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### AUTHOR(S)

Tomás P. Carroll, Catherine M. Greene, Noel G. McElvaney

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**Chapter title:** Measurement of the unfolded protein response (UPR) in monocytes.

Tomás P. Carroll,<sup>\*</sup> Catherine M. Greene,<sup>\*</sup> and Noel G. McElvaney<sup>\*</sup>

<sup>\*</sup>Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland.

**Running title:** Measuring UPR activation in the monocyte

Corresponding author: Dr. Tomás Carroll

Phone +353-1-8093800

Fax +353-1-8093808

Email: [tcarroll@rcsi.ie](mailto:tcarroll@rcsi.ie)

## **Abstract**

In mammalian cells the primary function of the endoplasmic reticulum is to synthesise and assemble membrane and secreted proteins. As the main site of protein folding and post-translational modification in the cell, the ER operates a highly conserved quality control system to ensure only correctly assembled proteins exit the ER, and misfolded and unfolded proteins are retained for disposal. Any disruption in the equilibrium of the ER engages a multifaceted intracellular signalling pathway termed the unfolded protein response (UPR) to restore normal conditions in the cell. A variety of pathological conditions can induce activation of the UPR, including neurodegenerative disorders such as Parkinson's disease, metabolic disorders such as atherosclerosis, and conformational disorders such as cystic fibrosis. Conformational disorders are characterised by mutations that modify the final structure of a protein and any cells that express abnormal protein risk functional impairment. The monocyte is an important and long-lived immune cell and acts as a key immunological orchestrator, dictating the intensity and duration of the host immune response. Monocytes expressing misfolded or unfolded protein may exhibit UPR activation and this can compromise the host immune system. Here we describe in detail methods and protocols for the examination of UPR activation in peripheral blood monocytes. This guide should provide new investigators to the field with a broad understanding of the tools required to investigate the UPR in the monocyte.

## **1. Introduction**

### **1.1. ER Stress and the Unfolded Protein Response (UPR)**

Newly synthesised secreted and transmembrane proteins are transported into the lumen of the endoplasmic reticulum (ER) where they are folded and correctly assembled. The homeostasis of this organelle is vital to continued normal cell function and survival. Perturbations that disrupt ER homeostasis, such as fluctuations in calcium storage or increased demand for protein folding arising from elevated production of secretory proteins, compromise ER protein folding capacity resulting in the accumulation of unfolded or misfolded protein within the lumen of the ER . An imbalance between the load of unfolded proteins entering the ER and the ability of the ER to process this load is termed ER stress, and can be detrimental to cell survival. ER stress can also be induced by a range of pathophysiological conditions, including stroke, ischaemia, diabetes, obesity, viral infection, and mutations that impair protein folding (Kaufman, 1999).

To maintain homeostasis, the ER has evolved highly specific signalling pathways collectively referred to as the unfolded protein response (UPR), which strives to adapt for survival or induce apoptosis. In the event of ER stress this protective pathway causes the translational attenuation of general protein synthesis, induces a transcriptional programme of ER-resident chaperones, and coordinates the degradation of misfolded proteins through ER-associated degradation (ERAD)(Mori, 2000). Three proximal ER-resident transmembrane sensors, protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6) form a tripartite management system that orchestrates the mammalian UPR (Ron and Walter, 2007). The luminal domain of these three molecules is sequestered by the ER chaperone glucose-regulated protein 78 (GRP78), however, upon misfolded protein accumulation, GRP78 dissociates from PERK, IRE1, and ATF6, leading to their activation (Malhotra and Kaufman, 2007).

The most immediate step in the UPR is translational attenuation to prevent further accumulation of unfolded proteins in stressed cells. This is mediated by PERK, through phosphorylation of eukaryotic initiation factor 2 (eIF2) at Serine 51 on its alpha subunit (eIF2 $\alpha$ ) (Harding et al., 1999). While reducing the rate of protein synthesis and the protein load in the ER, eIF2 $\alpha$  phosphorylation paradoxically promotes the translation of ATF4 mRNA, a basic leucine zipper (bZIP) transcription factor. ATF4 induces a pro-survival programme of genes essential for amino acid import, glutathione biosynthesis, and resistance to oxidative stress (Harding et al., 2003).

