

# **The role of mitochondria in innate immunity and inflammation**

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## Abstract

Deactivation of blood monocytes during sepsis is associated with increased mortality and susceptibility to secondary infections. Septic monocytes may also have mitochondrial DNA (mtDNA) depletion and mitochondrial respiratory dysfunction. Two principal approaches explored the link between these phenomena in THP-1 cells, a human leukaemia cell line resembling monocytes, to test the hypothesis that mtDNA depletion is important in the pathophysiology of monocytic cell immune deactivation.

Firstly, the consequences of immune deactivation for mitochondria was assessed using an endotoxin tolerance model in which repeated exposures to lipopolysaccharide (LPS) trigger diminishing inflammatory responses. In parallel with the induction of endotoxin tolerance, LPS treatment lead to increased mitochondrial respiration due to the activation of mitochondrial biogenesis. These results could not be confirmed in healthy volunteers following inhalation of LPS as this model failed to induce endotoxin tolerance in blood monocytes.

Secondly, the effects of depleting mtDNA, by treatment with ethidium bromide or transfection with short-interfering RNA targeted against mitochondrial transcription factor A, on immunity were measured. THP-1 cells with mtDNA depletion displayed the key phenotypic feature of deactivated septic monocytes, a decreased LPS-induced release of the pro-inflammatory cytokine tumour necrosis factor- $\alpha$ . Furthermore, there were significant alterations in the nuclear transcriptome of mtDNA-depleted THP-1 cells, with a particular inhibition of key innate immune signalling pathways and a marked blunting of the transcriptomic response to LPS.

These investigations confirm that there are complex but vital links between mitochondria and innate immunity. Compensatory responses following an inflammatory insult include the simultaneous induction of mitochondrial biogenesis and shift to an anti-inflammatory phenotype. Moreover, when sepsis disrupts mitochondrial homeostasis the negative effects of mtDNA depletion on innate immunity may exacerbate monocyte immune deactivation. Further investigations should focus on exploring the fundamental processes coupling mitochondria with immunity and confirming these findings in blood monocytes during sepsis.

This thesis is dedicated to Claire and Nora

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## Abbreviations

ACoA – acetyl coenzyme-A; ADP – adenosine diphosphate; ANOVA – analysis of variance; AS – autologous serum; Atg7 – autophagy-related protein-7; ATP – adenosine triphosphate; BSA – bovine serum albumin; B2M –  $\beta$ 2-microglobulin; CD14/16/36/206 – cluster of differentiation-14/16/36/206; cDNA – complementary deoxyribonucleic acid; CII – mitochondrial complex II; CIV – mitochondrial complex IV; CLP – caecal ligation and puncture; CO – carbon monoxide; CO<sub>2</sub> – carbon dioxide; CpG – cytosine-phosphate-guanine; CQ – chloroquine; CS – citrate synthase; DAMP – damage-associated molecular pattern; DAPI - 4'6'-diamidino-2-phenylindole; DCF-DA – dichloro dihydro fluorescein diacetate; DDM - N-dodecyl- $\beta$ -D-maltoside; DMEM - Dulbecco's Modified Eagle's Medium; DNA – deoxyribonucleic acid; dsRNA – double-stranded ribonucleic acid; DTNB – 5,5'-dithiobis-2-nitrobenzoic acid;  $\epsilon$  – extinction co-efficient; ECL – enhanced chemiluminescence; EGTA - ethylene glycol tetraacetic acid; ELISA – enzyme linked immunosorbent assay; EtBr – ethidium bromide; FCCP - carbonyl cyanide p-trifluoromethoxy-phenylhydrazone; FCS – foetal calf serum; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; GM-CSF – granulocyte macrophage colony stimulating factor; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hg19 – human genome version 19; HLA-DR – human leucocyte antigen-DR; HMGB1 – high mobility group box-1; HO-1 – haem-oxygenase-1; HRP – horseradish peroxidase; H<sub>2</sub>O – water; IFN $\alpha$ 1 – interferon- $\alpha$ 1; IFN $\gamma$  – interferon- $\gamma$ ; IFN $\Lambda$ 1 – interferon- $\Lambda$ 1; IL-1RA – interleukin 1 receptor antagonist; IL-1 $\beta$  – interleukin-1 $\beta$ ; IL-6 – interleukin-6; IL-8 – interleukin-8; IL-10 – interleukin-10; IL-18- interleukin-18; IMDM – Iscove's modified Dulbecco's medium; iNOS – inducible nitric oxide synthase; IPA<sup>®</sup> - ingenuity pathway analysis; IQR – interquartile range; IRF-3 – interferon regulatory factor-3; IRF-7 – interferon regulatory factor-3/7; JC-1 – tetraethylbenzimidazololyl-carbocyanine iodide; LC3 – microtubule-associated protein 1A/1B, light chain-3 ; LF- Lipofectamine; LPS – lipopolysaccharide; MAVS – mitochondrial antiviral signalling protein ; MEM - Minimal Essential Medium; MHC – major histocompatibility complex; mRNA – messenger RNA; MT-CO1 – mitochondrial complex IV subunit 1; mtDNA – mitochondrial deoxyribonucleic acid; MT-ND1 – mitochondrial nicotamide adenine dinucleotide dehydrogenase subunit 1; NAD<sup>+</sup> -oxidised nicotinamide dinucleotide; NADH - nicotinamide adenine dinucleotide dehydrogenase; NAO – nonyl-acridine

orange; NF- $\kappa$ B – nuclear factor- $\kappa$ B; NLRP3 – nucleotide-binding oligomerisation domain-like receptor family, pyrin domain containing-3; NRF-1/2 – nuclear respiratory factor 1/2; Nrf2 – nuclear factor (erythroid derived-2) like-2; OCR – oxygen consumption rate; OXPHOS – oxidative phosphorylation; O<sub>2</sub> – oxygen;  $\rho$ 0 – cells lacking mtDNA; PAGE – polyacrylamide gel electrophoresis; PAMP – pathogen-associated molecular pattern; PBMC – peripheral blood mononuclear cells; PBS – phosphate buffered saline; PCA – principle component analysis; PGC-1 $\alpha$  – peroxisome proliferator-activated receptor- $\gamma$  co-activator 1- $\alpha$ ; PI3K – phosphoinositide-3-kinase; PINK1 – phosphatase and tensin homolog-induced putative kinase-1; PKC $\theta$  – protein kinase  $\theta$ ; PMA – phorbol 12-myristate 13-acetate; PMSF – phenylmethane sulphonylfluoride; POLG – DNA polymerase- $\gamma$ ; PPAR – peroxisome proliferator activator receptor; PRR – pattern recognition receptor; PVDF – polyvinylidene difluoride;  $\rho$ 0 cells – cells lacking mitochondrial deoxyribonucleic acid; qPCR – quantitative polymerase chain reaction; RISC – ribonucleic acid-induced silencing complex; RNA – ribonucleic acid; RNA-Seq – ribonucleic acid sequencing; rRNA – ribosomal ribonucleic acid; RIG1- retinoic acid inducible gene-1; ROS – reactive oxygen species; RPMI 1640 – Roswell Park Memorial Institute 1640 medium; RTqPCR – reverse transcription quantitative polymerase chain reaction; SA – Staphylococcus aureus; SDHA – succinate dehydrogenase subunit A; SDS – sodium dodecyl sulphate; siRNA – short-interfering ribonucleic acid; SIRT1 – silent information regulator (sirtuin)-1; SIRT3 – sirtuin-3; SOD2 – superoxide dismutase-2; SPSS – statistical package for the social sciences; STAT1 – signal transducer and activation of transcription-1; STAT3 – signal transducer and activation of transcription-3 TFAM - mitochondrial transcription factor A; TGF $\beta$  – transforming growth factor- $\beta$ ; TLR-2 - toll-like receptor-2; TLR-3 - toll-like receptor-3; TLR-4 – toll-like receptor-4; TLR-7 - toll-like receptor-7; TLR-9 – toll-like receptor-9; TNB – 5-thio-2-nitrobenzoate; TNF $\alpha$  – tumour necrosis factor- $\alpha$ ; TP53 – tumour protein p53; TREM1 – triggering receptor expressed on myeloid cells-1; TSP1 – thrombospondin-1; 95% CI – 95% confidence interval,  $\Delta\psi_m$  – mitochondrial membrane potential.

## Units of measurement

bp – base pairs; Ct – threshold cycle; G – relative centrifugal force; kDa – kilo-Daltons; l – litre ml – millilitre;  $\mu$ l – microlitre; g – gram; mg – milligram;  $\mu$ g – microgram; ng – nanogram; pg – picogram; M – molar; mM – millimolar;  $\mu$ M – micromolar; nM – nanomolar; nmol – nanomole; pMole/min/mg protein – picomole per minute per milligram protein; cm – centimetre; mm – millimetre; nm – nanometre;; N – equivalent hydrogen ions per litre; RFU – relative fluorescent units; RPKM – reads per kilobase per million mapped reads; U – unit;  $^{\circ}$ C – degrees Celsius; IU/IU CS – units per unit citrate synthase activity.

# Chapter 1 Introduction

## 1.1 Overview

This chapter provides a detailed review of the themes explored in this PhD thesis. It begins with an overview of sepsis and the evidence for the role of immune suppression, in particular deactivation of blood monocytes, in adverse clinical outcomes in septic patients. Following this the different aspects of mitochondrial dysfunction that are associated with sepsis are detailed, including the evidence for these processes occurring in septic monocytes. Next, the potential importance of restoring mitochondrial function, through a combination of mitochondrial biogenesis and mitophagy, in the recovery from sepsis is explored. Finally the potential links between immune suppression, mitochondrial dysfunction and the compensatory responses triggered by sepsis are discussed.

## 1.2 The clinical problem of sepsis

While infectious diseases remain a major cause of premature mortality worldwide, advances in their prevention and treatment have substantially reduced the burden of infection in the developed world (Lozano *et al.*, 2012). However, such improvements have not translated into significantly better outcomes when an infection results in sepsis. Sepsis occurs when an infection triggers a systemic inflammatory response (Levy *et al.*, 2003). It is frequently complicated by organ dysfunction, the presence of which indicates severe sepsis. Septic shock is the most serious manifestation of sepsis and is characterised by the development of circulatory failure that is unresponsive to fluid resuscitation (Annane *et al.*, 2005).

Large epidemiological studies indicate that sepsis is a common and growing cause of critical illness which results in considerable morbidity and mortality (Vincent *et al.*, 2006; Vincent *et al.*, 2014). It has been reported that sepsis is responsible for 29.5% of admissions to critical care units across the world, including 28.7% of admissions in the United Kingdom (Harrison *et al.*, 2006; Vincent *et al.*, 2014). In the United States a longitudinal analysis indicated that there was an annual increase of 8.7% in the incidence of sepsis between 1979 and 2000 (Martin *et al.*, 2003). In 2004 the mortality rate for patients with severe sepsis in the United Kingdom was estimated to be 44.7%

while the 2014 international study found an in-hospital mortality rate of 35.6% (Harrison *et al.*, 2006; Vincent *et al.*, 2014). The modest improvements in sepsis outcomes in recent years have been outstripped by the rising incidence so that the number of deaths due to sepsis continues to increase (Martin *et al.*, 2003). Moreover, survivors often have an incomplete recovery, with the sepsis illness causing long term adverse effects on quality of life and life expectancy (Yende and Angus, 2007).

The current international guidelines for the management of sepsis focus on the two interventions which have been proven to improve survival; early resuscitation and the timely administration of broad spectrum antibiotics (Rivers *et al.*, 2001; Kumar *et al.*, 2006; Dellinger *et al.*, 2008; Peake *et al.*, 2014). Despite numerous clinical trials there are few other therapies with any proven benefits (Russell, 2006). In addition to the stubbornly high mortality rates, the increasing challenge of managing infections due to antibiotic resistant micro-organisms, combined with the scarcity of new antibiotics in the drug development pipeline, highlight the importance of developing novel treatments for sepsis (Davies, 2013). However, it is clear that the development of interventions that can effectively improve the prognosis of critically ill septic patients requires an improved understanding of the fundamental pathophysiology of sepsis (Monneret *et al.*, 2008).

### 1.3 Immune dysfunction in sepsis

Rather than the direct effects of the invading pathogen, the host inflammatory response to an infection appears to be the most critical determinant of the clinical course of sepsis (Kox *et al.*, 2000). Pattern recognition receptors (PRRs) on the surface of innate immune cells recognise conserved pathogen-associated molecular patterns (PAMPs) on invading micro-organisms and trigger an immune response (van der Poll and Opal, 2008). Recognition of PAMPs activates pro-inflammatory responses aimed at attracting and activating other immune cells and controlling the infection. In sepsis this initial inflammatory response can become excessive and dysregulated, leading to tissue damage, organ dysfunction and circulatory failure (Rittirsch *et al.*, 2008). As a result, a number of therapeutic interventions aimed at attenuating inflammatory responses in sepsis have been trialled. Nonetheless, these interventions, which often involve trying to inhibit the action of specific pro-inflammatory cytokines, have failed

to produce any significant impact on patient outcomes (Kox *et al.*, 2000). Despite the absence of effective immunomodulatory interventions, improvements in supportive care mean that more patients are surviving this early phase of sepsis (Hotchkiss *et al.*, 2013a).

In addition to triggering pro-inflammatory responses, recognition of PAMPs by innate immune cells simultaneously initiates compensatory anti-inflammatory responses aimed at limiting tissue damage (Scheffold *et al.*, 2008a). These anti-inflammatory responses may also become excessive and lead to a prolonged state of immune deactivation in which the host is unable to clear the primary infection and is vulnerable to developing secondary hospital-acquired infections (Hotchkiss *et al.*, 2009). The majority of deaths in sepsis occur later in the course of the illness and are associated with evidence of immune deactivation (Hotchkiss *et al.*, 2013a).

There is increasing evidence from clinical studies of the importance of immune deactivation in sepsis. A post mortem study of 235 patients dying due to post-surgical sepsis found an unresolved septic focus, despite appropriate antibiotic therapy, in the majority of patients (Torgersen *et al.*, 2009). Other studies have found that a significant proportion of previously immune competent critically ill patients will have evidence of reactivation of latent viral infections and that this is associated with prolonged hospitalisation and increased mortality (Luyt *et al.*, 2007; Limaye *et al.*, 2008). In addition, in an analysis of 464 consecutive patients presenting with fever the presence of an anti-inflammatory cytokine profile on admission was associated with increased mortality (Van Dissel *et al.*, 1998). Smaller studies have also indicated that a reduced ability to secrete pro-inflammatory cytokines in response to an *ex-vivo* inflammatory stimulus can predict mortality in children and adults with sepsis (Heagy *et al.*, 2003; Hall *et al.*, 2013).

The exact balance between pro- and anti-inflammatory responses in a particular individual with sepsis depends on a number of factors, including the time course of the infection, pathogen virulence, host genetics and the presence of co-morbid diseases (Skrupky *et al.*, 2011). Thus, effective interventions to alter host responses in sepsis need to be targeted to the prevailing inflammatory state of the individual (Monneret *et al.*, 2008). Given the significant morbidity and mortality related to immune deactivation in sepsis, the development of novel immune stimulating interventions

have the potential to produce significant improvements in outcomes of patients with evidence of immune suppression (Hotchkiss *et al.*, 2013a).

#### 1.4 Blood monocytes and sepsis

The precise mechanisms underlying immune deactivation in sepsis are not completely understood. There is evidence of generalised deactivation of innate and adaptive immunity with increased apoptosis and hypo-responsiveness of lymphocytes, a shift in cytokine release towards an anti-inflammatory profile and impairment of monocyte function (Hotchkiss and Karl, 2003). In particular, this deactivation of blood monocytes appears to play an important role in the aetiology of sepsis-induced immune paralysis.

##### 1.4.1 Monocyte functions

Monocytes are bone marrow-derived innate immune cells which represent 5-10% of circulating leukocytes and persist in the peripheral blood for several days (Gordon and Taylor, 2005). While monocytes ultimately differentiate to supply tissues with macrophages and dendritic cells, during an infection they perform a number of additional important effector functions (Serbina *et al.*, 2008). Monocytes limit an infection by acting as phagocytic cells to ingest pathogens and scavenge toxins (Hume, 2006). Furthermore, through the release of cytokines and the presentation of antigens, monocytes play a key role in the co-ordination of innate immunity and the stimulation of lymphocytes to trigger adaptive immune responses targeted at the specific invading pathogen (Geissmann *et al.*, 2008; Auffray *et al.*, 2009).

There are three main subtypes of monocytes based on the surface expression of cluster of differentiation-14 (CD14, a component of the lipopolysaccharide (LPS) receptor complex) and CD16 (the FcγRIII immunoglobulin receptor); classical (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate (CD14<sup>++</sup>CD16<sup>+</sup>) and non-classical (CD14<sup>+</sup>CD16<sup>++</sup>) monocytes (Ziegler-Heitbrock *et al.*, 2010). In blood the majority are classical monocytes which have a predominantly phagocytic phenotype (Serbina *et al.*, 2008). Non-classical monocytes have an increased ability to produce pro-inflammatory cytokines and present antigens, while intermediate monocytes form a small proportion of blood monocytes and have a transitional phenotype (Mukherjee *et al.*, 2015). Several small observational studies have indicated that there is a significant expansion of pro-inflammatory non-classical monocytes in patients with severe sepsis (Fingerle *et*



*al.*, 1993; Schinkel *et al.*, 1998; Schinkel *et al.*, 1999; Mukherjee *et al.*, 2015). However, the exact proportion and phenotype of monocyte subsets in sepsis appears to vary depending on the nature of the infection and the underlying conditions within the tissues (Strauss-Ayali *et al.*, 2007; Colo Brunialti *et al.*, 2012).

#### *1.4.2 THP-1 cells as model of blood monocytes*

Rather than primary blood monocytes, THP-1 cells are used in the majority of the investigations presented in this thesis. The THP-1 cell line was isolated and cultured from the blood of a male infant with acute monocytic leukaemia over 30 years ago (Tsuchiya *et al.*, 1980). These cells have similar morphology, surface antigens and secretory products to blood monocytes and display more mature monocyte markers than other similar cell lines (Altieri and Edgington, 1988; Auwerx, 1991). Due to their stability and homogeneity and the difficulty of isolating blood monocytes in large numbers, THP-1 cells are frequently used to investigate monocyte functions in disease models (Qin *et al.*, 2014). In particular, THP-1 cells are useful in the study of inflammatory conditions as they have similar gene expression and cytokine release profiles to monocytes following stimulation with PRR ligands, including LPS from the outer membrane of Gram negative bacteria (Perez-Perez *et al.*, 1995; Sharif *et al.*, 2007).

#### *1.4.3 Monocyte deactivation in sepsis*

A large number of observational clinical studies have found an association between evidence of monocyte deactivation and the risk of adverse outcomes in patients with sepsis. In general, deactivated monocytes are identified by the detection of either functional or phenotypic biomarkers of immune suppression. The two most commonly identified features of dysfunctional monocytes in sepsis are discussed below.

##### *1. Reduced ex-vivo release of tumour necrosis factor- $\alpha$ (TNF $\alpha$ ) by septic monocytes.*

TNF $\alpha$ , a pro-inflammatory cytokine produced by monocytes and macrophages, acts as a central regulator of the inflammatory response to an infection through nuclear factor- $\kappa$ B (NF- $\kappa$ B)-mediated activation of inflammatory gene expression (Aggarwal *et al.*, 2012). *Tnfa*<sup>-/-</sup> knockout mice have an increased susceptibility to bacterial infection and clinical trials of TNF $\alpha$  inhibition in patients with sepsis have failed to show significant benefits (Fisher Jr *et al.*, 1996; Pasparakis *et al.*, 1996; Reinhart and Karzai,

2001). It has been consistently shown that the release of TNF $\alpha$  in response to an *ex vivo* stimulation with LPS is significantly impaired in monocytes isolated from patients with sepsis (Sfeir *et al.*, 2001; Cavaillon and Adib-Conquy, 2007; Monneret *et al.*, 2008). Furthermore, small observational studies have indicated that there is both a greater reduction in LPS-induced TNF $\alpha$  release and a failure to increase TNF $\alpha$  production over the course of the illness in non-surviving sepsis patients compared to survivors (Munoz *et al.*, 1991; Ploder *et al.*, 2006). In a study of 70 septic children, a decrease in TNF $\alpha$  release in response to LPS by monocytes was strongly associated with the risk of persistent nosocomial infection and death (Hall *et al.*, 2011). These researchers also carried out an innovative pilot study in which 7 children had restoration of *ex vivo* LPS-induced TNF $\alpha$  release and suffered no episodes of nosocomial infection after treatment with granulocyte-macrophage colony stimulating factor (GM-CSF), a cytokine that enhances many monocyte functions.

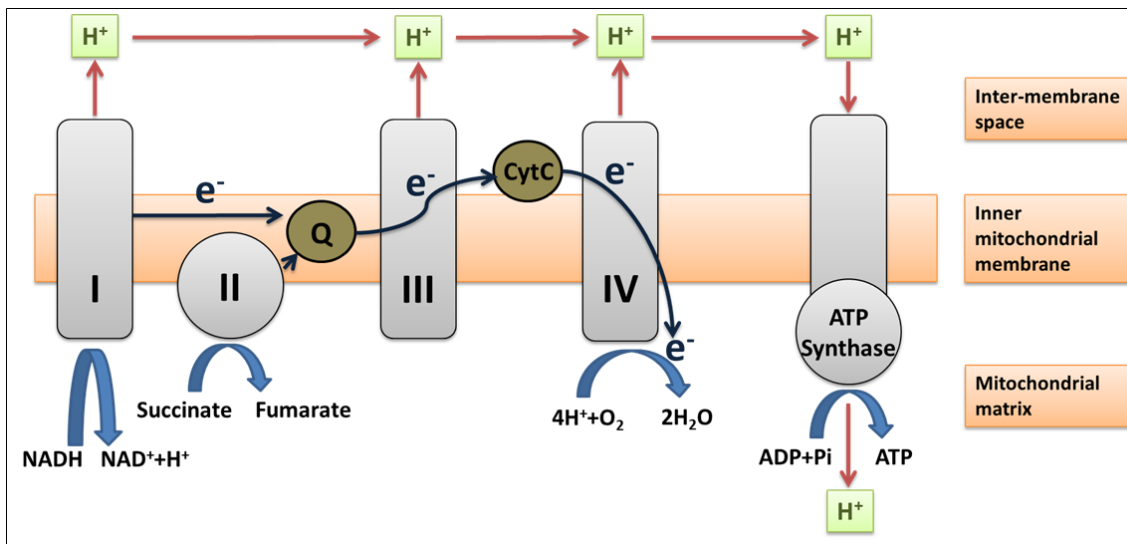
## 2. *Decreased surface expression of human leukocyte antigen-DR (HLA-DR).*

A reduction in the expression of HLA-DR, a class II major histocompatibility complex (MHC) protein involved in antigen presentation to lymphocytes, has also been identified as a biomarker of monocyte deactivation (Monneret *et al.*, 2008). In 153 patients with septic shock decreased monocyte HLA-DR expression was found to be strongly correlated with an increased risk of developing nosocomial infections (Landelle *et al.*, 2010). Longitudinal studies have shown that mortality is increased in septic patients with low monocyte HLA-DR and that a failure to recover HLA-DR expression during the sepsis illness is also associated with non-survival (Monneret *et al.*, 2006; Wu *et al.*, 2011). In a pilot intervention study 9 septic patients with low monocyte HLA-DR were treated with the potent monocyte activator interferon- $\gamma$  (IFN $\gamma$ ) and had stabilisation in their clinical condition in association with recovery of both monocyte HLA-DR expression and LPS-induced TNF $\alpha$  release (Döcke *et al.*, 1997). Similarly, a randomised, placebo-controlled trial of 38 patients with low monocyte HLA-DR and severe sepsis found that treatment with GM-CSF resulted in normalisation of HLA-DR expression and pro-inflammatory cytokine production by monocytes and resulted in shorter length of mechanical ventilation and hospitalisation (Meisel *et al.*, 2009).

Thus, there is considerable evidence that patients with sepsis-induced monocyte immune deactivation are more likely to have adverse clinical outcomes. However, these observational clinical studies do not assess the underlying mechanisms behind monocyte deactivation in sepsis. There have been suggestions that effects of anti-inflammatory cytokines, an impairment of chemotaxis or an unresponsiveness to co-stimulation by helper T-lymphocytes may be implicated (Sfeir *et al.*, 2001; Pachot *et al.*, 2008; Sinistro *et al.*, 2008; Xu *et al.*, 2012). Alternatively, an emerging possibility is that monocyte deactivation may be caused by sepsis-induced depletion and dysfunction of their mitochondria.

### 1.5 Mitochondrial biology

Mitochondria are double-membrane organelles that carry out several critical cellular functions. They are involved in calcium homeostasis, cell signalling pathways, apoptosis regulation and the biosynthesis of a number of essential compounds (West *et al.*, 2011b; Greaves *et al.*, 2012). However, the primary role of mitochondria is in the generation of cellular energy in the form of adenosine triphosphate (ATP), a process which occurs at five enzyme complexes on the inner mitochondrial membrane (DiMauro and Schon, 2003) (Figure 1.1). Through the oxidation of electron carriers these complexes generate an electrochemical gradient across the inner mitochondrial membrane which drives the synthesis of ATP. This process is termed oxidative phosphorylation (OXPHOS) and utilises over 90% of cellular oxygen (Qian and Van Houten, 2010).



**Figure 1.1 Schematic diagram of the mitochondrial oxidative phosphorylation system.** At five enzyme complexes on the inner mitochondrial membrane the oxidation of the electron donors nicotinamide adenine dinucleotide (NADH) and succinate, which are formed in the breakdown of glucose, fats and amino acids, is coupled to the phosphorylation of adenine diphosphate (ADP) to produce energy in the form of ATP. Step-wise redox reactions lead to the transfer of electrons to complex IV which reduces molecular oxygen to water. During this process complexes I, III and IV actively pump protons from the mitochondrial matrix into the inter-membrane space. The resultant proton gradient across the inner mitochondrial membrane drives the generation of ATP by ATP synthase (complex V). The diagram includes; I – NADH dehydrogenase, II – succinate dehydrogenase, III – cytochrome bc1 complex, IV – cytochrome c oxidase, Q – ubiquinone, CytC – cytochrome C, navy arrows – electron transfer, red arrows – proton transfer. This figure is adapted from (Brealey and Singer, 2003).

Uniquely among animal cell organelles, each mitochondrion contains multiple copies of its own genome in the form of circular mitochondrial DNA (mtDNA) (Chandel and Schumacker, 1999). The vast majority of mitochondrial proteins are encoded by nuclear genes but mtDNA encodes 13 essential subunits of the OXPHOS complexes in addition to the RNA required for the translation of these genes within the mitochondrion (Chinnery and Hudson, 2013). Deletions and mutations in mtDNA cause a variety of human diseases that preferentially affect tissues with high metabolic requirements, such as neurones and skeletal muscle (DiMauro and Schon, 2003). The accumulation of mtDNA defects over time is also thought to contribute to the ageing process and be involved in the pathogenesis of neurodegenerative diseases (Greaves *et al.*, 2012). Experimental decreases in mtDNA copy number compromise mitochondrial respiration leading to a dependence on anaerobic metabolism and

ultimately to impaired cellular functions (Chandel and Schumacker, 1999; Jeng *et al.*, 2008).

## 1.6 Mitochondrial dysfunction in sepsis

In clinical studies and animal models assessing sepsis and critical illness there is extensive evidence of mitochondrial dysfunction in a wide range of tissues and cell types, including monocytes. The adverse effects of sepsis on mitochondria are manifested in a number of ways.

### 1.6.1 Mitochondrial respiratory dysfunction in sepsis

The findings from a range of clinical studies and animal sepsis models suggest that mitochondrial respiration may be compromised during severe sepsis. It has been shown that septic tissues have reduced oxygen consumption and ATP generation despite the presence of an adequate oxygen supply, a condition termed cellular dysoxia (Levy and Deutschman, 2007; Singer, 2007). The mechanisms underlying this loss of respiratory activity are not fully understood, although inhibition of OXPHOS complexes by reactive oxygen and nitrogen species and alterations in the availability of respiratory substrates have been implicated (Jeger *et al.*, 2013; Saeed and Singer, 2013).

In a novel experimental medicine study the expression of genes involved in ATP generation during mitochondrial OXPHOS was found to be down-regulated when the transcriptome of 8 healthy volunteers was analysed at serial time points following the administration of intravenous LPS (Calvano *et al.*, 2005). These findings are consistent with an earlier clinical study which found that muscle biopsies from septic patients had significantly reduced mitochondrial respiratory activity, as evidenced by decreased ATP levels and OXPHOS complex I activity, with a particularly severe impairment measured in those who were ultimately non-survivors (Brealey *et al.*, 2002). Similarly, mitochondrial mass, complex I activity and ATP levels were all significantly lower in muscle biopsies from 10 patients with sepsis and multi-organ failure compared to 10 matched controls (Fredriksson *et al.*, 2006). In addition, a larger study revealed that 198 severe sepsis patients had significantly reduced platelet OXPHOS complex IV activity throughout the first week of illness compared to 96 age-matched controls (Lorente *et al.*, 2015). The same authors had also previously shown that patients

surviving severe sepsis had higher platelet complex IV activity which increased over the course of the illness when compared to non-survivors (Lorente *et al.*, 2014).

In several small observational clinical studies mitochondrial respiration has also been shown to be compromised in monocytes from septic patients. A study of 19 patients with sepsis found that peripheral blood mononuclear cells (PBMCs, predominantly monocytes and lymphocytes) had a significant impairment of oxygen consumption and mitochondrial OXPHOS complex I, II and IV activity, effects that were reproduced by exposing healthy PBMCs to serum from the sepsis patients (Garrabou *et al.*, 2012). Elsewhere, PBMCs isolated from 20 patients with septic shock had decreased mitochondrial respiration due to impaired complex V activity and this dysfunction was associated with an increased risk of organ failure and death (Japiassú *et al.*, 2011). Similarly, a further study found that monocytes from 18 patients with severe sepsis had both impaired mitochondrial respiration and decreased HLA-DR expression (Belikova *et al.*, 2007).

### *1.6.2 Mitochondria and oxidative stress in sepsis*

Sepsis-induced OXPHOS dysfunction is also associated with excessive mitochondrial reactive oxygen species (ROS) production (Galley, 2011). Mitochondria are the major source of cellular ROS production due to reactions between molecular oxygen and electrons leaking from OXPHOS complexes I, II and III (West *et al.*, 2011b).

Mitochondrial ROS are required for a number of cell signalling processes and for bacterial killing by phagocytes, but excessive production can overwhelm antioxidant defences and lead to oxidative stress (Galley, 2011; West *et al.*, 2011a; West *et al.*, 2011b). There is considerable evidence that generalised oxidative stress is present in patients with sepsis, with both animal and cell culture sepsis models confirming that this is linked to the induction of mitochondrial respiratory dysfunction (Crimi *et al.*, 2006; Andrades *et al.*, 2011; Drabarek *et al.*, 2012; Cherry *et al.*, 2014). Furthermore, ROS-mediated oxidative damage to OXPHOS complexes and mtDNA may then exacerbate mitochondrial dysfunction, leading to further oxidative stress and, ultimately, to the induction of cell death by apoptosis (Brealey and Singer, 2003; Lee *et al.*, 2012).

### 1.6.3 Depletion of mtDNA in sepsis

As well as causing impairment of mitochondrial respiratory function and excessive mitochondrial ROS production, sepsis may also lead to damage and depletion of mtDNA. As mtDNA lacks protective histones, has limited capacity for repair and is directly exposed to mitochondrial ROS generated by dysfunctional OXPHOS complexes, it is particularly vulnerable to oxidative damage (Lee and Wei, 2005). A mouse model of sepsis involving intra-peritoneal administration of LPS found that the resultant oxidative damage to mtDNA in hepatocytes lead to specific mtDNA deletions and a reduction in mtDNA copy number (Suliman *et al.*, 2003a). In 28 critically ill patients a clinical longitudinal study found that whole blood mtDNA copy number was significantly reduced compared to controls and that persistent mtDNA depletions were strongly associated with increased mortality (Côté *et al.*, 2007). A subgroup analysis in a larger observational study of 147 patients with sepsis identified a more specific significant decrease in mtDNA copy number in monocytes and lymphocytes, the degree of which correlated with the severity of the sepsis illness (Pyle *et al.*, 2010).

### 1.6.4 Mitochondria and inflammasome formation

While sepsis may lead to impairment of mitochondrial functions and mtDNA depletion, it has become apparent that the persistent presence of damaged and dysfunctional mitochondria can, in turn, act as a potent stimulus for on-going inflammation. Dysfunctional mitochondria may become more permeable and release their contents into the cytosol (Kepp *et al.*, 2011). Mitochondria contain two bacterial molecular signatures that act as damage-associated molecular patterns (DAMPs); hypo-methylated cytosine-phosphate-guanine repeats in mtDNA and the formylated N-terminals of mitochondrial proteins (Manfredi and Rovere-Querini, 2010). In the cytosol these mitochondrial DAMPs bind to intracellular PRRs to promote the formation of the nucleotide-binding oligomerisation domain-like receptor, pyrin domain containing-3 (NLRP3) inflammasome, a protein scaffold that facilitates the release of the pro-inflammatory cytokines interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 (Escames *et al.*, 2012).

Furthermore, mitochondrial DAMPs may be released from damaged cells, for example during trauma or chronic inflammation, and activate innate immune cells to trigger

brisk inflammatory responses (Cossarizza *et al.*, 2011; Zhao *et al.*, 2014). Observational studies in patients with sepsis and critical illness have found elevated levels of mitochondrial DAMPs in plasma, the magnitude of which is associated with the presence of multi-organ dysfunction and a higher risk of mortality (Kung *et al.*, 2012; Simmons *et al.*, 2013) The pro-inflammatory effect of extracellular mitochondrial DAMPs has been highlighted in animal models in which their systemic administration leads to the activation and degranulation of neutrophils and induction of a systemic inflammatory response that results in cardiovascular collapse (Zhang *et al.*, 2010; Wenceslau *et al.*, 2015). In addition, incubation of primary human monocytes with a combination of mitochondrial peptides also leads to immune activation and stimulates the release of pro-inflammatory cytokines (Crouser *et al.*, 2009). Conversely, other studies have indicated that longer exposures to mitochondrial DAMPs may actually suppress innate immune responses to subsequent inflammatory stimuli, including LPS-induced TNF $\alpha$  release by human monocytes (Fernández-Ruiz *et al.*, 2014; Zhao *et al.*, 2014). These findings suggests that the release of mitochondrial DAMPs during sepsis may both exacerbate initial exuberant inflammatory responses and also, if sustained, contribute to immune deactivation later in the course of the illness.

## 1.7 The role of mitochondria in the recovery from sepsis

During sepsis there is evidence of mitochondrial OXPHOS dysfunction, excessive mitochondrial ROS production and mtDNA depletion, while the presence of dysfunctional mitochondria can modulate inflammatory responses. In order to compensate for these adverse effects cells must be able to selectively remove dysfunctional organelles and generate new mitochondria to replace them. This compensatory response occurs by the coordinated induction of mitophagy and mitochondrial biogenesis (López-Armada *et al.*, 2013).

### 1.7.1 Mitochondrial biogenesis

Mitochondrial biogenesis is a highly dynamic process during which pre-existing mitochondria grow and divide. It requires the coordinated expression and interaction of a number of mitochondrial genes encoded by both nuclear DNA and mtDNA (Lee and Wei, 2005). Alterations in physiological conditions or cellular energy requirements trigger a complex network of hormones and signalling pathways that lead to the



expression of a number of mitochondrial transcription factors (Weitzel and Alexander Iwen, 2011). Peroxisome proliferator-activated receptor- $\gamma$  co-activator-1 $\alpha$  (PGC-1 $\alpha$ ) acts as a master regulator of mitochondrial biogenesis through the induction of nuclear transcription factors such as nuclear respiratory factors 1 and 2 (NRF-1 and -2) (Lee and Wei, 2005; Crouser, 2010). These transcription factors promote the expression of nuclear genes the products of which form essential mitochondrial constituents or act to influence important mitochondrial functions. In turn, the replication of mtDNA is controlled by mitochondrially-targeted proteins encoded on the nuclear genome, including polymerase- $\gamma$  (POLG), the DNA polymerase responsible for mtDNA replication, and mitochondrial transcription factor A (TFAM), which directly interacts with mtDNA to promote replication and the transcription of mtDNA-encoded genes (Kang *et al.*, 2007).

There are an increasing number of diverse studies in animal models which indicate that expanding the mitochondrial population is an important process in promoting survival and recovery from sepsis. In one highly innovative study the intra-tracheal instillation of bone marrow derived stromal cells increased alveolar ATP generation and protected against LPS-induced acute lung injury through the direct transfer of mitochondria to alveolar epithelial cells (Islam *et al.*, 2012). Elsewhere, the recovery of metabolic function and mtDNA copy number in hepatocytes was found to be dependent on the sustained expression of the activators of mitochondrial biogenesis in a murine model of bacterial peritonitis (Haden *et al.*, 2007). In addition, up-regulation of PGC-1 $\alpha$  expression has been shown to promote recovery from LPS-induced acute kidney injury in mice and the restoration of mitochondrial and cellular function following oxidant injury in rabbit renal proximal tubular cells (Rasbach and Schnellmann, 2007; Tran *et al.*, 2011).

In animal sepsis models the induction of mitochondrial biogenesis appears to be directly triggered by the binding of ligands to PRRs and the resultant activation of inflammatory signalling pathways (Drabarek *et al.*, 2012). Mice treated with a sub-lethal dose of heat-killed *Escherichia coli* were found to have an initial loss of mtDNA and mitochondrial proteins, which was rapidly reversed following the activation of mitochondrial biogenesis in a process dependent on the stimulation of toll-like receptor-4 (TLR-4, the PRR for LPS) (Reynolds *et al.*, 2009). Similarly, after exposure to

*Staphylococcus aureus* *Tlr-2*<sup>-/-</sup> and *Tlr-4*<sup>-/-</sup> knock-out mice had increased mortality in association with a diminished induction of PGC-1 $\alpha$  expression and persistent mtDNA depletion (Sweeney *et al.*, 2010). In another study pharmacological inhibition of the inflammatory transcription factor NF- $\kappa$ B significantly delayed the increases in NRF-1, TFAM and mtDNA copy number that occurred in response to LPS-induced inflammation in mice and also in human cell lines (Suliman *et al.*, 2010).

In contrast to these animal studies, the evidence for mitochondrial biogenesis induction in human patients with sepsis is less well established. A study of 16 muscle biopsies from patients with sepsis-induced multi-organ failure showed that the survivors had early increases in expression of PGC-1 $\alpha$  and mitochondrial antioxidants (Carré *et al.*, 2010). However, a similar investigation looking at muscle cell transcriptome revealed that incomplete and uncoordinated expression of mitochondrial transcription factors and genes during critical illness may lead to a failure to maintain adequate mitochondrial function (Fredriksson *et al.*, 2008). Additional longitudinal studies will, therefore, be important to clarify the precise role of mitochondrial biogenesis, particularly in human monocytes, during the recovery of critically ill patients following an inflammatory insult.

### 1.7.2 Mitophagy

Through encapsulation in an autophagosome and subsequent lysosomal degradation, damaged and dysfunctional cellular contents are catabolised during autophagy (Lee *et al.*, 2012). Mitophagy is a specialised form of autophagy which involves the removal of mitochondria from a cell in response to developmental demands or in order to maintain quality control (Youle and Narendra, 2011). Dysfunctional mitochondria, particularly those generating excessive ROS or with depolarisation of the mitochondrial membrane potential ( $\Delta\psi_m$ , generated by the transport of protons across the inner mitochondrial membrane during OXPHOS), are selectively targeted for mitophagy by the accumulation of phosphatase and tensin homologue-induced putative kinase-1 (PINK1) which leads to the recruitment of the mitophagy activator Parkin (Narendra *et al.*, 2008; Frank *et al.*, 2012; Gilkerson *et al.*, 2012; Hill *et al.*, 2012).

There is emerging evidence that mitophagy is a critical compensatory response in sepsis. In general there is a lack of clinical data from human patients with sepsis;

although one study found that PINK1 levels were higher (suggesting increased mitophagy) in PBMCs from 8 septic patients compared to 14 critically ill controls (Mannam *et al.*, 2014). This is consistent with murine models in which a septic insult has been found to lead to the induction of mitophagy in association with the recovery of mitochondrial function (Carchman *et al.*, 2013; Chang *et al.*, 2015). Conversely, in a rabbit model of critical illness non-survivors had evidence of inadequate autophagy alongside increased organ damage and a greater impairment of mitochondrial respiration (Gunst *et al.*, 2013). Other studies have indicated that inhibiting autophagy leads to the accumulation of damaged mitochondria and the persistence of oxidative stress following an inflammatory stimulus (Nakahira *et al.*, 2011; Zhou *et al.*, 2011; Motori *et al.*, 2013). Furthermore, defective or inhibited mitophagy may also result in a failure to clear the mitochondrial DAMPs arising from dysfunctional mitochondria, which can exacerbate inflammation due to an increase in NLRP3 inflammasome formation (Nakahira *et al.*, 2011; van der Burgh *et al.*, 2014).

Murine sepsis models also suggest that the induction of mitophagy is closely integrated with the activation of mitochondrial biogenesis following an inflammatory insult. After an intra-abdominal *Staphylococcus aureus* infection there was concurrent up-regulation of mitophagy and the transcription of the key biogenesis regulators *Pgc-1 $\alpha$*  and *Tfam* in the pulmonary tissue of mice (Chang *et al.*, 2015). Elsewhere, after both caecal ligation and puncture (CLP) and LPS exposure, mice in which the resultant contemporaneous induction of mitophagy and mitochondrial biogenesis was blunted had a higher mortality (Mannam *et al.*, 2014). Separately, the simultaneous activation of mitophagy and mitochondrial biogenesis occurring after either CLP or treatment with LPS has been shown to be abolished by inhibiting TLR-4 signalling (Carchman *et al.*, 2013). Intriguingly, this study also suggests that the presentation of mtDNA to the intracellular PRR TLR-9 during mitochondrial degradation may activate mitochondrial biogenesis, as inhibiting either mitophagy or TLR-9 signalling prevented the LPS-induced up-regulation of PGC-1 $\alpha$ , NRF-1 and TFAM.

Thus, there is growing evidence from animal models to suggest that mitophagy is an essential part of the adaptive response to sepsis which is closely integrated with mitochondrial biogenesis. However, there is a clear need for clinical studies in order to confirm the relevance of these findings to human sepsis.

## 1.8 Integration of compensatory responses and inflammation in sepsis

The recovery from sepsis and restoration of cellular functions requires the coordinated activation of a number of compensatory responses. These include the induction of anti-inflammatory cytokine production to promote tissue repair, antioxidant defences to protect against oxidative stress, and recovery of mitochondrial function through mitophagy and mitochondrial biogenesis. While the regulation of these responses is not completely understood, there is emerging evidence that they may share fundamental common processes. In the following sections, two potential mechanisms that may link these compensatory responses during sepsis are reviewed in more detail.

### 1.8.1 Redox-sensitive pathways and compensatory responses during sepsis

In mouse models the induction of both mitochondrial biogenesis and mitophagy appear to be intrinsically linked to the activation of other fundamental homeostatic pro-survival responses, including anti-inflammatory cytokine release, through redox-sensitive pathways (Piantadosi and Suliman, 2012). Haem-oxygenase-1 (HO-1) is an inducible antioxidant enzyme which, through the production of carbon monoxide (CO) during haem detoxification, can stimulate the expression of the transcription factor nuclear factor (erythroid-derived-2)-like-2 (Nrf2) (Alam and Cook, 2003). Nrf2 has the ability to bind to anti-oxidant response elements on gene promoters for transcription factors regulating both mitochondrial biogenesis and anti-inflammatory cytokine production (Piantadosi *et al.*, 2008).

The induction of HO-1 in mouse hepatocytes and macrophages following treatment with LPS has been found to result in the activation of mitochondrial biogenesis, by PGC-1 $\alpha$  and NRF-1, and a simultaneous shift to an anti-inflammatory phenotype that is characterised by increased *Il-10* and decreased *Tnfa* expression (Piantadosi *et al.*, 2011). In a murine peritonitis model of sepsis inhaled CO was found to significantly increase survival through the up-regulation of HO-1 and Nrf2, which resulted in a higher mtDNA copy number and increased IL-10 release (MacGarvey *et al.*, 2012). In another study, the induction of mitochondrial biogenesis following pneumonia was inhibited in *Nrf2*<sup>-/-</sup> knockout mice, resulting in increased pro-inflammatory cytokine release and more severe acute lung injury (Athale *et al.*, 2012). In addition to activating mitochondrial biogenesis, Nrf2 may also be important in the regulation of mitophagy

during sepsis, with *Nrf2*<sup>-/-</sup> mice having a significantly impaired ability to up-regulate mitophagy after *Staphylococcus aureus* infection compared to wild-type mice (Chang *et al.*, 2015).

While the above studies suggest that HO-1/Nrf2 signalling may link mitochondrial biogenesis, mitophagy and anti-inflammatory cytokine production with the induction of antioxidant defences, it should be noted that these investigations have all been carried out by a single research group. The findings, therefore, require verification by independent investigators and subsequent validation in studies on human cells and patients with sepsis.

### *1.8.2 Sirtuins and the link between metabolism and immunity*

Mitochondrial function and turnover may also be integrated with immunity through the action of a group of deacetylases termed silent information regulators (sirtuins) (Preyat and Leo, 2013). Through the modulation of gene expression and protein activity, sirtuins are involved in the regulation of multiple fundamental biological processes. As their deacetylase activity is dependent on the presence of the oxidised form of the respiratory chain co-enzyme nicotinamide adenine dinucleotide (NAD<sup>+</sup>), sirtuins can act as sensors of cellular energy status and link metabolism with other cellular processes (Parihar *et al.*, 2015). There are seven mammalian sirtuins each of which have distinct principal sub-cellular locations, with nuclear SIRT1 and mitochondrial SIRT3 appearing to be particularly important in regulating the responses to an inflammatory stimulus (Liu *et al.*, 2012b).

Within the nucleus SIRT1 acts as a key activator of both mitochondrial biogenesis, by increasing the activity of PGC-1 $\alpha$ , and autophagy in response to cellular stressors, including inflammation (Takeda-Watanabe *et al.*, 2012; Brenmoehl and Hoeflich, 2013). Furthermore, there is increasing evidence that SIRT1 also has an important role in the resolution of inflammation through the negative regulation of pro-inflammatory responses. This effect may be due to the direct inhibition of inflammatory signalling pathways or specific effects of SIRT1 on the transcription of pro-inflammatory genes (Capiralla *et al.*, 2012; Preyat and Leo, 2013). For example, it has been shown in THP-1 cells that TLR-4 signalling leads to a rapid accumulation of SIRT1 at the promoters of the genes that encode the pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$ , with the

resultant epigenetic changes leading to the inhibition of NF- $\kappa$ B-dependent transcription of these genes and reduced cytokine production (Liu *et al.*, 2011). In a separate study this SIRT-1-mediated impairment of pro-inflammatory responses was found to occur in tandem with the induction of mitochondrial biogenesis after treatment of THP-1 cells with LPS, with SIRT1 inhibition leading to LPS-induced mitochondrial depletion and impaired cellular respiration (Liu *et al.*, 2015).

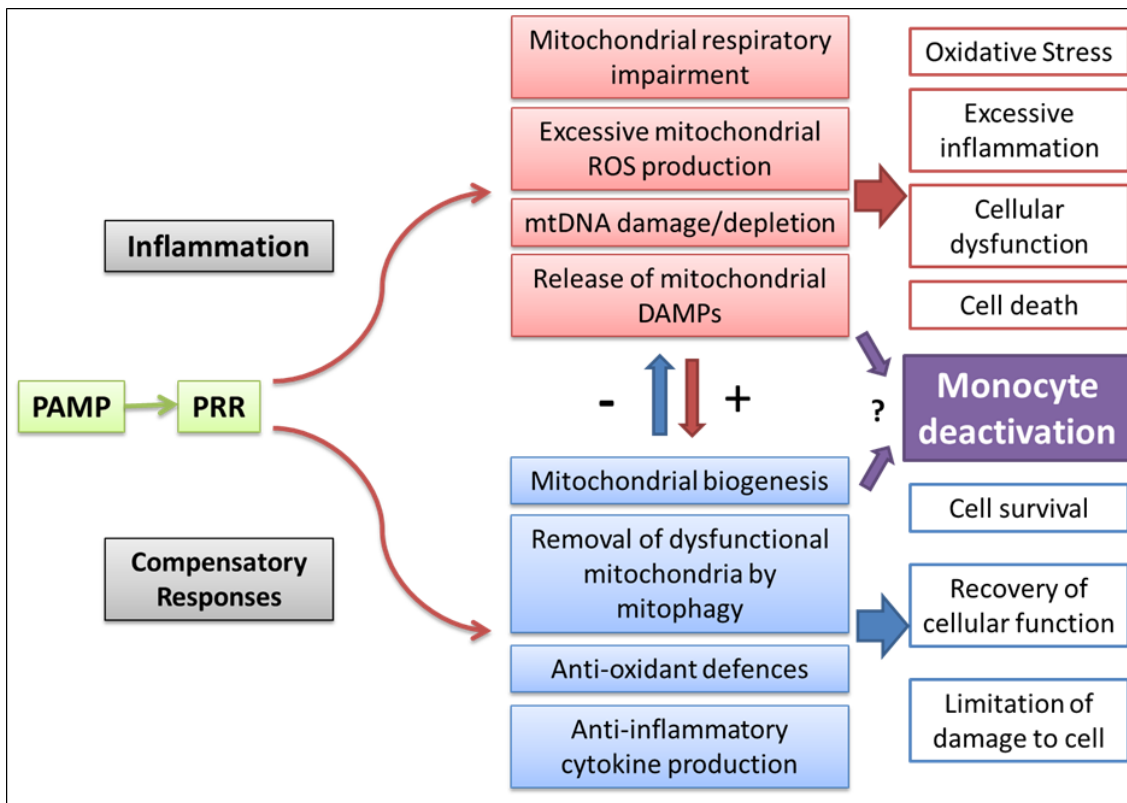
In addition to these effects on immunity and mitochondria, the activation of SIRT1 also leads to the up-regulation of SIRT3, which is the major deacetylase that is active within mitochondria (Liu *et al.*, 2015). SIRT3 is required for effective mitochondrial biogenesis and SIRT3-mediated deacetylation reactions also enhance the function of OXPHOS proteins, leading to increased mitochondrial oxygen consumption (Brenmoehl and Hoeflich, 2013). This is illustrated by the finding that *Sirt3*<sup>-/-</sup> mice have an impaired ability to generate ATP in a variety of tissues in association with hyperacetylation of mitochondrial proteins, including those forming OXPHOS complex I (Ahn *et al.*, 2008). SIRT3 is also critical for the compensatory responses to oxidative stress within the mitochondria. In HEK293 cells increased ROS levels have been shown to lead to the activation of the mitochondrial antioxidant SOD2 through deacetylation reactions catalysed by SIRT3 (Chen *et al.*, 2011). In another study the induction of oxidative stress in human umbilical vein endothelial cells was found to result in the SIRT3-mediated up-regulation of genes involved in mitochondrial biogenesis and mitophagy, with inhibition of SIRT3 leading to impaired mitochondrial respiration (Tseng *et al.*, 2013).

