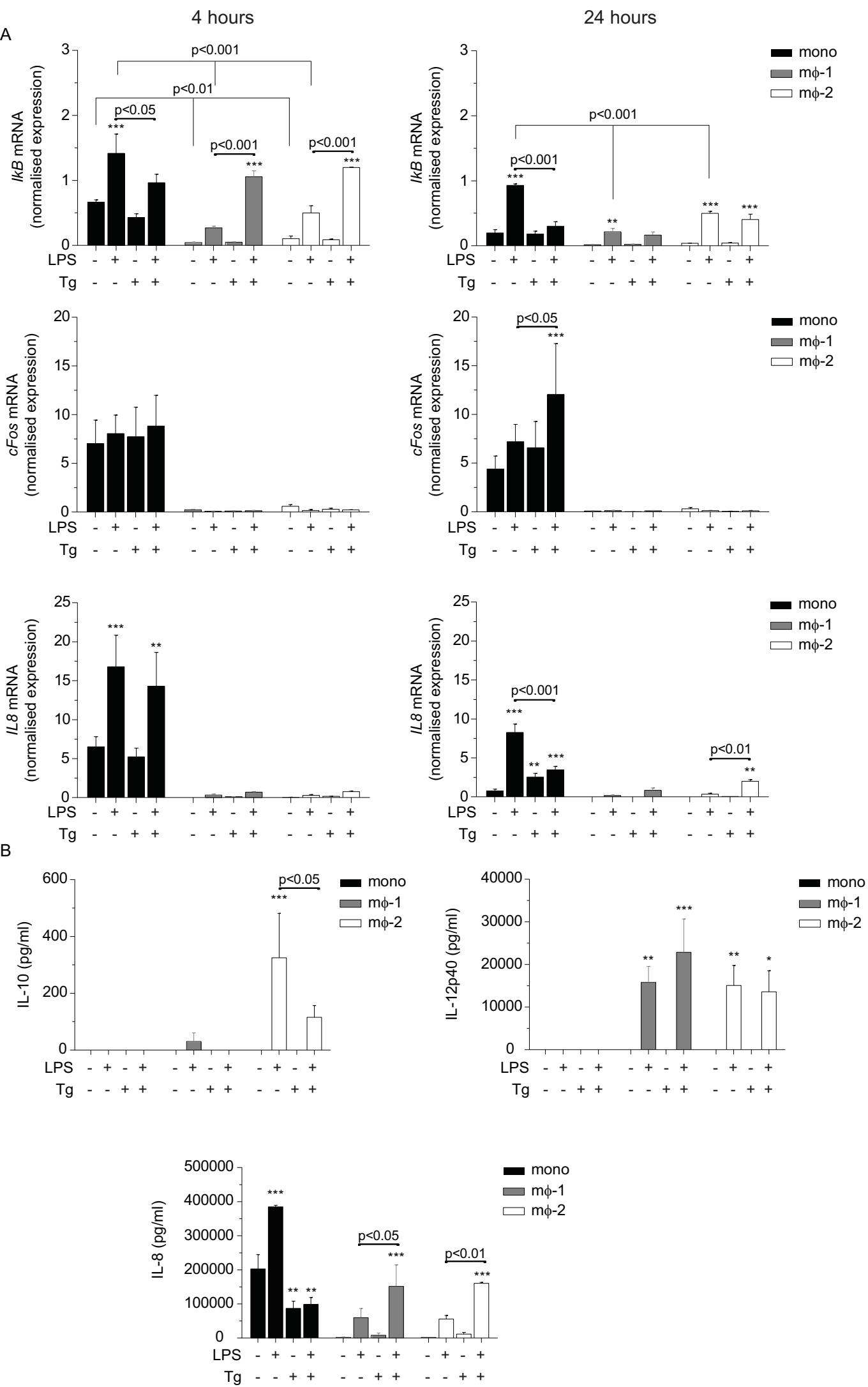
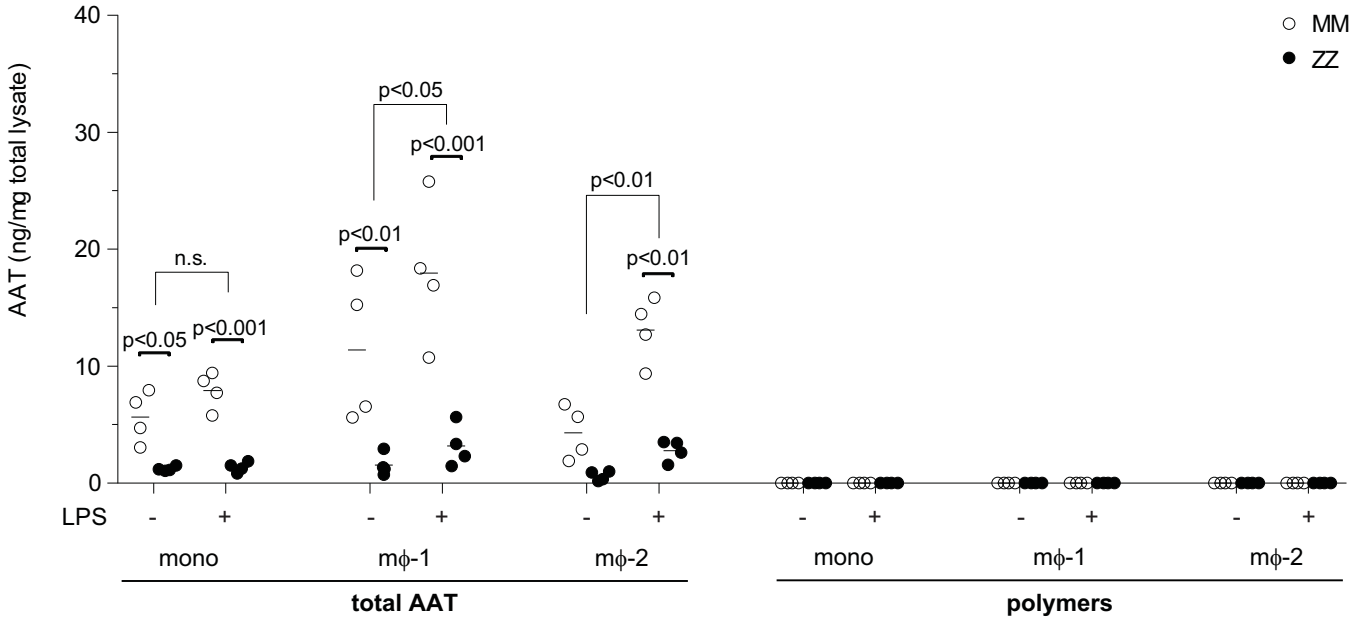