The next step in the UPR employs IRE1 and ATF6, which regulate chaperone induction, expansion of the ER in response to increased client protein load and ER-associated degradation (ERAD) (Mori, 2000). Upon UPR engagement, IRE1 is activated and its endoribonuclease activity causes removal of a 26-nucleotide intron from X-box binding protein 1 (XBP-1) mRNA (Tirasophon et al., 1998). The spliced XBP-1 mRNA is translated into a potent bZIP transcription factor which translocates to the nucleus and acts as a key regulator of ER folding capacity. Concurrently, another bZIP transcription factor ATF6 is released from GRP78 and transported to the Golgi where it undergoes sequential cleavage by site-1 protease (S1P) and site-2 protease (S2P). Cleaved ATF6 cooperates with spliced XBP-1 to induce the expression of ER chaperones, ER quality control genes, folding enzymes and ERAD (Yoshida et al., 1998; Yoshida et al., 2001). ERAD ensures the removal of terminally misfolded proteins from the ER lumen to the cytoplasm for ultimate degradation by the ubiquitin-proteasome system (Travers et al., 2000).

## **1.2. The Monocyte**

The human immune system has been historically divided into innate immunity and acquired or adaptive immunity. Monocytes and their multitude of differentiated offspring fulfil important

regulatory and effector roles in both arms of the immune system (Medzhitov and Janeway, 2000). Peripheral blood monocytes in the circulation migrate through blood vessel walls into various organs and differentiate into more specialised macrophages. Monocytes and their macrophage progeny act as immunological orchestrators and serve three major functions, antigen presentation, phagocytosis, and immunomodulation (Dale et al., 2008). Upon activation, monocytes and macrophages produce large amounts of cytokines and chemokines that recruit other immune cells such as neutrophils to sites of infection and inflammation.

The first reports of ER stress and UPR activation in immune cells were in professional antibody-secreting plasma cells (Calfon et al., 2002). This developmental ER stress is a natural consequence of the transition from B cell to plasma cell, which produces huge amounts of antibodies and requires a massive expansion in ER folding capacity (Iwakoshi et al., 2003). However, there is emerging evidence that UPR activation in monocytes and macrophages may play a role in the pathogenesis of a number of diseases. In mouse models of atherosclerosis, macrophages overloaded with free cholesterol were shown to exhibit UPR activation (Li et al., 2005), and monocytes from Type II diabetes patients showed elevated expression of GRP78 (Komura et al., 2010). Monocytes from cystic fibrosis (CF) patients were shown to be intrinsically abnormal in their cytokine responses (Zaman et al., 2004), but this can be reversed by inhibitors designed to prevent  $\Delta F508$  CFTR (cystic fibrosis transmembrane conductance regulator) degradation by ERAD and increase the secretion of CFTR (Vij et al., 2006). In addition, work from this group has demonstrated intracellular accumulation of Z alpha-1 antitrypsin (AAT) in the ER of monocytes, and this causes sustained activation of the UPR with subsequent effects on immune function (Carroll et al., 2010).

As the tissue macrophage can present a challenge in terms of isolation with unreliable sampling techniques or lengthy differentiation protocols from monocytes that risk cell activation,

the monocyte is a useful tool for investigating immune system derangement in a variety of disorders.

## **2.1. Isolation, culture and treatment of peripheral blood monocytes**

In this section we will provide details for isolating monocytes from freshly-drawn whole blood from human donors. It must be noted that studying the behaviour and function of human monocytes is a difficult undertaking. Monocytes constantly observe their surroundings and swiftly react to changes, as is their nature. The isolation, purification and culture of these cells *in vitro* can affect their phenotype, and care must be taken not to stimulate the cells. The faster the isolation process the less chance of cell activation, and all buffers to be used should be pre-warmed to 37°C. We use Lymphoprep (Axis-Shield) to perform density gradient separation but Ficoll-Paque can also be used (Yeo et al., 2009).

