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Mechanisms of human innate immune cell death and consequent adaptive immune responses during *Mycobacterium tuberculosis* infection

A thesis submitted for the degree of
Doctor of Philosophy

by

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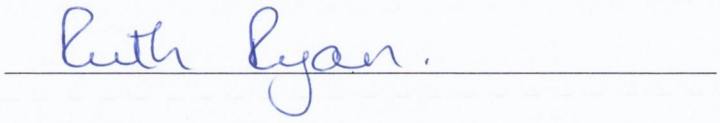
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Summary

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis, is a highly successful pathogen, infecting an estimated one-third of the global population. Mtb infection leads to the deaths of approximately 1.3 million people each year. Although 90% of immunocompetent infected people do not develop active tuberculosis disease upon infection, Mtb persists within the lungs and can lead to active disease decades after initial infection. An improved understanding of the interaction of Mtb with the human immune system is urgently required in order to effectively target new treatments and vaccines.

Mtb infects and replicates within alveolar macrophages. A major host defence against Mtb infection is death of the infected macrophages. Apoptosis of Mtb-infected macrophages aids in bacillary killing, antigen presentation and cross-presentation to $CD4^+$ and $CD8^+$ T cells. Conversely, necrosis of macrophages during Mtb infection is believed to lead to unimpeded release of Mtb from the dying macrophages, facilitating its dissemination to uninfected cells. Mitochondria can be involved in both apoptotic and necrotic cell death pathways. Previous studies have revealed the mitochondria as potential mediators of Mtb-induced macrophage death. The present work investigated the role of mitochondrial death pathways in the death of Mtb-infected human macrophages. In parallel, the ERK1/2 and p38 mitogen-activated protein kinases (MAPK) were assessed for potential signalling roles in the previously reported pro-survival effect of IL-10 on Mtb-infected macrophages. A human cell line and both primary human monocyte-derived macrophages and alveolar macrophages were studied during infection with Mtb H37Ra. Macrophage death was assessed by propidium iodide exclusion and detection of fragmented DNA. Pharmacological inhibition of the ERK1/2 or p38 MAPK signalling had no effect on the viability of Mtb-infected macrophages. In the present work, IL-10 did not maintain the viability of macrophages during Mtb infection.

Dendritic cells (DCs) are vital to the response to tuberculosis infection, stimulating $CD4^+$ and $CD8^+$ T cell activation. They are a major source of IL-12, initiating T_H1 immunity and potentiating T cell release of IFN-γ and TNF-α, which increase the anti-mycobacterial activity of phagocytes. Mtb infects DCs and impairs their function both in mouse models and in human monocyte-derived DCs. Macrophages undergo cell death following Mtb infection, facilitating bacillary killing. However, DC viability in Mtb infection has not previously been investigated. Mtb-induced DC death may impair DC migration and T cell priming. DC viability was assessed during Mtb infection to determine the extent and mechanism of DC death, and its effect on Mtb viability.

Human monocyte-derived DCs were infected with Mtb H37Ra and H37Rv. Live bacilli were required to induce DC maturation and death. A significant decrease in DC viability and increase in DNA fragmentation was observed after 72 h infection with Mtb, with increased expression of DC maturation markers. Pan-caspase blockade did not inhibit cell death following Mtb infection. In fact, Mtb-induced cell death lacked some important features of classical apoptosis: death proceeded in the absence of caspase 3 and 7 activation, and without nuclear fragmentation. It was also noted that Mtb replicated within infected DCs. The persistence of the bacilli within DCs could allow the dissemination of Mtb from migrating infected DCs to other target cells. However, the death of infected DCs may enhance cross-presentation by uninfected DCs to $CD8^+$ T cells and ultimately aid the adaptive immune response to Mtb. The lack of caspase activity may also contribute to the host response by allowing damage-associated molecular pattern molecules to drive anti-tuberculosis immunity without caspase neutralisation.

The adaptive immune response is critical to the control of Mtb infection. Cell death can influence the inflammatory response of DCs and the subsequent T cell phenotype directed by these DCs. Phagocytosis of dead cells by DCs in the absence of bacterial pattern-associated molecular pattern molecules (PAMPs) can induce a tolerogenic DC and T cell phenotype, while DC ingestion of dead cells containing bacterial PAMPs can drive immunogenic DC and T cell responses. The present work studied the contribution of dying Mtb-infected human macrophages to T cell responses during *in vitro* Mtb infection. Human DCs were cultured with dying Mtb-infected macrophages or sterile apoptotic macrophages. The DCs were then co-cultured with allogeneic T cells. The cytokine release profiles of the DC-macrophage co-cultures and subsequent DC-T cell co-cultures were assessed by ELISA. The activation and proliferation of T cells was assessed by flow cytometry following co-incubation with macrophage-stimulated DCs. Live Mtb, but not dead bacilli, induced macrophage cell death. Co-culture of dying Mtb-infected macrophages and DCs resulted in an inflammatory cytokine response, similar to that previously observed with direct DC infection. Conversely, sterile apoptotic macrophages, which underwent cell death in the absence of bacterial PAMPs, did not contribute to cytokine release upon co-culture with autologous DCs. Co-culture of macrophage-stimulated DCs with allogeneic T cells led to T cell activation and proliferation. DCs stimulated with Mtb-infected macrophages caused T cell activation and proliferation above levels induced by DCs exposed to apoptotic macrophages. DC-infected macrophage and subsequent DC-T cell co-cultures elicited T_H1 and T_H17 cytokine responses, supporting the concept that induction of macrophage cell death by live Mtb favours the robust initiation of innate and adaptive immune responses.

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Abbreviations

$\Delta\Psi_m$:	Inner mitochondrial transmembrane potential
7-AAD:	7-aminoactinomycin D
ABTS:	2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt
AFB:	Acid-fast bacteria
AIDS:	Acquired immunodeficiency syndrome
AIF:	Apoptosis inducing factor
AM:	Alveolar macrophage
ANOVA:	Analysis of variance
APAF1:	Apoptotic peptidase activating factor 1
APC:	Antigen presenting cell
APC:	Allophycococyanin (fluorescent dye)
ASC:	Apoptosis-associated speck-like protein containing a CARD
ATP:	Adenosine triphosphate
BAD:	Bcl-2 antagonist of cell death
BAK:	Bcl-2 homologous antagonist/killer
BAL:	Bronchoalveolar lavage
BAX:	Bcl-2-associated X
BCA:	Bicinchoninic acid
BCG:	Bacillus Calmette-Guérin
Bcl:	B-cell lymphoma
BH:	Bcl-2 homology
BID:	Bcl-2 interacting domain
BIK:	Bcl-2 interacting killer
BIM:	Bcl-2 interacting mediator of cell death
BMF:	Bcl-2 modifying factor
BOK:	Bcl-2-related ovarian killer
BSA:	Bovine serum albumin
CAD:	Caspase-activated deoxyribonuclease
Calpain:	Calcium-activated neutral protease
CAPS:	3-(cyclohexylamino)-1-propanesulfonic acid
CD:	Cluster of differentiation (e.g. CD14)
CFSE:	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
CFU:	Colony forming unit
CHX:	Cycloheximide
CTL:	Cytotoxic T lymphocyte
DAMPs:	Damage-associated molecular pattern molecules
DAPI:	4',6-diamidino-2-phenylindole

DCs	Dendritic cells
DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (also: CD209)
DISC:	Death-inducing signalling complex
DMSO:	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
EndoG:	Endonuclease G
ER:	Endoplasmic reticulum
ERK:	Extracellular signal-regulated kinase
ESAT-6:	Early secreted antigenic target, 6 kDa
FACS:	Fluorescence-activated cell sorting
FADD:	Fas-associated protein with death domain
FasL:	Fas ligand
FBS	Foetal bovine serum
FITC:	Fluorescein isothiocyanate
GM-CSF:	Granulocyte-monocyte colony-stimulating factor
H_2O_2:	Hydrogen peroxide
HIV:	Human immunodeficiency virus
HMGB1:	High-mobility group box 1 protein
HRP:	Horseradish peroxidase
IAP:	Inhibitor of apoptosis protein
IFN-γ:	Interferon gamma
iH37Rv:	γ-irradiated H37Rv
IL:	Interleukin
IMM:	Inner mitochondrial membrane
iNOS:	Inducible nitric oxide synthase
ipr1:	Intracellular pathogen resistance 1
IRF8:	Interferon regulatory factor 8
KMS DCs:	DCs stimulated with streptomycin-killed H37Ra-infected macrophages
LAM:	Lipoarabinomannan
LH37Ra:	Live H37Ra
LMS DCs:	DCs stimulated with live H37Ra-infected macrophages
LN:	Lymph node
LPS:	Lipopolysaccharide
LTBI:	Latent tuberculosis infection
MΦ:	Macrophage
ManLAM:	Mannosylated lipoarabinomannan
MAP kinases:	Mitogen-activated protein kinases

Mcl-1:	Myeloid cell leukemia 1
MDMs:	Monocyte-derived macrophages
MDR-TB:	Multidrug-resistant tuberculosis
MLR:	Mixed leukocyte reaction
MMP-1:	Matrix metalloproteinase-1
MOI	Multiplicity of infection
MOMP:	Mitochondrial outer membrane permeabilisation
MPT:	Mitochondrial permeability transition
MPTP:	Mitochondrial permeability transition pore
Mtb:	*Mycobacterium tuberculosis*
MTR:	MitoTracker Red
NAD^+:	Nicotinamide adenine dinucleotide
NaDCC:	Sodium dichloroisocyanurate
NKT:	Natural killer T cells
NLR:	Nucleotide binding domain leucine-rich repeat
NOD2:	Nucleotide-binding oligomerization domain containing 2, also known as the caspase recruitment domain family, member 15 (CARD15)
nuoG:	NADH dehydrogenase subunit G
OMM:	Outer mitochondrial membrane
PAMP:	Pathogen-associated molecular pattern
PAR:	Poly(ADP-ribose)
PARP-1:	Poly(ADP-ribose) polymerase-1
PBMC:	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE:	Phycoerythrin
PGE_2:	Prostaglandin E_2
PI:	Propidium iodide
PIM:	Phosphatidylinositol mannoside
pknE:	Protein kinase E
PMA:	Phorbol 12-myristate 13-acetate
PPD:	Purified protein derivative
PRR:	Pattern recognition receptor
PS:	Phosphatidylserine
pSTAT (1/3):	Phosphorylated signal transducer and activator of transcription (1/3)
PUMA:	p58 upregulated modulator of apoptosis
Q-VD-OPh:	Quinolyl-valyl-O-methylaspartyl-[-2,6-difluorophenoxy]-methyl ketone
RIP1/3:	Receptor-interacting protein 1/3

ROS:	Reactive oxygen species
RPMI:	Roswell Park Memorial Institute (medium)
SEM:	Standard error of mean
sH37Ra:	Streptomycin-killed H37Ra
SMAC/DIABLO:	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
Spp:	Species
STAT (1/3):	Signal transducer and activator of transcription
STS:	Staurosporine
TB:	Tuberculosis
TBST:	Tris-buffered saline–Tween
tBID:	Truncated BID
TCR:	T cell receptor
TdT:	Terminal deoxynucleotidyl transferase
TGF-β:	Transforming growth factor beta
T_H:	T helper
THP-1:	Human acute monocytic leukaemia cell line
TLR:	Toll-like receptor
TMR:	Tetramethylrhodamine
TNF-α:	Tumour necrosis factor alpha
TNFR:	Tumour necrosis factor receptor
TRADD:	TNF receptor-associated death domain
TRAF:	TNFR-associated factor
TRAIL:	TNF-related apoptosis-inducing ligand
T_{reg}:	T regulatory
TTP:	Time to positivity
TUNEL:	Terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labelling
U:	Uninfected
US:	Unstimulated
WHO:	World Health Organisation
XDR-TB:	Extensively drug-resistant tuberculosis
Z-VAD-fmk:	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Publications

Ryan, R. C. M., O'Sullivan, M. P. & Keane, J. 2011. *Mycobacterium tuberculosis* infection induces non-apoptotic cell death of human dendritic cells. *BMC Microbiology*, 11:237.

1 Introduction

1.1 Tuberculosis

Tuberculosis (TB) is the disease caused by *Mycobacterium tuberculosis* (Mtb), a Gram-positive bacterium of the order Actinomycetales (Cole et al., 1998; National Center for Biotechnology Information, 2011). Mtb is one of the world's most successful pathogens: it is estimated that it infects one-third of the global population (World Health Organisation, 2010a). Mtb has been a leading cause of global morbidity and mortality throughout human civilisation (Daniel, 2006; Donoghue, 2011). An ancient ancestor of modern Mtb was discovered in 9000-year-old Neolithic human remains (Hershkovitz et al., 2008). Mtb infection existed throughout the ancient Egyptian kingdom (Zink et al., 2003). TB swept across Europe and North America in the mid-nineteenth and early twentieth century, with death rates of up to 1000 per 100,000 of the population per year across both continents (Krause, 1928; Grigg, 1958; Daniel, 2006). In Ireland during the nineteenth and early twentieth century, TB caused extensive mortality, often wiping out entire families (M'Intire Falkiner, 1892; Barr, 1956; Breathnach and Moynihan, 2003). In the first Irish census, carried out in 1841, TB was listed as the leading cause of death in Ireland at that time (Deeny, 1962; Breathnach and Moynihan, 2003; Breathnach and Moynihan, 2009). The death rates from TB remained high into the early twentieth century, declining only after the end of the Second World War with the widespread availability of antibiotic treatments in the United States and Europe (Deeny, 1962; Daniel, 2006).

TB primarily affects the lungs (85% of cases in the immunocompetent), although extra-pulmonary disease is also widely reported. Symptoms of active TB disease include a cough with sputum (sometimes haemoptysis), weight loss, fatigue and night sweats (World Health Organisation, 2011a). Mtb enters the body via droplet inhalation (Russell et al., 2010). Within the lung, the mycobacteria are phagocytosed by alveolar macrophages and dendritic cells (DCs). Although the majority of TB infections are controlled by the innate and the adaptive immune response, with only 5-10% of infected individuals developing active TB disease (World Health Organisation, 2010a; Natarajan et al., 2011; Russell, 2011), Mtb can persist within the lungs for decades without eradication. Mtb survives within granulomas, structures within the lung formed by immune cells, which segregate infected cells and prevent dissemination of the bacteria to other areas of the body (Figure 1-1) (Saunders and Britton, 2007). Alveolar macrophages are among the first immune cells to encounter Mtb upon its entry to the airway. They phagocytose the bacilli and induce recruitment of immune cells from the bloodstream to the site of infection (Figure 1-1). The granuloma

develops as the infected macrophages become surrounded by uninfected macrophages and lymphocytes. At this early stage, the granuloma is well vascularised. As time progresses, a fibrous cuff develops at the periphery of the granuloma, which becomes less well vascularised, causing the interior to become hypoxic and necrotic (the caseum; Figure 1-1) (Reece et al., 2010). If the immune system becomes impaired, the granuloma can lose its integrity, allowing release of Mtb and reactivation of TB disease.

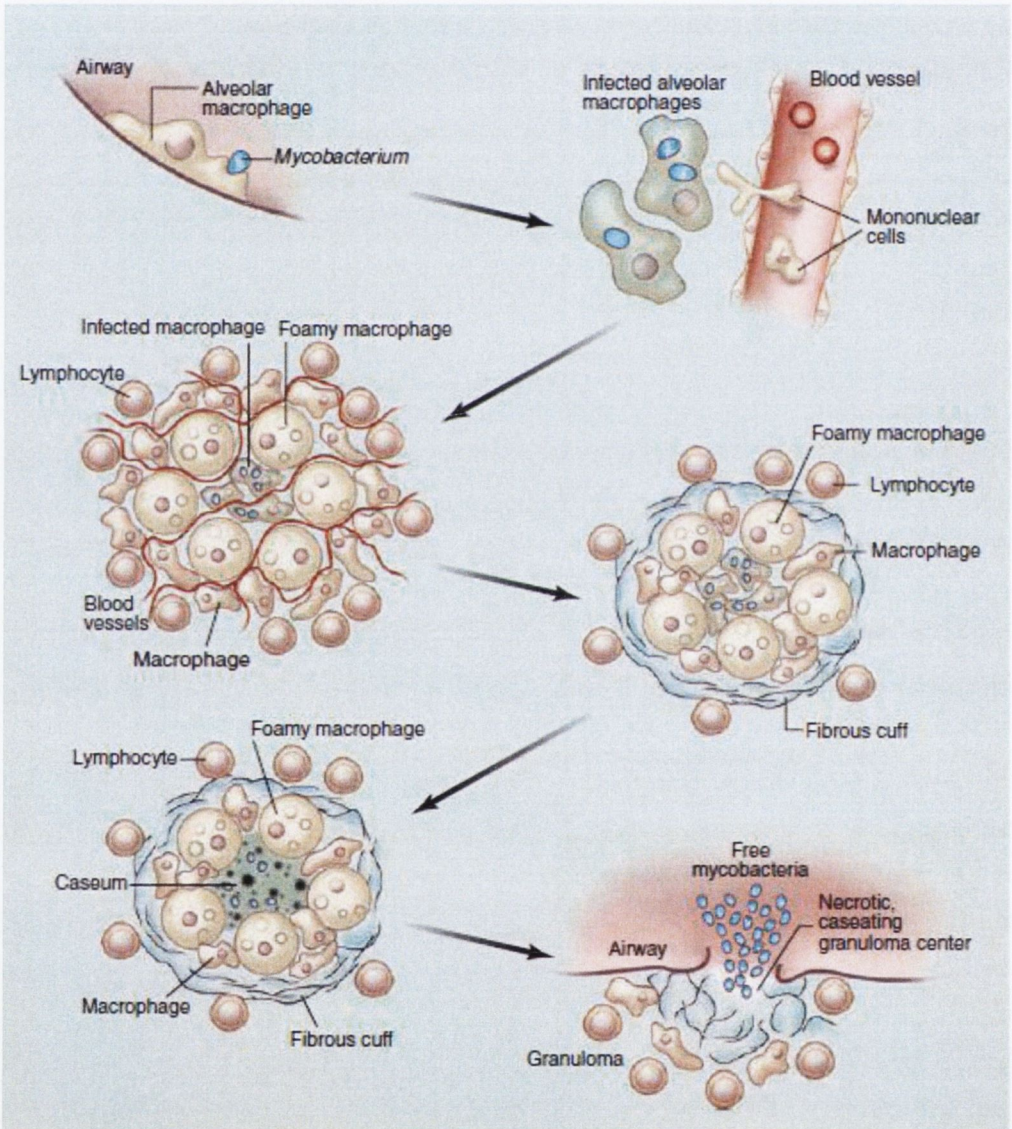


Figure 1-1: Formation of the granuloma in TB disease.

Image from Russell et al. (2010).

Within the granuloma, Mtb lies almost dormant, walled off but not eradicated: a state known as latent TB infection (LTBI) (Hett and Rubin, 2008). In this manner, Mtb retains a reservoir for infection, as individuals with LTBI are healthy and frequently unaware of their condition (Lin and Flynn, 2010). Years or decades after initial infection, LTBI can reactivate, generally due to dampening or compromise of the

immune system (e.g. from malnutrition, disease or immunosuppressive therapy), by mechanisms that are poorly understood (Russell, 2007; Ahmad, 2010). Additionally, individuals successfully treated for TB disease can readily become re-infected, as the memory response to Mtb is insufficient to protect against infection (Cooper, 2009).

Although just 5-10% of otherwise healthy individuals who become infected with Mtb progress to active TB infection, TB is a leading cause of death in people co-infected with human immunodeficiency virus (HIV): almost one-quarter of HIV-related deaths in 2009 were attributable to Mtb infection (World Health Organisation, 2011b). Additionally, strains of Mtb that are resistant to first-line drugs (multidrug-resistant; MDR-TB) and both first- and second-line drugs (extensively drug-resistant; XDR-TB) are emerging, which create major challenges to TB treatment, particularly in those co-infected with HIV (World Health Organisation, 2007). The backdrop of these emerging crises should drive research to better understand the immunobiology of this disease, which can inform therapy and vaccine strategies.

1.1.1 TB and Ireland

Current statistics indicate that in 2009 Ireland had a TB prevalence of approximately 11 cases per 100,000 of the population (Figure 1-2) (Health Protection Surveillance Centre, 2011b). The low rate of drug-resistant (5.8% of total cases) and multidrug-resistant TB (0.4% of total cases), together with a relatively high rate of successful treatment (76.5%) meant that less than 2% of deaths in people diagnosed with TB in Ireland in 2008 were attributable to TB disease (a rate of less than 1 per 100,000 population) (European Centre for Disease Prevention and Control/WHO Regional Office for Europe, 2011; Health Protection Surveillance Centre, 2011a). These rates however, fail to demonstrate that both the number and complexity of cases in Ireland has been increasing in the last decade, with increasing complicated MDR cases (Kennedy et al., 2011).

1.1.2 The global TB epidemic

Although some countries in Europe, such as Romania and the Russian Federation, have a comparatively high prevalence of TB (Figure 1-2), the World Health Organisation (WHO) reports that the WHO European Region accounts for only 5% of all TB cases and has lower incidence, prevalence and mortality than countries in Africa and Asia (World Health Organisation, 2011a).

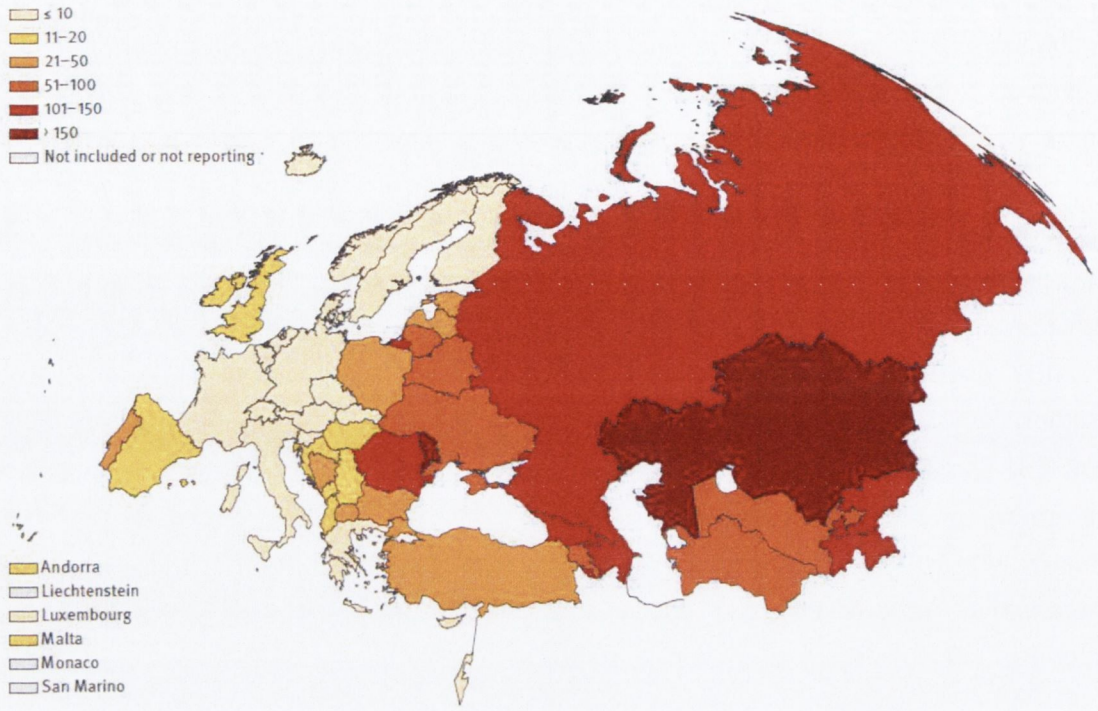


Figure 1-2: Tuberculosis prevalence (per 100,000 population) in Europe in 2009.

Image and statistics from European Centre for Disease Prevention and Control/WHO Regional Office for Europe (2011).

The greatest number of new TB cases occurs in Africa and Asia (World Health Organisation, 2010b). Figures from the WHO reveal that in countries such as South Africa, there were approximately 300 new TB cases per 100,000 population in 2009 (Figure 1-3). Within the African WHO region, the prevalence of TB in 2009 was 450 per 100,000 and the mortality rate from TB was 50 per 100,000 of the population (World Health Organisation, 2010a).

Globally, the WHO estimates that one-third of the world's population is infected with Mtb (World Health Organisation, 2010a), amounting to approximately 2.3 billion people (United States Census Bureau, 2011). This staggering figure points to the success of the pathogen and the need for the development of improved vaccine, detection and treatment strategies.

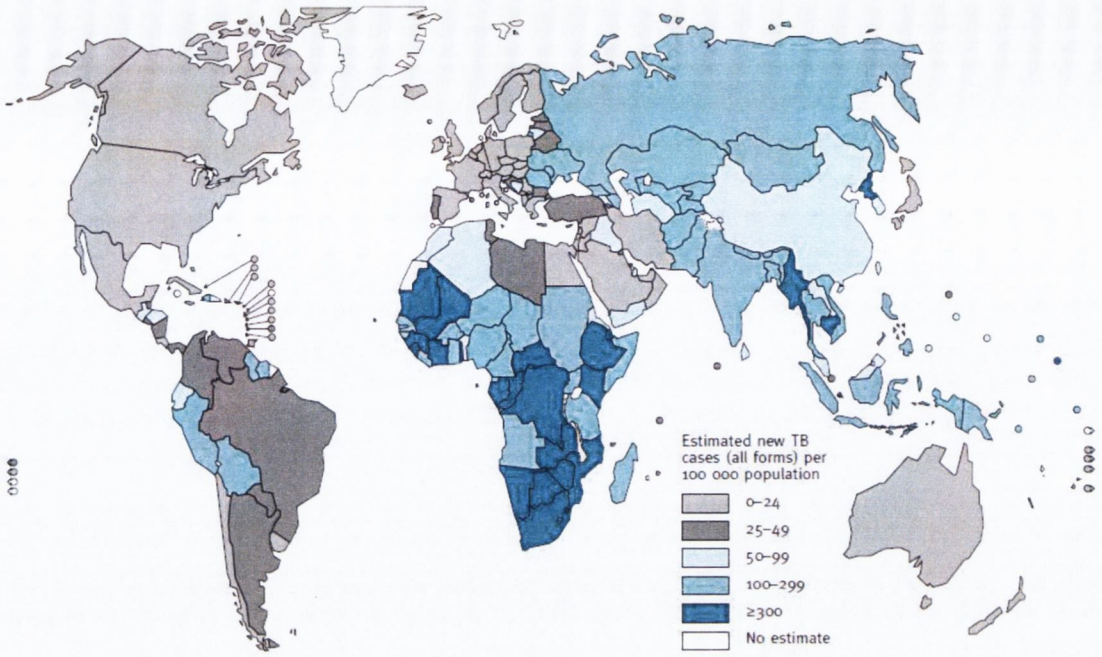


Figure 1-3: Global incidence of TB in 2009.

Image from World Health Organisation (2010b).

The current challenges facing clinicians and policymakers in the prevention and treatment of TB are multi-faceted. The only TB vaccine currently available is the BCG (bacillus Calmette-Guérin; an attenuated strain of the Mtb-related *Mycobacterium bovis*) (Milstien and Gibson, 1990; Grange, 1998). An improved TB vaccine has not become available since the initial development of the BCG in 1921 (Daniel, 2006). Although BCG protects infants from miliary (disseminated) and meningeal TB, it affords little to no protection against disease caused by Mtb in adults (Hussey et al., 2007; Sterling et al., 2007; Sadagopal et al., 2009; Venkataswamy et al., 2009). The available drug regimes require a lengthy treatment duration and are becoming less effective at treating MDR-TB and XDR-TB (Almeida Da Silva and Palomino, 2011; Liu et al., 2011). Finally, there is a need for simple and effective tests to identify individuals with LTBI likely to progress to active disease (Berry et al., 2010; Wallis et al., 2010). Our understanding of the mechanism of Mtb survival within the human body is limited. Overcoming the deficits in vaccine and treatment options requires greater insight into the interaction between Mtb and the human immune system.

One of the key host responses to Mtb infection is death of the infected macrophages. The host-protective benefits of infected cell death include removal of the mycobacterial niche cell, concomitant bacterial killing and enhanced antigen presentation (Oddo et al., 1998; Schaible et al., 2003; Winau et al., 2006; Randhawa et al., 2008; Divangahi et al., 2010). An enhanced knowledge of the cell death process undergone by infected immune cells, together with the consequences for adjacent

leukocytes, may help to inform and direct new vaccine and treatment development (Schaible et al., 2003; Ríos-Barrera et al., 2006; Behar et al., 2011).

In the work presented herein, the mechanism of cell death in TB infection was explored in primary human macrophages and DCs, together with the consequences of macrophage death for the adaptive immune response. The various known modes of cell death are reviewed in the following sections. Current knowledge on the mechanisms and consequences of cell death during infection, particularly Mtb infection, are subsequently discussed.

1.2 Mechanisms of cell death

Cell death was initially described in the literature by researchers in the nineteenth century, who catalogued the morphological features they observed (Lockshin and Zakeri, 2001). There was a resurgence of interest in the area in the 1950s and 60s, when it was recognised that cell death is a regulated process with an important role in development, and the term 'programmed cell death' was coined to reflect this (Glucksmann, 1951; Lockshin and Williams, 1965; Häcker and Vaux, 1997). In the 1970s, Kerr and colleagues (1972) combined observations of morphological features of cell death with the consequences of that death to form a paradigm of cell death which has endured to the present day. They proposed the term 'apoptosis' to describe a form of programmed – or regulated – cell death with a specific set of morphological features; in contrast to cell death by 'necrosis', morphologically distinct from apoptosis and considered an unregulated (or accidental) form of cell death associated with injury or disease. It is now recognised that many forms of cell death previously described as necrotic are highly regulated and that programmed necrosis can occur (Vanlangenakker et al., 2008; McCall, 2010; Moquin and Chan, 2010).

Activity in cell death research continues, as it has a pivotal role in many diverse physiological processes: from development and homoeostasis, to the functioning of the immune system and its response to disease. Cell death is necessary during development and throughout life, ensuring the correct development of the brain, nerves and digits (Abud, 2004; Vanderhaeghen and Cheng, 2010). Cell death also maintains the homeostasis of the immune system by removing self-reactive immune cells, thus preventing autoimmune diseases (Ludwig-Portugall et al., 2008; Bonilla and Oettgen, 2010; Chen and Wang, 2010). However, excess cell death can lead to pathology and disease. Neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and glaucoma, are caused by neuronal death in the developed nervous system (Lin and Beal, 2006; Baltmr et al., 2010). During myocardial

infarction, the loss of blood supply causes myocardial cell death, leading to ischaemia-reperfusion injury once blood supply is restored (Chen and Lesnefsky, 2011).

1.2.1 Cell death and infection

Cell death plays a significant role in the host response to many infections, particularly those caused by intracellular organisms. Pathogens that reside within host cells can be destroyed by the death of their resident cell. For this reason, cell death is an important host-protective response to infection, removing the pathogen's niche and aiding in its elimination (Labbe and Saleh, 2008). To combat this, cell death may be modulated by pathogens to aid their own spread and survival, as evident from the strategies employed by both viruses and bacteria (Böhme and Rudel, 2009; Upton et al., 2010).

1.2.2 Cell death and viruses

Viruses invade and use the host cells' own machinery to replicate within infected cells. Cell death during viral infection can be a protective host response and many viruses actively promote host cell viability in order to allow time for them to replicate. Epstein-Barr virus is a human herpes virus which infects B cells and sets up latent infection. In order to maintain infection, the virus produces homologues of the human pro-survival Bcl-2 protein to prevent B cell death and its own demise (Kalla and Hammerschmidt, 2011). Chronic infection by hepatitis C virus is also maintained, in part, by prevention of B cell death by the virus (Ayers et al., 2011). Viruses can also promote cell death in order to escape the infected cell and spread, or in order to counteract the host immune response (Galluzzi et al., 2008). During the early stages of HIV infection, host cell viability is maintained to allow viral replication. HIV later induces death of the infected cells to escape and colonise further cells. In addition, the virus induces the release of toxins from infected host cells that causes death of uninfected bystander immune cells, leading to depletion of $CD4^+$ and $CD8^+$ T cells and the development of acquired immunodeficiency syndrome (AIDS) (Gougeon and Piacentini, 2009).

1.2.3 Cell death and bacteria

Although bacteria differ from viruses, in that they can replicate without the aid of host cells, they also frequently interfere with host cell death to facilitate their propagation and survival. One such example is chlamydia, which manipulates host cell death to promote infection. Chlamydiae are obligate intracellular bacteria that cause diseases of the genital tract, eye, vascular system and respiratory tract (Byrne and Ojcius,

2004). During infection, the bacterium can alternately prevent or induce cell death. In the acute phase of infection, *Chlamydia* species induce cell lysis to spread to neighbouring cells. Once the infection shifts to a chronic phase, the bacteria inhibit cell death by degradation of the host cell's pro-death BH3-only proteins, and in so doing, establish long-term colonisation (Miyairi and Byrne, 2006; Sharma and Rudel, 2009). Other bacteria, such as *Neisseria gonorrhoeae*, actively induce cell death to degrade epithelial barriers and spread to the sub-epithelial tissues (Muller et al., 1999).

1.2.4 Cell death and *Mycobacterium tuberculosis*

In *M. tuberculosis* (Mtb) infection, cell death and its modulation by the bacilli is a major topic of research (Keane et al., 1997; Hinchey et al., 2007; Lee et al., 2009b; Behar et al., 2010; Behar et al., 2011). Findings in this field have led to the development of vaccines designed to promote death of infected cells in an effort to boost the protective immune response (Kernodle and Bochan, 2002; Winau et al., 2006; Hinchey et al., 2007; Ranganathan et al., 2009; Sadagopal et al., 2009; Hinchey et al., 2011). Although Mtb can infect a variety of cells, its primary niche cell is the alveolar macrophage (Martino, 2008). Typically, material phagocytosed by macrophages is enclosed within phagosomes, which acidify as they mature, thus degrading their contents. Mtb prevents the acidification process so, as an alternate strategy for bacterial removal, the infected macrophage can undergo cell death (Behar et al., 2011; O'Leary et al., 2011). Macrophage death aids both in antigen presentation and killing of Mtb (Chan et al., 1992; Molloy et al., 1994; Oddo et al., 1998; Keane et al., 2002; Winau et al., 2006; Herbst et al., 2011). Therefore, macrophage death is an important protective response against Mtb. The type of cell death undergone by Mtb-infected macrophages is also of significance in the host immune response. Macrophage apoptosis aids in antigen presentation and bacillary killing (Oddo et al., 1998; Schaible et al., 2003; Winau et al., 2006; Divangahi et al., 2010), while macrophage death by necrosis is believed to allow escape of Mtb from the dying cell, facilitating dissemination of Mtb to uninfected cells (Molloy et al., 1994; Chen et al., 2006; Chen et al., 2008).

1.2.5 The type of cell death matters immunologically

Pathogens can induce varying forms of cell death, which may have significant consequences for the host immune response. The mode of death undergone by infected cells can be beneficial or detrimental to the host response to infection: apoptosis can induce immune tolerance in bystander cells (Griffith et al., 2007; Tassiulas et al., 2007), while both apoptotic and necrotic forms of cell death have

been shown to induce an immunogenic response (Zychlinsky and Sansonetti, 1997; Scaffidi et al., 2002; Torchinsky et al., 2009) (further reviewed in Section 1.6). There are diverse initiation and signalling pathways involved in cell death, as well as a variety of morphological features. Traditionally, cell death has been divided into one of the two initially identified cell death categories: apoptosis or necrosis. As cell death research has advanced, however, a number of modes of cell death have been identified that do not fit completely within either category. The types of cell death, and the cellular machinery involved in their induction and execution, are discussed in the following sections.

1.3 Apoptosis

Apoptosis is a form of cell death, first defined by Kerr and colleagues (1972), which involves specific morphological changes within the dying cell. Apoptotic cells undergo changes to their nucleus and cytoplasm as they break down in a distinctive fashion. Cells undergoing apoptosis shrink in volume (termed pyknosis), become smaller and rounded, and display nuclear fragmentation (termed karyorrhexis) (Doonan and Cotter, 2008). The integrity of the plasma membrane is maintained during apoptosis (Kroemer et al., 2009). Apoptotic cells undergo membrane blebbing, breaking into apoptotic bodies which become engulfed by phagocytic cells, such as macrophages or DCs (Figure 1-4) (Lodish et al., 2004).

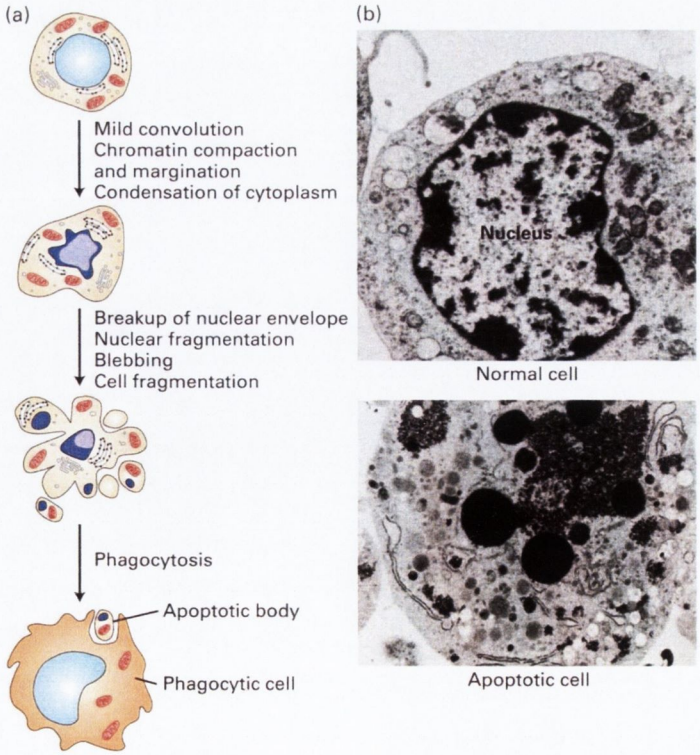


Figure 1-4: Morphological features of apoptotic cells.

Image from Lodish et al., (2004).

Most of the morphological features of apoptosis are due to the proteolytic activity of a family of cysteine proteases, known as caspases. DNA fragmentation is mediated by a caspase-activated deoxyribonuclease (CAD), which is activated by cleavage of its inhibitor protein by caspase 3 (Sakahira et al., 1998). Caspase 6 activity results in nuclear shrinkage by cleaving nuclear structural proteins (Orth et al., 1996). These proteases were originally identified in the nematode worm, *Caenorhabditis elegans*, and are highly evolutionarily conserved (Ellis and Horvitz, 1986; Li and Yuan, 2008). They exist in an inactive form as zymogens, termed procaspases, and can be rapidly activated by cleavage, either by themselves (autocleavage) or by other caspases (Alberts et al., 2002).

Caspases can be classified into three categories based on their function: initiator caspases, executioner (or effector) caspases and inflammatory caspases. The initiator caspases (caspases 8, 9 and 10) participate in early signalling events during cell death and can activate the executioner caspases. Executioner caspases (caspases 3, 6 and 7) are responsible for the proteolysis involved in dismantling the apoptotic cell (Launay et al., 2005; Li and Yuan, 2008). The inflammatory caspases (human caspases 1, 4, 5; mouse caspases 11 and 12) are involved in regulating inflammation through their ability to activate the cytokines IL-1β and IL-18 (Martinon et al., 2002; Salskov-Iversen et al., 2011; Walsh et al., 2011). Caspase 4 is also instrumental as an initiator caspase in apoptosis induced by endoplasmic reticulum stress, indicating that caspases can serve multiple roles (Hitomi et al., 2004; Yang et al., 2011). Apoptosis is mediated by one of two main pathways: the extrinsic pathway, or the intrinsic pathway.

1.3.1 The extrinsic apoptosis pathway

The extrinsic apoptotic pathway commences with the cell surface binding of a death ligand to a transmembrane death receptor (Figure 1-5). Death ligands are members of the tumour necrosis factor (TNF) superfamily, including TNF-α, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL). Binding of these ligands to their cognate receptors (TNF-receptor 1 (TNF-R1), TRAIL receptor or Fas/CD95) causes recruitment of the adapter molecule, Fas-associated protein with death domain (FADD), and procaspase 8 to assemble the death-inducing signalling complex (DISC). Procaspase 8 possesses some protease activity and undergoes autocleavage within the DISC to form active caspase 8: the initiator caspase (Alberts et al., 2002). Caspase 8 directly activates the executioner caspases, caspases 3 and 7, which mediate proteolysis and cell death (Taylor et al., 2008; Wiezorek et al., 2010). Caspase 8 can also indirectly activate caspase 3/7 via the intrinsic pathway, which is discussed in the next section.

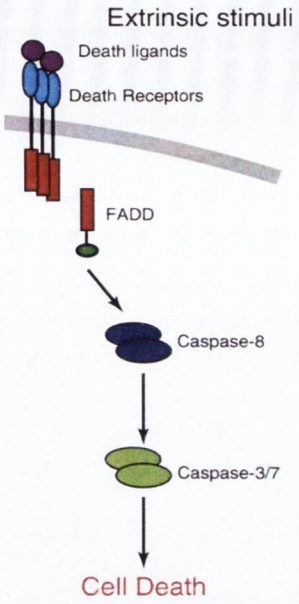


Figure 1-5: The extrinsic apoptosis pathway is initiated by extracellular signalling via death receptors.

Image adapted from Danthi (2011).

1.3.2 The intrinsic apoptosis pathway

The intrinsic (or mitochondrial) pathway of apoptosis is triggered intracellularly in response to various stressors, such as DNA damage, hypoxia, withdrawal of growth factors, or infection (Figure 1-6). It is mediated by a family of proteins known as the Bcl-2 (B-cell lymphoma 2) proteins (Vaux et al., 1988). The Bcl-2 family consists of both pro- and anti-apoptotic proteins, and it is the balance of signalling among them that determines the fate of the cell (Oltval et al., 1993).

Anti-apoptotic proteins include Bcl-2, Bcl-w, Bcl-xL, Mcl-1 (myeloid cell leukemia 1) and A1, each of which contains four BH (Bcl-2 homology) domains (Boise et al., 1993; Veis et al., 1993; Gibson et al., 1996; Hamasaki et al., 1998; Rinkenberger et al., 2000). Pro-apoptotic proteins fall into two categories, distinguished by the number of BH domains they contain. The multi-domain effector proteins are BAX (Bcl-2-associated X), BAK (Bcl-2 homologous antagonist/killer) and BOK (Bcl-2-related ovarian killer) (Oltval et al., 1993; Chittenden et al., 1995; Kiefer et al., 1995; Hsu et al., 1997). The remaining pro-apoptotic proteins contain three BH domains and are collectively known as BH3-only proteins (Willis and Adams, 2005; Lomonosova and Chinnadurai, 2009). This group includes PUMA (p58 upregulated modulator of apoptosis), BIM (Bcl-2 interacting mediator of cell death), BAD (Bcl-2 antagonist of cell death), NOXA, BIK (Bcl-2 interacting killer), BID (Bcl-2 interacting domain), BMF (Bcl-2 modifying factor) and HRK (Boyd et al., 1995; Yang et al., 1995; Wang et al.,

1996; Inohara et al., 1997; O'Connor et al., 1998; Oda et al., 2000; Puthalakath et al., 2001; Yu et al., 2001).

Initiation of the intrinsic pathway by cellular stress or infection leads to binding of the BH3-only proteins to the pro-survival Bcl-2 proteins, allowing activation of pro-apoptotic BAX and BAK through an as yet undefined mechanism (Willis and Adams, 2005; Leibowitz and Yu, 2010). Active BAX and BAK insert into the mitochondrial outer membrane and oligomerise, causing mitochondrial outer membrane permeabilisation (MOMP) (Narita et al., 1998; Nechushtan et al., 2001) (Figure 1-6). MOMP allows the release of pro-apoptotic mitochondrial proteins: cytochrome *c*, apoptosis-inducing factor (AIF) and SMAC/DIABLO (Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI) (Susin et al., 1999; Du et al., 2000; Verhagen et al., 2000). Once in the cytoplasm, cytochrome *c* binds to APAF1 (apoptotic peptidase activating factor 1), forming heterodimers to develop a structure known as the apoptosome (Zou et al., 1999). Assembly of the apoptosome causes recruitment and activation of procaspase 9, which cleaves and activates caspase 3, leading to proteolysis of cellular proteins (Li et al., 1997). SMAC/DIABLO contributes to the activation of caspases by blocking the inhibitory IAPs (inhibitor of apoptosis proteins) (Du et al., 2000; Verhagen et al., 2000). AIF translocates to the nucleus, where it mediates DNA fragmentation in conjunction with other proteins (such as endonuclease G (EndoG) and cyclophilin A) (Susin et al., 1999; Cande et al., 2004).

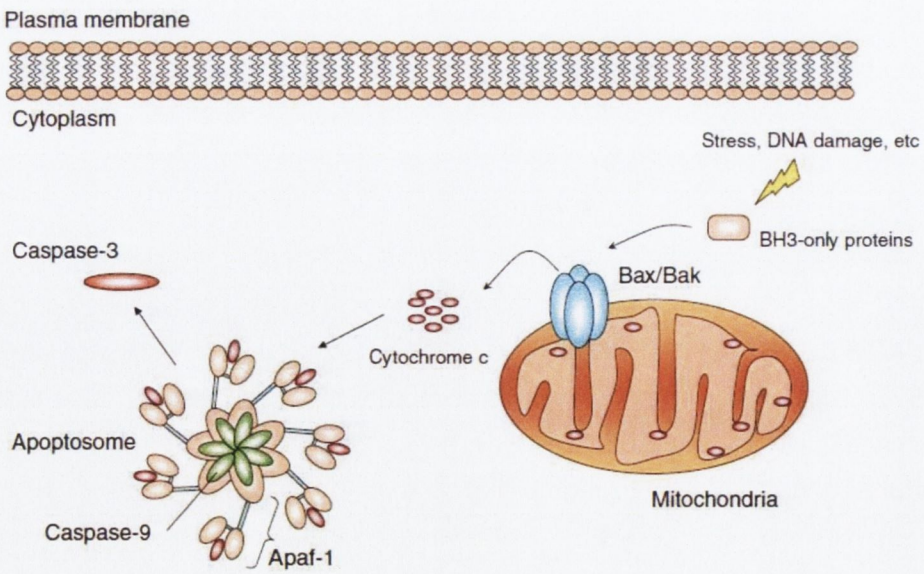


Figure 1-6: The intrinsic apoptotic pathway mediates cell death in the absence of extracellular death signalling.