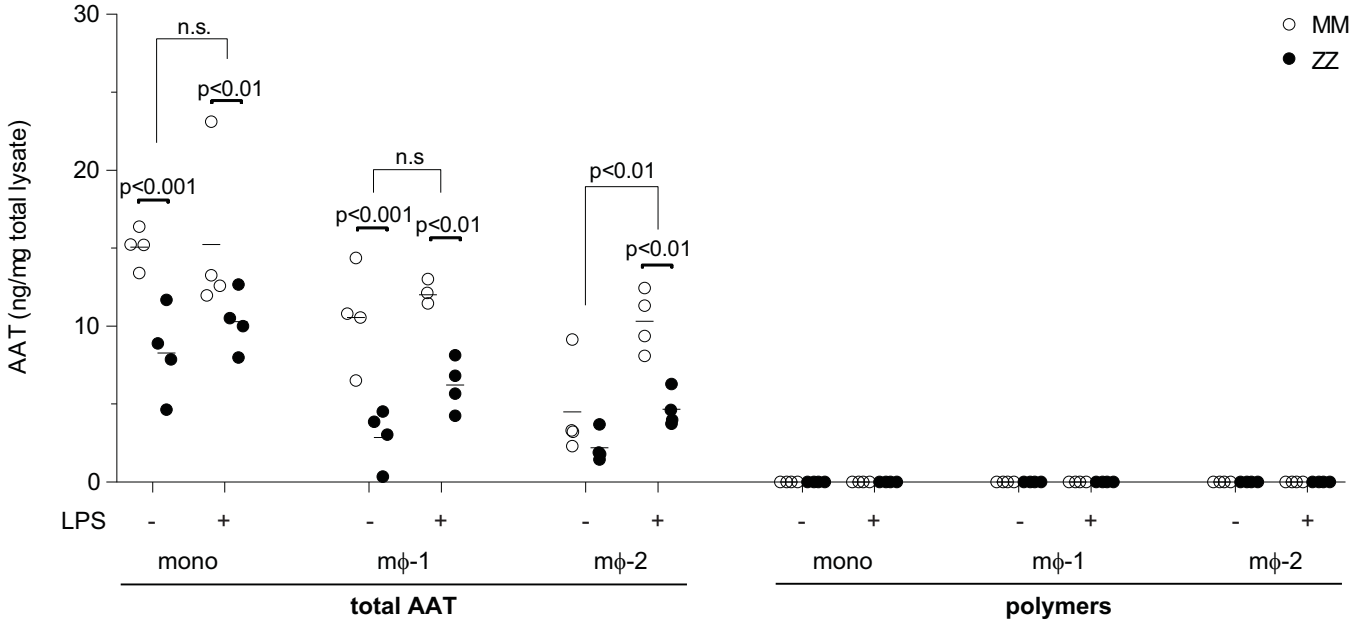