### **2.1.1. Required Materials**

- Lymphoprep (Axis-Shield)
- 1X saline (0.9% NaCl)
- Hanks Balanced Salt Solution (Biosciences)
- EasySep Human CD14 positive selection kit (Stemcell Technologies) and EasySep magnet (Stemcell Technologies). The kit is designed to isolate CD14 positive cells from fresh or previously frozen peripheral blood mononuclear cells by positive selection.
- 5 mL polystyrene round bottom Falcon tube (BD Biosciences, product)
- RPMI 1640 (Biosciences) supplemented with 10% FCS, 2% glutamine and penicillin/streptomycin (necessary for culturing monocytes)

- Thapsigargin (Molecular Probes) and tunicamycin (Sigma) for chemically inducing UPR activation. Thapsigargin depletes calcium stores in the ER by inhibiting  $\text{Ca}^{2+}$  ATPase, while tunicamycin inhibits N-linked glycosylation of newly-synthesised proteins.

### **2.1.2. Monocyte Isolation**

Mononuclear cells are isolated from venous peripheral blood obtained from donors by density gradient separation. The blood must be collected in tubes containing an anticoagulant (EDTA or heparin). Once drawn, blood is immediately placed in 50 mL tube and mixed with an equal volume of 0.9% NaCl (1X saline). The diluted blood is then layered over Lymphoprep in a fresh 50 mL tube. For example, if 15 mL blood is obtained, mix with 15 mL 1X saline and then slowly and carefully layer over 15 mL Lymphoprep. Alternatively, Lymphoprep can be under-layered below the diluted blood using a long pipette. It is essential to avoid mixing of blood and Lymphoprep before centrifugation. Centrifuge the Lymphoprep and blood at 800 x g for 10 minutes at room temperature, with the centrifuge brake switched off. After centrifugation, the mononuclear cells (which include monocytes and lymphocytes) form a distinct band at the blood/Lymphoprep interface. Carefully remove this band using a Pasteur pipette and place in a fresh tube. Add an equal volume of 1X saline or HBSS to reduce the density of the solution and pellet these cells by gentle centrifugation (300 x g, 10 minutes). Resuspend cells in 1mL EasySep recommended medium (1 mM EDTA, 2% Foetal Calf Serum in PBS Dulbecco) and transfer this 1mL cell suspension to a 5 mL Falcon tube. It is useful to count the number of mononuclear cells isolated at this step. Monocytes should typically represent 10% of this total mononuclear fraction.

From this point, we follow the EasySep CD14 kit protocol exactly as described in order to isolate monocytes from the mononuclear cell population. In our experience, the most critical step in the Lymphoprep protocol is to ensure the centrifuge brake is switched off as this will ensure a tight band of mononuclear cells forms at the interface between serum and Lymphoprep. Another

critical step is the careful layering of diluted blood over Lymphoprep to ensure no mixing occurs between the layers. Leaving the centrifuge brake on or inefficient layering technique will result in a diffuse mononuclear band at the interface, and a poor monocyte yield. Once monocytes are successfully isolated and counted they can be used in a variety of applications. For example, if performing stimulation experiments, monocytes can be cultured in RPMI containing 10% (v/v) foetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere for up to 24 hours. However, it is essential that monocytes are cultured in polypropylene tubes, and not polystyrene tubes, as polystyrene promote significant adherence and probable activation of monocytes.

## **2.2. Enzyme-linked Immunosorbent Assay (ELISA)**

In addition to its role in maintaining ER homeostasis, it has become apparent that ER stress-induced UPR activation can influence the expression of a subset of inflammatory genes. The spliced form of XBP-1 has been shown to control the production of IL-6 in the mouse B cell (Iwakoshi et al., 2003). XBP-1 was also shown to regulate the production of IFN- $\beta$  in mouse macrophages subjected to ER stress (Smith et al., 2008). Another study in a mouse model of atherosclerosis demonstrated that accumulation of free cholesterol in macrophages caused UPR activation and the secretion of significant amounts of TNF- $\alpha$  and IL-6 (Li et al., 2005). Thus, the underestimated technique of ELISA is a reliable method of investigating an aberrant immune response in monocytes, often a consequence of UPR activation. This quantitative method is highly reproducible, relatively cheap and easy to perform. However, due to the large quantities of chemokines and cytokines secreted by monocytes, dilution of supernatants is often required and as little as 2  $\mu$ L of supernatant can be sufficient in some cases, for example when assaying IL-8. Another application for ELISA is in the investigation of impaired secretion of specific proteins. For

example, we have used this technique to show impaired secretion of AAT from monocytes isolated from alpha-1 antitrypsin deficient-individuals (Carroll et al., 2010).