Thus, the sequential activation of SIRT1 and SIRT3 following an inflammatory stimulus may link several compensatory responses, including mitochondrial biogenesis, autophagy, antioxidant defences and the down-regulation of pro-inflammatory cytokine production (Liu *et al.*, 2015). At this point, the importance of sirtuins during sepsis has been investigated in animal models but there is very limited data on their role in human sepsis. Studies in animal sepsis models have indicated that activation of SIRT1 may limit the negative effects of inflammation. In mice the effects of ischaemic-reperfusion injury were found to be abrogated by treatment with the SIRT1 activator SRT1770 due to the simultaneous induction of mitochondrial biogenesis and inhibition of pro-inflammatory signalling and TNF $\alpha$  release (Khader *et al.*, 2014). Similarly,

following intraperitoneal injection with LPS, mice treated with the sirtuin activator resveratrol were protected against acute lung injury and produced lower levels of pro-inflammatory cytokines, effects that were reversed by inhibiting SIRT1 (Li *et al.*, 2013). Conversely, while SIRT1 activation may limit the negative effects of excessive inflammation, in specific situations the inhibition of SIRT1 may also be beneficial during sepsis. For example, treatment of mice with the SIRT1 inhibitor EX527 has been shown to lead to the reversal of sepsis-induced immune suppression and result in increased survival after CLP (Vachharajani *et al.*, 2014).

### 1.9 Sepsis, mitochondria and immunity

The interactions between inflammation, mitochondrial dysfunction and the biogenesis and autophagy of mitochondria appear to be highly complex and variable (Figure 1.2). On the one hand, inflammatory responses can impair mitochondrial function by a number of mechanisms. These dysfunctional mitochondria may, in turn, be important in both sustaining excessive inflammatory responses and in the inhibition of cellular functions. On the other hand, signalling pathways activated following an inflammatory stimulus also appear to simultaneously initiate a range of compensatory mechanisms aimed at maintaining cell viability during stress conditions. In addition to the induction of anti-inflammatory responses and antioxidant defences, there is a coordinated up-regulation of mitophagy and mitochondrial biogenesis which leads to the selective removal and replacement of dysfunctional mitochondria. While these processes are associated with the recovery from an inflammatory insult, the sustained stimulation of mitochondrial biogenesis during conditions of on-going mitochondrial damage such as sepsis could also contribute to immune suppression through the concomitant promotion of excessive anti-inflammatory cytokine release or the prolonged inhibition of pro-inflammatory cytokine production (Piantadosi and Suliman, 2012). Investigations which explore the links between the effects of both inflammatory and compensatory responses on mitochondria and immunity in human monocytes will provide valuable insights into the importance of changes to the function and turnover of mitochondria in sepsis-induced monocyte deactivation.



**Figure 1.2 A summary of the potential interactions between inflammation, mitochondria and compensatory responses in monocytes during sepsis.**

Recognition of PAMPs by PRRs simultaneously triggers both inflammatory and compensatory pro-survival responses. Inflammation can produce negative effects in mitochondria including impairment of respiration, excessive ROS production and depletion of mtDNA, which can result in cellular dysfunction. In addition the release of mitochondrial DAMPs can trigger further inflammation. Compensatory responses involve the co-ordinated stimulation of anti-oxidant and anti-inflammatory responses along with the selective removal and replacement of dysfunctional mitochondria through mitophagy and mitochondrial biogenesis. These responses are aimed at mitigating the effects of excessive inflammation and mitochondrial dysfunction in order to allow recovery of cell functions and survival. Monocyte immune deactivation in sepsis may result from the effects of mitochondrial damage and respiratory impairment which lead to cellular dysfunction. However, excessive compensatory anti-inflammatory responses triggered, in association with mitochondrial biogenesis, by on-going PRR stimulation and mitochondrial damage may also exacerbate the impairment of monocyte immune responses.



## 1.10 Conclusion

Sepsis is an increasing problem in which much of the morbidity and mortality occurs in patients with evidence of immune suppression. In particular, immune deactivation in blood monocytes appears to be critical and is consistently associated with inferior clinical outcomes in septic patients. There is widespread evidence of mitochondrial dysfunction and depletion of mtDNA in sepsis, including in monocytes. In addition, it is increasingly apparent that the coordinated stimulation of mitochondrial biogenesis and mitophagy is important in the recovery from sepsis. These processes appear to be initiated by inflammatory signalling and may also be integrated with the activation of both antioxidant defences and the anti-inflammatory responses that can exacerbate immune suppression. An improved understanding of the relationship between inflammatory responses and mitochondrial function and turnover in monocytes may provide important insights into the mechanisms leading to immune deactivation in monocytes. Ultimately, this has the potential to identify novel therapies that can stimulate the innate immune system and improve clinical outcomes in septic patients with deactivated monocytes.

## Chapter 2 Aims and Objectives

### 2.1 Overview

This chapter begins with a statement of the fundamental research hypotheses before listing the principal aims and objectives that are addressed in the remaining chapters of this thesis.

### 2.2 Hypothesis

After separate findings of impaired immune responses and reduced mtDNA copy number in blood monocytes during sepsis, this thesis aims to address the overarching hypothesis that mtDNA depletion is an important process in the pathophysiology underlying immune deactivation in human monocytic cells. More specifically, the following four hypotheses will be tested;

1. THP-1 cells with immune deactivation will also have evidence of mtDNA depletion and mitochondrial respiratory dysfunction.
2. Following an inflammatory insult there will be a regulated induction of compensatory responses, which include mitochondrial biogenesis and mitophagy, in THP-1 cells.
3. Depleting mtDNA will lead to an impaired ability of THP-1 cells to produce immune responses, in a similar manner to the immune deactivation seen in septic monocytes.
4. There will be significant alterations in the expression of nuclear genes, particularly those involved in inflammatory and immune signalling pathways, in THP-1 cells following mtDNA depletion.

### 2.3 Aims and Objectives

Two main approaches will be used to explore the relationship between mitochondria and immunity in THP-1 cells and blood monocytes. Firstly, in Chapter 4 and Chapter 5 the effect of inducing a state of immune deactivation on the mitochondrial functions of monocytic cells will be assessed. These experiments will have the following principal aims and objectives;

1. To produce a model of monocytic cell immune deactivation by pre-incubating THP-1 cells with LPS to render them endotoxin tolerant.
2. To explore the time-course and dynamics of changes in immunity and mitochondria in THP-1 cells following exposure to lipopolysaccharide (LPS).
3. To assess the effects of exposure of THP-1 cells to LPS on compensatory pro-survival responses, including mitochondrial biogenesis and mitophagy.
4. To determine the effects of inhaling LPS on immunity and mtDNA copy number in the blood monocytes of healthy volunteers.

Subsequently, in Chapter 6, Chapter 7 and Chapter 8 the consequences of depleting mtDNA on the immune functions of THP-1 cells will be determined. The major aims and objectives of these investigations will be;

1. To deplete mtDNA from THP-1 cells using treatment with ethidium bromide and transfection with short-interfering RNA targeted against key genes involved in mtDNA replication.
2. To assess mitochondrial and immune functions in THP-1 cells after depletion of their mtDNA.
3. To determine whether restoration of mtDNA copy number leads to a recovery of cellular respiration and immunity in THP-1 cells.
4. To measure the effects of mtDNA depletion on the expression of nuclear genes by THP-1 cells.
5. To identify any differences in the transcriptomic response of THP-1 cells to LPS caused by mtDNA depletion.

## Chapter 3 Methods

### 3.1 Overview

This chapter describes the principles and protocols of the methods used to generate the data presented in the subsequent chapters of this thesis. In addition, details of the suppliers of the reagents, consumables and equipment used in the experiments are provided. The chapter finishes with an overview of the statistical tests used to analyse the data generated using these methods.

### 3.2 Materials and Equipment

#### 3.2.1 Materials and Reagents

The THP-1 human monocytic cell line was kindly donated by Dr John Taylor's laboratory in the Institute of Cellular Medicine, Newcastle University.

The following reagents were obtained from Life Technologies (Paisley, UK); 4'6'-diamidino-2-phenylindole (DAPI), 2'7'-dichlorofluorescein diacetate (DCF-DA), DNA-free DNase Treatment Kit, Dulbecco's Modified Eagle's Medium (DMEM), *Escherichia coli* (K-12 strain) fluorescein conjugate, foetal calf serum (FCS), iBlot2<sup>®</sup> Transfer Stacks, High Capacity complementary DNA (cDNA) Reverse Transcription Kit, Iscove's Modified Dulbecco's Medium (IMDM), Lipofectamine RNAiMAX, Minimal Essential Medium (MEM), Negative Control siRNA number 1, nonyl-acridine orange (NAO), Novex<sup>®</sup> enzyme-linked immunosorbent assay (ELISA) antibody pair kits (details in Table 3.3), Novex<sup>®</sup> 4-20% Tris-Glycine pre-cast protein gels, Novex<sup>®</sup> Native PAGE<sup>™</sup> 3-12% Bis-Tris gel, Novex<sup>®</sup> Native PAGE<sup>™</sup> 4x sample buffer, Novex<sup>®</sup> Native PAGE<sup>™</sup> 5% G-250 sample additive, Novex<sup>®</sup> Native PAGE<sup>™</sup> 20x running buffer, Novex<sup>®</sup> Native PAGE<sup>™</sup> 20x cathode buffer additive, nuclease-free water, Opti-MEM reduced serum medium, penicillin and streptomycin, propidium iodide, Roswell Park Memorial Institute (RPMI) 1640 medium, SeeBlue Plus2 pre-stained protein ladder, *Staphylococcus aureus* (Wood Strain without protein A) fluorescein conjugate, Silencer<sup>®</sup> Select siRNA (details in Table 3.1), Taqman<sup>®</sup> Gene Expression Assay (details in Table 3.8), Taqman<sup>®</sup> Gene Expression master mix and tetraethylbenzimidazolylcarbocyanine iodide (JC-1).

The following reagents were purchased from Sigma-Aldrich, St Louis, MO, USA; Acetyl co-enzyme-A (ACoA), agarose, antimycin A, bafilomycin A1 from *Streptomyces griseus*, bovine serum albumin (BSA), bromophenol blue, cytochrome C, D-glucose, N-dodecyl- $\beta$ -D-maltoside (DDM), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), ethidium bromide, ethylene glycol tetraacetic acid (EGTA), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (fCCP), galactose, Giemsa, glycerol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hydrogen peroxide, L-glutamine, lipopolysaccharide (LPS) from *Escherichia coli* 026:B6, magnesium chloride, milk powder,  $\beta$ -nicotinamide adenine dinucleotide (NADH), nitrotetrazolium blue chloride (NTB), oligomycin, phenylmethanesulfonyl fluoride (PMSF), phosphate-buffered saline (PBS), phorbol 12-myristate 13-acetate (PMA), potassium phosphate, rotenone, sodium borohydrate, sodium chloride, sodium citrate, sodium dodecyl sulphate, sodium pyruvate, sucrose, sulphuric acid, Triton X, trizma base, trizma-hydrochloride, trypan blue, tween, uridine, valinomycin and zymosan particles from *Saccharomyces cerevisiae*.

6, 24 and 96 well plates and 25cm<sup>3</sup> and 75cm<sup>3</sup> tissue culture flasks were obtained from Greiner Bio-One (Stonehouse, UK).

The following reagents were acquired from Thermo-Fisher Scientific (Rockford, IL, USA); enhanced chemiluminescent (ECL) substrate, 16% formaldehyde solution (methanol-free), NUNC MaxiSorp 96 well microplates and tetramethylbenzidine substrate.

The DNeasy Blood and Tissue Kit, QiA Quick Gel Extraction Kit and RNeasy mini kit were all purchased from Qiagen (Valencia, CA, USA).

Coomassie Brilliant Blue reagent, iQ™SYBR® Green supermix, microseal B plate sealers and 96 well PCR plate were obtained from BioRad (Hercules, CA, USA).

Seahorse Biosciences (Chicopee, MA, USA) provided the XF Assay Cartridges, XF Calibrant Solution and XF Cell Culture 96 well microplates.

CD14 MicroBeads, MACS® MS columns and the Mini-MACS Separator were purchased from Miltenyi Biotec (Auburn, CA, USA).

Dextran was obtained from Pharmacosmos (Holbaek, Denmark) and Percoll was from GE Healthcare Biosciences (Little Chalfort, UK).

The biotinylated protein ladder was from Cell Signalling Technology (Beverly, MA, USA), the Complex IV human specific activity microplate assay kit was purchased from Abcam (Cambridge, UK), Vectashield Hard Set Mounting Medium was acquired from Vector laboratories (Peterborough, UK) and the Agilent 6000 RNA Pico Kit was from Agilent Technologies (Santa Clara, CA, USA).

The tables provide details of the antibodies used in flow cytometry (Table 3.2), Western blotting (Table 3.6) and confocal microscopy (Table 3.9), along with the primers used in quantitative polymerase chain reactions (Table 3.4) and the Taqman<sup>®</sup> gene expression probes (Table 3.8) used in reverse transcription quantitative polymerase chain reactions.

### *3.2.2 Equipment*

The Shandon Cytospin 3, NanoDrop 2000 spectrophotometer and MultiSkan Ascent plate reader were all from Thermo-Fisher Scientific while the FACSCanto II and LSRFortessa X20 flow cytometers and BD Lyse-Wash machine were from Beckton Dickinson (BD) Biosciences (Franklin Lakes, NJ, USA). Leica Microsystems (Heidelberg, Germany) provided the Leica SB2 UV confocal microscope and the 3000 B inversion microscope while the iBlot2<sup>®</sup> Gel Transfer Device and 7500 Fast Real Time PCR System were from Life Technologies (Paisley, UK). The automatic inhalation-synchronised dosimeter nebuliser for the LPS inhalation study was provided by Spira (Hameenlinna, Finland). The other equipment included; the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA), FLUOStar Omega Plate Reader (BMG Labtech, Ortenberg, Germany), HiSeq 2500 (Illumina, San Diego, CA, USA), MultiSpectral Imaging System (UVP, Upland, CA, USA), the MyiQ<sup>™</sup> PCR machine (BioRad, Hercules, CA, USA), the Sanyo MCO-19AIC CO<sub>2</sub> incubator (Sanyo Electric Biomedical, Osaka, Japan) and the XF96<sup>e</sup> extracellular flux analyser (Seahorse Biosciences, Chicopee, MA, USA).

### *3.2.3 Software*

Data were collected and analysed using the following software; Microsoft Office Excel 2013 (Microsoft, Redmond, WA, USA), BD FACSDiva for flow cytometry data, BioRad

iQ5™ optical software v2.0 for quantitative polymerase chain reaction data, Image J software (National Institute of Health, Bethesda, MD, USA) for quantification of Western Blot bands and Volocity (PerkinElmer, Waltham, MA, USA) for analysis of confocal microscopy images. Details of the software used to analyse the transcriptomics data are included in section 3.12.2. GraphPad Prism 6 (GraphPad, La Jolla, CA, USA) and Statistical Package for the Social Sciences (SPSS) for Windows 19 (IBM, New York, USA) were used for statistical analysis.

### 3.3 Cell culture and monocyte isolation

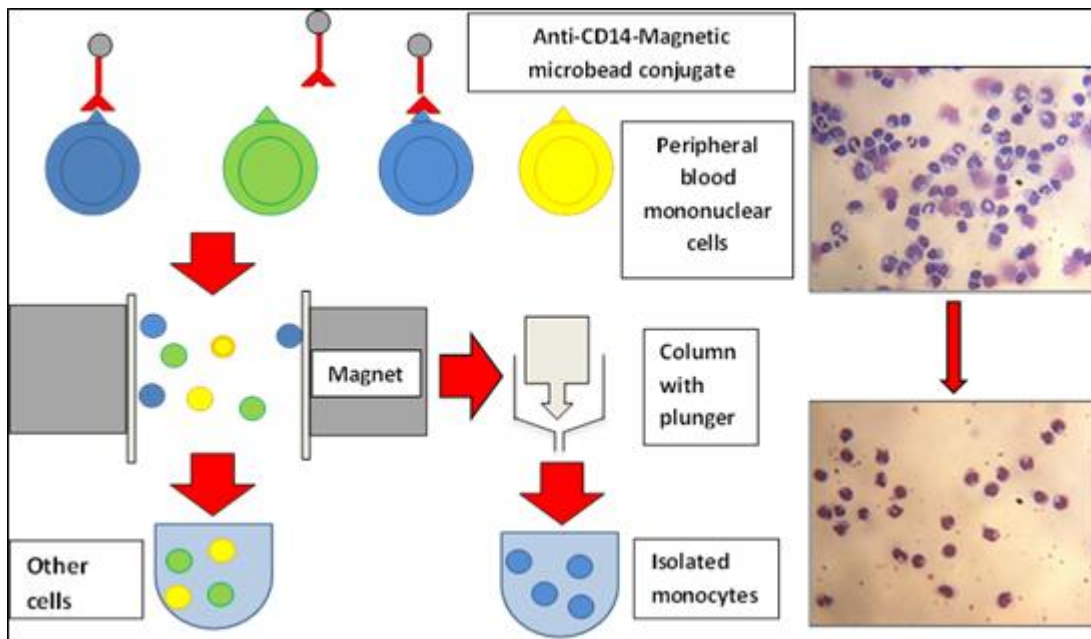
#### 3.3.1 THP-1 cell culture

THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2mmol/l L-glutamine, 11.11mM D-glucose, 100U/ml penicillin and 100µg/ml streptomycin (termed growth medium). THP-1 cell culture was carried out in a humidified incubator at 37°C with 5% carbon dioxide (CO<sub>2</sub>) and maintained at a concentration of less than 1x10<sup>6</sup>cells/ml.

#### 3.3.2 Human monocyte isolation and culture

Human PBMCs were isolated from citrated whole blood by dextran sedimentation followed by Percoll density-gradient centrifugation (Haslett *et al.*, 1985). Cytospin slides stained with Giemsa were reviewed to ensure that the purity of the isolated PBMCs was greater than 95% prior to further processing.

Positive selection was then used to isolate monocytes from the PBMC layer, as this method has been shown to be most effective at increasing the purity of the selected cells without significantly altering gene expression (Lyons *et al.*, 2007). Monocytes were labelled with CD14-MicroBeads and then retained in a MACS® MS column in the magnetic field from the Mini-MACS® Separator (Figure 3.1). The purity of the isolated monocytes was assessed on Giemsa-stained cytospin slides and only samples with greater than 90% monocytes were used in further experiments. The isolated human monocytes were cultured in IMDM containing 10% autologous serum in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

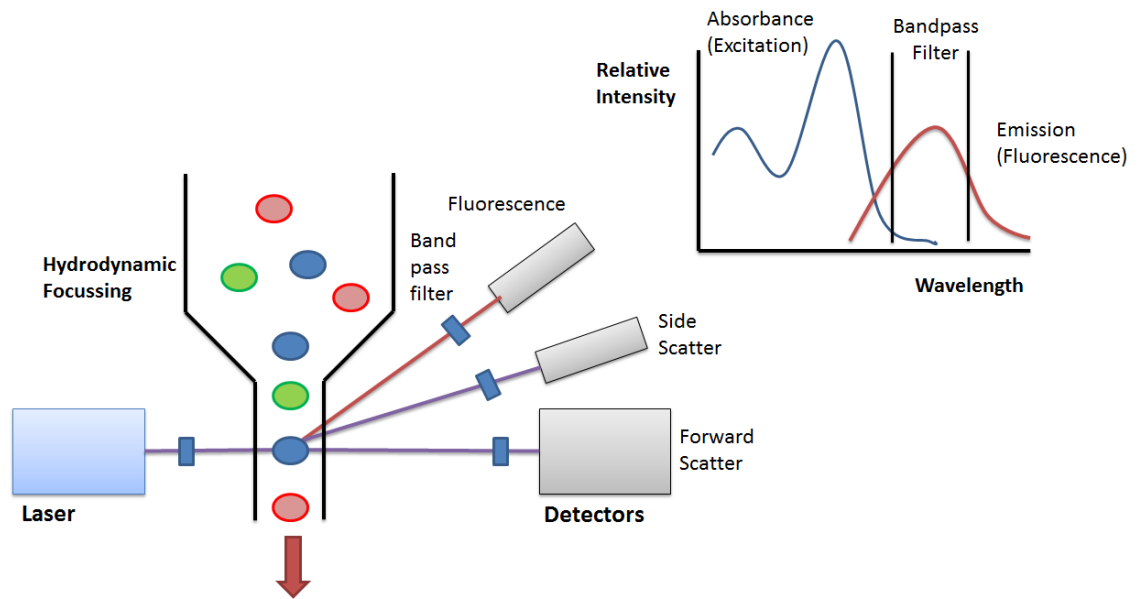


**Figure 3.1 Schematic diagram representing monocyte isolation from the peripheral blood mononuclear cell fraction by positive selection using CD14-MicroBeads.** Monocytes expressing CD14 are coated in microbeads and retained in a MACS® MS column within the magnetic field from the miniMACS® separator, while the other unlabelled cells pass through the column and are discarded. The MACS® MS column is then removed from the magnet and the monocytes flushed out using a plunger. Images show Giemsa stained cytopspin slides at x40 magnification before and after positive selection of monocytes.

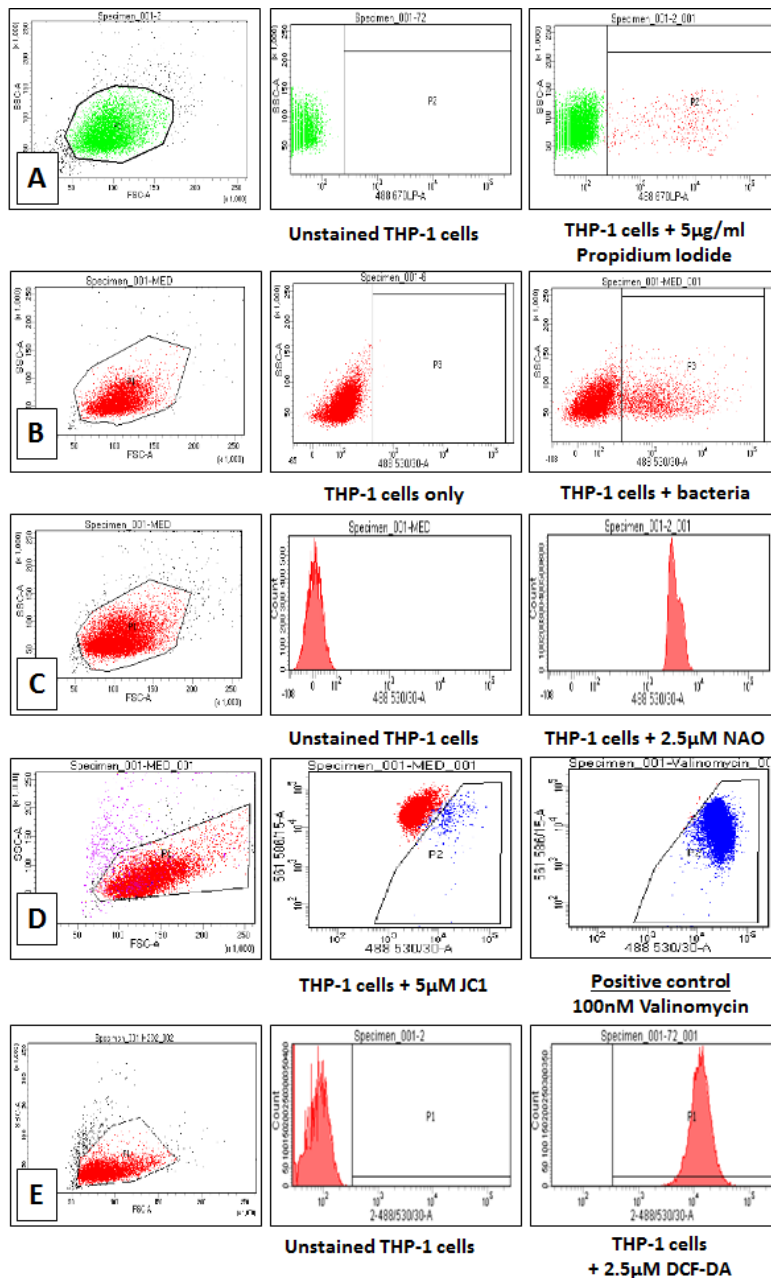
### 3.3.3 Assessment of cell viability

Cell concentration and viability were determined by an assessment of 0.4% trypan blue exclusion using a haemocytometer. The viability of THP-1 cells was further tested using flow cytometry (Figure 3.2) to measure the proportion of cells taking up propidium iodide, a dye that is excluded by viable cells with intact cell membranes.  $5 \times 10^5$  THP-1 cells were suspended in 500µl PBS and incubated with 0.5µg/ml propidium iodide for 1 minute at room temperature before assessing fluorescence using the FACSCanto II flow cytometer (Figure 3.3 A).





**Figure 3.2 Schematic diagram indicating the principles of flow cytometry.** Flow cytometry is a technique in which a hydrodynamically-focussed stream of single cells passes through a laser of a particular wavelength. The degree to which the laser beam is scattered by each cell in a forward and side direction is measured by different detectors. Cellular characteristics can be distinguished using these data for forward scatter, which indicates size, and side scatter, which represents complexity (Brown and Wittwer, 2000). Fluorochromes, which may be conjugated to antibodies, are used to provide a fluorescent label to specific cellular constituents. The laser excites any fluorochromes retained within or bound to the surface of the cell to a higher energy level. The light that is subsequently emitted when the fluorochrome returns to its ground state is measured by a detector (Maecker *et al.*, 2004). A bandpass emission filter is used to prevent light outside the emission spectrum of the particular fluorochrome from reaching the detector.



**Figure 3.3 Scatter plots and histograms indicating the measurement of THP-1 cell viability, phagocytosis, mitochondrial mass, mitochondrial membrane potential and reactive oxygen species production by flow cytometry.** (A) Cell viability was determined by the proportion of THP-1 cells excluding propidium iodide. (B) The phagocytic ability of THP-1 cells was assessed by measuring the proportion of cells internalising fluorescein-conjugated bacteria. (C) Mitochondrial mass was determined by the fluorescence due to uptake of NAO into THP-1 cell mitochondria. (D) THP-1 cells with a depolarised mitochondrial membrane potential had a shift from red to green JC-1 fluorescence as highlighted by treatment with valinomycin. (E) Production of reactive oxygen species by THP-1 cells was measured by the fluorescence produced by the oxidation of DCF-DA. When assessing relative fluorescence of positive populations the data were normalised for the background fluorescence by calculating the signal intensity ( $= [\text{mean}(\text{positive}) - \text{mean}(\text{background})] / [2 \times \text{standard deviation}(\text{background})]$ ) (Maecker *et al.*, 2004)

### 3.4 Experimental models of inflammation

#### 3.4.1 Endotoxin tolerance in THP-1 cells

Repeated stimulation of monocytes with LPS generates diminishing inflammatory responses, a phenomenon termed endotoxin tolerance (Cavaillon and Adib-Conquy, 2006). Endotoxin tolerance can be reliably induced by pre-incubating monocytic cells with LPS at doses above 10ng/ml and has been extensively used as a model of monocyte deactivation (Randow *et al.*, 1995; Li *et al.*, 2004). In endotoxin tolerance experiments  $1 \times 10^6$  THP-1 cells were incubated in  $25\text{cm}^3$  tissue flasks containing 5mls growth medium to which 100ng/ml LPS from *Escherichia coli* O26/B6 was added either 72 (t=0 hours), 48 (t=24 hours), 24 (t=48 hours), 6 (t=66 hours) or 2 (t=70 hours) hours prior to the end of a 72 hour pre-incubation period. After this pre-incubation the THP-1 cells were then pelleted, washed with PBS and re-suspended in fresh medium before comparing immune and mitochondrial functions to those in control cells pre-incubated for the previous 72 hours in growth medium without LPS.

#### 3.4.2 LPS Inhalation in healthy human volunteers

The inhalation of LPS is an established *in vivo* model that reliably induces safe, self-limiting acute pulmonary and systemic inflammation in healthy volunteers (Janssen *et al.*, 2013). Twelve volunteers aged between 18 and 40 years were randomly allocated to inhalation of 60 $\mu\text{g}$  LPS or a placebo of endotoxin-free 0.9% saline, which was delivered over 5 inhalations using an automatic inhalation-synchronised dosimeter nebuliser. Symptoms, clinical observations and spirometry were monitored and blood samples were taken at 0 hours (pre-inhalation) and at 6 and 24 hours post-inhalation. At each of these time points monocytes were isolated from the peripheral blood and immune functions and mtDNA copy number measured.

The LPS inhalation study design and approvals were carried out by Dr Sarah Wiscombe (Clinical Research Fellow, Institute of Cellular Medicine, Newcastle University). Dr Wiscombe was also responsible for the recruitment of volunteers, administration of nebulised LPS/saline and initial processing of blood samples to isolate PBMCs. The LPS inhalation study was approved by the local research ethics committee and the Newcastle-upon-Tyne Hospitals NHS Foundation Trust.

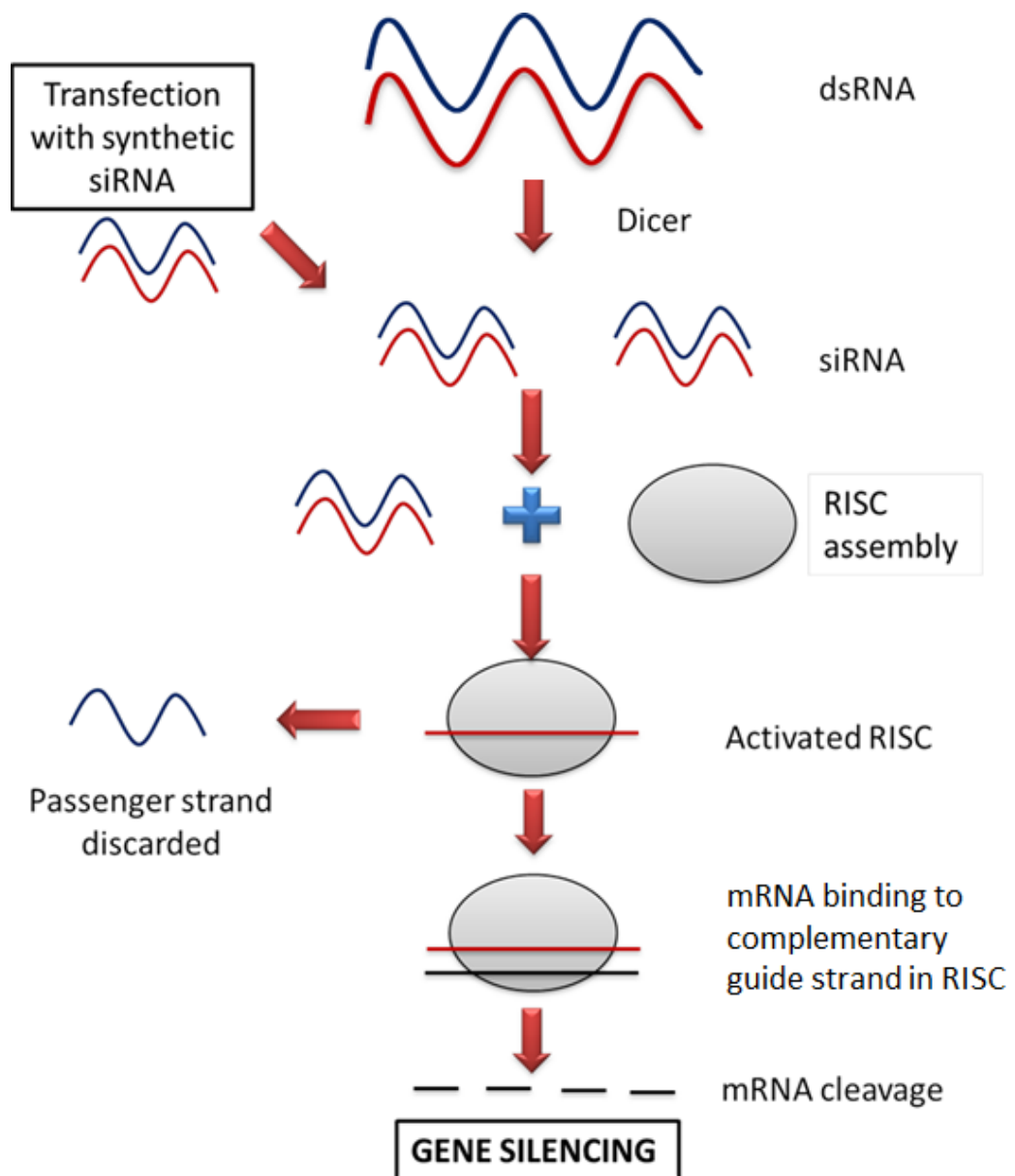
### 3.5 Depletion of mtDNA in THP-1 cells

#### 3.5.1 Treatment with ethidium bromide

Ethidium bromide is an intercalating agent that is commonly used for the detection of DNA. Chronic exposure of cells to low concentrations of ethidium bromide leads to the selective inhibition of mtDNA replication and the generation of cell lines that lack mtDNA (termed  $\rho 0$  cells) (Chandel and Schumacker, 1999). THP-1 cells lacking mtDNA were generated by incubation in RPMI 1640 medium containing 50ng/ml ethidium bromide for 8 weeks (Hashiguchi and Zhang-Akiyama, 2009). To maintain cell viability the medium was supplemented with 2mM L-glutamine, 110 $\mu$ g/ml sodium pyruvate, 50 $\mu$ g/ml uridine and 10% FCS. After the 8 week exposure ethidium bromide was removed from the growth medium 48 hours prior to any subsequent analysis (Marchetti *et al.*, 1996). The characteristics of ethidium bromide-treated THP-1 cells were compared to control cells that were incubated in the same growth medium lacking ethidium bromide for 8 weeks.

#### 3.5.2 Transfection with short-interfering RNA (siRNA) against POLG and TFAM

RNA interference is a process in which short double-stranded RNA fragments induce the specific destruction of messenger RNA (mRNA) containing a complementary nucleotide sequence (see Figure 3.4) (Carthew and Sontheimer, 2009). The transfection of cells with synthetic short-interfering RNA (siRNA) is a well-established method to produce post-transcriptional silencing of specific genes (Whitehead *et al.*, 2009).



**Figure 3.4 The principal of RNA interference by siRNA.** RNA interference is a form of post-transcriptional gene silencing in which a short segment of RNA induces destruction of mRNA containing a complementary sequence of nucleotides. Within cells, siRNA is formed by the cleavage of double-stranded RNA (dsRNA) into 19-23 nucleotide long fragments by the Dicer enzyme. Alternatively, synthetic siRNA can be introduced into the cell using various transfection techniques. This siRNA is incorporated into the RNA-induced silencing complex (RISC) and unwound by the Argonaute 2 protein. The passenger (sense) siRNA strand is then discarded, leading to the formation of a functional, activated RISC. Finally, the guide (antisense) siRNA strand within the RISC selectively binds to messenger RNA (mRNA) with the complementary nucleotide sequence and this mRNA is cleaved and degraded, leading to silencing of a particular gene in a sequence-specific manner (Carthew and Sontheimer, 2009). This figure is adapted from (Whitehead *et al.*, 2009).

In order to inhibit mitochondrial biogenesis THP-1 cells were transfected with Silencer® Select siRNA targeted against the *POLG* and *TFAM* genes (detailed in Table 3.1). The siRNA was delivered to the cells using the Lipofectamine RNAiMAX liposomal reagent as this has been shown to produce effective transfection in THP-1 cells without significant effects on cell viability (Gantier *et al.*, 2008). Silencer® Select Negative Control siRNA number 1, a 21-mer double-stranded RNA with a sequence designed to have minimal effects on the human transcriptome, was used in order to control for the effects of transfection.

Reverse transfection of siRNA into THP-1 cells was carried out. Liposome-siRNA complexes were first formed by mixing 30nM siRNA, 10µl Lipofectamine RNAiMAX and 990µl Opti-MEM medium in a 25cm<sup>3</sup> tissue culture flask and incubating at room temperature for 20 minutes. 5x10<sup>5</sup> THP-1 cells were then suspended in 4mls RPMI medium supplemented with 10% FCS, 50µg/ml uridine and 110µg/ml sodium pyruvate. This cell suspension was slowly added to the liposome-siRNA complex solution and the resultant mixture incubated at 37°C with 5% CO<sub>2</sub>. The transfection was repeated every 48 hours for 8 days before comparing mitochondrial and immune functions to controls transfected with a 30nM Silencer® Select Negative Control siRNA number 1 or incubated with Lipofectamine RNAiMAX or growth medium alone.

	Strand	Sequence (5'→3')	Length (mer)	Molecular Weight (g/mol)	Manufacturer Catalogue No.
TFAM	Sense	GAAGAGAU AAGCAGAUUUAtt	21	6800	Life-Technologies s14001
	Antisense	UAAAUCUGCUUAUCUCUUCtt	21	6500	
POLG	Sense	GUUGACUACUACACCUCAtt	21	6600	Life-Technologies s10787
	Antisense	UGAGGUGUAAGUAGUCAACag	21	6800	

**Table 3.1 Details of Silencer® Select siRNA used in THP-1 cell transfection.**

### 3.6 Assessment of differentiation of THP-1 cells to macrophage-like cells

THP-1 cells can be induced to differentiate into macrophage-like cells that resemble primary human macrophages using various chemical stimuli (Schwende *et al.*, 1996; Daigneault *et al.*, 2010). In order to determine whether THP-1 cells had differentiated into macrophage-like cells during the endotoxin tolerance model, the findings from a range of assessments were compared to a positive control of THP-1 cells stimulated to differentiate by a 72 hour incubation in growth medium supplemented with 10nM PMA (Park *et al.*, 2007). The following cell characteristics were analysed;

1. A qualitative assessment of THP-1 cell morphology was carried out by imaging a minimum of 100 cells in three separate wells on a 6 well plate using a DMI3000 B inversion microscope.
2. The adherence of THP-1 cells to a 6 well plate after a 72 hour incubation was assessed by serial cell counts using a haemocytometer. Cell counts were taken after an initial aspiration of the supernatant in each well (count A) and then after detaching any adherent cells using a cell scraper and washing with an equal volume of PBS (count B). The counts were used to calculate the percentage of non-adherent cells present;

$$\% \text{ non-adherent cells} = [\text{Count A} / (\text{Count A} + \text{Count B})] \times 100$$

3. The expression of macrophage differentiation markers on the surface of THP-1 cells was measured using the BD FACSCanto-II flow cytometer. For each of the differentiation markers a minimum of 10,000 events were recorded and each condition was assessed in triplicate. Details of the markers that were measured are given in Table 3.2.

Marker	Function, Change during macrophage differentiation	Fluorochrome	Clone	Host species, isotype	Manufacturer catalogue number	Excitation wavelength -Bandpass filter (nm)
CD14	LPS recognition and binding Down-regulated	PE-Cy7	M5E2	Mouse, IgG2a	BD Biosciences, 561385	488-780/60
CD36	Scavenger receptor Up-regulated	PE	5-271	Mouse, IgG2a	BioLegend, 336206	488-585/42
CD206	Mannose receptor Up-regulated	PE	15-2	Mouse, IgG1	BioLegend, 321106	488-585/42

**Table 3.2 Fluorochrome-conjugated antibodies used to assess cell surface expression of macrophage differentiation markers by flow cytometry.** During monocyte-macrophage differentiation there is evidence that the cell surface expression of CD14 is down-regulated (Kruger *et al.*, 1996; Spano *et al.*, 2013), while the expression of CD36 (Huh *et al.*, 1996; Schuierer *et al.*, 2006) and CD206 (Porcheray *et al.*, 2005; Daigneault *et al.*, 2010) are up-regulated. The fluorochrome-conjugated antibodies were provided by BD Biosciences and BioLegend (San Diego, CA, USA).

### 3.7 Immune functions of THP-1 cells and monocytes

#### 3.7.1 Cytokine release assay

THP-1 cells ( $2.5 \times 10^5$  THP-1 cells/well) or human monocytes ( $1 \times 10^5$  cells/well) were re-suspended in growth medium containing 10% FCS and seeded onto a 24 well plate. Half of the wells were then treated with LPS, at a concentration of 100ng/ml for THP-1 cells and 10ng/ml for monocytes. Following incubation at 37°C for 4 hours (pro-inflammatory cytokines) or 16 hours (anti-inflammatory cytokines) the supernatants from each well were collected and stored at -80°C.

Cytokine concentrations in these supernatants were subsequently determined using Novex® ELISA antibody pair kits (Table 3.3). After coating NUNC MaxiSorp® 96 well microplates with the detection anti-cytokine antibody overnight, non-specific binding was blocked by incubation with assay buffer containing 0.5% BSA for 1 hour. The sample supernatants (triplicates), along with standards of known concentration and blank wells containing assay buffer (both duplicates), were then incubated with the detection biotin-conjugated anti-cytokine antibody for 2 hours at room temperature. After washing, a streptavidin-horseradish peroxidase solution was added for 30



minutes, before a final wash and the addition of the tetramethylbenzidine substrate. The subsequent colorimetric reaction was stopped after 30 minutes by the addition of 2N sulphuric acid.

A FLUOStar Omega Plate Reader was used to measure the absorbance of each well at 450nm. A standard curve was generated by plotting a 4-parameter curve fit of the cytokine concentrations in the standards against their measured blank-corrected optical density. Cytokine concentrations in the unknown samples were calculated by plotting their mean blank-corrected optical densities onto the linear region of the standard curve. The LPS-induced cytokine release for each condition was then determined using the equation;

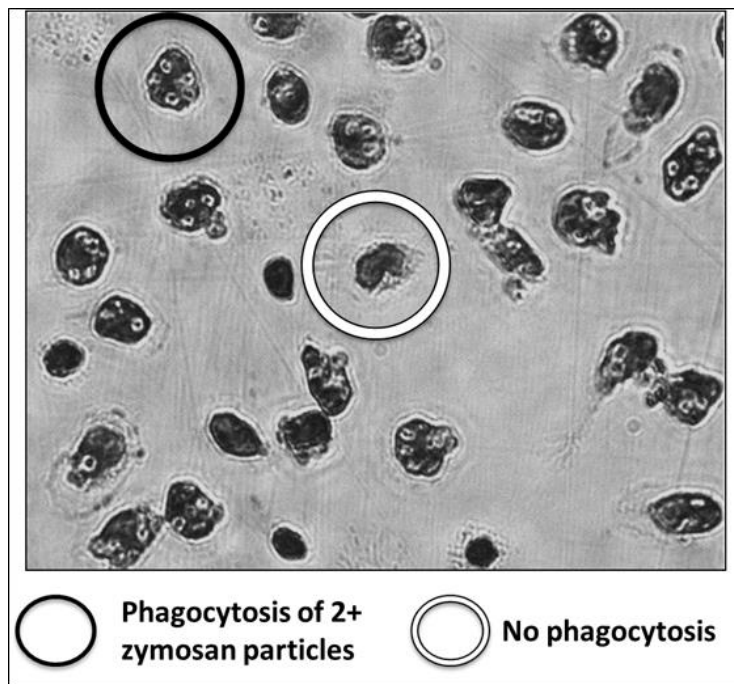
$$\text{LPS-induced cytokine release} = \text{Cytokine release with LPS} - \text{Cytokine release with medium}$$

Cytokine	Range of detectable concentrations (pg/ml)	Catalogue number
IL-1 $\beta$	31.25-1000	CHC1213
IL-1RA	31.25-1000	CHC1183
IL-6	15.6-1000	CHC1263
IL-8	12.5-800	CHC1303
IL-10	31.25-1000	CHC1323
TNF $\alpha$	15.6-1000	CHC1753

**Table 3.3 Summary of range of detectable concentrations for the Novex® ELISA antibody pair kits used to detect cytokines released by THP-1 cells and monocytes.**

### 3.7.2 Phagocytosis of zymosan particles by human monocytes

Monocytes were adhered to a 96 well plate during a 30 minute pre-incubation of  $5 \times 10^4$  cells per well in 50 $\mu$ l IMDM containing 10% autologous serum at 37°C. 1 $\mu$ g of zymosan particles from *Saccharomyces cerevisiae*, opsonised by pre-incubation in 50% autologous serum for 30 minutes, was then added and a further 1 hour incubation at 37°C carried out (Wolf *et al.*, 1988). Excess zymosan was washed off and the monocytes were air-dried, fixed with methanol and stained with Giemsa. The proportion of monocytes that had internalised two or more zymosan particles was determined using the Leica 3000 B inversion microscope (Figure 3.5). A minimum of 100 monocytes were counted in three separate wells for each experimental condition.



**Figure 3.5 Representative image of phagocytosis of zymosan particles by monocytes.** The proportion of monocytes that have internalised two or more zymosan particles (black circle) was determined using the Leica 3000 B inversion light microscope. Image taken at 40x magnification.

### 3.7.3 Phagocytosis of fluorescent killed bacteria by THP-1 cells

The ability of a suspension of THP-1 cells to internalise fluorescein-labelled killed bacteria (*Escherichia coli* (K-12 strain) or *Staphylococcus aureus* (Wood Strain without protein A)) was determined by flow cytometry (Figure 3.2). The fluorescein-labelled bacteria were opsonised by pre-incubation with 10% human serum for 30 minutes and  $1 \times 10^7$  bacteria were then added to a suspension of  $1 \times 10^6$  THP-1 cells in 500  $\mu$ l growth medium. Following incubation in a tube rotator at 37°C for 1 hour non-internalised bacteria were removed by washing with PBS. The THP-1 cells were then re-suspended in 500  $\mu$ l PBS to which 500  $\mu$ l 0.1% trypan blue was added in order to quench any remaining extracellular fluorescence.

The proportion of THP-1 cells phagocytosing bacteria was determined by measuring the fluorescence (excitation wavelength 488nm, band pass filter 530/30nm) using a FACSCanto II Flow Cytometer. A minimum of 10,000 events was recorded and each condition was assessed in triplicate. A negative control of THP-1 cells without bacteria was used to determine the baseline cellular fluorescence and define the negative population. The THP-1 cells that had internalised bacteria formed a separate

population that stained positive for fluorescein and the proportion of THP-1 cells in this population was measured (Figure 3.3 B).

### 3.8 Mitochondrial assessments

#### 3.8.1 Mitochondrial DNA copy number by quantitative polymerase chain reaction (qPCR)

DNA was extracted from pellets of  $1 \times 10^6$  THP-1 cells or monocytes using the DNeasy Blood and Tissue Kit. The NanoDrop 2000 spectrophotometer was used to determine the concentration (by ultraviolet absorbance at 260nm) and purity (by the ratio of absorbance at 260nm and 280nm) of the extracted DNA. Prior to analysis the DNA solutions were diluted with nuclease-free water to produce a starting DNA concentration of 10ng/ $\mu$ l.

The relative mtDNA copy number was determined using real-time qPCR. The reaction compared the level of the mtDNA gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*MT-ND1*) to that of the nuclear reference gene  $\beta$ 2-microglobulin (*B2M*) (Payne *et al.*, 2011). The *B2M* and *MT-ND1* primers are listed in Table 3.4.

	Gene	Forward (5'3')	Reverse (5'3')	Anneal Temp (°C)	Size (bp)
Template	<i>B2M</i>	CGCAATCTCCAGTGACAGA A	GCAGAATAGGCTGCTGTTCC C	60	1092
	<i>MT-ND1</i>	CAGCCGCTATTAAGGTTCC G	AGAGTGCATCATATGTTGT TC	60	1040
qPCR	<i>B2M</i>	CACTGAAAAAGATGAGTAT GCC	AACATTCCTGACAATCCC	62.5	231
	<i>MT-ND1</i>	ACGCCATAAACTCTTCACC AAAG	GGGTTTCATAGTAGAAGAGC GATGG	62.5	111

**Table 3.4 *B2M* and *MT-ND1* primer sequences for template generation and mtDNA copy number measurement by quantitative PCR.**

Templates covering the *MT-ND1* and *B2M* target sequences were first amplified by standard PCR and separated using agarose gel electrophoresis. The QiA Quick Gel Extraction Kit was used to extract DNA from the gel and the DNA concentration quantified using the NanoDrop 2000 spectrophotometer. This DNA concentration was then used to calculate the DNA copy number of the template using the equation;

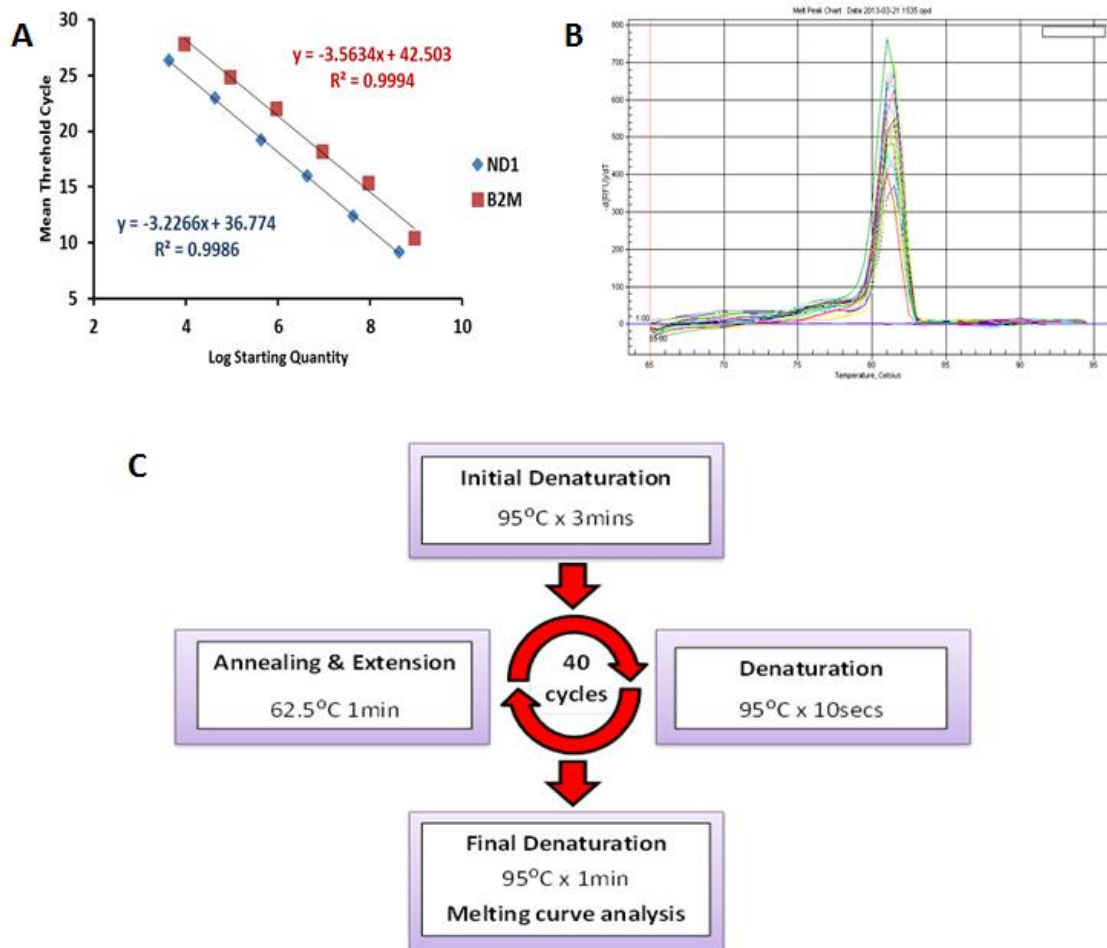
$$\text{Copy number} = [\text{DNA concentration (g/l)} \div (\text{Amplicon length (bp)} \times 2 \times 330)] \times \text{Avogadro's number}$$

Serial dilutions of the template DNA enabled the generation of standard curves for the qPCR reaction covering copy numbers in the range  $10^8$ - $10^3$  copies/ $\mu$ l. These standard curves were used to ensure that each reaction achieved a linear curve ( $r^2 > 0.98$ ) with an amplification efficiency within the optimal range (90-110%) (Figure 3.6 A). Template negative controls and melting curve analysis of the amplified DNA product were used to confirm the absence of DNA contamination (Figure 3.6 B).