Image adapted from Li and Yuan (2008).

The extrinsic and intrinsic apoptotic pathways can exhibit crosstalk via caspase 8, when this caspase is activated by the extrinsic pathway, but mediates death via an intrinsic pathway mechanism. In this situation, caspase 8 cleaves the pro-apoptotic BH3-only protein, BID (Bcl-2 interacting domain). Truncated BID (tBID) causes release of cytochrome *c* from the mitochondria, assembly of the apoptosome and activation of caspase 3 and 7.

The plasma membrane of apoptotic cells retains its integrity during apoptosis, even after caspase activation. Plasma membrane rupture following the apoptotic programme is known as secondary necrosis (Silva, 2010). This is observed most commonly *in vitro*, where there is no facility for removal of dead cells. Following apoptosis *in vivo*, neighbouring phagocytes recognise and rapidly remove cell debris, although apoptosis of large numbers of cells may lead to secondary necrosis *in vivo* if apoptotic cells cannot be removed quickly enough by phagocytes (Gregory and Pound, 2010).

1.3.3 Granzyme B-mediated apoptosis

Cancerous or infected cells are targeted by cytotoxic T cells and natural killer (NK) cells to undergo apoptosis mediated by granzyme B (Hoves et al., 2010). Cytotoxic lymphocytes secrete perforin, which forms pores in the target cell membrane and facilitates entry of granzyme B to the cytoplasm (although the exact mechanism of granzyme delivery is currently unknown) (Anthony et al., 2010). Granzyme B is a serine protease and can initiate apoptosis by directly processing caspase 3 and causing its auto-activation, or by cleaving BID to tBID, allowing formation of the apoptosome and activation of executioner caspases in this manner (Afonina et al., 2010).

1.3.4 Granulysin-mediated apoptosis

Granulysin is a lytic protein secreted by cytotoxic ($CD8^+$) and natural killer T cells (Pena et al., 1997). In conjunction with other lytic proteins (granzymes and perforin) it mediates killing of target cells, such as tumour cells, virus-infected cells and cells infected with intracellular bacteria (Kaufmann et al., 1986; Hata et al., 2001; Zhang et al., 2009b). Granulysin plays a significant role in anti-mycobacterial defence: in concert with perforin it lyses Mtb-infected cells and mediates direct killing of Mtb, in addition to killing the infected cell (Stenger et al., 1998b; Saini et al., 2011).

1.4 Necrosis

Necrosis is a form of cell death distinct from apoptosis. It differs in terms of morphological changes within the dying cell, some initiating stimuli and also in its cell death effector molecules. Necrotic cells display an increase in cell volume and rupture of the cell membrane (Ziegler and Groscurth, 2004). Notably, induction of necrosis does not rely on caspase activation or release of mitochondrial cell death proteins, such as cytochrome *c* (Zong et al., 2004; Krysko et al., 2008). Necrosis has historically been described as an unregulated, or 'accidental', form of cell death, but it is now recognised that necrosis can also be a highly regulated, programmed process (Darzynkiewicz et al., 1997; Golstein and Kroemer, 2007; Golant et al., 2008; Hitomi et al., 2008; Galluzzi et al., 2009; McCall, 2010; Galluzzi et al., 2011; Kung et al., 2011). In *C. elegans*, necrosis is controlled by genes involved in plasma membrane and endoplasmic reticulum Ca^{2+}-permeable channels, calreticulin, calpains and cathepsins, demonstrating a high level of regulation in this form of cell death (Xu et al., 2001; Syntichaki et al., 2002; Bianchi et al., 2004).

A number of different forms of regulated necrotic death have also been observed in mammals. The term 'necrosis' has, until recently, been used to describe all non-apoptotic cell death. The awareness that non-apoptotic death – as well as classical apoptosis – can occur in a regulated manner has exposed this broad use of 'necrosis' as inaccurate and redundant (Fink and Cookson, 2005). While the term 'programmed necrosis' exists within the literature, there appears to be no current consensus on its precise meaning and it is varyingly used to refer to one particular form of regulated necrotic death, or to regulated necrotic death as a whole (Galluzzi et al., 2011). It is clearer, therefore, to refer to modes of regulated necrotic, non-apoptotic death by individual names (Galluzzi et al., 2011), and this is the convention used in the current work.

To date, there are four identified pathways of mammalian regulated non-apoptotic cell death: pyroptosis, pyronecrosis, necroptosis and parthanatos (Cookson and Brennan, 2001; Willingham et al., 2007; Hitomi et al., 2008; David et al., 2009). It is important also to note that cell death displaying features of accidental necrosis is described in many pathologies and infections, including TB, which nonetheless appears highly regulated (O'Sullivan et al., 2007). Cell death research is a rapidly evolving field and it is likely that additional modes of regulated necrotic death will be defined in the future. The apparently regulated, non-apoptotic death observed in TB is not limited to the exact mechanisms and mediators of currently described modes of programmed necrotic death. The different form of necrotic death can vary in terms of their mediators and consequences, and are described in the following sections.

1.4.1 Accidental necrosis

Accidental necrosis occurs as a result of severe cellular insult, such as trauma, burn injuries, pressure injuries, exposure to detergents, or sepsis (Choumar et al.; Galluzzi et al., 2011; Krysko et al., 2011). Hypoxia, extremes in temperature and lack of cell nutrients can also cause accidental necrosis (Vanlangenakker et al., 2008). The process of necrotic death is a series of catastrophic events that overcome normal cellular function and result in rupture of the affected cell. Accidental necrosis is initiated via calcium influx to the cytoplasm, either from extracellular sources or from intracellular calcium stores (such as the endoplasmic reticulum). Excessive Ca^{2+} concentrations can be due to cellular dysfunction (such as that induced in neurodegenerative diseases or by mechanical/extreme temperature stress) or as a consequence of defective ion transport due to ATP depletion (which can occur during ischaemia/reperfusion injury) (Giorgi et al., 2008; Halestrap, 2009; Lemasters et al., 2009). The increase in cytoplasmic calcium leads to a number of fatal changes within the cell. Mitochondria enter the mitochondrial permeability transition (MPT), with formation of mitochondrial permeability transition pores (MPTP) in the inner mitochondrial membrane (Abdallah et al., 2010; Li et al., 2010). MPTP cause breakdown of the mitochondrial membrane potential ($\Delta\Psi_m$) – the electrical potential difference between the mitochondrial matrix and inter-membrane space which drives ATP production from ADP – leading to mitochondrial swelling and rupture. Loss of $\Delta\Psi_m$ (and the subsequent breakdown of the mitochondria) results in cessation of ATP production (Halestrap et al., 2004). Elevated cytoplasmic calcium concentration also activates calpains (calcium-activated neutral proteases) and leads to lysosomal membrane permeability, allowing release of cathepsin proteases and increasing cytoplasmic acidity. The proteolytic activity of calpains and cathepsins causes wide scale cellular damage that, together with the cellular energy crisis, culminates in rupture of the cell (Christofferson and Yuan, 2010). Necrosis typically features release of damage-associated molecular pattern molecules (DAMPs), such as HMGB1 (high mobility group box 1), which can lead to an inflammatory immune response.

The mitochondrial pathway mediating necrosis features some of the same components as the apoptotic mitochondrial pathway. The difference between the two pathways is the type of mitochondrial permeabilisation observed in the two cell death modalities (Figure 1-7). In apoptosis, mitochondrial outer membrane permeabilisation (MOMP) occurs via activity of BAK and BAX, triggering apoptotic death via release of mitochondrial death proteins. The mitochondrial necrosis pathway is mediated by MPTP formation in the inner mitochondrial membrane (Kroemer et al., 2007; Javadov et al., 2011; Kinnally et al., 2011), leading to cessation of ATP production and death of the cell.

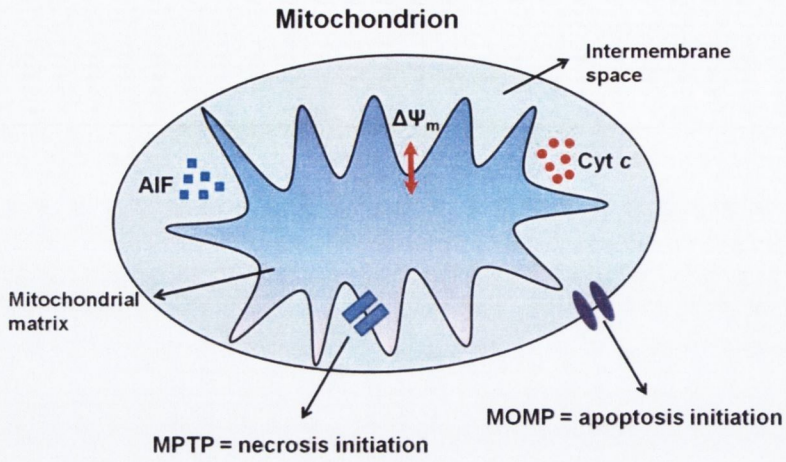


Figure 1-7: The mechanism and location of mitochondrial membrane permeabilisation determines the cell death modality.

1.4.2 Pyroptosis

Pyroptosis is a specific form of programmed necrotic cell death induced in macrophages which is dependent on caspase 1 activity (Cookson and Brennan, 2001). Active caspase 1 converts pro-interleukin-1β (pro-IL-1β) and pro-IL-18 to their active forms (Franchi et al., 2009; Brodsky and Medzhitov, 2011). Other caspases are not active during pyroptosis and mitochondrial death proteins are retained within the mitochondria (Hilbi et al., 1998; Brennan and Cookson, 2000; Jesenberger et al., 2000; Lamkanfi et al., 2008). *Salmonella* species (spp.) and *Shigella* spp. induce pyroptosis in macrophages (Hilbi et al., 1998; Hersh et al., 1999; Fink and Cookson, 2007). Pyroptosis has also been described during infection with the intracellular organisms, *Legionella pneumophila* and *Burkholderia thailandensis* (Miao et al., 2010). Pyroptosis is believed to be an antimicrobial mode of cell death due to the resulting release of inflammatory IL-1β and lysosomal antimicrobial factors (observed during *in vivo* murine *Salmonella* infection) (Bergsbaken et al., 2011).

Caspase 1 forms protein complexes known as inflammasomes in order to activate IL-1β and IL-18 (Martinon et al., 2002; Tschopp et al., 2003; Petrilli et al., 2005). Inflammasomes are comprised of caspase 1, a nucleotide binding domain leucine-rich repeat (NLR) protein, and apoptosis-associated speck-like protein containing a CARD (ASC) (van de Veerdonk et al., 2011). There are three currently known variants of the inflammasome, dependent on the component NLR protein: the NLRP1 (NALP1), NLRP3 (NALP3) or NLRC4 inflammasome (Davis et al., 2011). Both IL-1β and ASC have been shown to be critical for host-protective responses and granuloma formation in tuberculosis (TB) infection in a murine model *in vivo* (Mayer-Barber et al., 2010; McElvania TeKippe et al., 2010). The NLRP3 inflammasome is activated by Mtb infection of human macrophages *in vitro* and is essential for the death of these cells

induced by Mtb (Mishra et al., 2010; Wong and Jacobs Jr, 2011). Although there are some reports that Mtb inhibits inflammasome activation, IL-1β can be produced in a caspase 1-independent manner during Mtb infection, where it mediates protective immunity (Master et al., 2008; Mayer-Barber et al., 2010).

Recent studies have indicated that the non-apoptotic macrophage cell death induced by Mtb is independent of caspase 1 activity (Lee et al., 2011; Welin et al., 2011). However, Ciaramella and colleagues reported that caspase 1 blockade significantly reduced death of Mtb-infected macrophages and monocytes (Ciaramella et al., 2002). Therefore, although it is clear that Mtb activates NLRP3 and Mtb infection results in the release of IL-1β, the debate regarding caspase 1 activity in Mtb-induced cell death requires resolution in order to confirm or exclude the involvement of pyroptosis during Mtb infection.

1.4.3 Pyronecrosis

This form of programmed necrotic death occurs with the involvement of components of the NLRP3 inflammasome (NALP3 and ASC), but independent of caspase 1 activity and is therefore distinct from pyroptosis (Willingham et al., 2007; Averette et al., 2009; Kroemer et al., 2009). Pyronecrosis due to infection with *Neisseria* is cathepsin B-dependent and results in the release of HMGB1 (Duncan et al., 2009). Cells undergoing death by pyronecrosis lack DNA fragmentation and PARP (poly(ADP-ribose) polymerase) cleavage. In addition to induction of pyroptosis, *Shigella flexneri* can also induce pyronecrosis in macrophages and monocytes (Willingham et al., 2007; Galluzzi and Kroemer, 2009). Pyronecrosis has also been described during infection with the intracellular parasite, *Toxoplasma gondii* (Zhao et al., 2009).

Lee and colleagues (2011) and Welin et al. (2011) both reported that Mtb-induced macrophage death is caspase 1-independent, but is not dependent on cathepsin B, a protease implicated in pyronecrosis during *Neisseria* infection. However, their studies conflicted regarding the necessity of the expression of the mycobacterial protein, early secreted antigenic target, 6 kDa (ESAT-6) for induction of cell death. ESAT-6 has been shown to activate the NLRP3 inflammasome and was noted by Welin et al. to be required for macrophage death induced by Mtb (Mishra et al., 2010; Welin et al., 2011). They also observed that Mtb-induced macrophage death resulted in HMGB1 release. Release of HMGB1 and the activation of NALP3 and ASC within the NLRP3 inflammasome, in the absence of caspase 1 activity, are features of pyronecrosis. In contrast, however, Lee et al. found that Mtb-induced macrophage death was independent of the mycobacterial ESX1 system, which is critical for ESAT-6 secretion (Lee et al., 2011). Based on the conflicting results of these studies, it is clear that

further research is required to clarify the mechanism of Mtb-induced macrophage death (which features in Chapter 3 of the current work).

1.4.4 Necroptosis

Necroptosis is a form of regulated necrotic death induced by death receptor signalling when caspase 8 is deleted or inhibited (Holler et al., 2000; Degterev et al., 2005; Degterev et al., 2008; Galluzzi and Kroemer, 2008; Hitomi et al., 2008; He et al., 2009; Zhang et al., 2009a). The key players in necroptosis are two members of the receptor interacting protein (RIP) family of kinases: RIP1 and RIP3 (He et al., 2009; Ch'en et al., 2011). These kinases are central to the regulation of both cell survival and cell death (Kaiser et al., 2011; McNeal et al., 2011; O'Donnell and Ting, 2011). In cells with functional caspase 8, the active caspase cleaves RIP1 and RIP3 in response to death stimuli transduced via tumour necrosis factor receptor 1 (TNFR1), thereby preventing necroptosis in favour of apoptosis. However, when caspase 8 has been genetically or pharmacologically inhibited, RIP1 functions in complex with RIP3 to mediate necroptosis in response to TNFR1 signalling (Vandenabeele et al., 2006; Hitomi et al., 2008; Kaiser et al., 2011; Oberst et al., 2011). Following TNFR1 ligation, RIP1 and RIP3 can form a complex known as the necrosome, which mediates a number of processes (including production of reactive oxygen species [ROS], highly damaging to proteins, lipids and DNA) that culminate in cell death (Kim et al., 2007; Vanden Berghe et al., 2007; Li et al., 2010; Kung et al., 2011).

Necroptosis can participate in the homoeostatic control of T cells. This form of cell death is responsible for the death of activated (antigen-stimulated) caspase 8-deficient T cells. It is postulated that this may represent a host-protective response to viral caspase inhibition and could result in removal of virally-infected T cells (Ch'en et al., 2008; Ch'en et al., 2011). Necroptosis is also involved in pathology and disease, such as neuronal cell death, retinal disorders, inflammatory bowel disease and vaccinia virus infection (Cho et al., 2009; Rosenbaum et al., 2010; Trichonas et al., 2010; Welz et al., 2011; Yamanaka et al., 2011). Necrostatin-1, a specific small molecule inhibitor of RIP1 which prevents necroptosis, has been shown to be protective in experimental ischaemic brain injury (which can occur during stroke) and ischaemic retinal damage (a consequence of glaucoma or hypertensive/diabetic retinopathy) (Degterev et al., 2005; Rosenbaum et al., 2010; Northington et al., 2011). Although necroptosis has not been described in the context of Mtb infection to date, these findings underline the importance of regulated necrosis in disease.

1.5 Parthanatos

Poly(ADP-ribose) polymerase-1 (PARP-1) controls DNA repair after low to moderate levels of DNA damage have occurred within the cell. PARP-1 is cleaved and inactivated by caspases during apoptosis and so is not active in apoptotic death (Lazebnik et al., 1994). However, when DNA damage is too severe to repair, PARP-1 can initiate a particular form of caspase-independent cell death, which has been termed parthanatos (David et al., 2009; Rouleau et al., 2010). Parthanatos is initiated by PARP-1 and is mediated by the product of PARP-1 activation, poly(ADP-ribose) (PAR). PARP-1 utilises nicotinamide adenine dinucleotide (NAD^+) as a source of ADP-ribose to produce PAR. Over-activation of PARP-1 following severe DNA damage leads to cell death by two mechanisms:

1. Excessive PAR is produced in the nucleus, which causes PAR translocation to the cytosol. Here, PAR interacts with the mitochondria, resulting in the release of AIF (Andrabi et al., 2008). Once released, AIF translocates to the nucleus where it mediates DNA fragmentation, resulting in cell death.

2. Cellular NAD^+ is depleted during PARP-1 over-activation in response to extensive DNA damage. In attempting to replenish NAD^+, the cell rapidly depletes its ATP and dies (Zong et al., 2004).

Parthanatos has been implicated in disease, particularly in neurodegenerative disorders (Ghezzi et al., 2010; Kuzhandaivel et al., 2010; Yu et al., 2011). *Mycobacterium bovis*-induced macrophage death is associated with caspase-independent AIF nuclear translocation (Vega-Manriquez et al., 2007). The release of AIF in the absence of caspase activation is a hallmark of parthanatos and the involvement of parthanatos in human macrophage death during Mtb infection was assessed in Chapter 3 of the current work.

1.6 Cell death during bacterial infections

Cell death, as noted previously, occurs during homoeostatic maintenance within the body. Physiological cell death is apoptotic, which retains immunogenic host cell-derived DAMPs within apoptotic bodies as the cell fragments and tends to promote phagocytosis of apoptotic cells in a silent or tolerogenic manner, without immune activation (Figure 1-8) (Devitt and Marshall, 2011). It must be noted however that, although apoptosis is frequently regarded as a silent form of cell death, this is not always the case, as demonstrated by immune responses to apoptotic cancer cells (Molinari et al.; Ronchetti et al., 1999; Boozari et al., 2010; Zitvogel et al., 2010). Similarly, necrosis is generally considered to be an exclusively immunogenic form of cell death but this may not always be accurate: Kushwah et al. (2010) demonstrated *in vitro* that necrotic DCs do not induce an immunogenic response.

1.6.1 Apoptosis in host defence against infection

As a countermeasure to bacterial invasion, infected cells can undergo apoptosis. Apoptosis is a successful host defence because it allows for the exposure of intracellular bacteria to the immune system and may also in itself aid in bacterial killing (Ying et al., 2008; Nogueira et al., 2009; Garrison et al., 2010; Miao et al., 2010; Bewley et al., 2011). The controlled disassembly of the plasma membrane promotes formation of apoptotic bodies, which are taken up by neighbouring antigen-presenting cells (APCs) (Albert et al., 1998a). Although apoptotic bodies from infected cells retain intracellular DAMPs, preventing their release and consequent inflammatory response, they also contain intact bacteria or bacterial components (Halicka et al., 2000; Torchinsky et al., 2010). Bacteria contain pathogen-associated molecular patterns (PAMPs), which are recognised by various pattern recognition receptors (PRRs) on innate immune cells and stimulate an immune response (Brereton and Blander, 2011). Phagocytosis of PAMP-containing apoptotic bodies by uninfected, bystander APCs aids in the induction of an adaptive immune response to the pathogen, as the bacterial antigens from the apoptotic cell are presented to T cells by the APC (Figure 1-8) (Albert et al., 1998b; Winau et al., 2006; Torchinsky et al., 2009).

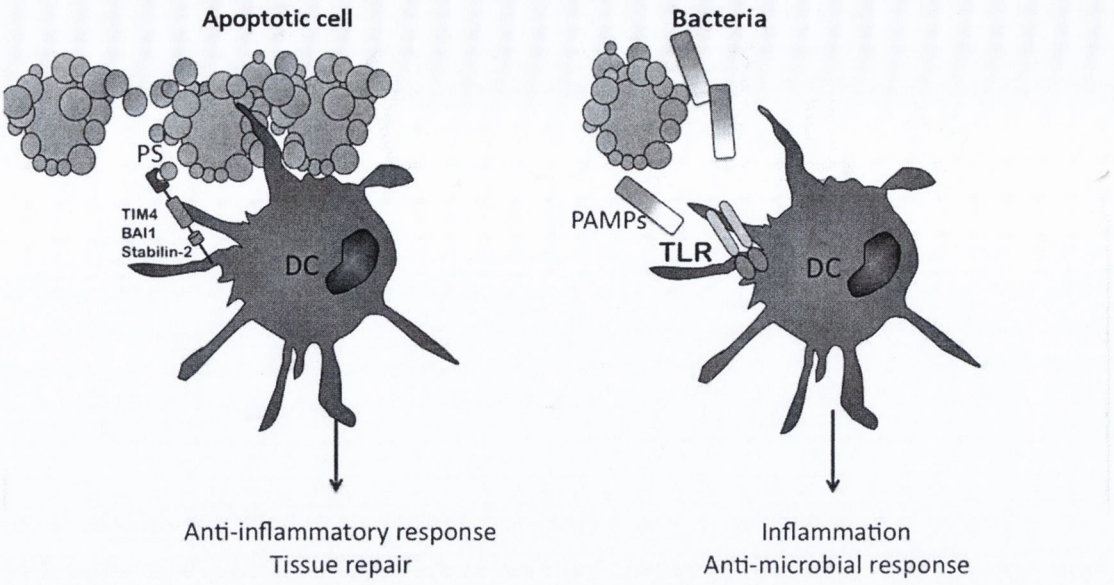


Figure 1-8: The presence of bacterial PAMPs in apoptotic cells promotes a host immune response.

Image adapted from Brereton and Blander (2011).

1.6.2 Apoptosis following bacterial and *M. tuberculosis* infection

Induction of apoptosis is an important host response during TB infection. Apoptosis of Mtb-infected cells benefits the host in three ways: removing the cell within which the bacteria are replicating, killing of the intracellular bacteria and aiding antigen presentation (Oddo et al., 1998; Keane et al., 2002; Winau et al., 2006; Hinchey et al., 2007; Rodrigues et al., 2009; Divangahi et al., 2010; Herbst et al., 2011).

The immune consequences of cell death during bacterial infection differ from homoeostatic cell death and bacteria have evolved mechanisms to interfere with host cell death to serve their own ends. Bacteria frequently reside within host cells during infection, either as a niche within which to evade the immune system and replicate, or in the course of dissemination to neighbouring uninfected cells or to the bloodstream. As organisms differ in their mechanisms of infection and colonisation, it can be beneficial to the bacterium to either inhibit or induce cell death (Labbe and Saleh, 2008).

Certain bacteria actively target components of cell death pathways for inhibition, in order to maintain survival of the infected cell. *Anaplasma*, *Coxiella* and *Shigella* can delay cell death by inhibiting caspases. The pro- and anti-apoptotic Bcl-2 proteins also form attractive bacterial targets to modulate host cell death. The bacteria *Anaplasma*, *Chlamydia*, *Neisseria*, *Shigella*, *Ehrlichia*, *Coxiella* and *Legionella* all up- or down-regulate various Bcl-2 family members in order to prevent apoptosis (Böhme and

Rudel, 2009). Mtb promotes activation of the pro-survival Bcl-2 protein, Mcl-1, to maintain survival of infected macrophages (Sly et al., 2003). By inhibiting or delaying host cell death, these bacteria preserve their replicative niche until they are ready to break free of the infected cell and spread to neighbouring cells. Virulent strains of Mtb have been shown to inhibit macrophage apoptosis, preserving their niche cell (Balcewicz-Sablinska et al., 1998; Keane et al., 2000; Sly et al., 2003; Sohn et al., 2009). The *nuoG* gene (encoding the type I NADH dehydrogenase) of Mtb inhibits TNF-α-mediated macrophage apoptosis (Velmurugan et al., 2007; Miller et al., 2010). Nitrate stress-mediated apoptosis of macrophages is similarly impeded by the product of the Mtb *pknE* (protein kinase E) gene (Kumar and Narayanan, 2011). Conversely, bacteria can actively promote host cell death to engineer their rapid escape and dissemination. In order to force their release from macrophages, *Salmonella* induces death by pyroptosis, while *Shigella* triggers pyronecrosis (Carneiro et al., 2009; Galluzzi and Kroemer, 2009). *Chlamydia*, *Ehrlichia*, *Shigella*, *Neisseria* and *Legionella* can all promote apoptosis by the formation of BAK-BAX mitochondrial pores and consequent caspase 3 activation (Böhme and Rudel, 2009). *Helicobacter pylori* induces a form of non-apoptotic cell death in gastric epithelial cells, possibly through activation of PARP-1 (parthanatos) (Nossa et al., 2009; Radin et al., 2011). Induction of necrotic death by bacteria can facilitate their spread within the host, as the infected cells' plasma membrane ruptures during necrotic cell death and allows bacterial escape. Once outside the host cell, bacterial stimulation of cell death can also facilitate their extracellular survival by eliminating nearby immune cells, as observed during infection with *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus* and *Streptococcus* (Nizet, 2007; Lamkanfi and Dixit, 2010).

Similar to other pathogens, Mtb can interfere with infected cells' intended mode of death to serve its own ends. A proposed virulence factor of Mtb is the ability of virulent strains to convert cell death from apoptosis to necrosis (Keane et al., 2000; Chen et al., 2006; Gan et al., 2008; Divangahi et al., 2009). Necrosis allows unimpeded escape of the bacilli from the infected cell, without bacterial killing or enhanced antigen presentation (Molloy et al., 1994; Sohn et al., 2009). The fact that attenuated strains of Mtb tend to induce abundant apoptosis of infected cells (rather than necrosis) may be another indication that the switch from apoptosis to necrosis is an Mtb virulence factor (Bhattacharyya et al., 2003; Hinchey et al., 2007; Bohsali et al., 2010). Elucidation of the specific type of cell death induced during Mtb infection is therefore of importance, as it has a bearing on whether the death benefits the host or the bacterium (Lee et al., 2009b; Behar et al., 2011). The current work aimed to shed light on the mechanisms of Mtb-induced cell death in human macrophages and DCs, and the consequences of that cell death on subsequent T cell responses. Macrophages,

DCs and T cells are reviewed individually (in the context of Mtb infection) in the following sections.

1.7 Immune response to *M. tuberculosis* infection

Innate immune cells, including macrophages and DCs, comprise the initial defence against Mtb infection. Macrophages engulf Mtb and can undergo death, which kills internalised bacilli (Molloy et al., 1994; Oddo et al., 1998; Rodrigues et al., 2009). Macrophages also possess potent bactericidal activity; however, to be fully effective this requires activation by T cells in the lungs, which occurs later in infection (Desai et al., 1989; Bonecini-Almeida et al., 1998; Jayaraman et al., 2010). Macrophages release chemokines to attract immune cells to the site of infection and are central to the development of the granuloma (Roach et al., 2002; Leemans et al., 2005; Ferrara et al., 2008; Khader et al., 2009; Wang et al., 2010). Like macrophages, DCs can also phagocytose Mtb and secrete chemokines and cytokines following Mtb infection (Henderson et al., 1997; Jang et al., 2008; Gonzalez-Juarrero et al., 2009; Wang et al., 2010). The key function of DCs is migration from the site of infection to the local draining lymph node to activate T cells (Janeway et al., 2005). T cells form a vital part of the adaptive response to Mtb (Chen et al., 2009). B cells, the other component of the adaptive immune response, are largely uncharacterised in Mtb infection, although Mtb-specific B cells have been identified in TB patients and appear to be associated with containment of infection (Feng et al., 2011; Zhang et al., 2011). The T cell response to Mtb infection is slow, occurring approximately 8-10 days after infection with Mtb in murine models (Gallegos et al., 2008; Reiley et al., 2008; Wolf et al., 2008). After 14 days, Mtb-specific T cells arrive in the lungs and activate macrophage bactericidal activity primarily through release of interferon (IFN)-γ (Bonecini-Almeida et al., 1998; Tascon et al., 1998), though Fas-mediated macrophage activation has also been reported (Brookes et al., 2003). T cells can also mediate apoptosis of infected cells (Keane et al., 2002; Martino et al., 2007; Herbst et al., 2011) and direct killing of Mtb itself (Canaday et al., 2001; Dieli et al., 2001; Cowley and Elkins, 2003; Bastian et al., 2008; Gallegos et al., 2011).

The adaptive immune response can be broadly categorised as T_H1/inflammatory, T_H2/anti-inflammatory or T_H17, based on the cytokines released by T cells. T_H1 and inflammatory cytokines include IFN-γ, IL-1β, IL-2, IL-8, IL-12 and TNF-α (Li et al., 2011). T_H2 and anti-inflammatory cytokines include IL-4, IL-5, IL-6, IL-10, IL-13 and TGF-β (Talat et al., 2011). The T_H17 cytokines include IL-17, IL-22 and (in humans) IL-26 (Dubin and Kolls, 2008).

Macrophages, DCs and T cells all contribute to cytokine release during Mtb infection (Portevin et al., 2011; Sable et al., 2011; Sanarico et al., 2011). Pro-inflammatory cytokines, in particular, perform important roles in the control of Mtb infection. TNF-α is critical to the maintenance of the granuloma and protection during LTBI: re-activation of TB disease was a noted problem encountered with the introduction of anti-TNF-α antibodies as therapies for autoimmune diseases (Mohan et al., 2001; Keane, 2005; Chakravarty et al., 2008). Additionally, TNF-α confers protection through induction of apoptosis of Mtb-infected macrophages and mycobacterial killing (Spira et al., 2003; Clay et al., 2008; Randhawa et al., 2008). IL-1β is also an important mediator of mycobacterial killing (Mayer-Barber et al., 2010). IL-12 secretion is required for sufficient IFN-γ release, which in turn increases the antimycobacterial action of macrophages (Cooper et al., 1995; Flynn et al., 1995a; Bonecini-Almeida et al., 1998; Herbst et al., 2011). The recent report of a case of fatal tuberculosis in a patient with IL-12 receptor deficiency, in spite of anti-tuberculosis treatment, highlights the requirement for IL-12 in human immunity against Mtb (Tabarsi et al., 2011). The T_H2 cytokine IL-6 is required for anti-TB immunity: it mediates induction of protective T cell responses and its deletion in mice leads to lethal Mtb infection (Appelberg et al., 1994; Ladel et al., 1997). IL-2 release directs and maintains T_H1 polarisation, while down-regulating T_H17 differentiation (Liao et al., 2011). IL-2 is also important for the generation of memory $CD8^+$ T cells (Feau et al., 2011). Inflammatory IL-17, secreted by T_H17 cells, is produced during Mtb infection (Torrado and Cooper, 2010). It plays an important role in the formation and maintenance of the granuloma and it has been proposed that IL-17 is required for optimal IFN-γ secretion in the lung during *in vivo* Mtb infection (Umemura et al., 2007; Okamoto Yoshida et al., 2010). However, excessive production of IL-17 has been suggested as a cause of the extensive lung tissue damage observed in patients with multidrug-resistant Mtb infection (Basile et al., 2011).

The anti-inflammatory cytokines IL-10 and TGF-β inhibit inflammation and the development of T_H1 or T_H17 responses (Bonecini-Almeida et al., 2004; Jiagang et al., 2011). TGF-β induces tolerance in macrophages and DCs, which can then suppress T cell proliferation in response to antigen (Wahl et al., 1988; Luo et al., 2007; Pallotta et al., 2011). TGF-β and IL-10 are produced by Mtb-infected macrophages and are associated with down-regulation of protective inflammatory responses (Rojas et al., 1999b; Biswas et al., 2007; Almeida et al., 2009; Schreiber et al., 2009; Redford et al., 2010). Additionally, IL-10 prevents maturation of Mtb-containing phagosomes within macrophages, aiding survival of the bacilli (O'Leary et al., 2011). However, complete absence of IL-10 during Mtb is detrimental, as chronic uncontrolled inflammation eventually leads to death of Mtb-infected IL-10 knockout mice (Higgins et al., 2009), indicating that a fine balance between pro- and anti-inflammatory

cytokine signalling must be maintained in order to control Mtb disease without inducing excess pathology (Chakravarty et al., 2008; Nguyen et al., 2011).

1.7.1 Macrophages

The alveolar macrophage (AM) plays a central role in the immune response to TB infection (Rajaram et al., 2010; Arcos et al., 2011; Russell, 2011). As innate immune cells, AMs form the first line of defence against inhaled pathogens, and readily ingest Mtb (Lawlor et al., 2011). Phagocytosis of Mtb is mediated by various macrophage cell surface receptors, such as CD14, scavenger receptors, complement receptors and the mannose receptor (Ernst, 1998). Mtb has evolved strategies to evade detection and elimination by the immune system. After the bacillus is phagocytosed, it arrests maturation of its phagosome within the AM through induction of IL-10 secretion from the infected macrophage (Philips, 2008; Flannagan et al., 2009; O'Leary et al., 2011). When this occurs, the phagosomal compartment does not become acidified and Mtb can survive within the cell, undetected by the immune system. As a counter-measure, infected AMs undergo cell death, ensuring that the bacilli are no longer concealed. Human AM apoptosis is observed in patients with active TB disease (Klingler et al., 1997). It has been demonstrated in human *in vitro* (using monocyte-derived macrophages; MDMs) and murine *in vivo* experiments that macrophage apoptosis facilitates killing of Mtb and aids antigen presentation via release of apoptotic bodies containing mycobacterial antigens from the dying macrophage (Schaible et al., 2003; Winau et al., 2006). Cell death of AMs, therefore, may be a critical host response in TB infection.

Mtb-infected macrophages can be induced to die by a number of exogenous factors. TNF-α can promote macrophage apoptosis in both murine macrophages and human AMs during Mtb infection, and also prevents macrophage death by necrosis (Flynn et al., 1995b; Keane et al., 1997; Rojas et al., 1999a; Spira et al., 2003; Clay et al., 2008). Fas-induced macrophage apoptosis (mediated by FasL release from T cells) aids in mycobacterial killing, and FasL up-regulation is observed in patients with Mtb-induced pleurisy (Oddo et al., 1998; Budak et al., 2008). Release of the cytotoxic molecules perforin, granulysin and granzymes from cytotoxic $CD8^+$ T cells also mediates apoptosis of Mtb-infected macrophages (Stenger et al., 1997; Lewinsohn et al., 1998; Woodworth et al., 2008). ATP, released from dying Mtb-infected human MDMs, causes macrophage apoptosis and has been implicated as a potential auto-induction mechanism of macrophage apoptosis in an *in vitro* study (Placido et al., 2006). Although IFN-γ has been shown to induce apoptosis during Mtb infection in murine macrophages, this effect is not reported in human macrophages (Rojas et al., 1999a; Randhawa et al., 2008; Lee and Kornfeld, 2010; Herbst et al., 2011).

Similarly, nitric oxide has been shown to contribute to macrophage cell death (and plays a major role in macrophage antimycobacterial defence) during Mtb infection in the mouse, but its contribution to human AM immunity is controversial (Arias et al., 1997; Herbst et al., 2011). While both NO and its inducer, inducible nitric oxide synthase (iNOS), are produced in human MDMs during Mtb infection *in vitro* and are found in human lung granulomas *in vivo*, no link between NO and apoptosis induction has yet been established in human Mtb infection (Aston et al., 1998; Choi et al., 2002; Schon et al., 2004; Lee et al., 2009a).

Macrophage necrosis can also be triggered by exogenous mediators. Complement activation during Mtb infection is associated with the induction of necrosis, which, unlike apoptotic death, does not reduce bacterial viability (Oddo et al., 1998). A switch in the Mtb-infected macrophage death phenotype from necrosis to apoptosis is associated with increased bacterial killing (Gan et al., 2005). Conversely, necrotic death allows the escape of viable bacilli from the dying macrophage (Lee et al., 2006). Virulent (rather than attenuated) strains of Mtb have been reported to promote necrosis of infected macrophages in preference to apoptosis (Chen et al., 2006; Park et al., 2006). Macrophage necrosis is induced by virulent Mtb by disrupting membrane repair of dying Mtb-infected macrophages, essential to apoptotic body formation. The bacilli released from the dying macrophage are thus not encapsulated within apoptotic bodies, and can freely disseminate to infect neighbouring cells (Gan et al., 2008; Divangahi et al., 2009). Genetic studies in mice have identified that the gene, *Intracellular pathogen resistance 1* (*ipr1*), mediates macrophage bacterial killing and affects the cell death phenotype of infected macrophages (Pan et al., 2005). Mice lacking this gene succumb to overwhelming Mtb infection, which is characterised by collections of necrotic macrophages within the lungs. Restoration of the functional *ipr1* gene to deficient mice causes a shift in the macrophage death phenotype from necrosis to apoptosis and re-establishes limitation of Mtb replication. The human homologue of *ipr1*, *SP110*, is currently under investigation for a role in susceptibility to human Mtb infection, with conflicting reports thus far: an association appears to exist in some populations, but not in others (Thye et al., 2006; Tosh et al., 2006; Babb et al., 2007; Liang et al., 2011).

It is apparent from the breadth of both human and murine experiments, however, that macrophage death (and the mode of that death) during Mtb infection is of critical importance in the host immune response. The macrophage cell death induced during Mtb infection is caspase-independent and involves the activity of tBID and cathepsin L (Lee et al., 2006; O'Sullivan et al., 2007). This necrotic form of cell death is not dependent on caspase 1 or cathepsin B, indicating that it is not pyroptosis, but it is not yet fully apparent whether it could be classified as pyronecrosis (O'Sullivan et al.,

2007; Lee et al., 2011; Welin et al., 2011). Much remains to be elucidated about the mode and associated signalling involved in Mtb-induced macrophage death. *Chapter 3 of the current work aimed to investigate the mechanism of macrophage death during Mtb infection by assessing the role of the intrinsic apoptosis pathway and of parthanatos.*

Non-pathogenic and attenuated mycobacteria induce cell death, while virulent Mtb can obstruct the macrophage cell death process (Keane et al., 1997; Keane et al., 2000; Sly et al., 2003; Loeuillet et al., 2006; Bohsali et al., 2010). It has previously been reported that the anti-inflammatory cytokine IL-10 mediates the survival of macrophages infected with virulent strains of Mtb (Balcewicz-Sablinska et al., 1998). IL-10 has a dampening effect on the immune system and limits inflammation. It is required to prevent pathological inflammation during Mtb infection (Higgins et al., 2009). However, its activity in Mtb infection is also associated with sub-optimal immune responses. IL-10 secretion from human macrophages causes arrest of phagolysosomal maturation, allowing Mtb persistence within AMs (O'Leary et al., 2011). Additionally, IL-10 release during Mtb infection is associated with impaired protective T_H1 responses (including reduced IL-12 and IFN-γ secretion) and failure to efficiently kill the bacilli (Hickman et al., 2002; Almeida et al., 2009; Schreiber et al., 2009; Redford et al., 2010; Yahagi et al., 2010). Although IL-10 has been implicated in macrophage apoptosis inhibition, the precise signalling pathway is unknown (Balcewicz-Sablinska et al., 1998). *Further experiments detailed in Chapter Three aimed to clarify the role of IL-10 and its signalling pathways in macrophage cell death during TB infection.*

1.7.2 Dendritic cells

The recent description of interferon regulatory factor 8 (IRF8) mutations associated with human dendritic cell (DC) immunodeficiency (Hambleton et al., 2011) and their involvement with disseminated mycobacterial disease has re-focused attention on this sentinel cell. The role of DCs is to capture antigen, migrate to the nearest draining lymph node (LN) and present the antigen to both naïve and memory T cells to initiate cell-mediated immunity. Prior to encountering antigen, DCs are immature and tissue-resident. Immature DCs are actively phagocytic, sampling their environment (Janeway et al., 2005). Pattern recognition receptors (PRRs) on DCs, such as DEC 205, DC-SIGN and dectin-1, recognise foreign antigens and mediate phagocytosis (Geijtenbeek et al., 2000; Kato et al., 2006; Schlesinger et al., 2008). DC-SIGN (murine homologue: SIGNR3), which is involved both in pathogen phagocytosis and in antigen presentation, is the dominant route of uptake of Mtb in both human and murine DCs (Geijtenbeek et al., 2003; Tailleux et al., 2003; Tanne et al., 2009). On encountering

antigen, DCs mature and travel to the local draining LN for antigen presentation to T cells (Blander, 2008). Mature DCs express high levels of adhesion molecules, such as DC-SIGN, ICAM-1, ICAM-2, CD58 and LFA-1, as well as co-stimulatory molecules CD80/CD86 and MHC class I and class II, which allow contact with T cells for antigen presentation and T cell activation (Janeway et al., 2005). DCs can be divided into a number of subsets. The major DC subsets are myeloid (or conventional) DCs and plasmacytoid DCs. Plasmacytoid DCs express the surface molecule BDCA-2 (CD303). Myeloid DCs can be subdivided into BDCA-1 $(CD1c)^+$ DCs and BDCA-3 $(CD141)^+$ DCs (Delamarre and Mellman, 2011). Plasmacytoid DCs are poor at antigen presentation but are important producers of type I interferon during viral infection (Palucka et al., 2010; Reizis et al., 2011). It is predominantly myeloid DCs which perform the important functions of antigen presentation and cytokine secretion (such as IL-12, crucial to protective immunity in TB disease) during Mtb infection (Cheong et al., 2010; de Beaucoudrey et al., 2010; Tabarsi et al., 2011), and which are modelled by monocyte-derived DCs in the current work. An imbalance in the ratio of myeloid to plasmacytoid DCs – with reduced myeloid DCs – has been reported in patients with active TB disease compared with healthy household contacts, underlining the importance of myeloid DCs in the control of Mtb (Gupta et al., 2010).

Both human and murine DCs can phagocytose Mtb, which leads to DC maturation (Henderson et al., 1997; Wolf et al., 2007). Mtb-infected DCs stimulate $CD4^+$ and $CD8^+$ T cell activation (Schreiber and Sandor, 2010). Dissemination of Mtb to the LN, which occurs in part via infected DCs, is an important precursor to T cell activation and aids initiation of adaptive immune responses (Chackerian et al., 2002b). During TB, like other chronic infections, the pool of DCs must be replenished in order to maintain antigen presentation. Throughout Mtb infection, peripheral circulating monocytes can be recruited to the lungs and to the pulmonary lymph nodes, and differentiate to DCs (Muller and Randolph, 1999; Randolph et al., 1999; Skold and Behar, 2008). DC viability during Mtb infection has not previously been studied in the context of the functional capacity or mycobactericidal capability of dying infected DCs. The important host-protective consequences of macrophage cell death in TB infection have been clearly demonstrated in the literature (Keane et al., 1997; Keane et al., 2000; Behar et al., 2011). A greater understanding of the cell death consequences of Mtb infection on DCs could indicate an equally significant effect of DC death in the immune response to Mtb. *Chapter 4 of the current work sets out the results of studies on the extent and mechanism of DC death during* in vitro *infection with Mtb.*

1.7.3 T cells

T cells form one arm of the adaptive immune response, with B cells and antibodies forming the other. In concert with phagocytes, such as macrophages, T cells act to kill invading Mtb and maintain immune homoeostasis. In some individuals, this results in clearance of the bacilli; however, for the many who proceed to LTBI, T cells play an important role in both the formation and maintenance of the granuloma (Russell, 2007). There is a number of T cell subsets and each has been shown to play a role during Mtb infection. $CD4^+$ T cells comprise the helper T cell (T_H) category, with the ability to activate macrophages and kill infected cells (Mutis et al., 1993; Bonecini-Almeida et al., 1998; Lewinsohn et al., 1998; Bastian et al., 2008; Gallegos et al., 2011). $CD8^+$ T cells are also known as cytotoxic T lymphocytes (CTLs), as they kill infected cells in an antigen-specific manner by granzyme-mediated apoptosis (Chinnaiyan et al., 1996; Hoves et al., 2010). In addition, they can also release potent antimicrobial peptides, capable of lysing both infected/cancerous cells and invading pathogens, including Mtb (Markham et al., 1984; Ochoa et al., 2001; Walch et al., 2005; Fujiwara and Takiguchi, 2007; Inoda et al., 2011; Thiel et al., 2011). Both $CD4^+$ and $CD8^+$ T cells are required for successful immunity to Mtb infection (Saunders et al., 2002; Chen et al., 2009). Via IFN-γ-dependent (Bonecini-Almeida et al., 1998; Tascon et al., 1998) and IFN-γ–independent mechanisms (Cowley and Elkins, 2003; Martineau et al., 2007; Gallegos et al., 2011), both $CD4^+$ and $CD8^+$ T cells potentiate the mycobactericidal action of macrophages. $CD4^+$ T cells are instrumental in the formation of granulomas (Saunders et al., 2002). $CD8^+$ T cells kill Mtb-infected cells by releasing granzyme and perforin, as well as mediating direct killing of Mtb bacilli with granulysin, particularly during the early stages of Mtb infection (Stenger et al., 1998a; Silva and Lowrie, 2000; Canaday et al., 2001; Lazarevic et al., 2005; Andersson et al., 2007; Woodworth et al., 2008). During the chronic stage of Mtb infection, $CD8^+$ T cells are an important source of IFN-γ and participate in maintenance of the granuloma (Lazarevic et al., 2005; Ordway et al., 2007b). Both $CD4^+$ and $CD8^+$ T cells participate in memory responses to Mtb (Wang et al., 2004; Kagina et al., 2009).

$CD4^+$ and $CD8^+$ T cells are known as αβ T cells, so called because of the α and β chains comprising their T cell receptor (TCR). An alternate and rarer type of T cells, known as γδ T cells due to their differing TCR (Hayday et al., 1999), has also been shown to contribute to Mtb immunity (Ladel et al., 1995a; Ladel et al., 1995b), through perforin-mediated killing of Mtb-infected monocytes and macrophages (Martino et al., 2007). Patients with active TB disease display a marked reduction in the size of their γδ T cell population compared with LTBI patients, underlining the importance of this subset of T cells in the control of Mtb infection (Li et al., 1996).