### **2.2.1. Required Materials for IL-8 ELISA**

- Monocyte supernatants
- Immulon 2HB 96 well high-binding microtitre plates (Thermo Electron Corporation)
- Voller's coating buffer (100mM Bicarbonate/carbonate buffer, pH 9.6)
- Wash buffer (0.05% Tween in PBS, pH 7.4)
- Blocking buffer (1% BSA, 0.05% Tween in PBS, pH 7.4)
- Recombinant human IL-8 (R&D Systems)
- Monoclonal antibody to human IL-8 (MAB208, R&D Systems)
- Biotinylated antibody to human IL-8 (BAF208, R&D Systems)
- Streptavidin-horseradish peroxidase (HRP) (Biolegend)
- ABTS substrate (Invitrogen)

### **2.2.2. ELISA to Measure IL-8**

Supernatants are recovered and protein concentrations determined by ELISA with specific antibodies to the secreted protein of interest, in this example the chemokine IL-8. The day before, a high-binding 96 well plate is coated with 100  $\mu$ L per well of capture antibody (MAB208, 1/500 dilution) diluted in Voller's buffer – this is a key point. If PBS is used as a diluent for the coating antibody, as stated in the manufacturer's datasheet, the ELISA will not work. The plate is sealed and incubated overnight at 4°C. Next day, each well is aspirated and filled with wash buffer, and repeated for a total of 3 washes using a multi-channel pipette. Complete removal of the liquid at each step is achieved by inverting the plate and blotting on clean tissue paper. Plate is blocked by

the addition of 200  $\mu$ L of blocking buffer and incubated for 1 hour at room temperature. Repeat aspiration and wash steps as before for 3 washes. Plates are filled with 100  $\mu$ L per well of monocyte supernatant (diluted). Serial dilutions of recombinant human IL-8 in serum-free RPMI medium are performed to construct a standard curve (31.25 – 2000 pg/mL) and the plate is incubated for 2 hours at room temperature. Plate is washed 3 times as before, 100  $\mu$ L of the detection antibody (BAF208, 1/2500 dilution) is added to each well, and plate is incubated for a further 2 hours. Next, plate is washed 3 times, 100  $\mu$ L streptavidin-HRP is added (1/2500 dilution) and incubated for 30 minutes at room temperature. Another 3 washes are performed, and the plate is incubated with 100  $\mu$ L ABTS for 5 – 30 minutes in a dark box or cupboard protected from direct light. It is useful to check the plate periodically for the development of a green colour, and once a significant colour is observed, measure the absorbance of each well at 405 nm on a Victor2 microplate reader (Wallac). Prepare a standard curve from the data produced from the serial dilutions with concentration on the X axis versus absorbance on the Y axis (linear). Interpolate the concentration of the sample from this standard curve using Prism 4.0 statistical analysis software (GraphPad).

As an ancillary step to ELISA, cytokine arrays can be employed to measure multiple proteins in the monocyte supernatants (RayBiotech, Inc). This technique, although expensive, allows the semi-quantitative measurement of over 100 cytokines, chemokines, proteases, antiproteases, and growth factors from a single sample. Any potential leads can then be confirmed by ELISA or quantitative RT-PCR.

### **2.3. RNA Isolation, cDNA synthesis and RT-PCR**

There are inherent difficulties involved in measuring the activation of the major UPR orchestrators. The phosphorylation of PERK and IRE1 and the proteolytic cleavage of ATF6 are hallmarks of their activation. However, these proteins are expressed at very low levels and detection is hindered

by a lack of high quality commercial antibodies. As surrogate markers of activation, a number of UPR genes downstream of these proximal sensors have been identified, and can be easily measured by real-time PCR. For example, ATF4 is selectively activated by PERK-induced phosphorylation of eIF2 $\alpha$ , and while IRE1 activation is difficult to reproducibly and consistently measure, the processing of unspliced XBP-1 mRNA into mature spliced XBP-1 (sXBP-1) mRNA is a convenient, widely-used indicator of IRE1 activation (Marciniak et al., 2004). The expression of several other UPR-responsive genes can be easily measured in this way, including calreticulin, calnexin, CHOP, ERdj5, p97/VCP, GRP78, and GRP94.