The real-time qPCR was carried out using a BioRad MyiQ™ PCR machine. A final 25 $\mu$ l reaction volume was produced containing 300nM of the target forward and reverse primers, iQ™SYBR® Green supermix and 10ng of DNA. The template standards and negative controls were assessed in duplicate while the unknown samples were analysed in triplicate. The reaction was carried out in 96 well PCR plates sealed with microseal B plate sealers. The SYBR 62.5 protocol was used and the fluorescence generated by the binding of iQ™SYBR® Green to amplified double-stranded DNA was measured during each of the 40 cycles (Figure 3.6 C). The cycle at which this fluorescence exceeded a threshold of 250 relative fluorescent units (RFU) while in the exponential phase of the amplification curve was used to define the threshold cycle (Ct). All data were analysed using the BioRad iQ5™ optical software v2.0 and the relative mtDNA copy number per cell calculated using the  $\Delta$ Ct data;

$$\Delta\text{Ct} = \text{Ct MT-ND1} - \text{Ct B2M}$$

$$\text{Relative mtDNA copy number per cell} = 2^{(-\Delta\text{Ct})}$$



**Figure 3.6 Standard curve, melting curve and protocol for quantitative polymerase chain reaction to measure mtDNA copy number.** (A) Representative scatter plot indicating parallel standard curves for serial dilutions of *MT-ND1* and *B2M*. (B) Representative melting curve analysis line graph showing a pure DNA amplification product (single peak) after quantitative polymerase chain reaction (qPCR) with primers for a sequence on the *MT-ND1* gene. (C) SYBR 62.5 protocol for determining mtDNA copy number by qPCR using the DNA-binding dye iQ™SYBR® Green.

### 3.8.2 Mitochondrial mass by uptake of nonyl-acridine orange

Mitochondrial mass was determined by measuring the uptake of NAO, a dye that localises to mitochondria regardless of the mitochondrial membrane potential (Cottet-Rousselle *et al.*, 2011).  $5 \times 10^5$  THP-1 cells were incubated with  $2.5 \mu\text{M}$  NAO at  $37^\circ\text{C}$  for 30 minutes before removing excess dye by washing twice using the BD Lyse-Wash machine and measuring the fluorescence (excitation wavelength 488nm, bandpass filter 530/30nm) using the BD FACSCanto-II flow cytometer (Figure 3.3 C). A minimum of 10,000 events was recorded and each condition was assessed in triplicate. As a positive control mitochondrial mass was increased in THP-1 cells by pre-incubation for 72 hours in DMEM supplemented with 5mM galactose and lacking glucose (Morán *et al.*, 2010).

### 3.8.3 Measurement of mitochondrial membrane potential depolarisation using JC-1

The serial reduction of electrons and transport of protons across the inner mitochondrial membrane by the OXPHOS complexes generates the mitochondrial membrane potential ( $\Delta\psi\text{m}$ ) (Brealey and Singer, 2003). Dysfunction of the OXPHOS complexes leads to depolarisation of the  $\Delta\psi\text{m}$  (Green *et al.*, 2011). JC-1 is a cationic dye which accumulates in mitochondria in a  $\Delta\psi\text{m}$ -dependent manner resulting in the formation of J-aggregates and a shift from green to red fluorescence. As a result the ratio of red: green JC1 fluorescence provides a useful measure of  $\Delta\psi\text{m}$  which can be used to assess the presence of dysfunctional mitochondria (Salvioli *et al.*, 1997).

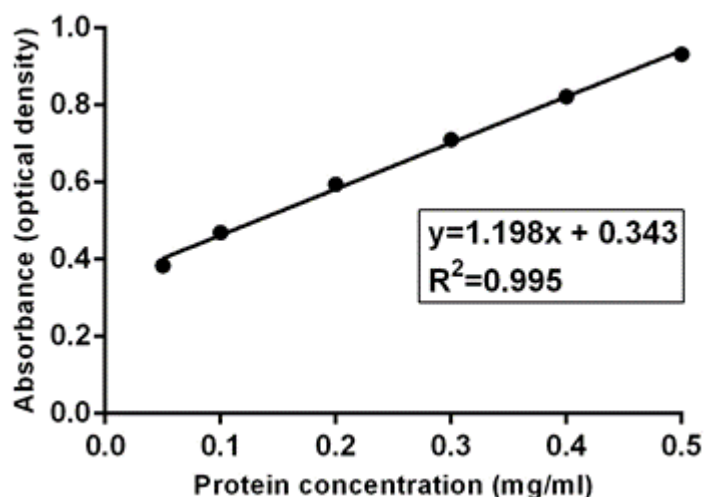
THP-1 cells were suspended at  $1 \times 10^6$  cells/ml in PBS and incubated with  $5 \mu\text{M}$  JC-1 for 30 minutes. As a positive control for  $\Delta\psi\text{m}$  depolarisation, cells were pre-treated for 10 minutes with 100nM valinomycin, a potassium-selective ionophore which dissipates the  $\Delta\psi\text{m}$  (Salvioli *et al.*, 1997). Following incubation with JC-1 the red (excitation wavelength 561nm, bandpass filter 186/15nm) and green (488nm, 530/30nm) fluorescence of THP-1 cells was measured using the LSRFortessa X20 flow cytometer. A minimum of 10,000 events was recorded and each condition was assessed in triplicate. The proportion of cells displaying green fluorescence was measured in order to identify cells with  $\Delta\psi\text{m}$  depolarisation (Figure 3.3 D).

### 3.8.4 Reactive oxygen species (ROS) production

The production of ROS by THP-1 cells was determined using DCF-DA. Following entry into cells DCF-DA is deacetylated by esterases to form 2'7' dichlorfluorescein, which becomes highly fluorescent after oxidation by hydrogen peroxide (Winterbourn, 2013). THP-1 cells were suspended at  $1 \times 10^6$  cells/ml in 1ml PBS and incubated at 37°C with 1µM DCF-DA for 30 minutes. As a positive control cells were then treated with 100µM hydrogen peroxide for 60 minutes. At the end of this incubation the fluorescence (absorption wavelength 488nm, band pass filter 530/30 nm) of the THP-1 cells was measured on the BD FACSCanto-II flow cytometer (Figure 3.3 E). A minimum of 10,000 events were recorded and each condition was assessed in triplicate.

### 3.9 Protein expression by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Western blot

Protein was extracted from pellets of  $3 \times 10^6$  THP-1 cells using a lysis buffer (formulation in Table 3.5). The protein concentration in the resultant lysate was determined using a Bradford Assay in which protein binding to an acidic solution of Coomassie Brilliant Blue reagent produces a change in absorbance at 595nm that can be compared to a standard curve of known concentrations of BSA (Figure 3.7) (Noble and Bailey, 2009).



**Figure 3.7** Example of Bradford assay standard curve showing linear regression of protein concentration against absorbance at 595nm

The expression of mitochondrial proteins was then determined by Western blotting, a technique in which proteins are separated on the basis of size and then detected using specific antibodies (Mahmood and Yang, 2012). Firstly, 20µg of protein was added to an equal volume of loading dye containing SDS, an anionic detergent which unfolds the proteins and confers a negative charge (formulation in Table 3.5). The proteins in each lysate sample were then separated by SDS-PAGE using Novex® 4-20% Tris-Glycine pre-cast protein gels. A SeeBlue Plus2 pre-stained protein ladder and a biotinylated protein ladder were added in order to allow estimation of the molecular weight of the resultant protein bands. Following SDS-PAGE the separated proteins were transferred onto a PVDF membrane using iBlot2® Transfer Stacks and the iBlot2® Gel Transfer Device.

The PVDF membrane was incubated with a blocking buffer (formulation in Table 3.5) for 1 hour at room temperature to prevent any non-specific antibody binding and the primary antibody was then added. Following incubation with the primary antibody (either for 1 hour at room temperature or overnight at 4°C) the membrane was washed and the secondary horseradish peroxidase-conjugated antibody then added. After a final 1 hour incubation at room temperature and a further wash the presence of bound secondary antibody was determined by adding an ECL substrate and detecting the resultant signal using the MultiSpectral Imaging System. In order to detect other proteins the ECL substrate was then washed off and a different primary antibody added. Tris-buffered saline with 0.1% tween was used for all washes (formulation in Table 3.5). The details of the primary and secondary antibodies used in the detection of proteins during Western blotting are listed in Table 3.6.

Image J software was used to analyse the relative densities of different protein bands. The relative expression of protein in each sample was then compared to that of β-actin, a ubiquitously expressed protein used as a loading control.



Buffer	Formulation
Lysis Buffer	50mM trizma hydrochloride pH 7.5, 130mM sodium chloride, 2mM magnesium chloride, 1mM PMSF, 1% triton X
Loading Dye (2x)	250mM trizma hydrochloride pH 6.8, 20% Glycerol, 4% sodium dodecyl sulphate, 0.1% bromophenol blue
Running Buffer	25mM trizma base, 200mM glycine, 0.1% sodium dodecyl sulphate
Tris-buffered saline – 0.1% tween (TBS-T)	20mM trizma hydrochloride pH 7.0, 0.5mM sodium chloride, 0.1% tween
Blocking Buffer	TBS-T, 5% milk powder

**Table 3.5 Formulations of Western blot buffers**

Antibody	Host species, isotype	Manufacturer, Catalogue Number	Final concentration	Band molecular weight (kDa)
<b>Primary antibody</b>				
$\beta$ -actin	Mouse, IgG2	Abcam, ab8226	0.5 $\mu$ g/ml	42
LC3-I/II	Rabbit, polyclonal	Cell Signalling, CS54995	1 $\mu$ g/ml	14/16
MTCO1	Mouse, IgG2a	Abcam, ab14705	1 $\mu$ g/ml	40
Mitoprofile® total OXPHOS antibody cocktail	Mouse	MitoSciences, MS604	6 $\mu$ g/ml	Complex I – 20, Complex II – 30 Complex III – 47 Complex IV – 39 Complex V - 53
POLG	Rabbit, polyclonal	Sigma-Aldrich, SAB2700005	1 $\mu$ g/ml	150
SDHA	Mouse, IgG1	Abcam, ab14715	0.2 $\mu$ g/ml	70
SOD2	Rabbit, polyclonal	Abcam, ab13533	0.2 $\mu$ g/ml	25
TFAM	Mouse, IgG2b	Novus Biological, NBP1-71648	1 $\mu$ g/ml	25
<b>Secondary antibody</b>				
Anti-mouse Ig-HRP	Rabbit	Dako, 0260	1:5000 dilution	N/A
Anti-rabbit Ig-HRP	Goat	Dako, 0448	1:2000 dilution	N/A

**Table 3.6 Primary and secondary antibodies used in Western blotting.**

Antibodies were provided by Abcam, Cell Signalling (Danvers, MA, USA), Dako (Cambridge, UK), MitoSciences (Eugene, OR, USA), Novus Biologicals (Cambridge, UK) and Sigma-Aldrich (St Louis, MO, USA).

### 3.10 Assessments of the oxidative phosphorylation (OXPHOS) system

#### 3.10.1 Spectrophotometric assessment of isolated OXPHOS complex IV and citrate synthase activity

Cellular homogenates containing mitochondrial respiratory chain enzymes were produced from a suspension of  $6 \times 10^6$  THP-1 cells in 120  $\mu$ l 20mM potassium phosphate buffer by four rapid freeze-thaw cycles using liquid nitrogen. Spectrophotometric measurements of the activity of citrate synthase and OXPHOS complex IV within the homogenates were taken using the MultiSkan Ascent plate reader. All reactions were carried out in triplicate and the results were normalised to the protein content of the homogenate (determined using the Bradford assay).

Citrate synthase is a nuclear DNA-encoded enzyme that catalyses the initial reaction in the citric acid cycle in the mitochondrial matrix and provides a quantitative marker of cellular mitochondrial content (Rodenburg *et al.*, 2012). The following reaction is catalysed by citrate synthase;



Citrate synthase activity was determined by measuring the subsequent reaction between CoA-SH and DTNB, which generates 5-thio-2-nitrobenzoate to produce a change in absorbance at 412nm. 2.5  $\mu$ l of cellular homogenate was suspended in 197.5  $\mu$ l of a 0.1mM Tris-hydrochloride buffer containing 0.1mM DTNB, 0.1mM ACoA and 0.1% Triton X. The reaction was initiated by mixing this solution with 0.25mM oxaloacetate and the rate of change of absorbance at 412nm measured before calculating the citrate synthase activity using the equation;

$$\text{Citrate Synthase activity (IU/l)} = [(\text{rate of change in absorbance per min}/13.6) \times (\text{Total volume}/\text{Homogenate volume})] \times 1000$$

(Where 13.6/cm/mM = Extinction co-efficient ( $\epsilon$ ) for DTNB at 412nm)

In a similar manner, OXPHOS complex IV activity was determined by measuring the rate of oxidation of cytochrome C (Kirby *et al.*, 2007). A 1% solution of cytochrome C in 10mM potassium phosphate was first reduced by incubation on ice with sodium borohydrate for 30 minutes. 20  $\mu$ l of the cellular homogenate was then suspended in 160  $\mu$ l of 10mM potassium phosphate. After the addition of 20  $\mu$ l of reduced

cytochrome C and an incubation at 38°C for 2 minutes the rate of decrease in absorbance at 550nm (due to cytochrome C oxidation) was measured and the following equation used to calculate the complex IV activity;

$$\text{Complex IV activity} = [(rate\ of\ change\ in\ absorbance\ per\ min)/18.7] \times (Total\ volume/Homogenate\ volume) \times 1000$$

(Where 18.7 =  $\epsilon$  for Cytochrome C at 550nm)

In addition, the activity of OXPHOS complex IV was also measured in THP-1 cell lysates using the Complex IV Human Specific Activity Microplate Assay Kit. Each experimental condition was assessed in triplicate by analysing 200µg of protein per well on a 96 well plate using the protocol specified by the manufacturer. Again, complex IV activity was determined by assessing the rate of oxidation of reduced cytochrome C, as reflected by the maximal rate of decline in the absorbance at 550nm.

### 3.10.2 In-gel activity of OXPHOS complex I

Blue-native PAGE was used to separate the OXPHOS complexes from mitochondrial lysates on the basis of their size and in-gel complex I activity was then assessed using a histochemical measurement of the oxidation of reduced NADH (Nijtmans *et al.*, 2002).

Firstly, mitochondria were extracted from pellets of  $24 \times 10^6$  THP-1 cells (Rodenburg *et al.*, 2012). The pellets were suspended in 1ml of Medium B (250mM sucrose, 2mM HEPES, 0.1mM EGTA, pH7.4) in a glass pestle and homogenised by 20 strokes of a homogeniser. After centrifugation at 1200G for 10minutes at 4°C the supernatant was set aside and the pellet re-suspended in 0.8mls of Medium B before repeating the homogenisation. The resulting solution was again centrifuged at 1200G for 10 minutes at 4°C before combining the supernatants from the two homogenisations and centrifuging this solution at 11,000G for 10 minutes at 4°C. After re-suspending the resulting mitochondrial pellet in 100µl Medium B, the mitochondria were lysed by an incubation on ice with 1.5% DDM for 15minutes followed by a 20 minute 20,000G centrifugation at 4°C. The protein concentration of the lysate was then determined using the Bradford assay.

A solution of 40µg of this mitochondrial protein lysate in Novex® Native PAGE™ 4x sample buffer and 5% G-250 sample additive was added to wells on a Novex® Native PAGE™ 3-12% Bis-Tris gel and blue-native PAGE was carried out. In order to measure

OXPHOS complex I activity the gel containing the separated OXPHOS complexes was subsequently incubated for 1 hour at 37°C in a solution of 45µM reduced NADH and 200µM NTB in 5mM Tris hydrochloride at pH7.2. Finally, the relative activity of complex I was determined by quantifying the density of staining of the gel caused by the oxidation of NADH by complex I using Image J.

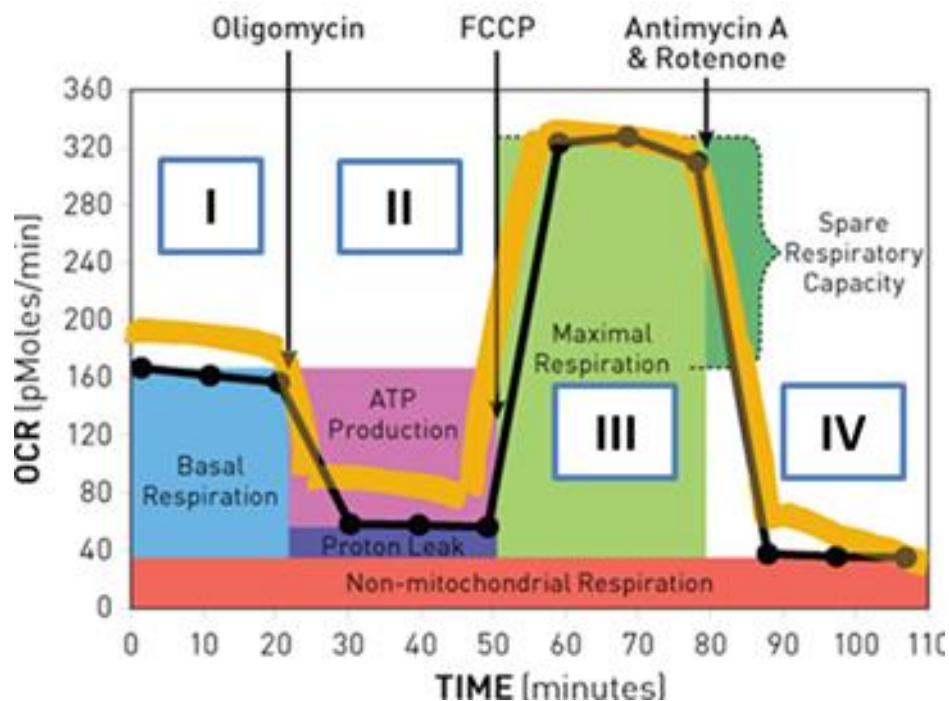
### 3.10.3 Oxygen consumption by the Seahorse XF96<sup>e</sup> Extracellular Flux analyser

The cellular respiration of THP-1 cells was assessed using the Seahorse XF96<sup>e</sup> extracellular flux analyser which allows real-time measurements of oxygen consumption rate (OCR) by live cells (Hill *et al.*, 2012). A solid-state sensor probe is used to measure the rate of change in dissolved oxygen concentration within a transient micro chamber that is created above a monolayer of cells at serial time points. Different metabolic inhibitors are sequentially injected into the chambers in order to assess different aspects of respiration (Table 3.7).

Metabolic inhibitor	Dose	Mechanism of Action	Measurements
Oligomycin	1µM	Blocks proton channel of OXPHOS complex V to inhibit ATP synthesis	Proportion of OCR devoted to ATP synthesis Proton leak through inner mitochondrial membrane
Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (fCCP)	0.5µM then 1µM	Uncoupling agent which allows protons to leak across the inner mitochondrial membrane	Maximal oxygen consumption Spare respiratory capacity
Rotenone and Antimycin A	1µM	Inhibit OXPHOS complex I and III to abolish mitochondrial respiration	Non-mitochondrial respiration

**Table 3.7 Metabolic inhibitors used to assess different aspects of cellular respiration with the Seahorse XF96<sup>e</sup> extracellular flux analyser.**

A sensor cartridge was pre-incubated with a calibration plate containing 200µl per well of XF Calibrant Solution at 37°C without CO<sub>2</sub> for at least 12 hours prior to starting the assay. Later, THP-1 cells were re-suspended in assay medium, consisting of MEM supplemented with 11.1mM D-Glucose and 2mM L-Glutamine and adjusted to pH 7.0, and seeded onto a 96 well plate at 80,000 cells in 175µl assay medium per well. Following incubation at 37°C in a humidified incubator without CO<sub>2</sub> for 45 minutes, this plate was centrifuged at 2000G for 15 minutes in order to produce a monolayer of cells on the base of the wells. Meanwhile, solutions of the metabolic inhibitors were prepared in assay medium and loaded into 4 injection ports in the sensor cartridge, which was then calibrated within the XF96<sup>e</sup> analyser. Following this the calibration plate was replaced with the 96 well microplate containing the THP-1 cell monolayer and the assay was commenced. After measurement of basal respiration, the metabolic inhibitors were added sequentially to the wells in order to assess different aspects of respiration (Figure 3.8). During each of the four stages of the assessment the OCR was measured in 16 wells per condition at 3 different time points. All OCR data was normalised to the total protein per well which was determined using the Bradford assay.



**Figure 3.8 Respiratory profile from the Seahorse XF96<sup>®</sup> extracellular flux analyser.** Serial measurements of OCR are taken from a transient micro chamber over a monolayer of cells following the sequential addition of different metabolic inhibitors. After assessing basal respiration (I), oligomycin is added to inhibit ATP synthesis (II). The addition of the uncoupling agent FCCP then induces maximal mitochondrial respiration (III) before mitochondrial respiration is abolished by rotenone and antimycin A (IV). The OCR due to the following aspects of cellular respiration can then be determined; Non-mitochondrial respiration = non-mitochondrial OCR (IV), Basal mitochondrial respiration = basal OCR (I) – non-mitochondrial OCR (IV), ATP production = basal OCR (I) – post-oligomycin OCR (II), Proton Leak = post oligomycin OCR (II) – non-mitochondrial OCR (IV) and Maximal Mitochondrial Respiration = maximal OCR (III) – non-mitochondrial OCR (IV). Figure is adapted from (Seahorse Biosciences).

### 3.11 Assessment of messenger RNA (mRNA) transcription by reverse transcription qPCR

RNA was extracted from pellets of  $4 \times 10^6$  THP-1 cells using the RNeasy mini kit. The NanoDrop 2000 spectrophotometer was used to determine the concentration and purity of the extracted RNA.

Single-stranded complementary DNA (cDNA) was synthesised from this RNA using the High Capacity cDNA Reverse Transcription Kit. The reaction used random hexamer primers to non-specifically initiate cDNA synthesis from all RNA transcripts and was catalysed by MultiScribe<sup>®</sup> reverse transcriptase. The manufacturer's protocol was followed and 5  $\mu$ g of RNA was added per reaction. The primers were annealed to the

RNA at 25°C for 10 minutes and cDNA was synthesised during an incubation at 37°C for 120 minutes before the reaction was terminated by 5 minutes incubation at 85°C.

Following this the relative transcription of specific genes was determined by reverse transcription-qPCR (RTqPCR) using the Taqman® Gene Expression Assay. This technique uses a Taqman® probe, containing a FAM™ fluorescent probe at the 5' end and a non-fluorescent quencher at the 3' end, which specifically anneals to the target cDNA sequence. Amplification by Taq polymerase is initiated by the binding of unlabelled forward and reverse primers to the target sequence and this leads to the cleavage of the annealed Taqman® probe. The resultant release of FAM™ from the quencher produces a fluorescence that is proportional to the amount of target cDNA sequence present (Medhurst *et al.*, 2000).

The RTqPCR was carried out on the 7500 Fast Real Time PCR System using a final 20µl reaction volume containing 10µl 2x Taqman® Gene Expression Master Mix, 1µl 20x Taqman® Gene Expression Assay, 7µl RNase free water and 2µl cDNA. The details of the Taqman® Gene Expression Assays that were used are listed in Table 3.8. The protocol involved an initial 2 minute incubation at 50°C followed by 20 seconds at 95°C and 40 cycles of 30 seconds at 60°C and 3 seconds at 95°C during which the fluorescence was measured. The cycle at which the fluorescence generated by cleavage of FAM™ from the Taqman® probe exceeded 0.25 RFU was used to define the threshold cycle (Ct). Each sample was assessed in triplicate and the reaction efficiency confirmed using a standard curve of cDNA serially diluted by a factor of 1:10 with RNase-free water from a starting amount of 2µl. The contamination of reagents was excluded through the use of cDNA negative controls. The relative amount of cDNA for each specific target was determined by comparison with a control housekeeping gene to generate  $\Delta Ct$  data;

$$\Delta Ct = Ct \text{ target gene} - Ct \text{ housekeeping gene}$$

$$\text{Relative RNA transcription} = 2^{(2^{-\Delta Ct})}$$

Gene Symbol	Gene product	Assay ID	Amplicon length (bp)
<i>ACTB</i>	Actin, beta	Hs01060665_g1	63
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	Hs02758991_g1	63
<i>HMOX1</i>	Haem-oxygenase-1	Hs01110250_m1	82
<i>IFIT1</i>	Interferon-induced protein with tetratricopeptide repeats-1	Hs03027069_s1	134
<i>IFITM1</i>	Interferon-induced transmembrane protein-1	Hs00705137_s1	93
<i>IL1B</i>	Interleukin-1 beta	Hs01555410_m1	91
<i>MTCO1</i>	Mitochondrially-encoded cytochrome C oxidase 1	Hs02596864_g1	94
<i>MYD88</i>	Myeloid differentiation primary response-88	Hs01573837_g1	94
<i>SDHA</i>	Succinate dehydrogenase complex, subunit A, flavoprotein	Hs00417200_m1	124
<i>STAT1</i>	Signal transducer and activator of transcription-1	Hs01013996_m1	66
<i>TLR4</i>	Toll-like receptor-4	Hs00152939_m1	89
<i>TNF</i>	Tumour necrosis factor	Hs01113624_g1	143
<i>TREM1</i>	Triggering receptor expressed on myeloid cells-1	Hs00218624_m1	80

**Table 3.8 Details of Taqman® Gene Expression Assays used in reverse transcription quantitative polymerase chain reactions**

### 3.12 Analysis of the transcriptome by RNA sequencing (RNA-Seq)

#### 3.12.1 RNA preparation and sequencing

RNA was extracted from pellets of  $4 \times 10^6$  THP-1 cells using the RNeasy mini kit and any residual DNA was then removed using the DNA-free DNase treatment kit. The concentration and quality of the RNA was determined by micro-capillary electrophoresis using the Agilent 6000 RNA Pico Kit and the Agilent 2100 Bioanalyser. All RNA samples were analysed in duplicate and only those with a RNA Integrity Number greater than 7 were used for RNA-Seq.

The RNA samples were sent to AROS Applied Biotechnology A/S (Aarhus, Denmark) where the RNA-Seq was carried out. Firstly, the total RNA was converted into a library of template cDNA suitable for sequencing using the Illumina TruSeq Stranded Total RNA Sample Prep kit. During the initial step in this process mRNA was enriched by



removing ribosomal RNA (rRNA) from the total RNA samples using RiboZero rRNA removal beads. Next, the remaining mRNA was broken up into small fragments by heating with divalent cations. These RNA fragments were then converted to cDNA, with first strand synthesis by reverse transcriptase using random primers and second strand synthesis by DNA polymerase I. Following this, specific adapters were attached to each end of the cDNA fragments in order to facilitate sequencing. The resultant products were then amplified by PCR to create a final cDNA library and the Illumina HiSeq 2500 machine was used to sequence this cDNA library (Liu *et al.*, 2012a).

### 3.12.2 Data processing and analysis

The RNA-Seq experiments produced data in the form of 100bp long paired-end (sequenced in both directions) reads, with a minimum of 60 million of these reads per sample. These data were analysed with the assistance of Jannetta Steyn from the Newcastle University Bioinformatics Support Unit. The data were first processed to remove any regions from the reads containing sequences from the adapters that were attached to each cDNA fragment using autoadapt software (Martin, 2011). After carrying out a quality control assessment with FastQC software, TopHat2 was then used to align the reads against the hg19 (human genome version 19, Genome Reference Consortium GRCh37.p13) reference genome (Kim *et al.*, 2013; Andrews, 2015). Next, HTSeq was used to annotate the aligned reads, in order to identify transcription units corresponding to particular genes, and produce counts of the number of reads for each gene (Anders *et al.*, 2015). The read count per gene was then normalised as reads per kilobase per million mapped reads (RPKM) and differential gene expression between samples and conditions determined using DESeq2 software (Mortazavi *et al.*, 2008; Sonesson and Delorenzi, 2013; Love *et al.*, 2014). The relationships between the transcriptome in different samples and conditions were explored by generating hierarchical clustering dendrograms, principal components analysis (PCA) plots, heat maps of relative gene expression and Venn diagrams indicating the overlap in gene expression.

Finally, the biological significance of the changes in gene expression on cellular processes and signalling pathways was investigated using Ingenuity® Pathway Analysis (IPA®) (Krämer *et al.*, 2014). In IPA® the differential expression data were analysed in the context of the Ingenuity® Knowledge Base, a large curated database of published

observations on mammalian biology, in order to identify the likely up-stream causes and down-stream effects of any changes in gene expression (Calvano *et al.*, 2005). Prior to the pathway analysis the normalised RPKM data was filtered to include only genes that had a greater than 0.5 log-fold change between conditions and were significantly differentially expressed, as defined by a p-value adjusted for multiple comparisons using the Benjamini and Hochberg method of less than 0.05 (Benjamini and Hochberg, 1995). The assessment of the effect of the changes in gene expression on canonical signalling pathways was also filtered to only include significantly altered pathways (adjusted p-value less than 0.05) that differed from the mean in the control sample by greater than two standard deviations (z-score greater than  $\pm 2$ ).

### 3.13 Autophagy and mitophagy

During autophagy the cytosolic form of microtubule-associated protein 1A/1B light chain 3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II (Tanida *et al.*, 2008). LC3-II is recruited to the autophagosome membrane and organelles and proteins targeted for degradation are then engulfed (Lee *et al.*, 2012). Inhibitors of late-stage autophagy, such as chloroquine and bafilomycin A1, prevent the subsequent fusion of autophagosomes with lysosomes, leading to a failure to degrade the autophagosome contents, including LC3-II. The resultant accumulation of LC3-II can then be measured in order to assess the autophagic flux (Klionsky *et al.*, 2012). Autophagy of particular organelles can also be determined by measuring the co-localisation of LC3-II on the autophagosome membrane with a marker specific to that organelle (Zinchuk *et al.*, 2007). In this way mitophagy, the autophagy of mitochondria, can be measured by assessing the co-localisation of LC3-II and a mitochondrial structure, such as OXPHOS complex II (Frank *et al.*, 2012).

#### 3.13.1 Detection of LC3-II by Western Blot

Following a 2 hour incubation in the presence or absence of 10 $\mu$ M chloroquine, protein was extracted from THP-1 cells and the amount of LC3-II relative to the housekeeping protein  $\beta$ -actin was determined by Western blot (see section 3.9). As a positive control autophagy was induced by serum starvation, which involved incubating THP-1 cells in RPMI 1640 medium without FCS for 24 hours prior to treatment with chloroquine.

### *3.13.2 Confocal microscopy to measure mitophagy by the co-localisation of LC3-II and OXPHOS complex II*

In order to assess mitophagy THP-1 cells were incubated in the presence or absence of 5nM bafilomycin A1 for 2 hours. Serum-starved THP-1 cells incubated in RPMI 1640 medium without FCS for 2 hours were used as a positive control for the induction of mitophagy.

After washing, the THP-1 cells were re-suspended in PBS at  $2.5 \times 10^5$  cells/ml and a cytopsin was carried out at 800rpm for 3 minutes. The cells were then fixed to the slides by incubation for 40 minutes at room temperature in a solution of 4% paraformaldehyde in PBS. Following this the cells were permeabilised by a 20 minute incubation at 4°C in PBS containing 0.1% Triton X and 0.1% citrate. After blocking non-specific antibody binding using a solution of 2% BSA in PBS, the slides were then incubated with the primary antibodies for LC3-II and mitochondrial complex II for 16 hours at 4°C. Following this overnight incubation, the slides were washed and incubated with fluorochrome-conjugated secondary antibodies and the nuclear dye DAPI for a further 2 hours at room temperature. Unbound secondary antibody was removed by washing and a cover slip applied using Vectashield Hard Set Mounting Medium. The details of the primary and secondary antibodies used in these experiments are listed in Table 3.9.

Antibody	Host species, isotype	Manufacturer, Catalogue Number	Final concentration
<b>Primary antibodies</b>			
Complex II	Mouse, IgG1	Invitrogen, 459200	1µg/ml
LC3-II	Rabbit, polyclonal	Cell Signalling, CS-54995	4µg/ml
<b>Secondary antibodies and fluorochromes</b>			
DAPI	-	Life Technologies, D3571	5µg/ml
Anti-mouse IgG Oregon Green®	Goat	Life Technologies, O6380	8µg/ml
Anti-rabbit IgG Alexa Fluor® 568	Goat	Life Technologies, A11011	8µg/ml

**Table 3.9 Primary and secondary antibodies and fluorochromes used in confocal microscopy experiments.**

Co-localisation of LC3-II and mitochondrial complex II was determined using the Leica SB2 UV confocal microscope and the 63x magnification X63 HCX PL APO lens. After setting up negative (unstained) and positive (stained for DAPI, LC3-II and complex II) control slides the microscope parameters were kept the same throughout the image collection. The pre-set lasers and filters were used to assess the fluorescence in the ultraviolet (for DAPI nuclear staining), fluorescein isothiocyanate (for complex II staining) and trimethylrhodamine (for LC3-II staining) channels.

Each experimental condition was assessed in triplicate with images taken for a minimum of 100 cells over 3 separate fields of view for each slide. The images were analysed using Volocity software and correction for background fluorescence was carried out on all images. The staining for the complex II and LC3-II was quantified by determining the volume of pixels staining for each target per 100 cells. The co-localisation of complex II and LC3-II was assessed by calculating the Mander's M1 and M2 co-localisation co-efficient (Table 3.10) (Zinchuk *et al.*, 2007).

	Measurement	Interpretation
Mander's M1 co-localisation co-efficient	Proportion of pixels with complex II staining that also stain for LC3-II	Mitophagy – proportion of mitochondria co-localised with the autophagosomes
Mander's M2 co-localisation co-efficient	Proportion of pixels with LC3-II staining that also stain for complex II	Contribution of mitophagy to total autophagy -proportion of autophagosomes co-localised with mitochondria

**Table 3.10 Mander's M1 and M2 co-localisation co-efficient for the assessment of mitophagy in confocal microscopy images**

### 3.14 Statistical analysis

All data were collated using Microsoft Office Excel 2013 and GraphPad Prism 6 was used to generate figures. Statistical analyses were carried out using GraphPad Prism 6 and SPSS for Windows 19.

All experiments were carried out on a minimum of 3 biological replicates; the number of replicates used to generate the data for a specific experiment is detailed in the legend of each figure. Where stated data are normalised relative to the mean in the control sample in order to facilitate representation on graphs. In these cases the statistical analysis was carried out on the original data and not the normalised values. Similarly when different parameters are displayed on the same graph the statistical significance of alterations in the original data for each individual parameter was analysed separately.

The Shapiro-Wilk test was used to determine the normality of the data. Normally-distributed data are presented as mean and 95% confidence interval in text or mean  $\pm$  standard deviation in figures, and were analysed using an independent t-test, one-way analysis of variance (ANOVA) with Dunnett's post-hoc analysis or two-way ANOVA with Tukey's post hoc analysis, depending on the data set. Non-normal data are presented as median and interquartile range in both text and figures, and were analysed using the non-parametric Mann-Whitney U test or Kruskal-Wallis analysis of variance with Dunn's post-hoc analysis, depending on the data set. Categorical data were analysed using Fisher's exact test and the relationship between variables was assessed by linear regression and Pearson's correlation co-efficient. A p-value of less than 0.05 was defined as the threshold for statistical significance.

## Chapter 4 A time course experiment investigating the relationship between compensatory responses activated by treatment of THP-1 cells with lipopolysaccharide

### 4.1 Overview

The precise mechanisms behind monocyte deactivation in human sepsis are not fully understood but there is increasing evidence of mitochondrial dysfunction and depletion of mtDNA in septic monocytes (Pyle *et al.*, 2010; Japiassú *et al.*, 2011; Garrabou *et al.*, 2012). In clinical studies and animal models survival and recovery of cellular functions in sepsis appears to be dependent on the induction of compensatory responses, including the restoration of mitochondrial function through the activation of mitochondrial biogenesis, mitophagy and antioxidant defences (Carré *et al.*, 2010; Piantadosi and Suliman, 2012; Carchman *et al.*, 2013).

Monocytic cells exposed to LPS display endotoxin tolerance, whereby subsequent stimulation with LPS triggers diminishing pro-inflammatory responses (Biswas and Lopez-Collazo, 2009). As endotoxin tolerant monocytes have a similar phenotype to that of deactivated monocytes in sepsis, this model was used to explore the changes in the function and turnover of mitochondria that occur in association with the inhibition of pro-inflammatory responses following an immune stimulus (Cavaillon and Adib-Conquy, 2006).

#### 4.1.1 Hypothesis

THP-1 cells exposed to LPS will display evidence of immune suppression, in the form of endotoxin tolerance, along with mitochondrial dysfunction and mtDNA depletion. The recovery of immune and mitochondrial functions will occur in association with the activation of compensatory responses, including mitochondrial biogenesis and mitophagy.

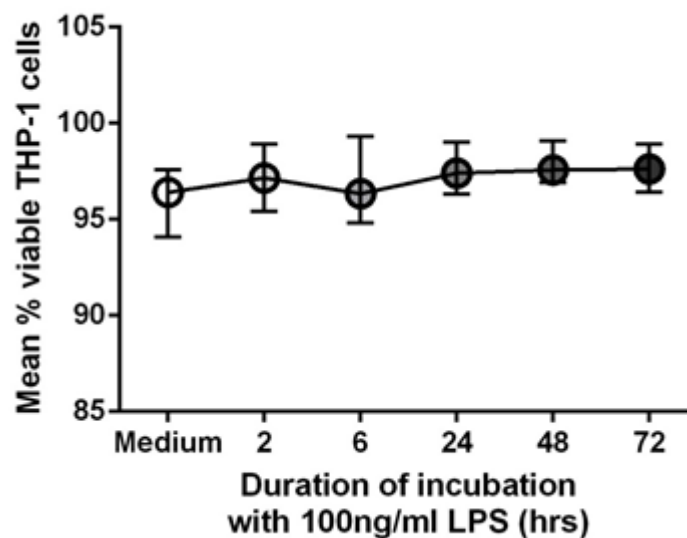
#### 4.1.2 Aims and Objectives

1. To produce a model of monocytic cell immune deactivation by incubating THP-1 cells with LPS to render them endotoxin tolerant.
2. To explore the time-course and dynamics of changes in immunity and mitochondria in THP-1 cells following exposure to LPS.
3. To assess the effects of treatment with LPS on compensatory responses including mitochondrial biogenesis and mitophagy in THP-1 cells.

#### 4.2 Results

##### 4.2.1 Exposure to LPS does not alter THP-1 cell viability

THP-1 cells were incubated with 100ng/ml LPS for 0, 2, 6, 24, 48 and 72 hours and cell viability was then assessed by using flow cytometry to measure the ability of cells to exclude propidium iodide. LPS treatment did not have any significant cytotoxic effect, with greater than 95% of cells remaining viable at all time points (Figure 4.1).



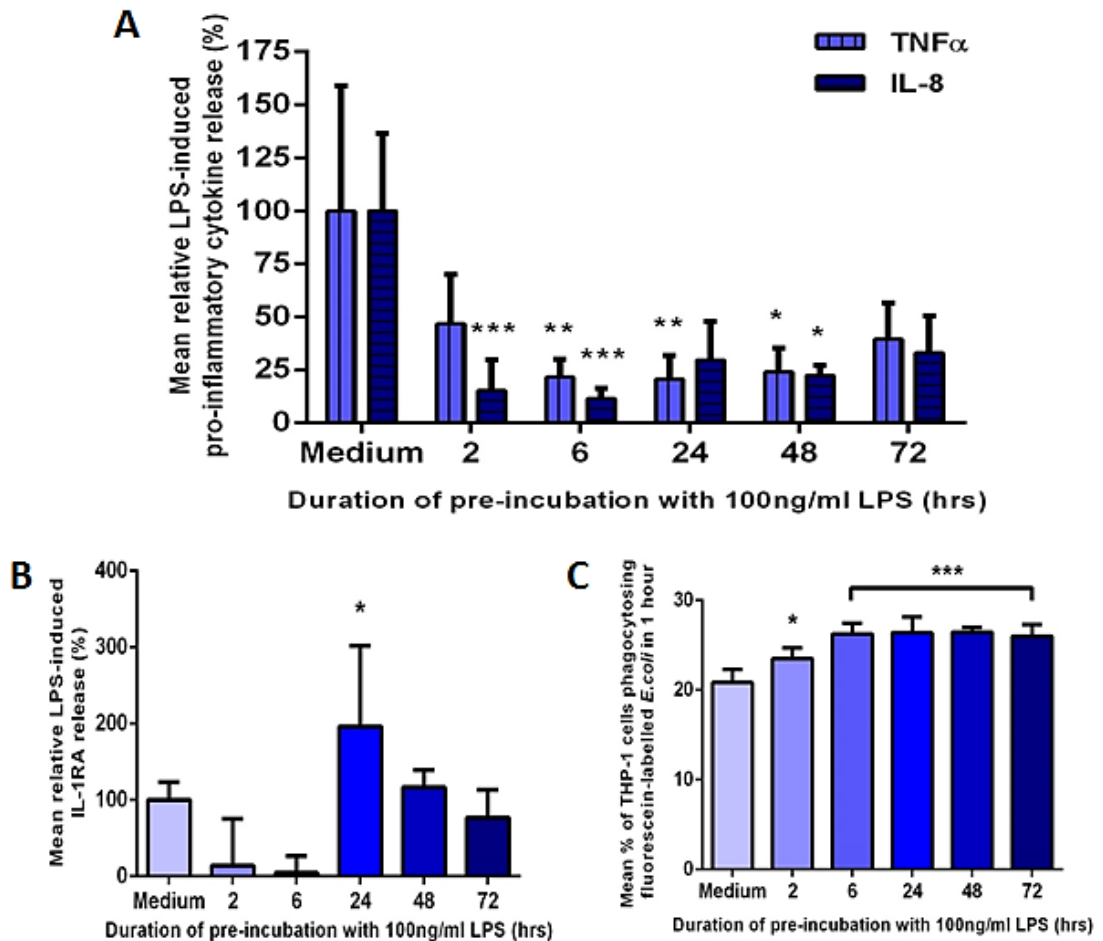
**Figure 4.1 Exposure to LPS does not affect THP-1 cell viability.** Cell viability was determined by measuring the proportion of cells excluding propidium iodide following incubation with 100ng/ml LPS for 0-72 hours (n=4). Data are presented as mean  $\pm$  standard deviation and analysed using one-way ANOVA ( $p=0.665$ ). All differences from the medium control are non-significant with Dunnett's multiple comparison test.

#### 4.2.2 Pre-incubation with LPS leads to a change in THP-1 cell immune phenotype consistent with endotoxin tolerance

In order to confirm that THP-1 cells were successfully rendered endotoxin tolerant, the initial investigations assessed the consequences of a prior exposure to 100ng/ml LPS for 0-72 hours on the immune responses to a second inflammatory stimulus. It was found that the release of the pro-inflammatory cytokines TNF $\alpha$  and IL-8 triggered by a second 4 hour exposure to 100ng/ml LPS was significantly reduced in THP-1 cells pre-incubated with LPS for the previous 2-48 hours (Figure 4.2 A). Conversely, THP-1 cells pre-incubated with LPS for 24 hours displayed an increased ability to release the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1RA) in response to a second LPS stimulus (Figure 4.2 B). There was also a significantly enhanced capacity to phagocytose *Escherichia coli* in THP-1 cells pre-incubated with LPS for the previous 2-72 hours (Figure 4.2 C). At all time points there was no detectable LPS-induced release of the pro-inflammatory cytokines IL-6 and IL-1 $\beta$  or the anti-inflammatory cytokine IL-10.

Thus, pre-incubation of THP-1 cells with LPS resulted in an altered immune response to a subsequent stimulus that was characterised by a change to an anti-inflammatory cytokine release profile and an increased phagocytic capacity. Having confirmed that pre-incubation with LPS altered the immune phenotype of THP-1 cells, the remainder of the assessments were carried out on THP-1 cells after a single exposure to 100ng/ml LPS for 0-72 hours. No second inflammatory stimulus was applied in these experiments.

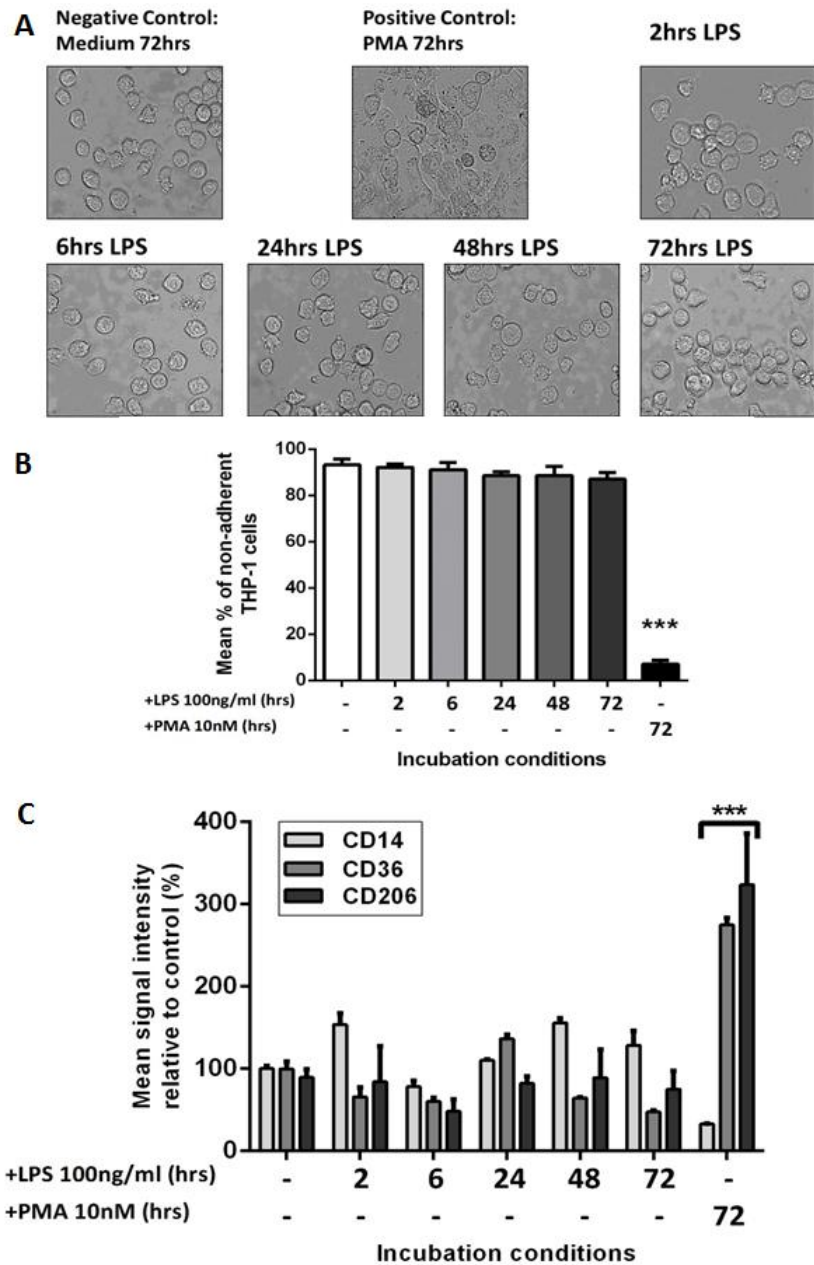




**Figure 4.2 Pre-incubation of THP-1 cells with LPS results in a change in immune phenotype consistent with endotoxin tolerance.** THP-1 cells were pre-incubated with 100ng/ml LPS for 0-72 hours and the ability to respond to a second inflammatory stimulus was then determined. (A) The release of the pro-inflammatory cytokines TNF $\alpha$  and IL-8 in response to a second 4 hour exposure to 100ng/ml LPS was measured by ELISA (n=6). (B) The release of the anti-inflammatory cytokine IL-1RA in response to a second 16 hour exposure to 100ng/ml LPS was determined by ELISA (n=5). (C) Phagocytosis of fluorescein-labelled *Escherichia coli* (*E.coli*) in 1 hour was determined by flow cytometry (n=4). All data are presented as mean  $\pm$  standard deviation with the values in panels A and B expressed as relative cytokine release compared to the mean in the medium control. Non-normal data were analysed by Kruskal Wallis test (A – TNF $\alpha$  p=0.002, IL-8 p<0.001). Normal data were analysed by one-way ANOVA (B - p<0.001, C - p<0.001). Differences from the medium control are non-significant with post-hoc analysis testing except; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

#### 4.2.3 Exposure to LPS does not induce macrophage differentiation in THP-1 cells

A number of studies have indicated that THP-1 cells can be differentiated into macrophage-like cells by culturing them with specific chemicals and cytokines (Schwende *et al.*, 1996; Daigneault *et al.*, 2010). As a result, an investigation was carried out to determine whether the change in the immune functions of THP-1 cells after exposure to LPS could be explained by macrophage differentiation, rather than the induction of endotoxin tolerance. At all time points following LPS treatment cell morphology, adherence and the expression of the differentiation markers CD14, CD36 and CD206 were all significantly different from the findings in a positive control of THP-1 cells incubated with 10nM PMA, a potent stimulus for *in vitro* macrophage differentiation (Park *et al.*, 2007) (Figure 4.3). The alteration of the immune phenotype of THP-1 cells occurring following an initial incubation with LPS, therefore, appears not to be due to differentiation into macrophage-like cells.

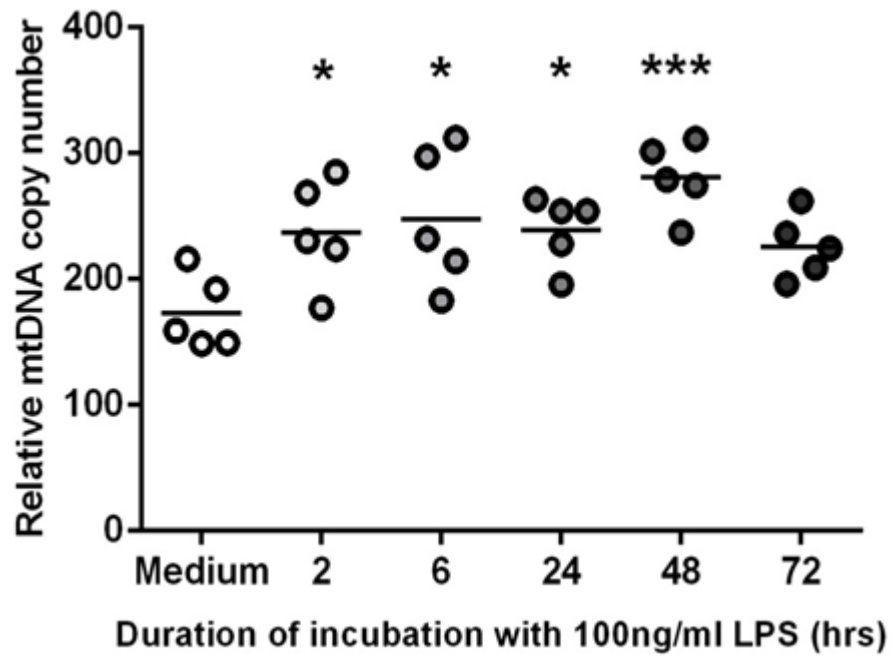


**Figure 4.3 There is no evidence of macrophage differentiation in THP-1 cells exposed to LPS.** THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours before measuring macrophage differentiation in comparison to a positive control of cells treated with 10nM PMA for 72 hours. (A) Representative images of THP-1 cell morphology on inversion light microscope at 40x magnification showing irregular, clumped amoeboid cells after treatment with PMA. (B) THP-1 cell adherence to a 6 well plate was determined by cell counts before and after removal of adherent cells using a cell scraper. (n=3) (C) Flow cytometry was used to determine the relative signal intensity for the expression of the markers of macrophage differentiation CD14, CD36 and CD206 (n=3). Data are presented as mean  $\pm$  standard deviation (relative to the mean of medium control in panel C) and analysed using one-way ANOVA ( $p < 0.001$  for adherence and expression of CD14, CD36 and CD206) with Dunnett's multiple comparison test. \*\*\* $p < 0.001$  – PMA treated cells are significantly different from all other conditions.