4 hours
24 hours
A
B
C
D
spliced XBP1 mRNA (normalised expression)
CHOP mRNA (normalised expression)
GADD34 mRNA (normalised expression)
GRP78 mRNA (normalised expression)
mono
mϕ-1
mϕ-2
LPS
Tg
p<0.001
p<0.01
p<0.05


A
4 hours
24 hours
mono
mϕ-1
mϕ-2
IkB mRNA (normalised expression)
cFos mRNA (normalised expression)
IL8 mRNA (normalised expression)
p<0.001
p<0.01
p<0.05
LPS
Tg
B
IL-10 (pg/ml)
IL-12p40 (pg/ml)
IL-8 (pg/ml)

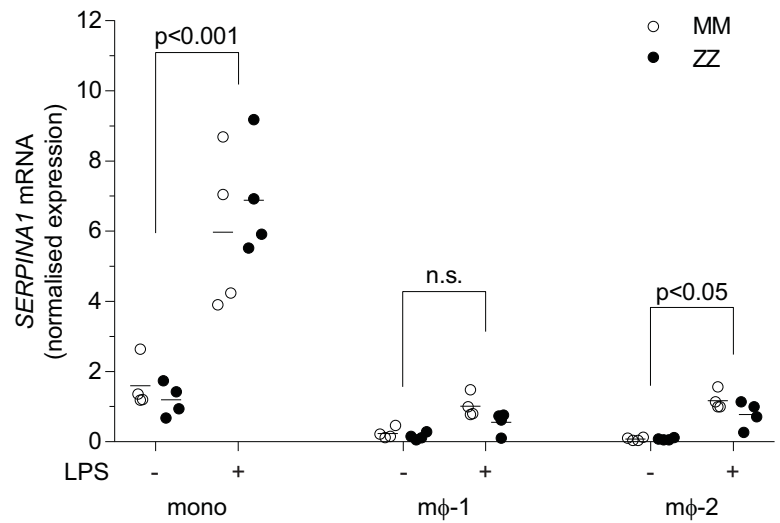
A

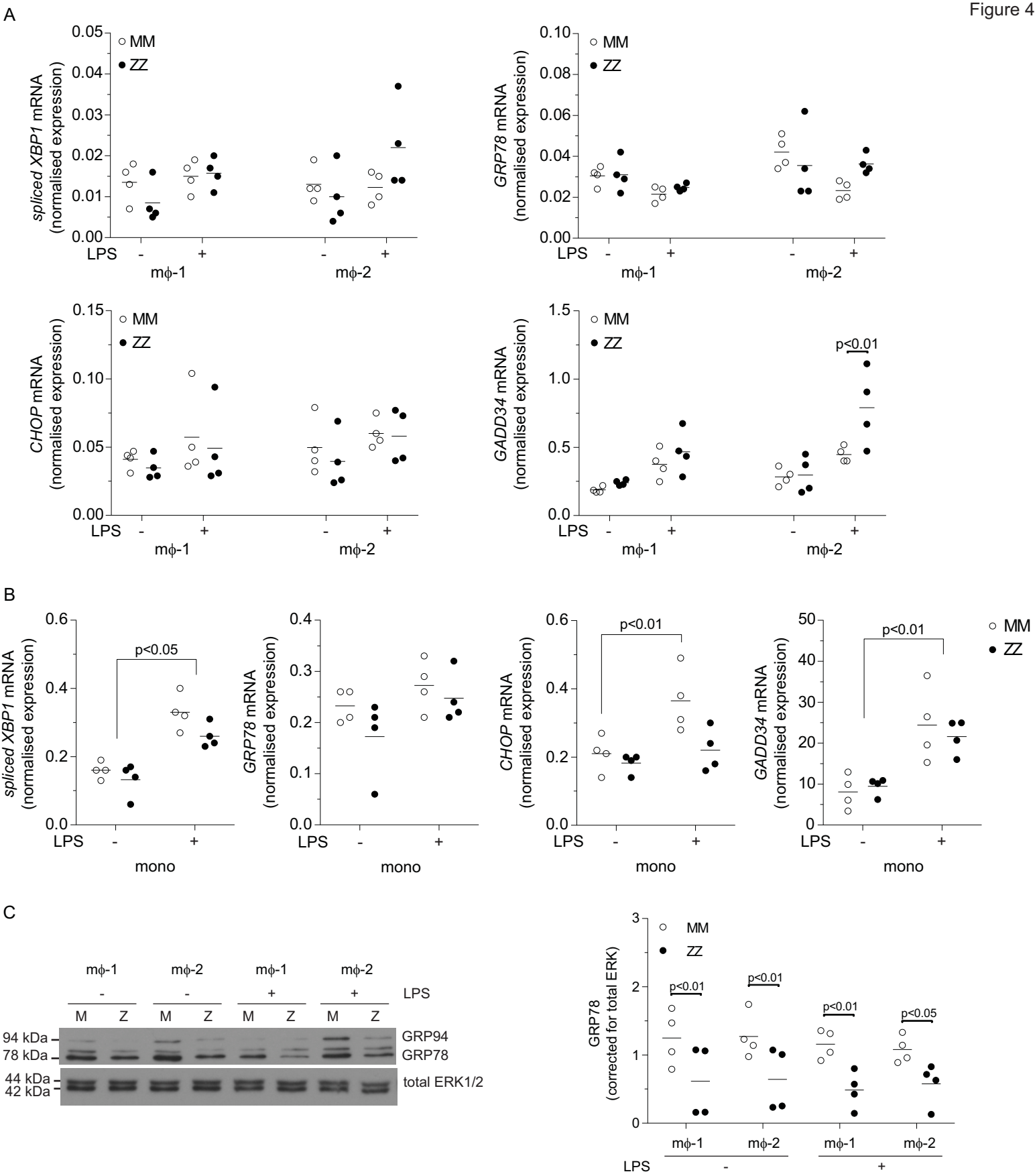

AAT (ng/mg total lysate)
MM
ZZ
p<0.05
p<0.001
p<0.01
n.s.
LPS
mono
mϕ-1
mϕ-2
total AAT
polymers