### **2.3.1. Required Materials**

- TRI reagent (Sigma)
- QuantiTect reverse-transcription cDNA synthesis kit (Qiagen)
- SYBR Green I Master mix (Roche) for real-time quantitative RT-PCR
- GoTaq Green Master mix (Promega) for conventional RT-PCR
- Oligonucleotide primers (Eurofins MWG Operon)

### **2.3.2. Real-time PCR**

For analysis of UPR markers, 500  $\mu$ L TRI reagent is added to the isolated monocytes (in a fume hood) and RNA is recovered according to the manufacturer's instructions. We prefer to resuspend RNA in 0.1% diethylpyrocarbonate-treated (DEPC) water, as DEPC is an efficient, nonspecific inhibitor of RNases. Store the isolated RNA at -80°C and avoid repeated freeze-thaw cycles. Quantify RNA concentration on a spectrophotometer prior to each separate cDNA synthesis. Equal quantities of RNA are then reverse transcribed into cDNA using the QuantiTect Reverse Transcription kit. The resulting cDNA is used as the template for quantitative real-time PCR.

Oligonucleotide primers are synthesised specific to each target UPR gene and quantitative PCR reactions performed containing 2 µl template cDNA, 10 µl 2X SYBR Green I master mix, 10 picomoles of forward and reverse primers, and nuclease-free water to give a final volume of 20 µl. Amplification is performed on the Roche LightCycler 480 PCR system with the expression of target genes relative to the housekeeping gene β-actin determined using the  $2^{-\Delta\Delta C_T}$  method (Livak and Schmittgen, 2001)(Figure 1.1.).

**Figure 1.1.** Quantitative RT-PCR analysis of calreticulin (CRT), sXBP-1 and GRP78 mRNA induction in peripheral blood monocytes after treatment with thapsigargin for 4 hours.

### 2.3.3. Analysis of XBP-1 mRNA cleavage

XBP-1 mRNA splicing is analysed in our laboratory using a conventional semi-quantitative RT-PCR assay developed by Harding *et al.* (Calfon *et al.*, 2002). RNA is isolated using TRI reagent and cDNA synthesised as described. The resulting cDNA is template in a reaction with GoTaq master mix and specific XBP-1 forward and reverse primers. The primers used to amplify XBP-1 cDNA: forward 5'-AAACAGAGTAGCAGCTCAGACTGC-3'; reverse 5'-TCCTTCTGGGTAGACCTCTGGGA-3'. PCR products are resolved on a 2.5% agarose gel with unspliced XBP-1 yielding a product of 480 bp, while spliced XBP-1 is 454 bp. Agarose gel electrophoresis may need to be performed for up to 2 hours to detect an appreciable difference between spliced and unspliced XBP-1.

## 2.4. Detection of UPR Markers by Immunoblotting

As previously described, the phosphorylation of PERK and IRE1 and the proteolytic cleavage of ATF6 are difficult to detect. However, the activation of a number of other downstream UPR proteins such as ATF4, CHOP, GRP78, and p97/VCP can be detected by Western blotting.

#### **2.4.1. Immunoblotting Method**

Monocytes are isolated as described, pelleted by gentle centrifugation (400 x g, 5 minutes) and cells resuspended in 1 mL hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM PMSF and 0.5 mM DTT) (Sigma). Cells are lysed for 30 minutes on ice before centrifugation at 14,000 x g for 10 min at 4°C. The supernatant is then recovered for immunoblotting and stored at -80°C until required. Protein concentration in the monocyte lysate is determined by the method of Bradford (Bradford, 1976). Whole cell lysates are separated by electrophoresis on SDS-polyacrylamide gel and transferred to a PVDF membrane (Sigma). The percent polyacrylamide used will depend on the size of the UPR protein being investigated. PVDF membranes can be cut to investigate the levels of multiple proteins if there is sufficient difference in the size of the proteins being examined, and this can eliminate the need to strip and reprobe membranes. Non-specific binding is blocked with 5% bovine serum albumin (Sigma) in PBS containing 0.1% Tween-20 (Sigma). Immunoreactive proteins are detected by incubating the membrane with specific antibodies to UPR proteins of interest, for example the excellent anti-KDEL antibody which detects GRP78 and GRP94 (Stressgen), and comparing with appropriate loading controls such as GAPDH or  $\beta$ -actin.