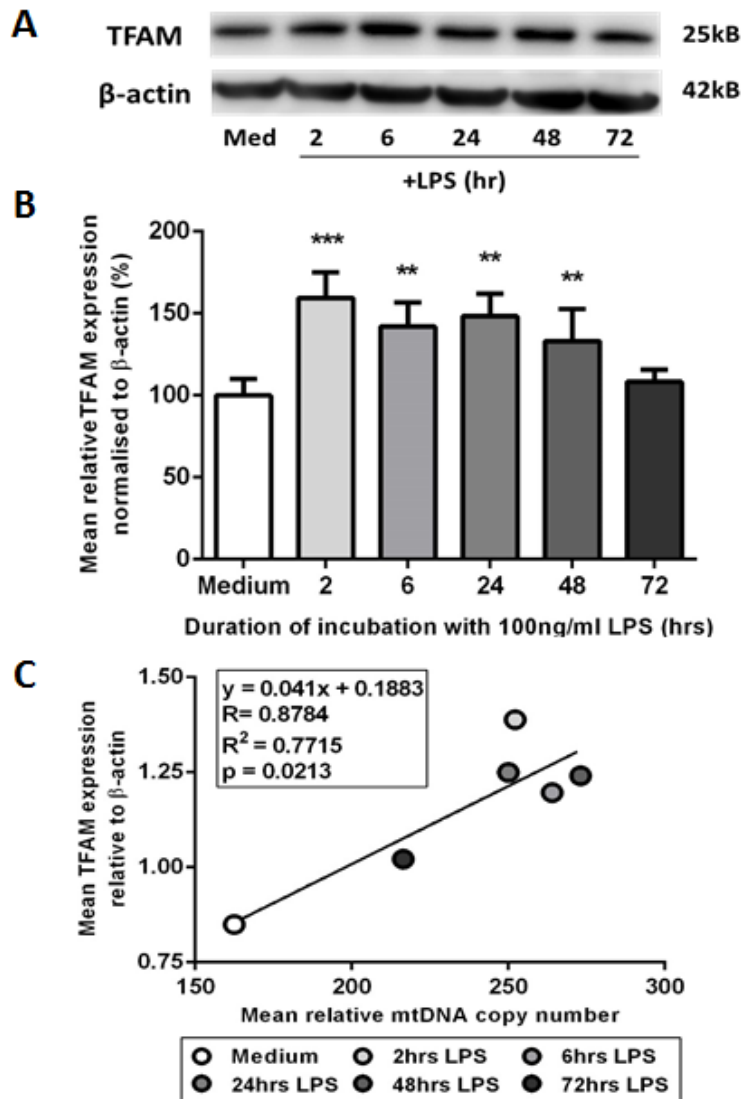
#### 4.2.4 Induction of mitochondrial biogenesis in THP-1 cells following exposure to LPS

After finding that the model successfully rendered THP-1 cells endotoxin tolerant, the effect of exposure to 100ng/ml LPS for 0-72 hours on mtDNA copy number was determined. In contrast to the findings in monocytes from patients with severe sepsis, THP-1 cells with evidence of immune deactivation after treatment with LPS did not have any depletion of mtDNA. Instead, mtDNA copy number was actually significantly increased after exposure to LPS for 2-48 hours, suggesting an early and sustained induction of mitochondrial biogenesis (Figure 4.4).

Within mitochondria mtDNA is coated by proteins to form nucleoids (Kukat *et al.*, 2011). As TFAM is the major protein constituent of these mitochondrial nucleoids and a key regulator of both mtDNA replication and mitochondrial biogenesis, the alterations in mtDNA copy number were verified by assessing TFAM protein expression (Kang *et al.*, 2007; Campbell *et al.*, 2012). In agreement with the changes in mtDNA copy number there was a significant increase in the level of TFAM protein after incubation of THP-1 cells with 100ng/ml LPS for 2-48 hours (Figure 4.5 A and B). As expected, there was also a significant positive correlation between mtDNA copy number and TFAM protein expression (Figure 4.5 C). Therefore, in this model, it appears that the down-regulation of pro-inflammatory cytokine release by THP-1 cells following exposure to LPS occurs in association with the activation of mitochondrial biogenesis and in the absence of any evidence of mtDNA depletion.



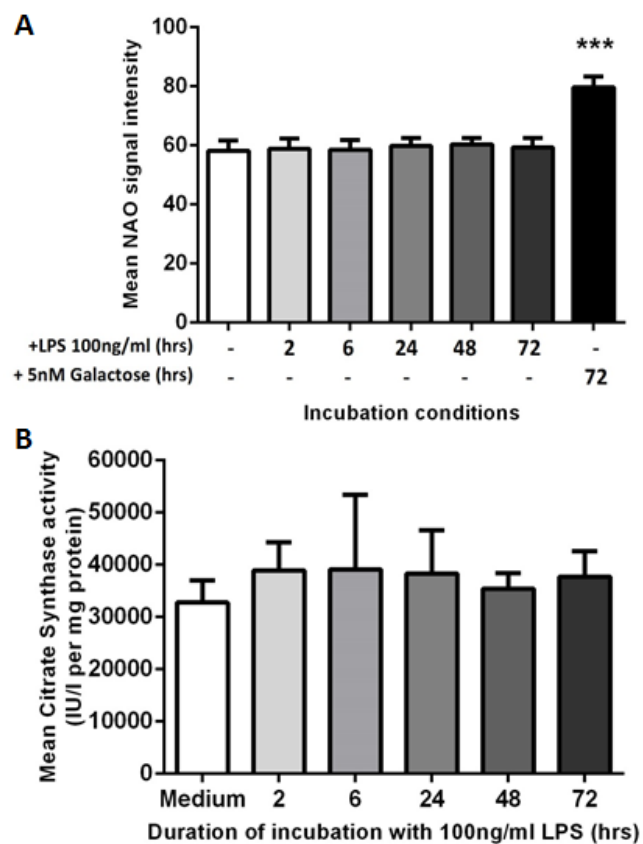
**Figure 4.4 Increased mtDNA copy number following exposure of THP-1 cells to LPS.** THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours and mtDNA copy number was determined by measuring levels of *MT-ND1* relative to *B2M* using qPCR (n=5). Normal data are represented as the mean (line) with individual measurements and analysed using one-way ANOVA (p=0.004). Differences from the medium control are non-significant with Dunnett's multiple comparison test except; \*p<0.05, \*\*\*p<0.001.



**Figure 4.5 Exposure of THP-1 cells to LPS leads to increased *TFAM* expression.** THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours and the level of TFAM protein then measured. (A) Representative images of protein bands for TFAM and  $\beta$ -actin on a PVDF membrane following Western blot. (B) The level of TFAM protein expression relative to  $\beta$ -actin was assessed by Western Blot (n=4). The data are presented as mean  $\pm$  standard deviation relative to the mean in the medium control and analysed using one-way ANOVA ( $p < 0.001$ ). Differences from the medium control are non-significant with Dunnett's multiple comparison test except; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . (C) Scatter plot, linear regression and Pearson's correlation of the relationship between mtDNA copy number and TFAM protein levels.

#### 4.2.5 No change in mitochondrial mass following exposure to LPS

The effect of this induction of mitochondrial biogenesis was first investigated by assessing overall mitochondrial mass in THP-1 cells after treatment with LPS. Two different techniques were used; measurement of the uptake of the mitochondrial dye NAO by flow cytometry and a colorimetric assessment of the activity of citrate synthase, a constitutively expressed mitochondrial matrix enzyme (Kirby *et al.*, 2007; Cottet-Rousselle *et al.*, 2011). In contrast to the effects on mtDNA copy number, there was no detectable increase in the mitochondrial mass of THP-1 cells treated with 100ng/ml LPS for 2-72 hours (Figure 4.6).

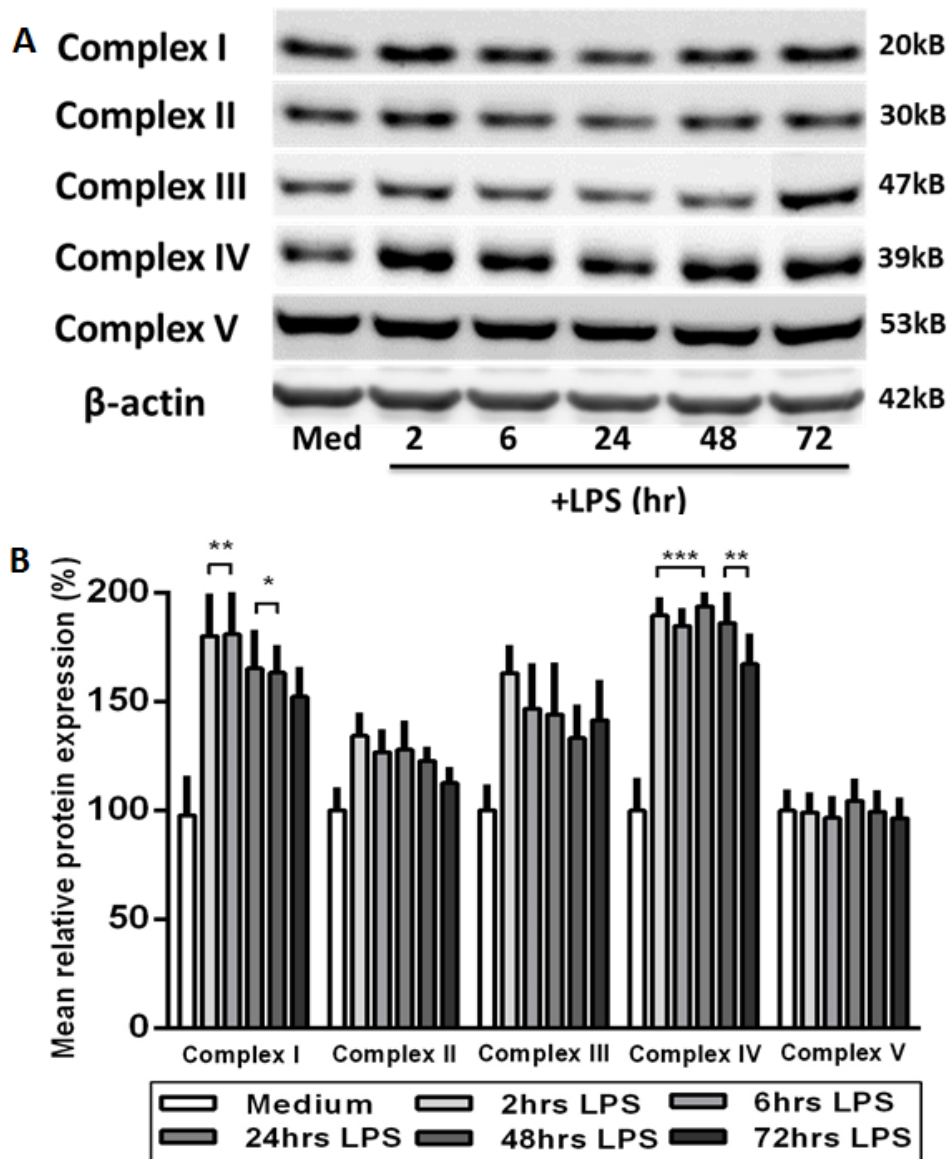


**Figure 4.6 Unchanged mitochondrial mass following exposure of THP-1 cells to LPS.** THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours before measuring mitochondrial mass. (A) Mitochondrial mass was assessed by measuring the uptake of NAO using flow cytometry, with THP-1 cells incubated in glucose-free medium supplemented with 5mM galactose for 72 hours as a positive control (n=4). (B) A colorimetric assay was used to assess the activity of the mitochondrial matrix enzyme citrate synthase (n=3). Normal data are presented as mean  $\pm$  standard deviation and analysed using one-way ANOVA (A –  $p < 0.001$ , B –  $p = 0.896$ ). Differences from the medium control are non-significant with Dunnett’s multiple comparison test except; \*\*\* $p < 0.001$ .

#### 4.2.6 Increased expression of mitochondrial OXPHOS complexes I and IV after exposure of THP-1 cells to LPS

As mtDNA contains 13 genes that encode vital subunits of OXPHOS complexes I, III, IV and V on the inner mitochondrial membrane, the consequences of the increase in mtDNA copy number after LPS treatment were further assessed by measuring the expression of protein subunits of each OXPHOS complex (Greaves *et al.*, 2012). It is shown in Figure 4.7 that the expression of OXPHOS complexes I and IV, the two complexes with the greatest number of mtDNA-encoded constituents, was significantly increased in THP-1 cells after exposure to LPS, while there were no significant changes in the levels of complexes II, III and V (Schon *et al.*, 2012). Thus, in parallel with the increase in mtDNA copy number, and despite the unchanged overall mitochondrial mass, treatment of THP-1 cells with LPS lead to an increase in the level of OXPHOS complexes I and IV.





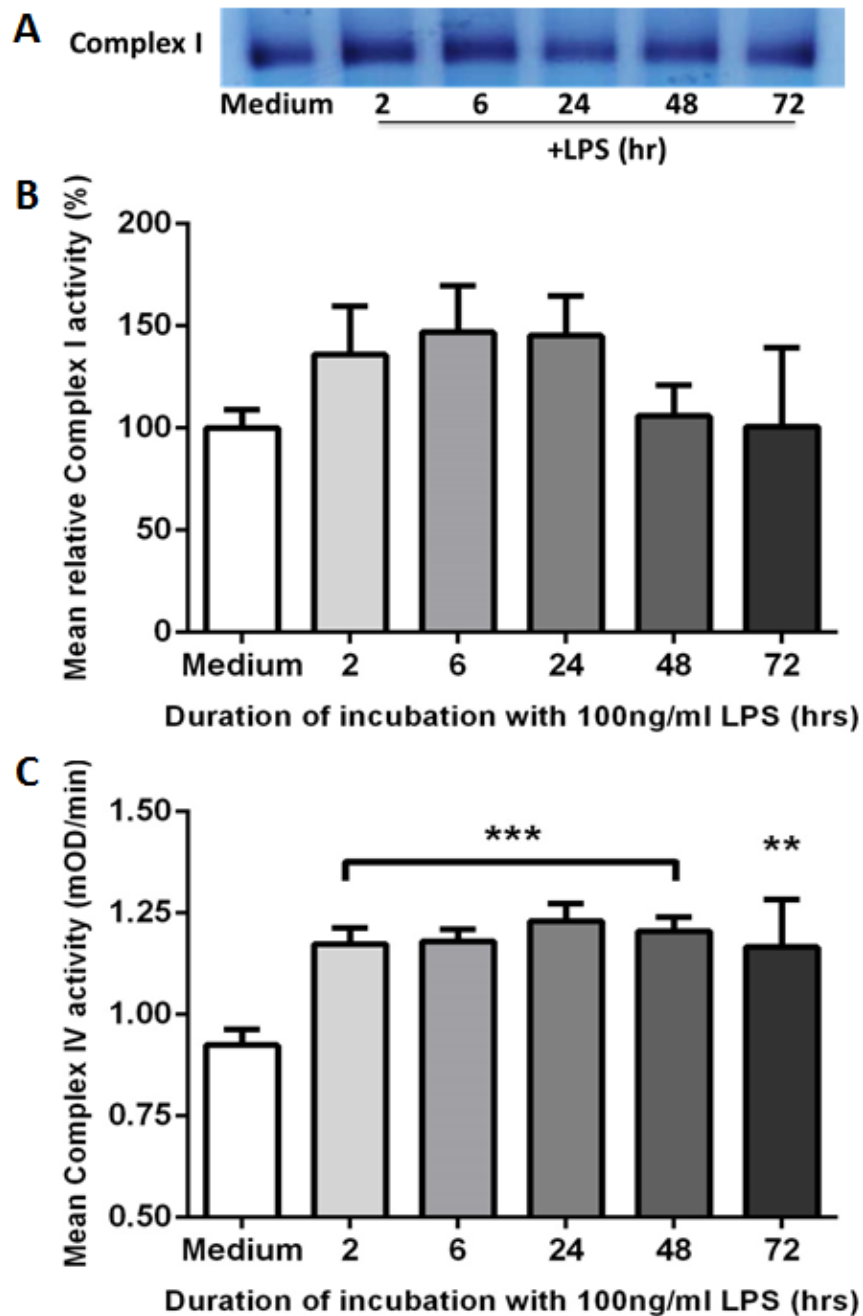
**Figure 4.7** The expression of protein subunits of mitochondrial OXPHOS complexes I and IV is increased in THP-1 cells following exposure to LPS. THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours and the level of protein subunits of the five mitochondrial OXPHOS complexes was then determined by Western blot. (A) Representative images of protein bands on PVDF membrane following Western blot. (B) The protein expression (relative to  $\beta$ -actin) of the mitochondrial OXPHOS complexes (n=4). The data are represented as mean  $\pm$  standard deviation relative to the mean in the medium control and analysed using one-way ANOVA (complex I - p=0.020, complex II - p=0.126, complex III - p=0.308, complex IV - p<0.001, complex V - p=0.986). The differences from the medium control are not significant with Dunnett's multiple comparison test except; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

#### 4.2.7 Increased mitochondrial respiration following treatment of THP-1 cells with LPS

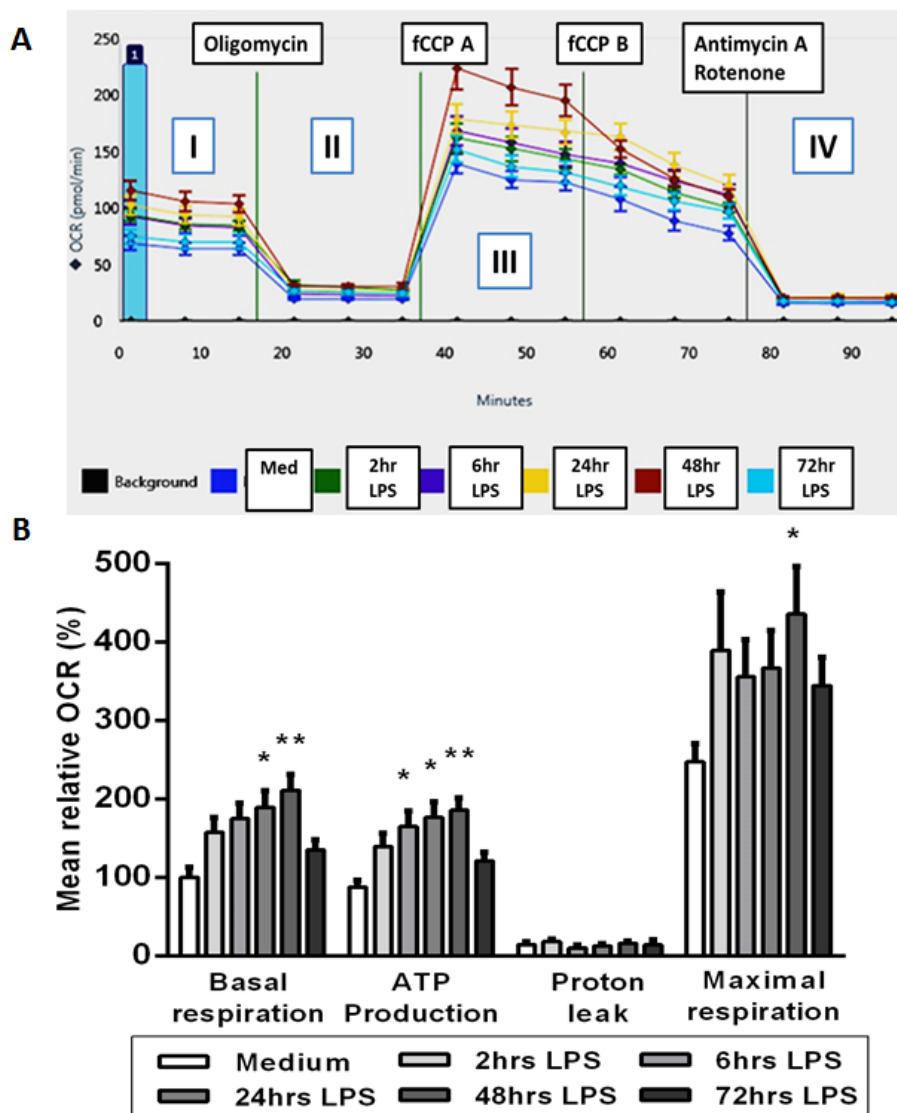
Next, the effects of exposure to LPS on mitochondrial respiration in THP-1 cells were determined. Firstly, the activity of isolated mitochondrial OXPHOS complexes I and IV were measured (Nijtmans *et al.*, 2002; Rodenburg *et al.*, 2012). While, in keeping with the up-regulation of protein expression, there was a significant increase in the enzymatic activity of complex IV, the activity of complex I did not significantly change at any of the time points following treatment with LPS (Figure 4.8).

Subsequently, different aspects of mitochondrial respiration were assessed in more detail by determining the effects of LPS treatment on oxygen consumption by THP-1 cells using the Seahorse XF96<sup>e</sup> extracellular flux analyser (Hill *et al.*, 2012). Exposure of THP-1 cells to 100ng/ml LPS for 6-48 hours resulted in increased mitochondrial oxygen consumption, particularly for basal mitochondrial respiration and mitochondrial ATP production (Figure 4.9). There were no significant LPS-induced alterations in oxygen consumption due to non-mitochondrial respiration or the leakage of protons across the inner mitochondrial membrane.

These results show that treatment of THP-1 cells with LPS leads to an increase in mitochondrial respiration that occurs in association with the activation of mitochondrial biogenesis and increased OXPHOS protein expression, but despite unchanged overall mitochondrial mass.



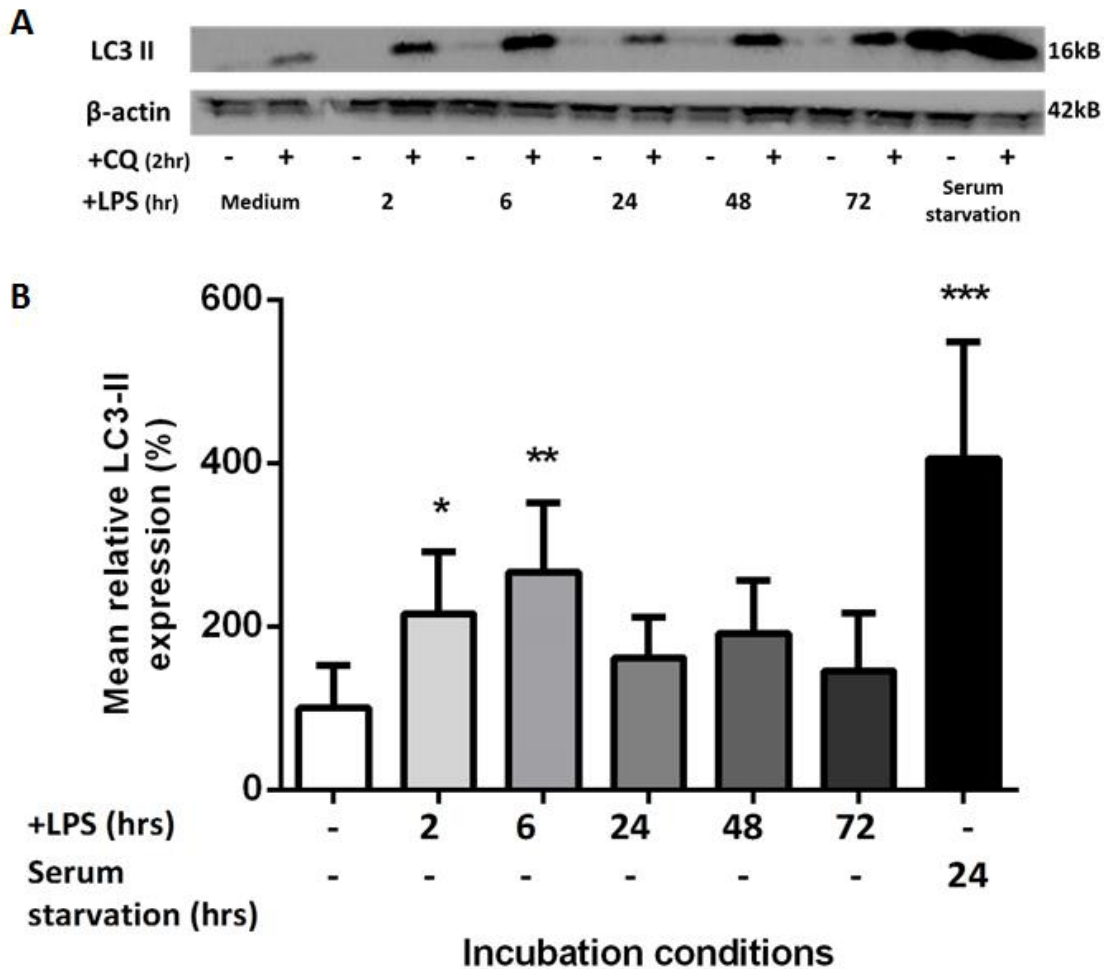
**Figure 4.8 Increased mitochondrial OXPHOS complex IV activity after treatment with LPS.** THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours before assessing the activity of OXPHOS complexes I and IV. (A) Representative image of a 3-12% Bis-Tris gel stained for complex I activity after blue native-PAGE. (B) In-gel complex I activity relative to the mean in the medium control (n=3). (C) Complex IV activity as determined by the rate of reduction of cytochrome C (n=3). Normal data are presented as mean  $\pm$  standard deviation and analysed using one-way ANOVA (B –  $p=0.069$ , C -  $p<0.001$ ). Differences from the medium control are non-significant with Dunnett's multiple comparison test except; \*\* $p<0.01$ , \*\*\* $p<0.001$ .



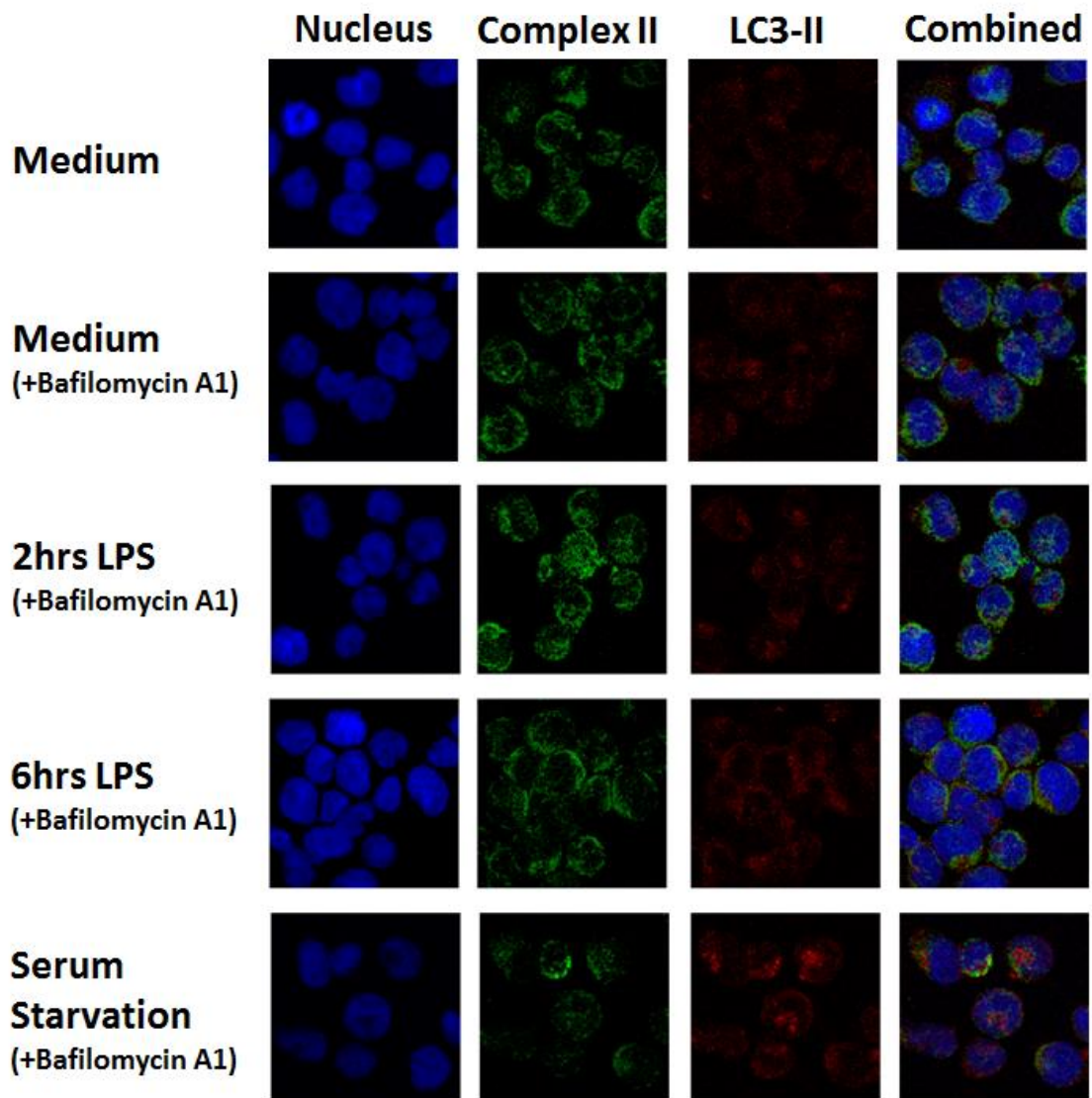
**Figure 4.9 Exposure of THP-1 cells to LPS leads to increased oxygen consumption due to mitochondrial respiration.** THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours and different aspects of respiration were then determined by measuring the OCR after the sequential addition of metabolic inhibitors using the Seahorse XF96<sup>e</sup> extracellular flux analyser. (A) An example of the respiratory profile of THP-1 cells following exposure to LPS for 0-72 hours. (B) The OCR was determined for the following aspects of cellular respiration; Basal mitochondrial respiration = basal OCR (I) – non-mitochondrial OCR (IV); ATP production = basal OCR (I) – post-oligomycin OCR (II); Proton leak = post-oligomycin OCR (II) – non-mitochondrial respiration (IV); Maximal mitochondrial respiration = maximal OCR (III) – non-mitochondrial OCR (IV). Data are presented as mean ( $\pm$  standard deviation) relative OCR compared to the mean basal mitochondrial respiration in the medium control (n=5 for all experiments). The data for each aspect of mitochondrial respiration was analysed separately using one-way ANOVA (Maximal respiration – p=0.192) or Kruskal Wallis test (Basal respiration – p=0.006, ATP production – p=0.007, Proton leak – p=0.312). Differences from medium control are non-significant with post-hoc testing except; \*p<0.05, \*\*p<0.01

#### *4.2.8 Early induction of autophagy and mitophagy in THP-1 cells exposed to LPS*

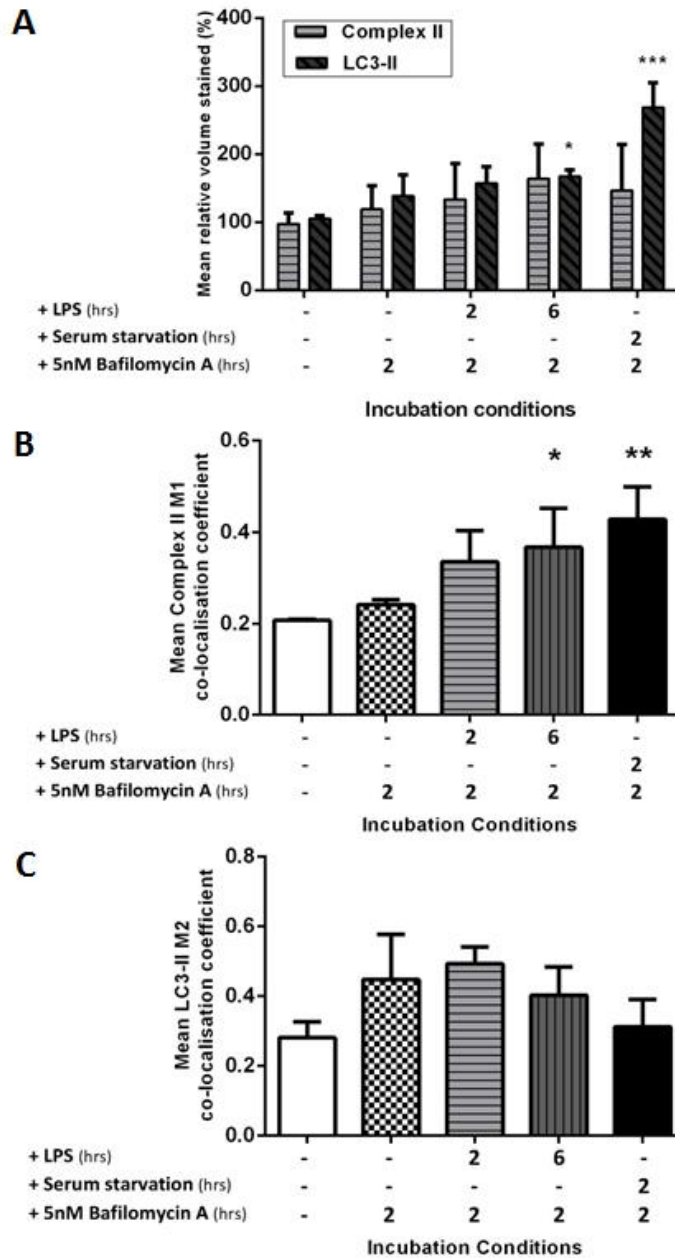
As previous studies have indicated that the removal of dysfunctional mitochondria may be up-regulated during inflammation, an assessment of the effects of treatment with LPS on both autophagy and mitophagy in THP-1 cells was carried out (Carchman et al., 2013; Chang et al., 2015). There was an early LPS-induced activation of autophagy, as indicated by a significantly increased accumulation of the autophagosome protein LC3-II in THP-1 cells exposed to 100ng/ml LPS for 2-6 hours (Tanida et al., 2008) (Figure 4.10). Using confocal microscopy to measure the co-localisation of mitochondria to autophagosomes it was then confirmed that this induction of autophagy in THP-1 cells after treatment with LPS for 6 hours was accompanied by a significant increase in mitophagy (Figure 4.11 and Figure 4.12) (Zinchuk et al., 2007). These results indicate that mitophagy is up-regulated in parallel with the activation of mitochondrial biogenesis in THP-1 cells after treatment with LPS.



**Figure 4.10 Induction of autophagy in THP-1 cells exposed to LPS.** THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours and autophagic flux was assessed by measuring the accumulation of LC3-II occurring during the final 2 hours after treatment with 10 $\mu$ M chloroquine (CQ). As a positive control for autophagy THP-1 cells were incubated in RPMI 1640 medium without FCS (serum starvation) for 24 hours. (A) Representative image of LC3-II and  $\beta$ -actin protein bands on a PVDF membrane following Western blot. (B) The expression of LC3-II relative to  $\beta$ -actin after treatment with CQ was determined by Western blot (n=5). Data are presented as mean  $\pm$  standard deviation relative to the mean in the medium control and analysed using one-way ANOVA ( $p < 0.001$ ). Differences from the medium control (white bar) are non-significant with Dunnett's multiple comparison test except; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 4.11 Confocal microscopy images indicating co-localisation of mitochondrial OXPHOS complex II and the autophagosome marker LC3-II.** THP-1 cells were incubated with 100ng/ml LPS for 0-6 hours and treated with 5nM bafilomycin A1 for the final 2 hours to allow accumulation of LC3-II (in all conditions except the 1<sup>st</sup> row, labelled medium). As a positive control for autophagy THP-1 cells were incubated in RPMI 1640 medium without FCS (5<sup>th</sup> row, labelled serum starvation) for 2 hours. Representative confocal microscopy images are displayed indicating staining of cytospin slides for the nucleus (1<sup>st</sup> column, blue), mitochondrial complex II (2<sup>nd</sup> column, green) and the autophagosomal marker LC3-II (3<sup>rd</sup> column, red). The final column indicates a composite image produced by overlaying the nuclear, mitochondrial and autophagosome staining.



**Figure 4.12 Induction of mitophagy following exposure of THP-1 cells to LPS for 6 hours.** Quantification of the confocal microscopy imaging was carried out following the assessment of mitochondrial complex II and autophagosome LC3-II staining in THP-1 cells. (A) Quantification of the volume of pixels staining for complex II and LC3-II per 100 THP-1 cells relative to the mean in the untreated control (1<sup>st</sup> bars) (n=3). (B, C) Co-localisation of complex II and LC3-II staining using Mander's M1 (the proportion of complex II positive pixels also staining for LC3-II, indicating mitophagy) and M2 (proportion of LC3-II positive pixels also staining for complex II, indicating the contribution of mitophagy to total autophagy) co-localisation co-efficient (n=4). Data are presented as mean  $\pm$  standard deviation and analysed using one-way ANOVA (A - complex II p=0.504, LC3-II p<0.001, B – p=0.001, C – p=0.022). Differences from bafilomycin A1-treated control (2<sup>nd</sup> bar) are non-significant with Dunnett's multiple comparison test except; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

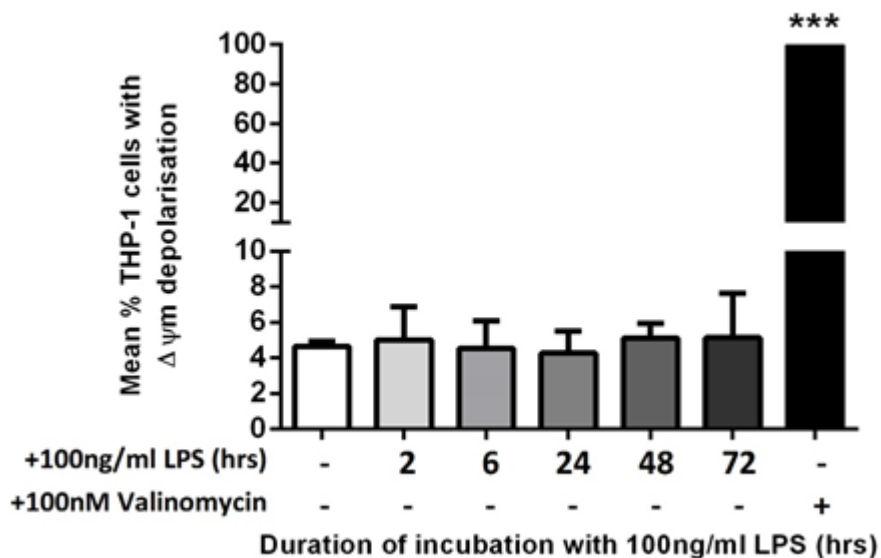


#### 4.2.9 Early oxidative stress and induction of antioxidant defences but no mitochondrial membrane potential depolarisation following treatment of THP-1 cells with LPS

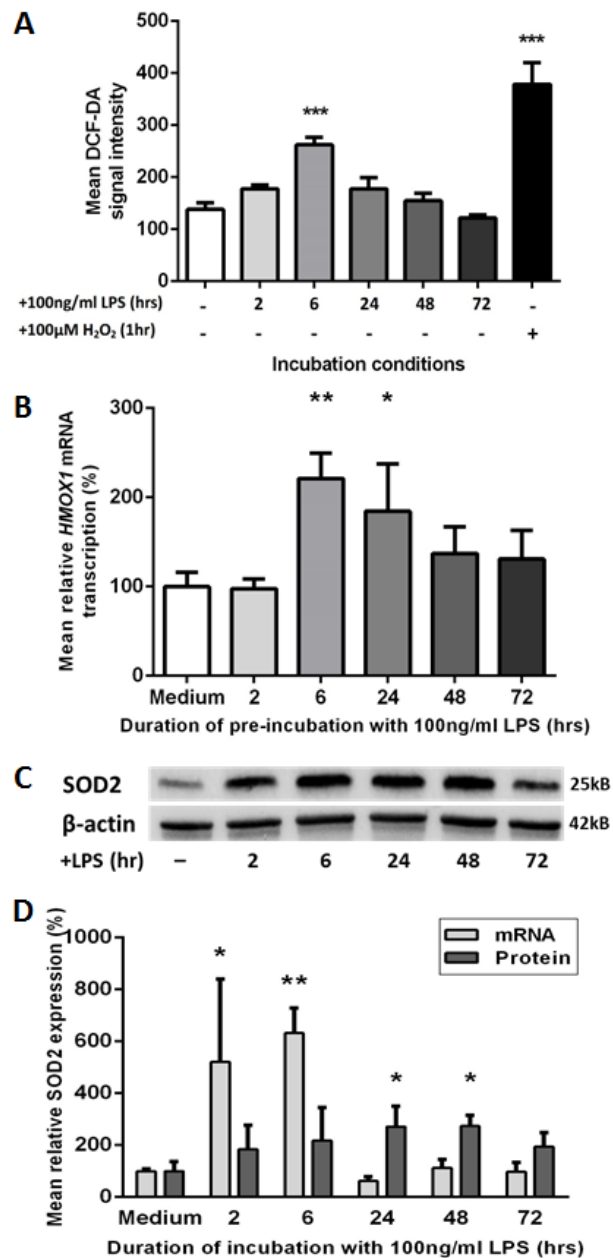
Finally, the effect of treatment with LPS on two markers of mitochondrial dysfunction, mitochondrial membrane potential ( $\Delta\psi_m$ ) depolarisation and oxidative stress, was determined.

Depolarisation of  $\Delta\psi_m$ , which is generated by the transport of electrons across the inner mitochondrial membrane by OXPHOS complexes I, III and IV, is associated with a loss of mitochondrial integrity and has been found to occur after exposure to LPS in cell culture and animal models (Narendra *et al.*, 2008; Bauerfeld *et al.*, 2012; Carchman *et al.*, 2013). However, in keeping with the findings of unchanged oxygen consumption for proton leak across the inner mitochondrial membrane (Figure 4.9), there was no evidence of  $\Delta\psi_m$  depolarisation in THP-1 cells after treatment with LPS (Figure 4.13).

Excessive mitochondrial ROS production, due to increased leakage of electrons from the OXPHOS system, is another feature of mitochondrial dysfunction that has been associated with inflammation, including exposure to LPS (Suliman *et al.*, 2003b; Galley, 2011). THP-1 cell ROS production was increased in the initial period after treatment with 100ng/ml LPS and this was followed by increased transcription of mRNA from the *HMOX1* gene which encodes HO-1, an enzyme that is induced by oxidative stress (Alam and Cook, 2003) (Figure 4.14 A and B). Measurements of the expression of the *SOD2* gene, which encodes the mitochondrial antioxidant superoxide dismutase-2 (SOD2), show that the resolution of this LPS-induced oxidative stress occurred in association with the activation of antioxidant defences. After exposure of THP-1 cells to LPS there was a rapid increase in the transcription of *SOD2* mRNA which mirrored the increase in ROS production and was followed by significantly increased SOD2 protein levels at 24 and 48 hours (Figure 4.14 C and D).



**Figure 4.13 Exposure to LPS does not significantly alter the mitochondrial membrane potential in THP-1 cells.** After incubation with 100ng/ml LPS for 0-72 hours the proportion of THP-1 cells with a depolarised mitochondrial membrane potential ( $\Delta\psi_m$ ) was determined by measuring the green (depolarised  $\Delta\psi_m$ ) and red (maintained  $\Delta\psi_m$ ) fluorescence from the JC1 dye by flow cytometry. As a positive control for  $\Delta\psi_m$  depolarisation THP-1 cells were incubated with 100nM valinomycin. The data are presented as mean  $\pm$  standard deviation and analysed using one-way ANOVA ( $p < 0.001$ ). All differences from the medium control are non-significant with Dunnett's multiple comparison test except; \*\*\* $p < 0.001$ .



**Figure 4.14 Resolution of early oxidative stress in association with the induction of antioxidant defences in THP-1 cells exposed to LPS.** THP-1 cells were incubated with 100ng/ml LPS for 0-72 hours and markers of oxidative stress and antioxidant responses measured. (A) ROS production was measured by oxidation of DCF-DA using flow cytometry. THP-1 cells incubated with 100μM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 1 hour provided a positive control (n=3). (B) The mRNA transcription of *HMOX1* relative to *GAPDH* was determined by RTqPCR (n=3). (C) Representative image of protein bands on a PVDF membrane following Western blot (D) The mRNA transcription (relative to *GAPDH*) and protein expression (relative to β-actin) of the mitochondrial antioxidant gene *SOD2* was measured (n=3). Data presented as mean ± standard deviation (mRNA and protein data are relative to the mean of the medium control) and analysed using one way ANOVA (A – p<0.001, B – p=0.002, D – mRNA p<0.001, protein p=0.030). Differences from the medium control are non-significant with Dunnett’s multiple comparison test except; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

## 4.3 Discussion

### 4.3.1 Incubation of THP-1 cells with LPS leads to the induction of endotoxin tolerance

Endotoxin tolerance, in which a prior exposure to LPS from Gram negative bacteria results in diminishing pro-inflammatory responses to subsequent inflammatory stimuli, appears to be an adaptive response aimed at limiting the harmful effects of excessive inflammation (Biswas and Lopez-Collazo, 2009). Features of endotoxin tolerance have been identified in blood monocytes from patients with a variety of acute and chronic inflammatory conditions (del Campo *et al.*, 2011; Lopez-Collazo and del Fresno, 2013). Deactivated monocytes isolated from critically ill patients with sepsis share a similar phenotype with endotoxin tolerant monocytes produced by *in vitro* or *in vivo* exposure to LPS (Escoll *et al.*, 2003; Draisma *et al.*, 2009). The archetypal feature of endotoxin tolerance is a marked reduction in the release of the pro-inflammatory cytokine TNF $\alpha$  by monocytes in response to subsequent exposures to LPS, a finding that is associated with adverse outcomes when detected in septic monocytes (Biswas and Lopez-Collazo, 2009; Hall *et al.*, 2011). Similarly, there is evidence of impaired antigen presentation by both endotoxin tolerant and septic monocytes, as reflected by a reduction in the expression of HLA-DR on their surface (Wolk *et al.*, 2000; Landelle *et al.*, 2010). In contrast, anti-inflammatory responses, particularly LPS-induced IL-10 and IL-1RA release, and phagocytic capacity have been found to be up-regulated in endotoxin tolerant monocytes and in those isolated from patients with sepsis (Sfeir *et al.*, 2001; Escoll *et al.*, 2003; del Fresno *et al.*, 2009; Doring *et al.*, 2014).

In this chapter it is shown that incubation of THP-1 cells with 100ng/ml LPS produces many of the features of both endotoxin tolerant and septic monocytes. In particular the exposure of THP-1 cells to LPS leads to a reduced ability to produce pro-inflammatory cytokines, including TNF $\alpha$ , but enhanced anti-inflammatory responses and improved phagocytosis. This suggests that LPS-treated THP-1 cells provide a good model for assessing the relationship between the induction of an immune deactivation that is similar to that seen in severe sepsis and the changes in monocyte cell mitochondria that occur following an inflammatory insult.

#### 4.3.2 Endotoxin tolerant THP-1 cells do not have evidence of mtDNA depletion or mitochondrial respiratory dysfunction

In a few small observational studies septic monocytes have been found to have evidence of mtDNA depletion and decreased mitochondrial respiratory activity (Pyle *et al.*, 2010; Japiassú *et al.*, 2011; Garrabou *et al.*, 2012). Animal models have also indicated that a septic insult can lead to oxidative damage to mtDNA, mtDNA depletion and a reduction in OXPHOS activity in a variety of tissues (Suliman *et al.*, 2003a; Haden *et al.*, 2007; Carchman *et al.*, 2013). In order to try to clarify the relationship between these findings the effects of inducing a state of immune deactivation on the mitochondrial functions of THP-1 cells was explored. Treatment with LPS, despite altering the immune phenotype of THP-1 cells by producing endotoxin tolerance, did not lead to any evidence of mtDNA depletion or mitochondrial respiratory impairment. On the contrary, there was an early and sustained increase in both mtDNA copy number and mitochondrial respiration in endotoxin tolerant THP-1 cells. This suggests that mtDNA depletion is not an essential pre-requisite for the induction of immune dysfunction in monocytic cells, and that this process may occur despite adequate mitochondrial respiration.

The differences between the findings from this model and those from clinical and animal sepsis studies, in particular the lack of mtDNA depletion and OXPHOS dysfunction, may reflect the limitations of using a single, sterile stimulus to model the overwhelming, multiple and persistent inflammatory triggers that are present during sepsis. The relatively mild nature of treatment with 100ng/ml LPS is highlighted by the absence of any measurable effect on THP-1 cell viability or  $\Delta\psi_m$  depolarisation, findings that contrast with those from septic monocytes (Adrie *et al.*, 2001). The consequences of treating THP-1 cells with 100ng/ml LPS may, therefore, be more reflective of the processes that are activated when a less severe infection is successfully cleared, rather than those occurring in the more unusual circumstances in which an infection leads to excessive systemic inflammation and sepsis. However, the evidence of increased mitochondrial ROS production and oxidative stress in this model of endotoxin tolerance does indicate that the effects of LPS on THP-1 cell mitochondria are not entirely benign. Furthermore, the rapid resolution of these effects also

suggests that there is an induction of very efficient compensatory responses that maintain mitochondrial function in THP-1 cells following exposure to LPS.

#### 4.3.3 Mitochondrial biogenesis is triggered by exposure to LPS

Rather than producing mtDNA depletion, treatment of THP-1 cells with LPS resulted in an early and sustained increase in mtDNA copy number due to the activation of mitochondrial biogenesis. Mitochondrial biogenesis appears to be an essential response that is required to compensate for the adverse effects of inflammation on the structure and function of mitochondria (Kozlov *et al.*, 2011). Animal models suggest that mitochondrial biogenesis is directly triggered by inflammatory signalling and is associated with a more rapid recovery of cellular respiration and improved survival during sepsis (Suliman *et al.*, 2003b; Sweeney *et al.*, 2010). Similarly, in critically ill patients survivors have been found to have an early up-regulation of the key mitochondrial biogenesis regulator PGC-1 $\alpha$ , along with evidence of a reduction in mitochondrial damage (Carré *et al.*, 2010).

The beneficial effects of stimulating mitochondrial biogenesis in response to an inflammatory insult may also extend beyond improving the capability of the cell to replace damaged and dysfunctional mitochondria. The results in this chapter indicate that the activation of mitochondrial biogenesis occurring in THP-1 cells following treatment with LPS was associated with a significant increase in mitochondrial respiratory activity. This finding is consistent with previous observations that treatment with LPS stimulates mitochondrial oxygen consumption by THP-1 cells and leads to enhanced ATP production by murine macrophages (Bauerfeld *et al.*, 2012; Liu *et al.*, 2015). In addition to allowing the cell to cope with the increased metabolic demands during an infection, there is evidence that this up-regulation of mitochondrial respiration can also lead to an increased resistance to the negative effects of excessive inflammation (Islam *et al.*, 2012; Stetler *et al.*, 2012).

While these findings suggest that the activation of mitochondrial biogenesis in THP-1 cells after exposure to LPS is likely to be broadly beneficial, this process may also potentially exacerbate endotoxin tolerance-related immune deactivation. The time-course experiments in this chapter show that there is a temporal association between the activation of mitochondrial biogenesis and the shift towards an anti-inflammatory

phenotype. Furthermore, at 72 hours after treatment with LPS the recovery of THP-1 cell immune functions occurs at a point when mtDNA copy number and TFAM levels have also returned to baseline. This association is consistent with findings from murine sepsis models in which there appears to be a co-regulation of mitochondrial biogenesis and anti-inflammatory cytokine production through the activation of redox-sensitive pathways following exposure to an inflammatory stimulus (Piantadosi *et al.*, 2011; MacGarvey *et al.*, 2012). It is, therefore, possible that mitochondria are linked with immune deactivation in monocytes through the excessive co-stimulation of mitochondrial biogenesis and anti-inflammatory pathways during sepsis, rather than, or perhaps in addition to, any adverse effects of mtDNA depletion (Piantadosi and Suliman, 2012).