Natural killer T (NKT) cells respond to lipid (rather than protein) antigens (Terabe and Berzofsky, 2012). Though they express αβ TCRs, they differ from other αβ T cells in that the α chain of their TCR is invariant and the β chain possesses only a limited repertoire (Godfrey et al., 2000; Godfrey et al., 2010). NKT cells recognise lipid antigens presented by APCs via the CD1d antigen presenting molecule (Exley et al., 1997), while other αβ T cells recognise protein antigen loaded on MHC class I and II molecules (Janeway et al., 2005). Although NKT cells are T lymphocytes and produce T_H1 and T_H2 cytokines as well as cytotoxic granulysin (Yoshimoto et al., 1995; Gansert et al., 2003; Devera et al., 2011), they respond rapidly to infection, a response resembling that of innate immune cells (Terabe and Berzofsky, 2012). For this reason, NKT cells are said to span both innate and adaptive immunity (Nishimura et al., 2000; Galli et al., 2003). Phosphatidylinositol mannoside (PIM), a mycobacterial lipid, is recognised by both human and murine NKT cells (Fischer et al., 2004). NKT cells mediate protection to Mtb infection *in vivo* (Chackerian et al., 2002a) and their activation during vaccination enhances the efficacy of TB vaccines (Venkataswamy et al., 2009). They can activate the anti-mycobacterial activity of macrophages during Mtb infection (Yoneda and Ellner, 1998) and also directly mediate killing of Mtb (Gansert et al., 2003; Sada-Ovalle et al., 2008).

Regulatory T cells (T_{reg} cells) form a counterbalance to the activity of immunogenic T cells (Cantor and Simpson, 1975). This subset of T cells comprises $CD4^+$ cells which express CD25 (the alpha chain of the IL-2 receptor) and the transcription factor FoxP3 ($CD4^+CD25^+FoxP3^+$ cells) (Fontenot et al., 2003). Regulatory T cells are present during Mtb infection and have been identified in high numbers in the peripheral blood and granulomas of tuberculosis patients (Hougardy et al., 2007; Rahman et al., 2009). Mtb promotes the expansion of T_{reg} cells (Liu et al., 2010; Periasamy et al., 2011), which can suppress protective inflammatory responses and inhibit mycobacterial clearance (Kursar et al., 2007; Ordway et al., 2007a; Scott-Browne et al., 2007; Ordway et al., 2011; Welsh et al., 2011). The activity of T_{reg} cells during Mtb infection also delays the proliferation and trafficking to the lung of Mtb-specific $CD4^+$ and $CD8^+$ effector T cells, facilitating bacterial replication in the early phase of infection (Shafiani et al., 2010).

It is clear from the persistence of Mtb within the granuloma that even the combined activities of innate and adaptive immunity are often insufficient to completely clear Mtb infection. The T cell represents an attractive target for manipulation for improved vaccine and treatment strategies, as its activation is central to anti-TB immunity (Chen et al., 2009; Reiley et al., 2010). Recent research indicates that the activation of T cells within the TB granuloma is inadequate, due to limited antigen presentation (Egen et al., 2011). Activation of T cells occurs through interaction with DCs in the LN.

DCs, dependent on the material they have phagocytosed (and the endocytic receptors engaged in the process), may have a tolerogenic or immunogenic phenotype, which influences the subsequent response they induce in T cells (Steinman et al., 2003; Cools et al., 2007; Torchinsky et al., 2010).

1.8 The type of cell death influences immune outcomes

Dead cells are routinely phagocytosed by DCs for processing and presentation to T cells (Albert et al., 1998a; Albert et al., 1998b). The cause of cell death, be it homoeostatic turnover or the result of infection, influences the phenotype acquired by the phagocytosing DC, which in turn helps to determine the consequent T cell phenotype activated by this DC (Figure 1-8 and Figure 1-9) (Torchinsky et al., 2010; Brereton and Blander, 2011).

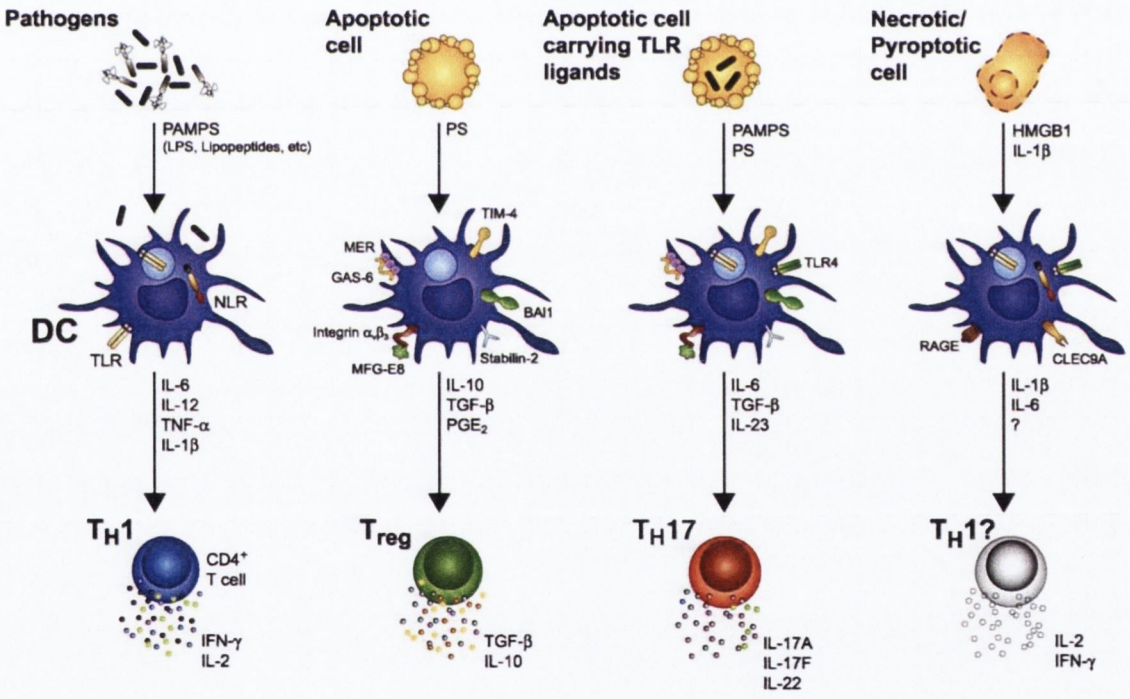


Figure 1-9: The cargo of dying cells directs the phenotype of phagocytosing DCs and the T cells they activate.

Image from Torchinsky et al. (2010).

DCs directly infected with pathogens secrete pro-inflammatory cytokines, such as IL-6, IL-12, TNF-α and IL-1β. The release of these cytokines directs the polarisation of T_H1 cells, which in turn secrete both pro-inflammatory IFN-γ and IL-2. In contrast to DC exposure to pathogens, DCs encountering uninfected apoptotic cells (which expose phosphatidylserine (PS) on their outer cell membrane: an early signal of apoptosis and a proposed tolerance-inducing molecule) can release the anti-inflammatory IL-10 and

TGF-β, together with prostaglandin E_2 (PGE_2) (Figure 1-9) (Chong et al., 2010; Tanaka et al., 2010). The combination of anti-inflammatory cytokines and PGE_2 from DCs encountering apoptotic cells can skew T cell polarisation to an anti-inflammatory, regulatory phenotype (T_{reg}) (Figure 1-9) (Torchinsky et al., 2010; Torres-Aguilar et al., 2010; Nguyen et al., 2011). PGE_2 has diverse effects on the immune system and is capable of exerting both pro- and anti-inflammatory actions (Legler et al., 2010). It can act on T cells to prevent production of T_H1 cytokines and increase secretion of IL-10 (which itself increases the anti-inflammatory action of PGE_2), as well as inhibiting T cell proliferation (Strassman et al., 1994; Kalinski et al., 1997; Li et al., 2008). The roles of PGE_2 during Mtb infection have been extensively researched. Elkington et al. (2005) found that virulent Mtb induced PGE_2 secretion, which in turn up-regulated the expression and secretion of matrix metalloproteinase-1 (MMP-1), involved in human lung cavitation (Elkington et al., 2011) and consequent spread of Mtb. PGE_2 has been shown to down-regulate macrophage responses to *M. avium* complex (Gan et al., 1995; Venkataprasad et al., 1996). PGE_2 also promotes the development of T regulatory cells (Baratelli et al., 2005; Garg et al., 2008). In a murine model of experimental tuberculosis, PGE_2 demonstrated a host-protective effect during the early phase of infection, augmenting iNOS responses and thus aiding in the control of mycobacterial growth. However, in the late or chronic stage of Mtb infection, PGE_2 down-regulated protective immune responses, leading to decreased secretion of IFN-γ, TNF-α and iNOS, and increased mycobacterial growth (Rangel Moreno et al., 2002). Chen and colleagues (2008) reported that PGE_2 secretion during Mtb H37Ra infection prevented necrosis and promoted apoptotic macrophage death by preventing mitochondrial inner membrane disruption. In their model, virulent H37Rv reduced PGE_2 production, promoting necrosis and mycobacterial growth. Divangahi et al. (2010) also demonstrated that inhibition of apoptosis by Mtb via blockade of PGE_2 production prevented T cell cross-priming, delaying adaptive immune responses to Mtb infection *in vivo*.

In the model proposed by Torchinsky et al. (2010) (Figure 1-9), DC phagocytosis of infected apoptotic cells (carrying TLR ligands from their ingested pathogens) leads to release of a combination of the pro- and anti-inflammatory cytokines IL-6/IL-23 and TGF-β, respectively, required for T_H17 polarisation (Zhou et al., 2007; Yu and Gallagher, 2010; Yabu et al., 2011). In addition, Torchinsky et al. (2010) suggest that uninfected necrotic and/or pyroptotic cells may induce a T_H1 response via their release of HMGB1 and IL-1β (Hersh et al., 1999; Brennan and Cookson, 2000; Scaffidi et al., 2002), leading to secretion of IL-6 and IL-1β (Andersson et al., 2000; Hreggvidsdottir et al., 2009; Garcia-Arnandis et al., 2010) from DCs. Hedlund and colleagues (2010) reported that exposure of human DCs to aged, apoptotic neutrophils resulted in a tolerogenic response with an absence of DC maturation, while culture of DCs with

dying, Mtb-infected neutrophils led to DC maturation, indicating an immunogenic response. These findings support the notion that DCs can distinguish between normal cell turnover and pathogenic death due to Mtb infection.

1.9 Dendritic cells and models of tuberculosis

As cell death is abundant within the TB granuloma, the activity of bystander DCs following ingestion of the remnants of Mtb-infected cells may represent a significant mode of T cell activation and polarisation (Cree et al., 1987; Fayyazi et al., 2000). Schaible and colleagues (2003) and Winau et al. (2006) demonstrated in human *in vitro* and murine *in vivo* systems (respectively) that cell death of Mtb-infected macrophages was crucial to $CD8^+$ T cell proliferation induced by bystander DCs co-cultured with the infected macrophages. *In vivo* experiments by Divangahi et al. (2010) determined that death of Mtb-infected macrophages aided both $CD4^+$ and $CD8^+$ T cell expansion in mice.

While this has identified cell death of infected macrophages as vital to the activation of T cells by bystander DCs in TB infection of mice, the attendant cytokine profiles of the DC/macrophage and the subsequent DC/T cell interactions is unknown during Mtb infection. Nor is it known whether macrophage cell death contributes to T cell activation in humans. It has been demonstrated *in vivo* that T cells display sub-maximal cytokine secretion within TB granulomas (Egen et al., 2011). Knowledge of the cytokine milieu associated with these important cell interactions can help to inform on the phenotype of the participant cells and perhaps also indicate their likely effects on surrounding cells and tissue. Targeting the activation of T cells by DCs and the effector function of these T cells (as evidenced by their secreted cytokines) may be beneficial to prevention or resolution of TB disease. As our understanding of the cellular interactions and phenotypes during TB increases, the available targets for vaccine and treatment options will also expand. *The current work examined the impact of the cell death stimulus of human macrophages on the consequent T cell response induced by uninfected bystander DCs. It compared the response induced by macrophages infected with live Mtb (which undergo cell death) with the response directed by macrophages infected with dead Mtb (which do not undergo cell death) and is detailed in Chapter 5.*

1.10 References

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2 Materials and Methods

This chapter contains a comprehensive list of the materials and methods used throughout the present work. Some methods are referred to in more detail in the individual chapters in which they were used.

2.1 Mycobacteria

2.1.1 *Mycobacterium tuberculosis* H37Ra and H37Rv

M. tuberculosis (Mtb) strains H37Ra and H37Rv were obtained from the American Type Culture Collection (Manassas, VA). Aliquots of Mtb were stored at -80°C. Before use in experiments, a 1 ml aliquot of Mtb was thawed and grown to log phase in 9 ml Middlebrook 7H9 broth (Difco/Becton Dickinson, Sparks, MD) supplemented with albumin-dextrose-catalase supplement (Becton Dickinson) and 0.05% Tween 80 (Difco) for a minimum of 7 days. The medium was refreshed every 7 days by removing 1 ml of Mtb from culture and adding to 9 ml fresh Middlebrook 7H9 medium. The bacteria were replaced with a fresh aliquot of Mtb after 30 days.

All waste having been in contact with Mtb (including Mtb suspension being discarded) was inactivated overnight in sodium dichloroisocyanurate solution (NaDCC; 5 g/L; Johnson & Johnson, New Brunswick, NJ). Work with Mtb H37Ra was carried out in a biosafety level II facility. Experiments utilising live Mtb H37Rv were performed by Dr. M. O'Sullivan in a biosafety level III facility with self-contained facilities, including waste inactivation.

2.1.2 Streptomycin treatment of *Mycobacterium tuberculosis* H37Ra

Log-phase H37Ra was treated with streptomycin sulphate (Sigma, St. Louis, MO; 0.1 mg/ml) for 48 h prior to infection. Streptomycin was thoroughly washed from Mtb prior to infection of cells. Mtb was centrifuged at 3,800 rpm for 10 min and the supernatant was discarded. An equal volume of sterile tissue culture-grade phosphate-buffered saline (PBS) was added to resuspend the pellet; this step was performed twice. Mtb was resuspended in 2 ml RPMI containing 10% pooled human serum type AB (Sigma) prior to infection of primary macrophages, or 2 ml RPMI containing 10% low-endotoxin ('defined') foetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA) prior to infection of dendritic cells (DCs) (Sections 2.3 and 2.4).

2.1.3 Gamma-irradiated *Mycobacterium tuberculosis* H37Rv

Gamma-irradiated H37Rv (iH37Rv) was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository under the following licence: NIAID, NIH: *Mycobacterium tuberculosis*, Strain H37Rv, Gamma-Irradiated Whole Cells, NR-14819. On receipt, iH37Rv was prepared in 1 ml aliquots by Dr. M. O'Sullivan and stored at -80°C for future use. To prepare iH37Rv for use in experiments iH37Rv was thawed and diluted in 5 ml sterile tissue culture-grade Dulbecco's PBS (calcium- and magnesium-free; Sigma). Bacteria were sonicated in a sonicating water bath for 15 minutes to disperse clumps.

A BD CrystalSpec nephelometer (BD Diagnostic Systems, Sparks, MD) was used to estimate iH37Rv bacterial concentration and calculate bacilli/ml of suspension (McFarland, 1907). The BD CrystalSpec nephelometer used was a small, portable device that measured the relative turbidity of bacterial suspension in a clear medium, such as Middlebrook 7H9 broth or PBS, by passing a beam of light through the sample. The light reflected from the bacteria in suspension was read by a detector. The greater the concentration of bacteria in the sample, the higher the turbidity of the suspension. The relative turbidity of the sample, compared to a clear blank liquid standard, was assigned a McFarland value, which correlated with a known concentration of bacteria per millilitre of liquid suspension (CFU/ml) (McFarland, 1907). A table of concentration ranges was supplied as part of the CrystalSpec nephelometer manual, allowing the bacilli/ml to be determined from the sample's McFarland value.

iH37Rv was centrifuged at 3,800 rpm for 10 minutes and the supernatant was discarded. The supernatant was replaced with an equal volume of RPMI containing 10% defined FBS to resuspend the bacterial pellet. Bacteria were syringed a minimum of 8 times through a 25G needle to minimise clumping prior to co-incubation with macrophages or DCs.

Media for mycobacteria

Mtb H37Ra and H37Rv were propagated in liquid culture in Middlebrook 7H9 broth prior to infection of macrophages or DCs. In order to quantify Mtb intracellular growth in DCs, Mtb was harvested from infected cells (detailed in Section 2.16) and grown on Middlebrook 7H10 agar plates.

Materials

- Middlebrook 7H9 broth (Difco)

- Middlebrook ADC enrichment (albumin, dextrose, catalase; Difco)
- Middlebrook 7H10 agar (Difco)
- Middlebrook OADC enrichment (oleate, albumin, dextrose, catalase; Difco)
- Tween 80 (Difco)
- Glycerol (Difco)
- L-asparagine, anhydrous (Sigma)
- Cycloheximide (Sigma); 1 g was dissolved in 50 ml sterile water to give 20 mg/ml stock solution; cycloheximide was stored at 4°C.

7H9 broth

7H9 powder (0.47 g) and Tween 80 (50 µl) were added to 90 ml ultra-pure water. The solution was autoclaved for 10 minutes at 120°C and 1 bar pressure, allowed to cool to 45°C and 10 ml ADC enrichment was added aseptically. The solution was mixed, allowed to cool and stored at 4°C for a maximum of 4 weeks.

7H10 agar

7H10 powder (1.9 g), L-asparagine (0.1 g) and glycerol (0.5 ml) were added to 90 ml ultra-pure water. The solution was autoclaved for 10 minutes at 120°C and 1 bar pressure and allowed to cool to 55°C in a water bath for 45 min. OADC enrichment (10 ml) and cycloheximide stock solution (50 µl) were added to the solution, which was gently mixed and plates were aseptically poured. Plates were allowed to cool and dry in the laminar flow cabinet and stored at 4°C for a maximum of 4 weeks. Before use, plates were incubated overnight at 37°C to check sterility and to dry the plates.

2.2 Cell Culture

Several different cell types were used in the current work, each requiring varying culture protocols, and are described individually in the following sections.

2.2.1 THP-1 cells

THP-1 cells, a human monocytic cell line, were obtained from the American Type Culture Collection. THP-1 cells were cultured in RPMI 1640 with L-glutamine, containing 10% FBS (both Gibco, Carlsbad, CA). The medium was refreshed every 2-3 days during culture. To differentiate THP-1 cells to macrophages, the cells were cultured with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 72-96 h at a density of 0.5×10^5 cells/ml, prior to mycobacterial infection, on 48-well plates and two-well Lab-Tek II glass chamber slides (Nunc, Roskilde, Denmark). For confocal analysis, cells were seeded on glass coverslips within 24-well plates at a higher

density of 1.0 x 10^5 cells/ml.

2.2.2 Human monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of anonymous healthy donors (provided, with permission, from the Irish Blood Transfusion Service) or from whole venous blood drawn from healthy donors and used to generate monocyte-derived macrophages (MDMs).

Isolation of PBMCs

Leucosep tubes (50 ml; Greiner Bio-One, Frickenhausen, Germany) were filled with 15 ml Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 400 x *g* for 5 min to allow the Lymphoprep to pass through the porous barrier to the bottom of the tube. Blood was slowly withdrawn from the Leukopak and carefully diluted 1:4 with tissue culture-grade Dulbecco's PBS. The diluted blood was carefully poured into the Leucosep tubes (25 – 30 ml blood per tube) and centrifuged at 400 x *g* for 25 min at room temperature with the brake off. The mononuclear cell fraction (lymphocytes and monocytes) accumulated as a white band at the interface between the plasma and the Lymphoprep layer and was aspirated using a plastic Pasteur pipette. The mononuclear cells were diluted with PBS to 20 ml and centrifuged at 400 x *g* for 10 minutes and this wash was repeated once. Cells were then resuspended in 2 ml serum-free RPMI and counted using a glass haemocytometer. Monocytes were isolated from the mononuclear cells using either plastic adherence, or magnetic bead separation. Both methods are described below.

Monocyte separation by plastic adherence

PBMCs were resuspended in serum-free RPMI 1640 at a density of 1 x 10^7 cells/ml, transferred to a T75 tissue culture flask and incubated for 2 hours in 5% CO_2 at 37°C. The monocytes adhered to the flask while the lymphocytes remained in suspension. The medium containing the lymphocytes was removed and the monocyte layer was washed once with 10 ml PBS. Ice-cold PBS/EDTA solution (PBS containing 10 mM sterile EDTA; ethylenediaminetetraacetic acid; Sigma) was added to the flask, which was then put on ice. The flask was periodically tapped to encourage detachment of the monocytes, for a maximum of 30 – 45 minutes. PBS/EDTA solution was also used to wash the cells from the flask. The cells were not scraped from the flask, to avoid prematurely activating them. Monocytes were centrifuged at 1200 rpm and resuspended in RPMI with 10% human serum. Monocytes were plated at the required

density: 1 x 10^6 cells/ml – 2 x 10^6 cells/ml. Two days following monocyte isolation, the medium was removed from the cells and replaced with fresh RPMI with 10% human serum. Where necessary, cells were washed once with serum-free RPMI before adding RPMI with 10% human serum. The medium was replenished again five days after initial isolation. Macrophages were used for Mtb experimental infection seven days after isolation.

Isolation of human monocytes from buffy coats using Invitrogen Dynabeads® Untouched™ Human Monocytes Kit

Invitrogen Dynabeads® Untouched™ Human Monocytes magnetic negative selection kit was used (Invitrogen, Carlsbad, CA). This kit depleted B cells, T cells, natural killer (NK) cells, granulocytes, erythrocytes and dendritic cells (Invitrogen, 2008), leaving the monocytes free from bound antibodies or magnetic beads. The protocol was provided by Invitrogen Corporation (Invitrogen, 2008) and was followed exactly.

Isolation Buffer

Calcium- and magnesium-free PBS supplemented with 0.1% bovine serum albumin (BSA; Sigma) and 2 mM EDTA. All buffers/solutions were kept cold and the centrifuge was maintained at 4°C.

Dynabeads

Dynabeads were vortexed in their vial for 30 seconds and the desired volume of Dynabeads was transferred to a 15 ml tube. An equal volume of Isolation Buffer was added to the beads and mixed. The tube was placed in Dyna-Mag-15 magnet (Invitrogen) for 1 min and the supernatant was discarded. The tube was removed from the magnet and the washed Dynabeads were washed in an equal volume of Isolation Buffer as the initial volume of Dynabeads.

PBMCs were prepared from buffy coats and resuspended at 1 x 10^8 PBMC per ml in Isolation Buffer. 1 ml (1 x 10^8) PBMCs in Isolation Buffer were transferred to a 50 ml tube, 200 µl Blocking Reagent was added, 200 µl Antibody Mix was added and the solution was mixed well and incubated for 20 min at 4°C. The cells were washed by adding 20 ml Isolation Buffer, mixed well by inversion and centrifuged at 350 x *g* for 8 min at 4°C. The supernatant was then discarded. Cells were resuspended in 1 ml Isolation Buffer and transferred to a 15 ml tube. 1 ml pre-washed Depletion MyOne SA Dynabeads were added and cells were incubated for 15 min at 4°C with gentle tilting and rotation. 1 ml Isolation Buffer was added and the bead-bound cells were resuspended by vigorously pipetting more than 10 times using a 2 ml serological

pipette with a narrow tip. 10 ml Isolation Buffer was added and the tube was placed in the magnet for 2 min. The supernatant, containing the monocytes, was transferred to a new 50 ml tube and the original tube was retained. 10 ml Isolation Buffer was added to the original 15 ml tube containing the Dynabeads, the bead-bound cells were resuspended by vigorously pipetting and the tube was placed in the magnet for 2 min. The two supernatants were then combined. To remove residual beads, the tube containing the combined supernatants was placed in the magnet for 2 min and the cells were transferred to a new tube.

Isolation of human monocytes from buffy coats using STEMCELL Technologies EasySep® Human Monocyte Enrichment Kit

As an alternative to the Invitrogen monocyte separation kit, the STEMCELL Technologies (Vancouver, BC) monocyte enrichment kit was used to isolate human monocytes by negative immunomagnetic separation. This kit contained CD2, CD3, CD16, CD19, CD20, CD56, CD66b, CD123, glycophorin A and dextran-coated magnetic particles, thus negatively selecting human monocytes (STEMCELL Technologies, 2009). The recommended protocol was followed as described below.

Recommended buffer

PBS containing 2% PBS (calcium- and magnesium-free) and 1 mM EDTA.

PBMCs were prepared at a concentration of 5 x 10^7 cells/ml in the recommended buffer in a 5 ml sterile polystyrene tube (BD Biosciences, Franklin Lakes, NJ). EasySep Human Monocyte Enrichment Cocktail was added at 50 µl/ml cells, mixed well and cells were incubated at 4°C for 10 minutes. EasySep D Magnetic Particles for Monocytes were vortexed and added to the cells at 50 µl/ml cells, mixed well and incubated at 4°C for 5 minutes. The cell suspension was brought to a total volume of 2.5 ml by adding the recommended buffer, mixed well and placed in an EasySep magnet (STEMCELL Technologies) for 2.5 minutes at room temperature (15-25°C). Leaving the tube in the magnet, the supernatant (containing the monocytes) was swiftly poured into a new 5 ml tube. Monocytes were then counted and plated at the desired concentration (typically 1 x 10^6 cells/ml).

2.2.3 Primary human alveolar macrophages

Alveolar macrophages (AMs) were obtained, with consent, from adults free from pulmonary disease by the Endoscopy Unit at St. James's Hospital, Dublin. Bronchoscopy was performed in patients to investigate coughing. Cells were used only

from patients with a normal chest X ray and who were found to be free from pulmonary disease on follow up. Alveolar macrophages were isolated from bronchoalveolar lavage (BAL), after volunteers had signed informed consent, approved by the St. James's Hospital/AMNCH ethics committee. AMs were isolated from the BAL fluid using the following technique:

BAL fluid was transferred from its syringe to a 50 ml tube. The fluid was filtered through a 100 µm nylon cell strainer into a fresh 50 ml tube and centrifuged at 1400 rpm for 15 minutes. The supernatant was discarded and the cells were resuspended in 2 ml RPMI containing 10% human serum, 50 µg/ml cefotaxime (Melford Laboratories, United Kingdom) and 50 U/ml amphotericin B (Gibco). Cells were counted and adjusted to 1 x 10^6 cells/ml. The viability of AMs was assessed using propidium iodide exclusion (described in Section 2.5). Where AMs were viable, cells were seeded on cell culture plates and Lab-Tek chamber slides for experiments. Cells were cultured overnight at 37°C with 5% CO_2 and were used for experiments the following day.

2.2.4 Human monocyte-derived dendritic cells

To generate human monocyte-derived dendritic cells (DCs), PBMCs were isolated from buffy coats by density centrifugation using Lymphoprep (Section 2.2.2). Monocytes were purified from PBMC by plastic adherence or by immunomagnetic separation using the STEMCELL Technologies EasySep® Human Monocyte Enrichment Kit. To generate immature DCs from monocytes, the monocytes were were seeded on 6-, 24- or 48-well culture dishes at a density of 1 x 10^6 cells/ml or 2 x 10^6 cells/ml for 6 days in RPMI containing 10% defined FBS with 40 ng/ml human IL-4 and 50 ng/ml GM-CSF (both ImmunoTools, Friesoythe, Germany). On day 3 after seeding, half the medium was carefully removed from the surface of the liquid, to avoid disturbing the cells. The medium was gently replenished with fresh medium containing double the cytokine concentration: 80 ng/ml IL-4 and 100 ng/ml GM-CSF. The DCs were used for experiments by day 6.

For cell death assessment, DCs were cultured from monocytes isolated by plastic adherence, which was a rapid and effective method for monocyte isolation and DC culture (Elkord et al., 2005; Kurlander et al., 2006). Cell death was analysed by fluorescence microscopy, ensuring that visual confirmation of DC size and morphology was performed, in order to exclude any potential contaminating cells (such as platelets, lymphocytes or erythrocytes) from analysis. Monocyte separation for DC generation was performed by negative immunomagnetic separation for caspase inhibition and caspase activity assays (Section 2.2.2). In this way, the absence of contaminating cells was ensured, to allow the measurement of caspase activity in DCs

alone.

2.2.5 Primary human T cells

Primary human T cells were isolated from PBMCs (generated from buffy coats by density centrifugation; Section 2.2.2). Human $CD3^+$ T cells were isolated from PBMCs using a human CD3 positive selection magnetic separation kit (STEMCELL Technologies). The PPD (purified protein derivative; from Mtb) status of the donors, which indicates previous exposure to Mtb (Dacso, 1990), was unknown. The separation kit protocol was followed as recommended and is included in the procedure outlined below.

Recommended buffer

PBS containing 2% PBS (calcium- and magnesium-free) and 1 mM EDTA.

PBMCs were prepared at a concentration of 1×10^8 cells/ml in buffer in a 5 ml sterile polystyrene tube. EasySep Positive Selection Cocktail was added at a concentration of 100 µl/ml cells, mixed well and cells were incubated at room temperature (15 – 25°C) for 15 minutes. EasySep Magnetic Nanoparticles were mixed by pipetting vigorously more than 5 times, added to the cells at 50 µl/ml, mixed well and incubated at room temperature for 10 minutes. The cell suspension was brought to a total volume of 2.5 ml by adding the recommended buffer, mixed well and placed in an EasySep magnet (STEMCELL Technologies) for 5 minutes. Leaving the tube in the magnet, the supernatant was swiftly discarded, leaving the desired magnetically-labelled cells in the tube. Recommended medium (2.5 ml) was added, the cell suspension was mixed gently, placed back in the magnet and set aside for 5 min. A total of three 5-minute magnetic separations were performed to maximise removal of unwanted cells. The cells were prepared for freezing by resuspending in defined FBS. Freezing medium (defined FBS containing 10% tissue culture grade dimethyl sulfoxide [DMSO]; Sigma) was slowly added drop-wise to the cells. Cells were split into aliquots, put in a freezing container (Nalgene/Thermo Fisher Scientific) containing isopropyl alcohol (Sigma) and placed in a -80°C freezer overnight. The following day, cells were removed from the freezing container and stored in a -150°C freezer until required for use.

2.3 Infection of macrophages with *M. tuberculosis*

Mtb, grown to log phase in Middlebrook 7H9 broth (Difco/Becton Dickinson, Sparks, MD) supplemented with albumin-dextrose-catalase supplement (Becton Dickinson) and 0.05% Tween 80 (Difco), was centrifuged at 3,800 rpm for 10 min and the

supernatant was discarded. Mtb was resuspended in 2 ml RPMI 1640 containing 10% FBS (for infection of THP-1 cells) or 2 ml RPMI containing 10% human serum (for infection of MDMs and AMs). Clumps of bacteria were dispersed by carefully passing the bacterial suspension through a 25 gauge needle eight times. Mtb was centrifuged at 800 rpm for 3 min to pellet any remaining clumps and the supernatant was removed and retained.

To determine the amount of Mtb necessary to achieve the required number of bacilli per cell (multiplicity of infection; MOI) and to assess the adequacy of dispersion, macrophages cultured in two-well Lab-Tek II glass chamber slides were infected with a range of volumes of resuspended Mtb suspension for 3 h. Extracellular bacteria were washed from the cells and macrophages were fixed for 5 min in 2% paraformaldehyde and stained with modified auramine O stain (Scientific Device Laboratory, Des Plaines, IL) for acid-fast bacteria. Macrophage nuclei were counterstained with 10 μg of Hoechst 33258/ml (Sigma). The percentage of infected cells and the number of bacilli per cell were determined by observing the slides under an inverted fluorescence microscope (Olympus IX51, Olympus Corporation, Center Valley, PA). This allowed determination of the volume of Mtb suspension required for the desired MOI. Based on this result, macrophages were infected at the desired MOI for 3 h. After this time, extracellular bacteria were removed by washing. The medium was gently aspirated up and down using a plastic Pasteur pipette then discarded carefully. Fresh serum-free medium was added to the cells and the wash was repeated. RPMI (containing 10% FBS or 10% human serum) was replaced following washing. Macrophages were maintained in culture at 37°C for 2 to 3 days before harvesting. Primary human AMs were maintained in culture for 4 days following infection, prior to harvesting. Where used, recombinant human IL-10 (eBioscience, Hatfield, UK) (20 ng/ml) was applied 20 min prior to infection with *M. tuberculosis* and replenished after washing. Low MOI refers to 1-5 bacilli per cell; high MOI refers to 10-20 bacilli per cell.

2.4 Infection of dendritic cells with *M. tuberculosis*

Determination of MOI for DCs differed from the macrophage protocol, due to the non-adherent nature of DCs. A nephelometer was used to calculate bacterial numbers in Mtb liquid culture on the day of each experiment. The relative turbidity of Mtb suspension (in clear Middlebrook 7H9 medium) was measured and the number of bacteria per millilitre of medium was calculated on the day of infection. The number of DCs in culture per millilitre of medium was known, and allowed calculation of the appropriate volume of Mtb suspension to use for the required MOI. Slides of infected DCs for retrospective confirmation of MOI by AFB staining were also prepared (see

Section 2.4.2).

2.4.1 Calculation of mycobacterial numbers for dendritic cell infection

Mycobacteria were suspended in Middlebrook broth, gently mixed to ensure even dispersion, placed in a sterile 5 ml glass tube in a nephelometer and McFarland reading was recorded. This was repeated twice and the average was used for calculation. The corresponding CFU/ml value for the nearest McFarland value was read. (For example, a McFarland value of 1.8 would be read from the manual as 2.0, which corresponded to 6×10^8 CFU/ml). To calculate the volume of bacterial suspension to apply to cells, the following formula was used:

$$\frac{\text{Bacterial concentration (CFU/ml)}}{\text{Cell concentration (cells/ml)}}$$

This determined the number of bacteria per cell in 1 ml bacterial suspension. The volume to use for the desired MOI, in the volume of the wells used in the experiment, was then calculated. Mycobacteria were incubated with dendritic cells for 24 h. When harvesting the cells for CFU or BacT experiments, the cells were centrifuged three times at 800 rpm in a micro-centrifuge, discarding the medium each time, to remove extracellular bacteria. As this agitation would mature the DCs, this was not suitable for experiments where DCs were to be left in culture for a number of days. In this case, half the medium was then removed from the wells and replaced with serum-free RPMI. Half the medium was removed again and replaced with RPMI containing 10% defined FBS, 100 ng/ml human GM-CSF and 80 ng/ml IL-4. Because only half the medium was replaced, this ensured that the final concentration of GM-CSF was 50 ng/ml and IL-4 was 40 ng/ml, to maintain cytokine activity and DC phenotype during Mtb infection.

2.4.2 Confirmation of MOI

Although MOI was calculated using a nephelometer prior to infection, the number of bacilli phagocytosed by DCs during infection was confirmed in every experiment by visualising stained slides of infected DCs by fluorescence microscopy. After 24 h infection with Mtb, cells were removed from the well by pipetting gently up and down to dislodge them from the plate, placed in a 2 ml microfuge tube (with a conical bottom) and an equal volume of 4% paraformaldehyde was added. After 10 minutes, the cells were spun at 5,600 rpm for 10 minutes on a micro-centrifuge. Most of the supernatant was removed, leaving only 20-50 µl. Cells were resuspended in this small volume and pipetted onto a poly-lysine coated glass slide (VWR, Radnor, PA). Slides

were left overnight to dry and then stained for acid-fast bacteria using modified auramine O stain as previously described (Section 2.3).

2.5 Viability staining

Cell viability was assessed using the propidium iodide (PI) exclusion method for plasma membrane integrity of cells, and the nuclei were counterstained with Hoechst stain. Cells were incubated with 5 µg of PI/ml, Hoechst 33342 (20 µg/ml), and Hoechst 33258 (50 µg/ml) for 10 min at room temperature. The number of PI-positive cells relative to the total number of nuclei per field was counted by automated fluorescence microscopy using the IN Cell Analyzer 1000 and IN Cell Investigator software (GE Healthcare, Pittsburgh, PA). Each condition was assayed in triplicate, and 8 fields were counted in each well. Staurosporine (Sigma) (1 µM, diluted in RPMI with 10% FBS for THP-1 cells, or in serum-free RPMI for all primary human cells) was applied for 24 h as a positive control for cell death. Results were displayed as percent PI-positive cells.

2.6 DNA fragmentation (Cell Death Detection ELISA)

The cell death detection ELISA^{PLUS} kit (Roche Applied Science, Mannheim, Germany) was used to quantify Mtb-induced DNA fragmentation as recommended by the manufacturer (Roche Applied Science, 2010). Triplicate wells were assayed for each condition. Staurosporine (1 µM, diluted in serum-free RPMI; Sigma) was applied for 24 h as a positive control for DNA fragmentation.

Cell culture plates were centrifuged at 200 x *g* for 10 min at room temperature with the centrifuge brake at zero to pellet the cells, medium was removed carefully with a plastic Pasteur pipette and lysis buffer (200 µl) was added to each well for 30 min to lyse the cells. The plates were centrifuged at 200 x *g* for 10 minutes with the brake off to pellet the lysed cells. Nucleosomes remained in the supernatant. The plate was gently removed from the centrifuge bucket in the tissue culture cabinet. From each well, 20 µl was transferred to a well on the ELISA plate. Incubation buffer (20 µl) was transferred to one well as a blank and 20 µl of the kit positive control was added to a separate well. Immunoreagent (80 µl) was added to each well and incubated for 2 hours on a plate shaker (300 rpm) at room temperature. The buffer was removed from the wells and the wells were washed 3 times with 150-200 µl of incubation buffer. ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt) substrate (100 µl) was added to the wells, mixed well, and the absorbance was read at 405 nm on a plate reader (Wallac Victor^2, Turku, Finland).

2.7 DNA fragmentation (TUNEL)

The *in situ* cell death detection kit, TMR red (Roche Applied Science), was used to detect apoptotic DNA fragmentation induced by *M. tuberculosis*, as per the manufacturer's instructions. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is a technique used to detect apoptosis by labelling the terminal ends of fragmented DNA (Gavrieli et al., 1992). The Roche kit used in this study caused polymerisation of tetramethylrhodamine (TMR)-labelled nucleotides to free 3'-OH DNA ends (fragmented DNA) by an enzymatic reaction, catalysed by terminal deoxynucleotidyl transferase (TdT) (Roche Applied Science, 1996). The incorporated TMR labelled nucleotides were then visualised by fluorescence microscopy. Each condition was assayed in triplicate and at least 600 cells were counted per well. Staurosporine-treated macrophages were used as a positive control for apoptosis. Macrophages were treated for 24 h with 1 µM staurosporine diluted in RPMI with 10% FBS for THP-1 cells, or in serum-free RPMI for primary human AMs. Fixed and permeabilised, uninfected macrophages incubated with Label Solution (not TUNEL reaction mixture) were used as negative controls.

Macrophages were centrifuged to pellet any floating dying and dead cells, and were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature. Cells were rinsed with PBS, permeabilised with 0.1% Triton X-100 for 2 minutes on ice and washed twice with PBS (200 µl/well). Cells were resuspended in 50 µl/well TUNEL reaction mixture and incubated for 1 h at 37°C in the dark. Cells were washed three times in PBS and analysed for apoptosis under a fluorescence microscope.

2.8 Protein quantification

The protein content of samples for Western blotting (Section 2.9) was quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce/Thermo Scientific, Rockford, IL). This assay relied on the detection of the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium using colorimetric detection of Cu^{+1} using a reagent containing bicinchoninic acid (Smith et al., 1985). The reaction created a purple colour that was almost linear with increasing protein concentrations (Pierce, 2008). Standards of known protein concentration were used to create a standard curve from which to calculate the concentration of protein in samples. The quantification of protein in each sample allowed for equal protein loading on Western blots.

Standards (bovine serum albumin) were diluted according to kit protocol to give a range of protein concentrations between 20-2,000 µg/ml, as well as a blank containing no protein. 25 µl sample or standard was pipetted into a well of a 96 well plate. Cell-

free RPMI 1640 containing 10% FBS, used for culture of the sample cells, was also added to a separate well. 200 µl Working Reagent was added to each well, the plate was mixed thoroughly on a plate shaker for 30 seconds, covered and incubated at 37°C for 30 minutes. The plate was cooled to room temperature and absorbance was read on a plate reader at 560 nm. The absorbance of the Blank standard was subtracted from all other standards and samples. A standard curve was plotted using the average blank-corrected absorbance measurement vs. its protein concentration in µg/ml. The standard curve was used to estimate the protein concentration in the samples. The protein concentration of cell-free RPMI 1640 containing 10% FBS (used for culturing the cells in each sample) was calculated and subtracted from each sample reading to account for the protein contribution of FBS.

2.9 Western blotting

Western blotting was carried out to confirm that the recombinant IL-10 used to treat macrophages was capable of inducing signalling (Section 2.3 and Chapter 3). Undifferentiated THP-1 cells were treated with IL-10 (20 ng/ml) for 10 minutes or 1 h. Pre-treatment with 1 µM prostaglandin E_2 (PGE_2) (Sigma) for 2 h prior to IL-10 treatment was used to increase pSTAT3 band intensity on the blot (Cheon et al., 2006). IFN-γ signalling results in STAT1 phosphorylation and was used as a positive control (200 U/ml for 10 min or 1 h; R&D Systems, Minneapolis, MN). After experimental exposure the cell culture plates were centrifuged at 200 x *g* for 10 min to sediment cells, the medium was removed from the plates and the cells were resuspended in lysis buffer. Lysates were vortexed, sonicated for 15 s, and incubated at 65°C for 15 min. Equal protein concentrations (10 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7% Tris glycine minigels. Protein was electrophoretically transferred at 28-30 V by using an Atto semidry blotter to 0.2 µm pore size polyvinylidene difluoride membrane (Immun-Blot PDVF membrane; Bio-Rad, Hercules, CA), which was then washed and blocked for 1 h with 5% non-fat milk in Tris-buffered saline–Tween (TBST) containing 0.01% sodium azide. The membrane was incubated with the appropriate primary antibody in TBST–5% milk, followed by incubation with horseradish peroxidase-conjugated secondary antibody (BD Biosciences). Signal was detected using enhanced chemiluminescence (Amersham ECL Advance Western blotting detection kit; GE Healthcare, Little Chalfont, Buckinghamshire) and exposure to Hyperfilm MP (GE Healthcare). The following primary antibodies were used: mouse anti-human phospho-STAT3 (Cell Signaling Technology, Beverly, MA), mouse anti-human STAT3 (clone 84; BD Biosciences Pharmingen, San Diego, CA), mouse anti-human STAT1 (BD Biosciences Pharmingen). STAT3 was used as a loading control.

Buffers and gels

20X TBST

1 M Tris (Sigma) pH 8.0	100 ml
NaCl (Sigma)	87.7 g
Tween 20 (Sigma)	10 ml
Distilled water	to 500 ml

Blocking buffer

5% milk in 1X TBST

10X CAPS buffer

CAPS (3-(cyclohexylamino)-1-propanesulfonic acid; Sigma)	22.13 g
Distilled water	900 ml

Solution was titrated with 2 N NaOH to pH 11 and made up to 1 L.

1X CAPS buffer

10X CAPS buffer	200 ml
Methanol	200 ml

Made to 2 L with distilled water

1% **Igepal** (Sigma), 10mM **Tris**, pH 8.0, 150mM **NaCl** (Sigma) (stored at 4°C).
Tris was dissolved and the pH was adjusted to 8. NaCl was added to 150mM (0.439g in 50ml Tris). Igepal was then added (500mg in 50ml solution)

1 M **Sodium fluoride** (Sigma) (2.1g in 50ml water)
10% **Sodium deoxycholate** (Sigma) (5g in 50ml water)

Lysis Buffer

1% Igepal, 10mM Tris, pH 8, 150mM NaCl (see above)	5 ml
Sodium fluoride (1M)	50 µl
EDTA (Sigma; 0.5M)	50 µl
Sodium deoxycholate (10%)	125 µl
Protease inhibitor cocktail (Sigma)	500 µl

containing: AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin, hemisulfate salt, Pepstatin A

Phosphatase inhibitor cocktail (Sigma)	50 µl

containing: Sodium orthovanadate, Sodium molybdate, Sodium tartrate, Imidazole

Lysis buffer (without protease and phosphatase inhibitors) was prepared prior to carrying out the experiment and stored at 4°C. Protease and phosphatase inhibitor

cocktails were added on the day of use.

Loading buffer

Distilled water	4.4 ml
0.5 M Tris-HCl, pH 6.8 (Sigma)	1.0 ml
100% Glycerol (Sigma; final concentration 10%)	0.8 ml
10% SDS (w/v) (Sigma)	1.6 ml
0.1% bromophenol blue (Sigma)	0.2 ml

Loading buffer was stored at room temperature. On the day of the experiment, DL-dithiothreitol (DTT; Fluka/Sigma) was added to the loading buffer to give a final concentration of 20 mM in loading buffer.

Stacking gel

0.5 M Tris pH 6.8	2.5 ml
10% SDS	0.1 ml
30% Acrylamide (Sigma)	1.5 ml
TEMED (Sigma)	0.01 ml
Distilled water	5.79 ml
10% Ammonium persulfate (Sigma)	0.1 ml

Separating/Resolving gel	**7%**
Distilled water	5.06 ml
1.5 M Tris pH 8.8	2.5 ml
10% SDS	0.1 ml
30% Acrylamide	2.3 ml
TEMED	0.01 ml
10% Ammonium persulfate	0.1 ml

2.10 Phosphatidylserine exposure and mitochondrial membrane potential detection

THP-1 cells were differentiated with PMA for 72 h and were then infected as described in Section 2.3. After 72 h infection, the cells were incubated for 15 min at room temperature with 10 µl of annexin V-Alexa Fluor 488 (Molecular Probes/Invitrogen, Carlsbad, CA) and MitoTracker Red CM-H_2XRos (MTR; 500 nM) mitochondrial potentiometric dye (Molecular Probes/Invitrogen). The macrophages were washed and the nuclei were counter-stained with Hoechst 33342 (20 µg/ml), and Hoechst 33258 (50 µg/ml). The cells were analyzed within 1 h using an IN Cell Analyzer 1000 automated fluorescence microscope and IN Cell Investigator software (GE Healthcare).