#### **2.5. Immunofluorescence**

Immunofluorescence is a powerful tool that can be used to demonstrate the accumulation of misfolded protein within the ER. For example, we have used this technique to show intracellular

accumulation of AAT within the ER of monocytes from alpha-1 antitrypsin deficient-individuals, as well as increased GRP78 and GRP94 expression (Carroll et al., 2010).

### **2.5.1. Required Materials**

- Poly-L-lysine coated slides (Sigma)
- 4% Paraformaldehyde (Sigma) diluted in PBS
- 0.2% Triton X-100 diluted in PBS (permeabilisation buffer)
- 4% BSA/1% gelatin in PBS (blocking buffer)
- Vectashield (Vector Laboratories)

### **2.5.2. Co-localisation using Immunofluorescence**

This method is used to investigate AAT localisation to the ER by double-staining monocytes with an antibody to AAT and an antibody to ER-resident chaperones containing the tetrapeptide KDEL motif. Monocytes are isolated as described and 100  $\mu$ L of monocyte suspension pipetted onto a poly-L-lysine glass slide ('P' side up). Score the slide with a diamond pen to indicate the position of cells. Poly-L-lysine coated slides should be used as they provide higher adhesion, reducing the chances of tissue or cell loss during processing. The cells are allowed to adhere to slide for 10 minutes and then fixed in 4% paraformaldehyde. The cell membranes are disrupted in 0.2% Triton X-100. Non-specific binding of antibodies and fluorescent conjugates are blocked by pre-incubation in 4% BSA/1% gelatin. Co-localisation of AAT and ER-specific chaperones is detected by immunofluorescence using goat polyclonal anti-AAT-FITC (Abcam) and mouse monoclonal anti-KDEL (Stressgen), with an anti-mouse tetramethylrhodamine isothiocyanate (TRITC) secondary conjugate (Abcam) for visualization of the anti-KDEL antibody. PBS washes (x 3) are performed after each incubation, and ensure the addition and removal of solutions is performed carefully in the

corner of the slides so as not to disturb or dislodge the monocytes. Cells are then mounted in Vectashield, containing 4', 6-diamidino-2-phenylindole di-lactate (DAPI), and examined using a LSM510 Meta laser scanning confocal microscope (Zeiss). Images are captured at  $\times 63$  magnification and  $\times 4$  zoom with excitation wavelengths for FITC, TRITC and DAPI of 488 nm, 543 nm and 364 nm respectively (Figure 1.2.).

**Figure 1.2.** Intracellular accumulation of AAT and increased KDEL expression in the ER of ZZ monocytes. The subcellular distribution of AAT in monocytes isolated from MM (normal) and ZZ (AAT-deficient) individuals was determined by immunofluorescence using antibodies for AAT (green) and the ER marker KDEL (red), with colocalisation indicated by yellow staining. Nuclei were stained with DAPI (blue). Imaging was acquired using a Zeiss LSM510 Meta confocal microscope and the images presented are single focal plane scans of 1  $\mu\text{m}$  depth at the mid section of the fixed cells.