#### *4.3.4 Mitochondrial quality control through the co-induction of mitochondrial biogenesis and mitophagy*

Mitophagy was also found to be significantly up-regulated following treatment of THP-1 cells with LPS. Previous studies have indicated that the removal of defective mitochondria through mitophagy is essential in order to maintain a healthy mitochondrial population during inflammation (Kim *et al.*, 2007). In animal models of inflammation inhibiting mitophagy allows damaged mitochondria to accumulate, with consequential adverse effects on cellular function and viability (Nakahira *et al.*, 2011; Motori *et al.*, 2013). Although data from human studies is limited, it appears that mitophagy may be insufficient during severe sepsis, resulting in the persistence of dysfunctional mitochondria that can drive oxidative stress, lead to deficient respiration and cause cell death through the induction of apoptosis (Carré *et al.*, 2010; Kozlov *et al.*, 2011; Gunst *et al.*, 2013).

The up-regulation of mitophagy in THP-1 cells exposed to LPS occurs in parallel with the stimulation of mitochondrial biogenesis. A co-ordinated activation of these two processes allows for the selective replacement of dysfunctional mitochondria, which can enable overall mitochondrial quality to be maintained in the face of significant inflammation (Hill *et al.*, 2012; Carchman *et al.*, 2013). Indeed, in THP-1 cells these processes may be particularly efficient as the results in this chapter show that mitochondrial OXPHOS activity is actually increased after treatment with LPS, despite unchanged mitochondrial mass.

The regulation of mitophagy appears to be very sensitive to alterations in mitochondrial function, with features of mitochondrial damage such as  $\Delta\psi_m$  depolarisation and increased ROS production acting as potent mitophagy activators (Youle and Narendra, 2011). Despite findings that  $\Delta\psi_m$  depolarisation occurs in animal sepsis models and monocytes from sepsis patients, exposure to LPS did not lead to significant alterations in the  $\Delta\psi_m$  of THP-1 cells (Adrie *et al.*, 2001; Carchman *et al.*, 2013). On the other hand, there was evidence of significant oxidative stress occurring at the same time as the activation of mitophagy in LPS-treated THP-1 cells. As previous studies have found that mitochondria producing excessive ROS are specifically targeted for clearance by mitophagy during inflammation, it is possible that this early oxidative stress is a major trigger for the LPS-induced up-regulation of mitophagy that is seen in THP-1 cells (Nakahira *et al.*, 2011; Chang *et al.*, 2015). Following the activation of mitophagy ROS production by THP-1 cells returns to baseline levels, suggesting that the removal of dysfunctional mitochondria may be important, along with the activation of antioxidant defences, in the resolution of LPS-induced oxidative stress.

#### 4.3.5 Conclusion

The treatment of THP-1 cells with 100ng/ml LPS for 0-72 hours provides a useful model for assessing the changes occurring in parallel with the induction of endotoxin tolerance, a process with important similarities to the immune deactivation of monocytes during sepsis. In contrast to sepsis, rather than respiratory impairment and mtDNA depletion, these endotoxin tolerant THP-1 cells had increased OXPHOS activity and an induction of mitochondrial biogenesis. Furthermore, after treatment with LPS there was an up-regulation of mitophagy which may, alongside the increase in mitochondrial biogenesis, lead to the resolution of oxidative stress and an improvement in overall mitochondrial respiratory efficiency, through the selective removal of dysfunctional mitochondria.

These observations provide important insights into the interactions between mitochondria and the innate immune response, as well as the co-ordination of compensatory responses that are required to maintain cell viability and function following an inflammatory insult. However, the underlying cellular mechanisms controlling these responses have not been explored and the precise nature of the



causality and relationships between the changes that have been observed remain unclear. In addition to confirming the findings in primary blood monocytes and patients with sepsis, further work is required to clarify the fundamental processes controlling the co-regulation of mitochondrial homeostasis and immune responses during inflammatory conditions.

## Chapter 5 The effect of LPS inhalation on blood monocyte immune functions and mitochondrial DNA copy number

### 5.1 Overview

LPS is a key constituent of the outer membrane of Gram negative bacteria which provokes a brisk inflammatory response when bound by TLR-4 on the surface of innate immune cells, including monocytes (Raetz and Whitfield, 2002). The inhalation of LPS by healthy volunteers produces self-limiting alveolar and systemic inflammation (Thorn, 2001). The technique, while widely used to provide an *in vivo* model of acute neutrophilic lung inflammation, also produces a transient systemic inflammatory response that is characterised by elevations in pro-inflammatory cytokines, acute phase proteins and leucocyte count in the peripheral blood (Kitz *et al.*, 2008; Fouassier *et al.*, 2009; Korsgren *et al.*, 2012). In view of these systemic effects, LPS inhalation was chosen as an acceptable experimental model in which to attempt to transiently induce mild features of endotoxin tolerance in peripheral blood monocytes and confirm the findings from THP-1 cells treated with LPS that are detailed in Chapter 4. A dose of 60µg of LPS was selected for inhalation by the volunteers as this has been previously shown to reliably induce an inflammatory response in a safe and well-tolerated manner (Barr *et al.*, 2013).

#### 5.1.1 Hypothesis

Following inhalation of LPS peripheral blood monocytes will display evidence of endotoxin tolerance, as indicated by impaired immune responses to *ex vivo* inflammatory stimuli, along with an associated induction of mitochondrial biogenesis.

#### 5.1.2 Aims and Objectives

1. To safely administer inhaled LPS or saline placebo to randomly allocated, well-matched groups of healthy volunteers.
2. To confirm the induction of systemic inflammation following inhalation of LPS by measuring changes in clinical observations and leucocyte counts.
3. To isolate monocytes from peripheral blood samples and assess the effects of LPS inhalation on immune functions and mtDNA copy number.

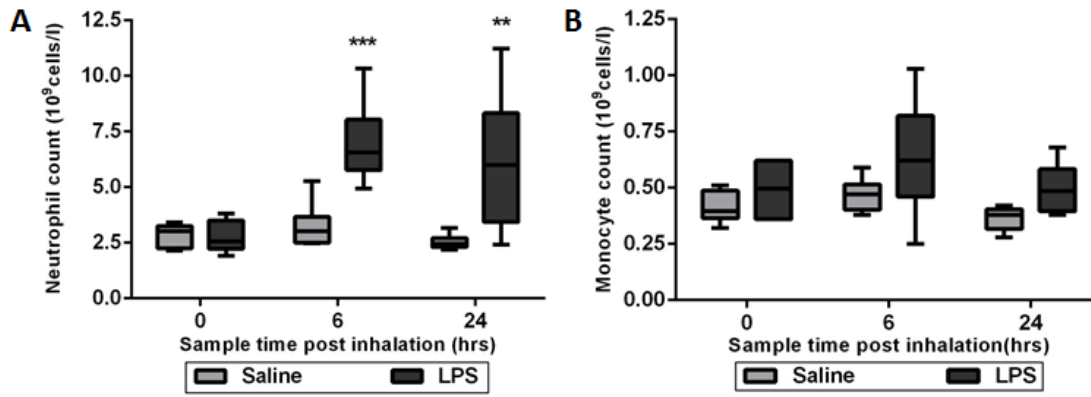
## 5.2 Results

### 5.2.1 Increased peripheral blood neutrophil count following inhalation of LPS

Twelve healthy volunteers were randomly allocated to inhalation of either 60µg LPS or a saline placebo (n=6 in each group). There were no significant differences in baseline demographic and clinical parameters between the two study groups (Table 5.1). At 6 and 24 hours post inhalation there was a significant increase in peripheral blood neutrophil count in the LPS group but not the saline group (Figure 5.1 A). However, peripheral blood monocyte count and changes in body temperature, heart rate and systolic blood pressure were not significantly altered by inhalation of either saline or LPS (Figure 5.1 B, Table 5.2). No serious adverse events were reported following inhalation in either group.

	LPS Group	Saline Group	p-value
Age (years)	23.5 (20.5-26.5)	20.5 (19-22)	0.12
Gender	3 Female, 3 Male	5 Female, 1 Male	0.221
Height (m)	1.75 (1.67-1.83)	1.73 (1.60-1.86)	0.78
Weight (kg)	74.8 (62-87.7)	65 (51.4-78.6)	0.206
Temperature (°C)	36.5 (36.1-36.9)	36.3 (35.6-36.9)	0.428
Heart rate (bpm)	72.7 (62.3-85.1)	76.5 (64.7-88.3)	0.544
Systolic blood pressure (mmHg)	115 (102-128)	111 (101-120)	0.485
Forced Expiratory Volume 1 second (l)	4.01 (3.34-4.67)	4.00 (2.86-5.14)	0.992
White cell count (10 <sup>9</sup> cells/l)	5.23 (4.47-6.01)	5.48 (4.39-6.57)	0.641
Neutrophil count (10 <sup>9</sup> cells/l)	2.77 (2.03-3.51)	2.85 (2.31-3.39)	0.838
Monocyte count (10 <sup>9</sup> cells/l)	0.50 (0.37-0.61)	0.41 (0.34-0.61)	0.188

**Table 5.1 Summary of baseline demographics and clinical parameters in the LPS inhalation study.** All normally distributed numerical data are presented as mean (95% confidence interval) and the differences between the groups analysed using independent t-tests. Age is not normally distributed and is presented as median (range) and the differences between the groups analysed using the Mann Whitney U test. The distribution of categorical data was assessed using Fisher's Exact Test.



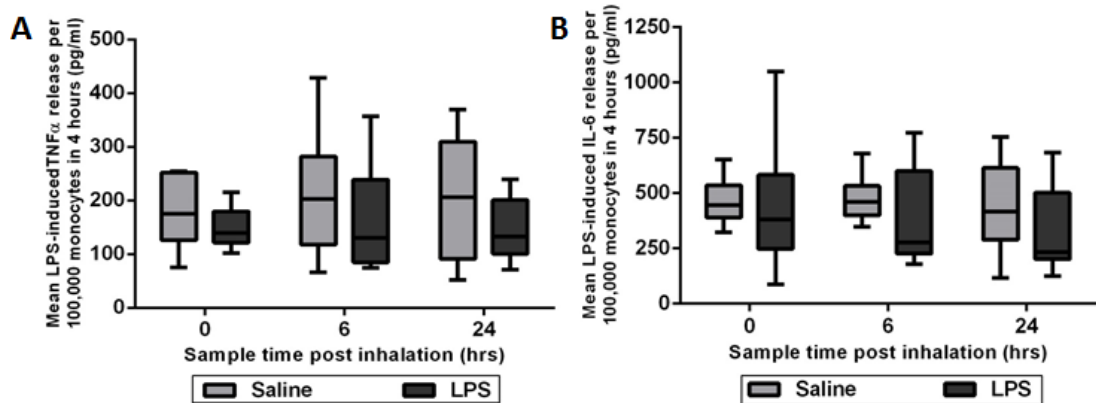
**Figure 5.1 Significantly increased peripheral blood neutrophil count following inhalation of LPS.** Peripheral blood neutrophil and monocyte counts were carried out at 0 (pre-inhalation), 6 and 24 hours following inhalation of LPS and saline. Data are represented by box plots indicating 25<sup>th</sup> quartile, median and 75<sup>th</sup> quartile and whiskers indicating the range and analysed by two-way ANOVA with Tukey’s post hoc analysis. All differences are non-significant except; \*\*p<0.01 and \*\*\*p<0.001 for the differences in neutrophil count from the 0 hour sample in the LPS group.

	LPS Group	Saline Group	p-value
Temperature (°C)	0.43 (0.12-0.74)	0.71 (0.04-1.39)	0.35
Heart rate (bpm)	6.7 (0.7-12.7)	7.7 (3.9-11.5)	0.725
Systolic blood pressure (mmHg)	-6.7 (-0.1- -13)	-3.7 (-0.2- -7.5)	0.589
White cell count (10 <sup>9</sup> cells/l)	5.53 (2.56-8.5)	0.46 (-0.18-1.10)	<b>0.002</b>
Neutrophil count (10 <sup>9</sup> cells/l)	4.32 (2.25-6.38)	0.04 (-0.33-0.47)	<b>0.002</b>
Monocyte count (10 <sup>9</sup> cells/l)	0.19 (0.03-0.34)	0.06 (-0.02-0.13)	0.077

**Table 5.2 Changes in clinical and peripheral blood leucocyte parameters following inhalation of LPS or saline.** Data indicate the maximum change from the baseline (t=0) measurement for all parameters. All normally distributed data are presented as mean (95% confidence interval) and the differences between the groups analysed using independent t-tests. The change in neutrophil count is not normally distributed and is presented as median (range) and the differences between the groups analysed using the Mann Whitney U test.

### 5.2.2 No significant effect of LPS inhalation on monocyte cytokine release

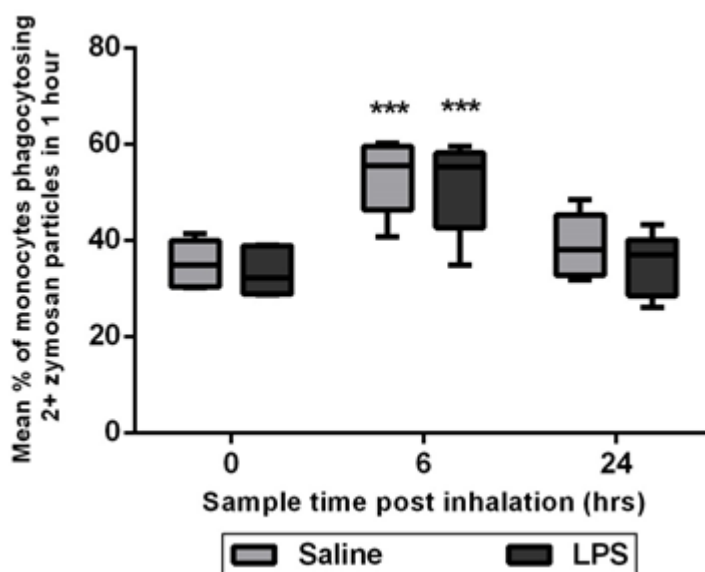
At each time point the ability of monocytes isolated from peripheral blood samples to produce an immune response to a second inflammatory stimulus was determined. Firstly the production of pro-inflammatory cytokines in response to a four hour incubation with 10ng/ml LPS was measured. The LPS-induced release of TNF $\alpha$  and IL-6 was not significantly different at either 6 or 24 hours after inhalation of LPS, indicating that this model failed to produce evidence of endotoxin tolerance in the peripheral blood monocytes of the healthy volunteers (Figure 5.2).



**Figure 5.2 Cytokine release by monocytes is not significantly altered by LPS inhalation** Monocytes were isolated from peripheral blood samples at 0 (pre-inhalation), 6 and 24 hours following inhalation of saline or LPS. The release of the pro-inflammatory cytokines TNF $\alpha$  (A) and IL-6 (B) in response to a 4 hour incubation with 10ng/ml LPS was determined by ELISA. Data are represented as box plots of the 25<sup>th</sup> quartile, median and 75<sup>th</sup> quartile with whiskers indicating the range and analysed by two-way ANOVA with Tukey's post hoc analysis. All differences are non-significant.

### 5.2.3 Diurnal variation in monocyte phagocytosis

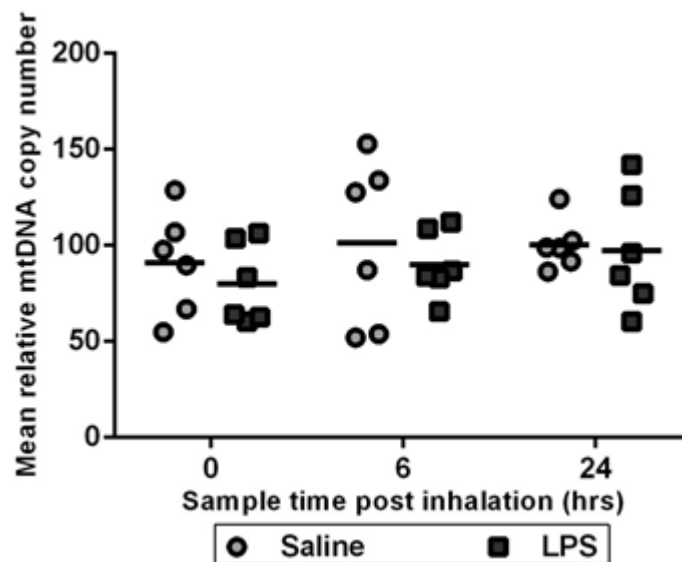
In addition to measuring cytokine release in response to LPS, the ability of blood monocytes to phagocytose serum-opsonised zymosan particles was determined at baseline and 6 and 24 hours post inhalation. There was no significant difference in the proportion of monocytes internalising zymosan between the groups randomised to inhalation of LPS or saline at each time point (Figure 5.3). However, in both groups phagocytosis was significantly greater in the 6 hour sample (taken at approximately 3pm) than in the 0 and 24 hours samples (taken at approximately 9am), suggesting a diurnal variation in the phagocytic ability of blood monocytes.



**Figure 5.3 Monocyte phagocytosis exhibits diurnal variation but is not significantly affected by LPS inhalation.** Monocytes were isolated from peripheral blood samples at 0 (pre-inhalation), 6 and 24 hours following inhalation of saline or LPS. The proportion of monocytes able to phagocytose serum opsonised zymosan in 1 hour was then determined. Data are represented as box plots of the 25<sup>th</sup> quartile, median and 75<sup>th</sup> quartile with whiskers indicating the range, and analysed using two-way ANOVA with Tukey's post hoc analysis. All differences are non-significant except; \*\*\* $p < 0.001$  for phagocytosis in both the saline and LPS inhalation groups at 6 hours compared to 0 and 24 hours.

#### 5.2.4 Monocyte mtDNA copy number is not significantly altered by inhalation of LPS

In view of the findings that sepsis has adverse effects on mitochondrial functions and that mitochondrial biogenesis is activated during the resolution of inflammation, mtDNA copy was also measured in monocytes isolated from peripheral blood samples at each time point. In keeping with the lack of effect on immune functions, there was no significant difference in monocyte mtDNA copy number over time or between the groups randomised to LPS or saline inhalation (Figure 5.4). This finding indicates that there is no evidence of the induction of mitochondrial biogenesis in blood monocytes following inhalation of 60µg LPS and that monocyte mtDNA copy number does not appear to exhibit diurnal variation.



**Figure 5.4 No significant difference in monocyte mtDNA copy number after inhalation of LPS.** Monocytes were isolated from peripheral blood samples at 0 (pre-inhalation), 6 and 24 hours following inhalation of saline or LPS and mtDNA copy number determined by measuring levels of *MT-ND1* relative to *B2M* using qPCR. Data are represented as individual points with lines indicating the mean mtDNA copy number. All differences are non-significant by two-way ANOVA with Tukey's post hoc analysis.

## 5.3 Discussion

### 5.3.1 LPS inhalation as a model of systemic inflammation

In agreement with previous findings, the inhalation of 60µg LPS by healthy participants was found to provide a well-tolerated model of acute inflammation in this randomly allocated, placebo-controlled study (Shyamsundar *et al.*, 2009; Barr *et al.*, 2013). There were no serious adverse events and no significant perturbation of clinical parameters was seen following inhalation of either saline or LPS. In addition there was a reliable induction of a degree of systemic inflammation, as indicated by peripheral blood leucocytosis and neutrophilia at 6 and 24 hours after inhalation of LPS. These results are consistent with other reports which have indicated that leucocyte counts, acute phase proteins and pro-inflammatory cytokines, are all significantly raised in peripheral blood samples after LPS inhalation (Fouassier *et al.*, 2009; Korsgren *et al.*, 2012; Janssen *et al.*, 2013).

It appears, however, that a milder degree of systemic inflammation may have been produced than that in certain previous LPS inhalation studies that used similar doses of LPS (Kitz *et al.*, 2008; Fouassier *et al.*, 2009). In contrast to these studies, there was a failure to produce any consistent changes in symptoms or clinical observations such as body temperature or heart rate following inhalation of LPS. This mild systemic effect may partly explain why LPS inhalation failed to achieve the primary objective of inducing endotoxin tolerance in peripheral blood monocytes. Endotoxin tolerance is classically defined as an attenuation in the release of TNF $\alpha$  in response to a second LPS stimulus, but the *ex vivo* LPS-induced release of both TNF $\alpha$  and IL-6 was not significantly altered in monocytes isolated from individuals after inhalation of LPS (Lopez-Collazo and del Fresno, 2013). In addition, there was no significant difference in peripheral blood monocyte count or monocyte phagocytosis between the groups inhaling LPS or saline at any time point. There was also no evidence of the induction of mitochondrial biogenesis in monocytes following LPS inhalation, but it is difficult to draw any firm conclusions from this finding given the lack of effects on monocyte immune parameters. It should be noted that the small numbers of volunteers included in the study and the limited time points analysed may mean that subtle differences in



immune responses or mtDNA copy number caused by LPS inhalation were not detected.

Given that LPS inhalation is primarily designed as a model of acute pulmonary inflammation, it is perhaps understandable that it did not induce endotoxin tolerance in blood monocytes (Michel *et al.*, 1997). Inhalation of LPS has been previously found to lead to the accumulation of monocyte-like cells within the lungs, and also to produce a significant up-regulation in the expression of a broad range of inflammatory genes in alveolar macrophages (Brittan *et al.*, 2012; Reynier *et al.*, 2012). In contrast to these findings in pulmonary monocytic cells, there are, to my knowledge, no published reports of LPS inhalation producing significant effects on the function of blood monocytes. It is likely that the dose of LPS that circulating monocytes are exposed to following LPS inhalation is insufficient to produce the profound changes in their phenotype that are seen in endotoxin tolerance (Cavaillon and Adib-Conquy, 2006).

Future studies may consider using an alternative translational research model that involves the intravenous administration of low-dose LPS to healthy volunteers. There are numerous publications reporting that the use of intravenous LPS is a safe technique which produces a significantly greater magnitude of systemic inflammation, characterised by a brisk febrile response and transient influenza-like symptoms, than LPS inhalation (Calvano *et al.*, 2005; Talwar *et al.*, 2006). In addition, it has been consistently shown that blood monocytes from healthy volunteers display the classical features of endotoxin tolerance following intravenous LPS administration, including a reduced ability to release TNF $\alpha$  in response to a second LPS exposure (van 't Veer *et al.*, 2007; Draisma *et al.*, 2009; Kox *et al.*, 2011). Measuring the effects of intravenous LPS on mitochondrial respiration and biogenesis may, therefore, provide important insights into the links between immunity and mitochondria in endotoxin tolerant primary human monocytes.

### *5.3.2 Diurnal variation in monocyte phagocytosis*

In the groups inhaling both saline and LPS the ability of blood monocytes to phagocytose serum-opsonised zymosan particles was significantly higher in the sample taken in the afternoon (6 hour sample) compared to those taken at 9am (0 and 24 hours samples). Previous studies have also shown that there are significant variations

in monocytes over the course of a day with changes in parameters including the absolute monocyte count in peripheral blood, the relative proportions of monocytes subsets and monocyte immune functions all being identified (Sennels *et al.*, 2011; Shantsila *et al.*, 2012). In particular, the phagocytic ability of monocytes and macrophages has been shown to be significantly altered depending on the time of day, with peak phagocytosis seen in the afternoon, or during the light period in animal models (Hayashi *et al.*, 2007; Shantsila *et al.*, 2012). More broadly, there is considerable evidence that many cellular and bodily functions including immunity vary throughout the diurnal cycle under the control of both central and cell-specific mammalian circadian clocks (Keller *et al.*, 2009; Scheiermann *et al.*, 2013). Therefore, it is clearly vital that the potential effects of the circadian rhythm and diurnal variation are considered and controlled for in investigations involving serial blood sampling at different times of the day, particularly when monocytes are being studied.

### *5.3.3 Conclusion*

In this small randomly allocated, placebo-controlled study the inhalation of LPS by healthy volunteers was well-tolerated and produced a mild systemic inflammatory response. However, there was no evidence of the induction of either endotoxin tolerance or mitochondrial biogenesis in blood monocytes after inhalation of LPS. Further investigations could consider using intravenous LPS as a more reliable method of producing endotoxin tolerant monocytes, but must take into account the potential for diurnal variation in monocyte characteristics.

## Chapter 6 Immune functions of p0 THP-1 cells generated by treatment with ethidium bromide

### 6.1 Overview

Mutations, deletions and depletions of mtDNA lead to defects in the assembly of OXPHOS subunits and impaired mitochondrial respiration (DiMauro and Schon, 2003). The resultant failure to meet cellular energy requirements causes clinical disease, particularly in cells with high energy requirements (Greaves *et al.*, 2012). In addition, evidence of mtDNA depletion and mitochondrial respiratory dysfunction in blood monocytes is associated with adverse clinical outcomes in sepsis (Pyle *et al.*, 2010; Japiassú *et al.*, 2011). However, the effects of these changes on monocyte cellular functions, particularly the ability to produce an immune response to an inflammatory stimulus, are not established (Levy and Deutschman, 2007).

In order to assess the effects of mtDNA depletion on immune functions, THP-1 cells were treated with ethidium bromide, a DNA intercalating agent which selectively inhibits mtDNA replication by POLG without significantly affecting the nuclear genome (Chandel and Schumacker, 1999). Long term treatment with low dose ethidium bromide is an established method of producing cells lacking mtDNA, termed p0 cells, in a variety of cell types including THP-1 cells (King and Attardi, 1989; Zuckerbraun *et al.*, 2007; Hashiguchi and Zhang-Akiyama, 2009). The depletion of mtDNA causes morphological and functional changes in mitochondria, including a loss of mitochondrial respiration and a compensatory increase in glycolytic metabolism (Holmuhamedov *et al.*, 2003). The p0 cells provide a valuable model in which to understand mitochondrial functions and the role of mitochondria and mtDNA-encoded genes in cellular processes and diseases (Chandel and Schumacker, 1999).

#### 6.1.1 Hypothesis

Treatment of THP-1 cells with ethidium bromide will produce p0 cells lacking mtDNA. These p0 THP-1 cells will have a reduced ability to produce an immune response to an inflammatory stimulus.

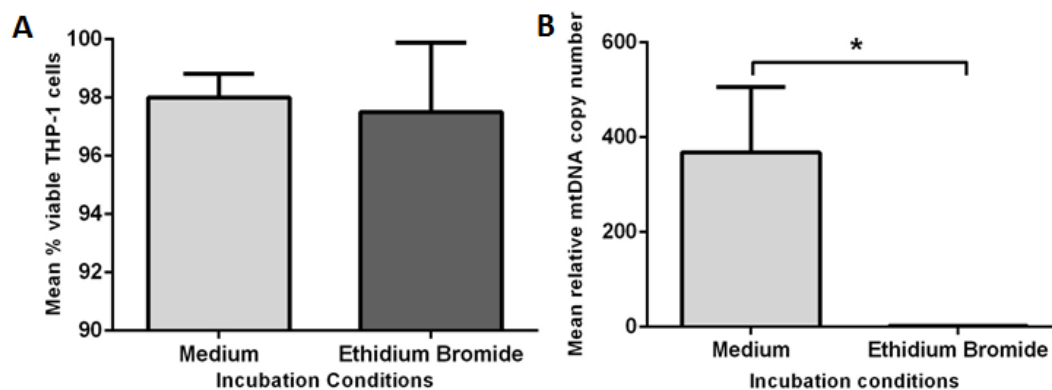
### 6.1.2 Aims and Objectives

1. To generate  $\rho 0$  THP-1 cells by incubation with low dose ethidium bromide.
2. To determine the effects of mtDNA depletion on mitochondrial respiratory functions.
3. To assess the immune functions of  $\rho 0$  THP-1 cells.

## 6.2 Results

### 6.2.1 Generation of $\rho 0$ THP-1 cells by incubation with ethidium bromide

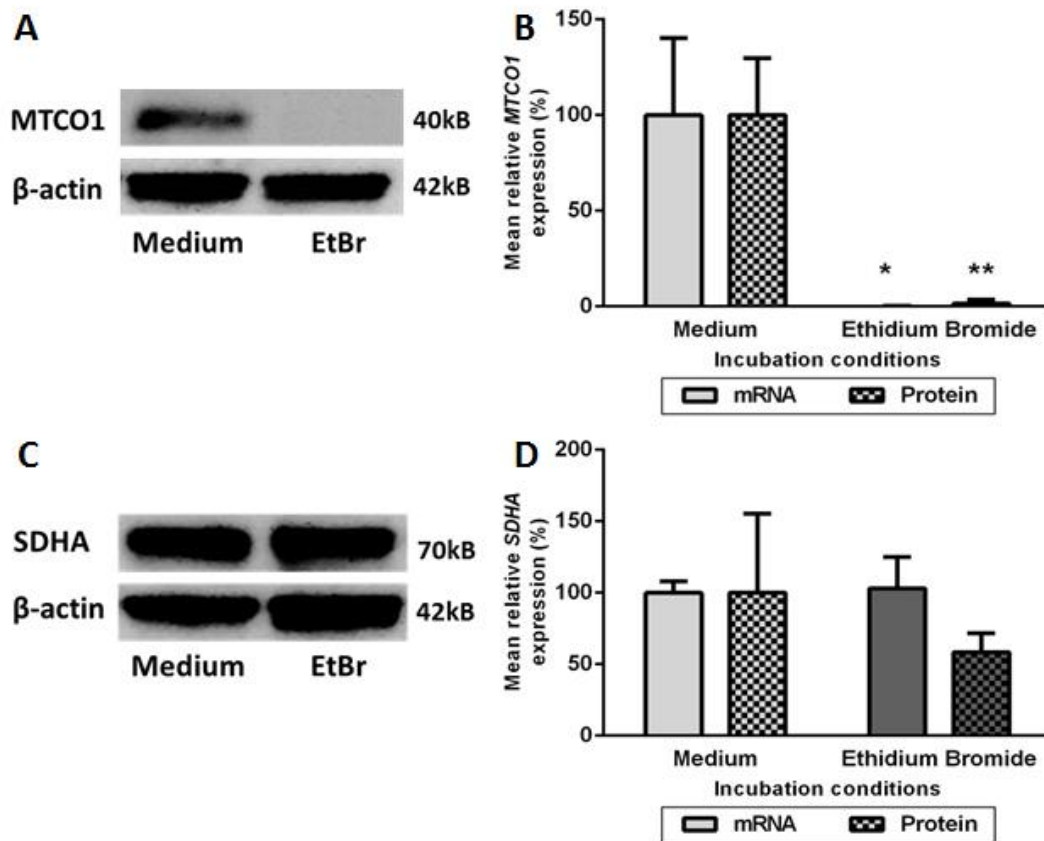
In order to completely deplete mtDNA, THP-1 cells were incubated with 50ng/ml ethidium bromide in growth medium supplemented with 50 $\mu$ g/ml uridine and 110 $\mu$ g/ml pyruvate for 8 weeks. This treatment with ethidium bromide did not produce any adverse effects on THP-1 cell viability but was successful in generating  $\rho 0$  THP-1 cells with almost complete depletion of relative mtDNA copy number (2.1 copies (95% CI 1.8-2.4) vs. 367.8 copies (95% CI 26-710),  $p=0.01$ ) (Figure 6.1).



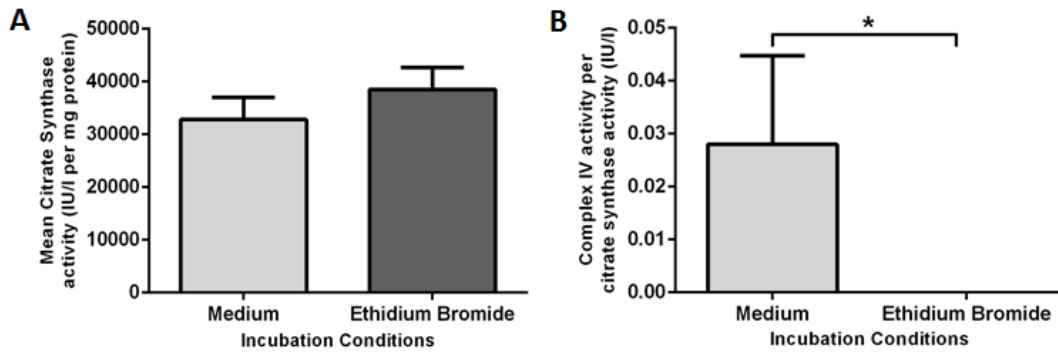
**Figure 6.1 Treatment with ethidium bromide for 8 weeks produces  $\rho 0$  THP-1 cells without altering cell viability.** THP- 1 cells were incubated for 8 weeks in medium containing 50ng/ml ethidium bromide and compared to control cells incubated in untreated medium. (A) Cell viability was determined by the counting the proportion of THP-1 cells excluding 0.4% trypan blue (n=4). (B) mtDNA copy number was determined by measuring levels of *MT-ND1* relative to *B2M* using quantitative polymerase chain reaction (n=3). All data are presented as mean  $\pm$  standard deviation (n=3). The significance of the differences between the groups was analysed using independent t-tests; (A)  $p=0.705$ , (B)  $p=0.010$ . The differences are non-significant except; \* $p<0.05$ .

### 6.2.2 A selective and functional loss of mtDNA in $\rho 0$ THP-1 cells

The effects of this mtDNA depletion on THP-1 cell mitochondria were subsequently confirmed by assessing mitochondrial gene expression and respiratory chain enzyme activity. The  $\rho 0$  THP-1 cells had almost complete loss of mRNA transcription and protein expression from the mtDNA-encoded *MT-CO1* gene but no significant alterations in mRNA or protein levels from the nuclear DNA-encoded mitochondrial *SDHA* gene (Figure 6.2). As expected, the activity of OXPHOS complex IV, which contains vital components that are encoded by mtDNA, was also completely lost in  $\rho 0$  THP-1 cells (Figure 6.3). However, the activity of citrate synthase, a nuclear DNA encoded mitochondrial matrix enzyme that is constitutively expressed and provides a quantitative marker of mitochondrial mass, was unchanged in  $\rho 0$  THP-1 cells (Kirby *et al.*, 2007). These results indicate that treatment with ethidium bromide preferentially affects mtDNA, with no significant changes seen in the expression of mitochondrial proteins encoded by nuclear genes, leaving  $\rho 0$  THP-1 cells with mitochondrial scaffolds that lack the mtDNA-encoded components (Holmuhamedov *et al.*, 2003).



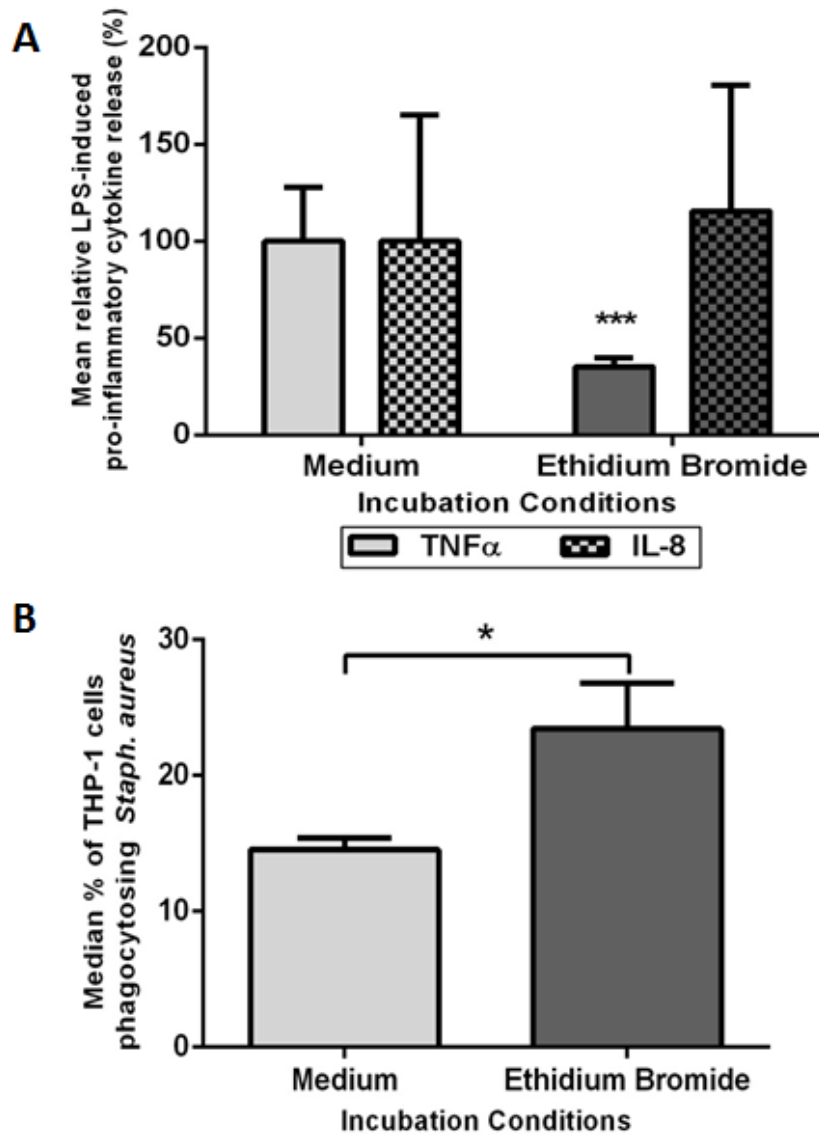
**Figure 6.2 Selective depletion of mtDNA-encoded mRNA and protein in p0 THP-1 cells.** THP-1 cells were incubated for 8 weeks with 50ng/ml ethidium bromide (EtBr) and compared to control cells incubated in medium. (A, C) Representative images of protein bands on a PVDF membrane following Western blot. (B) Relative mRNA transcription (compared to *GAPDH*) and protein expression (compared to  $\beta$ -actin) from the mtDNA-encoded *MT-CO1* gene (n=3). (C) Relative mRNA transcription (compared to *GAPDH*) and protein expression (compared to  $\beta$ -actin) from the nuclear DNA-encoded mitochondrial gene *SDHA* (n=3). All data are presented as mean  $\pm$  standard deviation relative gene expression compared to the mean in the control (medium) condition. The significance of the differences between the groups was analysed using independent t-tests (B - *MT-CO1* mRNA p=0.012, protein p=0.008; D - *SDHA* mRNA p=0.823, protein p=0.630). Significant differences are displayed as; \*p<0.05, \*\*p<0.01.



**Figure 6.3 Loss of OXPHOS complex IV activity in p0 THP-1 cells.** THP-1 cells were incubated for 8 weeks with 50ng/ml ethidium bromide and compared to control cells incubated in medium. The activity of (A) citrate synthase and (B) OXPHOS complex IV was determined by spectrophotometric measurements of isolated enzyme activity in cellular homogenates. All experiments were carried out in 3 independent replicates and are presented as mean  $\pm$  standard deviation. The significance of the differences between the groups was analysed using independent t-tests (A -  $p=0.171$ , B -  $p=0.045$ ). All differences are not significant except; \* $p<0.05$ .

### 6.2.3 Alterations in immune function in p0 THP-1 cells

Having found that incubation with 50ng/ml ethidium bromide for 8 weeks produces p0 THP-1 cells with a specific loss of mtDNA-encoded mitochondrial gene expression and OXPHOS complex activity, the ability of these cells to produce an immune response was then assessed. The mean release of the pro-inflammatory cytokine TNF $\alpha$  in response to a 4 hour incubation with 100ng/ml LPS was significantly reduced in p0 THP-1 cells compared to controls (147.9pg/ml (95% CI 128-168) vs. 419.2pg/ml (95% CI 297-541),  $p<0.001$ ) (Figure 6.4 A). However, mtDNA depletion did not lead to a generalised impairment in immune function as mean LPS-induced IL-8 release was not significantly different in p0 cells compared to those incubated in medium (113.9pg/ml (95% CI 47-181) vs. 98.6pg/ml (95% CI 31-166),  $p=0.688$ ). Furthermore, the median proportion of p0 THP-1 cells phagocytosing fluorescent *Staphylococcus aureus* in 1 hour was actually significantly higher than in control cells (23.7% (IQR 21.5-25.3) vs. 14.7% (IQR 14-15),  $p=0.03$ ) (Figure 6.4 B).



**Figure 6.4 Significantly reduced LPS-induced TNF $\alpha$  but increased phagocytosis of *Staphylococcus aureus* by p0 THP-1 cells.** THP- 1 cells were incubated for 8 weeks with 50ng/ml ethidium bromide and compared to control cells incubated in medium. (A) The LPS-induced release of TNF $\alpha$  and IL-8 following 4 hour incubation with 100ng/ml LPS was determined by ELISA (n=3). (B) The phagocytosis of serum-opsonised fluorescein-labelled *Staphylococcus aureus* in 1 hour was determined by flow cytometry (n=3). The cytokine release data (A) are presented as mean  $\pm$  standard deviation relative to the mean in the medium control while the phagocytosis data (B) are presented as median  $\pm$  interquartile range. The significance of the differences between the groups was analysed using independent t-tests (A - TNF $\alpha$  p<0.001, IL-8 p=0.688) or the Mann Whitney test (B - p=0.029). Significant differences are displayed as; \*p<0.05, \*\*\*p<0.001.



### 6.3 Discussion

Evidence of monocyte immune deactivation has been consistently associated with adverse outcomes in patients with sepsis (Monneret *et al.*, 2006; Landelle *et al.*, 2010). Separately, it has been found that septic monocytes also have mitochondrial respiratory impairment and depletion of mtDNA (Pyle *et al.*, 2010; Garrabou *et al.*, 2012). However, a causal link between these findings has not been established and it has been unclear whether immune deactivation and mitochondrial impairment in septic monocytes are rather epiphenomena that reflect a general cellular dysfunction in the face of severe and sustained inflammatory stimuli (Levy and Deutschman, 2007).

To my knowledge this investigation represents the first attempt to investigate the potential effects of mtDNA depletion on immune functions in a human monocytic cell culture model.  $\rho 0$  THP-1 cells with a loss of functional mitochondrial respiration were successfully generated by treatment with 50ng/ml ethidium bromide for 8 weeks. These  $\rho 0$  cells displayed evidence of immune deactivation with a significantly reduced ability to release TNF $\alpha$  in response to LPS, a key functional defect that has been associated with adverse clinical outcomes when detected in deactivated septic monocytes (Ploder *et al.*, 2006; Hall *et al.*, 2011).

While these results provide some initial insights into the interplay between immunity and mitochondria, a causal relationship between mtDNA depletion and alterations in the immune phenotype of THP-1 cells is not established. Given the growing appreciation of the importance of mitochondria in the regulation of cell signalling pathways and effector innate immune responses, any negative effects of mtDNA depletion on these processes could directly lead to a reduced ability of THP-1 cells to produce inflammatory responses (West *et al.*, 2011b; Tait and Green, 2012; Weinberg *et al.*, 2015). However, there are a number of other competing potential conclusions that can be drawn from these preliminary investigations.

Firstly, although ethidium bromide has been shown to produce a selective depletion of mtDNA, it is clear that this treatment is not completely targeted to the mitochondria. Ethidium bromide is a cytotoxic DNA intercalating agent which has a preferential effect on the rapidly replicating mtDNA (King and Attardi, 1989). However,  $\rho 0$  cells generated by treatment with ethidium bromide may also have significant alterations in nuclear

gene transcription, including in key inflammatory signalling pathways (Magda *et al.*, 2008). Such effects appear to be variable depending on the cell type and culture conditions and it is as yet unclear whether they are caused by ethidium bromide or occur as a consequence of the loss of functional mitochondria (Miceli and Jazwinski, 2005). Thus, it is possible that the impaired ability of p0 cells to produce TNF $\alpha$  may occur due to 'off-target' effects of ethidium bromide treatment on pathways involved in LPS-induced inflammatory signalling, rather than due to the consequences of mtDNA depletion.

A further possible explanation for the alterations in immune function in p0 THP-1 cells is that they do not relate directly to changes in mtDNA copy number but may rather occur due to the functional consequences of this depletion. Cells depleted of mtDNA lack a functional OXPHOS system and are dependent on glycolytic metabolism for ATP production (Qian and Van Houten, 2010). The metabolic changes within the cell resulting from the switch to glycolysis appear to have complex effects on the ability of the cell to mount an effective immune response. For example, cells that are dependent on glycolysis produce large amounts of lactic acid during pyruvate metabolism (Garcia-Alvarez *et al.*, 2014). Lactic acid has been shown to inhibit LPS-induced TNF $\alpha$  release by human monocytes, an effect that can be reversed by inhibiting lactic acid production, and patients with severe sepsis frequently have evidence of a systemic lactic acidosis that predicts severity and outcome (Dellinger *et al.*, 2008; Dietl *et al.*, 2010). On the other hand, studies indicate that macrophages up-regulate glycolysis in association with pro-inflammatory responses and that metabolites produced in glycolytic respiration, such as succinate, may also augment pro-inflammatory cytokine production (O'Neill and Grahame Hardie, 2013; Tannahill *et al.*, 2013).

Finally, the altered immune responses of p0 THP-1 cells may occur due to differentiation of the cells during the treatment with ethidium bromide. The findings of reduced inflammatory cytokine production but enhanced phagocytosis in p0 THP-1 cells are similar to the change in immune phenotype seen during differentiation to alternatively-activated macrophages, cells with an anti-inflammatory phenotype and important roles in tissue repair and the resolution of inflammation (Murray and Wynn, 2011; Sica and Mantovani, 2012). While macrophage differentiation in p0 THP-1 cells was not assessed in this chapter, it has been shown that THP-1 cells can be induced to

undergo alternative macrophage differentiation *in vitro* through the addition of specific drugs and cytokines (Schwende *et al.*, 1996; Gordon and Martinez, 2010). However, although alterations in metabolism do occur during macrophage differentiation, in contrast to the loss of mitochondrial respiration in  $\rho 0$  cells, the anti-inflammatory properties of alternatively activated macrophages have been previously found to be linked with an up-regulation of OXPHOS respiration (Rodríguez-Prados *et al.*, 2010).

In summary, it appears that mtDNA depletion induced by treatment with ethidium bromide may be associated with an alteration in the immune phenotype of THP-1 cells. However, this needs to be confirmed in further investigations that use a more targeted and specific method of depleting mtDNA and focus on identifying the potential mechanisms linking mtDNA depletion with monocytic cell immune dysfunction.

## Chapter 7 The effects of transfection with siRNA targeted against *POLG* and *TFAM* on mitochondria and immunity in THP-1 cells

### 7.1 Overview

The link between the separate findings of immune deactivation and mitochondrial depletion in septic monocytes has not been well understood. The results in Chapter 6 suggest that depleting mtDNA from THP-1 cells, by incubation with ethidium bromide to generate p0 cells, results in an impaired ability to release TNF $\alpha$  in response to LPS. However, ethidium bromide, which acts as a non-specific DNA intercalating agent, is a cytotoxic compound that may alter nuclear gene expression (Miceli and Jazwinski, 2005; Magda *et al.*, 2008; Hashiguchi and Zhang-Akiyama, 2009). In addition, the mtDNA depletion produced in p0 cells is significantly more profound than that seen in sepsis or in patients with mtDNA depletion disorders, making extrapolations to human diseases more difficult (Cohen, 2013).

In view of these limitations, a more specific approach aimed at producing a partial depletion of mtDNA was used to validate the findings from p0 THP-1 cells. This method involved using siRNA to selectively silence the transcription of two nuclear genes encoding key proteins that are essential for the maintenance and replication of mtDNA within the mitochondria (Carthew and Sontheimer, 2009). Firstly, siRNA was targeted against *POLG*, which encodes the catalytic subunit of polymerase- $\gamma$  (*POLG*), the only DNA polymerase that is known replicate mtDNA (Hudson and Chinnery, 2006). In addition, THP-1 cells were also transfected with siRNA directed against *TFAM*, which encodes mitochondrial transcription factor A (*TFAM*), a protein that directly interacts with mtDNA to facilitate replication and gene transcription (Kang *et al.*, 2007).

#### 7.1.1 Hypothesis

The transfection of THP-1 cells with siRNA targeted against *POLG* and *TFAM* will lead to mtDNA depletion, mitochondrial respiratory dysfunction and impaired immune functions. Following removal of the siRNA there will be recovery of all of these parameters in association with the restoration of *POLG* and *TFAM* expression.

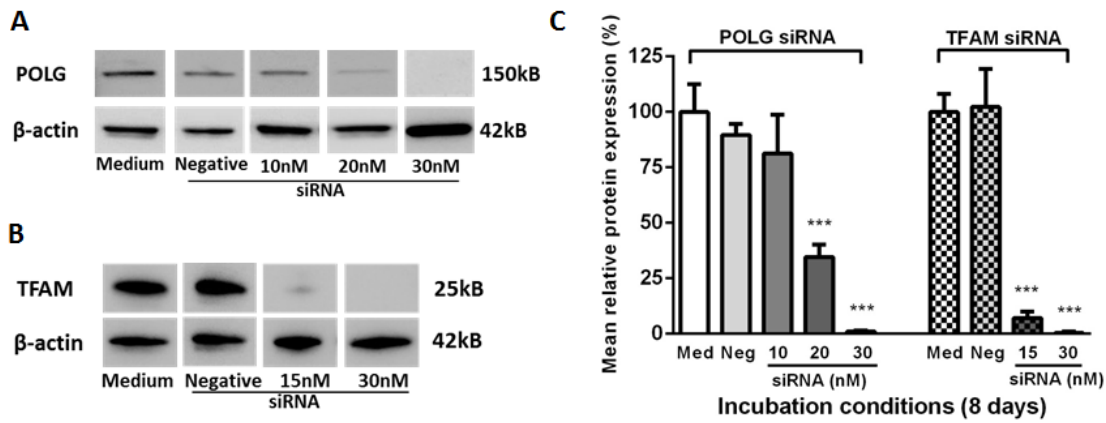
### 7.1.2 Aims and Objectives

1. To inhibit the expression of proteins essential for mtDNA replication by transfecting THP-1 cells with siRNA targeted against *POLG* and *TFAM*.
2. To confirm whether silencing of *POLG* and *TFAM* leads to mtDNA depletion in THP-1 cells.
3. To assess the effects of transfection with *TFAM* and *POLG* siRNA on THP-1 cell respiratory and immune functions.
4. To determine the ability of changes in mtDNA copy number, cellular respiration and immunity to recover after removal of the siRNA.