2.11 Cytochrome *c*/AIF immunostaining

THP-1 cells were differentiated on glass coverslips within 24-well plates and infected with Mtb H37Ra as outlined in Section 2.3. Cells were treated with 500 nM hydrogen peroxide (H_2O_2; Sigma-Aldrich) for 24 h as a positive control for macrophage apoptosis (Laochumroonvorapong et al., 1996; Ogawa et al., 2003). On days 1, 2 and 3 post-infection, cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 and blocked with 2% fish gelatine (Sigma) before incubating overnight at 4°C (or at room temperature for 1 h) in primary antibody at 1/100 dilution in 2% fish gelatine (mouse anti-human monoclonal cytochrome *c*; Invitrogen, or rabbit anti-human monoclonal AIF; Cell Signaling Technology/New England BioLabs, Danvers, MA). After thorough washing of primary antibody from the cells, the secondary antibody was added at 1/400 dilution (goat anti-rabbit Alexa Fluor 488 for AIF staining; donkey anti-mouse Alexa Fluor 488 for cytochrome *c* staining; both Invitrogen/Molecular Probes). The nuclei were counterstained with 10 µg of Hoechst 33258/ml, 5 µM DRAQ5 (BioStatus, Leicestershire, England), or 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) and visualised by confocal microscopy (Section 2.15).

2.12 MAP kinase inhibition

Differentiated THP-1 cells or primary human monocyte-derived macrophages were treated with the ERK 1/2 inhibitor, PD98059 (Sigma) or the p38 MAP kinase inhibitor, SB203580 (Sigma) 30-60 min prior to infection with Mtb. THP-1 cells were incubated with SB203580 at concentrations of 3 µl and 10 µl; and with PD98059 at 10 µl and 25 µl. Primary human MDMs were incubated with SB203580 at a concentration of 3 µl and with PD98059 at 10 µl. Cells were then infected using the method described in Section 2.3. Cell death was measured using propidium iodide exclusion after 48 h infection (Section 2.5).

2.13 Caspase inhibition

The pan-caspase inhibitor, quinolyl-valyl-O-methylaspartyl-[-2,6-difluorophenoxy]-methyl ketone (Q-VD-OPh) (20 µM; Enzo Life Sciences AG, Lausen, Switzerland), was added to DCs 4 h prior to infection with H37Ra and replenished every 24 h for the duration of infection.

2.14 Detection of caspase 3 and 7 activity

Caspase 3 and 7 activity was assesed using the luminescent Caspase-Glo assay system (Promega, Madison, WI), which measured luminescence produced by caspase-mediated cleavage of Z-DEVD-aminoluciferin substrate (Promega Corporation, 2011). Cycloheximide (5 µg/ml for 3 days in parallel with infection; Sigma) and staurosporine (1 µM for 24 h) were used as positive controls for caspase 3 and 7 activation and cell death. DCs were cultured in 96-well plates and the assays were carried out in a total volume of 200 µl. Caspase-Glo reagent was added to each well and gently mixed using a plate shaker at 300 rpm for 30 s. The plate was incubated at room temperature for 30 minutes and luminescence was measured using a Wallac Victor^2 plate reader.

2.15 Laser scanning confocal microscopy

Analysed using a Zeiss LSM 510 laser confocal microscope equipped with an Argon (488 nm excitation line; 510 nm emission detection) laser and a diode pulsed solid state laser (excitation 561 nm; emission 572 nm long pass filter) (Carl Zeiss MicroImaging GmbH). Images were generated and viewed using LSM Image Browser (Carl Zeiss MicroImaging).

2.16 Colony forming units

Dendritic cells were harvested 24 h (Day 1) or 72 h (Day 3) after infection with Mtb. Cells were centrifuged at 800 rpm for 5 min and the supernatant was discarded. This step was repeated twice to remove extracellular bacteria. Cells were lysed using 0.1% Triton X-100 for 10 min and samples were centrifuged at 10,800 rpm for 10 min to pellet mycobacteria released from lysed DCs. The supernatant was discarded, the mycobacteria were resuspended in Middlebrook medium and passed through a 25G needle eight times to disperse clumps. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} were prepared in Middlebrook 7H9 broth and 10 µl aliquots were plated in triplicate on Middlebrook 7H10 agar for colony forming unit (CFU) determination. Agar plates were incubated at 37°C for 21 days, CFUs were counted and the average number of CFUs per plate was calculated.

2.17 BacT/ALERT® 3D

The BacT/ALERT® 3D automated microbial detection system was used to detect mycobacterial growth (in parallel with colony forming unit assessment on agar plates). Samples for analysis were inoculated into sealed bottles containing Mtb liquid culture

medium. The base of each bottle housed a sensor that underwent colour change on contact with carbon dioxide and other metabolites produced by growing Mtb. The detection system detected alterations in light reflectance due to the colorimetric changes in the sensor (BioMerieux SA, 2011). The BacT/ALERT® 3D system took readings from each bottle at 10 minute intervals for 41 days. On reaching a threshold reflectance value (unknown; proprietary information of the company) during this time, the BacT/ALERT® 3D system marked the bottle 'positive'. If the threshold reflectance had not been reached at 41 days (sufficient time for Mtb growth), the bottle was deemed negative for mycobacterial growth.

In parallel with CFUs, 500 µl of bacterial suspension was inoculated into each bottle of the bioMérieux BacT/ALERT® 3D automated microbial detection system (bioMérieux, Durham, NC) and time to reach positivity was recorded. Percentage time to positivity (TTP) was calculated using the following equation:
((TTPDay1-TTPDay3)/TTPDay3)x100 as previously published for the BD BACTEC liquid culture platform (Keane et al., 2002). In this equation TTP Day 1 is the time to culture positivity for infected DC lysates at Day 1, and TTP Day 3 is the time to positivity for infected DC lysates at Day 3. A positive change in percentage TTP indicated mycobacterial growth.

2.18 Flow Cytometry

Dendritic cell surface markers, T cell surface markers and T cell proliferation were analysed by flow cytometry on a CyAn ADP flow cytometer (Dako/Beckman Coulter, Brea, CA). Cells were gated by forward scatter and side scatter to exclude dead cells and debris. Results were analysed using Summit software version 4.3 (Dako/Beckman Coulter).

Antibodies

- FITC mouse anti-human CD14 (clone M5E2), FITC mouse IgG2a, κ isotype control (BD Pharmingen)
- FITC mouse anti-human CD40 (clone 5C3), FITC mouse IgG1 κ isotype (BD Pharmingen)
- APC mouse anti-human CD83 (clone HB15e), APC mouse IgG1 κ isotype control (BD Pharmingen)
- PE mouse anti-human CD86 (clone 2331; FUN-1), PE mouse IgG1 κ isotype control (BD Pharmingen)
- FITC mouse anti-human CD86 (clone 2331; FUN-1), FITC mouse IgG1 κ isotype control (BD Pharmingen)

- PE mouse anti-human CD209 (DC-SIGN; clone DCN46), PE mouse IgG2b κ isotype control (BD Pharmingen)
- PerCP/Cy5.5 mouse anti-human CD209 (DC-SIGN; clone 9E9A8), PerCP/Cy5.5 mouse IgG2a, κ isotype control (BioLegend, San Diego, CA)
- APC mouse anti-human CD2 (clone LT2), APC mouse IgG2a isotype control (ImmunoTools)
- Pacific Blue mouse anti-human CD4 (clone RPA-T4), Pacific Blue mouse IgG1, κ isotype control (BD Pharmingen)
- V450 mouse anti-human CD4 (clone RPA-T4), V450 mouse IgG1, κ isotype control (BD Horizon, San Diego, CA)
- FITC mouse anti-human CD4 (clone L3T4), FITC mouse IgG1, κ isotype control (eBioscience)
- PE mouse anti-human CD8 (clone MEM-31), PE mouse IgG2a isotype control (ImmunoTools)
- APC mouse anti-human CD25 (clone BC96), APC mouse IgG1, κ isotype control (eBioscience)
- APC/Cy7 mouse anti-human CD25 (clone BC96), APC/Cy7 mouse IgG1, κ isotype control (BioLegend)
- PE mouse anti-human CD71 (clone MEM-75), PE mouse IgG1 isotype control (ImmunoTools)

2.19 Cell surface marker staining

Cells were kept on ice and centrifuged at 4°C unless otherwise indicated. Cells were transferred from the cell culture plate well to a labelled FACS (fluorescence-activated cell sorting) tube and the well was washed once with 0.5 ml of PBS. (These steps were omitted if the cells were stained *in situ* on their tissue culture plate.) Cells were centrifuged (1500 rpm, 5-10 min) and washed once with 500 µl of FACS buffer. The supernatant was aspirated and the pellet was resuspended in 100 µl of FACS buffer. Antibody (10 µl, or as directed by the manufacturer) was added to the tubes/wells. 7-aminoactinomycin D (7-AAD; Sigma), when used, was added as a viability stain at this stage (20 µg/ml). Cells were mixed gently and incubated for 20 minutes at room temperature in the dark. Cold FACS buffer (500 µl) was added to the cells, centrifuged at 1500 rpm for 5 min, the supernatant was discarded and the wash was repeated once. Cells were resuspended in 300 µl of FACS buffer with 100 µl 4% paraformaldehyde, mixed and stored on ice protected from light. If 7-AAD was used, actinomycin D (Sigma) was added at this stage at the same concentration as 7-AAD (20 µg/ml) to prevent leaching of 7-AAD from the cells. Cells were fixed for at least 2 h prior to flow cytometry.

FACS buffer (Filter sterilised and stored at 4°C):

Ca2+/Mg2+-free PBS	500 ml
Sodium azide (0.02%)	0.1 g
BSA (0.2%)	1 g

2.20 T cell labelling with CFSE

T cells were labelled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Invitrogen). The staining protocol described was adapted from Lyons and Doherty (2004). Prior to mixed leukocyte reaction with DCs (Section 2.21), previously isolated and frozen $CD3^+$ T cells (Section 2.2.5) were thawed quickly at 37°C, centrifuged to remove freezing medium and resuspended in PBS/0.1% bovine serum albumin (BSA; Sigma) at a final concentration of between 3×10^6 cells/ml and 6×10^6 cells/ml. CFSE (5 mM; 2 µl) was added per millilitre of cells (final concentration of 10 µM CFSE) in a 50 ml tube and the cells were incubated for 10 min at 37°C in the dark. Following incubation, the CFSE staining was stopped by adding a 5 x greater volume than cells of ice-cold RPMI with 10% defined FBS and incubating on ice for 5 min. The cells were washed three times in RPMI with 10% defined FBS and resuspended in RPMI containing 10% defined FBS and penicillin-streptomycin (Sigma; 10 µl/ml). An aliquot of labelled cells was fixed and stored at 4°C for flow cytometer calibration and the remainder of the labelled T cells were incubated with stimulated DCs (Section 2.21.3) for 3-5 days.

2.21 Mixed leukocyte reaction

To determine the responses of $CD3^+$ T cells to DCs stimulated by uninfected, apoptotic macrophages or Mtb-infected, dying macrophages (Chapter 5), a mixed leukocyte reaction (MLR) was performed. This was a three-stage process:

Step 1: Human macrophages were infected with live Mtb H37Ra or streptomycin-killed H37Ra for 3 days to induce cell death. In parallel, separate macrophages were incubated for 3 days with cycloheximide (5 µg/ml). Uninfected macrophages were also used as a control.
Step 2: Autologous DCs were incubated with the macrophages for 2 days.

Step 3: After 2 days, DCs were removed and cultured with allogeneic T cells for 3-5 days. DCs matured with LPS alone (1 µg/ml; Sigma) were used as controls. Unstimulated DCs (not incubated with macrophages) were also used as controls in one experiment.

The activation and proliferation of the T cells was measured by flow cytometry and the cytokine response was measured by ELISA.

2.21.1 Infection and treatment of macrophages

Primary human monocyte-derived $CD14^+$ monocytes were selected by negative magnetic separation (Section 2.2.2) and differentiated. Macrophages were infected at MOI 20, as in Section 2.3, or treated with cycloheximide (5 μg/ml) for 3 days. The following mycobacteria were used: live H37Ra and streptomycin-killed H37Ra. Prior to addition of DCs after 3 days, cycloheximide was thoroughly washed and any detached macrophages were added back into the well. Macrophage cell death was analysed by PI exclusion (Section 2.5) after 3 days.

2.21.2 Macrophage and dendritic cell co-culture

Autologous DCs were generated from the same pool of $CD14^+$ monocytes used to generate macrophages, as in Section 2.2.4. They were allowed to differentiate for 8 days. After 3 days of Mtb infection or treatment of macrophages, the plate was centrifuged to sediment any detached cells. DCs were supplemented with fresh RPMI 1640 containing 10% defined FBS, IL-4 (final concentration 40 ng/ml), GM-CSF (final concentration 50 ng/ml) and penicillin-streptomycin (10 μl/ml; to kill extracellular Mtb), and then added to the macrophages. DCs were co-cultured with macrophages to allow DC stimulation for 2 days.

2.21.3 Dendritic cell and T cell co-culture

Allogeneic $CD3^+$ T cells (labelled with CFSE (Section 2.20) or unlabelled) were plated on 96 well V-bottom plates. DCs were gently removed from their wells and added to T cells. Co-culture was maintained in RPMI containing penicillin-streptomycin (10 μl/ml). The medium (RPMI with 10% defined FBS and penicillin-streptomycin (10 μl/ml) was replenished every 3 days. Cells were cultured for up to 5 days. Proliferation was measured by CFSE dilution after 4 days by flow cytometry. Surface expression of CD25 and CD71 was measured at day 5 by flow cytometry.

2.22 Cytokine analysis using MesoScale Discovery platform

Cytokine secretion was measured in cell-free supernatants by ELISA by Dr. M. O'Sullivan using the Meso Scale Discovery SECTOR Imager 2400 and the following assays: human IL-6, human TGF-β, human IL-17 assays and the human Th1/Th2 10-

cytokine multiplex assay, capable of detecting IFN-γ, IL-1β, IL-10, IL-12 p70, IL-13, IL-2, IL-4, IL-5, IL-8 and TNF-α (all Meso Scale Discovery, Gaithersburg, MD).

Buffers and ELISA plate were prepared as per manufacturer's instructions. Calibrators (standards) were prepared to allow calculation of a standard curve (with a detection range of 0-10,000 pg/ml). Blocking Buffer was applied for 1 h at room temperature and washed 3 times with PBS containing 0.05% Tween-20. 25 µl calibrator or sample was pipetted into a separate well of the ELISA plate, sealed and incubated for 1-2 h on a microplate shaker (Cellestis, Victoria, Australia) at 300-1000 rpm. 25 µl Detection Antibody Solution was added to each well, the plate was sealed and incubated for 1-2 h at room temperature on a microplate shaker at 300-1000 rpm. The plate was washed 3 times with PBS containing 0.05% Tween and 150 µl 2X Read Buffer T was added to each well. The plate was read on a SECTOR Imager 2400, which adjusted for dilution and generated a standard curve. The following samples were probed by ELISA:

Dendritic cells directly infected with Mtb: live H37Ra or streptomycin-killed H37Ra at MOI 1 for 24 or 48 h. LPS was applied for 24 h (Sigma; 1 µg/ml) as a positive control for DC maturation and cytokine secretion.

Co-cultures of dendritic cells with Mtb-infected, dying macrophages: Macrophages were infected and co-cultured with DCs (Section 2.21.2). After 2 days of co-culture, supernatants were harvested for ELISA. LPS was applied for 24 h (1 µg/ml) to DCs in the absence of macrophage co-culture as a positive control.

Co-cultures of dendritic cells and allogeneic T cells: Following co-culture with infected macrophages (above), DCs were co-cultured with T cells (Section 2.21.3). After 3 days, supernatants were removed for ELISA analysis.

2.23 Statistical analysis

Results are expressed as means ± the standard errors of the mean (SEM). The data were analysed using GraphPad Prism 5 (La Jolla, CA) statistical software using repeated measures one way analysis of variance (ANOVA) with Tukey's post test. The non-parametric Friedman test, followed by the Wilcoxon signed-rank test, was used where indicated. A P value of <0.05 was considered statistically significant. Graphs were compiled using GraphPad Prism 5 software.

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3 Characterisation of the human macrophage cell death mechanism during *Mycobacterium tuberculosis* infection

3.1 Introduction

Mycobacterium tuberculosis (Mtb) infects and multiplies within alveolar macrophages (AMs), evading destruction by the immune system. Mtb-infected macrophages can undergo cell death. The programmed death of macrophages deprives Mtb of a protected environment, prevents dissemination, causes bacillary killing and leads to activation and cross-priming of Mtb-specific $CD4^+$ and $CD8^+$ T cells, respectively (Molloy et al., 1994; Oddo et al., 1998; Schaible et al., 2003; Winau et al., 2006; Randhawa et al., 2008; Divangahi et al., 2010). Programmed cell death, therefore, is an important host immune response during tuberculosis infection. The mode of cell death undergone by infected cells can also have a significant impact on the immune response of bystander cells (Liu et al., 2002; Griffith et al., 2007; Li et al., 2009; Chong et al., 2010); therefore the mechanisms mediating macrophage death during Mtb infection are of considerable interest. The recognition of the influence of cell death on the immune response has led to the development of candidate tuberculosis vaccines capable of inducing cell death, which exhibit enhanced immunogenicity and efficacy *in vivo* compared with the current *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine (Hinchey et al., 2007; Gartner et al., 2008; Ranganathan et al., 2009; Hinchey et al., 2011). A modified form of BCG, with the capacity to induce macrophage death, has also demonstrated increased immunogenicity over the BCG strain used in the existing tuberculosis vaccine (Kernodle and Bochan, 2002; Snyder, 2006; Sadagopal et al., 2009). The precise mechanisms mediating human macrophage death during Mtb infection are currently undefined. Both apoptotic and non-apoptotic modes of macrophage death have been reported during Mtb infection (Danelishvili et al., 2003; Chen et al., 2006; Behar et al., 2011). Previous studies have indicated the involvement of the mitochondria in Mtb-induced macrophage death (Duan et al., 2002; Sly et al., 2003; Gan et al., 2005; O'Sullivan et al., 2007). The present work sought to elucidate the role, if any, of the mitochondria in Mtb-induced macrophage cell death.

The mitochondria are involved in a number of cell death pathways, including the intrinsic apoptosis pathway, the caspase-independent form of cell death known as parthanatos and necrosis. Apoptosis occurring via the intrinsic pathway leads to the insertion of active BAK and BAX proteins into the outer mitochondrial membrane, causing mitochondrial outer membrane permeabilisation (MOMP). MOMP allows

release of the mitochondrial proteins, cytochrome *c* and apoptosis-inducing factor (AIF). Cytochrome *c* mediates formation of the apoptosome, causing downstream activation of the executioner caspase 3. AIF participates in DNA fragmentation. Truncated BID (tBID) promotes mitochondrial cytochrome *c* release during apoptosis and was detected by O'Sullivan et al. (2007) in macrophages infected with Mtb, suggesting cytochrome *c* as a candidate involved in the execution of Mtb-induced macrophage death. Parthanatos is a caspase-independent form of cell death initiated by PARP-1 and mediated, in part, by AIF released from the mitochondria (David et al., 2009; Zhang et al., 2010; Yu et al., 2011). Macrophage death during Mtb infection *in vitro* is caspase-independent, suggesting the possible involvement of parthanatos, which proceeds via a mitochondrial pathway in the absence of caspase activation (Lee et al., 2006; O'Sullivan et al., 2007; Wang et al., 2009). Necrosis is also mediated by a mitochondrial pathway. Formation of mitochondrial permeability transition pores (MPTP) in the inner mitochondrial membrane during necrosis can cause loss of mitochondrial membrane potential ($\Delta\Psi_m$) prior to MOMP, leading to cell death (Halestrap, 2009).

Mtb has evolved strategies to interfere with successful macrophage death, allowing the bacilli to remain shielded from the immune system. There is evidence that attenuated strains of Mtb – such as H37Ra – promote macrophage death, while virulent strains – such as H37Rv – lead to comparatively lower death of infected macrophages (Keane et al., 1997; Fratazzi et al., 1999; Ríos-Barrera et al., 2006). Keane et al. (2000) observed abundant death of primary human AMs infected with the attenuated strain H37Ra, with significantly less cell death induced by infection with the virulent H37Rv. Others have reported similar findings in human macrophage and alveolar epithelial cell lines (Danelishvili et al., 2003). It is postulated that this inhibition of macrophage death by virulent Mtb constitutes a virulence factor, aiding its survival within infected cells (Sly et al., 2003; Loeuillet et al., 2006; Behar et al., 2011).

Equally, however, there are numerous reports of the induction of abundant cell death by virulent Mtb strains (Chen et al., 2006; Gan et al., 2008; Grover et al., 2008; Abebe et al., 2011; Lee et al., 2011). There are several possible reasons for this dichotomy. Varying cell types (cell lines vs. primary cells, peripheral vs. alveolar macrophages) and differing species (human, mouse or guinea pig) utilised in studies may account for some differences. There is also wide variation in the Mtb bacilli:cell ratio (multiplicity of infection; MOI) used by different research groups, ranging from 1:1 (Herbst et al., 2011) to 50:1 (Lee et al., 2006) and higher (Danelishvili et al., 2010). This disparity in MOI could also have a significant effect on the viability of infected cells. Another proposed explanation for these conflicting reports is that the

mode of macrophage death induced by virulent and attenuated Mtb strains differs: attenuated strains are suggested to promote death via macrophage apoptosis, while virulent strains may induce macrophage necrosis and simultaneously inhibit apoptosis (Chen et al., 2006; Wong and Jacobs Jr, 2011). Necrosis is proposed to allow unimpeded release of Mtb from the dying cell, in contrast to the mycobactericidal consequence of apoptosis (Molloy et al., 1994). However, the use of the terms apoptosis and necrosis in the context of Mtb-induced cell death are not clearly defined. It is increasingly recognised that each mode of cell death comprises a number of characteristics (as discussed in the introductory chapter of the current work), including morphological observations (for example, nuclear fragmentation, DNA fragmentation or phosphatidylserine exposure) and activity of differing classes of proteases (such as caspases or cathepsins) (Kroemer et al., 2009). No single cell death assay can inform on the entire spectrum of characteristics of any mode of cell death. For this reason, it is necessary to perform a number of assays to determine and define the type of cell death being observed. Defining the mode of Mtb-induced macrophage death as apoptosis or necrosis based on an insufficient number of assays may be misleading and could further contribute to the lack of consensus regarding the macrophage death phenotype in Mtb infection.

The current work aimed to identify the cell signalling pathways responsible for a reported association between IL-10 and inhibition of human macrophage death during virulent Mtb H37Rv infection. IL-10 secretion is greater from macrophages infected with virulent *Mycobacterium bovis* compared with macrophages infected with attenuated *M. bovis* strains, with a simultaneous reduction in cell death (Rodrigues et al., 2009). Virulent Mtb H37Rv infection is proposed to be associated with reduced human macrophage death, due to increased IL-10 production during infection (Balcewicz-Sablinska et al., 1998). This pro-survival effect of IL-10 was also observed by Balcewicz-Sablinska et al. (1998) during attenuated H37Ra infection of macrophages with the addition of exogenous IL-10. The signalling pathways involved in IL-10-mediated macrophage cell death inhibition during Mtb infection remain to be elucidated. A study using a human monocytic cell line (U937) reported that p38 mitogen-activated protein (MAP) kinase inhibition reduced levels of apoptosis during *E. coli* infection (Wang et al., 2007). *Mycobacterium avium* induces murine macrophage death via p38 signalling, a pathway that can be inhibited by IL-10 (Bhattacharyya et al., 2003). Additionally, recent evidence suggests that p38 signalling is involved in murine macrophage death during Mtb H37Ra infection (Wojtas et al., 2011). In contrast, ERK1/2 MAP kinase signalling has been implicated in the inhibition of murine macrophage death observed during Mtb H37Rv infection (Wojtas et al., 2011). The present study sought to investigate the involvement (if any) of the p38 and ERK1/2

pathways, which may be inhibited or activated, respectively, by IL-10 signalling, in Mtb-induced human macrophage death (Niiro et al., 1998; Zhou et al., 2001).

Hypothesis

- Mtb-induced macrophage death occurs via a mitochondrial pathway.

- IL-10 and its downstream signalling pathway promotes macrophage survival during Mtb infection. IL-10 inhibition of the p38 MAP kinase signalling pathway is responsible for the pro-survival effects of IL-10 on human macrophages during Mtb infection, while IL-10 induction of ERK1/2 signalling promotes macrophage viability during Mtb infection of human macrophages.

Aims of this section

The aims of this work were to determine the role of mitochondrial cell death pathways within human Mtb-infected macrophages and to elucidate the downstream signalling that mediates the reported pro-survival effects of IL-10 in Mtb-infected human macrophages.

Summary of results

- Although loss of mitochondrial membrane potential ($\Delta\Psi_m$) was observed in Mtb-infected macrophages, both cytochrome *c* and AIF were retained within the mitochondria – indicating the absence of mitochondrial outer membrane permeabilisation (MOMP) – even while high levels of cell death occurred within the macrophage cultures.

- Although it had been previously suggested that IL-10 reduced macrophage death during Mtb infection, this effect was not observed either in primary human macrophages or in a cell line model.

- Inhibition of either the p38 or ERK1/2 MAP kinase pathways had no significant effect on the viability of Mtb-infected macrophages.

3.2 Methods

The materials and methods used are more fully described in Chapter 2. This section outlines the basis for the choice of methods utilised herein.

3.2.1 Cell culture

The current work studied the effect of IL-10 on cell death of three types of human macrophage during Mtb infection: an immortalised monocytic cell line (THP-1), primary human peripheral monocyte-derived macrophages (MDMs), and primary human AMs. Work was initially carried out in THP-1 cells, as they have been demonstrated to be a suitable model in which to study human macrophage cell death during Mtb infection (Riendeau and Kornfeld, 2003). Following optimisation, experiments were repeated in primary human MDMs, generated from buffy coats received from the Irish Blood Transfusion Service. Being primary human cells, these were a relevant model in which to study macrophage cell death responses to Mtb infection. The most representative macrophage in which to study Mtb infection was the human AM. These cells were isolated from patients who underwent bronchoscopy for persistent cough. Macrophages were used only from patients with a normal chest X ray and who were found on follow-up to be free from pulmonary disease. As these cells were a valuable reagent, and not always readily available, they were used once experiments had been fully optimised.

3.2.2 Infection with Mtb

The attenuated Mtb strain H37Ra induces a form of cell death that displays some features of apoptotic death (exposure of phosphatidylserine), but differs from classical apoptosis, in that it is not mediated by the activity of caspases (O'Sullivan et al., 2007). The involvement of a mitochondrial death pathway was suggested by the report of O'Sullivan et al. due to their detection of cleaved BID, which can mediate the release of cytochrome *c* from the mitochondria, leading to apoptosis via the intrinsic pathway. In contrast, AIF release from the mitochondria can mediate cell death in the absence of caspase activation, which may be pertinent due to the caspase-independent nature of Mtb-induced macrophage death. Macrophages were infected with Mtb H37Ra in order to assess the localisation of cytochrome *c* and AIF during infection, to investigate the involvement of the intrinsic apoptosis pathway and parthanatos during Mtb-induced macrophage death. Similarly, in order to examine the role of IL-10 in macrophage cell death inhibition during Mtb infection, macrophages were infected with the attenuated Mtb strain H37Ra, as this laboratory strain of Mtb was shown to induce abundant cell death – which could be inhibited by addition of exogenous human IL-10 – by Balcewicz-Sablinska et al. (1998).

Particular attention was paid to ensuring an accurate and consistent number of phagocytosed bacilli per macrophage (MOI) for each experiment to exclude variation in the phagocytosis of Mtb between experiments. Any phagocytic variation in the THP-1 cell line was most likely due to batch variations in foetal bovine serum (used in the cell culture medium). In primary cells, donor variation also led to varying phagocytosis of Mtb between individual donors. Therefore, as part of each experiment, a number of monocytes/macrophages were removed from the total cell sample volume on day one of the experiment and propagated directly on glass slides. On the day of infection these macrophages were infected with a range of volumes of mycobacterial suspension for 3 hours prior to the experimental infection. The slides were then stained for Mtb using acid-fast bacteria (AFB) staining to visualise and manually count, under fluorescence microscopy, the number of Mtb bacilli phagocytosed by the macrophages. The appropriate volume of mycobacterial suspension was thus determined in order to ensure a consistent MOI for all experiments. Low MOI is defined as 1-5 phagocytosed Mtb bacilli per macrophage and high MOI refers to 10-20 bacilli per cell.

IL-10 was added 20 minutes prior to infection (in keeping with the method used by Balcewicz-Sablinska et al. (1998)). IL-10 was replenished after washing off non-phagocytosed Mtb (three hours following Mtb incubation with macrophages; see Methods Section 2.3) in order to maintain IL-10 within the macrophage cultures for the duration of Mtb infection.

3.2.3 Detection of phosphatidylserine exposure, $\Delta\Psi_m$ and localisation of AIF and cytochrome *c*

The cellular localisation of cytochrome *c* and AIF was assessed by confocal microscopy. Cell death was assessed by phosphatidylserine (PS) exposure and detection of $\Delta\Psi_m$, which were measured by fluorescence microscopy. Loss of $\Delta\Psi_m$ can initiate cell death, or occur as a consequence of cell death initiated by another mechanism, such as cytochrome *c* release (Zamzami et al., 1995; Bossy-Wetzel et al., 1998; Kruman et al., 1998; Fimognari et al., 2011). Exposure of PS, a lipid normally found on the inner leaflet of the plasma membrane, on the outer plasma membrane is an early marker of apoptosis and is also reported during parthanatos (Wang et al., 2009). Annexin V is a protein that binds to PS and can be used to indirectly probe for exposed PS (Koopman et al., 1994). Fluorescently-tagged annexin V binding was used here to assess PS exposure in THP-1 cells infected with Mtb H37Ra, as O'Sullivan et al. (2007) demonstrated that PS exposure occurred as THP-1 cells died during Mtb infection. Additionally, the dye used to detect $\Delta\Psi_m$ (which loses its fluorescence in parallel with

the loss of $\Delta\Psi_m$) possessed red fluorescence, preventing the use of propidium iodide (also a red fluorescent dye, described in the next section) in simultaneous viability assessment.

The GE IN Cell Analyzer 1000 automated high content analysis fluorescence microscopy system was utilised for the detection and analysis of PS exposure and $\Delta\Psi_m$. The system consisted of an automated fluorescence microscope, which captured images, and analysis software (IN Cell Investigator) for automated identification and quantification of cells of interest. A minimum of eight fields of view within each cell culture plate well were subjected to imaging. The fields were manually set to evenly sample from the whole of the well (rather than cluster in one area), and this same set of fields was used for each well of the culture plate being analysed. User-adjustable proprietary analysis programs within the IN Cell Investigator software package were then used to analyse the captured images. The analysis program used in the current work was Dual Area Object Analysis, which allowed for simultaneous detection of the $\Delta\Psi_m$ and PS exposure (Figure 3-1). The software initially detected and quantified total cells by their nuclear fluorescence (using blue fluorescent Hoechst staining, with an emission wavelength of approximately 461 nm). Cellular PS exposure was detected by green fluorescence (emission wavelength approximately 488 nm) and $\Delta\Psi_m$ was detected by red fluorescence (emission wavelength approximately 599 nm). Viable cells displayed intense fluorescence in the red ($\Delta\Psi_m$) channel. The PS exposure by dying cells appeared in the green channel. As cells died, fluorescence dimmed in the red ($\Delta\Psi_m$) channel.

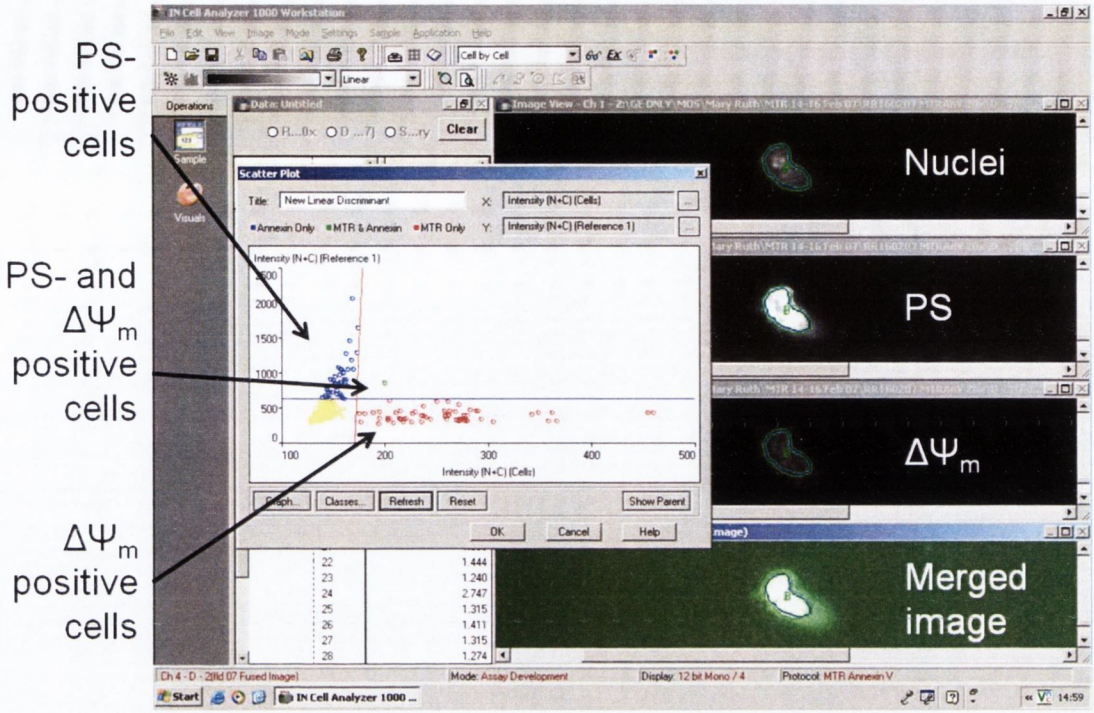


Figure 3-1: Classification of PS and $\Delta\Psi_m$ positive cells using GE IN Cell Investigator analysis software.

The figure above shows an example of cells being assessed for PS and $\Delta\Psi_m$ using GE IN Cell Investigator software. THP-1 cells were differentiated to macrophages using 100 nM phorbol 12-myristate 13-acetate (PMA) for 72 h. Macrophages were infected at MOI 5-10 for 72 h, then were incubated for 15 min at room temperature with 10 µl of annexin V-Alexa Fluor 488, MitoTracker Red (MTR; 500 nM) mitochondrial potentiometric dye, Hoechst 33342 (20 µg/ml) and Hoechst 33258 (50 µg/ml). Cells were initially identified by the software using their nuclear fluorescence (provided by Hoechst staining). From this population of cells, the fluorescence intensity of MitoTracker Red (MTR; $\Delta\Psi_m$; x-axis) and annexin V (phosphatidylserine (PS); y-axis) were plotted on a scatter plot to define PS-positive (blue circles), $\Delta\Psi_m$ positive (red circles), PS and $\Delta\Psi_m$ double-positive (green circles) and negative (yellow circles) sub-populations. The software then quantified the number of cells in each sub-population.

The GE Investigator software was manually observed and monitored throughout its analysis, to ensure that the program successfully detected and correctly identified each parameter.

3.2.4 Macrophage viability assessment

The propidium iodide (PI) exclusion method was chosen to assess viability of Mtb-infected macrophages in experiments where $\Delta\Psi_m$ was not being measured. This fluorescent, DNA intercalating agent stains the DNA and RNA of dead and dying cells that have lost their plasma membrane integrity, but does not permeate the membrane of viable cells, thus discriminating dead cells from live cells (Suzuki et al., 1997). It is particularly useful for detecting death of adherent cells by microscopy (Zamai et al., 2001) and was used here to identify death of Mtb-infected macrophages by automated fluorescence microscopy (using a GE IN Cell Analyzer 1000 with the Dual Area Object

Analysis program described above, optimised to detect nuclear Hoechst staining and PI staining of dead cells). Figure 3-2 below is a screenshot from the IN Cell Analyzer 1000 showing an example of Hoechst and PI staining in uninfected differentiated THP-1 cells.

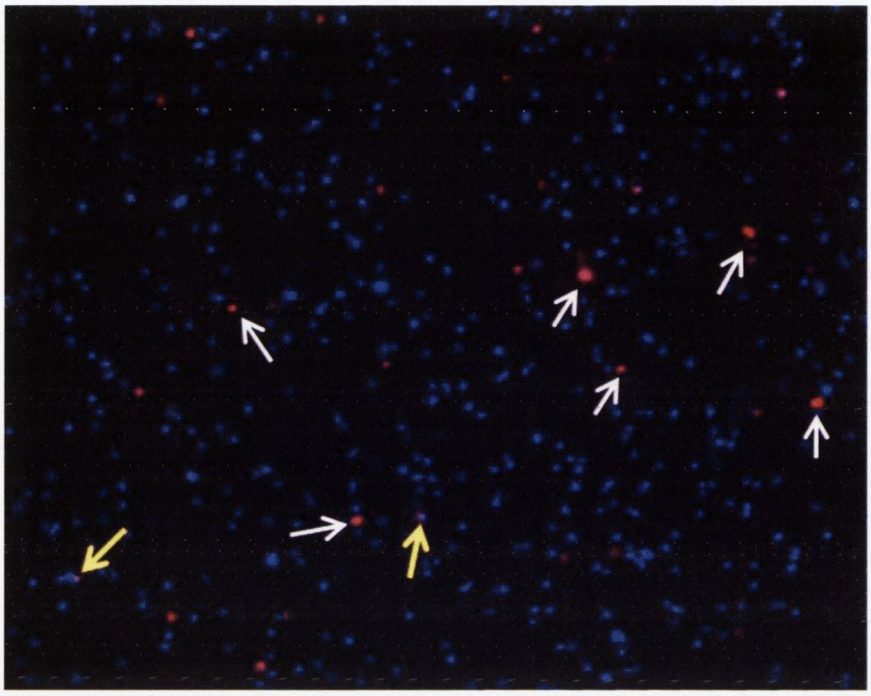


Figure 3-2: Cell viability assessment by propidium iodide exclusion using a GE IN Cell Analyzer 1000.

The figure above shows an example of uninfected macrophages stained with propidium iodide to assess viability. THP-1 cells were differentiated to macrophages using 100 nM PMA for 72 h. The uninfected macrophages were then incubated with 10 µg of PI/ml, Hoechst 33342 (20 µg/ml), and Hoechst 33258 (50 µg/ml) for 10 min at room temperature. The cells were visualised on a GE IN Cell Analyzer 1000 and analysed to detect viable (blue nuclear staining; Hoechst) and dead (blue and red nuclear staining; Hoechst and PI co-staining) cells. The intense fluorescence of PI frequently obscured the fainter Hoechst fluorescence of dead and dying cells in the merged images, with the result that only the red fluorescence of dead cells was visible. Some dead cells are indicated in the image above. The yellow arrows indicate cells with discernible Hoechst and PI co-staining.

The PI exclusion method does not allow discrimination of the mode of cell death (i.e. apoptotic vs. non-apoptotic cell death). It was used here to detect any alterations in macrophage viability due to exogenous IL-10 application or blockade of p38/ERK1/2 signalling during Mtb infection.

DNA fragmentation occurs during both apoptotic and non-apoptotic cell death, and is a feature of the non-apoptotic macrophage death induced by Mtb (O'Sullivan et al., 2007). DNA fragmentation was also observed in Mtb-infected AMs in the work reported by Balcewicz-Sablinska et al. (1998). In the current work, DNA fragmentation was measured in two ways:

1. The Roche Cell Death Detection ELISA^{PLUS} kit. This method, used by O'Sullivan et al. (2007), detected free nucleosomes released into the cytoplasm from fragmented DNA of dead and dying cells.

2. The terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labelling (TUNEL) technique, assessed by fluorescence microscopy. This method was used by Balcewicz-Sablinska et al. (1998) to detect DNA fragmentation in Mtb-infected human AMs and was replicated in the current work.

3.2.5 Statistical analysis

The data were analysed by GraphPad Prism 5 statistical software using repeated measures one-way analysis of variance (ANOVA) (to detect the existence of variance within the means of the treatment groups), followed by Tukey's post test (to inform on which treatment groups differed from each other).

3.3 Results

3.3.1 Loss of ΔΨm is an early event in Mtb-induced macrophage death

Cell death was assessed in Mtb-infected macrophages by detection of PS exposure and loss of $\Delta\Psi_m$. Annexin V binding was used to assess PS exposure in THP-1 cells infected with Mtb H37Ra (Figure 3-3A). Mitochondria were stained with MitoTracker Red (MitoTracker/MTR), which fluoresces in the presence of an intact $\Delta\Psi_m$ and loses fluorescence with $\Delta\Psi_m$ depolarisation. With increasing levels of Mtb infection (low to high MOI), increasing numbers of cells underwent PS exposure and simultaneously lost $\Delta\Psi_m$ (Figure 3-3B). PS expression on the cell surface is an early event in apoptosis; thus the simultaneous loss of $\Delta\Psi_m$ indicates that $\Delta\Psi_m$ is lost early in Mtb-induced macrophage death (Fadok et al., 1992; Martin et al., 1995). Early apoptosis is an energy-dependent process which requires ATP production and thus an intact $\Delta\Psi_m$ (Ziegler and Groscurth, 2004; Kushnareva and Newmeyer, 2010). Therefore, apoptotic cells can express PS on their surface prior to loss of $\Delta\Psi_m$. Hydrogen peroxide (H_2O_2) treatment induced an apoptotic death phenotype, as many dying cells exposed PS while their $\Delta\Psi_m$ was still intact (Figure 3-3B). In contrast, Mtb-induced cell death, with simultaneous $\Delta\Psi_m$ depolarisation and PS exposure, was non-apoptotic.

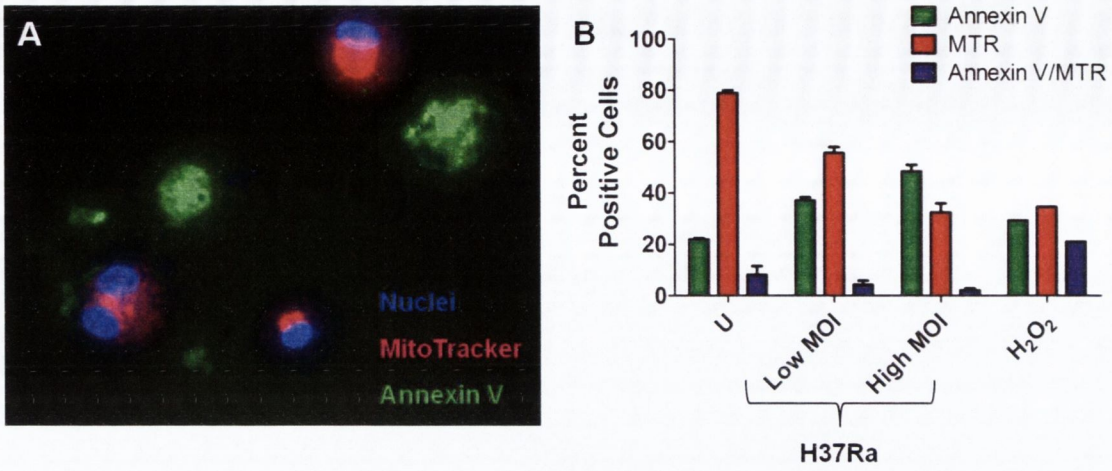


Figure 3-3: Human macrophages simultaneously expose phosphatidylserine and lose mitochondrial membrane potential during Mtb infection.

Human monocytic THP-1 cells were differentiated to macrophages with PMA (100 nM) for 72 h, then infected with Mtb H37Ra at low MOI (1-5 bacilli/cell) or high MOI (10-20 bacilli/cell) for 72 h. As a positive control for apoptosis, macrophages were treated with hydrogen peroxide (H_2O_2; 500 nM for 24 h). Phosphatidylserine (PS) exposure was assessed using fluorescently labelled annexin V and mitochondrial membrane potential was detected with the potentiometric dye, MitoTracker Red (MTR). Both annexin V and MitoTracker were detected by automated fluorescence microscopy using a GE IN Cell Analyzer 1000 and quantified with IN Cell Investigator software. U = uninfected macrophages. (A) Dying macrophages during Mtb infection exposed PS and lost their mitochondrial membrane potential. (B) Uninfected cells maintained their mitochondrial membrane potential (indicated by MitoTracker-positive cells) and were largely negative for annexin V (indicating lack of PS exposure). With Mtb infection, simultaneous PS exposure and loss of mitochondrial membrane potential was observed in an MOI-dependent manner. In contrast, many cells undergoing hydrogen peroxide-induced death exposed PS while their $\Delta\Psi_m$ was still intact (far right; blue bar). The images depict one representative experiment of three.

3.3.2 Mtb-induced macrophage death is not mediated by the intrinsic apoptosis pathway or by parthanatos

The early loss of $\Delta\Psi_m$, as well as previous reports within the literature, indicated the possible involvement of the mitochondria in the death of Mtb-infected macrophages (Duan et al., 2002; O'Sullivan et al., 2007). The macrophage death observed during *in vitro* Mtb infection displays both apoptotic (PS exposure) and non-apoptotic (caspase inactivity) features, indicating that an associated mitochondrial death pathway may be apoptotic or non-apoptotic (O'Sullivan et al., 2007). Cytochrome *c* (with or without parallel release of AIF) mediates the intrinsic apoptosis pathway. In the absence of cytochrome *c* release, AIF mediates a form of caspase-independent, necrotic cell death known as parthanatos (David et al., 2009; Wang et al., 2011). Confocal microscopy was carried out to visualise the location of cytochrome *c* and AIF within THP-1 cells infected with Mtb. Figure 3-4A-D shows cytochrome *c* staining within macrophages infected with Mtb at high MOI. MTR (which stained mitochondria) and cytochrome *c* were co-localised, indicating that cytochrome *c* was retained within the mitochondria.

In contrast, treatment of macrophages with hydrogen peroxide, which induces apoptosis of human monocytes during mycobacterial infection (Laochumroonvorapong et al., 1996), caused release of cytochrome *c* into the cytosol (Figure 3-4E).