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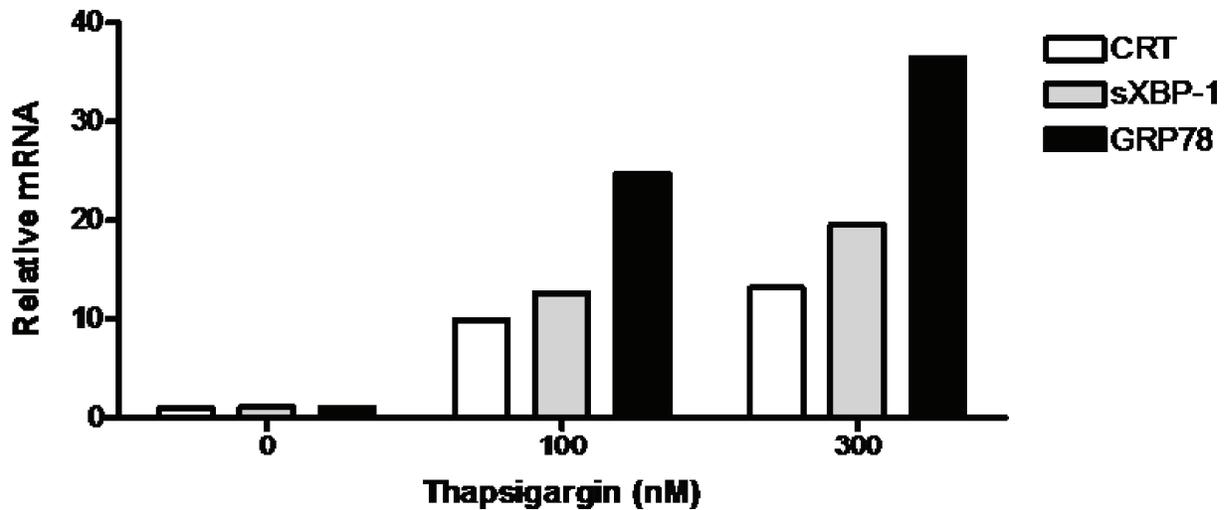
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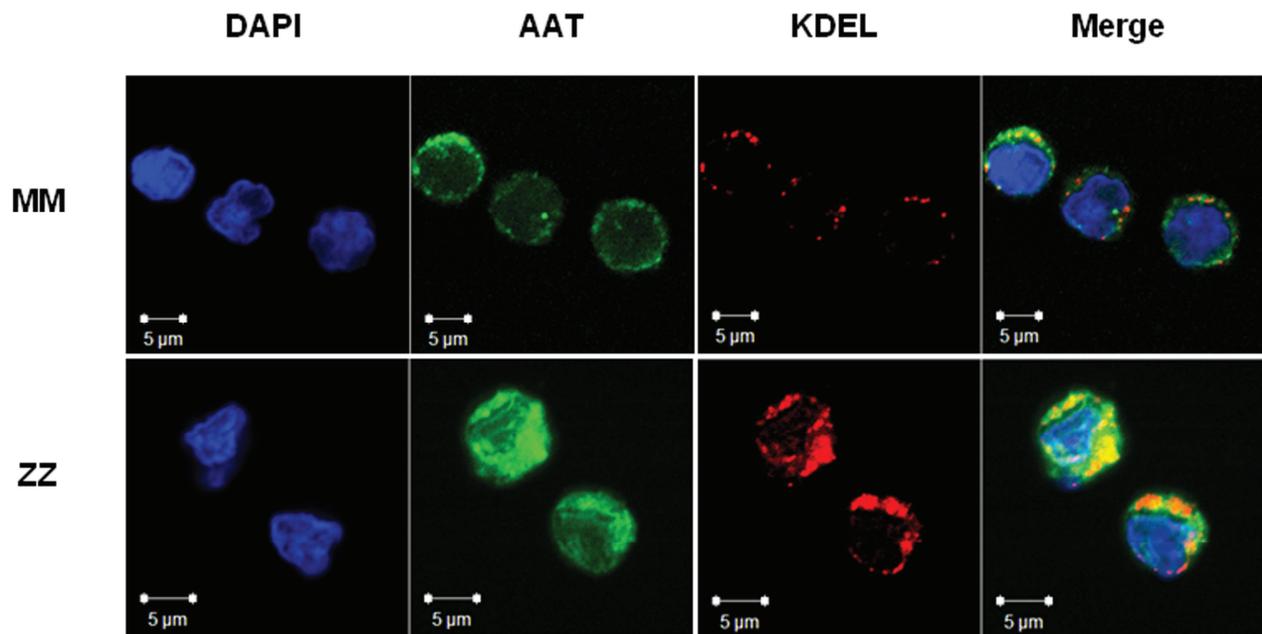
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## **Footnotes**

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**Figure 1.1.** Quantitative RT-PCR analysis of calreticulin (CRT), sXBP-1 and GRP78 mRNA induction in peripheral blood monocytes after treatment with thapsigargin for 4 hours.



**Figure 1.2.** Intracellular accumulation of AAT and increased KDEL expression in the ER of ZZ monocytes. The subcellular distribution of AAT in monocytes isolated from MM (normal) and ZZ (AAT-deficient) individuals was determined by immunofluorescence using antibodies for AAT (green) and the ER marker KDEL (red), with colocalisation indicated by yellow staining. Nuclei

were stained with DAPI (blue). Imaging was acquired using a Zeiss LSM510 Meta confocal microscope and the images presented are single focal plane scans of 1  $\mu\text{m}$  depth at the mid section of the fixed cells.