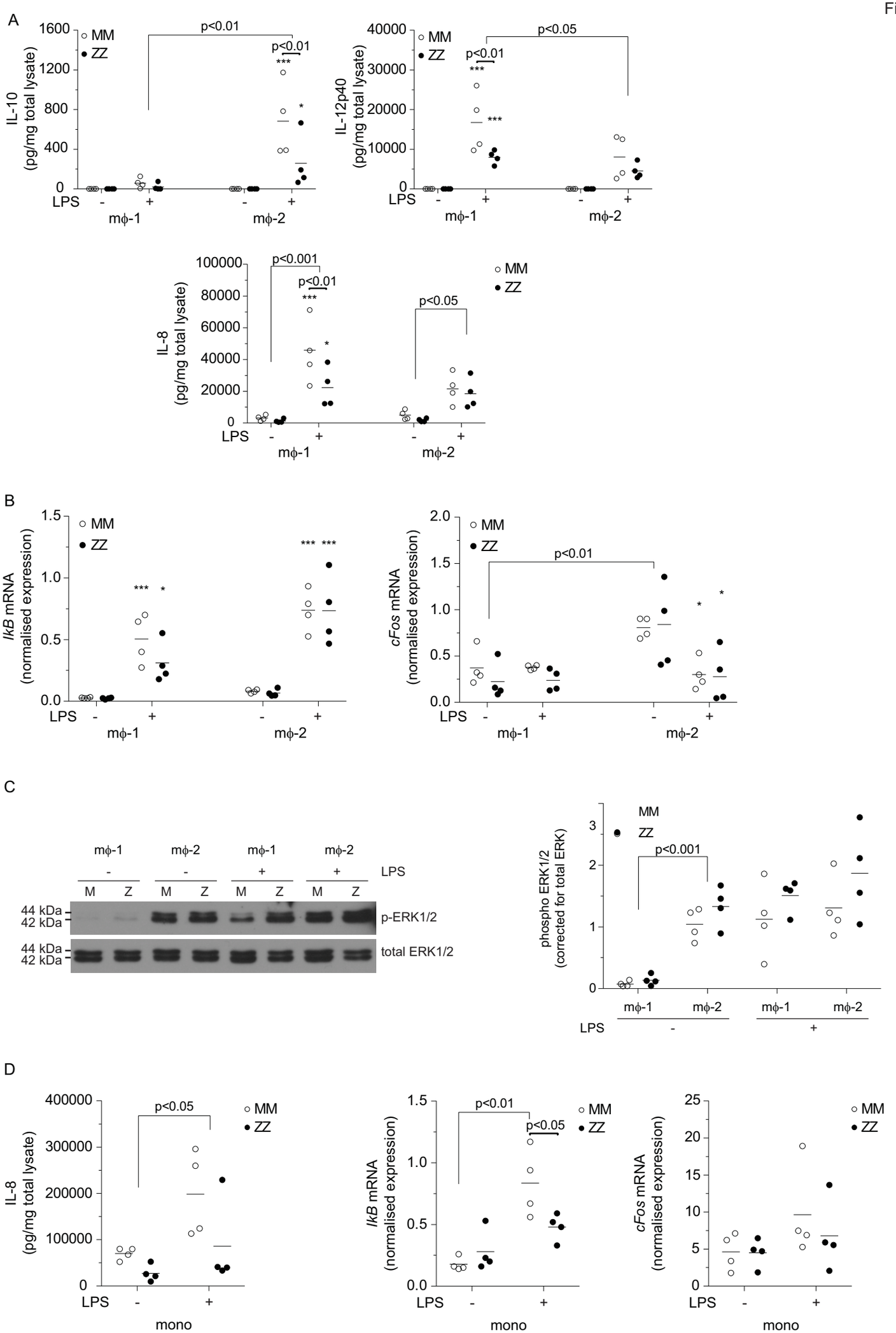
B


AAT (ng/mg total lysate)
MM
ZZ
n.s.
p<0.01
p<0.001
LPS
mono
mϕ-1
mϕ-2
total AAT
polymers

C


SERPINA1 mRNA
(normalised expression)
MM
ZZ
p<0.001
n.s.
p<0.05
LPS
mono
mϕ-1
mϕ-2


A
spliced XBP1 mRNA (normalised expression)
GRP78 mRNA (normalised expression)
CHOP mRNA (normalised expression)
GADD34 mRNA (normalised expression)
MM
ZZ
LPS
mϕ-1
mϕ-2
p<0.01
B
p<0.05
p<0.01
mono
C
mϕ-1
mϕ-2
LPS
M
Z
94 kDa
78 kDa
44 kDa
42 kDa
GRP94
GRP78
total ERK1/2
GRP78 (corrected for total ERK)
p<0.05


A
IL-10 (pg/mg total lysate)
IL-12p40 (pg/mg total lysate)
IL-8 (pg/mg total lysate)
MM
ZZ
p<0.01
p<0.05
p<0.001
LPS
mϕ-1
mϕ-2
B
IkB mRNA (normalised expression)
cFos mRNA (normalised expression)
C
mϕ-1
mϕ-2
LPS
M
Z
44 kDa
42 kDa
p-ERK1/2
total ERK1/2
phospho ERK1/2 (corrected for total ERK)
D
IL-8 (pg/mg total lysate)
IkB mRNA (normalised expression)
cFos mRNA (normalised expression)
mono