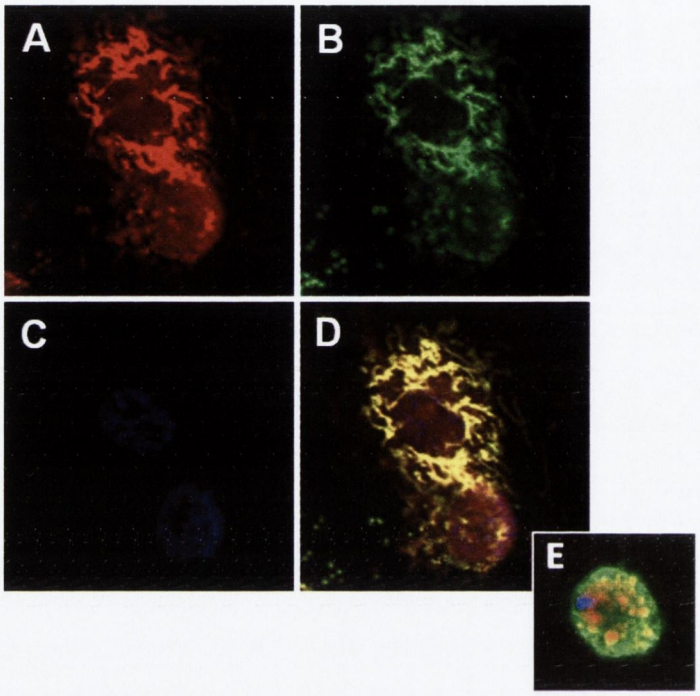


Figure 3-4: Cytochrome *c* is retained within mitochondria of Mtb-infected macrophages.

Differentiated THP-1 cells were infected with Mtb H37Ra at high MOI for 24 h and cytochrome *c* localisation was assessed by confocal microscopy. (A) MTR, indicating mitochondria. (B) cytochrome *c*. (C) Hoechst staining of nuclei. (D) Merged image. (E) Positive control for cytochrome *c* release: an uninfected macrophage treated with hydrogen peroxide (500 nM for 24 h), which induces apoptosis. Images from one representative experiment of three.

AIF localisation within Mtb-infected macrophages is shown in Figure 3-5. Similar to cytochrome *c*, AIF remained co-localised with the mitochondria of Mtb-infected macrophages and did not appear in the cytosol or translocate to the nucleus, where it mediates DNA degradation (Susin et al., 1999; Delavallée et al., 2011). The morphology of Mtb-infected macrophages was dramatically different to that of uninfected cells. Mtb-infected macrophages displayed marked cellular shrinkage and pyknosis, morphological features of apoptosis (Figure 3-5). The retention of cytochrome *c* and AIF within the mitochondria of dying Mtb-infected macrophages indicates that Mtb H37Ra infection does not induce MOMP, and that Mtb-induced macrophage death is not mediated by either the intrinsic apoptosis pathway or by parthanatos.

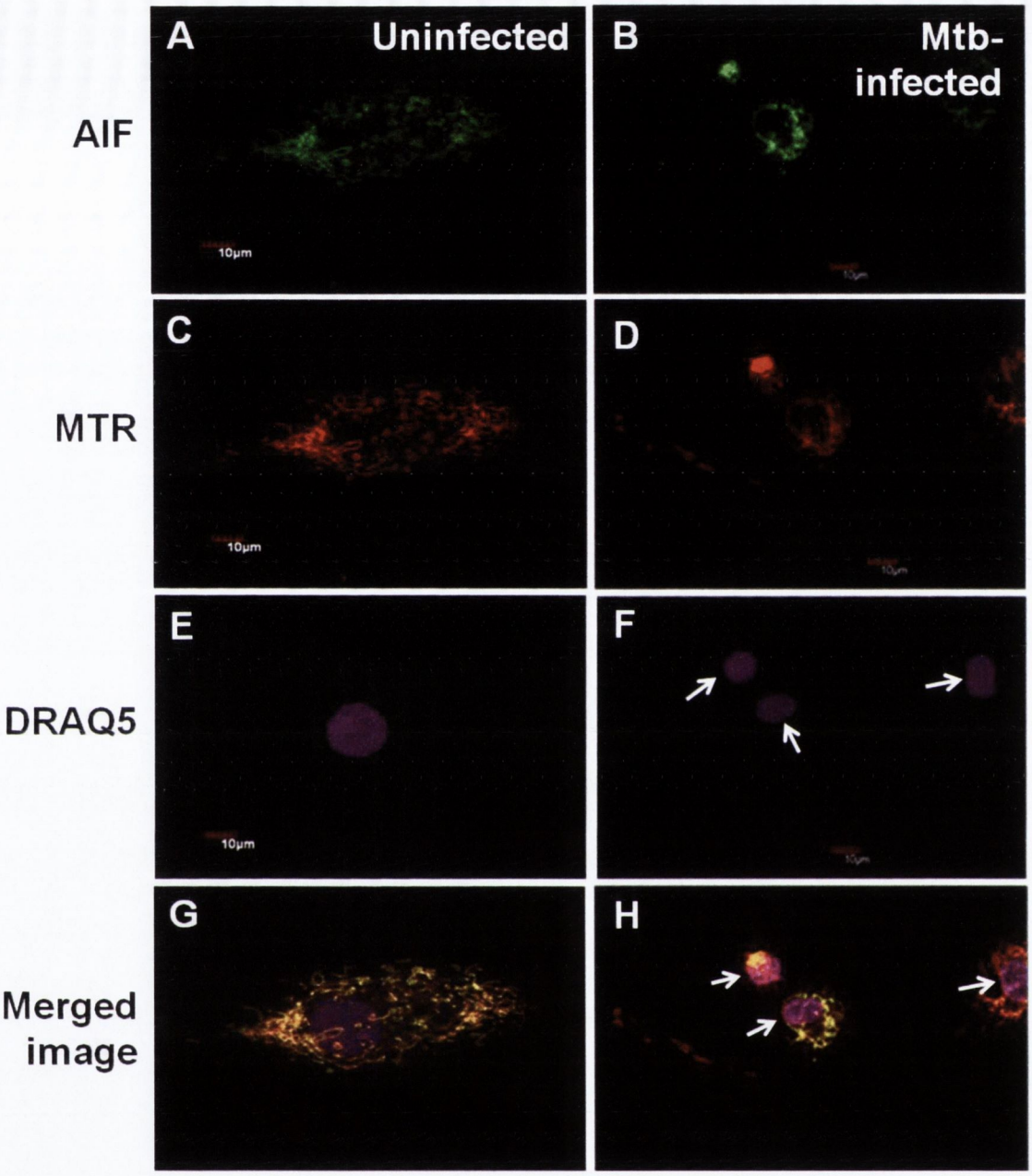


Figure 3-5: Mtb infection causes pyknosis of macrophages, but AIF remains within the mitochondria.

Differentiated THP-1 cells were infected with Mtb H37Ra at high MOI for 72 h and AIF localisation was assessed by confocal microscopy. Arrows indicate pyknotic nuclei. (A-B) AIF. (C-D) MTR (MitoTracker Red; mitochondria). (E-F) DRAQ5 (nuclei). (G-H) Merged image. Images show one representative experiment of three.

3.3.3 IL-10 does not influence macrophage cell death during Mtb infection

The anti-inflammatory cytokine, IL-10, has previously been reported to inhibit cell death of human macrophages infected with virulent Mtb (Balcewicz-Sablinska et al., 1998). The promotion of cell survival by IL-10 release during virulent Mtb infection

was recapitulated by Balcewicz-Sablinska and colleagues during attenuated Mtb infection by addition of exogenous human IL-10 to infected macrophages. Macrophage death represents an important host response to Mtb infection, as apoptosis of the infected macrophage can kill the internalised bacilli and aid activation of an adaptive immune response (Molloy et al., 1994; Oddo et al., 1998; Sly et al., 2003; Winau et al., 2006; Randhawa et al., 2008). The effect of IL-10 on Mtb-infected macrophage viability was initially studied in THP-1 cells. Cell death was measured by PI exclusion (to assess plasma membrane integrity) and the TUNEL technique (to detect DNA fragmentation). Loss of plasma membrane integrity and DNA fragmentation were significant in Mtb-infected cells ($p < 0.001$ and 0.05, respectively; Figure 3-6A and B). However, addition of exogenous IL-10 did not maintain THP-1 viability during Mtb infection.

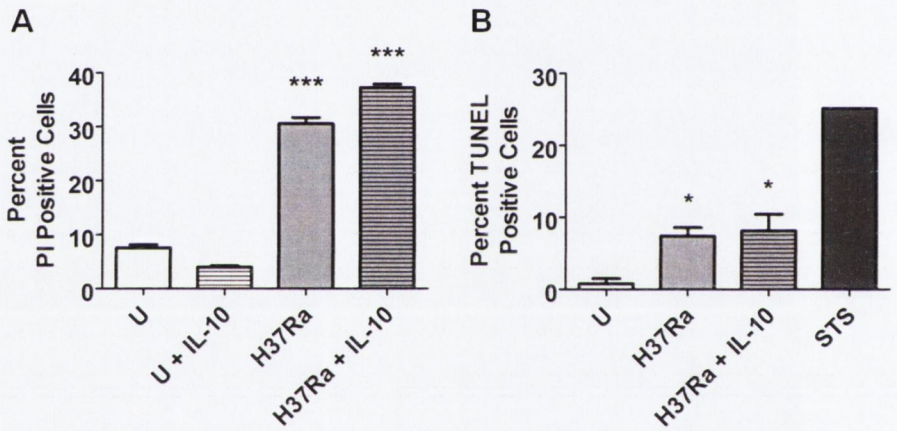


Figure 3-6: IL-10 does not influence THP-1 macrophage death during Mtb infection.

Differentiated THP-1 macrophages were infected with Mtb H37Ra at low MOI (1-5 bacilli/cell). Recombinant human IL-10 was added 20 min prior to infection and replenished after washing off extracellular Mtb to maintain its presence in the culture throughout infection. Staurosporine (STS; 1 µM treatment for 24 h) was used as a positive control for apoptosis. U = uninfected macrophages. (A) After 72 h infection, macrophages were incubated with propidium iodide (PI). Cell death was calculated as percent PI-positive cells. No significant effect of IL-10 on cell viability was observed. ***, $p < 0.001$ vs. both uninfected and uninfected with IL-10. The graph shows one representative donor of four. (B) After 96 h infection, macrophages were analysed for DNA fragmentation by TUNEL assay. There was a significant increase in DNA fragmentation of infected cells compared with uninfected cells. No significant difference in DNA fragmentation was observed with addition of IL-10. *, $p < 0.05$ vs. uninfected. One representative experiment of three is shown.

As primary cells may be more representative of *in vivo* responses, these experiments were then performed in primary human MDMs. Mtb-induced cell death was initially investigated in MDMs using PI exclusion. The level of PI staining was very low, which may have been due to detachment of macrophages from the cell culture plate, preventing their detection by fluorescence microscopy. As macrophages die, they lose their adherence, which has been observed to occur during Mtb infection (Keane et al., 1997; Vogt and Nathan, 2011). For this reason, the more sensitive Cell Death

Detection ELISA, which could detect fragmented DNA from both adherent and detached dead and dying cells, was used to study the effect of IL-10 on cell death of MDMs (Figure 3-7). Significant DNA fragmentation was observed in Mtb-infected cells ($p < 0.01$), which was not altered by addition of IL-10.

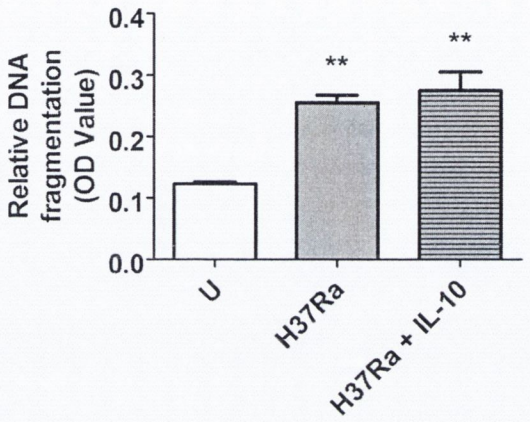


Figure 3-7: IL-10 does not influence human monocyte-derived macrophage cell death during Mtb infection.

Human monocyte-derived macrophages (MDMs) were infected with Mtb H37Ra for 96 h at low MOI. Recombinant human IL-10 (20 ng/ml) was added 20 min prior to infection and maintained in the culture throughout the duration of the experiment. After 96 h, DNA fragmentation was measured as a marker of cell death by Cell Death ELISA. Significant cell death was observed in infected cells compared with uninfected cells. No significant effect of IL-10 on macrophage viability was observed. **, $p < 0.01$ vs. uninfected. Data from one representative donor of three.

3.3.4 IL-10 does not rescue Mtb-infected primary human alveolar macrophages from death

Since exogenous IL-10 did not influence Mtb-induced death of MDMs, and as there may be differences in the response of macrophages derived from peripheral blood and the lung, the experiments were repeated in primary human AMs. The effect of addition of IL-10 on cell death of Mtb-infected AMs was examined by TUNEL to detect DNA fragmentation (Figure 3-8), as per the experiments carried out by Balcewicz-Sablinska et al. (1998). There was a significant increase in the percentage of TUNEL-positive AMs following infection with Mtb ($p < 0.01$), which was not reduced by exposure to exogenous human IL-10.

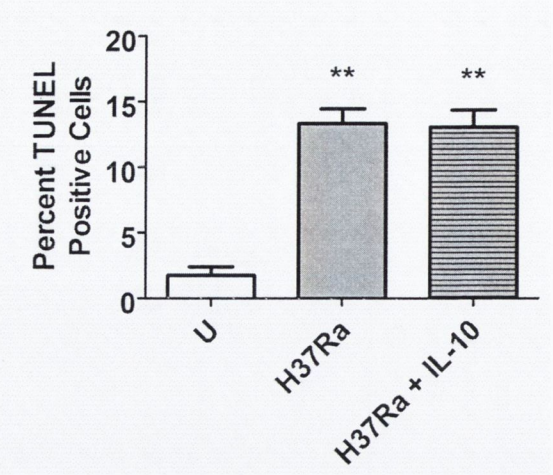


Figure 3-8: IL-10 does not rescue Mtb-infected primary human alveolar macrophages from cell death.

Primary human alveolar macrophages were infected with Mtb H37Ra for 96 h at low MOI. Recombinant human IL-10 (20 ng/ml) was added 20 min prior to infection and maintained in the cultures throughout the duration of the experiment. Fragmented DNA was labelled using the TUNEL technique and expressed as a percentage of total cell numbers. Significant DNA fragmentation was induced by Mtb, which was not altered by addition of IL-10. **, $p < 0.01$ vs. uninfected. The graph shows one representative of three individual donors.

3.3.5 Recombinant IL-10 is functional

In order to confirm that the recombinant human IL-10 was capable of inducing a signal within the macrophages, phosphorylation of STAT3 was assessed by Western blotting. IL-10 signalling causes phosphorylation of STAT3; therefore phosphorylated STAT3 (pSTAT3) was chosen as an indicator of IL-10 signalling.

Prostaglandin E_2 (PGE_2) has been shown to up-regulate IL-10 signalling (Cheon et al., 2006) and was used to increase pSTAT3 band intensity on the blot. IFN-γ signalling causes STAT1 phosphorylation and was included as a positive control. The loading control was total STAT3. With PGE_2 pre-treatment, pSTAT3 was present after 10 minutes and 1 hour treatment with IL-10 (Figure 3-9), indicating that the IL-10 was functional.

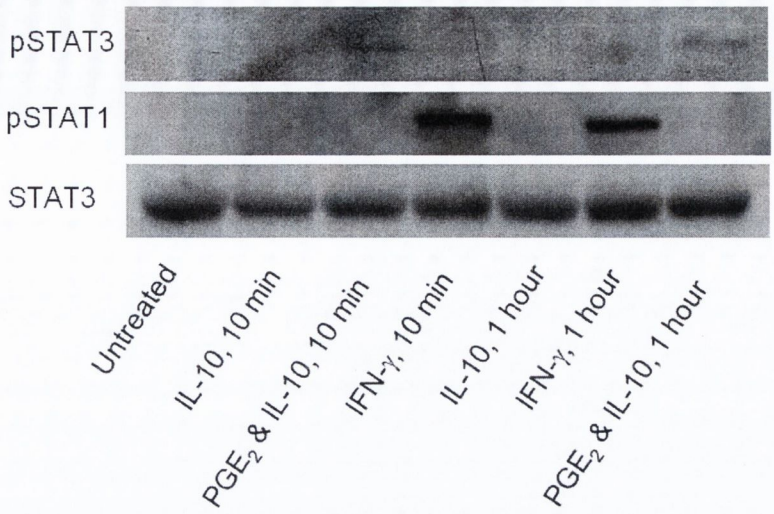


Figure 3-9: Signalling is functional in recombinant IL-10.

Undifferentiated THP-1 cells were treated with recombinant human IL-10 (20 ng/ml) for 10 min or 1 h. Two hour pre-treatment of THP-1 cells with prostaglandin E_2 (PGE_2) prior to IL-10 application was used to intensify IL-10 signalling. IFN-γ signalling causes phosphorylation of STAT1, which was used as a positive control. Total STAT3 was used as a loading control. The presence of bands corresponding to IL-10 with PGE_2 pre-treatment for 10 min and 1 h indicated that the recombinant IL-10 protein was functional.

3.3.6 ERK1/2 and p38 MAP kinase pathways do not mediate macrophage survival during Mtb infection

Mitogen-activated protein kinases (MAPKs) are cellular proteins which co-ordinate and execute extracellular signals, with predominant roles in cell survival and proliferation. They function within a cascade, consisting of the effector MAPK and two upstream kinases, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK) (Kumar et al., 2003). The current work focused on two of the three MAPK pathways: the ERK1/2 pathway and the p38 pathway. Both have pro- and anti-apoptotic roles, depending on the upstream signalling (Yang et al., 2007; Royuela et al., 2008; Cagnol and Chambard, 2010). The p38 MAPK also promotes cell death of *M. avium*- and Mtb H37Ra-infected murine macrophages (Bhattacharyya et al., 2003; Wojtas et al., 2011). Conversely, ERK1/2 signalling has been shown to prevent cell death of virulent Mtb-infected murine macrophages (Wojtas et al., 2011). As IL-10 can inhibit p38 signalling and induce ERK1/2 signalling, it was postulated that these may be mechanisms by which IL-10 could preserve macrophage viability during Mtb infection (Niiro et al., 1998; Zhou et al., 2001). The effect of inhibition of these MAPK pathways on the viability of human MDMs during Mtb infection was studied using the chemical inhibitors PD98059 (ERK1/2 inhibitor) and SB203580 (p38 inhibitor).

An initial toxicity study (Figure 3-10A and B) indicated that the inhibitors did not cause significant macrophage death above background levels at any of the concentrations tested. The optimal duration of inhibitor treatment was 48 h, as beyond this time point the macrophages underwent cell death due to the presence of the inhibitors. Macrophage viability was assessed by PI exclusion during the toxicity study and it was not possible to obtain an accurate assessment of the level of cell death induced by the inhibitors after 48 h, as cell death caused the macrophages to detach from the cell culture plates, preventing their detection. Significant cell death of MDMs was induced by Mtb infection ($p < 0.001$). Inhibition of the p38 and the ERK1/2 MAPK pathways did not significantly alter Mtb-infected MDM viability (Figure 3-10C). These results suggest that the ERK1/2 and the p38 MAP kinase pathways are not involved in human macrophage viability during Mtb H37Ra infection.

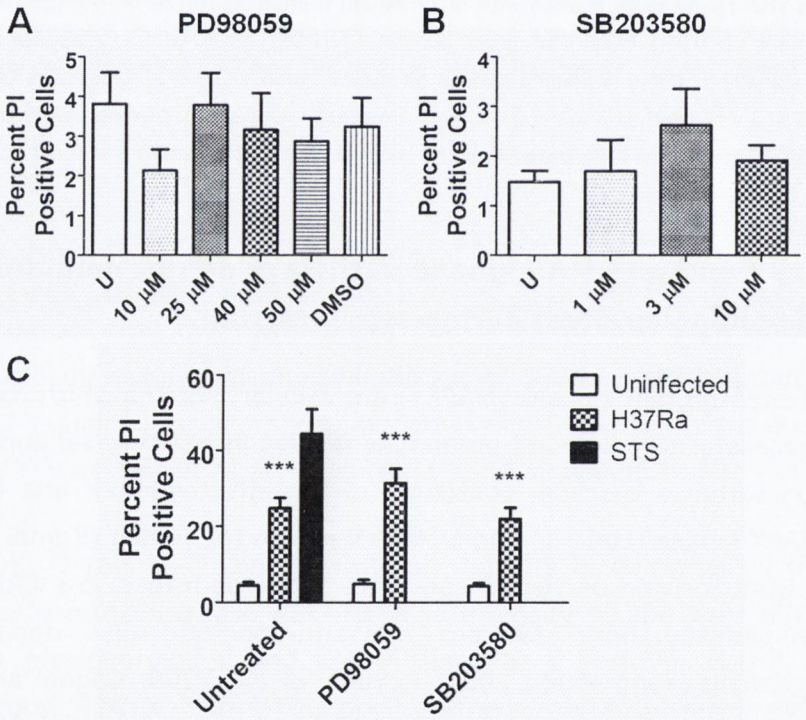


Figure 3-10: Inhibition of ERK 1/2 and p38 MAP kinases does not alter the viability of Mtb-infected human monocyte-derived macrophages.

Primary human monocytes were differentiated to macrophages in the presence of human serum for 7 days. MAPK inhibitors were applied 30 – 60 min prior to infection. U = untreated MDMs. A range of concentrations were applied to MDMs initially to investigate toxicity of (A) PD98059 (ERK1/2 inhibitor) and (B) SB203580 (p38 inhibitor) in the absence of Mtb. Neither inhibitor caused significant cell death at 48 h at any concentration. DMSO, the vehicle used with PD98059, also did not cause cell death at this time point (A). SB203580 was reconstituted in tissue culture-grade water. Both (A) and (B) represent data from three separate donors. (C) MDMs were infected with Mtb at low MOI for 48 h. SB203580 was used at a concentration of 3 μM and PD98059 was used at 10 μM. Staurosporine (STS; 1 μM for 24 h) was used as a positive control for cell death. Neither inhibitor had an effect on Mtb-induced macrophage death. ***, $p < 0.001$ vs. uninfected. The graph is compiled from pooled data from three individual donors.

3.4 Discussion

Infection of human macrophages with attenuated Mtb H37Ra caused significant macrophage death, as previously described (Keane et al., 1997; Ríos-Barrera et al., 2006; O'Sullivan et al., 2007). In this study dying macrophages underwent PS exposure, loss of $\Delta\Psi_m$ and nuclear pyknosis during Mtb infection. The loss of $\Delta\Psi_m$ and PS exposure occurred simultaneously in Mtb-induced macrophage death. O'Sullivan et al. (2007) noted that during this form of Mtb-induced death, PS exposure occurred prior to loss of plasma membrane integrity. The current work suggests that loss of $\Delta\Psi_m$ is an early event in Mtb-induced macrophage death, occurring alongside PS exposure, which may precede the loss of plasma membrane integrity. Early loss of $\Delta\Psi_m$ may be an indicator of a necrotic form of cell death, as apoptotic cells retain the ability to produce ATP until the later stages of death, which requires a functional $\Delta\Psi_m$ (Chou et al., 1995; Kass et al., 1996; Eguchi et al., 1997). In contrast to the macrophage death induced by Mtb, death caused by H_2O_2 led to the exposure of PS in many macrophages with an intact $\Delta\Psi_m$. Caspase-independent cell death, observed to occur during Mtb infection of macrophages (O'Sullivan et al., 2007), has been previously associated with simultaneous exposure of PS and loss of $\Delta\Psi_m$ (van Delft et al., 2010).

Both cytochrome *c* and AIF were retained within the mitochondria during Mtb infection, indicating an absence of MOMP in Mtb H37Ra-induced macrophage death. MOMP occurs during apoptosis and its absence from the macrophage death observed during Mtb infection is a further indication that Mtb H37Ra induces a non-apoptotic form of macrophage death. AIF mediates parthanatos, a form of caspase-independent programmed cell death (Moubarak et al., 2007; Andrabi et al., 2008; David et al., 2009; Artus et al., 2010; Delavallée et al., 2011). As the macrophage death observed during Mtb infection by O'Sullivan et al. (2007) was caspase-independent, yet displaying some morphological features of apoptosis and indicating possible involvement of a mitochondrial pathway, AIF was a promising candidate for investigation. In addition, AIF was implicated by Vega-Manriquez (2007) in *M. bovis*-induced cell death of bovine macrophages. However, AIF does not appear to contribute to human macrophage death during Mtb H37Ra infection, as it was retained within the mitochondria of Mtb-infected macrophages even after significant macrophage death had occurred. This suggests that Mtb-induced human macrophage death is not mediated by parthanatos. Similarly, Mtb H37Ra infection of macrophages did not result in cytochrome *c* release from the mitochondria, indicating that the intrinsic apoptosis pathway was not responsible for death of Mtb-infected macrophages. Mitochondrial retention of cytochrome *c* was also reported by Abarca-

Rojano et al. (2003) during Mtb H37Ra infection of murine macrophages. They found that cytochrome *c* release occurred only with virulent Mtb H37Rv infection and proposed that this may be indicative of the virulence of the mycobacterial strain. Release of cytochrome *c* has been associated with a reduction in the antimycobacterial capacity of infected macrophages; therefore, its retention within mitochondria during macrophage death may be a host-protective response (Duan et al., 2002).

Taken together, the simultaneous exposure of PS and collapse of $\Delta\Psi_m$ in addition to the retention of cytochrome *c* and AIF within the mitochondria (indicating an absence of MOMP) during Mtb H37Ra-induced human macrophage death suggests a non-apoptotic, necrotic form of cell death, which correlates with the findings of O'Sullivan et al. (2007). The following chapter of the present work demonstrates for the first time that Mtb H37Ra similarly induces a caspase-independent, non-apoptotic form of cell death in human dendritic cells (although Mtb-induced dendritic cell death is currently less well characterised than that of macrophages).

It has been reported that virulent Mtb induces macrophage necrosis, while attenuated Mtb strains, such as H37Ra, induce apoptosis (Chen et al., 2006; Lee et al., 2006; Gan et al., 2008). However, this phenotype is not observed by all groups; some have reported macrophage death with features of both apoptosis and necrosis during attenuated Mtb infection (Danelishvili et al., 2003; O'Sullivan et al., 2007) and others have noted macrophage apoptosis during virulent Mtb infection (Castaño et al., 2011). Indeed, the occurrence of both apoptotic and necrotic cell death has been reported in AMs from pulmonary tuberculosis patients challenged *in vitro* with virulent Mtb (Gil et al., 2004). The current work, in conjunction with the findings of O'Sullivan et al. (2007), suggests that human macrophage infection with Mtb H37Ra induces a necrotic, rather than an apoptotic, cell death phenotype. Non-apoptotic death has been observed in other infections, such as pyroptosis induced by *Chlamydia*, *Salmonella* and *Shigella* (Hilbi et al., 1998; Hersh et al., 1999; Fink and Cookson, 2007; Olivares-Zavaleta et al., 2011), pyronecrosis induced by *Neisseria* and *Toxoplasma gondii* (Duncan et al., 2009; Zhao et al., 2009) and vaccinia virus induction of necroptosis (Cho et al., 2009). It has been suggested that virulent Mtb induces NALP3-dependent pyronecrosis (Welin et al., 2011), but this is yet to be studied in Mtb H37Ra infection. As the necrotic death induced by Mtb H37Ra is caspase-independent, it may also be beneficial to examine the activity of the RIP1 and RIP3 kinases, to determine whether the observed macrophage death phenotype is necroptosis.

This study also aimed to elucidate the signalling pathways involved in the reported IL-10-mediated cell death inhibition during Mtb infection of human macrophages

(Balcewicz-Sablinska et al., 1998). Replication of the original experiments was required in order to perform this work. The effect of exogenous recombinant human IL-10 was investigated in a macrophage cell line and in both primary human MDMs and AMs during Mtb infection. No effect of recombinant human IL-10 on cell viability during Mtb infection was observed in any macrophage type. The IL-10 used in these experiments was confirmed as functional based on its ability to stimulate phosphorylation of STAT3. IL-10 had previously been linked to increased cell viability during infection with H37Ra (Balcewicz-Sablinska et al., 1998). The current work found, however, that IL-10 did not contribute to macrophage survival during Mtb infection.

Other groups, namely Rojas et al. (1999) and Feng et al. (2002), have shown a pro-survival effect of IL-10 in Mtb-infected murine macrophages. However, the former incubated the macrophages with increasing concentrations of IL-10 over a period of 24 hours prior to Mtb infection, which was a longer duration than that used by Balcewicz-Sablinska et al. and in the current work (20 minutes). The latter group studied macrophages from human IL-10-expressing transgenic mice, which may not completely correspond with the behaviour of human primary AMs during Mtb infection. The work carried out by Balcewicz-Sablinska et al. has been replicated with modification within the literature. Patel et al. (2009) also reported an increase in Mtb-infected human macrophage viability with addition of exogenous IL-10 at an identical concentration to that used by Balcewicz-Sablinska et al. (and in the present work) but revealed that a significantly greater exposure period to IL-10 prior to Mtb infection (24 hours vs. 20 minutes) was required in order to preserve macrophage viability. IL-10 has also demonstrated the propensity to induce, rather than inhibit, apoptosis in human macrophages and monocytes (Estaquier and Ameisen, 1997; Schmidt et al., 2000; Wang et al., 2001; Bailey et al., 2006), indicating that its action on these cells is not exclusively pro-survival.

The investigation of the p38 and ERK1/2 MAPK pathways did not reveal a role for either pathway in Mtb-induced human macrophage death. Blocking either pathway in primary human MDMs did not significantly affect macrophage viability during Mtb infection. Although both ERK1/2 and p38 are reported to exert significant effects on the cell death of murine macrophages during Mtb H37Ra infection (Wojtas et al., 2011), based on the current study this does not appear to extend to Mtb-infected human macrophages. The p38 MAPK has been implicated in caspase 8-mediated, TNF-dependent apoptosis of primary murine macrophages and a human cell line during Mtb infection (Kundu et al., 2009). TNF ordinarily acts as a pro-survival signal in human macrophages (Mangan and Wahl, 1991), but Mtb infection of human macrophages can render them susceptible to TNF-α-induced apoptosis (Filley and Rook, 1991; Filley et

al., 1992; Keane et al., 1997). However, because human macrophages undergo caspase-independent cell death during infection with Mtb (Lee et al., 2006; O'Sullivan et al., 2007), this may account for the variance between the results of Kundu et al. and the current work.

In conclusion, Mtb-induced macrophage death displays both apoptotic and non-apoptotic features, and does not conform to the morphology of classical apoptosis. It is not mediated by the intrinsic apoptosis pathway, or by parthanatos. Although IL-10 had been a proposed mediator of cell death inhibition during human Mtb infection, this was not borne out by the current work. The ERK1/2 and the p38 MAPK pathways do not appear to exert an effect on human macrophage viability during Mtb H37Ra infection.

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4 *Mycobacterium tuberculosis* infection induces non-apoptotic cell death of human dendritic cells

4.1 Introduction

The dendritic cell (DC) plays an important role in immunity during tuberculosis by initiating cell-mediated immune responses, which, in the majority of cases, lead to the resolution of infection (Dorhoi et al., 2011). Compelling evidence for the requirement of DCs in protection against mycobacterial infection has recently been provided by two reports detailing the consequences of DC deficiencies in patients with rare genetic mutations (Bigley et al., 2011; Hambleton et al., 2011). The initial report by Bigley et al. (2011) described a DC deficiency (the genetic basis for which is currently unknown) resulting in two of the four reported cases presenting with infections caused by attenuated or non-pathogenic mycobacterial strains. Hambleton and colleagues (2011) more recently reported on three subjects with mutations in interferon regulatory factor 8 (IRF8) associated with an absence of DCs, which resulted in susceptibility to disseminated BCG-osis (Bigley et al., 2011). BCG-osis is disseminated mycobacterial disease occurring as a result of vaccination with the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG) (Grange, 1998). BCG is currently the only available vaccine against tuberculosis and ordinarily does not cause disease in healthy individuals (Feldmann, 1932; Milstien and Gibson, 1990). These reports attest to the critical role DCs play in protection against mycobacterial infection.

It is well documented that Mtb infection leads to the death of infected macrophages (Keane et al., 1997; O'Sullivan et al., 2007; Rodrigues et al., 2009; Sohn et al., 2009); however, little is known about DC viability during Mtb infection. While the cell death response to Mtb infection is regarded as host-protective in the setting of the macrophage, due both to the ability of dying macrophages to kill the invading bacilli and to increased antigen presentation facilitated by dying, infected macrophages (Oddo et al., 1998; Schaible et al., 2003; Winau et al., 2006; Divangahi et al., 2010), it is unclear whether death of infected DCs could serve in a similar protective manner. Reduced DC viability during Mtb infection has been reported, but the mechanism of DC death has not previously been investigated (Mohagheghpour et al., 2000; Hanekom et al., 2003). In this chapter, the occurrence and mechanism of DC death was assessed during Mtb infection. Initial experiments indicated that cell death was induced in Mtb-infected DCs. The maturation status and cytokine production of these cells was assessed. It was endeavoured to characterise the type of cell death that occurred in Mtb-infected DCs, given that the mode of cell death of an infected cell has important downstream effects on immune induction or tolerance (Salio et al., 2000; Liu et al.,

2002; Green et al., 2009; Torchinsky et al., 2010). Finally, as the death of infected macrophages can reduce the viability of intracellular Mtb (Keane et al., 2002; Rodrigues et al., 2009), the ability of dying, infected DCs to kill intracellular Mtb was examined.

Hypothesis

- DCs undergo cell death following Mtb infection, which may reduce the viability of intracellular Mtb.

- Mtb-infected DCs mature and release cytokines, thus aiding the initiation of adaptive immune responses.

Aims of this section

The aims of this section were to examine the effect of Mtb infection on the viability of human monocyte-derived DCs. Once it was established that DC death occurred during Mtb infection, the mechanism of cell death was explored; to examine the role of caspases and, more particularly, the executioner caspases 3 and 7. The viability of Mtb following infection of DCs was also assessed, to ascertain whether dying DCs could limit Mtb growth.

Summary of results

- Infection with live, but not dead, Mtb caused DC death.

- Cell death was caspase-independent and did not involve activity of caspase 3 or 7.

- Although destined to die, Mtb-infected DCs successfully matured and secreted cytokines.

- Death of the host DCs did not kill intracellular Mtb.

4.2 Methods

The materials and methods used in this chapter are described in detail in Chapter 2. The rationale for use of the particular methodologies and reagents chosen in this chapter is outlined here.

4.2.1 Dendritic cells

Human monocyte-derived DCs were used in this study. They were generated from human peripheral blood monocytes, isolated from buffy coats and cultured in the presence of IL-4 (40 ng/ml) and GM-CSF (50 ng/ml) for 6 days. This method reliably generated large numbers of DCs. Initially, monocytes were isolated from fresh whole blood (approximately 10 – 20 ml) for the purpose of DC generation. However monocyte numbers from this volume of fresh blood were insufficient to generate enough DCs for use in an experiment. The monocyte yield from buffy coats (obtained, with permission, from the on-site Irish Blood Transfusion Service) was significantly higher and so was chosen in favour of isolating DCs from fresh blood. DCs produced in this way steadily expressed DC-SIGN and lost expression of CD14 from their surface after 6 days of differentiation (measured by flow cytometry; Figure 4-1 and Figure 4-5A). DC-SIGN^{+}CD14^{-} cells were considered to be DCs and had the characteristic DC appearance on microscopy (Figure 4-2). Figure 4-1 shows the process of gating DCs. The cells were initially gated based on their size and granularity, measured by forward scatter and side scatter. Unstained cells were used to set the negative portion (which corresponded also with isotype controls). DC-SIGN expression was detected using a PE-conjugated antibody, while the CD14 antibody was FITC-conjugated.

To detect and designate positive staining on the flow cytometer, PE and FITC single positive controls were used. These were DCs (from the same batch used for the whole experiment) incubated with an antibody against a surface molecule known to be expressed on DCs, conjugated to the same fluorochrome used to detect DC-SIGN (PE) or CD14 (FITC) expression. In this case, FITC-conjugated CD40 was used as a positive control for FITC staining, as CD40 is expressed on all antigen-presenting cells, including DCs (Lievens et al., 2009). As a positive control for PE staining, PE-conjugated CD86 was used to stain DCs; CD86 is constitutively expressed on DCs (Chorny et al., 2006). Single positive controls were particularly useful in these experiments, as CD14 (and, therefore, FITC staining) was absent from the surface of DCs, so the FITC positive control was advantageous in determining successful detection of FITC fluorescence by the chosen flow cytometer parameters. DC-SIGN/CD14 expression remained unchanged after infection of DCs with Mtb.

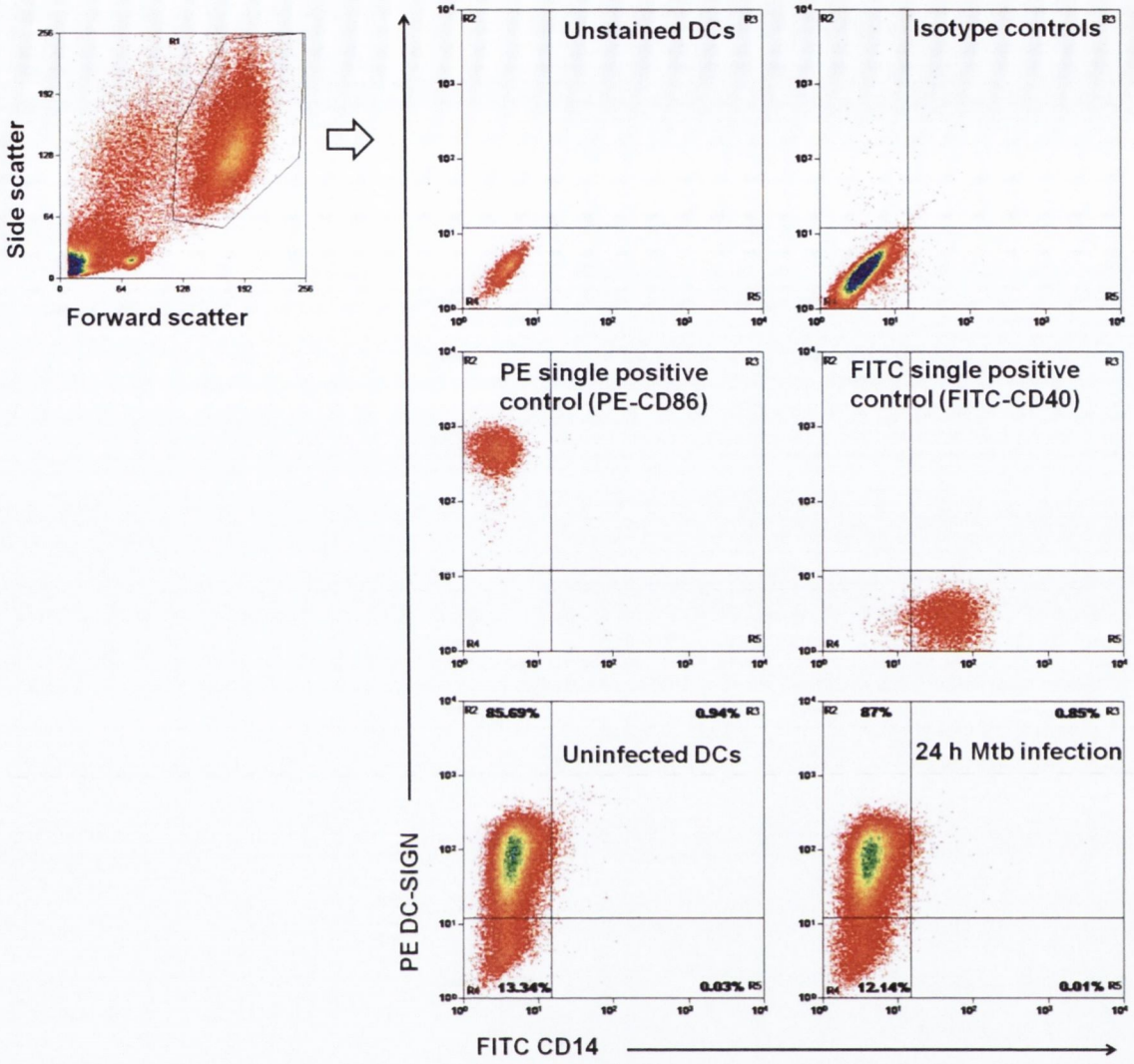


Figure 4-1: Gating of DC-SIGN^{+}CD14^{-} dendritic cells for flow cytometry analysis.

Total cells (excluding clumps and debris) were selected based on forward scatter and side scatter. Unstained DCs and DCs stained singly with each fluorochrome (conjugated to CD86 (PE) or CD40 (FITC); surface molecules known to be expressed on DCs) were used to set cut-off points for positive and negative staining. Both uninfected and Mtb-infected DCs showed similar levels of DC-SIGN expression and lack of CD14 expression.

DCs generated from monocytes also displayed a characteristic DC appearance after exposure to live Mtb; producing dendrites when challenged with the live (but not dead) bacilli (Figure 4-2).

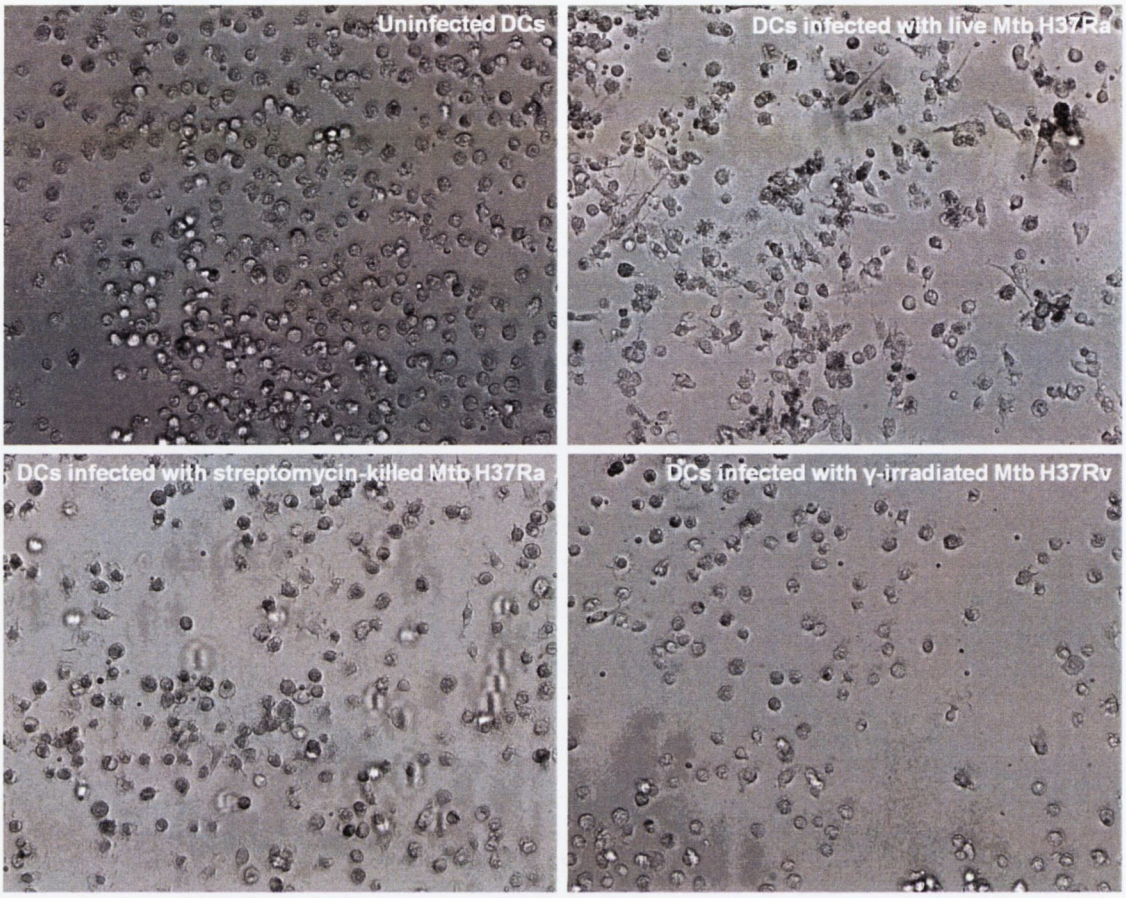


Figure 4-2: Live Mtb-infected DCs produce dendrites.

DCs were infected with live or killed Mtb at MOI 1 for 24 h. After live Mtb infection, DCs displayed characteristic dendrites, compared with uninfected DCs, or DCs infected with dead Mtb (streptomycin-killed H37Ra or γ-irradiated H37Rv) at the same time point.

4.2.2 Infection of DCs

In many experiments, DCs were infected with live or dead Mtb (streptomycin-killed H37Ra; sH37Ra, or γ-irradiated H37Rv; iH37Rv). This allowed for identification of any differing effects on DC death induced by viable or non-viable Mtb. Additionally, because dead Mtb did not induce DC death but still contained Mtb-specific pathogen-associated molecular patterns (PAMPs), dead bacilli acted as controls for infected DC viability. In this way, the viability of the DCs (rather than the presence of intracellular PAMPs) could be isolated and interrogated for its effect on caspase activity, maturation and cytokine secretion during Mtb infection.

The number of mycobacteria per DC (multiplicity of infection; MOI) was calculated using a nephelometer. (The detailed protocol is contained in Chapter 2, Section 2.4.) The method used for calculating MOI in macrophages (discussed in Chapter 3) was altered for DCs, as they phagocytosed mycobacteria at a slower rate than macrophages, requiring 24 h incubation before washing to remove extracellular bacilli,

compared to 3 h incubation for macrophages. The live Mtb in culture would continue to replicate during this time, rendering calculations based on the previous day's DC infection invalid. For this reason, a nephelometer was used to calculate the number of mycobacteria in liquid culture. From this calculation, the volume of liquid Mtb culture required to give the desired MOI could be determined and applied to the DCs. (Determination of MOI by microscopy was performed retrospectively for each experiment to ensure that the nephelometer-calculated MOI was indeed phagocytosed by the DCs.) The accuracy of nephelometer readings was confirmed by directly plating nephelometer-calculated numbers of mycobacteria on Middlebrook 7H10 plates (Chapter 2, section 2.15) and counting the resultant colony forming units (CFUs). The numbers of CFUs were comparable to those predicted by the nephelometer readings (Table 4-1, Table 4-2 and Table 4-3; displaying nephelometer estimates of CFU/ml of Mtb, together with the actual CFU count of that sample analysed by plating Mtb on Middlebrook agar plates).