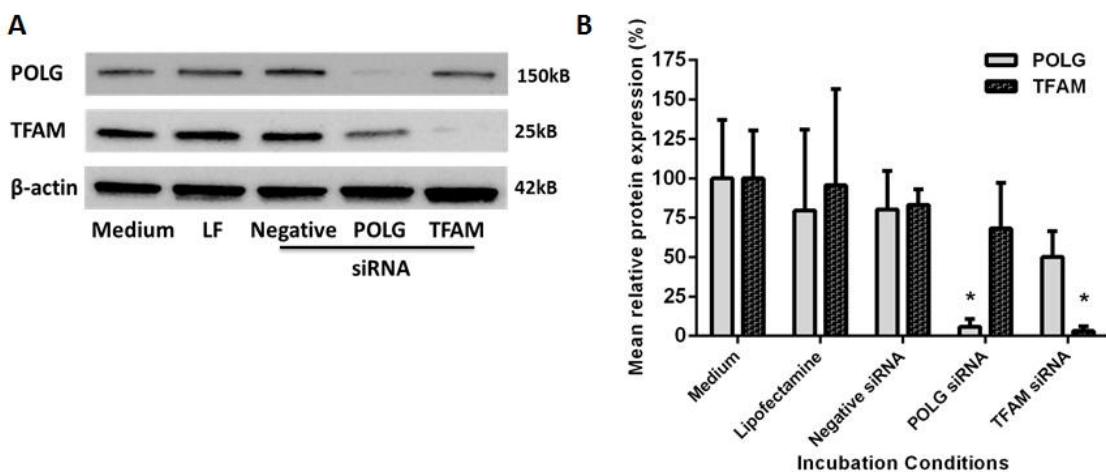
## 7.2 Results of the mtDNA depletion experiments

### 7.2.1 Abolition of *POLG* and *TFAM* expression without significant effects on cell viability or proliferation following siRNA transfection

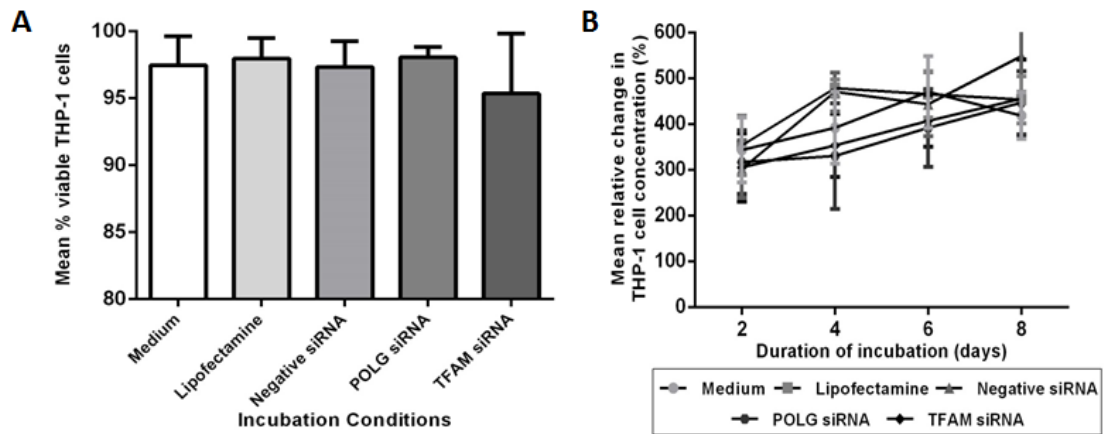
The results of preliminary dose-finding experiments indicated that repeated transfection with 30nM of siRNA targeted against *POLG* and *TFAM* every 48 hours for 8 days produced an optimal knock-down in the levels of the target proteins and all subsequent experiments were carried out using these conditions (Figure 7.1). In each case the effects of this transfection were assessed by comparing these cells with THP-1 cells incubated in either medium alone, treated with the transfection reagent Lipofectamine RNAiMAX or transfected with 30nM Silencer® Select Negative Control siRNA number 1 every 48 hours for 8 days. The expression of both *POLG* and *TFAM* proteins was almost completely abolished after 8 days transfection with 30nM of siRNA, with no effects on protein expression seen in the control conditions (Figure 7.2). This transfection with 30nM *POLG* and *TFAM* siRNA did not have any significant cytotoxic effects, with a mean of greater than 95% of THP-1 cells remaining viable in all conditions (Figure 7.3 A). In addition the ability of THP-1 cells to proliferate was not significantly altered over the course of the transfection period or between conditions (Figure 7.3 B).



**Figure 7.1 Transfection with 30nM *POLG* and *TFAM* siRNA produces optimal silencing of target protein expression.** THP-1 cells were transfected with 10nM, 20nM or 30nM *POLG* siRNA and 15nM or 30nM *TFAM* siRNA every 48 hours for 8 days. As controls THP-1 cells were incubated with 30nM of negative control siRNA or medium alone. (A, B) Representative images of protein bands on a PVDF membrane following Western blot (C) The expression of *POLG* and *TFAM* proteins relative to  $\beta$ -actin was determined by Western blot (n=3). Normal data are represented as mean  $\pm$  standard deviation relative to the mean in the medium control. The data were analysed using one-way ANOVA (*POLG* –  $p < 0.001$ , *TFAM* –  $p < 0.001$ ). Differences from the medium control are non-significant with Dunnett’s multiple comparison test except; \*\*\* $p < 0.001$ .



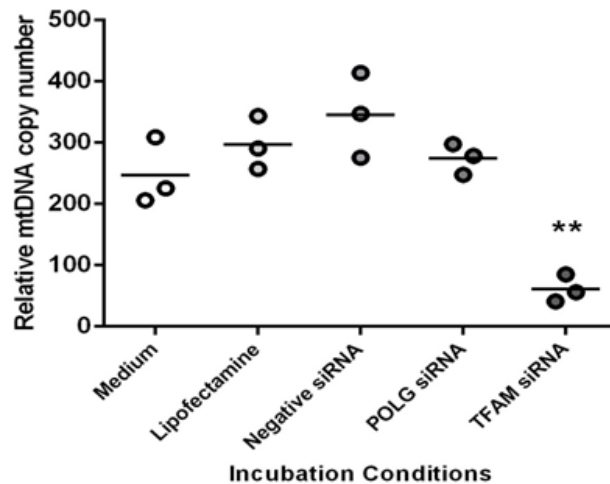
**Figure 7.2 Transfection with 30nM *POLG* and *TFAM* siRNA for 8 days leads to loss of target protein expression.** For 8 days THP-1 cells were incubated with the 30nM *TFAM* or *POLG* siRNA and compared to controls incubated with medium or the transfection reagent Lipofectamine RNAiMAX (LF) or transfected with 30nM of negative control siRNA. (A) Representative images of protein bands on a PVDF membrane following Western blot. (B) The expression of *POLG* and *TFAM* proteins relative to  $\beta$ -actin was determined by Western blot (n=3). Normal data are represented as mean  $\pm$  standard deviation relative to the mean in the medium control. The data were analysed using one-way ANOVA (*POLG* –  $p = 0.106$ , *TFAM* –  $p = 0.031$ ). Differences from the medium control are non-significant with Dunnett’s multiple comparison test except; \* $p < 0.05$ .



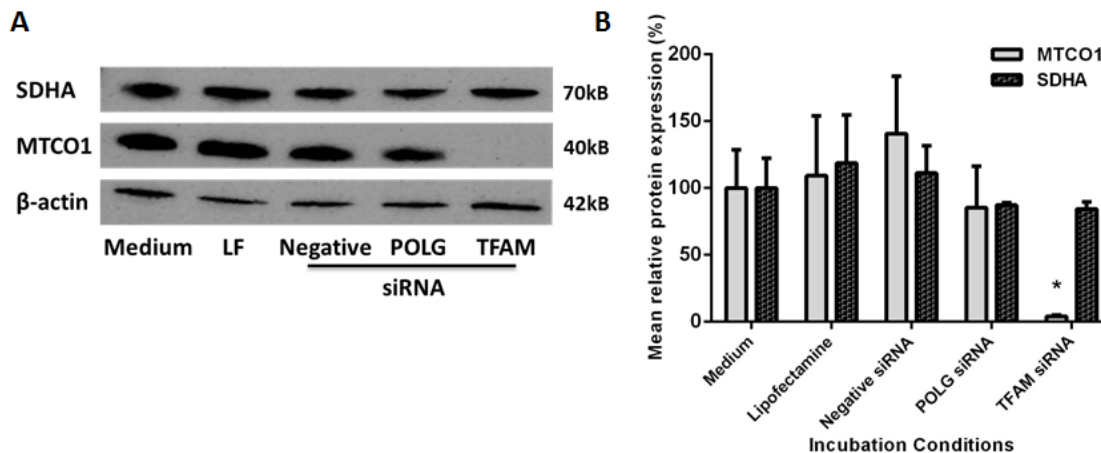
**Figure 7.3 Transfection with 30nM *POLG* and *TFAM* siRNA does not have adverse effects on THP-1 cell viability and proliferation.** For 8 days THP-1 cells were incubated with the 30nM *TFAM* or *POLG* siRNA and compared to controls THP-1 cells incubated with medium alone or the transfection reagent Lipofectamine RNAiMAX or transfected with 30nM of negative control siRNA. (A) Cell viability was determined by measuring the proportion of THP-1 cells excluding propidium iodide using flow cytometry (n=3) (B) Serial cell counts were used to determine the relative change in THP-1 cell concentration every 48 hours prior to each transfection (n=4). Normal data are represented as mean  $\pm$  standard deviation. The data were analysed using one-way ANOVA (p=0.678) with Dunnett's multiple comparison test (A) or two-way ANOVA with Tukey's multiple comparison test (B). Differences from the medium control are non-significant.

### 7.2.2 Depletion of mtDNA in THP-1 cells transfected with *TFAM* siRNA

Having established that siRNA transfection effectively silenced the target genes, the effect of abolishing *POLG* and *TFAM* protein expression on mtDNA copy number was determined. There was a significant depletion of relative mtDNA copy number in THP-1 cells after inhibition of *TFAM* expression (61 copies (95% CI 5-111) vs 247 copies (95% CI 117-328) in medium control cells, p=0.002) (Figure 7.4). This mtDNA depletion was associated with a selective loss of expression of the mtDNA-encoded protein MT-CO1, without any significant effect on the nuclear DNA-encoded mitochondrial protein SDHA (Figure 7.5). However, despite abolishing *POLG* expression, there was no significant change in either mean relative mtDNA copy number (275 copies (95% CI 212-338) vs 247 copies (95% CI 111-383) in medium control cells, p=0.875) or the expression of mitochondrial proteins in THP-1 cells transfected with *POLG* siRNA for 8 days (Figure 7.4 and Figure 7.5).



**Figure 7.4 Depletion of mtDNA after transfection with *TFAM* but not *POLG* siRNA.** For 8 days THP-1 cells were incubated with 30nM *TFAM* or *POLG* siRNA, or with the transfection reagent Lipofectamine RNAiMAX, 30nM of negative control siRNA or medium alone. mtDNA copy number was determined by measuring the level of *MT-ND1* relative to *B2M* using qPCR. Normal data are represented as mean with individual measurements (n=3). The data were analysed using one-way ANOVA ( $p < 0.001$ ). Differences from the medium control are non-significant with Dunnett's multiple comparison test except; \*\* $p < 0.01$ .

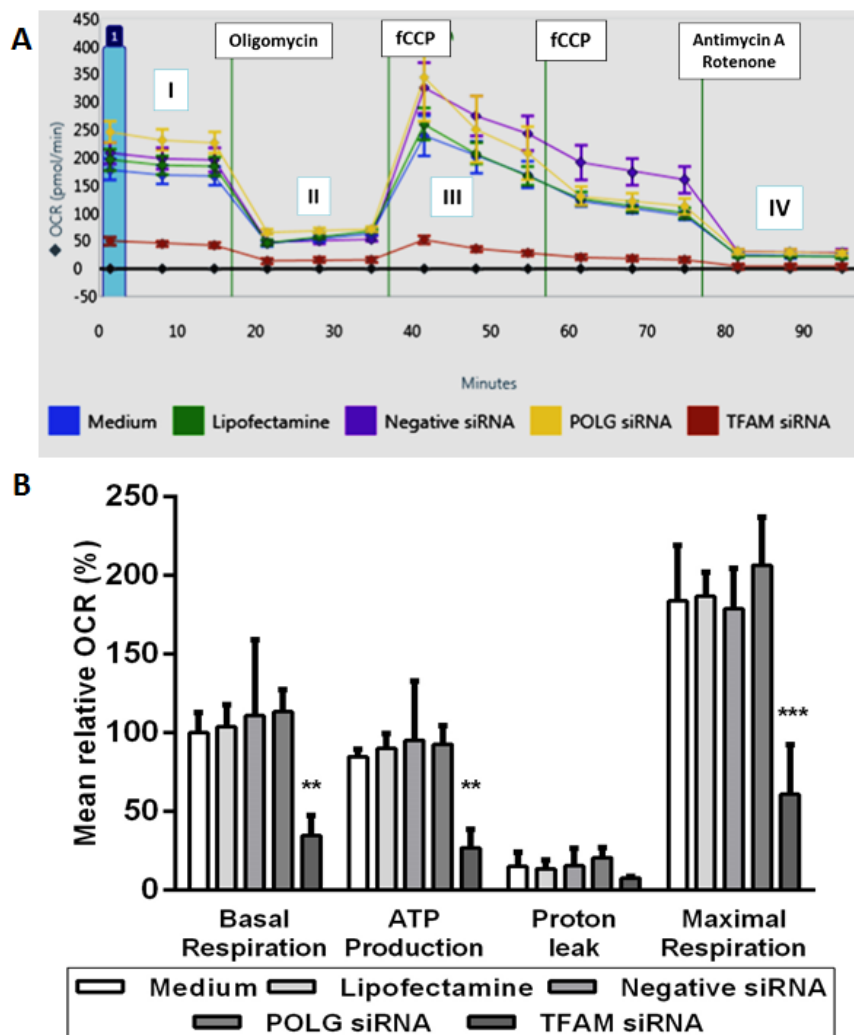


**Figure 7.5 Depletion of mtDNA-encoded proteins after transfection with *TFAM* but not *POLG* siRNA.** For 8 days THP-1 cells were incubated with 30nM *TFAM* or *POLG* siRNA, or with the transfection reagent Lipofectamine RNAiMAX (LF), 30nM of negative control siRNA or medium alone. (A) Representative images of protein bands on a PVDF membrane following Western blot. (B) The relative expression of the mitochondrial proteins MT-CO1 (encoded by mtDNA) and SDHA (encoded by nuclear DNA) relative to  $\beta$ -actin was measured by Western blot (n=3). Normal data are represented as mean  $\pm$  standard deviation relative to the mean of the medium control. The data were analysed using one-way ANOVA (MT-CO1 –  $p = 0.031$ , SDHA –  $p = 0.278$ ). Differences from the medium control are non-significant with Dunnett's multiple comparison test except; \* $p < 0.05$ .



### 7.2.3 Loss of mitochondrial oxygen consumption in THP-1 cells transfected with *TFAM* siRNA

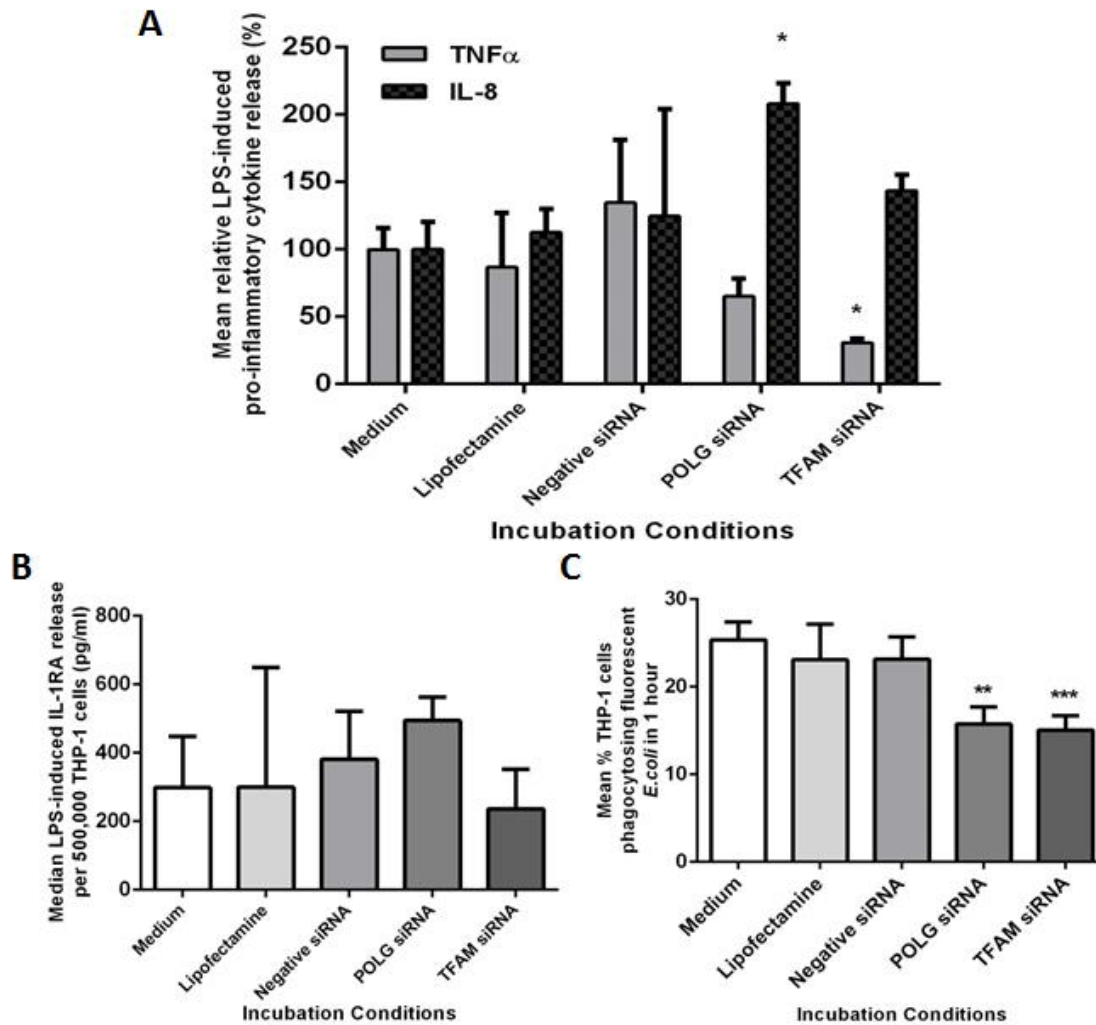
The depletion of mtDNA in THP-1 cells that was caused by transfection with *TFAM* siRNA produced a functional inhibition of mitochondrial respiration, as indicated by significantly reduced oxygen consumption by these cells (Figure 7.6). In particular, oxygen consumption for basal mitochondrial respiration (1663 pMole/min/mg protein (95% CI 706-2620) vs 4780 pMole/min/mg protein (95% CI 2794-5767) in the medium control,  $p=0.009$ ), mitochondrial ATP production (1291 pMole/min/mg protein (95% CI 410-2172) vs. 4060 pMole/min/mg protein (95% CI 3697-4423,  $p=0.003$ ) and maximal uncoupled mitochondrial respiration (2915 pMole/min/mg protein (95% CI 525-5304) vs. 8789 pMole/min/mg protein (95% CI 6100-11477),  $p<0.001$ ) was significantly lower in THP-1 cells after transfection with *TFAM* siRNA. In contrast, mitochondrial oxygen consumption was not significantly altered in THP-1 cells transfected with *POLG* siRNA, a finding that is consistent with the lack of effect of decreasing *POLG* protein expression on mtDNA copy number or mtDNA-encoded protein expression (Figure 7.6).



**Figure 7.6 Reduced oxygen consumption by THP-1 cells transfected with *TFAM* but not *POLG* siRNA.** THP-1 cells were incubated with 30nM *TFAM* or *POLG* siRNA and with the transfection reagent Lipofectamine RNAiMAX, 30nM of negative control siRNA and medium alone as controls. After 8 days different aspects of respiration were then determined by measuring OCR following the sequential addition of metabolic inhibitors using the Seahorse XF96<sup>e</sup> extracellular flux analyser. (A) An example of the respiratory profile of THP-1 cells following transfection with siRNA for 8 days. (B) The OCR for the following aspects of cellular respiration was determined; Basal mitochondrial respiration = basal OCR (I) – non-mitochondrial OCR (IV); Adenosine triphosphate (ATP) production = basal OCR (I) – post-oligomycin OCR (II); Proton leak = post-oligomycin OCR (II) – non-mitochondrial respiration (IV); Maximal mitochondrial respiration = maximal OCR (III) – non-mitochondrial OCR (IV). Data are presented as mean  $\pm$  standard deviation relative to the mean basal mitochondrial respiration in the medium control (n=4 for all experiments). The data for each aspect of mitochondrial respiration were analysed separately using one-way ANOVA (Basal respiration – p=0.005, ATP production – p=0.001, Proton leak – p=0.320, Maximal respiration – p<0.001). Differences from medium control are non-significant with Dunnett’s multiple comparison test except; \*\*p<0.01, \*\*\*p<0.001

#### 7.2.4 Impaired LPS-induced TNF $\alpha$ release and phagocytosis in THP-1 cells transfected with TFAM siRNA

After establishing the effects of transfection with 30nM *POLG* and *TFAM* siRNA on mitochondria, the ability of these THP-1 cells to produce an immune response to an inflammatory stimulus was assessed. THP-1 cells transfected with siRNA targeted against *TFAM*, but not *POLG*, for the previous 8 days had a significantly reduced ability to release the pro-inflammatory cytokine TNF $\alpha$  in response to a 4 hour treatment with 100ng/ml LPS (mean 23.5pg/ml (95% CI 20-27) vs 76.5pg/ml (95% CI 57-96) in the medium control,  $p=0.015$ ) (Figure 7.7 A). In contrast, the LPS-induced release of IL-8, another pro-inflammatory cytokine, was significantly higher in THP-1 cells transfected with *POLG* siRNA (median 211pg/ml (IQR 210-244) vs. 97.2pg/ml (IQR 97.1-114.3) in the medium control,  $p=0.025$ ), but unchanged in those transfected with *TFAM* siRNA (Figure 7.7 A). In addition, there were no significant changes in the release of the anti-inflammatory cytokine IL-1RA in response to a 16 hour incubation with 100ng/ml LPS in any of the experimental conditions (Figure 7.7 B). Finally, a significantly lower proportion of cells transfected with either *POLG* or *TFAM* siRNA were able to phagocytose *Escherichia coli* particles over the course of 1 hour (mean 15.7% (95% CI 10.9-20.6,  $p=0.011$ ) with *POLG* siRNA and 15.1% (95% CI 12.5-17.6,  $p<0.001$ ) with *TFAM* siRNA vs 25.4% (95% CI 22-28.6) in the medium control) (Figure 7.7 C).



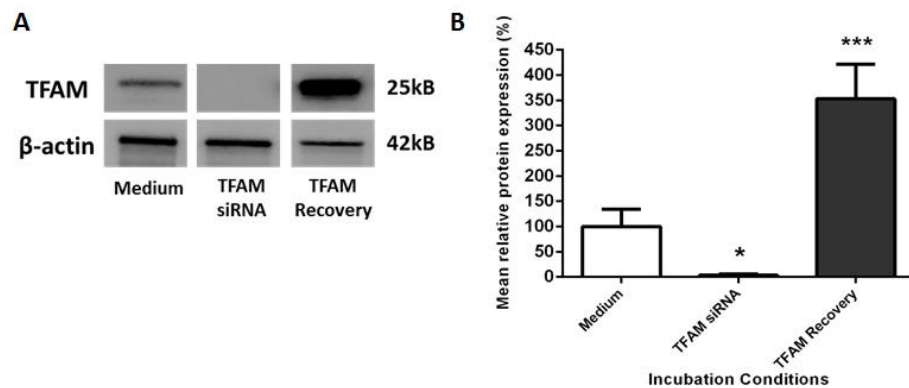
**Figure 7.7 The effect of transfection with *POLG* and *TFAM* siRNA on THP-1 cell immune functions.** The ability of THP-1 cells to produce an immune response was measured after incubation with 30nM *TFAM* or *POLG* siRNA, or with the transfection reagent Lipofectamine RNAiMAX, 30nM of negative control siRNA or medium alone, for 8 days (A) The release of the pro-inflammatory cytokines TNF $\alpha$  and IL-8 in response to a 4 hour incubation with 100ng/ml LPS was measured by ELISA (n=4). (B) The release of the anti-inflammatory cytokine IL-1RA in response to a 16 hour incubation with 100ng/ml LPS was measured by ELISA (n=4). (C) The proportion of cells phagocytosing fluorescein-labelled *Escherichia coli* in 1 hour was determined by flow cytometry (n=3). The cytokine release data (A, B) are relative to the mean of the medium control. Normal data are represented as mean  $\pm$  standard deviation and analysed using one way ANOVA with Dunnett's multiple comparison test (TNF $\alpha$  – p=0.002, IL-8 – p=0.013, Phagocytosis p<0.001). Non-normal data are presented as median  $\pm$  interquartile range and analysed using the Kruskal-Wallis test with Dunn's multiple comparison test (IL-1RA – p=0.289). Differences from the medium control are non-significant except; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

### 7.3 Results of the mtDNA recovery experiments

In order to determine the reversibility of the effects of silencing the target protein expression, the siRNA was removed following the initial 8 day transfection and the THP-1 cells were then incubated in growth medium for a further 8 days. After this recovery period the ability of THP-1 cells to restore their mtDNA copy number, cellular oxygen consumption and immune functions was measured. Given the lack of effect of *POLG* siRNA on the mitochondrial assessments, the mtDNA recovery experiments were only carried out on cells initially transfected with 30nM *TFAM* siRNA. The control for these experiments involved THP-1 cells incubated in growth medium for 16 days, as the mtDNA depletion experiments confirmed that Lipofectamine RNAiMAX and transfection with Silencer® Select Negative Control siRNA number 1 did not have any detectable effect on the readouts from the assays for THP-1 cell mitochondrial and immune functions used in these experiments.

#### 7.3.1 Increased *TFAM* levels following removal of *TFAM* siRNA

Transfection with *TFAM* siRNA produces only transient gene silencing as, 8 days after removal of *TFAM* siRNA, there was a recovery of *TFAM* protein expression to levels significantly higher than those in the control conditions (Figure 7.8).

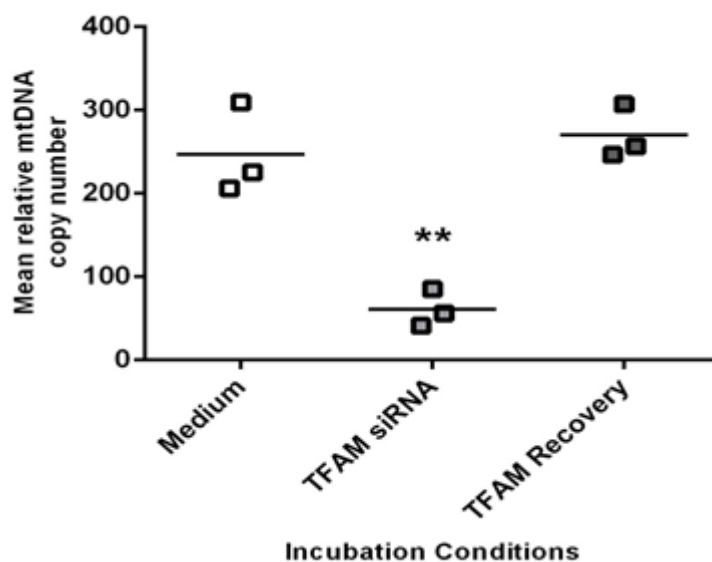


**Figure 7.8 Increased *TFAM* protein levels following removal of *TFAM* siRNA.**

THP-1 cells were incubated with 30nM *TFAM* siRNA and after 8 days the *TFAM* siRNA was removed and the cells incubated in growth medium for a further 8 days. (A) Representative images of *TFAM* and  $\beta$ -actin protein bands on a PVDF membrane following Western blot. (B) The expression of *TFAM* relative to  $\beta$ -actin was determined by Western blot (n=3). Normal data are represented as mean  $\pm$  standard deviation relative to the mean in the medium control and analysed using one-way ANOVA ( $p < 0.001$ ). Differences from the medium control are non-significant with Dunnett's multiple comparison test except; \* $p < 0.05$ , \*\*\* $p < 0.001$ .

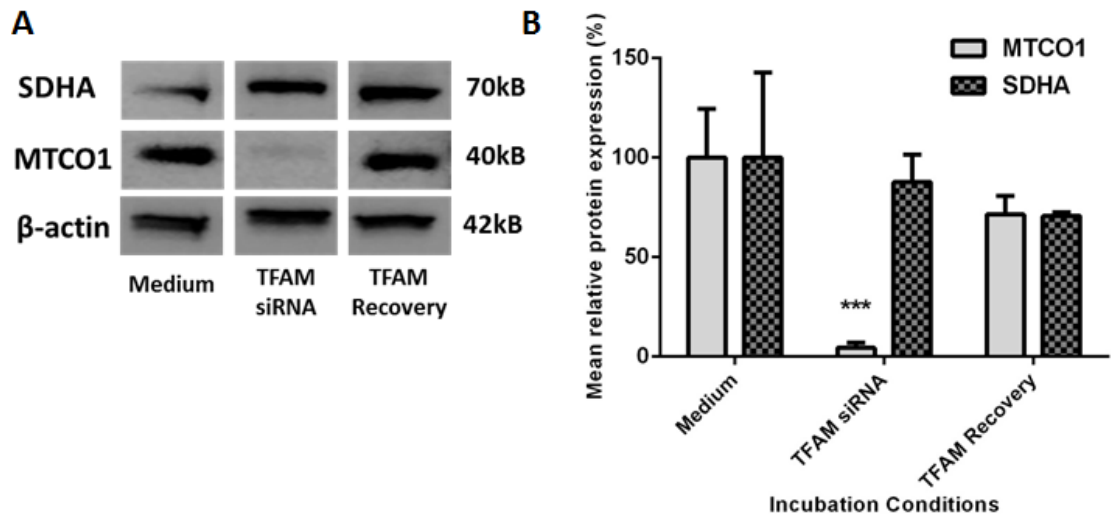
### 7.3.2 Functional recovery of mtDNA following removal of *TFAM* siRNA

The increased levels of the *TFAM* protein in THP-1 cells after the removal of *TFAM* siRNA are suggestive of the induction of a homeostatic response that aims to correct the effects of the period during which the *TFAM* gene was silenced. In keeping with this apparent compensatory response, there was a recovery of both relative mtDNA copy number (Figure 7.9) and the expression of the mtDNA-encoded MT-CO1 protein back to baseline levels 8 days after removal of *TFAM* siRNA (Figure 7.10). In addition, this recovery of mtDNA levels lead to a functional improvement in THP-1 cell bioenergetics, with oxygen consumption for all aspects of mitochondrial respiration restored back to the levels measured in the control conditions (Figure 7.11).

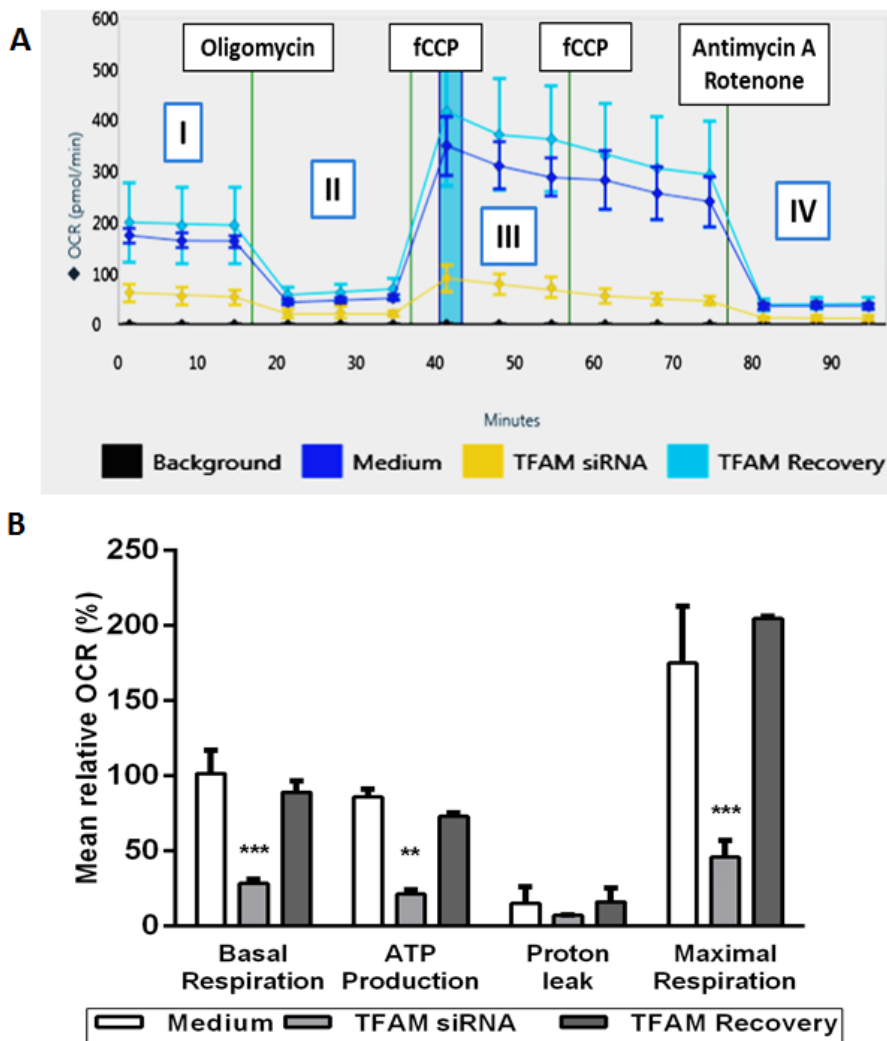


**Figure 7.9 Recovery of mtDNA copy number following removal of *TFAM* siRNA.**

THP-1 cells were incubated with 30nM *TFAM* siRNA and after 8 days the *TFAM* siRNA was removed and the cells incubated in growth medium for a further 8 days. mtDNA copy number was determined by measuring the level of *MT-ND1* relative to *B2M* using qPCR. Normal data are represented as mean with individual measurements (n=3). Analysis of the data was carried out using one-way ANOVA (p=0.001). Differences from the medium control are non-significant with Dunnett's multiple comparison test except; \*\*p<0.01.



**Figure 7.10 Recovery of mtDNA-encoded protein expression following removal of *TFAM* siRNA.** THP-1 cells were incubated with 30nM *TFAM* siRNA and after 8 days the *TFAM* siRNA was removed and the cells incubated in growth medium for a further 8 days. (A) Representative images of protein bands on a PVDF membrane following Western blot. (B) The relative expression of the mitochondrial proteins MT-CO1 (encoded by mtDNA) and SDHA (encoded by nuclear DNA) compared to  $\beta$ -actin was measured by Western blot (n=3). Normal data are represented as mean  $\pm$  standard deviation compared to the mean of the medium control. Analysis of the data was carried out using one-way ANOVA (MT-CO1 –  $p < 0.001$ , SDHA –  $p = 0.432$ ). Differences from the medium control are non-significant with Dunnett’s multiple comparison test except; \*\*\* $p < 0.001$ .

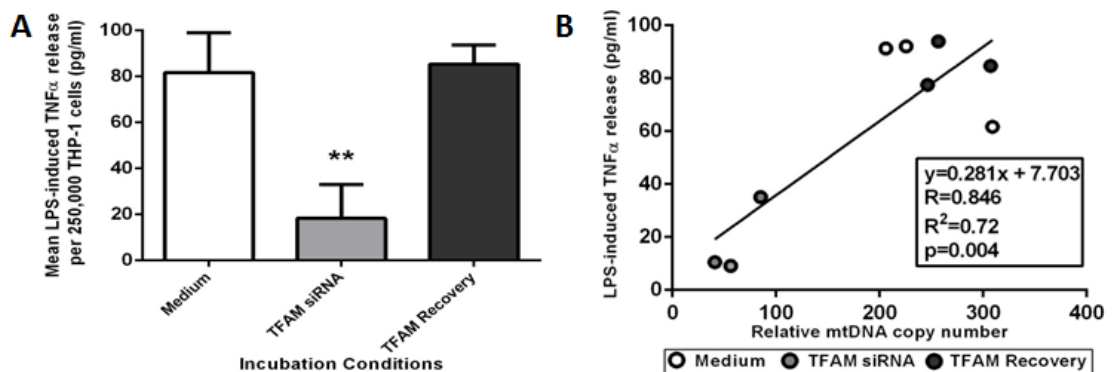


**Figure 7.11 Recovery of oxygen consumption by THP-1 cells following removal of *TFAM* siRNA.** THP-1 cells were incubated with 30nM *TFAM* siRNA and after 8 days the *TFAM* siRNA was removed and the cells incubated in growth medium for a further 8 days. Different aspects of respiration were then determined by measuring the OCR after the sequential addition of metabolic inhibitors using the Seahorse XF96<sup>e</sup> extracellular flux analyser. (A) An example of the respiratory profile of THP-1 cells in the recovery experiments. (B) The OCR for the following aspects of cellular respiration was determined; Basal mitochondrial respiration = basal OCR (I) – non-mitochondrial OCR (IV); Adenosine triphosphate (ATP) production = basal OCR (I) – post-oligomycin OCR (II); Proton leak = post-oligomycin OCR (II) – non-mitochondrial respiration (IV); Maximal mitochondrial respiration = maximal OCR (III) – non-mitochondrial OCR (IV). Data are presented as mean  $\pm$  standard deviation relative to the mean basal mitochondrial respiration in the medium control (n=3 for all experiments). The data for each aspect of mitochondrial respiration were analysed separately using one-way ANOVA (Basal respiration – p<0.001, ATP production – p<0.001, Proton leak – p=0.523, Maximal respiration – p<0.001). Differences from medium control are non-significant with Dunnett’s multiple comparison test except; \*\*p<0.01, \*\*\*p<0.001.

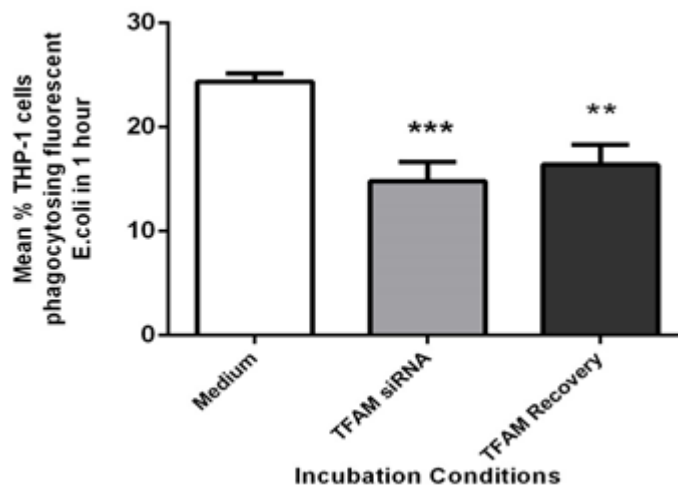


### 7.3.3 Restoration of LPS-induced TNF $\alpha$ release following removal of TFAM siRNA

Having established that both mtDNA levels and mitochondrial oxygen consumption could recover in association with an increase in TFAM protein expression following removal of TFAM siRNA, the restoration of THP-1 cell immune functions was then assessed. 8 days after the transfection with TFAM siRNA was discontinued the production of TNF $\alpha$  in response to a 4 hour treatment with 100ng/ml LPS returned to the levels of the control samples (Figure 7.12 A). In keeping with this finding, there is a significant positive correlation ( $R^2=0.72$ ,  $p=0.004$ ) between relative mtDNA copy number and LPS-induced TNF $\alpha$  release by THP-1 cells (Figure 7.12 B). In contrast to this recovery of TNF $\alpha$  release, there was only a partial improvement in the ability of THP-1 cells to phagocytose fluorescent *Escherichia coli* with the proportion of cells internalising bacteria remaining significantly below the levels in the control conditions (16.4% (95% CI 11.7-21.1) in TFAM recovery samples vs. 24.4% (95% 22.4-26.3) in the medium control,  $p=0.007$ ) (Figure 7.13).



**Figure 7.12 Recovery of LPS-induced TNF $\alpha$  release following removal of TFAM siRNA.** THP-1 cells were incubated with 30nM TFAM siRNA and after 8 days the TFAM siRNA was removed and the cells incubated in growth medium for a further 8 days. (A) The LPS-induced release of TNF $\alpha$  over 4 hours was measured by ELISA ( $n=3$ ). The data are represented as mean  $\pm$  standard deviation and analysed using one-way ANOVA ( $p=0.002$ ). Differences from the medium control with Dunnett's multiple comparison test are non-significant except; \*\* $p<0.01$ . (B) Scatter plot, linear regression and Pearson's correlation of the relationship between TNF $\alpha$  release and relative mtDNA copy number in THP-1 cells.



**Figure 7.13 Phagocytosis does not recover 8 days after removal of *TFAM* siRNA.**

THP-1 cells were incubated with 30nM *TFAM* siRNA and after 8 days the *TFAM* siRNA was removed and the cells incubated in growth medium for a further 8 days. The phagocytosis of fluorescein-labelled *Escherichia coli* in 1 hour was determined by flow cytometry (n=3). The data are represented as mean  $\pm$  standard deviation and analysed using one-way ANOVA ( $p < 0.001$ ). Differences from the medium control with Dunnett's multiple comparison test are represented as; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## 7.4 Discussion

### 7.4.1 Depletion of mtDNA in THP-1 cells after transfection with siRNA targeted against *TFAM*

*TFAM* is a highly-conserved protein containing two DNA-binding high mobility group box domains, which is encoded by nuclear DNA before localising to mitochondria due to the presence of a mitochondrial-targeting sequence (Campbell *et al.*, 2012). Within mitochondria *TFAM* binds non-specifically to the entire mitochondrial genome to form a major constituent of mtDNA-protein structures termed nucleoids (Garrido *et al.*, 2003; Kukat *et al.*, 2011). It is thought that *TFAM* bound in this manner has an important role in maintaining mtDNA stability (Kanki *et al.*, 2004). In addition, *TFAM* binds with high affinity to specific sequences overlying promoter regions to facilitate the initiation of RNA transcription from mtDNA, including the transcription of the RNA primers that promote mtDNA replication (Campbell *et al.*, 2012). Thus, *TFAM* appears to have a fundamental role in both mtDNA maintenance and replication, a conclusion supported by the consistent and strong correlation between *TFAM* levels and mtDNA

copy number that has been previously reported and now confirmed in this chapter (Kang *et al.*, 2007).

Studies in knockout mice suggest that defects in TFAM have serious consequences. Homozygous *Tfam*<sup>-/-</sup> mice lack any mtDNA and die during embryogenesis while *Tfam*<sup>+/-</sup> heterozygotes have a significant reduction in mtDNA copy number and RNA transcription from mtDNA-encoded genes across a range of tissues (Larsson *et al.*, 1998). In addition, although *TFAM* mutations have yet to be described as a cause of mitochondrial disease or mtDNA defects in humans, polymorphisms in the *TFAM* gene have been associated with an increased risk of neurodegenerative diseases (Belin *et al.*, 2007; Gaweda-Walerych *et al.*, 2010).

The results presented in this chapter indicate that transfection with siRNA targeted against *TFAM* is a specific, non-cytotoxic method of depleting mtDNA from THP-1 cells that leads to an impairment of mitochondrial respiration. This is in agreement with other studies that have found that transfection with *TFAM* siRNA using various techniques can reduce mtDNA copy number in a number of cell types (Kanki *et al.*, 2004; Pohjoismäki *et al.*, 2006; Kasashima *et al.*, 2011). Furthermore, the results from the recovery experiments show that this process is rapidly reversible with a recovery in mtDNA copy number and mitochondrial respiration occurring 8 days after removal of *TFAM* siRNA in association with the presence of increased levels of TFAM protein. Again, these findings are consistent with previous reports indicating that the recovery of mtDNA copy number is closely correlated with a restoration of TFAM expression and that an over-expression of TFAM may be beneficial in certain cell culture disease models (Jeng *et al.*, 2008; Thomas *et al.*, 2011).

#### ***7.4.2 Transfection with POLG siRNA does not affect THP-1 cell mtDNA copy number***

POLG consists of two subunits produced by the expression of nuclear genes, a catalytic subunit encoded by *POLG* and an accessory subunit encoded by *POLG2*. It is part of the mtDNA replication complex that associates with nucleoids during mitochondrial biogenesis (Hudson and Chinnery, 2006). POLG is understood to be the only DNA polymerase that is active within human mitochondria and, as such, appears to be crucial for both replication and repair of mtDNA (Chan and Copeland, 2009). As with TFAM, knockout mice studies indicate that POLG is essential for mammalian life as

homozygous *Polg*<sup>-/-</sup> mice have almost no mtDNA and die during embryogenesis (Hance *et al.*, 2005).

In view of this, it is perhaps surprising that transfection with *POLG* siRNA, despite almost completely abolishing *POLG* protein expression, had no effect on mtDNA copy number, mtDNA encoded protein expression or mitochondrial oxygen consumption in THP-1 cells. However, there are no consistent reports of the use of *POLG* siRNA as a reliable method of depleting mtDNA. In contrast to *TFAM*, *POLG* appears to be constitutively expressed across a range of tissues irrespective of their mtDNA copy number or the level of mitochondrial biogenesis (Schultz *et al.*, 1998). In addition, heterozygous *Polg*<sup>+/-</sup> mice have normal mtDNA copy numbers and no clinical phenotype despite a 50% reduction in *Polg* mRNA levels (Hance *et al.*, 2005). Furthermore, these *Polg*<sup>+/-</sup> mice are able to increase mtDNA levels in response to an over-expression of *TFAM* in the same manner as wild-type mice. Thus, it appears that both the enzymatic activity of *POLG* and cellular mtDNA copy number are not dependent on the level of *POLG* expression and that there is significant spare capacity in this system. As a result, it is possible that transfection with 30nM *POLG* siRNA for 8 days failed to deplete mtDNA in THP-1 cells because there was sufficient enzymatic activity in the residual *POLG* (<5% of expression in control samples) to maintain mtDNA replication. It may, therefore, be necessary to completely abolish all *POLG* expression within a cell, using longer transfections or higher siRNA concentrations, in order to definitively exclude *POLG* siRNA as a viable technique of depleting mtDNA.

However, given the consistent lack of correlation between the amount of *POLG* and mtDNA copy number, a potentially more productive alternative method could instead focus on the effects of inducing *POLG* dysfunction. Such an approach is suggested by the fact that mutations in *POLG* result in mtDNA instability and lead to a number of clinically important human mtDNA depletion and deletion syndromes (Chan and Copeland, 2009). In keeping with this, a previous study has shown that transfecting human HEK293 cells with a mutated, non-functional form of *POLG* leads to rapid, reversible mtDNA depletion with functional consequences for the mitochondria (Jazayeri *et al.*, 2003). Furthermore, studies indicate that *Polg* mutator mice, which express a proof-reading deficient variant of *Polg*, have significantly reduced mtDNA copy number and mitochondrial OXPHOS activity in association with evidence of

increased oxidative damage to mitochondrial constituents and a shift to glycolytic respiration (Dai *et al.*, 2013; Kolesar *et al.*, 2014; Saleem *et al.*, 2015).

#### 7.4.3 Immune dysfunction in THP-1 cells with mtDNA depletion

The depletion of mtDNA copy number induced by transfection with *TFAM* siRNA every 48 hours for 8 days resulted in a significant impairment of both mitochondrial oxygen consumption and immune functions in THP-1 cells. These results confirm the findings from Chapter 6 in which p0 THP-1 cells, lacking mtDNA due to treatment with ethidium bromide, also had a significantly impaired ability to release TNF $\alpha$  in response to LPS and a complete loss of mtDNA-dependent OXPHOS complex activity. A concern with the interpretation of the results from p0 cells is that ethidium bromide is a non-specific DNA intercalating agent that has been shown to significantly alter nuclear gene expression in addition to depleting mtDNA (Magda *et al.*, 2008). However, given the consistency of the findings between different techniques and the more targeted action of *TFAM* siRNA, the reduced LPS-induced TNF $\alpha$  release by THP-1 cells lacking mtDNA does not appear to occur as a result of 'off target' effects of the method of mtDNA depletion.

In Chapter 6 it was also noted that the p0 THP-1 cells generated by incubation with ethidium bromide had an immune phenotype consistent with differentiation into alternatively-activated macrophage-like cells, with reduced pro-inflammatory cytokine release but increased phagocytosis of *Staphylococcus aureus* (Tiemessen *et al.*, 2007; Murray and Wynn, 2011). However, unlike alternatively-activated macrophages, THP-1 cells with *TFAM* siRNA-induced mtDNA depletion did not release more anti-inflammatory cytokines and actually had an inhibition of *Escherichia coli* phagocytosis. The differences in the phagocytic ability of p0 THP-1 cells and those depleted of mtDNA after transfection with *TFAM* siRNA may reflect effects of the different methods of depleting mtDNA. Alternatively, it is possible that there are specific differences in the ability of the THP-1 cells to phagocytose Gram positive and Gram negative bacteria (Skovbjerg *et al.*, 2010). This theory is supported by the significantly lower median proportion of control THP-1 cells internalising bacteria when exposed to *Staphylococcus aureus* compared to *Escherichia coli* (14.7% (IQR 14-15) vs 24.8% (IQR 23.8-27.5),  $p=0.03$ ). It may, therefore, be valuable to measure the effects of mtDNA depletion on the ability of THP-1 cells to phagocytose a variety of bacterial species.

In addition to these key differences in immunological phenotype, the alterations in THP-1 cell metabolism in response to changes in mtDNA levels also refute the theory that differentiation into macrophage-like cells is occurring. Firstly, after transfection with *TFAM* siRNA THP-1 cells had a significant impairment of mitochondrial respiration, which contrasts with the up-regulation of OXPHOS activity seen in alternatively-activated macrophages (Rodríguez-Prados *et al.*, 2010). Furthermore, after removal of *TFAM* siRNA a recovery in the ability of THP-1 cells to release TNF $\alpha$  occurred in parallel with the restoration of mtDNA copy number and mitochondrial oxygen consumption. Although there is a degree of plasticity in macrophage differentiation, an increase in the ability to release pro-inflammatory cytokines happens during the shift towards a classically-activated macrophage phenotype, a process that involves a switch to glycolytic respiration rather than the recovery of OXPHOS activity that is seen in THP-1 cells after removal of *TFAM* siRNA (Murray and Wynn, 2011; O'Neill and Grahame Hardie, 2013).

The impairment of TNF $\alpha$  production by THP-1 cells after transfection with *TFAM* siRNA or treatment with ethidium bromide, therefore, appears to be due to the consequences of mtDNA depletion. A reduction in TNF $\alpha$  release in response to an *ex vivo* treatment with LPS is a key phenotypic feature of deactivated septic monocytes that is linked to an increase risk of developing secondary infections and dying (Cavaillon and Adib-Conquy, 2007; Hall *et al.*, 2011). Small pilot studies have suggested that immunotherapies that restore LPS-induced TNF $\alpha$  release by septic monocytes may improve clinical outcomes (Döcke *et al.*, 1997; Meisel *et al.*, 2009). In this context the recovery of TNF $\alpha$  release in association with restoration of mtDNA copy number in THP-1 cells following removal of *TFAM* siRNA is potentially interesting. If this finding is confirmed in primary human monocytes, it is possible that critically ill patients with evidence of monocyte deactivation and mtDNA depletion could benefit from interventions to stimulate mitochondrial biogenesis. Such an approach would provide a novel, biomarker-targeted therapy aimed at stimulating a specific aspect of the innate immune response that is dysfunctional in sepsis (Dare *et al.*, 2009; Hotchkiss *et al.*, 2013b). However, stimulating mitochondrial biogenesis may not provide a panacea for sepsis-induced monocyte dysfunction, as phagocytosis did not fully recover after removal of *TFAM* siRNA, despite restoration of mtDNA copy number.

#### 7.4.4 Conclusion

Inhibiting *POLG* expression by transfection of THP-1 cells with 30nM *POLG* siRNA for 8 days did not result in significant changes in mtDNA copy number or mitochondrial respiration. In contrast, transfection with siRNA targeted against *TFAM* produced a decrease in mtDNA copy number that occurred in parallel with an impairment of THP-1 cell immune functions, including a reduced ability to release TNF $\alpha$  in response to LPS, a key phenotypic feature of deactivated septic monocytes. Furthermore, the restoration of THP-1 cell mtDNA copy number after removal of *TFAM* siRNA was associated with the recovery of LPS-induced TNF $\alpha$  release. These results support the suggestion in Chapter 6 that immune dysfunction in THP-1 cells may be caused by depletion of mtDNA, a finding of potential importance to understanding sepsis-induced monocyte deactivation.

The underlying mechanisms behind the association between impaired immunity and mtDNA depletion will be explored in more detail in Chapter 8 . It remains possible that the effects on the ability of THP-1 cells to release TNF $\alpha$  are due to the metabolic consequences of the loss of mitochondrial respiration or that they occur due to a direct impact of the loss of mtDNA on immune and inflammatory signalling pathways (Dietl *et al.*, 2010; West *et al.*, 2011b).