Experiment 1	Nephelometer Estimate (CFU/ml)	Actual CFU Count (CFU/ml)
MOI 1	1×10^5	3×10^5
MOI 5	5×10^5	7×10^5
MOI 10	10×10^5	8×10^5

Table 4-1: Nephelometer estimates and actual CFU counts (experiment 1 of 3)

Experiment 2	Nephelometer Estimate (CFU/ml)	Actual CFU Count (CFU/ml)
MOI 1	2×10^6	5×10^6
MOI 5	10×10^6	8×10^6
MOI 10	2×10^7	1×10^7

Table 4-2: Nephelometer estimates and actual CFU counts (experiment 2 of 3)

Experiment 3	Nephelometer Estimate (CFU/ml)	Actual CFU Count (CFU/ml)
MOI 1	2×10^6	7×10^6
MOI 5	10×10^6	5×10^6
MOI 10	2×10^7	5×10^6

Table 4-3: Nephelometer estimates and actual CFU counts (experiment 3 of 3)

4.2.3 Cell death assays and caspase inhibition

There are many ways in which a cell can undergo death, each with its own phenotype. There is no one assay which can fully discriminate the manner of cell death. For this reason, a number of cell death assays were chosen to identify particular aspects of DC death following Mtb infection.

Propidium iodide (PI) exclusion was used initially to assess cell viability following infection of DCs with Mtb. The IN Cell™ assay (utilised for macrophage experiments

detailed in Chapter 3) was adapted to investigate cell death in non-adherent DCs. DCs were first subjected to gentle centrifugation (500 rpm for 5 minutes) to allow them to collect at the base of their cell culture wells for optimum detection by the IN Cell automated fluorescence microscopy system. The Dual Area Object Analysis program, used to detect live and dead macrophages (Chapter 3), was also used in the detection of live and dead DCs, with some adjustment of nuclear size parameters to account for differences between the different cell types. (Unlike Mtb-infected macrophages, nuclei of Mtb-infected DCs did not undergo pyknosis and this nuclear size difference required an adjustment of the analysis program.) The PI exclusion assay effectively discriminated live and dead cells, informing on the viability of Mtb-infected DCs.

To gain further information on the cell death process underway in Mtb-infected dying DCs, DNA fragmentation was measured using the Roche Cell Death Detection ELISA^{PLUS} kit. This method identified fragmented DNA in dead and dying cells, by detecting free nucleosomes released into the cytoplasm. DNA fragmentation is a feature of apoptosis, but is not seen in pyronecrosis (Willingham et al., 2007). DNA fragmentation can be mediated by caspase-dependent DNAse, so DNA fragmentation can indicate activity of caspases (Kroemer and Martin, 2005; Kitazumi and Tsukahara, 2011). Caspase activity is a feature of both apoptosis and pyroptosis, but is absent from pyronecrosis (Kroemer et al., 2009; Kepp et al., 2010). In the absence of caspase activity, other proteases, such as apoptosis-inducing factor (AIF) and cathepsins, can mediate DNA fragmentation (Moubarak et al., 2007; O'Sullivan et al., 2007; Luo et al., 2010; Zhao et al., 2010). In order to assess the requirement for caspases in the cell death observed in Mtb-infected DCs, a caspase inhibitor was employed to prevent activation of these proteases. The pan-caspase inhibitor, quinolyl-valyl-O-methylaspartyl-(-2,6-difluorophenoxy)-methyl ketone (Q-VD-OPh) (Caserta et al., 2003), was chosen to explore the role of caspases in Mtb-induced cell death, as another commonly used broad spectrum caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), was previously noted to cause death of primary monocyte-derived macrophages (Dr. Mary O'Sullivan, unpublished observations).

4.2.4 Detection of caspase 3/7 activity

To further elucidate whether caspases were involved in Mtb-induced DC death, the pan-caspase blockade experiments were followed by assessment of the activity of the executioner caspases 3 and 7 during Mtb infection. Caspases 3 and 7 are the terminal effector proteases of both the intrinsic and extrinsic caspase-dependent apoptosis pathways. Their activation during Mtb infection could indicate caspase-dependent cell death, although the activation of caspases can also occur late in cell death that has

been initiated in a caspase-independent manner (Kroemer and Martin, 2005; Tang et al., 2008). Caspase 3/7 activity was assessed in parallel with cell death, at time points before, during and after the occurrence of cell death. The Promega Caspase-Glo assay was selected for these experiments because of its high sensitivity of detection, allowing for the use of lower cell numbers. This was useful for caspase activity experiments, as cell numbers were limited. The assay's sensitivity could be attributed to its luminescence output, which is reported to be more sensitive than fluorescence-based assays (Corey, 2009). Luminescence was produced by caspase-mediated cleavage of the substrate Z-DEVD-aminoluciferin and was directly proportional to caspase activity (Promega Corporation, 2011). Caspase 3/7 activity detection was carried out in parallel with cell death assays, requiring a large number of cells in order to carry out all experiments at 24 h, 48 h and 72 h time points.

4.2.5 DC maturation and cytokine detection

Two central functions of DCs are maturation (which facilitates DC-T cell interaction) and cytokine release. Mtb-infected DCs were compared with LPS-treated (1 μg/ml for 24 h) DCs as a positive control, because LPS is known to reliably induce DC maturation and cytokine secretion (Elkord et al., 2005). To verify that DC maturation occurred in dying, Mtb-infected DCs, flow cytometry was performed to measure up-regulation of two co-stimulatory molecules, CD83 and CD86, which have been reported in the literature to reliably indicate DC maturation. CD86 becomes up-regulated when DCs mature, and CD83 is expressed solely on DCs that have matured (Tsuji et al., 2000; Elkord et al., 2005; Della Bella et al., 2008). Cytokine release was measured by ELISA using the Meso Scale Discovery SECTOR Imager 2400 to detect the following cytokines: IFN-γ, IL-1β, IL-13, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12p70 and TNF-α. (Samples were prepared by R. Ryan and the ELISA was performed by Dr. M. O'Sullivan.)

4.2.6 Colony forming units and BacT/ALERT® 3D

Killing of intracellular Mtb is a feature of macrophage cell death and in this study DCs were assessed for their mycobactericidal capacity. CFUs are a reliable method for determining mycobacterial growth (Lorian and Finland, 1969). This method involved plating serial dilutions (10^{-2} – 10^{-4}) of mycobacteria isolated from infected DCs – washed to remove extracellular bacilli – (cells were then lysed to release intracellular Mtb), and manually counting individual mycobacterial colonies grown on Middlebrook 7H10 agar plates. Because Mtb is a slow-growing organism, it took approximately 21

days before all colonies had appeared on the agar. An example of mycobacterial growth on CFU plates is shown in Figure 4-3.

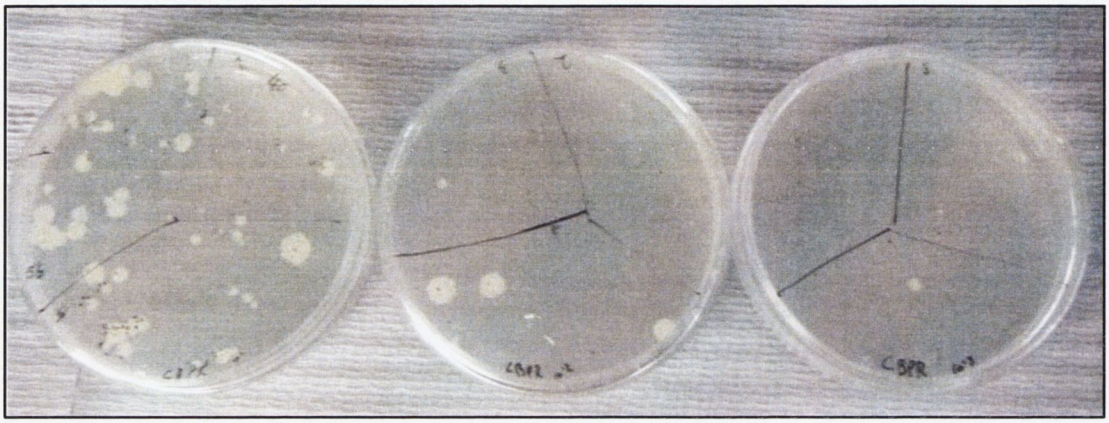


Figure 4-3: Mtb colony forming units.

Mtb H37Ra was grown on Middlebrook 7H10 agar for 21 days and colony forming units were manually counted to assess mycobacterial growth.

In parallel with CFUs, the bioMérieux BacT/ALERT® 3D automated microbial detection system was used to assess Mtb growth. (The principle behind this system is outlined in more detail in Chapter 2, Section 2.15.) A representative graph (generated from BacT/ALERT® 3D automated readings of reflectance) recording growth, or lack of growth, in liquid culture samples is shown in Figure 4-4.

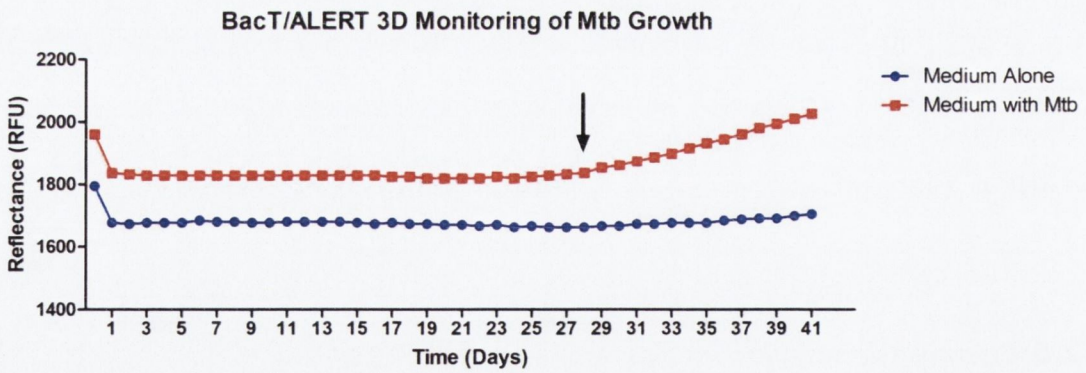


Figure 4-4: Example of BacT/ALERT® 3D mycobacterial growth monitoring.

Live Mtb H37Ra in Middlebrook medium, or medium alone (negative control), was inoculated into culture bottles and incubated for 41 days in a BacT/ALERT® 3D automated microbial detection system. Bacterial growth was detected by changes in reflectance of the liquid culture medium. After 41 days, any bottles that did not contain growing mycobacteria were labelled 'negative'. Bottles containing replicating Mtb had previously scored 'positive' (see arrow), as reflectance readings began to increase.

Bacterial growth was detected by monitoring changes in reflectance from the liquid culture. This allowed for the generation of a growth index (Percent change in Mtb growth; Figure 4-10) for Mtb isolated from infected DCs. The pattern of growth

observed in Mtb incubated in the BacT/ALERT® 3D system was consistent with that seen in parallel CFU plates.

4.2.7 Statistical analysis

Results are expressed as means ± the standard errors of the mean (SEM). The data were analysed with GraphPad Prism 5 statistical software (GraphPad Software, Inc., La Jolla, CA) by repeated measures ANOVA with Tukey's post test (which are parametric statistical tests for paired observations, e.g. uninfected vs. infected cells from the same population and the same donor). Cytokine secretion was analysed by the Friedman test followed by the Wilcoxon signed rank test (non-parametric statistical tests for paired observations). A P value of < 0.05 was considered statistically significant. Graphs were compiled using GraphPad Prism 5 software.

4.3 Results

4.3.1 Dendritic cells mature after they phagocytose Mtb

Dendritic cells form an important link between the innate and the adaptive immune response, so their viability during infection may have consequences for the host. DCs, differentiated from human monocytes for 6 days, were analysed by flow cytometry for surface expression of CD14 and DC-SIGN (Figure 4-1 and Figure 4-5A). DCs were routinely greater than 85% DC-SIGN^{+}CD14^{-}, which was not altered by Mtb infection, and displayed characteristic dendrites after exposure to live Mtb (Figure 4-2 and Figure 4-5B). To assess the effect of Mtb infection on DC viability, human DCs were infected with Mtb strains H37Ra and H37Rv. Confocal or fluorescence microscopy (to assess phagocytosis of mycobacteria) and PI staining (to measure cell death) were carried out in DCs infected with either H37Ra or H37Rv. All other experiments were performed with H37Ra only. Figure 4-5C shows DCs infected with live H37Rv and stained with auramine to detect mycobacteria, and demonstrates that Mtb was phagocytosed by the DCs. Similar results were obtained with iH37Rv, live H37Ra and sH37Ra.

Maturation was assessed in DCs infected with H37Ra (Figure 4-5D). DCs were infected with live or dead Mtb H37Ra at MOI 1 for 24 h. Approximately 60% of cells had phagocytosed mycobacteria at this time point. The cells were washed to remove extracellular mycobacteria and either analysed or incubated for a further 24 h before analysis. As reported in previous studies, live Mtb caused maturation of DCs, demonstrated by their up-regulation of the co-stimulatory molecules, CD83 and CD86 by 48 h post-infection (Figure 4-5D and E) (Henderson et al., 1997; Tsuji et al., 2000; Mihret et al., 2011). Streptomycin-killed H37Ra did not induce DC maturation.

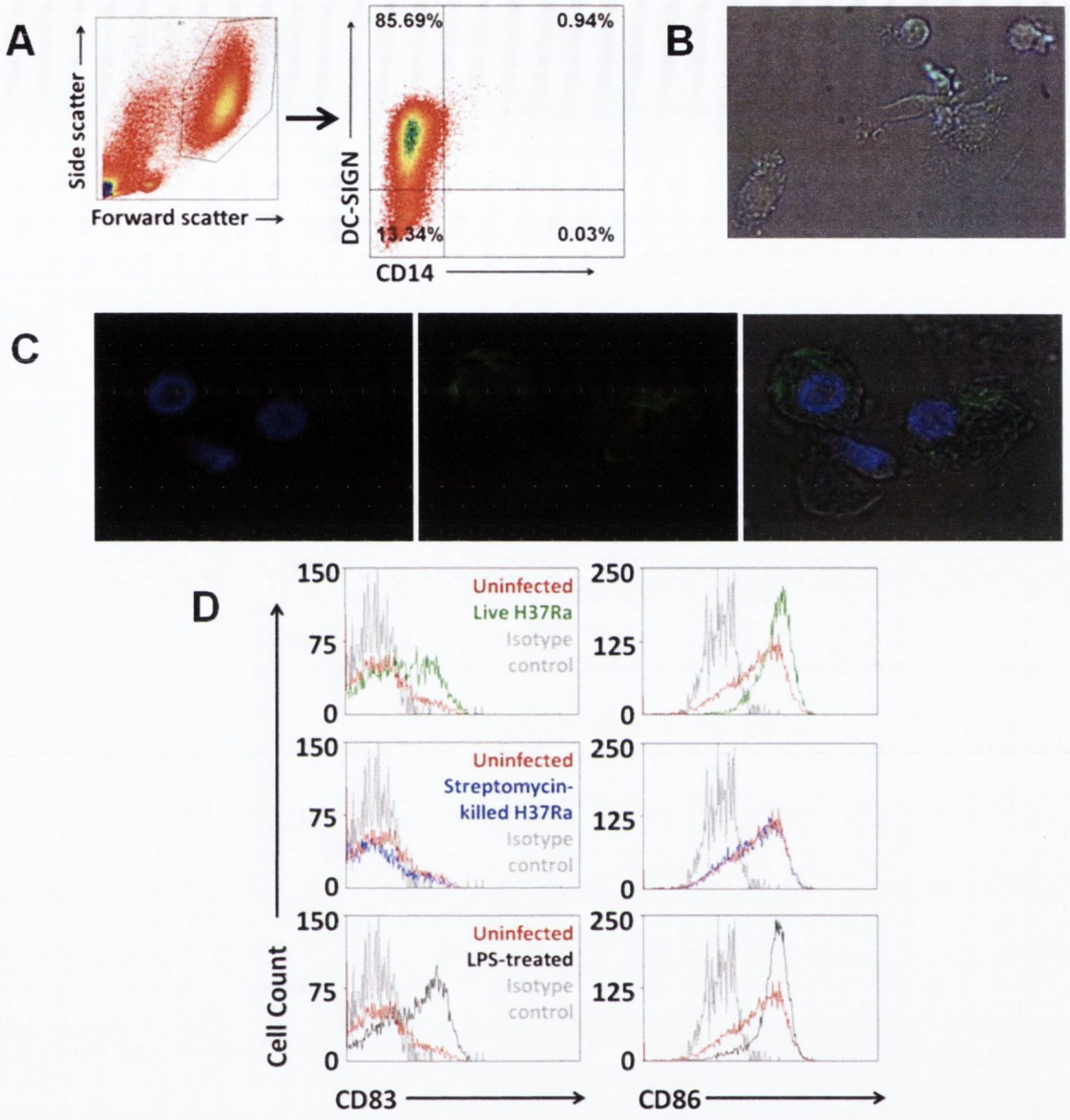


Figure 4-5: DCs mature after they phagocytose live Mtb.

Human monocytes were separated from buffy coats by plastic adherence and cultured for 6 days in the presence of recombinant human IL-4 (40 ng/ml) and GM-CSF (50 ng/ml) to allow differentiation to DCs. (A) Cells were analysed for CD14 and DC-SIGN expression by flow cytometry. DCs were $CD14^-$ and $DC\text{-}SIGN^+$ (typically > 85% of gated cells; both before and after infection with Mtb). Plots show uninfected, immature DCs after 6 days of cytokine treatment from one representative donor of three. (B) DCs were infected with live Mtb H37Ra at MOI 1 for 24 h and visualised by light microscopy. (C) DCs were infected with live Mtb H37Rv at MOI 10 overnight. Bacteria were stained with auramine and nuclei with Hoechst and were visualised by confocal microscopy. Similar results were obtained with γ-irradiated H37Rv (iH37Rv), live H37Ra and streptomycin-killed H37Ra (sH37Ra) (data not shown). (D) DCs were infected with live Mtb H37Ra or streptomycin-killed H37Ra at MOI 1 for 24 h or 48 h. LPS treatment (1 μg/ml for 24 h) was used as a positive control for DC maturation. Surface expression of CD83 and CD86 was assessed by flow cytometry. The histograms show one representative donor of three at 48 h post-infection.

4.3.2 Live Mtb infection causes DC death

To assess the relationship between intracellular infection and DC viability, human DCs were infected with live or dead Mtb H37Ra or H37Rv at MOI 10. Viability of infected DCs was assessed by propidium iodide exclusion and quantified on a GE IN Cell Analyzer 1000. Infection of DCs with either live strain was followed by significant cell death after 24-72 hours (Figure 4-6A and B), whereas dead bacilli (sH37Ra or iH37Rv) did not elicit this response. Incubation times with each strain were optimised to provide a significant increase in the percentage of PI positive cells above background (40-60%), while at the same time minimising the cellular disintegration that occurs in the late stages of cell death and would lead to an underestimate of the numbers of dead cells. Longer incubation times led to the death of the majority of infected cells (>95%). The virulent H37Rv strain induced cell death at a faster rate than an equivalent MOI of the attenuated H37Ra strain and, as a consequence, the PI exclusion assay was carried out 24 h after infection in H37Rv-infected DCs and 72 h after infection in H37Ra-infected cells. Cell death also occurred at 72 h with live H37Ra Mtb infection at the lower MOIs of 1 and 5 (Figure 4-6C).

Having observed that infection with live mycobacteria reduced the viability of DCs, the mechanism of cell death was investigated in H37Ra-infected DCs. It has previously been reported that macrophage cell death after Mtb infection results in DNA fragmentation (O'Sullivan et al., 2007). By ELISA, it was observed that DNA fragmentation was also a feature of the DC response to viable Mtb H37Ra infection (Figure 4-6D). Significant DNA fragmentation was induced in DCs infected with 10 bacilli per cell ($p < 0.05$). Apoptosis results in nuclear condensation, pyknosis and, eventually, fragmentation of the nucleus into apoptotic bodies (Kerr et al., 1972; Mevorach et al., 2010). To determine whether this occurs during Mtb infection, the nuclear morphology of DCs was examined by epifluorescent microscopy. The nuclei did not undergo fragmentation (Figure 4-6E). Visualised with Hoechst staining, the nuclei of infected cells were similar in appearance to those of uninfected cells at 72 h after infection, a time at which they had undergone significant cell death. DCs treated with cycloheximide and staurosporine displayed extensive nuclear fragmentation, indicating that the cells are capable of undergoing this process when treated with apoptotic stimuli (Figure 4-6E).

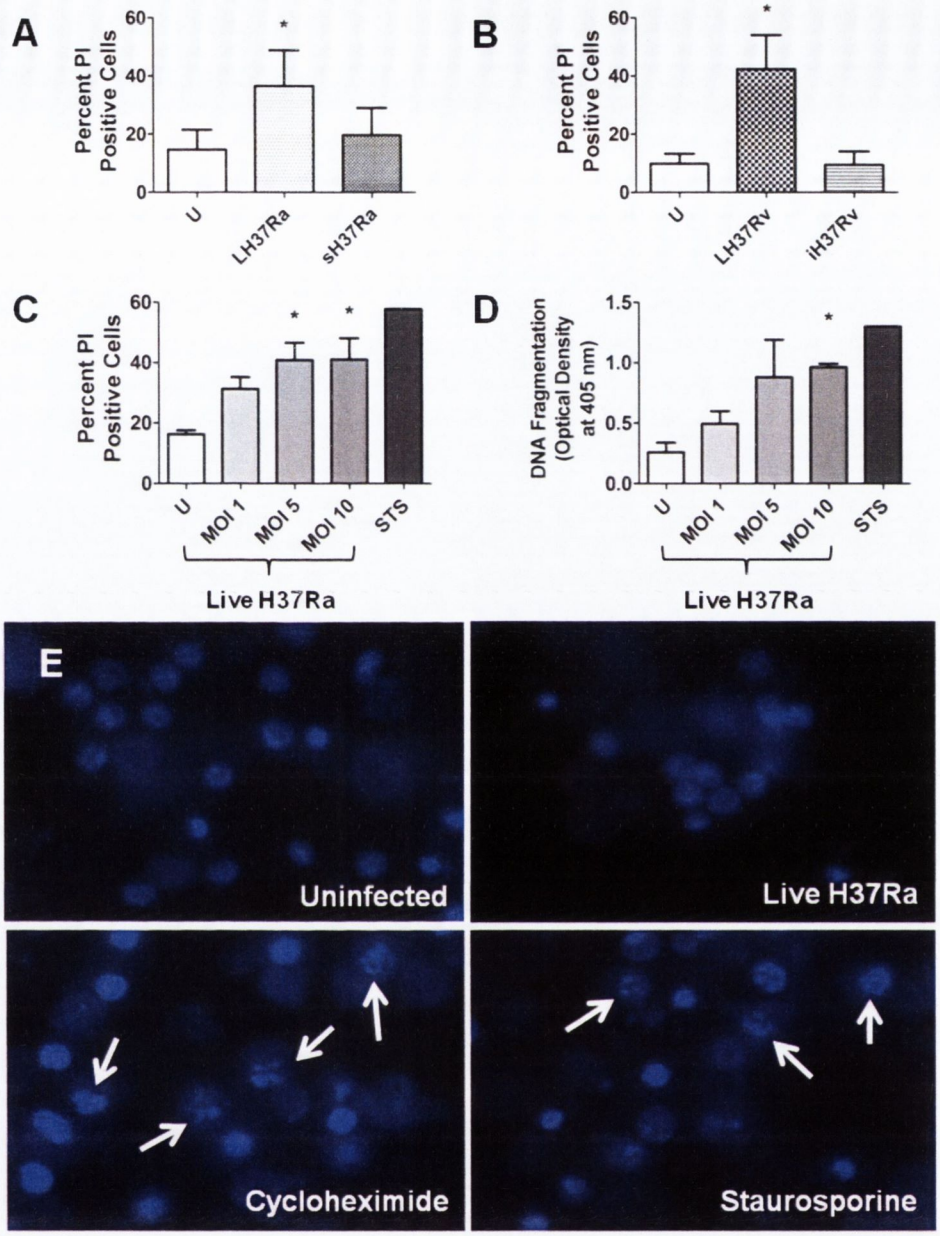


Figure 4-6: Live Mtb infection causes DC death and DNA fragmentation, without nuclear fragmentation

A – B. Dendritic cells (DCs) were infected, at MOI 10, with live/dead H37Ra or live/dead H37Rv. (U = uninfected, LH37Ra = live H37Ra, sH37Ra = streptomycin-killed H37Ra, LH37Rv = live H37Rv, iH37Rv = γ-irradiated H37Rv.) Cell death was measured by propidium iodide exclusion (A) 72 h post-infection or (B) 24 h post-infection on a GE IN Cell Analyzer 1000. (A-B) are means (± SEM) of 3 pooled donors. * $p < 0.05$ vs. uninfected. (C) DCs were infected with live H37Ra at MOI 1, 5 or 10. Cell death was measured by propidium iodide exclusion 72 h after infection. Staurosporine was used as a positive control for cell death. * $p < 0.05$ vs. uninfected. (D) DCs were infected with live H37Ra at MOI 1, 5 or 10. DNA fragmentation was measured by Cell Death ELISA 72 h after infection. * $p < 0.05$ vs. uninfected. (E) DCs were infected with live H37Ra at MOI 10 for 72 h. Nuclei were stained with Hoechst and visualised by fluorescence microscopy. Cycloheximide and staurosporine were used as positive controls for nuclear fragmentation. (C – E) are one representative donor of three, showing means (± SEM) of three independent wells.

4.3.3 Dendritic cell death after Mtb H37Ra infection is caspase-independent and proceeds without the activation of caspase 3 and 7

Activation of caspases is considered to be essential for classical apoptosis (Kroemer et al., 2009). Therefore, it was investigated whether DC death following Mtb infection was caspase dependent. Cells were treated with the pan-caspase inhibitor Q-VD-OPh, infected with H37Ra (at an MOI of 10) and cell death was assessed using IN Cell fluorescent microscopy and analysed as before. DCs were incubated with Q-VD-OPh 4 h prior to infection with Mtb and the inhibitor was replenished every 24 h during the experiment. Cell death was assessed at 72 h post-infection with Mtb H37Ra. The inhibition of caspases by Q-VD-OPh did not interfere with the level of cell death after Mtb infection (Figure 4-7A), although the inhibitor did prevent the nuclear fragmentation induced in DCs by cycloheximide and staurosporine treatment (Figure 4-7B).

The results thus far indicated that Mtb-infected DC death occurred with DNA fragmentation, but without nuclear fragmentation, and was caspase-independent. Caspase-independent cell death can occur with or without caspase activation depending on the mechanism of cell death (Kroemer and Martin, 2005; Auner et al., 2010; Park et al., 2011). In order to more closely examine the role of caspases in DC death induced by Mtb H37Ra infection, the activity of the executioner caspases 3 and 7 was analysed in parallel with cell death at 24 h, 48 h and 72 h post-infection with Mtb (Figure 4-7C). Staurosporine (24 h treatment at all time points) and cycloheximide (24, 48 and 72 h treatment in parallel with infection) were used as positive controls for caspase activity, inducing significantly increased caspase 3 and 7 activity at all time points examined (Figure 4-7C). Caspase activity was measured before and after significant cell death had occurred. Cell death due to Mtb H37Ra was apparent at 72 h post-infection (Figure 4-7C) and occurred with live Mtb infection only, as in previous experiments (Figure 4-6). Caspase 3 and 7 were not active above levels recorded in uninfected DCs at any time point examined, indicating that these caspases are not activated during Mtb H37Ra-induced DC death.

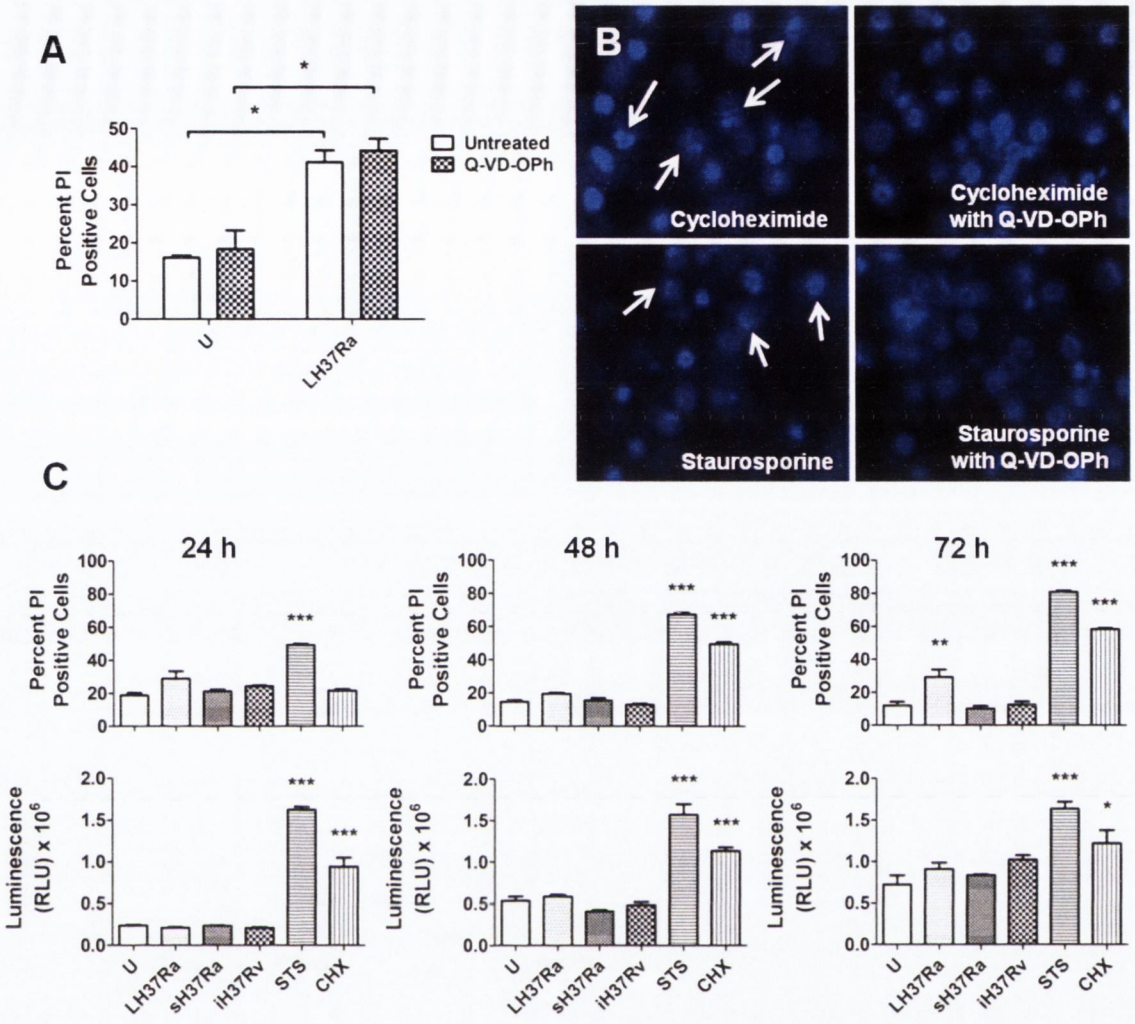


Figure 4-7: DC death after Mtb H37Ra infection is caspase-independent and proceeds without the activation of caspase 3 and 7

(A) DCs were infected with live Mtb H37Ra at MOI 10, in the presence or absence of the pan-caspase inhibitor, Q-VD-OPh (20 μM). Cell death was measured by propidium iodide exclusion 72 h post-infection. U = uninfected DCs, LH37Ra = live H37Ra. * $p < 0.05$ vs. uninfected. Data represent means ± SEM of three separate donors. (B) As a positive control, DCs were treated with cycloheximide (5 μg/ml for 72 h) or staurosporine (1 μM for 24 h) in the presence or absence of Q-VD-OPh. Nuclei were stained with Hoechst and visualised by fluorescence microscopy. (C) DCs were infected with live/dead Mtb H37Ra or γ-irradiated H37Rv at MOI 10. Caspase 3/7 activity was assessed at 24 h, 48 h and 72 h. Cell death was measured in parallel by propidium iodide exclusion (upper panels). U = uninfected DCs, LH37Ra = live H37Ra, sH37Ra = streptomycin-killed H37Ra, iH37Rv = γ-irradiated H37Rv, STS = staurosporine, CHX = cycloheximide. * $p < 0.05$ vs. uninfected. ** $p < 0.01$ vs. uninfected. *** $p < 0.001$ vs. uninfected. Data represent means (± SEM) of five donors.

4.3.4 Secretion of cytokines by Mtb H37Ra-infected DCs

Although macrophages and neutrophils die after Mtb infection, these dying and dead cells have been shown to play a role in host immune responses (Molloy et al., 1994; Laochumroonvorapong et al., 1996; Alemán et al., 2007; Persson et al., 2008; Rodrigues et al., 2009; Hedlund et al., 2010). To test if infected DCs contribute to immunity by secreting cytokines, DCs were infected at a low MOI of 1 bacillus per DC and assessed for cytokine production (assayed by Meso Scale Discovery multiplex ELISA). The release of the following cytokines was measured in the cell-free supernatants of infected and uninfected DCs: IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α. Low MOI caused 40-60% death after 72 h (shown in Figure 4-6C) and was chosen to allow assessment of cytokine release at 24 and 48 hours post-infection before excessive cell death had occurred. Infection of DCs from each donor (n = 3) with H37Ra consistently stimulated the release of pro- and anti-inflammatory cytokines including TNF-α, IL-6, IL-8, IL-10, IL-1β and a modest increase in secretion of IL-12p70 (Figure 4-8). There was a tendency for DCs infected with killed H37Ra to produce less IL-10, TNF-α, IL-6 and IL-1β than cells infected with live H37Ra, but these results did not reach statistical significance when the data was pooled due to donor variation. Other cytokines were unchanged after infection: IL-2, IFN-γ, IL-5 and IL-13 (Figure 4-9).

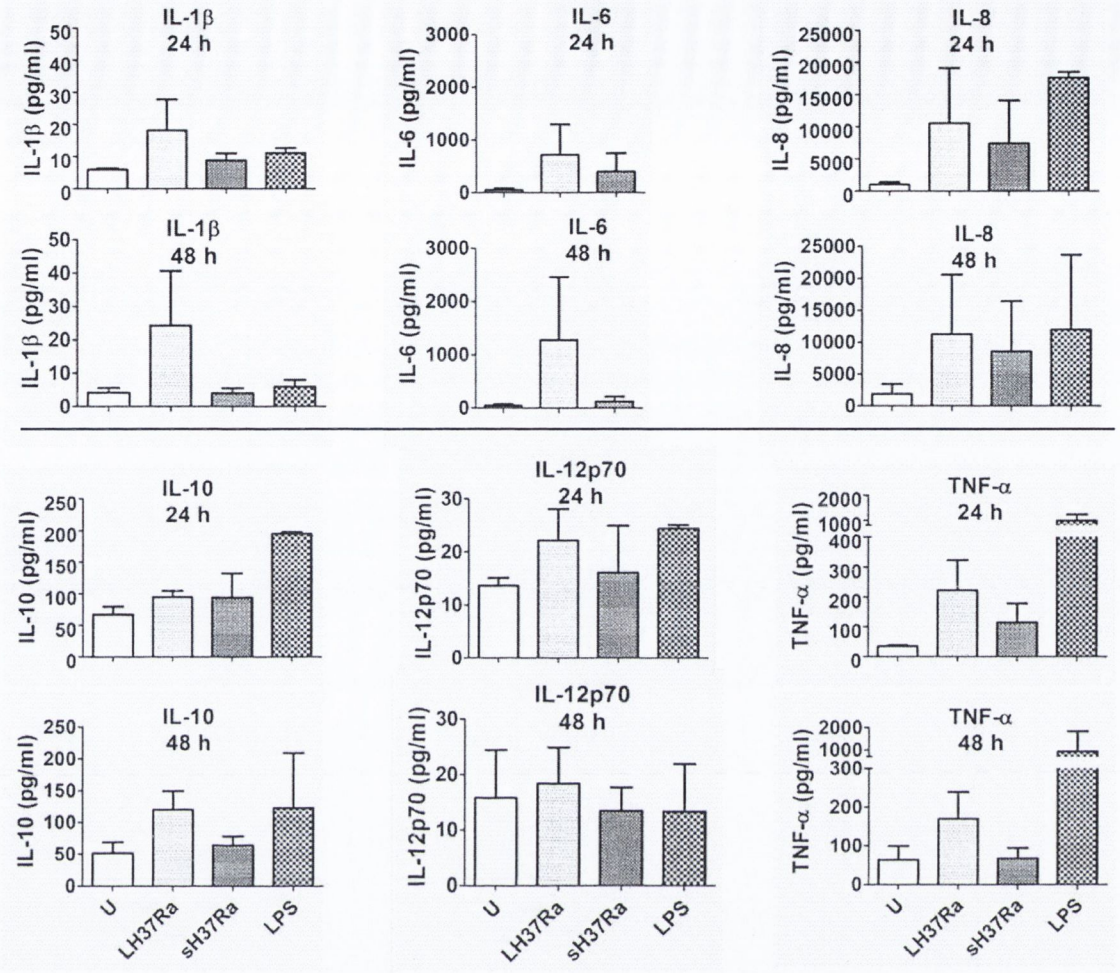


Figure 4-8: Dying Mtb-infected DCs secrete cytokines.

DCs were infected with live/dead Mtb H37Ra at MOI 1 for 24 h or 48 h, or treated with LPS (1 µg/ml) for 24 h. U = uninfected DCs, LH37Ra = live H37Ra, sH37Ra = streptomycin-killed H37Ra. Cytokine levels were measured in cell-free supernatants by ELISA. Data were analysed using the Friedman test followed by the Wilcoxon signed rank test and represent the means (± SEM) of three individual donors.

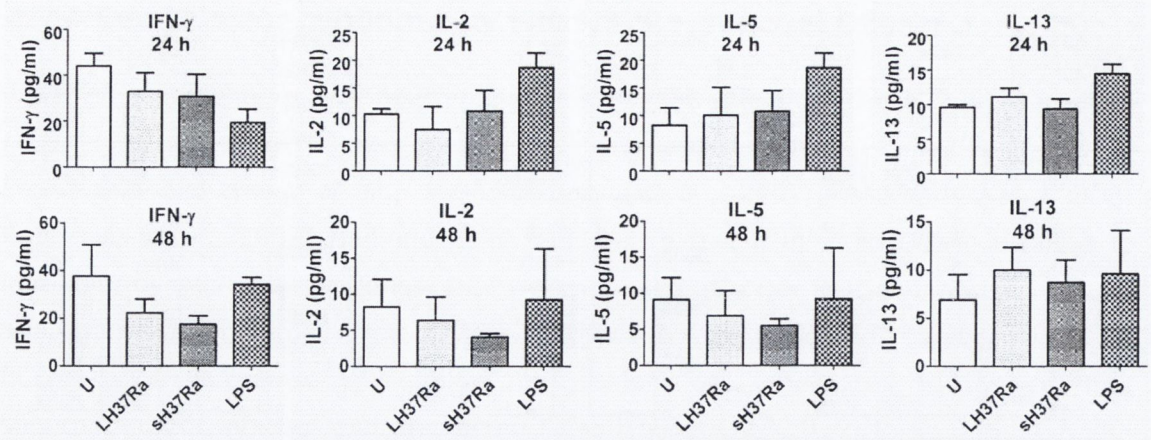


Figure 4-9: Low IFN-γ, IL-2, IL-5 and IL-13 secretion from Mtb-infected DCs.

DCs were infected with live/dead Mtb H37Ra at MOI 1 for 24 h or 48 h, or treated with LPS (1 μg/ml) for 24 h. Cytokine levels were measured in cell-free supernatants by ELISA. U = uninfected DCs, LH37Ra = live H37Ra, sH37Ra = streptomycin-killed H37Ra. Data were analysed using the Friedman test followed by the Wilcoxon signed rank test and represent the means (± SEM) of three individual donors.

4.3.5 Dendritic cells are permissive for growth of Mtb H37Ra

Alveolar macrophages also die after Mtb infection and yet are capable of restricting the growth of avirulent Mtb (Keane et al., 2000). Dendritic cells are responsible for activation of $CD4^+$ and $CD8^+$ cells to enable killing of intracellular Mtb, but infected DCs could also limit Mtb growth directly. There are conflicting reports within the literature regarding the fate of Mtb strains within DCs. In the present study, the ability of Mtb H37Ra to replicate within human DCs, in the sustained presence of GM-CSF and IL-4, was studied using two separate methods: CFU counts and the bioMérieux BacT/ALERT 3D automated microbial detection system (Figure 4-10). At MOIs of 1, 5 and 10 bacilli per DC, Mtb grew over the course of 3 days, as indicated by greater numbers of colony forming units by Day 3, compared with Day 1 (Figure 4-10A). At the same time point, a similar dose-response was observed for bacillary growth using liquid Middlebrook media in a BacT/ALERT system; a growth index (percent change in Mtb growth) was generated using 'time to positivity' data (see Chapter 2, section 2.15) (Figure 4-10B). Apoptosis has been linked to improved mycobactericidal effects in macrophages (Oddo et al., 1998; Randhawa et al., 2008; Rodrigues et al., 2009), whereas the present work demonstrates that Mtb H37Ra replicates within DCs despite (or perhaps because of) the abundant non-apoptotic cell death that occurs during infection.

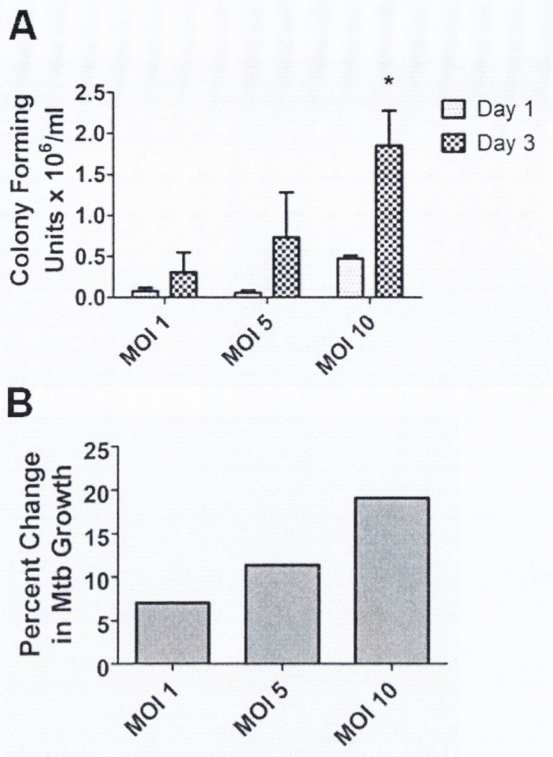


Figure 4-10: Dendritic cells are permissive for growth of Mtb H37Ra

(A) DCs were infected with live Mtb H37Ra for 20 h (Day 1) or 72 h (Day 3), at varying MOI. Colony-forming units were counted after 21 days. * $p < 0.05$ vs. Day 1. The graph represents the mean (± SEM) of three separate donors. (B) DCs were infected as above for 3 days and incubated in a bioMérieux BacT/ALERT 3D automated microbial detection system. Detection of bacterial growth was labelled 'positive' and time to reach positivity (TTP) was recorded. Percentage time to positivity was calculated using the formula: ((TTPDay1-TTPDay3)/TTPDay1)x100. In this equation TTP Day 1 is the time to culture positivity for infected DC lysates at Day 1, and TTP Day 3 is the time to positivity for infected DC lysates at Day 3. A positive change in percentage time to positivity was indicative of bacterial growth. The results shown are from one representative donor of three.

4.4 Discussion

As potent antigen presenting cells, DCs play an important role during tuberculosis infection. This study aimed to shed further light on the interaction between the cell and the bacterium. The impact of Mtb infection on the viability and function of human monocyte–derived DCs was investigated. In these experiments, DCs matured upon infection with live Mtb. There are numerous studies of the effects of Mtb on DC maturation. Certain Mtb components, such as purified protein derivative (PPD), induce DC maturation (Bagheri et al., 2008), while lipoarabinomannan (LAM) and Mtb heat shock protein 70 impede maturation of DCs (Dulphy et al., 2007; Motta et al., 2007). There are also conflicting reports of the effect of the whole bacterium; with studies demonstrating either enhanced or impaired DC maturation following Mtb infection (Henderson et al., 1997; Hanekom et al., 2003; Rajashree et al., 2008). Different clinical strains of Mtb have varying effects on human DC maturation. Impairment of DC maturation may be a function of virulence; with highly virulent Mtb strains causing reduced DC maturation compared with less virulent strains (Rajashree et al., 2008).

DC death followed infection with both the H37Ra and H37Rv strains of Mtb, required viable bacilli, and could be detected at 24 hours co-incubation. The type of cell death was atypical of apoptosis, because it lacked nuclear fragmentation and was caspase-independent, although it did proceed with DNA fragmentation. Caspase activation was not detected by substrate assay analysis. Although this type of cell death did not interfere with earlier DC maturation events or cytokine release, it was not associated with any detectable mycobactericidal effect of DCs. In this regard, DC death differs from infected macrophage cell death, which can kill the invading parasite (Keane et al., 2002). Data from Alaniz et al. suggest that DCs can serve as a niche cell that promotes intracellular bacterial replication (Alaniz et al., 2004). Other groups have examined DC mycobactericidal capacity using different models, with varying results (Fortsch et al., 2000; Bodnar et al., 2001; Tailleux et al., 2003). Fortsch et al. and Bodnar et al. found that DCs were permissive for growth of intracellular Mtb, while Tailleux et al. reported constraint of Mtb replication within DCs. The difference in findings was suggested to be due to removal of the cytokines, GM-CSF and IL-4, from DCs upon infection with Mtb in the initial study (Fortsch et al., 2000), while the latest work (Tailleux et al., 2003) maintained cytokine levels throughout infection. Removal of GM-CSF from DCs *in vitro* has been shown to increase spontaneous DC death (Chen et al., 2007). In the present study, the GM-CSF/IL-4 supplementation of DCs in culture was maintained to preserve the DC phenotype and to prevent spontaneous cell death. Maintenance of GM-CSF/IL-4 supplementation did not support infected DC viability or their ability to limit intracellular bacterial replication. Similar findings were reported in murine Mtb-infected DCs maintained in IL-4, which were unable to control

mycobacterial growth in the absence of exogenous IFN-γ (Bodnar et al., 2001). The current work models the early stages of Mtb infection in the lung, where newly arrived DCs may become infected before being activated by exposure to T_H1 cytokines (such as IFN-γ), allowing uncontrolled proliferation of mycobacteria. After the initiation of a T cell response and the formation of the granuloma, infected DCs are more likely to be exposed to IFN-γ and may be better able to control the growth of mycobacteria.

Unlike macrophages, DCs in isolation failed to kill bacilli, in the absence of T cell help. A key role for DCs is antigen presentation, which results in activation of T cell populations that can lead to efficient phagocyte killing of the intracellular bacillus, via granulysin-induced phagocyte death, or by cytokine release (e.g. IFN-γ) that supports the mycobactericidal capacity of phagocytes (Canaday et al., 2001; Dieli et al., 2001; Martino et al., 2007; Bastian et al., 2008). Although outside the scope of the current work, it is possible that dying DCs share some properties of dying macrophages, and contribute to this T cell response. In murine DCs the consequences of cell death after infection with *Legionella pneumophila* link caspase activity and bacterial killing (Nogueira et al., 2009), however caspase 3 or 7 activity, or association with Mtb killing, were not observed in dying, Mtb-infected DCs. DC death may constitute an important host response during Mtb infection, by enhancing antigen presentation to $CD4^+$ T cells and cross-presentation to $CD8^+$ T cells, thereby facilitating a maximally efficient adaptive immune response. It has been proposed that the apoptosis induced by the non-pathogenic mycobacterium, *M. smegmatis*, is a reason for its lack of pathogenicity (Bohsali et al., 2010). Cross-presentation of mycobacterial antigens from apoptotic macrophages is an important mode of activation of $CD8^+$ lymphocytes during TB infection (Schaible et al., 2003). Similarly, cross-presentation of antigens from dying Mtb-infected DCs by uninfected bystander DCs could be another key activator of $CD8^+$-mediated immunity.

In the present study both the attenuated H37Ra and virulent H37Rv strains caused death of human DCs. The caspase-independent cell death observed in H37Ra-infected DCs appears to be neither apoptosis nor pyroptosis (both of which require caspase activity), as the use of a pan-caspase inhibitor did not reduce DC death (Fernandes-Alnemri et al., 2007; Kroemer et al., 2009). There are various modes of non-apoptotic cell death, such as pyronecrosis and necroptosis, which can occur with or without caspase activation. While pyronecrosis is caspase-independent, DNA fragmentation is absent in this form of cell death. As DNA fragmentation is a feature of DC death during Mtb infection, it does not appear to be pyronecrosis. It is unclear whether the DC death induced by Mtb is necroptosis; this mode of cell death is dependent on RIP1 activation, which is inhibited by the pro-survival necrostatin proteins (Hitomi et al., 2008). Interrogation of RIP1 involvement in Mtb-infected DC death could be achieved

using commercially available necrostatin-1, the addition of which would inhibit necroptosis of cells (Christofferson and Yuan, 2010). The way in which cells die shapes the response of the immune system; death can be immunogenic, tolerogenic or silent (Green et al., 2009; Torchinsky et al., 2010). Therefore, the type of cell death undergone by Mtb-infected DCs is of interest, as it may either prompt or inhibit cytotoxic and helper T cell responses. Macrophage apoptosis appears to be beneficial for the host response to tuberculosis by having direct bactericidal effects on intracellular mycobacteria and also in the stimulation of protective immunity.

The genome of *M. tuberculosis* encodes for genes that actively inhibit macrophage apoptosis and enhance its intracellular survival, including *nuoG*, *pknE* and *secA* (Briken and Miller, 2008). It is likely that the products of these genes would also inhibit apoptosis of DCs, possibly steering the cells towards the non-apoptotic mode of cell death seen in the present study. Interestingly, foamy macrophages (which are positive for DC markers) in granulomas in the lungs of mice infected with *M. tuberculosis* have been found to express high levels of TNFR-associated factors (TRAFS) 1-3, which are associated with resistance to apoptosis (Ordway et al., 2005). Although H37Ra and H37Rv are highly related, being derived from the same parental H37 strain, they differ in important respects at the genetic (Zhang et al., 1998), transcriptional (Li et al., 2010) and post transcriptional (Frigui et al., 2008) levels. As a result, H37Ra displays several characteristics that are different from H37Rv (e.g. variations in PE/PPE/PE-PGRS proteins (Zheng et al., 2008), decreased survival inside human macrophages (Silver et al., 1998; Zhang et al., 1998; Keane et al., 2000), differences in the composition of mannose caps on LAM (Nigou et al., 2000) and impaired ability to secrete ESAT-6 (Frigui et al., 2008), each of which could have an impact on the mode of cell death (Barry and Beaman, 2006; Welin et al., 2011). Indeed, similar to the findings of O'Sullivan et al. (2007) in human macrophages, H37Rv infection killed DCs at a significantly faster rate than H37Ra. Further work will be needed to determine whether infection of DCs with H37Rv causes a similar caspase-independent mode of cell death.

Caspases can have variable effects on the immunogenic potential of dying cells. Exposure of damage-associated molecular pattern molecules (DAMPs), such as calreticulin, high-mobility group box 1 (HMGB1) or heat-shock proteins, by dying cells are associated with immunogenic cell death. Although these molecules are cellular components (and, therefore, 'self'), when exposed during cell death they function as 'find-me' or 'eat-me' signals; promoting phagocytosis, immune activation and an inflammatory response. Calreticulin exposure has been shown to be of particular importance in the induction of immunogenic cell death (Zitvogel et al., 2010). Exposure of calreticulin is caspase-dependent; however caspases can also mitigate the

pro-inflammatory release of DAMPs from dying cells and cell death that proceeds without the activity of caspases may generate more immune-activating DAMPs (Kazama et al., 2008; Green et al., 2009; Lüthi et al., 2009). Such an outcome might benefit the host response. These DAMPs could escape from the cell, unimpeded by caspase-neutralisation, and proceed to work in concert with the pro-inflammatory cytokine profile of live Mtb-infected DCs, to generate a better inflammatory response in the lymph node. It has been shown that the exposure of calreticulin on tumour cells, induced by anti-cancer agents, leads to an enhanced tumoricidal inflammatory immune response (Martins et al., 2011). On the other hand, cross-priming of T cells is associated with caspase-dependent macrophage apoptosis (Pang et al., 2009). It remains to be seen whether DC death that occurs without caspase activation could elicit a $CD8^+$ T cell response.

It is also possible that DC death could interfere with important DC functions such as migration to local lymph nodes for efficient antigen presentation. Others have shown that DC migration to local lymph nodes and activation of T cells is impaired in Mtb infection, which would delay stimulation of T cell responses (Khader et al., 2006; Gagliardi et al., 2007; Wolf et al., 2007; Wolf et al., 2008; Gagliardi et al., 2009). Depletion of DCs delays $CD4^+$ T cell responses and exacerbates TB infection *in vivo* (Tian et al., 2005). Although it cannot be extrapolated directly from the present *in vitro* experiments to the complex environment that these cell are exposed to *in vivo*, infected DCs are known to traffic from the lung to lymph nodes (Wolf et al., 2007). Recent work by Schreiber et al. (2011) has demonstrated for the first time that monocyte-derived inflammatory DCs traffic in and out of Mtb granulomas *in vivo*. They found that labelled DCs from experimentally transplanted granulomas could exit the granuloma and travel to the local draining lymph node. Interestingly, although no DC reaching the lymph node from the granuloma was observed to contain Mtb bacilli, Mtb-specific T cell proliferation occurred. The authors postulated that T cell priming was due to granuloma-derived DC transfer of mycobacterial antigens to lymph node-resident DCs, which then presented these to T cells. The source of the Mtb antigens was indeterminate, as granuloma-derived DCs reaching the lymph node did not contain whole Mtb bacilli. The current work suggests the hypothesis that, like dying Mtb-infected macrophages (Schaible et al., 2003), Mtb-infected DCs undergoing cell death might provide a source of mycobacterial antigens for uninfected bystander DCs within the granuloma, which could then travel to the lymph node to participate in T cell priming. Although DC death during Mtb infection has not yet been investigated *in vivo*, it is possible that death of Mtb-infected DCs could contribute to the maintenance of antigenic sampling and T cell priming throughout chronic Mtb infection *in vivo* (Tailleux et al., 2003; Schuck et al., 2009; Schreiber et al., 2011).

Elimination of the infected DCs could deprive the host response of an important source of cytokines; though data from Alaniz et al. (2004) suggest that DCs can serve, like macrophages, as a niche cell that promotes intracellular bacterial replication. DC release of the pro-inflammatory cytokines (such as IL-12) – necessary for T cell activation and control of bacillary growth – is reduced in TB infection (Nigou et al., 2001; Gagliardi et al., 2005). IL-12 is similarly important to DC function, promoting migration to the lymph node in response to the chemokine CCR7 (Khader et al., 2006). Mtb-infected DCs released IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α as reported previously (Floto et al., 2006; Rajashree et al., 2008; Bansal et al., 2010; Remoli et al., 2010; Wang et al., 2010), despite the fact that the majority of the cells eventually die. The cytokine secretion profile of Mtb-infected DCs would successfully drive differentiation of T_H1 and T_H17 responses (Torchinsky et al., 2009). The induction of inflammation during infection (as occurs with T_H1 responses) is important in promoting a successful memory $CD8^+$ T cell response (Wirth et al., 2011). Cultures containing dying DCs (infected with viable Mtb) induced a better inflammatory response than those containing viable DCs (infected with killed Mtb), which may indicate that dying DCs (infected with viable Mtb) may contribute to the generation of a memory $CD8^+$ response to Mtb infection. Infection with live bacilli elicited a more pro-inflammatory cytokine profile, which supports the use of live vaccines for tuberculosis.

Mtb and the human immune system have co-evolved, so that one third of the global population has been colonised by this pathogen, yet the immune system is adequate at preventing disease 90% of the time (WHO, 2007, 2009). The DC is the central cell that regulates this host response, and consequently is it increasingly viewed as a target for new therapeutic and vaccine strategies (Nakano et al., 2006; Kong et al., 2011). A greater understanding of the DC death response to Mtb infection – pro-inflammatory, and without the activation of caspases – may inform further research that defines the T cell consequences of this innate response.

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5 Mtb infection of human macrophages promotes a T_H1 and T_H17 response

5.1 Introduction

Macrophage death occurs during *M. tuberculosis* infection (Fayyazi et al., 2000; Leong et al., 2008; Seimon et al., 2010; Behar et al., 2011a). While cell death has immediate consequences for both the affected cell and – in the case of death during infection – associated bacterial cargo, it also exerts an effect on wider innate and adaptive immune responses (Green et al., 2009; Torchinsky et al., 2010). Macrophage cell death during tuberculosis (TB) infection, as described previously (Chapter 3), aids in bacterial clearance, cross-presentation and T cell activation (Schaible et al., 2003; Winau et al., 2006; Divangahi et al., 2010). In other infections that feature host cell death, such as *E. coli* and *Citrobacter rodentium*, the presence of bacterial PAMPs within infected dying cells directs an immunogenic T cell phenotype, while cells dying in the absence of PAMPs induce a tolerant or regulatory T cell response (Gallucci et al., 1999; Salio et al., 2000; Larsson et al., 2001; Liu et al., 2002; Lucas et al., 2003; Green et al., 2009; Torchinsky et al., 2009; Brereton and Blander, 2010; Torchinsky et al., 2010; Brereton and Blander, 2011). Similarly, necrotic tumour cells induce DC maturation, leading to strong immunogenic $CD4^+$ and $CD8^+$ T cell responses (Sauter et al., 2000). Depending on the trigger for their death, dying cells cause secretion of varying cytokines from the DCs which phagocytose them, thus signalling the expansion of immunogenic or tolerogenic T cell populations (Steinman et al., 2000). Mycobacterial infection can induce immune tolerance, which can be detrimental to the host. Inoculation of mice with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) leads to expansion of tolerogenic T regulatory cells (Li and Shen, 2008). BCG-treated human cord blood monocyte-derived DCs direct polarisation of T regulatory cells (Liu et al., 2010). Additionally, macrophages treated with mycolic acid, a component of the Mtb cell wall, induce a tolerogenic response in sensitised mice (Korf et al., 2006).

The granuloma is central to the control of TB infection. Mtb bacilli are contained – in some cases for decades – within this structure; however sterile immunity with complete elimination of the pathogen is often not achieved. Granulomas feature extensive macrophage death (Keane et al., 1997; Fayyazi et al., 2000; Saunders and Britton, 2007; Leong et al., 2008; Sanchez et al., 2009; Seimon et al., 2010). Macrophages, dendritic cells and T cells are in intimate contact within the granuloma during the course of TB infection (Welsh et al., 2011). It has been demonstrated *in vitro* and *in vivo* that DCs phagocytose exosomes and vesicles from Mtb-infected macrophages and cross-present the macrophage-derived mycobacterial antigens to T

cells (Schaible et al., 2003; Winau et al., 2006; Divangahi et al., 2010; Giri et al., 2010). Investigation of the cytokines released during these interactions, and the associated T cell responses, could contribute to our understanding of the signalling events that shape the adaptive immune response to Mtb. *The current work examined the effect of Mtb-induced macrophage death on the cytokine release from co-cultures with bystander DCs and the subsequent cytokine secretion profile directed by co-culture of these stimulated DCs with T cells, as well as the concurrent T cell activation and proliferation.*

The cytokines released by innate immune cells during their interaction with dying or dead cells can have profound effects on adaptive bystander cells. Dying cells containing no bacterial PAMPs can lead to differentiation of regulatory T cells, via secretion of anti-inflammatory cytokines such as IL-10 and TGF-β from DCs. Conversely, DC recognition of PAMPs within dying cells can cause DC secretion of cytokines including IL-6 and IL-23, with subsequent T_H17 polarisation (Torchinsky et al., 2010) (see Chapter 1, Figure 1-9). Following phagocytosis of apoptotic cells containing no PAMPs, resident liver macrophages release IL-10, which attenuates pathogenic liver inflammation (Zhang et al., 2011). An anti-inflammatory response associated with phagocytosis of dead cells can be detrimental during infection, as it can inhibit LPS-induced IL-6 and TNF-α production, down-regulate secretion of chemokines required to attract immune cells to the site of infection, and reduce the IFN-γ responsiveness of macrophages (which is required for optimum macrophage bactericidal activity) (Tassiulas et al., 2007). TNF-α is essential to immune cell recruitment to the lung, granuloma formation and mycobactericidal activity during Mtb infection (Roach et al., 2002; Clay et al., 2008). IL-6 and IL-1β are required for human T_H17 polarisation; IL-17 has important roles during Mtb infection in supporting IFN-γ production and granuloma formation (Umemura et al., 2007; Zhou et al., 2007; Kimura and Kishimoto, 2010; Okamoto Yoshida et al., 2010; Wozniak et al., 2010). Indeed, IL-6-deficient mice succumb to lethal Mtb infection, underlining the importance of this cytokine in the host-protective response to Mtb (Ladel et al., 1997). *As presented in this chapter, an* in vitro *system was designed to investigate the consequences of DC-macrophage interaction and subsequent DC-T cell interaction in the setting of macrophage cell death during TB infection.*

Hypothesis

Macrophage cell death contributes to the T cell response to Mtb infection.

Aims of this section

The cross-talk between infected macrophages, DCs and T cells is crucial to the efficacy of vaccines and the immune response to Mtb after initial infection, but is poorly understood. The overall aim of this work was to begin to evaluate the role of cell death on human DC and T cell responses to Mtb-infected macrophages. To this end, the *in vitro* T cell response to DCs cultured with macrophages undergoing cell death from (a) Mtb infection (death with presence of bacterial PAMPs) or (b) cycloheximide (CHX) exposure (apoptotic death in the absence of bacterial PAMPs) was studied. Macrophage infection with streptomycin-killed Mtb served as a control for the presence of bacterial PAMPs with the absence of cell death (as killed Mtb does not induce cell death; Chapter 4 and Figure 5-3). The cytokine secretion profiles of DC-macrophage co-cultures, and subsequent stimulated DC-T cell co-cultures were analysed, together with the activation (CD25 and CD71 up-regulation) and proliferation (CFSE dilution) of T cells following co-culture with stimulated DCs.

Summary of results

- Macrophage infection with live Mtb caused cell death.

- Co-culture of dying Mtb-infected macrophages and DCs resulted in an inflammatory cytokine response, similar to that observed with direct DC infection (Chapter 4).

- Conversely, macrophages treated with CHX (uninfected apoptotic macrophages), which underwent cell death in the absence of bacterial PAMPs, did not contribute to cytokine release upon co-culture with autologous DCs.

- Dead Mtb bacilli did not induce macrophage cell death. Cytokine release from DC-macrophage co-cultures where macrophages were infected with live mycobacteria was greater than that from co-cultures with macrophages infected with dead bacilli.

- Co-culture of macrophage-stimulated DCs with allogeneic T cells led to T cell activation and proliferation. DCs stimulated with Mtb-infected macrophages caused T cell activation and proliferation above levels induced by DCs exposed to uninfected macrophages or uninfected apoptotic macrophages.

- DC-infected macrophage and subsequent DC-T cell co-cultures supported release of T_H1 and T_H17 cytokines.

Abbreviations

LMS DCs: 'Live Mtb Stimulated DCs'; DCs stimulated by co-culture with live Mtb H37Ra-infected macrophages.

KMS DCs: 'Killed Mtb Stimulated DCs'; DCs stimulated by co-culture with streptomycin-killed Mtb H37Ra-infected macrophages.

Graphs are labelled with the following treatments:

U: uninfected macrophages

LH37Ra: macrophage infection with live Mtb H37Ra

sH37Ra: macrophage infection with streptomycin-killed Mtb H37Ra

CHX: cycloheximide-treated macrophages

US: unstimulated cells (DCs or T cells)

LPS: DCs treated with LPS

5.2 Methods

The materials and methods used in this chapter are described in detail in Chapter 2. The rationale for their use is outlined in this section.

5.2.1 Experimental design

The methodologies used for this work were chosen with the aim of assessing the downstream effect on the adaptive immune response of innate immune cell death in the setting of tuberculosis infection. An *in vitro* system was employed in order to perform this work in human cells (Figure 5-1). The experimental procedure outlined here is detailed in Chapter 2, Sections 2.18 and 2.19. Briefly, human monocytes were isolated from buffy coats and differentiated to macrophages and DCs. The macrophages were then infected with Mtb to induce macrophage death, or treated with cycloheximide (CHX) to induce sterile apoptosis (to mimic homoeostatic cell turnover). The autologous DCs were then added to the Mtb-infected dying macrophages and the apoptotic macrophages, and maintained in culture for two days. At this time, the culture supernatants were removed for cytokine secretion analysis. The DCs were then removed from the macrophages and added to allogeneic T cells, where they were maintained in co-culture for 3-5 days. The culture supernatants were assessed for cytokine release and the activation and proliferation of the T cells were investigated.

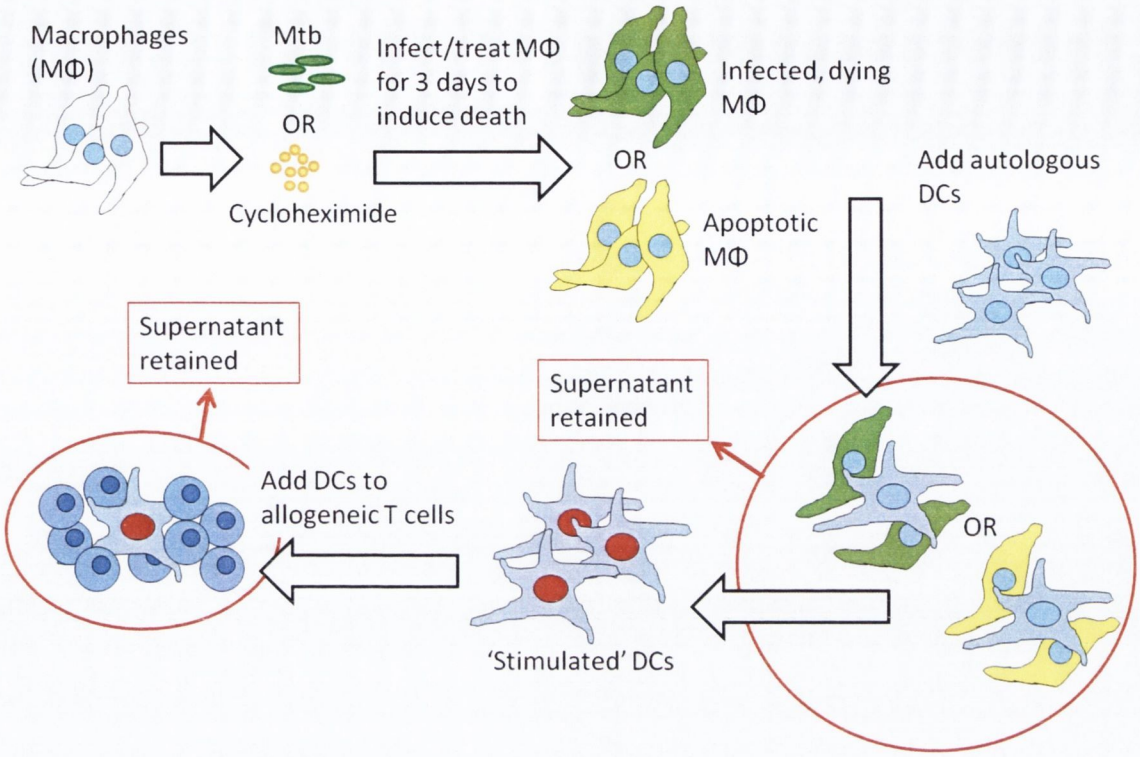


Figure 5-1: Experimental design.

Primary human monocytes were isolated from buffy coats and differentiated to either macrophages or DCs. Macrophages were treated with live or dead Mtb (MOI 20), or cycloheximide (5 µg/ml), for 3 days to induce cell death. The autologous DCs were then added to the macrophages and maintained in co-culture for 2 days. After this time, the macrophage-stimulated DCs were removed from the macrophages and the supernatant was retained for cytokine analysis. DCs were then cultured in a mixed leukocyte reaction (MLR) with allogeneic T cells for 3 – 5 days (detailed in Section 5.2.3). Cytokine release from the MLR, T cell activation and T cell proliferation were then analysed by ELISA and flow cytometry, respectively.

The experimental setup was designed to reflect an *in vivo* situation during TB infection or vaccination, where infected macrophages undergo cell death and interact with neighbouring bystander DCs (Winau et al., 2006). These DCs then communicate with T cells to direct an adaptive response to infection (Schreiber and Sandor, 2010). With this in mind, macrophages were infected with Mtb H37Ra at a high MOI of 20 bacilli per cell to induce cell death and maintained in culture for 3 days (Chapter 3). As a control for sterile apoptotic death – representing homoeostatic cell turnover – CHX was added to macrophages in parallel with infection (Donovan et al., 2009). Streptomycin-killed Mtb were used as a control for the presence of mycobacterial PAMPs in the absence of cell death, as dead bacilli do not induce macrophage death (Figure 5-3). Autologous DCs were then added to the infected/CHX-treated macrophages (after thorough washing of CHX from the macrophages) and cultured for 2 days: sufficient time for DC maturation (see Chapter 4 and Figure 5-5), required for communication with T cells.

5.2.2 Macrophages and DCs

Macrophages and DCs were isolated and cultured as described in Chapters 3 and 4. Chapters 2 and 3 describe the macrophage infection technique used in the present work. Macrophage cell death was measured using PI exclusion and analysed on a GE IN Cell Analyzer 1000.

5.2.3 Allogeneic mixed leukocyte reaction

An allogeneic mixed leukocyte reaction (MLR) was used in this work to determine the proliferative response of T cells to stimulation by DCs exposed to dying macrophages with the presence or absence of mycobacterial PAMPs. The allogeneic MLR was chosen as it delivers a higher proliferative response than autologous MLR; thus maximising separation of any differences in response to macrophage treatments (i.e., Mtb infection or CHX exposure) (Weksler and Kozak, 1977). The MLR can utilise detection of [^{3}H]thymidine incorporation to assess T cell proliferation (Green and Jotte, 1985). However, this method does not allow for simultaneous detection of proliferation and cell phenotype. In the current work, carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescent dye was used in place of [^{3}H]thymidine (see Section 5.2.4). The dividing cells, fluorescently labelled with CFSE, could also be labelled with a fluorochrome-conjugated antibody, allowing detection of division of specific cell populations (in this case, $CD2^+$ T cells) by flow cytometry.

5.2.4 T cells

T cells were isolated from buffy coats by positive immunomagnetic separation for $CD3^+$ cells (detailed in Chapter 2, Section 2.2.5). The technique of positive immunomagnetic separation maximises purity of the desired cells, but due to binding of the immunomagnetic beads to the identifying molecule (in this case, CD3), it cannot subsequently be detected by flow cytometry following separation by this method (STEMCELL Technologies, 2009; Willasch et al., 2010; Klyuchnikov et al., 2011). To overcome this, following $CD3^+$ cell separation, CD2 was used as a T cell marker for flow cytometric analysis of these cells in place of CD3 (Figure 5-2), as CD2 is present on all T cells (Rubin, 2009). The percentage of $CD4^+$ and $CD8^+$ T cells within the total T cell population was also assessed. Activation and proliferation of $CD2^+$ T cells was measured by flow cytometry. The gating strategy for T cells varied slightly depending on the assay (described fully in the following sections).

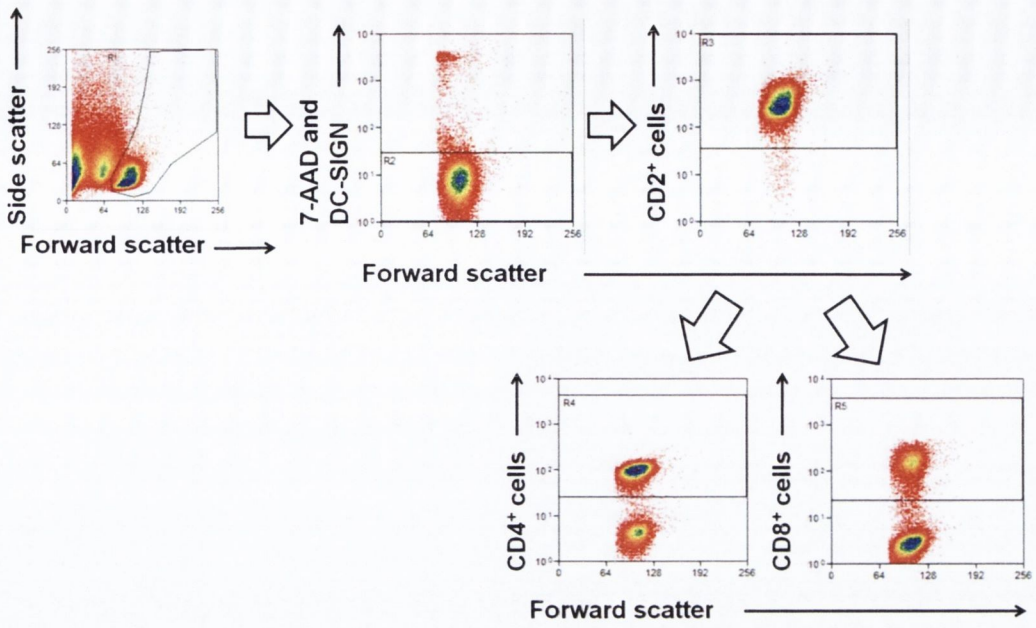


Figure 5-2: Gating strategy for T cells.

Cells were initially gated by forward scatter and side scatter to exclude clumps and debris. Dead cells (stained with 7-AAD) and DCs (DC-SIGN$^+$ cells) were excluded from analysis. Total T cells were identified by CD2 expression. T cells were further classified into CD4$^+$ or CD8$^+$ populations. Scatter plots show unstimulated T cells and are from one representative donor of three.

T cell activation (CD25 and CD71 expression)

The cell surface molecules, CD25 (IL-2 receptor α chain) and CD71 (transferrin receptor), were chosen to study the T cell immune response, as they are established markers of T cell activation (Canivet et al., 2008; Canivet et al., 2009; Porporatto et al., 2009; Alijotas-Reig et al., 2010). Up-regulation of CD25 and CD71 indicates activation of T cells in response to a stimulus and denotes an immunogenic response. Their expression can also correlate with T cell proliferation (Prémaud et al., 2011). For these experiments, dead cells were labelled with 7-aminoactinomycin D (7-AAD) and DCs were detected by labelling DC-SIGN, to eliminate both dead cells and DCs from analysis. Expression of CD25 and CD71 was then assessed in CD2$^+$ T cells.

T cell proliferation (CFSE dilution)

In response to infection, T cells undergo proliferation (Reiley et al., 2010). Conversely, apoptotic cells promote T cell tolerance, suppressing inflammatory T cell proliferation (Chong et al., 2010; Tanaka et al., 2010; Devitt and Marshall, 2011). To assess the proliferative response to macrophages dying under conditions of infection or sterile apoptotic cell death, T cell division was identified by dilution of the stable cytoplasmic fluorescent dye, CFSE. CFSE is divided equally among daughter cells upon cell

division; thus individual cell divisions are detectable by distinct reductions in CFSE fluorescence (Lyons and Parish, 1994). Gating of T cells for CFSE analysis differed slightly to that employed for CD25/CD71 analysis. For this protocol, cells were initially selected by size and granularity (forward and side scatter) and $CD2^+$ cells were then gated for CFSE fluorescence assessment. This gating strategy excluded DCs, which were neither labelled with CFSE, nor express CD2. Dilution of CFSE, which denotes cell division, was evident from multiple peaks in CFSE fluorescence, with each peak representing one division (Figure 5-10A).

5.2.5 Cytokine detection

Cytokine detection was performed by ELISA, as in Chapter 4, using a Meso Scale Discovery SECTOR Imager 2400 to simultaneously detect up to ten cytokines. The cytokines assayed were IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, TNF-α and TGF-β. Samples for cytokine analysis were prepared by R. Ryan and the ELISA was performed by Dr. M. O'Sullivan.

5.2.6 Statistical analysis

The data were analysed by GraphPad Prism 5 statistical software using repeated measures ANOVA with Tukey's post test. Cytokine release data were analysed using the Friedman test followed by the Wilcoxon signed rank test. A p value of < 0.05 was considered statistically significant.

5.3 Results

5.3.1 Live Mtb infection is required to cause macrophage death

Macrophages were infected for 72 h with live or dead Mtb H37Ra at an MOI of 20 bacilli per cell (confirmed for each experiment by acid-fast bacilli staining viewed under fluorescence microscopy; as detailed in the methods section of Chapters 3 and 4) to induce cell death. Parallel CHX treatment was used to induce sterile cell death. Almost 40% of live Mtb-infected macrophages underwent cell death by 72 h, with approximately 50% cell death of CHX-treated macrophages at this time point (Figure 5-3). Streptomycin-killed Mtb did not induce macrophage death above background uninfected levels, as observed previously with DCs (Chapter 4).

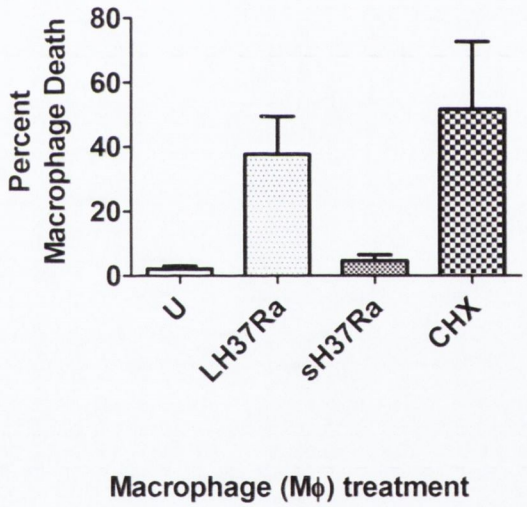


Figure 5-3: Live Mtb and cycloheximide cause macrophage death.

Human monocyte-derived macrophages were treated with live or dead Mtb H37Ra (MOI 20), or with cycloheximide (5 μg/ml), for 72 h to induce cell death. Macrophage death was measured by propidium iodide exclusion using a GE IN Cell automated fluorescence microscope. Graph depicts mean cell death (± SEM) of four donors. U = uninfected macrophages; LH37Ra = infection with live Mtb H37Ra; sH37Ra = infection with streptomycin-killed H37Ra; CHX = cycloheximide treatment.

5.3.2 Dendritic cells mature after exposure to Mtb-infected macrophages

As a preliminary study, DC maturation in response to live/dead Mtb-infected or uninfected/apoptotic macrophages was assessed in one donor. Autologous DCs were added to Mtb-infected or CHX-treated macrophages and cultured together for two days. Up-regulation of the cell surface molecules, CD83 and CD86, was used to investigate DC maturation by flow cytometry (as detailed in Chapter 2 and Chapter 4). To exclude macrophages from flow cytometry analysis, cells positive for CD14 were omitted (Figure 5-4).

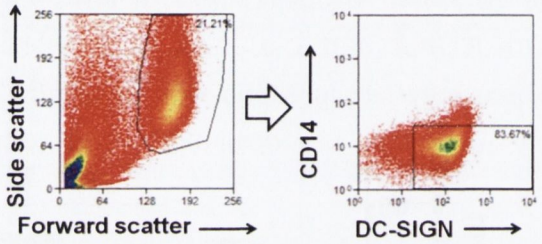


Figure 5-4: Exclusion of macrophages from DC maturation flow cytometric analysis.

Following co-culture of DCs and macrophages, DC maturation was analysed by detection of increased CD83 and CD86 expression by flow cytometry. Macrophages were omitted from DC maturation analysis by selecting only $CD14^{-}DC\text{-}SIGN^{+}$ cells, as macrophages are $CD14^{+}$ cells.

CD83-expressing DCs increased three-fold and the percentage of CD86-expressing DCs doubled in response to exposure to live Mtb-infected macrophages, compared with DCs exposed to uninfected macrophages (Figure 5-5). DCs did not increase CD83 or CD86 expression in response to culture with uninfected apoptotic (CHX-treated) macrophages.

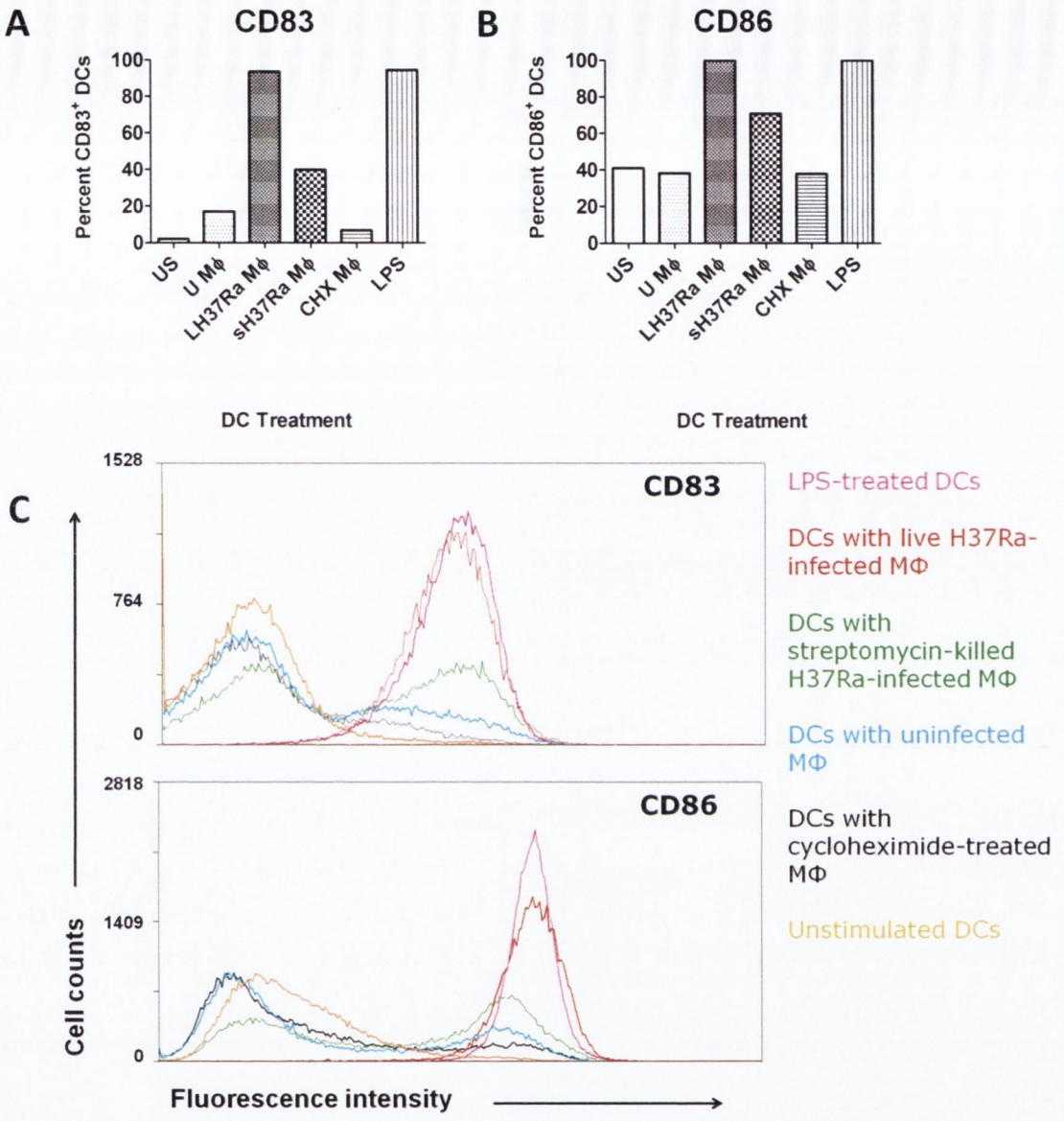


Figure 5-5: Mtb-infected macrophages induce DC maturation.

Human monocyte-derived macrophages (MΦ) were treated with live or dead Mtb (MOI 20), or with cycloheximide (5 μg/ml), for 72 h to induce cell death. After 72 h macrophage infection/treatment, autologous DCs were added to the macrophages and co-incubated for 48 h. As a positive control for DC maturation, LPS was added to DCs (1 μg/ml for 24 h) in the absence of macrophage co-culture. Surface expression of CD83 and CD86 on DCs was then measured by flow cytometry. Cells were gated to include only $CD14^{-}DC\text{-}SIGN^{+}$ DCs, excluding $CD14^{+}$ macrophages. Percentage $CD83^{+}$ (A) and $CD86^{+}$ DCs (B) and fluorescence intensity of CD83 and CD86 (C; indicating the relative surface expression on all gated cells) was analysed. All conditions were analysed in a fixed cell number of 40,000. Graphs and histograms show one donor. US = unstimulated DCs, cultured alone as a negative control; U = uninfected MΦ; LH37Ra = MΦ infection with live Mtb H37Ra; sH37Ra = MΦ infection with streptomycin-killed H37Ra; CHX = cycloheximide treatment of MΦ. Experiment performed with cells from one donor.

5.3.3 Co-cultures of DCs and Mtb-infected macrophages release inflammatory cytokines

Following 72 h macrophage infection/CHX treatment, autologous DCs were added to the macrophages. The DC-macrophage co-culture was maintained for 48 h and cytokine release was then analysed by ELISA. T_H1 and pro-inflammatory cytokines are shown in Figure 5-6; T_H2 and anti-inflammatory cytokines are shown in Figure 5-7. Co-cultures of DCs and live Mtb-infected macrophages released significant amounts of TNF-α ($p < 0.05$, $n = 5$) compared with uninfected or apoptotic macrophage co-cultures, and was also greater (711.6 pg/ml ± 420.8 pg/ml) than that secreted by co-cultures containing dead Mtb (104.1 pg/ml ± 30.02 pg/ml). Live Mtb co-cultures also secreted greater amounts of IL-1β, IL-6 and IL-10 than either uninfected, apoptotic or killed Mtb co-cultures, though the differences between the groups did not reach statistical significance (Figure 5-6 and Figure 5-7). Conversely, IL-8 release was moderately higher from co-cultures containing killed Mtb than those with live Mtb-infected macrophages, or uninfected/apoptotic macrophages (Figure 5-6). TGF-β secretion was highest from DC co-cultures with uninfected macrophages (339.7 pg/ml ± 110.7 pg/ml) and was comparatively (though not statistically significantly) lower from both co-cultures containing live/dead Mtb (265.5 pg/ml ± 68.97 pg/ml for live Mtb co-cultures and 251 pg/ml ± 64.84 pg/ml for killed Mtb co-cultures) and those containing CHX-treated apoptotic macrophages (229.2 pg/ml ± 64.95 pg/ml) (Figure 5-7). Some cytokines, namely IFN-γ, IL-2, IL-5, IL-12p70 and IL-13, were secreted only in very low concentrations from DC-macrophage co-cultures (Figure 5-6 and Figure 5-7).

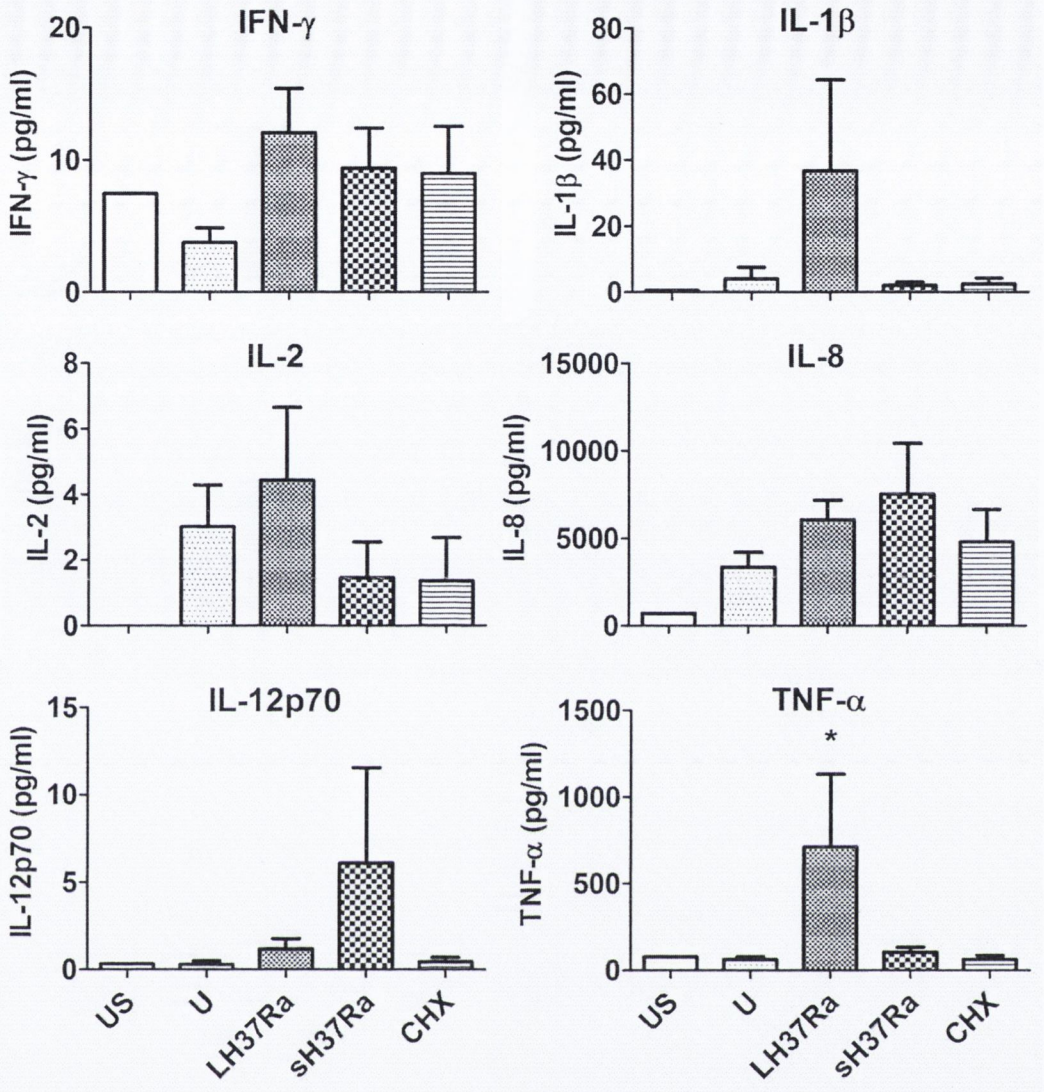


Figure 5-6: Co-cultures of DCs and Mtb-infected macrophages release T_H1 and pro-inflammatory cytokines.

Macrophages were infected with live or dead Mtb at MOI 20, or with cycloheximide (5 μg/ml), for 72 h to induce cell death. Autologous DCs were then co-cultured with the infected/cycloheximide-treated macrophages for 48 h. As a positive control for maturation and cytokine release, DCs were treated with LPS (1 μg/ml) for 24 h in the absence of macrophage co-culture. Cytokine levels from the co-cultures were measured in cell-free supernatants by ELISA. Graphs were compiled from mean cytokine production (± SEM) of five separate donors. US = unstimulated DCs, cultured without macrophages; U = uninfected macrophages; LH37Ra = macrophages infected with live H37Ra; sH37Ra = macrophages infected with streptomycin-killed H37Ra; CHX = cycloheximide-treated macrophages. Comparisons were performed among DC-macrophage co-culture conditions only (unstimulated DCs were not included in statistical comparison) using the Friedman test, followed by the Wilcoxon signed-rank test. * $p < 0.05$ vs. uninfected.