## Chapter 8 The effect of transfection of THP-1 cells with *TFAM* siRNA on nuclear gene expression and the transcriptomic response to LPS

### 8.1 Overview

TFAM is a key regulator of mtDNA replication and gene transcription and also a major component of the mitochondrial nucleoid (Campbell *et al.*, 2012). In Chapter 7 it was shown that down-regulation of *TFAM* expression by transfection with siRNA leads to mtDNA depletion, mitochondrial respiratory dysfunction and impaired immune responses in THP-1 cells. In order to assess these mechanisms linking mtDNA depletion to immune dysfunction, RNA sequencing (RNA-Seq) was used to determine the effects of mtDNA depletion by transfection with *TFAM* siRNA on THP-1 cell gene expression, both at baseline and following exposure to an inflammatory stimulus in the form of LPS. RNA-Seq uses high-throughput next generation sequencing in order to provide an assessment of the cellular transcriptome, the complete set of RNA transcribed from functional genes during particular conditions (Wang *et al.*, 2009). During RNA-Seq RNA is converted to a library of cDNA fragments that is then sequenced to produce millions of short read sequences (Wolf, 2013). These reads are then aligned to the appropriate reference genome and analysed qualitatively, to identify and describe the properties of the transcripts, and quantitatively, to assess differential gene expression between conditions (Ozsolak and Milos, 2011).

#### 8.1.1 Hypothesis

Transfection with *TFAM* siRNA will produce significant changes in THP-1 cell nuclear gene expression and alter the transcriptomic response to treatment with LPS. In particular, there will be negative effects on the expression of genes involved in inflammatory signalling pathways and effector immune responses.

#### 8.1.2 Aims and Objectives

1. To determine whether transfection with Silencer® Select Negative Control siRNA number 1 using Lipofectamine RNAiMAX results in any significant change to the THP-1 cell transcriptome.
2. To measure the effects of mtDNA depletion following transfection with *TFAM* siRNA on the expression of nuclear genes by THP-1 cells



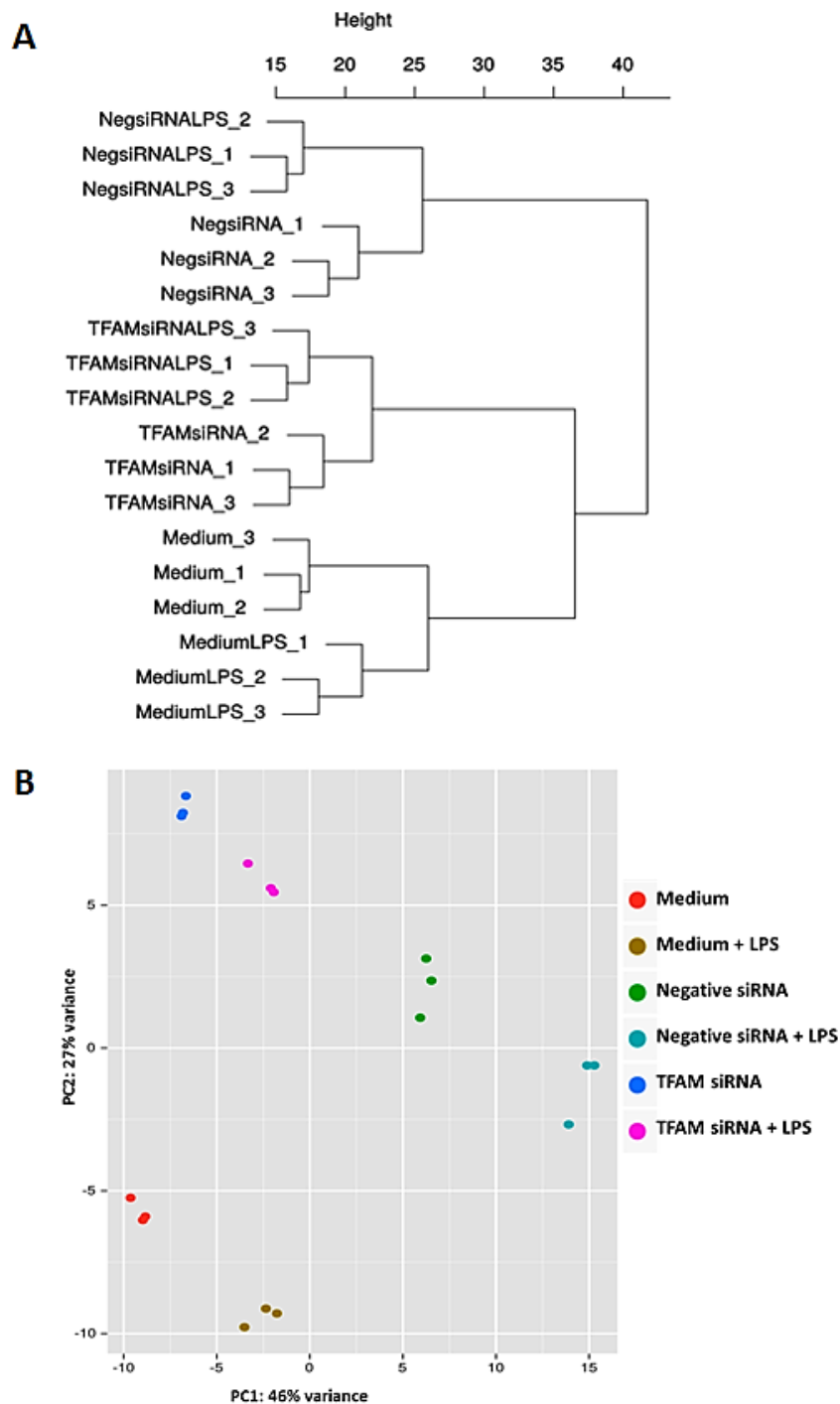
3. To identify any changes in the transcriptomic response to LPS in THP-1 cells after transfection with *TFAM* siRNA.

## 8.2 Results

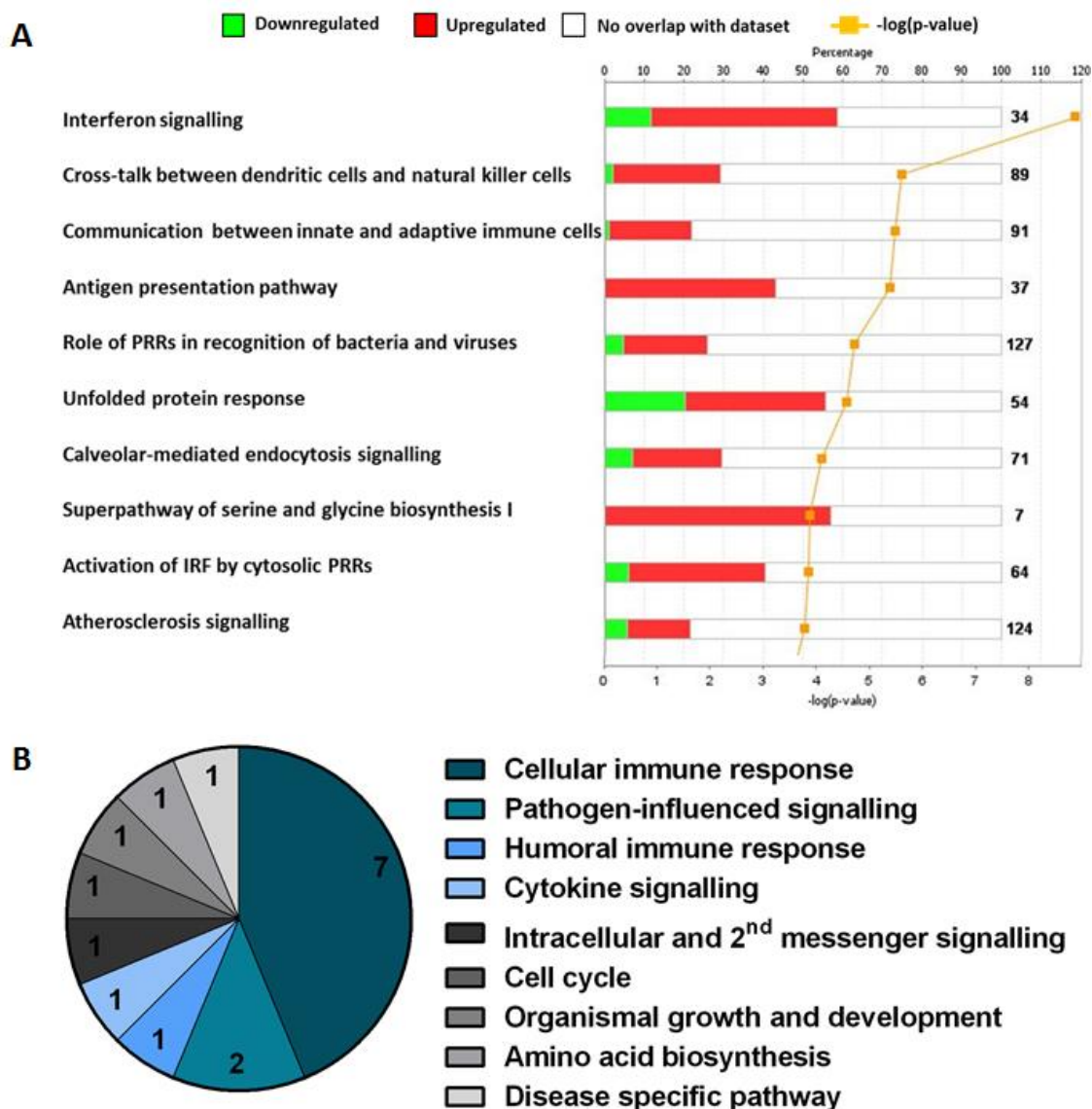
### *8.2.1 The process of siRNA transfection significantly alters the THP-1 cell transcriptome*

In Chapter 7 it was found that transfection with Silencer® Select Negative Control siRNA number 1 did not have any significant effect on THP-1 cell viability, proliferation and mitochondrial or immune functions. However, the transcriptomic analysis reveals that transfection of THP-1 cells with 30nM of this negative siRNA for 8 days does have a widespread effect on their gene expression. A hierarchical clustering dendrogram and PCA plot indicate that, while there is good agreement between the three biological replicates for each condition, there are considerable differences in the transcriptome of THP-1 cells after transfection with the negative siRNA compared to those cultured in growth medium (Figure 8.1). In total 1115 genes displayed significant differential expression after transfection with the negative siRNA, 749 were up-regulated and 366 down-regulated. These differentially expressed genes were predominantly involved in immune signalling pathways, with most of these pathways being up-regulated in the negative siRNA transfected cells (Figure 8.2).

Following these findings in subsequent analyses the transcriptome of *TFAM* siRNA-treated THP-1 cells was directly compared to that of cells transfected with the negative siRNA in order to correct for the effects of siRNA transfection.



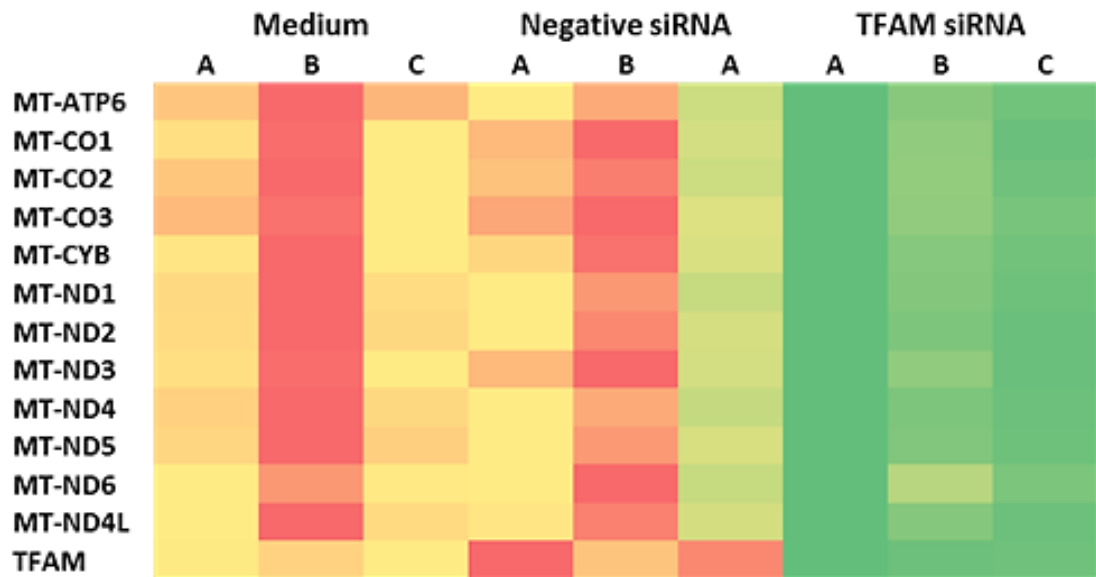
**Figure 8.1 The transcriptome of THP-1 cells is altered by transfection with negative siRNA and *TFAM* siRNA.** For 8 days THP-1 cells were incubated in growth medium or transfected with 30nM of negative or *TFAM* siRNA. After a final 4 hour incubation with or without 100ng/ml LPS gene expression was assessed by RNA-Seq. (A) A hierarchical clustering dendrogram in which the most similar samples are sequentially paired with the height of the link indicating the dissimilarity between the samples. (B) PCA plot in which the samples are plotted against two composite principle components that describe their variation.



**Figure 8.2 Transfection with negative siRNA alters immune pathways in THP-1 cells.** IPA<sup>®</sup> software was used to carry out a gene ontology analysis of the canonical signalling pathways significantly affected by genes differentially expressed in THP-1 cells transfected with 30nM negative siRNA compared to those incubated in medium for 8 days. (A) Bar chart of the number of up-regulated (red) and down-regulated (green) genes in the top ten canonical signalling pathways most significantly altered by transfection with negative siRNA. (B) Pie chart indicating the signalling pathway categories of the top ten canonical pathways most significantly altered by transfection with negative siRNA. (Abbreviations: IRF – interferon regulatory factors, PRR – pattern recognition receptor).

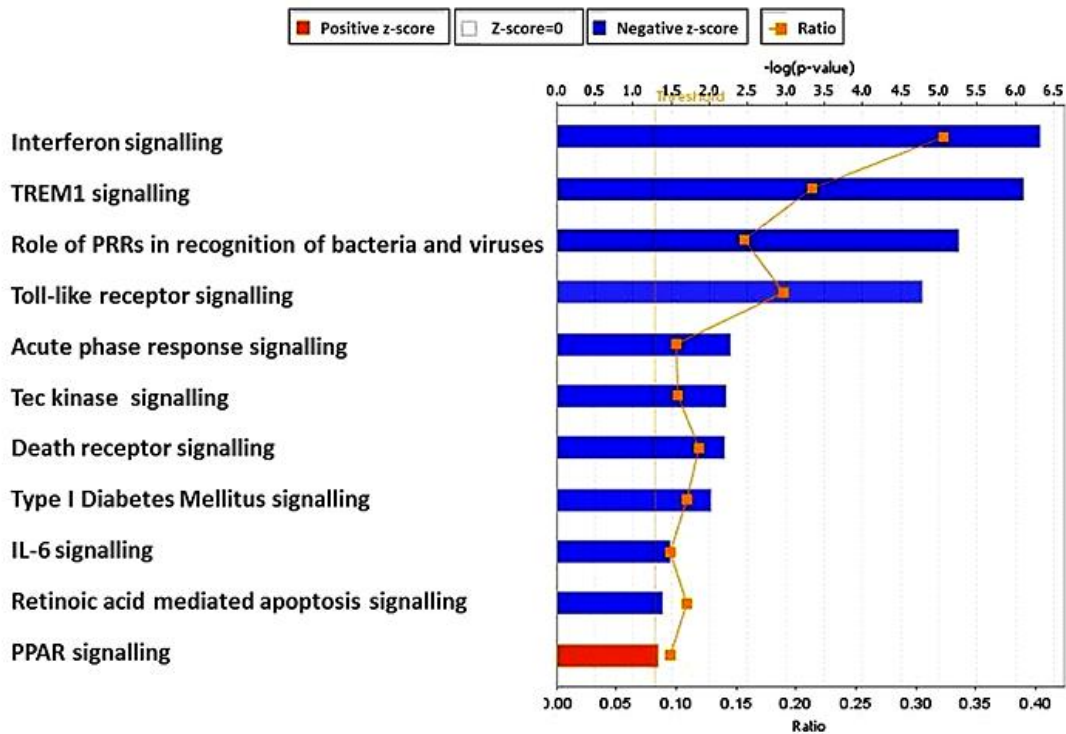
### *8.2.2 Transfection with TFAM siRNA produces significant changes to the THP-1 cell transcriptome*

A comparison between the transcriptome of THP-1 cells transfected with *TFAM* siRNA and those transfected with the negative siRNA revealed that 1166 genes were significantly differentially expressed. Of these genes 499 had increased expression and 667 had decreased expression in *TFAM* siRNA transfected cells. Further analysis confirmed that the mtDNA depletion in THP-1 cells transfected with *TFAM* siRNA caused a decrease in the expression of those genes encoded by mtDNA (Figure 8.3). Transfection with *TFAM* siRNA was also found to lead to a significant inhibition of a number of canonical signalling pathways, particularly those involved in the immune response and, to a lesser degree, apoptosis (Figure 8.4). The particular down-regulation of signalling pathways involved in the recognition of pathogens and the initiation of pro-inflammatory responses is consistent with the impaired immune responses generated by THP-1 cells following transfection with *TFAM* siRNA that was observed in Chapter 7 (Table 8.1). Furthermore, an analysis of the likely effect of these changes in gene expression on the ability of the cells to respond to upstream regulators also strongly predicts that mtDNA depletion due to treatment with *TFAM* siRNA will result in a diminished ability of THP-1 cells to react to LPS, pro-inflammatory cytokines and important transcriptional regulators of the inflammatory response (Table 8.2).

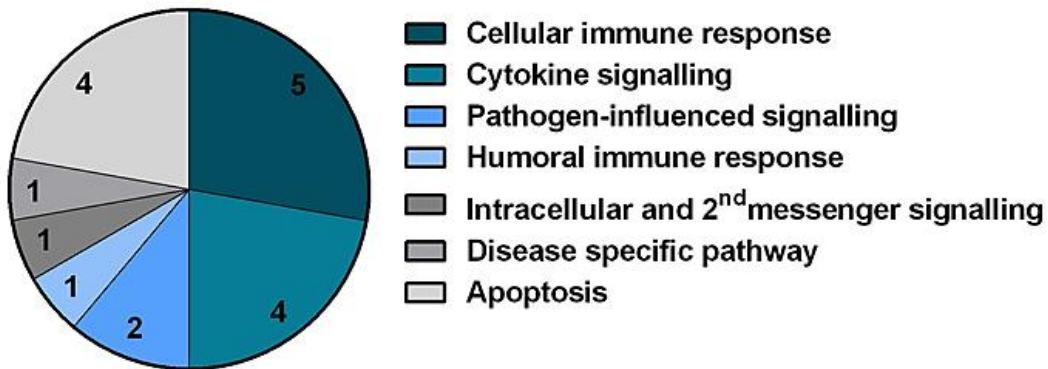


**Figure 8.3 Heat map indicating decreased relative expression of mtDNA-encoded genes and *TFAM* in THP-1 cells transfected with *TFAM* siRNA.** RNA-Seq was used to assess gene expression in THP-1 cells following transfection with 30nM of *TFAM* or negative siRNA or incubation in growth medium for 8 days. The heat map represents the normalised reads per kilobase per million mapped reads (RPKM) for the expression of each gene, with red indicating a higher read count and green a lower read count.

A



B



**Figure 8.4** Transfection with *TFAM* siRNA alters immune pathways in THP-1 cells. IPA® software was used to carry out a gene ontology analysis of the pathways significantly affected by genes differentially expressed in THP-1 cells transfected with *TFAM* siRNA compared to those transfected with negative siRNA. (A) Bar chart of significantly up-regulated (red) and down-regulated (blue) canonical signalling pathways, filtered for adjusted p-value <0.05 and z-score >±2. (B) Pie chart indicating the signalling pathway categories of the significantly altered canonical pathways after transfection with *TFAM* siRNA. (Abbreviations: IL-6 – interleukin-6; PPAR – peroxisome proliferator activated receptors; PRR – pattern recognition receptor; TREM1 – triggering receptor expressed on myeloid cells-1)

Function	z-score	p-value	Genes with altered expression		
			Proportion of genes in pathway	Up-regulated	Down-regulated
<b>Interferon signalling</b>					
Cellular immune response Cytokine signalling	-3.16	4.9x10 <sup>-7</sup>	11/34	-	<i>IFI35, IFNB1, IFIT1, IFITM1, IFITM2, IFITM3, IFIT3, IRF9, OAS1, STAT2, STAT1,</i>
<b>Triggering receptor expressed on myeloid cells-1 signalling</b>					
Cellular immune response Cytokine signalling	-3.50	7.9x10 <sup>-7</sup>	16/75	<i>MPO</i>	<i>CCL3, CD83, CIITA, IL1B, ITGAX, MYD88, NLRC4, NLRP12, TLR1, TLR3, TLR6, TLR7, TNF, TREM1, TYROBP</i>
<b>Role of pattern recognition receptors in recognition of bacteria and viruses</b>					
Cellular immune response Pathogen-influenced signalling	-3.74	5.5x10 <sup>-6</sup>	20/127	<i>IL12A</i>	<i>C3AR1, C5AR1, DDX58, EIF2AK2, IFIH1, IFNB1, IL1B, IRF7, MYD88, NLRC4, OAS1, OAS2, OAS3, PTX3, TLR1, TLR3, TLR6, TLR7, TNF</i>
<b>Toll-like receptor signalling</b>					
Apoptosis Cellular immune response Humoral immune response Pathogen-influenced signalling	-2.33	1.7x10 <sup>-5</sup>	14/74	<i>IL12A, PPARA</i>	<i>EIF2AK2, FOS, IL1B, IL1RN, MYD88, NFKBIA, TLR1, TLR3, TLR6, TLR7, TNF, TNFAIP3</i>
<b>Acute phase response</b>					
Cytokine signalling	-2.67	5.2x10 <sup>-3</sup>	17/169	<i>FTL, HMOX1, ORM1, ORM2, SOCS2</i>	<i>A2M, AGT, CEBPB, FOS, IKBKE, IL1B, IL1RN, MYD88, NFKBIA, SERPINE1, SOCS3, TNF</i>

**Table 8.1 The five pathways most significantly affected by transfection with *TFAM* siRNA** The details of the top five canonical pathways identified by IPA® to be most significantly down-regulated in THP-1 cells transfected with *TFAM* siRNA compared to those transfected with negative siRNA.

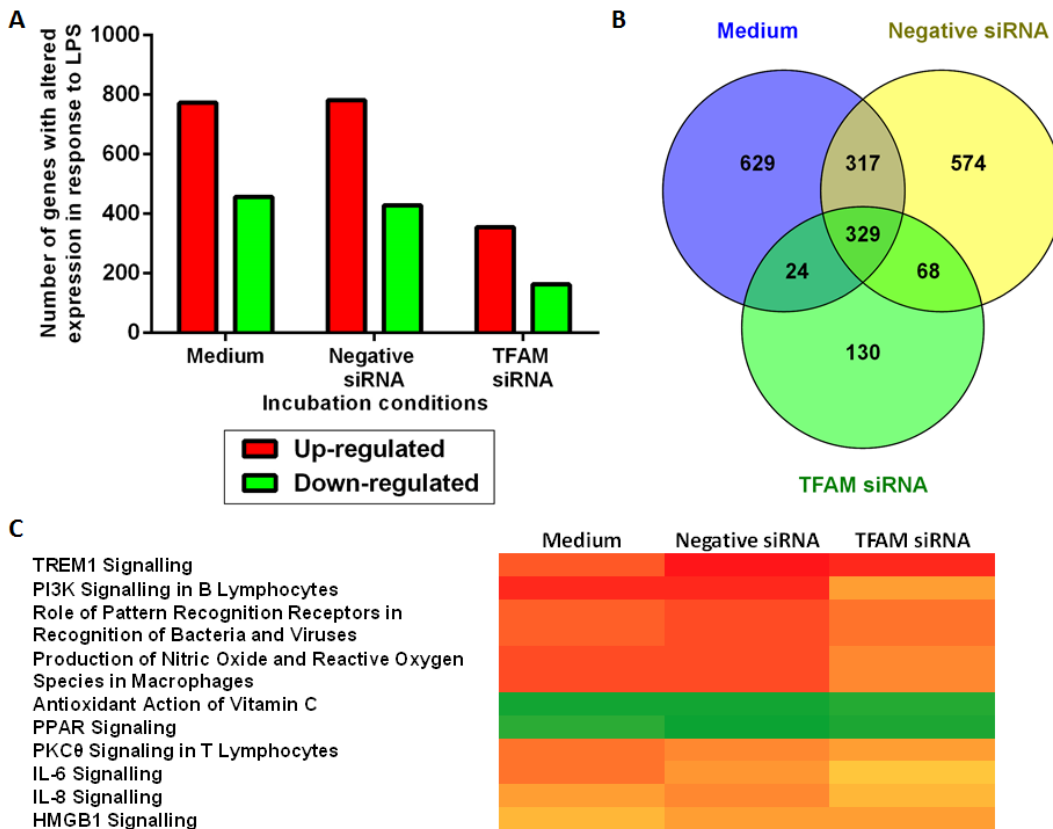
Upstream regulator	Function	Predicted effect	Activation z-score	Overlap p-value	Relevant genes	
					Activated	Inhibited
<b>Top 5 regulators</b>						
LPS	Pathogen associated molecular pattern	Inhibited	-7.06	$7.7 \times 10^{-68}$	64	164
IFN- $\gamma$	Cytokine	Inhibited	-7.83	$3.4 \times 10^{-52}$	12	41
TNF	Cytokine	Inhibited	-6.53	$4.9 \times 10^{-52}$	57	144
IFNA2	Cytokine	Inhibited	-7.22	$3.1 \times 10^{-50}$	5	65
IFNL1	Cytokine	Inhibited	-6.44	$1.2 \times 10^{-39}$	0	42
<b>Top 5 transcriptional regulators</b>						
STAT3	Response to cytokines and growth factors	Inhibited	-2.85	$8.3 \times 10^{-38}$	19	38
STAT1	Response to interferon	Inhibited	-5.98	$4.5 \times 10^{-32}$	5	50
IRF7	Interferon-mediated immune response	Inhibited	-6.67	$1.5 \times 10^{-28}$	1	49
TP53	Tumour suppressor gene	Inhibited	-2.30	$4.2 \times 10^{-24}$	53	73
IRF3	Interferon-mediated immune response	Inhibited	-4.97	$1.7 \times 10^{-23}$	5	37

**Table 8.2 The predicted effect of transfection with *TFAM* siRNA on the response of THP-1 cells to upstream regulators.** The effects of the genes differentially expressed between THP-1 cells transfected with *TFAM* siRNA and negative siRNA on the predicted response to upstream regulators was assessed using IPA<sup>®</sup>. (Abbreviations: IFNA2 - interferon- $\alpha$ 2; IFN- $\gamma$  - interferon- $\gamma$ , IFNL1 - interferon- $\lambda$ 1, IRF3/7 - interferon regulatory factor-3/7, LPS – lipopolysaccharide, STAT1/3 - signal transducer and activation of transcription-1/3, TNF - tumour necrosis factor, TP53 - tumour protein p53)

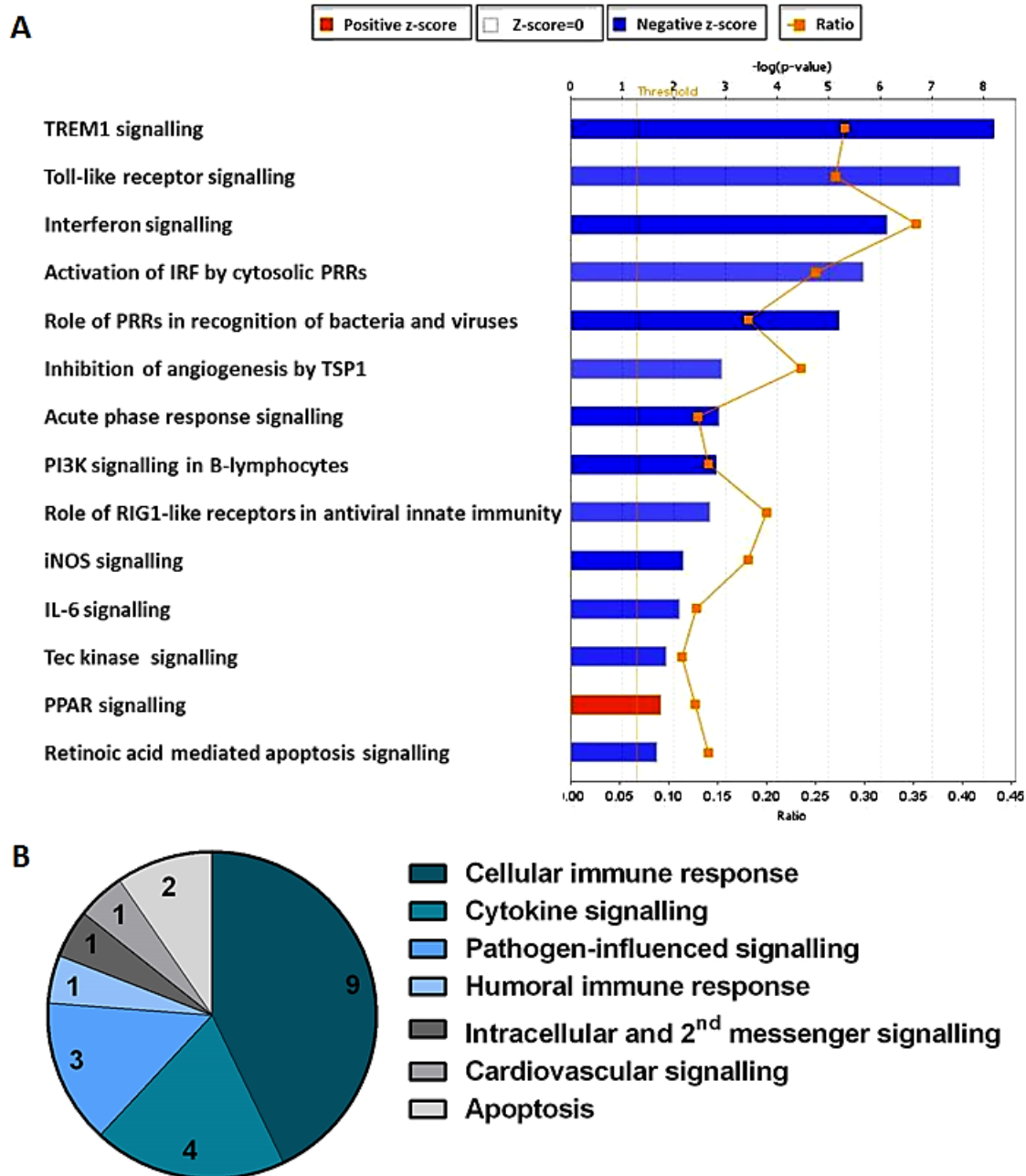


### *8.2.3 Transfection with TFAM siRNA significantly alters the transcriptomic response of THP-1 cells to LPS*

Next, the effect of exposure to 100ng/ml LPS for 4 hours on the THP-1 cell transcriptome was assessed. Firstly, a comparison of the genes that exhibited differential expression after LPS treatment in each condition was carried out. While, as expected, LPS was found to produce profound effects on the THP-1 cell transcriptome, there were considerably fewer genes with altered expression in the cells transfected with *TFAM* siRNA compared to those incubated in growth medium or transfected with the negative siRNA for the previous 8 days (Figure 8.5 A). Despite clear differences in the particular genes that were differentially expressed, similar canonical signalling pathways were activated by LPS treatment in all conditions, with the variation mainly apparent in the degree to which specific pathways were activated (Figure 8.5 B and C). Subsequently, a direct comparison between the transcriptome of LPS-treated THP-1 cells that were transfected with either *TFAM* siRNA or the negative siRNA for the previous 8 days revealed that 1528 genes were significantly differentially expressed. Of these genes 692 had increased expression and a further 836 genes were down-regulated in *TFAM* siRNA treated cells. In a similar manner to the changes seen before LPS treatment, these alterations in gene expression in *TFAM* siRNA transfected THP-1 cells resulted in a significant inhibition of a number of canonical signalling pathways, especially those involved in pathogen recognition and the generation of innate immune and inflammatory responses (Figure 8.6 and Table 8.3).



**Figure 8.5 The transcriptomic response of THP-1 cells to LPS.** The effect of treatment with 100ng/ml LPS for 4 hours was assessed in THP-1 cells incubated in medium or transfected with 30nM negative or TFAM siRNA for the previous 8 days. (A) Bar chart of the number of genes either up- or down-regulated following treatment with LPS. (B) Venn diagram of the overlap of differentially expressed genes after LPS treatment in each of the three conditions. (C) Heat map of the effect of the differentially expressed genes on the ten most altered canonical pathways on a scale where red squares indicate the most activated pathways, yellow squares indicate unaffected pathways and green squares indicate the most inhibited pathways (Abbreviations: HMGB1 – high mobility group box-1, IL-6/8 – interleukin-6/8; PI3K – phosphoinositide-3 kinase, PKCθ – protein kinase C-θ, PPAR – peroxisome proliferator activated receptors; TREM1 – triggering receptor expressed on myeloid cells-1).



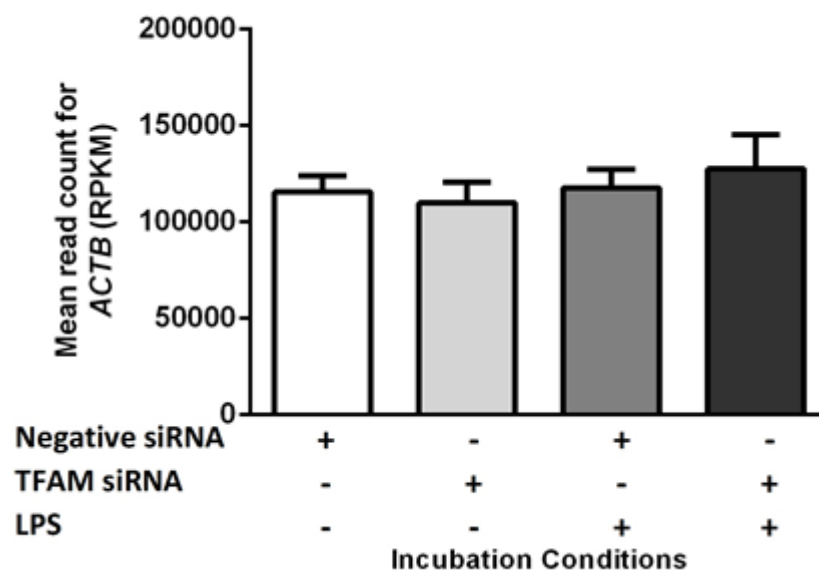
**Figure 8.6 Down-regulation of LPS-induced immune signalling pathways in THP-1 cells after transfection with *TFAM* siRNA.** IPA<sup>®</sup> software was used to carry out a gene ontology analysis of the canonical signalling pathways significantly affected by the differential expression of genes in THP-1 cells transfected with *TFAM* siRNA compared to those transfected with negative siRNA after treatment with 100ng/ml LPS for 4 hours. (A) Bar chart of significantly up-regulated (red) and down-regulated (blue) canonical signalling pathways, filtered for adjusted p-value <0.05 and z-score >±2. (B) Pie chart indicating the signalling pathway categories of the significantly altered LPS-induced canonical pathways after transfection with *TFAM* siRNA. (Abbreviations: IL-6 – interleukin-6; iNOS – inducible nitric oxide synthase, IRF – interferon regulatory factor, PI3K – phosphoinositide-3-kinase, PPAR – peroxisome proliferator activated receptors; PRR – pattern recognition receptor; RIG1 – retinoic acid-inducible gene-1, TREM1 – triggering receptor expressed on myeloid cells-1; TSP-1 – thrombospondin-1).

Function	z-score	p-value	Genes with altered expression		
			Proportion	Up-regulated	Down-regulated
<b>Triggering receptor expressed on myeloid cells-1 (TREM1) signalling</b>					
Cellular immune response Cytokine signalling	-4.15	6.3x10 <sup>-9</sup>	21/75	<i>MPO</i>	<i>CCL2, CCL3, CD40, CD83, CIITA, ICAM1, IL1B, ITGAX, MYD88, NLRC4, NLRP12, NOD2, TLR1, TLR3, TLR4, TLR6, TLR7, TNF, TREM1, TYROBP</i>
<b>Toll-like receptor signalling</b>					
Apoptosis Cellular immune response Humoral immune response Pathogen-influenced signalling	-2.13	2.8x10 <sup>-8</sup>	20/74	<i>ELK1, IL12A, MAP3K14, PPARA, TRAF4</i>	<i>EIF2AK2, FOS, IL1B, IL1RN, IRAK2, JUN, MYD88, NFKBIA, TLR1, TLR3, TLR4, TLR6, TLR7, TNF, TNFAIP3</i>
<b>Interferon signalling</b>					
Cellular immune response Cytokine signalling	-3.32	7.2x10 <sup>-7</sup>	12/34	-	<i>IFI35, IFIT1, IFIT3, IFITM1, IFITM3, IFNB1, IRF9, MX1, OAS1, STAT2, STAT1, TAP1</i>
<b>Activation of interferon regulatory factors by cytosolic pattern recognition receptors</b>					
Cellular immune response	-2.00	2.1x10 <sup>-6</sup>	12/34	-	<i>ADAR, CD40, DDX58, DHX58, IFIH1, IFIT2, IFNB1, IKBKE, IRF7, IRF9, ISG15, JUN, NFKBIA, STAT1, STAT2, TNF</i>
<b>Role of pattern recognition receptors in recognition of bacteria and viruses</b>					
Cellular immune response Pathogen-influenced signalling	-3.74	5.5x10 <sup>-6</sup>	20/127	<i>IL12A</i>	<i>C3, C3AR1, C5AR1, DDX58, EIF2AK2, IFIH1, IFNB1, IL1B, IRF7, MYD88, NLRC4, NOD2, OAS1, OAS2, OAS3, PTX3, TLR1, TLR3, TLR4, TLR6, TLR7, TNF</i>

**Table 8.3** The five pathways most significantly affected by the altered transcriptomic response to LPS following transfection with *TFAM* siRNA. The details of the top five canonical pathways identified by IPA® to be most significantly down-regulated as a result of the altered transcriptomic response to a LPS of THP-1 cells transfected with *TFAM* siRNA compared to those transfected with negative siRNA.

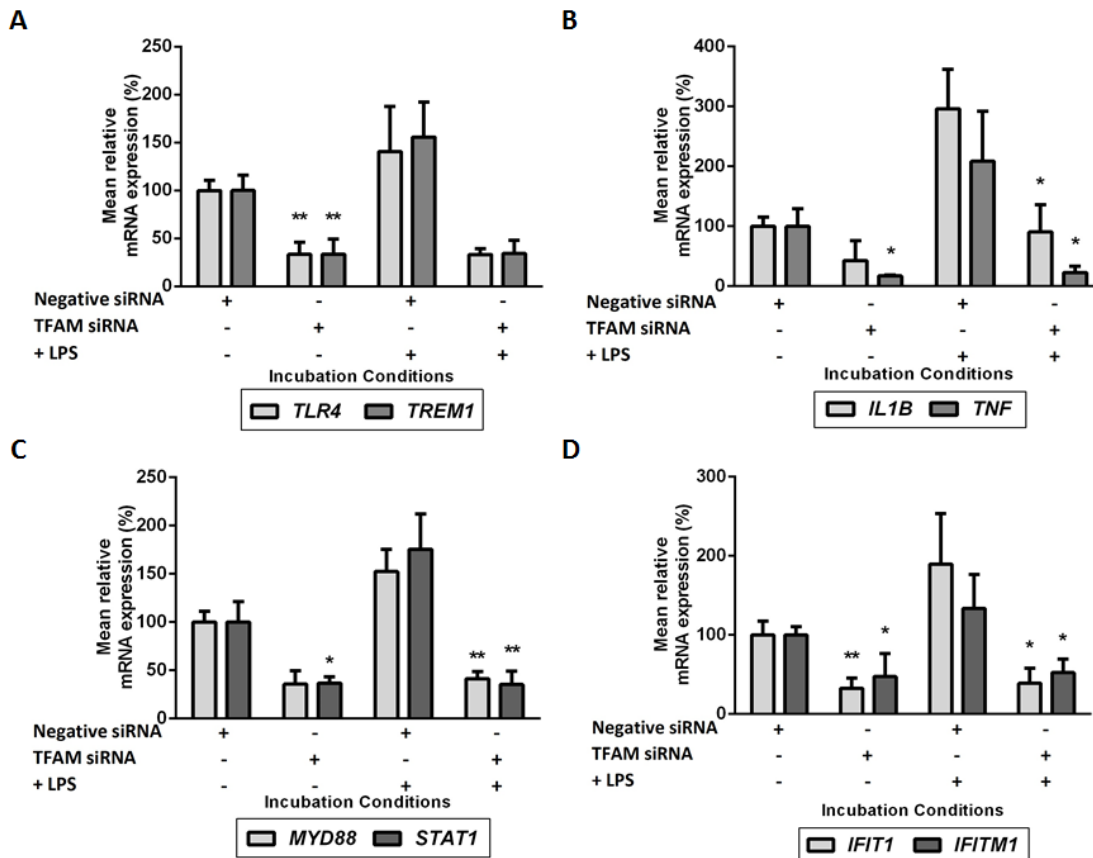
### 8.2.4 Confirmation of the down-regulation of gene expression in *TFAM* siRNA transfected THP-1 cells

Following the analysis of the RNA-Seq data the levels of mRNA transcription of specific genes identified to be significantly differentially expressed between the different conditions was confirmed using RTqPCR. The expression of these genes was compared between THP-1 cells transfected with the negative siRNA and *TFAM* siRNA both before and after exposure to 100ng/ml LPS for 4 hours. The mRNA levels were normalised to those of the housekeeping gene *ACTB*, as this was found to be stably expressed between conditions in the RNA-Seq data (Figure 8.7). For immunologically relevant genes encoding cell membrane receptors (*TLR4*, *TREM1*), pro-inflammatory cytokines (*IL1B*, *TNF*), inflammatory signalling molecules (*MYD88*, *STAT1*) and interferon signalling molecules (*IFIT1*, *IFITM1*) the RTqPCR data followed the trends seen in the RNA-Seq analysis. In general THP-1 cells with mtDNA depletion due to transfection with *TFAM* siRNA had reduced expression of these genes and an impaired ability to up-regulate this gene expression in response to treatment with LPS (Figure 8.8).



**Figure 8.7 Stable expression of *ACTB* between conditions in RNA-Seq data.**

THP-1 cells were transfected with 30nM negative or *TFAM* siRNA for 8 days and the gene expression assessed by RNA-Seq following a final 4 hour treatment with 100ng/ml LPS or medium. The mean normalised read count for the number of transcripts from the *ACTB* housekeeping gene is displayed. The significance of the differences between conditions was assessed by one-way ANOVA ( $p=0.924$ ).



**Figure 8.8 The effect of transfection with TFAM siRNA on inflammatory gene transcription.** THP-1 cells were transfected with 30nM of negative siRNA or *TFAM* siRNA for 8 days and then treated with 100ng/ml LPS or medium for a further 4 hours. The level of inflammatory gene mRNA transcription relative to the housekeeping gene *ACTB* was determined by RTqPCR (n=3). The data is displayed as mean gene expression normalised to the mean in the medium-treated negative siRNA condition (1<sup>st</sup> bars). The statistical significance of the differences in gene expression between THP-1 cells transfected with negative siRNA and those transfected with *TFAM* siRNA was determined separately for medium and LPS-treated cells by an independent t-test for normal data and a Mann Whitney U test for non-normal data. (A) Cell membrane receptor genes: *TLR4* - pre-LPS p=0.002, post-LPS p=0.100; *TREM1* – pre-LPS p=0.007, post-LPS p=0.100. (B) Cytokine genes: *IL1B* - pre-LPS p=0.055, post-LPS p=0.011; *TNF* – pre-LPS p=0.038, post-LPS p=0.018. (C) Inflammatory signalling genes: *MYD88* – pre-LPS p=0.100, post-LPS p=0.008; *STAT1* – pre-LPS p=0.026, post-LPS p=0.133. (D) Interferon signalling genes: *IFIT1*: pre-LPS p=0.006, post-LPS p=0.017; *IFITM1* – pre-LPS p=0.043, post-LPS p=0.039. All differences are non-significant except; \*p<0.05, \*\*p<0.01.

## 8.3 Discussion

### *8.3.1 Transfection with siRNA alters the THP-1 cell transcriptome*

Despite the lack of effect on the mitochondrial and immune function assays in Chapter 7, transfection with 30nM Silencer® Select Negative Control siRNA number 1 resulted in significant alterations to the THP-1 cell transcriptome. In particular the process of transfection appeared to activate canonical signalling pathways involved in pathogen recognition and cellular immunity. The commercial negative control siRNA used in these experiments has been designed to minimise any interaction with the nucleotide sequences on mRNA transcribed from the human genome. Nonetheless, studies have indicated that siRNA can have numerous 'off-target' effects on gene expression as well as potentially acting as a stimulus for antiviral immune responses (Jackson *et al.*, 2003; Jacobsen *et al.*, 2009). Furthermore, a delivery system is required to facilitate the uptake of siRNA into cells and studies have also suggested that these systems can have cytotoxic effects (Gilmore *et al.*, 2006; Akhtar and Benter, 2007). In particular cationic lipid delivery methods, including the Lipofectamine® system used in these experiments, have been found to produce significant effects on the transcriptome, including alterations in signalling pathways involved in the cellular stress response, immunity, inflammation and autophagy (Jacobsen *et al.*, 2009; Fiszler-Kierzkowska *et al.*, 2011; Mo *et al.*, 2012). These findings highlight the limitations of using siRNA transfection techniques, particularly when assessing cellular immune functions, and the vital importance of controlling for the effects of siRNA transfection during the experimental design.

### *8.3.2 Down-regulation of inflammatory signalling pathways in TFAM siRNA transfected THP-1 cells*

Elsewhere in this thesis, the depletion of mtDNA, through treatment with ethidium bromide or transfection with siRNA targeted against *TFAM*, has been found to reduce the ability of THP-1 cells to generate an immune response to an inflammatory stimulus. In agreement with these findings in this chapter it is shown that, in parallel with the depletion of mtDNA, transfection with *TFAM* siRNA produces striking effects on the THP-1 cell transcriptome. In particular, there is a significant down-regulation of key immune signalling pathways which summatively predict the reduced response to

LPS that is seen in the cytokine release experiments in Chapter 7. Furthermore, there are significant quantitative, but not qualitative, differences in the transcriptomic response of *TFAM* siRNA-transfected THP-1 cells to treatment with LPS. In these cells considerably fewer genes display altered expression after exposure to LPS and similar canonical signalling pathways are activated but to a lesser degree than is seen in the control conditions.

### 8.3.3 Linking mtDNA depletion with down-regulation of immune signalling pathways

The profound changes in the THP-1 cell nuclear transcriptome that are seen following mtDNA depletion suggest that the disruption of communication between the mitochondrial and nuclear genomes may lead to significant effects on nuclear gene expression. Cross-talk between the genomes is already known to be a pre-requisite for effective mitochondrial biogenesis and respiration, as this process ensures that the expression of mitochondrial genes on nuclear DNA and mtDNA is closely coordinated (Lee and Wei, 2005). Nuclear genes encode vital proteins that govern many mitochondrial functions, for example the antioxidants that control mitochondrial ROS levels and the transcription factors that regulate the replication and transcription of mtDNA (Kotiadis *et al.*, 2014). Conversely, signals generated by mitochondria can also alter nuclear gene expression (Liu and Butow, 2006). This process is termed retrograde signalling and is particularly important for the generation of homeostatic responses during conditions causing mitochondrial stress and dysfunction (Jazwinski, 2013). While the precise pathways involved in this mitochondria-to-nucleus communication are not established, the changes in the nuclear transcriptome that occur in *TFAM* siRNA transfected THP-1 cells suggest that retrograde signalling may influence cellular processes that are not directly involved in mitochondrial homeostasis (Kotiadis *et al.*, 2014). In particular the mtDNA depletion-mediated alterations in retrograde signals appear to lead to a striking down-regulation in the expression of genes involved in canonical pathways that are involved in immune and inflammatory responses.

This inhibition of immune signalling pathways in THP-1 cells following mtDNA depletion is consistent with the emerging understanding that mitochondria are able to exert important influences on the innate immune response in a number of different ways (Weinberg *et al.*, 2015). The outer mitochondrial membrane provides a vital platform for inflammatory signalling interactions, including the transduction of



antiviral responses via the mitochondrial antiviral signalling (MAVS) protein (Sun *et al.*, 2006; Tait and Green, 2012). The release of cytokines is modified by alterations in the levels of respiratory chain metabolites and ROS generated by mitochondria (West *et al.*, 2011b; Tannahill *et al.*, 2013). Moreover, during conditions of cellular stress the release of mitochondrial contents, including mtDNA, into the cytosol acts as a potent stimulus for both pro-inflammatory cytokine production, through NLRP3 inflammasome formation, and the induction of antiviral responses (Zhou *et al.*, 2011; West *et al.*, 2015). In addition to altered retrograde signalling, it is possible that the loss or disruption of these mitochondrially-driven processes may contribute to the impaired immune signalling that is found in THP-1 cells depleted of mtDNA following transfection with *TFAM* siRNA.

In the following sections the specific immune pathways that are particularly affected by mtDNA depletion are analysed.

#### ***8.3.4 Pattern recognition receptor (PRR) signalling***

During an infection the recognition of the presence of an invading pathogen is fundamental to the induction of an immune response. In monocytes pathogen recognition occurs due to the binding of PAMPs or DAMPs to cell surface or cytosolic PRRs (Czerkies and Kwiatkowska, 2014). Therefore, the reduced expression of genes encoding PRRs and their associated signalling pathways in *TFAM* siRNA transfected cells has the potential to profoundly inhibit the THP-1 cell immune response.

In particular, the inhibition of signalling via TLR-4, the PRR for LPS, could explain the blunted response to treatment with LPS that is seen in these cells. During the induction of endotoxin tolerance a transient fall in the cell surface expression of TLR-4 by monocytes has been reported (Moreno *et al.*, 2004; Sun *et al.*, 2014). However, this finding is inconsistent and there are a number of studies which indicate that monocyte TLR-4 expression is actually up-regulated during sepsis, an observation that does not appear to vary with either the duration or severity of the sepsis injury (Brandl *et al.*, 2005; Brunialti *et al.*, 2006; Schaaf *et al.*, 2009). This suggests that the functional significance of the potential down-regulation of TLR-4 signalling in *TFAM* siRNA transfected THP-1 cells is uncertain and requires further investigation.

In addition to the changes in *TLR-4* expression, *TFAM* siRNA transfected THP-1 cells also had a down-regulation of intracellular PRR signalling pathways. There has been considerable interest in the ability of mitochondrial proteins and mtDNA released from damaged mitochondria to act as DAMPs and induce inflammation through binding to intracellular PRRs (Galluzzi *et al.*, 2012). In particular, mtDNA has been found to trigger TLR-9 signalling pathways during the initiation of NLRP3 inflammasome formation (Zhang *et al.*, 2010). While altered TLR-9 signalling in mtDNA depleted THP-1 cells would, therefore, be of potential relevance to the mechanism underlying the impaired immune responses of these cells, the *TLR-9* gene is known to be expressed at a low level in monocytes and the transcription of mRNA from this gene was not significantly altered by transfection with *TFAM* siRNA (Hornung *et al.*, 2002). In addition, when assessing the significance of the changes in signalling via other cytosolic PRRs there is a concern that the introduction of foreign RNA into the cell during siRNA transfection may be an important confounding factor, particularly when assessing PRRs that detect cytosolic RNA such as TLR-3 and TLR-7 (Jacobsen *et al.*, 2009).

### ***8.3.5 Interferon signalling and monocyte immunity***

Two major interferon signalling pathways are significantly down-regulated in THP-1 cells transfected with *TFAM* siRNA. Interferons are important cytokines that modulate the immune response during an infection (Schroder *et al.*, 2004). The production of type I interferons by infected cells stimulates key antiviral defences in surrounding cells and activates the adaptive immune system (Ivashkiv and Donlin, 2014). On the other hand, interferon- $\gamma$  (IFN $\gamma$ ), the only type II interferon, is released by natural killer cells and T-lymphocytes and has a crucial role in the activation of monocytes and macrophages (Schroder *et al.*, 2004). The priming of human monocytes and THP-1 cells by *in vitro* treatment with IFN- $\gamma$  has been found to enhance pro-inflammatory responses, including LPS-induced TNF $\alpha$  release, and prevent the induction of endotoxin tolerance (Adib-Conquy and Cavaillon, 2002; Kurihara and Furue, 2013). In *ex vivo* studies treatment with IFN- $\gamma$  has also been shown to restore the ability of PBMCs and monocytes isolated from patients with severe sepsis to produce TNF $\alpha$  in response to LPS (Turrel-Davin *et al.*, 2011; Allantaz-Frager *et al.*, 2013). Furthermore, two small clinical trials have suggested that IFN- $\gamma$  treatment may both restore monocyte immune functions, including LPS-induced TNF $\alpha$  release, and potentially lead

to improved clinical outcomes in septic patients with evidence of monocyte deactivation (Döcke *et al.*, 1997; Nakos *et al.*, 2002). These findings suggest that the specific down-regulation of interferon signalling pathways seen in THP-1 cells with mtDNA depletion may contribute to their impaired ability to respond to inflammatory stimuli. Consequently, the stimulation of these interferon signalling pathways by treatment with IFN $\gamma$  has the potential to improve the ability of mtDNA-depleted cells to generate an adequate immune response.

### ***8.3.6 Triggering receptor expressed on myeloid cells-1 (TREM1) signalling and monocyte deactivation***

TREM1 is an activating receptor that is found on the cell surface of monocytes and neutrophils (Arts *et al.*, 2013). During an infection the stimulation of TREM1 on monocytes activates signalling pathways that result in the induction of phagocytosis and the release of pro-inflammatory cytokines and chemokines (Tessarz and Cerwenka, 2008). TREM1 activity is down-regulated by matrix metalloproteases which cleave it from the cell membrane to produce soluble TREM1, a molecule with anti-inflammatory effects (Gomez-Pina *et al.*, 2012).

The importance of TREM1 in the immune response has been highlighted by a murine pneumonia sepsis model in which the activation of TREM1 was associated with an enhanced inflammatory response, earlier bacterial clearance and improved survival (Lagler *et al.*, 2009). On the other hand, the down-regulation of TREM1 expression on the surface of monocytes has been implicated in the induction of endotoxin tolerance both in *in vitro* studies and in patients with cystic fibrosis (del Fresno *et al.*, 2008; Gomez-Pina *et al.*, 2012). This is consistent with findings from studies on patients with sepsis which indicate that an early decrease in TREM1 expression on the surface of monocytes and neutrophils is associated with adverse outcomes, including increased mortality (Oku *et al.*, 2013; Marioli *et al.*, 2014). Thus, the down-regulation of TREM1 signalling pathways could contribute to the impairment of THP-1 cell immune responses that occurs in parallel with mtDNA depletion after transfection with *TFAM* siRNA.

## 8.4 Conclusion

This analysis of the THP-1 cell transcriptome provides novel and powerful insights into the effects of siRNA transfection and mtDNA depletion on nuclear gene expression. After allowing for the 'off-target' effects of siRNA transfection there are significant alterations in the transcriptome of THP-1 cells with *TFAM* siRNA-induced mtDNA depletion. These changes lead to a marked down-regulation of immune canonical signalling pathways and a blunted activation of these pathways after treatment with LPS, findings that are consistent with the impaired immune phenotype of these cells. Further investigations are required to clarify the precise mechanisms by which mtDNA depletion alters nuclear gene expression and determine the particular immune pathways that are most important in the aetiology of immune deactivation in THP-1 cells with mtDNA depletion.

## Chapter 9 Discussion

### 9.1 Overview

This chapter begins with a review of the context and implications of the principal findings presented in this thesis, which complements the detailed discussions that have been included in each of the results chapters. The main strengths and limitations of the research are then detailed before considering the most relevant further investigations that will be required to confirm and explore the novel findings that have been discussed.

### 9.2 The relationship between mitochondria and immunity in human monocytic cells

Many of the adverse clinical outcomes in sepsis appear to occur as a consequence of an aberrant host immune response rather than direct effects of the invading pathogen (Kox *et al.*, 2000). The present paucity of therapies that effectively modulate this immune response reflects the incomplete understanding of the underlying pathophysiology that is present when an infection causes sepsis (Angus, 2011). Many patients with sepsis have evidence of immune suppression and it appears that deactivation of blood monocytes is particularly important in this process (Scheffold *et al.*, 2008b; Hotchkiss *et al.*, 2013a). While septic patients with monocyte deactivation are more likely to suffer nosocomial infections and die, the mechanisms by which monocyte immunity is impaired are currently poorly understood (Monneret *et al.*, 2006; Hall *et al.*, 2011). Small observational studies have suggested that septic monocytes may have mtDNA depletion and impaired mitochondrial respiration but the association between these findings and immune deactivation has been unclear (Pyle *et al.*, 2010; Japiassú *et al.*, 2011).

In this thesis the hypothesis that monocyte deactivation may be caused by mitochondrial dysfunction and mtDNA depletion has been examined. Two separate approaches, predominantly using *in vitro* models in THP-1 cells, were used to test this hypothesis. In Chapter 4 and Chapter 5 the effect of inducing immune deactivation on mitochondrial functions was explored, before carrying out an assessment of the consequences of mtDNA depletion for immunity in Chapter 6, Chapter 7 and Chapter

8. The two approaches produced contrasting results that highlight the complexities of the relationships between mitochondria and innate immunity during sepsis.

In Chapter 4 the endotoxin tolerance model was successfully used to induce a temporary state of immune deactivation in THP-1 cells resembling that seen in septic monocytes. However, there was no evidence of mtDNA depletion in the endotoxin tolerant THP-1 cells. Instead, a sustained induction of mitochondrial biogenesis was observed after treatment with LPS, which occurred in parallel with an activation of mitophagy and a shift to an anti-inflammatory immune phenotype. The resultant selective replacement of dysfunctional mitochondria may have contributed to the resolution of LPS-induced oxidative stress and the apparent improvement in mitochondrial efficiency, as evidenced by an increase in mitochondrial oxygen consumption despite an unchanged mitochondrial mass, which occurred during the time course experiment. These results could not be confirmed in human monocytes from healthy volunteers during the LPS inhalation study detailed in Chapter 5, as this technique failed to produce any significant change in the phenotype of blood monocytes.

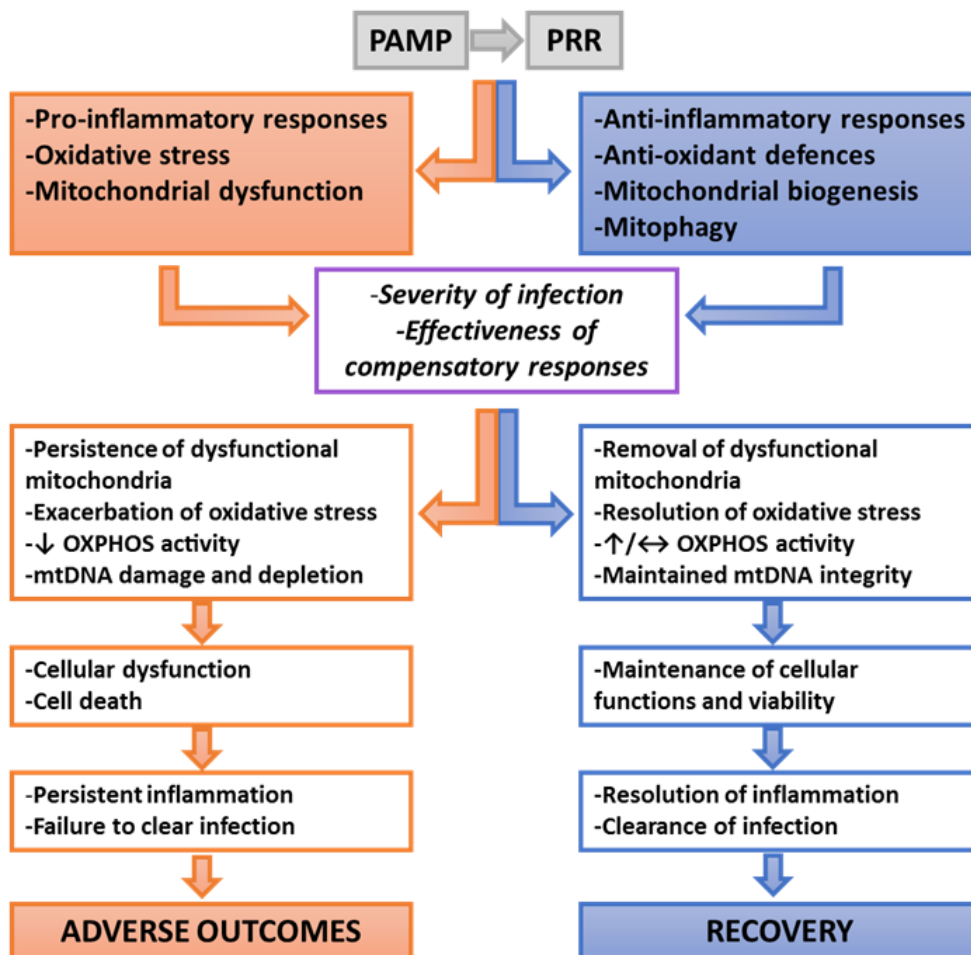
Thus, exposure of THP-1 cells to an inflammatory stimulus was found to trigger the simultaneous induction of a number of compensatory pro-survival responses, including those aimed at limiting inflammation and those involved in maintaining mitochondrial homeostasis. These results support the observation that survivors from sepsis have evidence of an early induction of mitochondrial biogenesis in muscle biopsy samples (Carré *et al.*, 2010). Moreover, they suggest that the findings from animal models, which indicate that the induction of mitochondrial biogenesis and mitophagy is an essential component of the recovery from an inflammatory insult that is linked with the activation of anti-inflammatory gene transcription, are relevant for human immune cells (Piantadosi *et al.*, 2011; MacGarvey *et al.*, 2012; Carchman *et al.*, 2013).

While mitochondrial biogenesis was activated during endotoxin tolerance, in Chapter 6 and Chapter 7 THP-1 cells with mtDNA depletion and impaired OXPHOS activity, induced by both treatment with ethidium bromide and transfection with *TFAM* siRNA, were found to have impaired immune responses. In particular, mtDNA depletion was associated with a reduction in the LPS-induced release of the pro-inflammatory cytokine TNF $\alpha$ , the key phenotypic biomarker of monocyte deactivation that is

associated with adverse outcomes in sepsis. Furthermore, the restoration of mtDNA copy number following removal of *TFAM* siRNA occurred in parallel with the recovery of the ability of the THP-1 cells to release TNF $\alpha$ . This link between mtDNA depletion and impaired immunity was supported by the analysis of gene expression data in Chapter 8, which indicated that transfection with *TFAM* siRNA had a significant inhibitory effect on key innate immune signalling pathways and lead to an attenuated transcriptomic response to LPS.

These investigations show for the first time that mtDNA depletion can directly impair the ability of human monocytic cells to produce an immune response and specifically inhibit the transcription of nuclear genes involved in innate immune signalling. This immune deactivation in mtDNA depleted THP-1 cells is consistent with the evidence that mitochondria have an important role in the propagation of inflammatory signals, the induction of anti-viral responses and the generation of effector innate immune responses (Sun *et al.*, 2006; West *et al.*, 2011b; Tait and Green, 2012; West *et al.*, 2015). The profound effect of mtDNA depletion on nuclear gene transcription also highlights the importance of cross-talk between the nuclear and mitochondrial genomes in the integration of metabolism with cellular processes, including inflammation and immunity (Kotiadis *et al.*, 2014).

In overall terms, the results presented in this thesis indicate that there are likely to be complex and variable interactions between mitochondria and the innate immune response during sepsis. Alterations in mitochondrial homeostasis have been found to produce significant effects on the immune functions of THP-1 cells. Both the activation of mitochondrial biogenesis, with a resultant increase in mtDNA copy number and mitochondrial respiration, and depletion of mtDNA, leading to impaired OXPHOS activity, have been found to be associated with evidence of immune deactivation. It may be the case that both of these processes are relevant in the mechanisms underlying sepsis-induced monocyte deactivation, with the exact contribution of each depending a number of factors, including the severity and duration of the sepsis illness (Figure 9.1). Further investigations that build on the key findings in this thesis have the potential to advance the understanding of the pathophysiology of sepsis-induced monocyte deactivation and the precise roles that mitochondria play in this process.



**Figure 9.1 Summary of the proposed consequences of the compensatory responses to an infection in monocytes.** During an infection the binding of PAMPs to PRRs leads to the recognition of the presence of an invading pathogen. Within monocytes this produces a simultaneous activation of both pro-inflammatory responses, which are aimed at eliminating the pathogen but also cause mitochondrial dysfunction and oxidative stress, and compensatory responses, which are aimed at maintaining homeostasis and include anti-inflammatory responses, antioxidant defences, mitochondrial biogenesis and mitophagy. The outcome depends on the severity of the infection and the effectiveness of these compensatory responses. In a milder infection with adequate compensation dysfunctional mitochondria are degraded and replaced, mitochondrial respiration and mtDNA integrity are maintained and the monocyte remains functional. However, in a severe infection the compensatory responses may be excessive, leading to a shift to an anti-inflammatory phenotype, or inadequate. With inadequate compensation dysfunctional mitochondria are not replaced, mitochondrial respiration is impaired, mtDNA becomes depleted and oxidative stress persists. In this situation, mtDNA depletion results in the inhibition of innate immune signalling which exacerbates the pre-existing down-regulation of pro-inflammatory responses, leading to monocyte deactivation and adverse clinical outcomes.



### 9.3 Strengths of research

The investigations described in this thesis provide important and novel insights into the potential interactions between monocyte immunity and mitochondria during inflammation and sepsis. Sepsis is an important clinical problem with persistently poor outcomes and a lack of effective treatments and these investigations have the potential to improve the understanding of the causes and consequences of sepsis-induced monocyte deactivation and mitochondrial dysfunction. A thorough assessment of THP-1 cell immune and mitochondrial functions has been carried out using a range of well-validated techniques and a variety of experimental models. Investigating these processes in human cells also makes the findings potentially more relevant for patients with sepsis than those from animal sepsis models, which have been found to translate poorly to human disease (Seok *et al.*, 2013).

The endotoxin tolerance model effectively produced a transient state of immune deactivation in THP-1 cells that closely resembles the phenotype of septic monocytes and consequently was able to provide insights into the role of mitochondria in this process (Cavaillon and Adib-Conquy, 2006; Hall *et al.*, 2013). The time course design of this experiment facilitated a detailed observation of the evolution of, and interactions between, various cellular reactions to an inflammatory stimulus, with a particular focus on a broad range of mitochondrial responses. Many of the effects of LPS on mitochondria that were measured support the observations from animal sepsis models, but critically do this in immunologically-relevant human cells.

The investigations into the effects of mtDNA depletion on THP-1 cell immunity provide original advances in the understanding of the associations between mitochondrial homeostasis and the immune response. In Chapter 6 and Chapter 7 mtDNA was effectively depleted from THP-1 cells using two very different techniques that produced generally consistent results. In addition to confirming the effects of mtDNA depletion on mitochondrial genetics and respiration, the immune function of THP-1 cells was assessed on both a functional and transcriptomic level. The use of RNA-Seq produced a holistic assessment of the effects of mtDNA depletion on the entire THP-1 cell transcriptome that avoided the bias of restricting the analysis to pre-selected genes of interest (Wang *et al.*, 2009). The observations that mtDNA depletion can

directly impair innate immunity and down-regulate immune signalling pathways offer important new insights into the consequences of sepsis-induced mitochondrial dysfunction for blood monocytes.

#### 9.4 Limitations of research

THP-1 cells provide a convenient, homogenous *in vitro* monocyte model that shares many phenotypic features with primary human monocytes (Altieri and Edgington, 1988). However, there are significant limitations that weaken the application of findings from THP-1 cells to human disease. THP-1 cells are an immortalised human pro-monocytic leukaemia cell line that were first isolated from man over 30 years ago (Tsuchiya *et al.*, 1980). In addition to the original oncogenic mutations the cells are likely to have undergone a substantial phenotypic and genetic drift as they have adapted to growth *in vitro* (Burdall *et al.*, 2003). As a result, there are potentially broad differences between a cell line, such as THP-1 cells, and the equivalent primary cells. For example, a study has shown that there were significant alterations in over half of the proteome, including proteins involved in metabolism and mitochondrial function, when mouse hepatocytes were compared to a murine hepatoma cell line (Pan *et al.*, 2009). More specifically for THP-1 cells, important differences in their immunological capabilities compared to primary monocytes have been described recently (Schildberger *et al.*, 2013). In particular it has been shown that, although the TNF $\alpha$  response is relatively preserved, THP-1 cells produce significantly less IL-8 and no IL-6 or IL-10 in response to LPS, findings that are consistent with the cytokine release experiments detailed in this thesis. It is clear, therefore, that all observations in THP-1 cells must be confirmed in primary human monocytes before any firm conclusions regarding their relevance to human disease can be made.

In addition to using THP-1 cells, the majority of the investigations in this thesis are also limited by their *in vitro* design. In general cell culture is carried out at low density using enriched medium in order to facilitate rapid cell division, conditions that are not usually present *in vivo* (Geraghty *et al.*, 2014). Furthermore, the lack of both tissue architecture and other cell types during each experiment mean that the conditions fail to model those occurring in a complex, multicellular organism (Hartung and Daston,

2009). These limitations can be addressed by translating the results from *in vitro* experiments into *in vivo* models or clinical samples from patients with sepsis.

There are also important problems with using the model of endotoxin tolerant THP-1 cells to investigate the changes occurring during the deactivation of septic monocytes. Although the immune phenotype of these cells does approximate that of septic monocytes, it is clear that this model does not adequately reflect the complex processes occurring during human sepsis (Lopez-Collazo and del Fresno, 2013). In particular, a single treatment with a sterile stimulus in the form of LPS does not resemble the sustained exposure to a myriad of potent inflammatory stimuli that occurs during sepsis (Kox *et al.*, 2000). Furthermore, the investigations into mitochondrial functions and turnover that were carried out on endotoxin tolerant THP-1 cells were mostly observational and did not assess the causation or mechanisms behind the changes that were seen. Finally, an attempt was made to confirm the results from the endotoxin tolerant THP-1 cells using the *in vivo* LPS inhalation model on healthy volunteers, but this proved to be unhelpful as it failed to produce any significant effects on blood monocytes.

In patients with sepsis a depletion of monocyte mtDNA copy number to around 75% of the level in healthy controls has been measured (Pyle *et al.*, 2010). However, the mtDNA depletion experiments presented in this thesis produced a significantly greater fall in THP-1 cell mtDNA copy number, with a complete loss of mtDNA after treatment with ethidium bromide and a reduction to around 25% of control samples after transfection with *TFAM* siRNA. This may mean that the effects on mitochondrial and immune functions seen in these cells is not reflective of those occurring in septic monocytes. Moreover, while the RNA-Seq data confirms that mtDNA depletion inhibits immune signalling pathways and blunts the transcriptomic response to LPS, these observations do not establish the precise mechanisms by which mtDNA depletion effects nuclear gene transcription and THP-1 cell immune responses.

## 9.5 Future work

### 9.5.1 *The link between immunity, mitochondrial biogenesis and mitophagy in THP-1 cells after LPS treatment*

Further investigations are required in order to identify the key mechanisms behind the activation of mitochondrial biogenesis and mitophagy, which occurred concurrently with a shift towards an anti-inflammatory, endotoxin tolerant immune phenotype, following exposure of THP-1 cells to LPS in Chapter 4. To this end, exploration of a number of promising areas could improve the understanding of the fundamental processes governing mitochondrial homeostasis and immunity during inflammation.

These areas include;

1. *TLR-4 signalling*: Animal sepsis models suggest that the induction of both mitochondrial biogenesis and mitophagy is dependent on signalling via PRRs, which also appears to simultaneously activate both inflammatory and compensatory responses (Sweeney *et al.*, 2010; Bauerfeld *et al.*, 2012). In order to confirm these findings the effects of siRNA-mediated knock-down of *TLR-4* expression or chemical inhibition of the intracellular domain of TLR-4 on the response of THP-1 cells to LPS could be investigated (Zhou *et al.*, 2012; Oda *et al.*, 2014).
2. *Redox-sensitive signalling*: There is a body of evidence which suggests that the induction of mitochondrial biogenesis and mitophagy in murine sepsis models is linked to the up-regulation of anti-inflammatory cytokines through the activation of the redox-sensitive HO-1/Nrf2 pathway (Piantadosi *et al.*, 2011; Chang *et al.*, 2015). If it is confirmed that the HO-1 and Nrf2 proteins are up-regulated in THP-1 cells after LPS exposure, chemical inhibitors, such as zinc protoporphyrin IX for HO-1 and brusatol for Nrf2, or siRNA targeted against the *HMOX1* and *NFE2L2* genes could then be used to determine the effects of inhibiting this pathway on the LPS-induced responses of THP-1 cells (Rushworth and MacEwan, 2008; Ren *et al.*, 2011; Abdalla *et al.*, 2015).
3. *Sirtuins*: Nuclear SIRT1 and mitochondrial SIRT3 have been found to be sequentially activated after exposure to LPS, leading to the concomitant inhibition of inflammatory signalling and activation of mitochondrial biogenesis (Liu *et al.*, 2015). Providing that SIRT1 and SIRT3 up-regulation is confirmed, the potential for

these NAD<sup>+</sup>-dependent deacetylases to link immunity and metabolism could be assessed by observing the effects of the SIRT1 inhibitor EX527 on the responses of LPS-treated THP-1 cells (Vachharajani *et al.*, 2014).

4. *Mitophagy*: The activation of mitophagy, leading to the removal of dysfunctional mitochondria, has been found to be vital for both the recovery from the adverse effects of inflammation and the induction of mitochondrial biogenesis (Lee *et al.*, 2012; Carchman *et al.*, 2013). As an initial exploration of the importance of mitophagy, THP-1 cells could be transfected with siRNA targeted against the key autophagy regulator autophagy-related protein-7 (Atg7) before determining the effects of transiently inhibiting autophagy on THP-1 cell viability, immunity, mitochondrial biogenesis and respiration after treatment with LPS (Pattison *et al.*, 2011).

#### 9.5.2 Translating the findings from endotoxin tolerant THP-1 cells into human monocytes

It will be essential to confirm that the LPS-induced changes in immunity, mitochondrial biogenesis and mitophagy seen in THP-1 cells are also found in human blood monocytes. Firstly, *in vitro* experiments could be carried out to assess the effects of LPS on immune and mitochondrial functions in primary blood monocytes isolated from healthy volunteers. However, as the duration of these experiments is likely to be limited by the propensity for monocytes to differentiate into macrophages and dendritic cells during cell culture, the isolation of blood monocytes during an *in vivo* model of systemic inflammation may provide a more useful assessment (Sánchez-Torres *et al.*, 2001; Eligini *et al.*, 2013). In Chapter 5 it was shown that the inhalation of 60µg LPS inhalation did not produce any significant effects on blood monocytes. An alternative strategy could involve the intravenous administration of LPS to healthy volunteers, as this has been found to reliably produce a brisk systemic inflammatory response along with significant effects on the phenotype of blood monocytes, including the induction of endotoxin tolerance (de Vos *et al.*, 2009; Draisma *et al.*, 2009). An experimental study could be carried out in which healthy volunteers are given intravenous LPS and immune functions, cellular respiration and mitochondrial turnover are measured in blood monocytes isolated at serial time points. RNA-Seq could also be used to investigate the mechanistic links between these processes by a

serial analysis of the transcriptomic changes occurring in monocytes following LPS exposure (Wang *et al.*, 2009; Fairfax *et al.*, 2014).

### 9.5.3 Investigating impaired immune signalling in THP-1 cells with mtDNA depletion

In order to establish the mechanisms by which mtDNA depletion causes immune deactivation the significance of the down-regulation of key immune signalling pathways in mtDNA-depleted THP-1 cells needs to be explored in more detail.

Investigating the effects of interventions to reverse these changes could also identify treatments with the potential to improve immune functions in patients with sepsis-induced monocyte deactivation and mtDNA depletion

The significant inhibition of the TREM1 signalling pathway that was seen in *TFAM* siRNA transfected THP-1 cells should be firstly correlated with TREM1 protein and cell surface expression in these cells. TREM1 is a cell surface receptor that enhances TLR-mediated innate immune responses and decreased TREM1 levels have been detected in endotoxin tolerant and septic monocytes (del Fresno *et al.*, 2008; Marioli *et al.*, 2014). Treatment with 1, 25 dihydroxyvitamin D3 or the metalloprotease inhibitor GM6001 has been shown to increase TREM1 mRNA and cell surface expression in human monocytic cells (Gomez-Pina *et al.*, 2012; Lee *et al.*, 2015). Therefore, if TREM1 down-regulation is confirmed, the effect of treatment with these compounds on THP-1 cell immunity could then be examined in order to determine whether the impairment of TREM1 signalling is vital for mtDNA depletion-mediated immune dysfunction.

Two major interferon signalling pathways and signalling via PRRs, including TLRs, are also among the principal canonical pathways that are down-regulated in THP-1 cells with mtDNA depletion following *TFAM* siRNA transfection. If these alterations are confirmed at a protein level by Western blot, an investigation into the potential for treatment with IFN- $\gamma$ , a major activating cytokine for monocytes and macrophages, to reverse these effects would be valuable (Schroder *et al.*, 2004). An *in vitro* exposure of human monocytes to IFN- $\gamma$  has been found to result in increased cell surface expression of TLR-4, enhanced LPS-induced pro-inflammatory cytokine release and a resistance to the induction of endotoxin tolerance (Adib-Conquy and Cavaillon, 2002; Bosisio *et al.*, 2002; Southworth *et al.*, 2012). Furthermore, in septic patients with monocyte deactivation *ex vivo* and *in vivo* treatment with IFN-  $\gamma$  can restore LPS

induced TNF- $\alpha$  release (Döcke *et al.*, 1997; Nakos *et al.*, 2002; Allantaz-Frager *et al.*, 2013).

#### *9.5.4 Assessing the effect of mtDNA depletion on immunity in human monocytes and in vivo models*

In addition to establishing the mechanisms linking mtDNA levels and immunity, the negative effects of mtDNA depletion on THP-1 cell immunity need to be reproduced in blood monocytes and confirmed *in vivo*. The propensity of blood monocytes to rapidly differentiate when cultured *in vitro* would present considerable challenges for experiments that attempt to replicate the techniques used to deplete mtDNA in THP-1 cells in this thesis. In view of these difficulties, an assessment of immune functions in blood monocytes isolated from patients known to have mtDNA depletion may be a preferable approach to validating the findings from THP-1 cells. For example, the immune functions of monocytes isolated from patients with diseases caused by mutations, deletions or depletion of mtDNA could be assessed (Greaves *et al.*, 2012).

Due to the relative rarity and variable, but often severe clinical phenotype of mtDNA diseases in humans, it may be most appropriate to attempt to confirm the effects of mtDNA depletion on the *in vivo* immune response using an animal sepsis model (DiMauro and Schon, 2003). Homozygous *Tfam*<sup>-/-</sup> mice die during the embryonic stage, while targeted *Tfam* knockout leads to tissue-specific mtDNA depletion and severe OXPHOS impairment (Larsson *et al.*, 1998; Wang *et al.*, 1999). On the other hand, *Tfam*<sup>+/-</sup> mice have a reduction in mtDNA copy number by 34-50% and more modest mitochondrial respiratory dysfunction (Larsson *et al.*, 1998). These *Tfam*<sup>+/-</sup> mice have been used to demonstrate the importance of mtDNA stability for the antiviral innate immune response (West *et al.*, 2015). A murine model of sepsis, such as caecal ligation and puncture, could be used to compare the responses of *Tfam*<sup>+/-</sup> mice to those of wild-type mice, in order to establish whether mtDNA depletion due to *Tfam* deficiency can cause impaired innate immune signalling and monocyte deactivation *in vivo* (Warren, 2009).

#### *9.5.5 Confirmation of findings in human sepsis*

Ultimately, it will be vital to confirm the findings from cell culture and experimental medicine models in human patients with sepsis. To date, most of the clinical literature

describing the function and turnover of mitochondria in sepsis consists of observations from small cross-sectional studies in which a restricted number of measurements are assessed (Jeger *et al.*, 2013). There are also very limited clinical data available on the concurrent assessment of mitochondria and immunity in blood monocytes during human sepsis (Belikova *et al.*, 2007). As a result, a well-designed longitudinal study in which serial measurements of monocyte immune and mitochondrial parameters are taken would be very valuable in understanding the dynamics and relationships of the changes occurring during human sepsis. In such a study the findings could be compared between groups with different illness severity and in survivors versus non-survivors, in order to identify those responses that are deficient or excessive in patients with adverse outcomes. Moreover, the immune and transcriptomic responses of monocytes from the subgroup of patients with sepsis-induced mtDNA depletion could be compared to those with maintained mtDNA copy number.

## 9.6 Concluding remarks

In this thesis an assessment of the role of mitochondrial dysfunction and mtDNA depletion in monocyte immune deactivation has been presented. Using *in vitro* models in THP-1 cells it has been shown that there is a complicated relationship between mitochondria and monocytic cell immunity. On the one hand, the novel discovery that mtDNA depletion produces a profound inhibition of innate signalling pathways that results in a blunted transcriptomic and functional immune response has been detailed. However, it has also been shown that the down-regulation of pro-inflammatory immune responses may be closely integrated with the activation of mitochondrial biogenesis during the compensatory responses to an inflammatory insult. Future research should focus on exploring the fundamental mechanisms by which mitochondria influence the immune response and on confirming the findings from THP-1 cells in human monocytes and patients with sepsis. Ultimately, an improved understanding of the interactions between mitochondria and immunity may suggest novel therapies to improve the currently poor outcomes of patients with sepsis-induced monocyte deactivation.



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## Appendix A: Abstracts for scientific meetings

Abstract for oral presentation at British Infection Association Spring Conference (London, UK; May 2014)

### *Title*

Reduced LPS-induced TNF $\alpha$  release by THP-1 cells depleted of mitochondrial DNA

### *Authors*

John D Widdrington <sup>1,2,3</sup>, Aurora Gomez Duran <sup>2</sup>, Angela Pyle <sup>2</sup>, Marie-Helene Ruchaud-Sparagano <sup>1</sup>, Patrick F Chinnery <sup>2,3</sup>, A John Simpson <sup>1,3</sup>

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### *Introduction*

In sepsis monocyte immune deactivation is associated with increased mortality and susceptibility to secondary infections. There is increasing evidence of depletion of mitochondrial DNA (mtDNA) and impaired mitochondrial respiration in monocytes from septic patients. This study aimed to assess the links between mtDNA depletion and immunity in human monocytic THP-1 cells.

### *Method*

To selectively deplete their mtDNA THP-1 cells were incubated with 50ng/ml ethidium bromide (EtBr) for 8 weeks. After confirming the effects of EtBr on mitochondria, immune responses were assessed by measuring lipopolysaccharide (LPS)-induced cytokine release and phagocytosis of fluorescent *Staphylococcus aureus* (SA).

### *Results*

Incubation with EtBr successfully generated THP-1 cells lacking mtDNA (termed p0 cells). There was a selective depletion of mtDNA-encoded RNA transcription and protein expression in these cells. In addition p0 THP-1 cells had complete loss of

activity in mitochondrial respiratory chain complex IV, which contains key mtDNA-encoded subunits. LPS-induced release of the pro-inflammatory cytokine tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) was significantly reduced but phagocytosis of SA was increased in  $\rho 0$  cells.

### *Discussion*

THP-1 cells depleted of mtDNA by treatment with EtBr produce significantly less TNF- $\alpha$  in response to LPS, a typical feature of deactivated monocytes in sepsis. However, mitochondrial depletion did not produce a global down-regulation of immune responses and phagocytosis was actually enhanced in  $\rho 0$  THP-1 cells. Further investigation into the complex effects of mitochondrial depletion on immunity may provide important insights into immune suppression in sepsis.

Abstract for poster presentation at ID Week 2014 Conference (Philadelphia, PA, USA; October 2014)

### *Title*

The Induction of Endotoxin Tolerance is Associated with the Activation of Mitochondrial Biogenesis in THP-1 Cells

### *Authors*

John D Widdrington <sup>1,2,3</sup>, Aurora Gomez Duran <sup>2</sup>, Angela Pyle <sup>2</sup>, Marie-Helene Ruchaud-Sparagano <sup>1</sup>, Patrick F Chinnery <sup>2,3</sup>, A John Simpson <sup>1,3</sup>

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### *Background*

In sepsis monocyte immune deactivation is associated with increased mortality and susceptibility to secondary infections. There is increasing evidence of mitochondrial depletion and dysfunction, including impaired respiration and oxidative stress, in monocytes in sepsis. Survival and recovery of cellular function following sepsis has been associated with the induction of mitochondrial biogenesis. Using endotoxin tolerance (ET), whereby repeated exposure to lipopolysaccharide (LPS) produces diminishing inflammatory responses, as a model of monocyte deactivation we investigated the link between immunity, respiration and mitochondrial biogenesis.

### *Methods*

THP-1 cells, a human monocytic cell line, were pre-incubated with 100ng/ml LPS from *Escherichia coli* O26:B6 for 0, 2, 6, 24, 48 and 72 hours. The ability of THP-1 cells to respond to a second inflammatory stimulus was then assessed in addition to measurements of oxygen consumption, oxidative stress and mitochondrial biogenesis.

## *Results*

Pre-incubation with LPS produced a change in THP-1 cell immune phenotype consistent with ET. In response to a second inflammatory stimulus there was reduced release of pro-inflammatory cytokines but increased anti-inflammatory cytokine release and phagocytosis. LPS exposure also resulted in evidence of early oxidative stress with recovery associated with the activation of antioxidant defences. Significant increases in mitochondrial DNA copy number and expression of mitochondrial transcription factor A following exposure to LPS suggest that mitochondrial biogenesis is induced during ET. In addition, after LPS exposure there was an increase in THP-1 cell oxygen consumption due to mitochondrial adenosine triphosphate generation.

## *Conclusions*

In association with a shift towards an anti-inflammatory phenotype there is evidence of the induction of mitochondrial biogenesis and anti-oxidant defences in ET THP-1 cells. Further investigation into the potential co-regulation of these pro-survival responses may provide important insights into the mechanisms of immune deactivation and cellular recovery in human monocytic cells following inflammatory insults in diverse conditions including sepsis.

# Poster No. 1353 The Induction of Endotoxin Tolerance is Associated with the Activation of Mitochondrial Biogenesis in THP-1 cells



John D Widdrington<sup>1,2</sup>, Aurora Gomez-Duran<sup>2</sup>, Angela Pyle<sup>2</sup>, Marie-Helene Ruchaud-Sparagano<sup>2</sup>, Patrick F Chinnery<sup>1,2</sup>, A John Simpson<sup>1,2</sup>  
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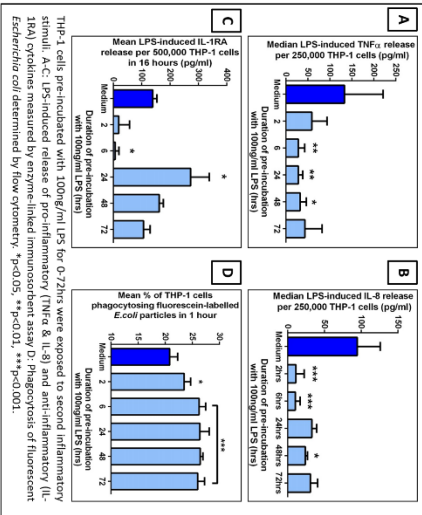
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## Introduction

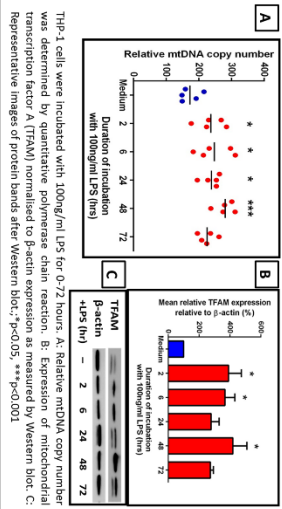
- Immune deactivation in blood monocytes is associated with increased mortality and susceptibility to nosocomial infection in patients with sepsis<sup>1</sup>.
- There is increasing evidence that septic monocytes also have impaired mitochondrial respiration and depletion of mitochondrial DNA, but the role of these changes in immune deactivation is not well understood<sup>2,3</sup>.
- In addition, survival and recovery of cellular functions in critical illness and animal sepsis models appears to be dependent on the induction of mitochondrial biogenesis<sup>4,5</sup>.
- The phenotype of deactivated septic monocytes resembles endotoxin tolerance (ET), whereby repeated exposure to lipopolysaccharide (LPS) triggers diminishing inflammatory responses<sup>6</sup>.
- A model of ET in THP-1 cells, a human monocyte leukaemia cell line, was used to explore the links between changes in immunity, cellular respiration and mitochondrial biogenesis after an inflammatory stimulus.

## Results

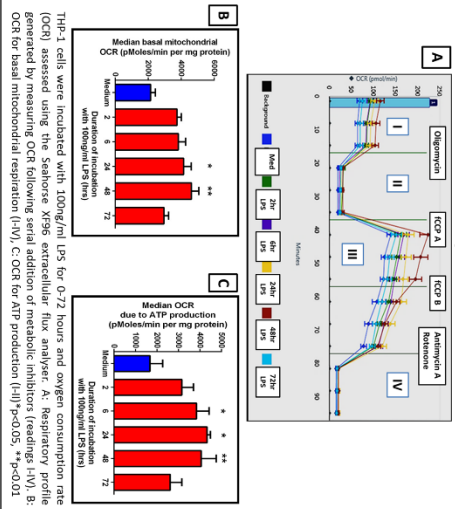
### Figure 1: Pre-incubation of THP-1 cells with LPS induces endotoxin tolerance with a shift towards an anti-inflammatory, pro-phagocytic phenotype



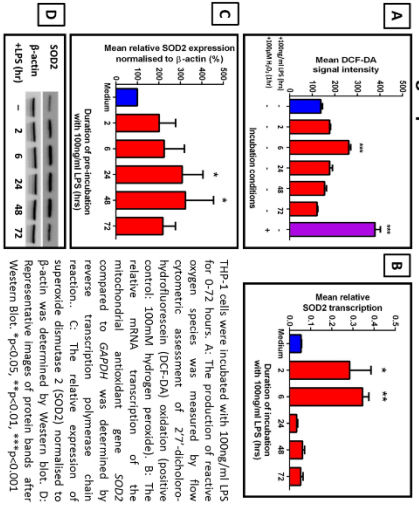
### Figure 2: Exposure to LPS leads to activation of mitochondrial biogenesis in THP-1 cells



### Figure 3: LPS increases THP-1 cell oxygen consumption due to mitochondrial ATP production



### Figure 4: The induction of antioxidant defences is associated with the resolution of oxidative stress following exposure of THP-1 cells to LPS



## Conclusions

- LPS stimulates a number of dynamic pro-survival mechanisms in THP-1 cells:

  - A shift towards an anti-inflammatory, pro-phagocytic immune phenotype consistent with endotoxin tolerance and resembling that displayed in deactivated septic monocytes<sup>7,8</sup>.
  - Stimulation of mitochondrial biogenesis leading to increased oxygen consumption during mitochondrial production of adenosine triphosphate
  - Induction of anti-oxidant defences to compensate for early oxidative stress

Further investigation into the potential co-regulation of these compensatory responses may provide important insights into the mechanisms of immune deactivation and cellular recovery in monocytes following inflammatory insults in diverse conditions including sepsis.

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Abstract for oral presentation at 25<sup>th</sup> European Conference of Clinical Microbiology and Infectious Diseases (Copenhagen, Denmark; April 2015)

*Title*

Mitochondrial DNA depletion in THP-1 cells is associated with impaired immune functions

*Authors*

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*Objectives*

This study aimed to assess the hypothesis that depletion of mitochondrial DNA (mtDNA) would lead to inhibition of THP-1 cell immune responses and had the following objectives;

1. To deplete mtDNA copy number in THP-1 cells by transfection with small-interfering RNA (siRNA) targeted against mitochondrial transcription factor A (TFAM).
2. To determine the effects of mtDNA depletion on THP-1 cell respiration and immune function.
3. To assess the ability of mtDNA copy number, cellular respiration and immune functions to recover following removal of TFAM siRNA.

*Methods*

THP-1 cells, a human monocytic leukaemia cell line, were transfected with 30nM TFAM siRNA. Control experiments included THP-1 cells transfected with a negative sequence siRNA and incubated with the transfection reagent Lipofectamine RNAiMAX. After 8 days mtDNA depletion was confirmed and the functional consequences of this

depletion were assessed by measuring mitochondrial protein expression and cellular oxygen consumption. THP-1 cell immunity was determined by quantifying lipopolysaccharide (LPS)-induced cytokine release and phagocytosis of fluorescent *Escherichia coli* (E.coli). The recovery of THP-1 cell functions was then assessed after a further 8 day incubation in the absence of TFAM siRNA.

### *Results*

Transfection with TFAM siRNA successfully depleted mtDNA from THP-1 cells. This was associated with a selective loss of mtDNA-encoded protein expression and a significant reduction in cellular oxygen consumption, particularly for mitochondrial respiration. Depletion of mtDNA was associated with a significantly reduced ability of THP-1 cells to both phagocytose E.coli and release the pro-inflammatory cytokine tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) in response to LPS. Removal of TFAM siRNA led to a compensatory increase in TFAM expression which resulted in recovery of mtDNA copy number, mtDNA-encoded protein expression and cellular oxygen consumption. LPS-induced TNF $\alpha$  release was restored in these cells but there was only a partial recovery in their phagocytic ability.

### *Conclusion*

Adverse outcomes in sepsis are associated with evidence of monocyte immune deactivation, including a reduced ability to release TNF $\alpha$  in response to LPS. However, the underlying mechanism behind this immune deactivation is not fully understood. Recent studies have indicated that septic monocytes have evidence of mtDNA depletion and mitochondrial respiratory dysfunction. Our results indicate that, in THP-1 cells, depletion of mtDNA and the resultant impaired mitochondrial respiration are associated with immune dysfunction. Furthermore, recovery of THP-1 cell mtDNA copy number and cellular respiration is associated with a restoration of LPS-induced TNF $\alpha$  release. Further research is required to determine the mechanisms linking mtDNA depletion to immune deactivation in monocytes. Ultimately therapeutic interventions to stimulate mitochondrial biogenesis may have the potential to provide novel interventions to restore immune function in septic monocytes.

## Appendix B: Lipopolysaccharide dose finding experiments

### Overview

In this appendix additional dose-finding experiments that were not included in the results section of Chapter 4 are detailed in order to display the data that informed the experimental design for the endotoxin tolerance time course experiments.

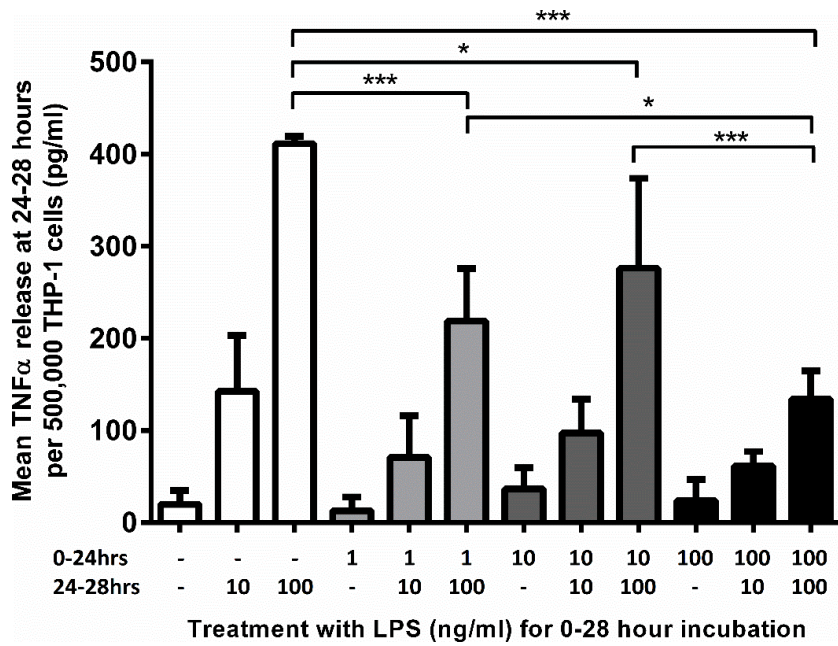
### Results

#### ***Optimal dose of LPS to induce endotoxin tolerance in THP-1 cells***

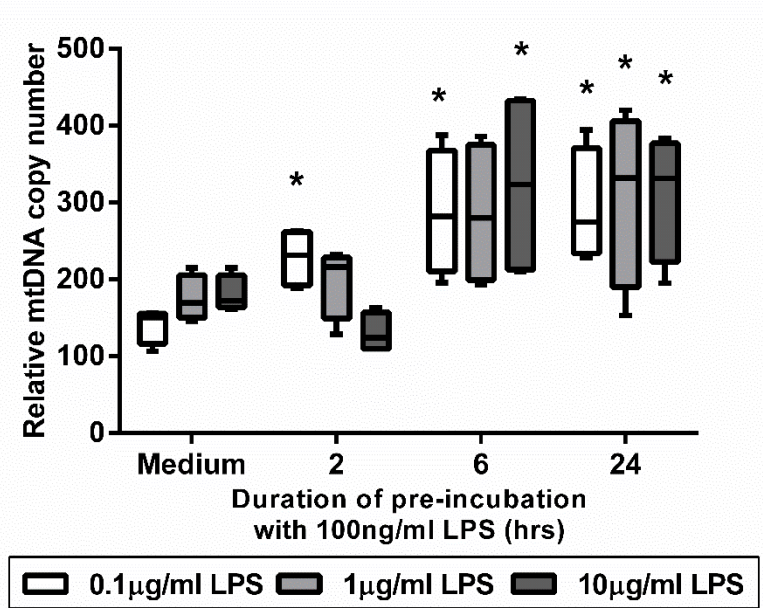
The dose of LPS that is required to produce endotoxin tolerance was optimised by pre-incubating THP-1 cells with 1, 10 and 100ng/ml for 24 hours and then measuring TNF $\alpha$  release in response to a second stimulus of 10 or 100ng/ml LPS for a further 4 hours. Pre-incubation with 100ng/ml produced the most significant decrease in TNF $\alpha$  secretion in response to a second exposure to 100ng/ml LPS (Supplementary Figure 1). These conditions were used in subsequent endotoxin tolerance experiments, with THP-1 cells being pre-incubated with 100ng/ml LPS for 2, 6, 24, 48 and 72 hours prior to measuring immune and mitochondrial functions.

#### ***The effect of LPS on mtDNA copy number***

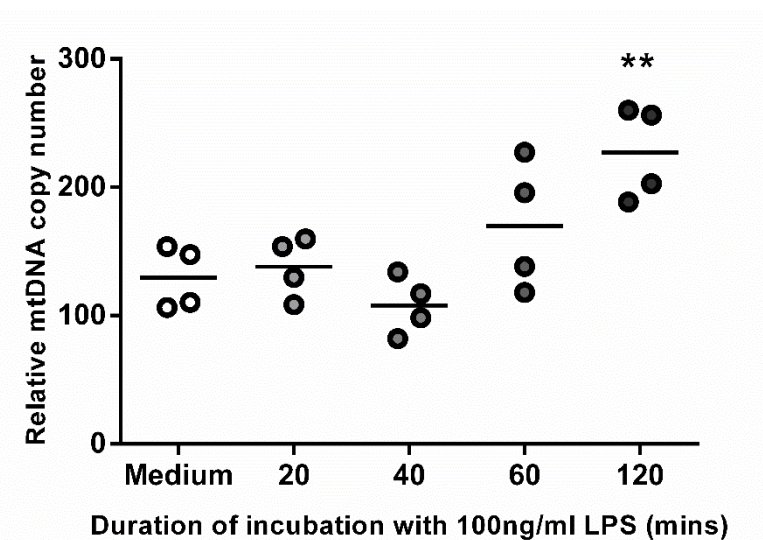
In contrast to findings in clinical and animal sepsis studies, in Chapter 4 exposure to 100ng/ml LPS did not result in any depletion of mtDNA, with mtDNA copy number instead increasing at 2-48 hours following treatment with LPS. To determine whether this effect was due to an inadequate dose of LPS, THP-1 cells were treated with 0.1 (100ng/ml), 1 and 10 $\mu$ g/ml LPS for 2, 6 and 24 hours. Although the LPS-induced increase in mtDNA copy number was delayed beyond 2 hours following exposure to LPS at concentrations above 100ng/ml there was no significant depletion of mtDNA copy number in any of the conditions (Supplementary Figure 2). The possibility that the initial 2 hour measurement time point missed an earlier depletion of mtDNA was also excluded as THP-1 cells treated with 100ng/ml LPS for 20, 40 and 60 minutes did not have any significant reduction in mtDNA copy number (Supplementary Figure 3).



**Supplementary Figure 1 Pre-incubation with 100ng/ml LPS produces optimal down-regulation of TNF $\alpha$  release in response to a subsequent LPS exposure.** THP-1 cells were incubated with medium or 1, 10 or 100ng/ml LPS for 24 hours before assessing TNF $\alpha$  release in response to a second stimulation with 10 or 100ng/ml LPS for a further 4 hours (24-28 hours) (n=6). The data are represented as mean  $\pm$  standard deviation TNF $\alpha$  release per 500,000 THP-1 cells and analysed by one-way ANOVA (p<0.001). All differences are non-significant with Tukey's multiple comparison test except; \*p<0.05, \*\*\*p<0.001.



**Supplementary Figure 2 Delayed increase in mtDNA copy number when THP-1 cells are exposed to LPS at concentrations greater than 100ng/ml.** THP-1 cells were incubated with LPS at a concentration of 0 (Medium), 0.1 (100ng/ml), 1 and 10μg/ml for 2, 6 and 24 hours and mtDNA copy number measured by quantitative PCR (n=4). Data are represented by box plots indicating 25<sup>th</sup> quartile, median and 75<sup>th</sup> quartile and whiskers indicating the range and analysed by two-way ANOVA ( $p < 0.001$ ). All differences are non significant with Tukey's multiple comparison test except; \*  $p < 0.05$ .



**Supplementary Figure 3 THP-1 cell mtDNA copy number is not significantly altered by exposure to 100ng/ml LPS for less than 120 minutes.** THP-1 cells were incubated with 100ng/ml LPS for 0, 20, 20, 60 and 120 minutes and mtDNA copy number was then determined by quantitative PCR (n=4). The data are represented as individual measurements with the lines indicating mean relative mtDNA copy number and analysed by one-way ANOVA ( $p = 0.001$ ). All differences are non-significant using Dunnett's multiple comparison test except; \*\*  $p < 0.01$ .