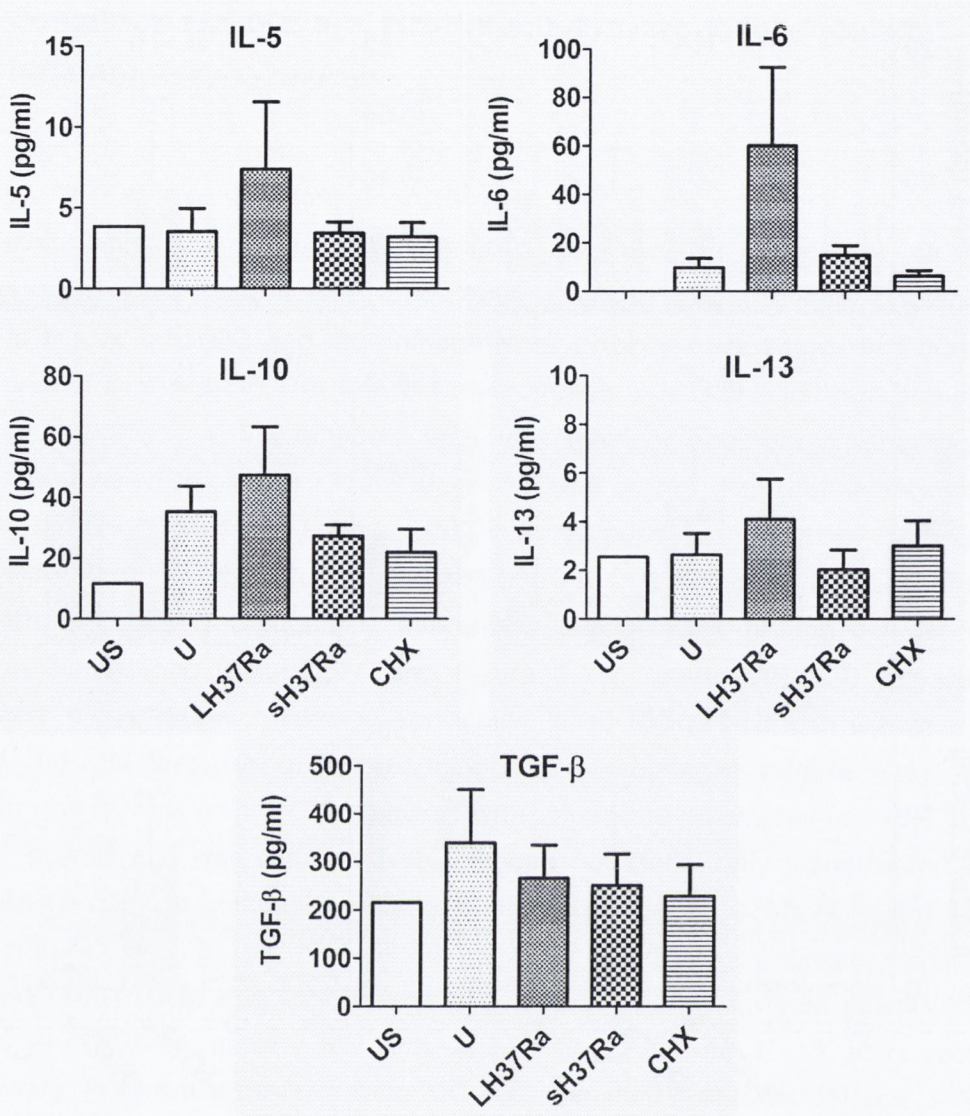


Figure 5-7: Co-cultures of DCs and Mtb-infected macrophages release T_H2 and anti-inflammatory cytokines.

Macrophages were infected with live or dead Mtb at MOI 20, or with cycloheximide (5 µg/ml), for 72 h to induce cell death. Autologous DCs were then co-cultured with the infected/cycloheximide-treated macrophages for 48 h. DCs were treated with LPS (1 µg/ml) for 24 h in the absence of macrophage co-culture as a positive control for maturation and cytokine release. Cytokine levels from the co-cultures were measured in cell-free supernatants by ELISA. Graphs were compiled from means (± SEM) of five individual donors. US = unstimulated DCs; U = uninfected macrophages; LH37Ra = macrophages infected with live H37Ra; sH37Ra = macrophages infected with streptomycin-killed H37Ra; CHX = cycloheximide-treated macrophages. Comparisons were performed among DC-macrophage co-culture conditions only (unstimulated DCs were not included in statistical comparison). Data were analysed using the Friedman test, followed by the Wilcoxon signed rank test.

5.3.4 T cells activate and proliferate in response to co-culture with macrophage-stimulated DCs

After stimulation of DCs by co-culture with macrophages, the DCs were removed from the macrophages and added to allogeneic T cells to assess T cell responses.

T cell activation

Activation of T cells was measured, after 5 days of co-culture with stimulated allogeneic DCs, by CD25 and CD71 up-regulation (Figure 5-5). The ratio of $CD4^+$:$CD8^+$ T cells was approximately 60:40 in all three donors, which remained stable and unchanged between unstimulated T cells and T cells exposed to DCs (regardless of DC treatment/co-culture).

CD2 was used as a global T cell marker. All T cell groups exposed to DCs, irrespective of the DC stimulus or treatment, demonstrated activation when compared to unstimulated T cells, which were cultured in parallel without DCs. T cells significantly increased expression (compared with unstimulated T cells) of CD25 and CD71 when exposed to DCs stimulated by live Mtb-infected macrophages (LMS DCs; CD25 and CD71 both $p < 0.01$) or killed Mtb-infected macrophages (KMS DCs; CD25 and CD71 both $p < 0.05$), but not after exposure to DCs stimulated by uninfected or apoptotic macrophages (Figure 5-8). LMS DCs stimulated more significant T cell activation (CD25 and CD71 up-regulation) than KMS DCs. DCs stimulated by uninfected apoptotic macrophages (treated with CHX) caused no increase in CD25 or CD71 expression over that induced by T cell exposure to uninfected macrophage-stimulated DCs.

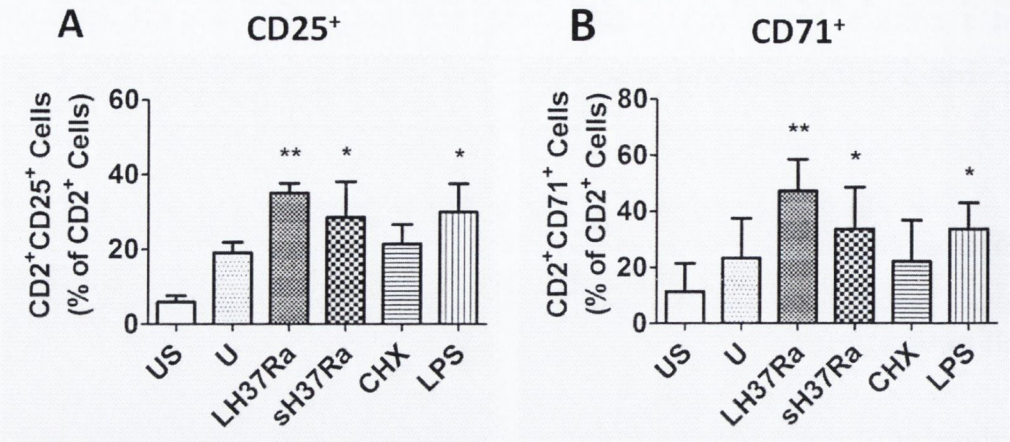


Figure 5-8: DCs stimulated by macrophages induce allogeneic T cell activation.

DCs were co-cultured with autologous Mtb-infected/cycloheximide-treated macrophages for 48 h, or treated with LPS (1 μg/ml) for 24 h as a positive control for DC maturation in the absence of macrophages. After 48 h stimulation, DCs were added to allogeneic T cells and co-cultured for 5 days. As a negative control, T cells were cultured in parallel without addition of DCs (unstimulated; US). T cell activation was measured by assessing up-regulation (compared to unstimulated T cells) of (A) CD25 and (B) CD71 by flow cytometry. U = uninfected macrophages; LH37Ra = macrophages infected with live H37Ra; sH37Ra = macrophages infected with streptomycin-killed H37Ra; CHX = cycloheximide-treated macrophages; LPS = LPS-treated DCs. The graphs show pooled data (± SEM) from three individual donors. * $p < 0.05$ vs. unstimulated T cells; ** $p < 0.01$ vs. unstimulated T cells.

T cell proliferation

T cell proliferation was assessed, after 4 days of co-culture with macrophage-stimulated allogeneic DCs, by CFSE dilution (Figure 5-9 and Figure 5-10). Four days was chosen for assessment, as initial experiments indicated that T cell proliferation was consistently detectable at this time point, but was not always evident earlier (an example of this is shown in Figure 5-9B and C). The gating strategy isolating $CD2^+$ T cells is shown in Figure 5-9A (and detailed in Section 5.2.4).

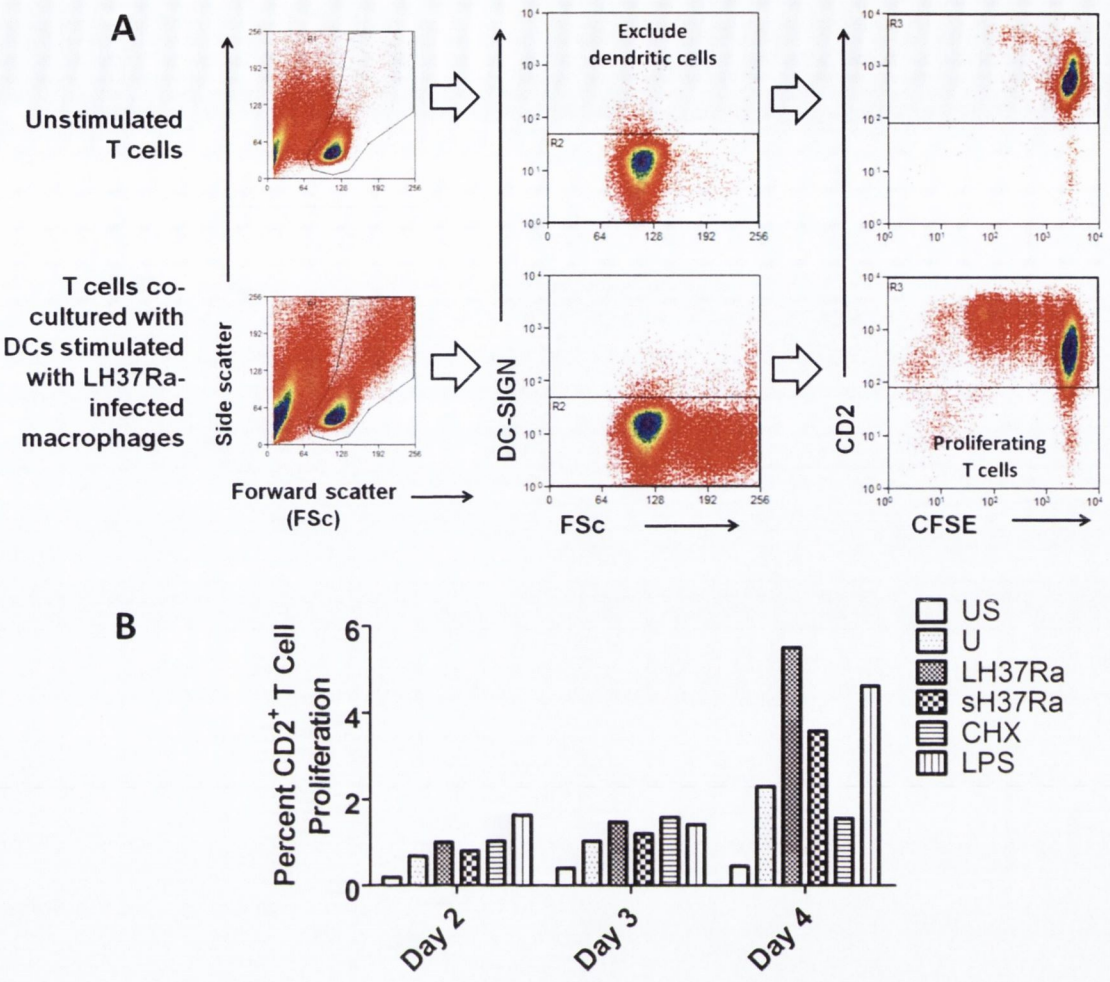


Figure 5-9: T cell proliferation was detectable after four days of culture with stimulated DCs.

T cells were labelled with CFSE and co-cultured with macrophage-stimulated, allogeneic DCs for 2, 3, or 4 days. Proliferation was then assessed by flow cytometric detection of CFSE dilution. (A) Cells were gated by forward scatter and side scatter to select T cells based on cell size and granularity. DC-SIGN$^+$ cells (DCs) were excluded from analysis. DC-SIGN$^-$CD2$^+$ T cells were assessed for proliferation. (B) DCs were co-cultured with autologous Mtb-infected/cycloheximide-treated macrophages for 48 h, or treated with LPS (1 µg/ml) for 24 h as a positive control for DC maturation in the absence of macrophages. After 48 h stimulation, DCs were added to allogeneic T cells and co-cultured for 2-4 days. Some T cells were cultured in isolation (unstimulated; US) as a negative control for 2-4 days. T cell proliferation was apparent from CFSE dilution in T cells co-cultured with DCs after 4 days. U = uninfected macrophages; LH37Ra = macrophages infected with live H37Ra; sH37Ra = macrophages infected with streptomycin-killed H37Ra; CHX = cycloheximide-treated macrophages; LPS = LPS-treated DCs. (A and B) each show a separate individual donor, representative of four.

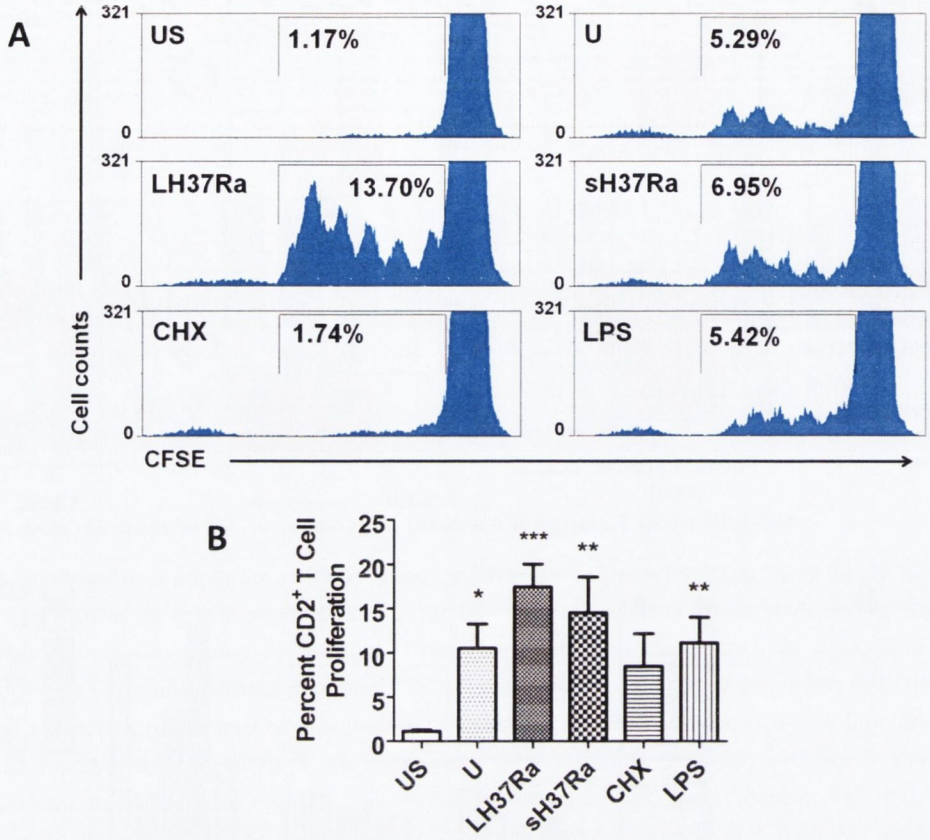


Figure 5-10: DCs stimulated by dying, live-Mtb-infected macrophages – but not uninfected apoptotic macrophages – induce increased T cell proliferation.

T cells were labelled with CFSE and co-cultured with macrophage-stimulated, allogeneic DCs for 4 days. Proliferation was then assessed by flow cytometric detection of CFSE dilution. Unstimulated (US) indicates T cells cultured in isolation. Mtb infection/cycloheximide exposure refers to macrophage treatment prior to 48 h co-culture with DCs. LPS denotes DCs not co-cultured with macrophages, but treated with LPS (1 μg/ml for 24 h) to induce maturation, as a positive control. U = uninfected macrophages; LH37Ra = macrophages infected with live H37Ra; sH37Ra = macrophages infected with streptomycin-killed H37Ra; CHX = cycloheximide-treated macrophages (5 μg/ml). (A) T cell divisions, each denoted by an individual CFSE peak, were observed when T cells were incubated with allogeneic DCs; particularly DCs stimulated with live Mtb-infected, dying macrophages (LMS DCs). (A) shows one representative donor of three; (B) denotes mean proliferation (± SEM) of three individual donors. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. unstimulated T cells. (Individual donor proliferation is shown in Table 5-1 below.)

	US	U	LH37Ra	sH37Ra	CHX	LPS
Donor 1	1.17	5.29	13.70	6.95	1.74	5.42
Donor 2	0.85	14.51	22.39	20.60	14.44	14.91
Donor 3	1.36	11.82	16.25	16.13	9.27	13.05

Table 5-1: Percentage of $CD2^+$ T cells proliferating in response to stimulated DCs: individual readings from three donors.

T cells were treated as described in Figure 5-10 above and proliferation was assessed. The table shows the data used to generate Figure 5-10B and demonstrates donor variation in T cell proliferation.

Significant $CD2^+$ T cell proliferation was induced by T cell co-culture with all DCs, except those stimulated by apoptotic macrophages (Figure 5-10). The greatest

proliferation was induced by LMS DCs ($p < 0.001$; $n = 3$) compared to unstimulated T cells cultured in isolation. KMS DCs also induced significant T cell proliferation ($p < 0.01$), which was slightly lower than that provoked by LMS DCs (with a mean proliferation of 14.56% ± 4.02% in KMS DC-T cell co-cultures compared with 17.45% ± 2.58% in LMS DC-T cell co-cultures; Figure 5-10 and Table 5-1). Reflecting their concomitant low activation (Figure 5-8), the proliferation of T cells cultured with uninfected apoptotic (CHX) macrophage-stimulated DCs was reduced compared with T cells exposed to all other stimulated DCs.

5.3.5 Cytokine secretion from co-cultures of macrophage-stimulated DCs and T cells

The cytokine secretion from stimulated DC-T cell co-cultures was measured by ELISA. T_H1 and pro-inflammatory cytokines are shown in Figure 5-11; T_H2 and anti-inflammatory cytokines are shown in Figure 5-12. IFN-γ and TNF-α secretion were significantly elevated (both $p < 0.01$; $n = 4$) in co-cultures of T cells with LMS DCs, compared to T cells co-cultured with DCs stimulated by uninfected macrophages (Figure 5-11). IL-17 secretion was significantly greater from LMS DC-T cell co-cultures ($p < 0.05$; $n = 4$) than from unstimulated T cells. IL-17 release was also higher from LMS DC-T cell co-cultures (78.73 pg/ml ± 51.51 pg/ml) compared with T cells co-cultured with DCs stimulated by uninfected macrophages (32.57 pg/ml ± 10.79 pg/ml), though the difference between these groups did not reach statistical significance. Other cytokines were also released at moderately higher levels from DC-T cell co-cultures containing LMS DCs compared with DC-T cell co-cultures containing uninfected macrophage-stimulated DCs: IL-1β, IL-2, IL-6 and IL-12p70. The greatest IL-8 and IL-10 secretion was observed from KMS DC-T cell co-cultures. There was a trend toward increased IL-8 and IL-10 in both LMS and KMS DC-T cell co-cultures compared with co-cultures containing DCs stimulated by uninfected macrophages (Figure 5-11 and Figure 5-12). Co-cultures of T cells with DCs stimulated by apoptotic macrophages (CHX) secreted greater (though not statistically significant) levels of pro-inflammatory IFN-γ, IL-12p70 and TNF-α than co-cultures from uninfected macrophages (Figure 5-11).

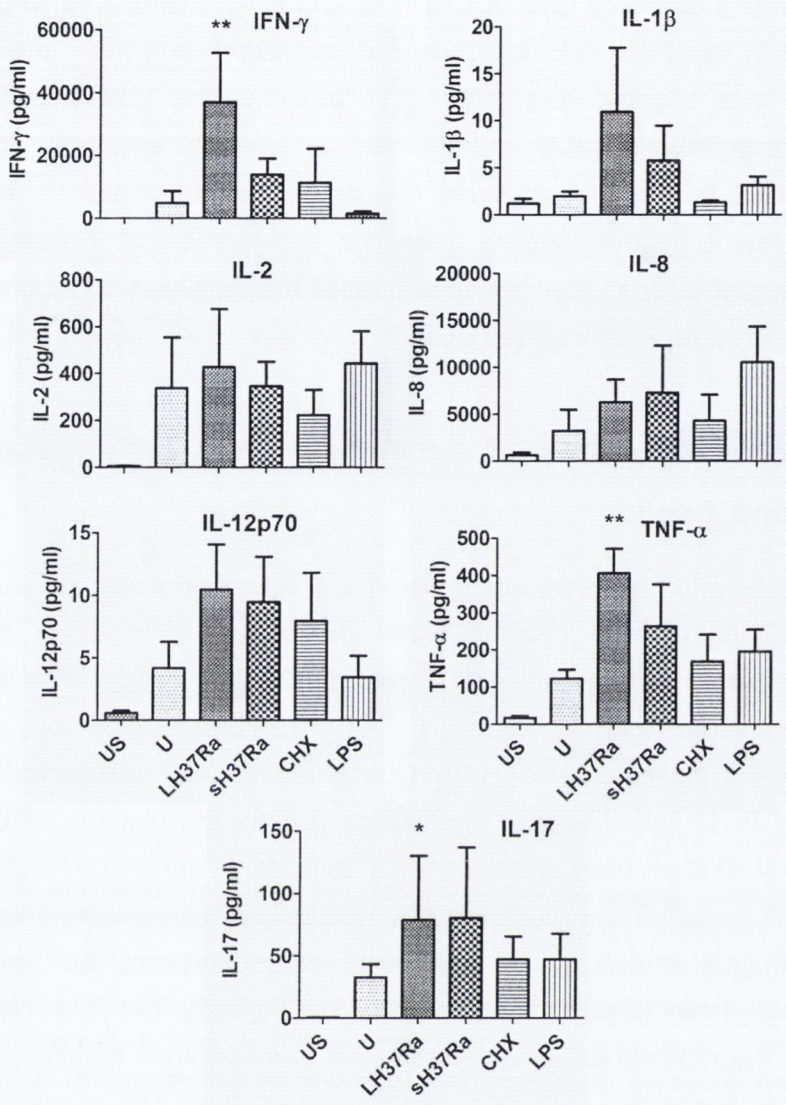


Figure 5-11: Co-cultures of T cells with DCs activated by Mtb-infected macrophages release T_H1 and pro-inflammatory cytokines.

DCs were co-cultured with uninfected, Mtb-infected, or cycloheximide-treated autologous macrophages for 48 h (as before). As a positive control for maturation and cytokine release, DCs were treated with LPS (1 μg/ml) for 24 h in the absence of macrophage co-culture. DCs were then removed from the macrophages, added to allogeneic T cells and the culture was maintained for 3 days. Cytokines were measured in cell-free supernatants by ELISA. Data represent mean cytokine secretion (± SEM) from four individual donors. US = unstimulated T cells, cultured without DCs; U = uninfected macrophages; LH37Ra = macrophages infected with live H37Ra; sH37Ra = macrophages infected with streptomycin-killed H37Ra; CHX = cycloheximide-treated macrophages; LPS = LPS-treated DCs. * $p < 0.05$ vs. unstimulated T cells; ** $p < 0.01$ vs. uninfected.

Incubation of all stimulated/treated DCs with allogeneic T cells led to the release of the T_H2 cytokines IL-5 and IL-13, together with the anti-inflammatory TGF-β, although not at concentrations statistically significantly different to unstimulated T cells (Figure 5-12). The release of IL-5 and IL-13 did not significantly vary between cultures exposed to uninfected, live/dead Mtb-infected or apoptotic macrophages. TGF-β secretion was similar from cultures of uninfected macrophage-stimulated DCs (201.2

pg/ml ± 33.31 pg/ml) and LMS DCs (181.9 pg/ml ± 64.23 pg/ml), but was reduced in cultures containing DCs stimulated with apoptotic macrophages (CHX) (117.3 pg/ml ± 30.65 pg/ml).

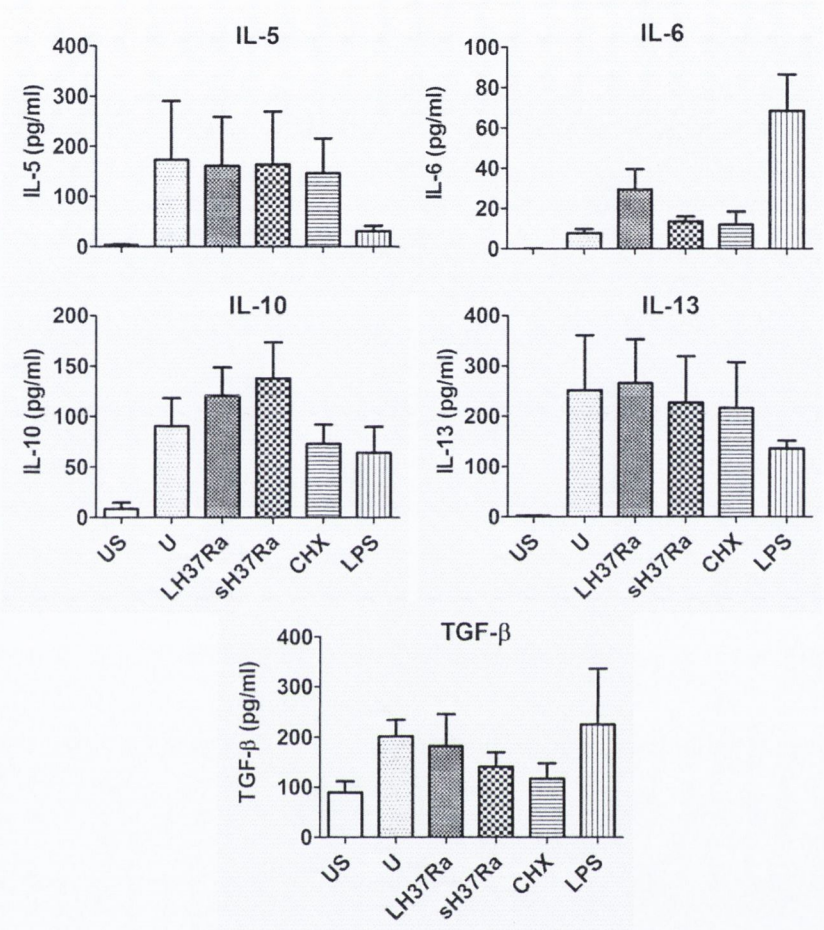


Figure 5-12: Co-cultures of T cells with DCs activated by Mtb-infected macrophages release T_H2 and anti-inflammatory cytokines.

DCs were cultured with uninfected, Mtb-infected, or cycloheximide-treated autologous macrophages for 48 h (as before). LPS treatment (1 μg/ml) was used to treat DCs in the absence of macrophage co-culture, as a positive control for maturation and cytokine secretion. DCs were then added to allogeneic T cells and the culture was maintained for 3 days. Cytokines were then measured in cell-free supernatants by ELISA. Data represent mean cytokine secretion (± SEM) from four individual donors. US = unstimulated T cells, cultured without DCs; U = uninfected macrophages; LH37Ra = macrophages infected with live H37Ra; sH37Ra = macrophages infected with streptomycin-killed H37Ra; CHX = cycloheximide-treated macrophages; LPS = LPS-treated DCs.

5.4 Discussion

Macrophage cell death is observed during Mtb infection and can be host-protective (Keane et al., 1997; Ríos-Barrera et al., 2006; Lee et al., 2009). The current work aimed to study, *in vitro*, the interaction between dying Mtb-infected human macrophages and the DCs that phagocytose them, together with the subsequent DC-T cell interaction. Similar to the response observed in Mtb-infected DCs in the previous chapter of the current work, macrophages infected with live, but not dead, Mtb underwent cell death. Upon co-culture of DCs with these macrophages, abundant TNF-α, as well as IL-1β and IL-6, were released. Maturation of the DCs by live Mtb-infected macrophages drove the production of inflammatory IFN-γ, TNF-α and IL-17 by T cells. These T cells also displayed increased activation and proliferation compared with uninfected macrophage-stimulated DC co-cultures, suggesting greater immunogenicity of live Mtb-infected macrophages (which underwent cell death), compared with killed Mtb-infected macrophages (which retained cell viability).

Conversely, macrophages treated with CHX, which underwent apoptotic cell death in the absence of bacterial PAMPs, did not contribute to cytokine release upon co-culture with autologous DCs. However, the subsequent allogeneic T cell co-culture with these DCs reflected a slight immunogenic response, secreting greater levels of IFN-γ, IL-12p70 and TNF-α, with reduced secretion of the anti-inflammatory TGF-β, compared with co-cultures containing uninfected macrophage-stimulated DCs (albeit at much lower concentrations than co-cultures with LMS or KMS DCs). Apoptotic cells have been shown to be immunogenic both *in vitro* and *in vivo* – the mechanism by which apoptotic tumour cells are recognised and cleared by the immune system – although they may have much lower immunogenicity than that of infected cells (Henry et al., 1999; Ronchetti et al., 1999; Fredly et al., 2011). Indeed, confirming their lower immunogenicity compared to DCs exposed to Mtb-infected macrophages, apoptotic macrophage-stimulated DCs did not support a proliferative T cell response greater than that induced by uninfected macrophage-stimulated DCs. DCs have been shown to distinguish between Mtb-infected and uninfected dying neutrophils, maturing only in response to recognition of the infected dying cells (Hedlund et al., 2010). This may also be true of uninfected vs. Mtb-infected dying macrophages (Figure 5-5) and could contribute to the lower cytokine secretion and T cell response to DCs stimulated with uninfected apoptotic macrophages.

DC-macrophage co-cultures containing live Mtb-infected macrophages elicited a different cytokine release profile to that of co-cultures containing killed Mtb-infected macrophages. TNF-α secretion was significantly elevated in co-cultures with live Mtb

compared with DC-uninfected macrophage co-cultures, but was not increased from co-cultures containing killed Mtb. Although not reaching statistical significance, IL-1β, IL-6 and IL-10 release was also greater from DC-live Mtb-infected macrophage co-cultures compared with killed Mtb co-cultures. The lack of statistical significance in the pooled data may be attributable to donor variation, which may be overcome in future studies by studying cytokine release from increased donor numbers. In contrast, IL-8 secretion, though not statistically significantly different, was moderately higher from killed Mtb co-cultures than from live Mtb co-cultures. In mice, IL-6 and TGF-β co-operate to stimulate IL-17 production from T cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Zhou et al., 2007). However, in humans, IL-6 and IL-1β are indispensable for the differentiation of T_H17 cells, while TGF-β inhibits T_H17 polarisation (Acosta-Rodriguez et al., 2007; Lasiglié et al., 2011). It is noteworthy that lower IL-6 and lower IL-1β secretion from killed Mtb-infected macrophage-DC co-cultures did not translate to lower IL-17 production from subsequent T cell co-cultures with these DCs. However, in addition to the relevant cytokine milieu, T cell receptor (TCR) engagement and co-stimulation of the T cell by the APC (in this case, the DC) are required for all T cell activation. Recent work has implicated the strength of TCR signalling in the development of T_H17 cells (Purvis et al., 2010). Though cytokines clearly play a vital role, additional factors contribute to T_H17 polarisation.

It is not possible to determine from the current work whether these cytokine secretion differences translate to altered T cell activation or proliferation, as DCs directly infected with Mtb were not cultured with T cells. It has been reported that direct DC infection drives protective T cell responses during Mtb infection (Tascon et al., 2000; Tsunetsugu-Yokota et al., 2002). However, these responses may not be optimal (Wolf et al., 2007; Meraviglia et al., 2010). Furthermore, DCs have been observed to down-regulate protective T cell responses in the chronic phase of Mtb infection, facilitating persistence of Mtb (Schreiber et al., 2010). This may be due to infiltrating monocytes, recruited to replenish the DC pool within the Mtb-infected lung, becoming infected with Mtb prior to their differentiation to DCs. Mtb infection of monocytes has been reported to result in partial differentiation of monocyte-derived DCs, leading to ineffective T cell priming capability (Mariotti et al., 2002; Mariotti et al., 2004; Makino et al., 2006; Rajashree et al., 2009).

A combination of T_H1 and T_H17 cytokines was released from LMS and KMS DC-T cell co-cultures. Inflammatory T_H1 cytokine release (IFN-γ, IL-1β, IL-6, IL-12p70 and TNF-α) from T cell-LMS DC co-cultures was greater than that induced by KMS DCs. IL-17 release was comparable between the two groups. Torchinsky et al. (2010) describe T_H1 cytokine release as a consequence of direct infection of DCs; a different T cell polarisation consequence to DC recognition of bacterial PAMPs from dying infected

cells, which (in *E. coli* infection) directs a T_H17 response. The mixture of T_H1 and T_H17 cytokines observed in the current study could be due to some DCs becoming directly infected with Mtb (released from dying macrophages) upon co-culture with infected macrophages, thereby stimulating a T_H1 response, in conjunction with other DCs ingesting bacterial PAMPs contained within dying Mtb-infected macrophages, thus inducing a T_H17 response. The inflammatory T_H1 cytokines IL-1β, IL-6, IL-12 and TNF-α are critical to the control of Mtb infection, while IL-10 can impede protective immunity during disease (Flynn et al., 1995; Ladel et al., 1997; Rojas et al., 1999; Robinson and Nau, 2008; Mayer-Barber et al., 2010; Redford et al., 2011). Moderately greater IL-12p70 secretion was observed from all DC-T cell co-cultures than from DC-macrophage co-cultures. IL-12p70, which is the bioactive form of IL-12, has important roles in potentiating IFN-γ release from T cells and maintaining the efficacy of memory T cells during *in vivo* Mtb infection (Feng et al., 2005; Cooper and Khader, 2008). The increased IL-12p70 secretion from DC-T cell co-cultures may represent part of the DC-mediated initiation of T cell responses to *in vitro* Mtb infection. A protective role for IL-17 has also been demonstrated in TB infection, in the formation and maintenance of the granuloma (Okamoto Yoshida et al., 2010; Torrado and Cooper, 2010) and in the development of secondary immune responses to Mtb following vaccination in mice (Khader et al., 2007). Some cytokines, such as TNF-α and IL-10, have been reported to be generated by cells treated with either mycobacterial antigens or the whole bacillus (Cyktor and Turner, 2011). However, the current work identified differences in the levels of cytokine secretion from DC-macrophage co-cultures depending on Mtb (and concomitant macrophage) viability. For example, live Mtb infection induced moderately greater IL-10 secretion from co-cultures than killed Mtb, while the significant TNF-α secretion observed following live Mtb infection was absent with killed Mtb infection. IL-6 and IL-1β secretion from DC-macrophage co-cultures was also dependent on viable Mtb, which induced macrophage cell death. Likewise, differences were observed in DC-T cell cytokine release induced by LMS DCs and KMS DCs, with a trend toward greater IL-10 and reduced inflammatory cytokine release from KMS DC co-cultures compared with LMS DC-T cell co-cultures.

Schaible et al (2003) conducted human *in vitro* experiments with vesicles from dying Mtb-infected macrophages and observed that these vesicles have the ability to transfer mycobacterial antigens to uninfected bystander cells. They cultured T cells with uninfected DCs in the presence of uninfected or Mtb-infected macrophages, which resulted in $CD8^+$ T cell proliferation and IFN-γ release. $CD8^+$ T cells proliferated only in the presence of DCs and infected macrophages. Vesicles alone, or infected macrophages alone were unable to cause $CD8^+$ T cell proliferation, indicating a dependence on the presence of uninfected bystander DCs. Caspase inhibition prevented the T cell response to bystander DCs co-cultured with infected

macrophages, suggesting that apoptotic death of the macrophages was required for cross-presentation of antigens to T cells. However, the pan-caspase inhibitor used in the study could also inhibit caspase 1-dependent activation of IL-1β and IL-18. As the action of the pan-caspase inhibitor may not have been confined to inhibition of effector caspases and apoptotic death, factors other than inhibition of apoptotic macrophage death could have contributed to the abrogation of T cell responses. It is possible that vesicles may also be released from dying cells undergoing non-apoptotic death, which could contribute to T cell cross-priming. It is now also recognised that Mtb-infected cells can release exosomes containing mycobacterial PAMPs, in the absence of caspase 3 activity, which can stimulate both innate and adaptive immune responses (Bhatnagar et al., 2007; Giri et al., 2010; Ramachandra et al., 2010; Singh et al., 2011). Given that Mtb can induce non-apoptotic macrophage death (O'Sullivan et al. (2007) and Chapter 3 of the current work), exosome- or vesicle-mediated antigen transfer from Mtb-infected macrophages undergoing non-apoptotic death to bystander DCs may constitute an important component of cross-presentation during Mtb disease. The *in vivo* occurrence of cross-presentation was confirmed by murine work carried out by Winau et al. (2006). They injected mice with vesicles from dying Mtb-infected macrophages and assessed the $CD8^+$ T cell response. They observed $CD8^+$ proliferation, dependent on migration of DCs to the vesicle injection site. The vesicles also conferred protection against TB challenge in mice. In further *in vivo* studies, Divangahi et al. (2010) revealed that cross-priming of $CD8^+$ T cells during TB infection was dependent on acquisition and presentation of bacterial PAMPs by DCs from dead and dying Mtb-infected macrophages. This 'detour' pathway of antigen presentation also aided in the expansion of Mtb-specific $CD4^+$ T cells (Winau et al., 2004; Divangahi et al., 2010).

Significant T cell activation (increased CD25 and CD71 expression) and proliferation, above that induced by allogeneic stimulation, was observed following culture with both LMS and KMS DCs. Reflecting maximal immunogenicity, T cell activation and proliferation was greatest in T cell co-cultures with LMS DCs. The differences observed between the T cell stimulatory capacity of LMS DCs and KMS DCs most likely stem from the fact that the macrophages they phagocytosed had been exposed to a live or killed organism. The live mycobacteria induced macrophage death, while the killed did not. It is clear from the lower DC and T cell responses to apoptotic macrophages (treated with CHX), which displayed similar levels of cell death to the live Mtb-infected macrophages, that cell death alone is not the immunogenic factor and the presence of bacterial PAMPs is required for an immune response to dying cells. However, mycobacterial PAMPs contained within the macrophages infected with killed Mtb were insufficient to induce the same level of immunogenicity as that of live Mtb macrophage infection. *These results suggest that a combination of mycobacterial PAMPs and the*

ability to induce cell death is required to elicit maximal innate and adaptive responses to TB infection. Orme reported on an *in vivo* study of the ability of four different strains of Mtb to elicit protective, Mtb-specific T cell responses, comparing the live and killed bacilli (Orme, 1988). He found that, while killed Mtb was capable of inducing non-specific and delayed-type hypersensitivity adaptive responses, only the live organism could provide Mtb-specific protective T cell responses. Incidentally, competent protection against subsequent Mtb challenge was provided only by inoculation with the live virulent H37Rv and Erdman Mtb strains, and not the live attenuated H37Ra strain used in the current work. It would be beneficial, therefore, to perform these experiments using a virulent Mtb strain, such as H37Rv.

While the macrophage death observed here may be beneficial to induction of T cell responses to Mtb, it is possible that it is not optimal. It is well documented that Mtb can interfere with host cell death and a number of Mtb genes have been implicated in this capacity. The Mtb NADH dehydrogenase subunit G (*nuoG*) gene inhibits TNF-α-mediated macrophage death (Velmurugan et al., 2007; Miller et al., 2010). Additionally, the *secA2* gene, which encodes an accessory secretion protein vital to Mtb protein export, aids Mtb growth within macrophages, inhibits macrophage death and reduces $CD8^+$ T cell priming (Kurtz et al., 2006). Deletion of these genes from Mtb significantly reduces its ability to inhibit macrophage death and (in the case of *secA2* deletion) results in increased priming of $CD8^+$ T cells, prompting research into use of Mtb mutants that promote cell death as new candidates for live TB vaccines (Hinchey et al., 2007; Velmurugan et al., 2007; Ranganathan et al., 2009; Hinchey et al., 2011). Blocking macrophage death during Mtb infection would be required in order to separate the relative contributions to the cytokine profiles and the T cell responses (observed in the current work) of the viability of the bacilli and the viability of the ingesting macrophage. One of the limitations of the current work is that it has not been possible to prevent macrophage death during Mtb infection. However, although this may be informative, it is perhaps unnecessary to the study of TB disease, where infection is caused by live bacilli and macrophage cell death is observed. The *in vitro* studies presented here describe a pro-inflammatory T_H1 reaction of both APCs and T cells, with an added T_H17 T cell response, to live Mtb infection. This phenotype is not observed with killed Mtb infection and points to a likely requirement for a live vaccine to prevent TB. It is also possible that the increased T cell responses to live Mtb infection were triggered by the presence of greater concentrations of mycobacterial PAMPs within infected macrophages, compared with killed Mtb-infected macrophages, due to intracellular replication of live Mtb bacilli. In order to address this issue, it would be necessary to generate an Mtb mutant incapable of replication, which may be possible in future studies through collaboration with a laboratory proficient in bacterial genetic manipulation.

Recent work by Sander and colleagues (2011) has shown that the innate immune system can distinguish between live and dead bacteria through recognition of viability-associated PAMPs (vita-PAMPs). Thus, a vigorous inflammatory response is reserved for live bacteria. They identified bacterial mRNA as a vita-PAMP, a key determinant used by the host immune system to identify and respond accordingly to viable bacteria. Murine *in vivo* studies showed that bacterial mRNA recognition by the innate immune system also led to a downstream adaptive immune response. Most importantly, the authors demonstrated that co-administration of bacterial mRNA and killed bacilli evoked a response similar to that of viable bacteria, indicating that vita-PAMPs may represent a promising adjuvant to current killed vaccines.

The *in vitro* immune reaction to live vs. dead Mtb reported here clearly indicates that human immune cells are capable of modifying their response to mycobacteria dependent on bacterial viability, which may have important consequences for TB vaccine development. Macrophage cell death is an important precursor to protective immunity in TB (Divangahi et al., 2010). Perhaps the induction of cell death is a signal of mycobacterial viability and its occurrence during infection may trigger immune responses. It has been proposed that the development of a vaccine that induces cell death may ensure a maximally efficient immune response, leading to competent protection against TB disease (Gartner et al., 2008; Behar et al., 2011b). This work supports the concept that induction of macrophage cell death by live mycobacteria – sensed as viable pathogens by host innate immune cells – favours the robust initiation of innate and adaptive immune responses to TB.

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5.6 Appendix

T cell viability is maintained throughout co-culture with DCs

T cell viability (all $CD2^+$ cells) was assessed after 5 days of co-culture with stimulated DCs (in parallel with CD25 and CD71 expression) (Figure 5-13). Some cell death was noted in T cells cultured with LMS DCs or DCs treated with LPS. However, these cultures also displayed T cell proliferation (Figure 5-10). T cells cultured with DCs stimulated by uninfected apoptotic (CHX) macrophages did not undergo death and maintained viability comparable to that of unstimulated T cells.

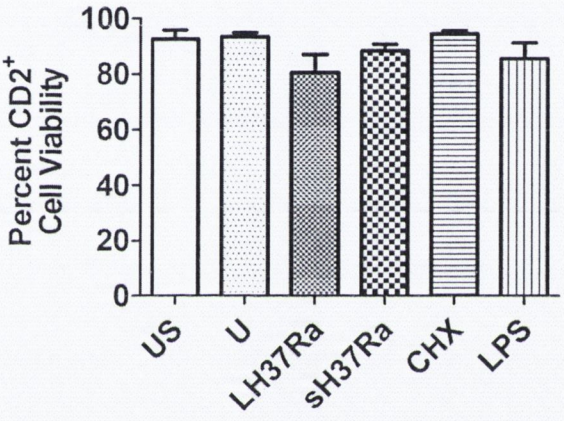


Figure 5-13: T cell viability is maintained throughout co-culture with DCs.

DCs were activated by co-culture with uninfected, Mtb-infected, or cycloheximide-treated (5 μg/ml) autologous macrophages for 48 h (as before). LPS treatment (1 μg/ml) was used to treat DCs in the absence of macrophage co-culture, as a positive control for maturation and cytokine secretion. DCs were then added to allogeneic T cells and the culture was maintained for 5 days. $CD2^+$ cell viability was measured by flow cytometry using 7-AAD exclusion. Data represent mean cell viability (± SEM) from three individual donors. US = unstimulated T cells, cultured without DCs; U = uninfected macrophages; LH37Ra = macrophages infected with live H37Ra; sH37Ra = macrophages infected with streptomycin-killed H37Ra; CHX = cycloheximide-treated macrophages; LPS = LPS-treated DCs.

6 General discussion

Mycobacterium tuberculosis (Mtb) infection has resulted in morbidity and mortality throughout human civilisation and remains a major global health concern to the present day (Nerlich et al., 1997; World Health Organisation, 2010; Donoghue, 2011). Both innate and adaptive immune responses play a critical role in protection and immunity against tuberculosis (TB) infection (Aston et al., 1998; Canaday et al., 2001; Tian et al., 2005; Jordao et al., 2008; Schaefer et al., 2008; Hambleton et al., 2011; Herbst et al., 2011). Chronic colonisation of the host by Mtb is achieved only by disruption of the optimal functioning of the immune system (Mariotti et al., 2002; Loeuillet et al., 2006; Mahon et al., 2009).

Death of infected macrophages occurs during Mtb infection (Keane et al., 1997; O'Sullivan et al., 2007; Wojtas et al., 2011). It is well documented that apoptotic death of Mtb-infected macrophages facilitates bacillary killing and aids in the induction of adaptive immune responses (Oddo et al., 1998; Winau et al., 2006; Rodrigues et al., 2009; Divangahi et al., 2010). The mechanism of human macrophage death during Mtb infection has not been fully elucidated. The mechanism mediating Mtb-infected macrophage death is of critical interest, as the mode of cell death can have a profound effect on the immune response of bystander cells (Liu et al., 2002; Griffith et al., 2007; Li et al., 2009; Chong et al., 2010), which could potentially be harnessed in the development of improved TB vaccines (Behar et al., 2011). A number of vaccines which cause increased cell death have been proposed for the prevention of TB and demonstrate enhanced immunogenicity and efficacy *in vivo* compared with the current BCG vaccine (Hinchey et al., 2007; Gartner et al., 2008; Ranganathan et al., 2009; Hinchey et al., 2011) Chapter 3 of the current work investigated the involvement of the intrinsic apoptosis pathway and parthanatos in Mtb-induced human macrophage death. Additionally, the ERK1/2 and the p38 MAPK pathways were examined as potential signalling pathways mediating the proposed pro-survival activity of IL-10 on macrophage viability during Mtb infection. Although IL-10 had previously been implicated in the maintenance of macrophage viability during Mtb infection, this was not observed in the current work (Balcewicz-Sablinska et al., 1998). Though both the ERK1/2 and the p38 MAPK pathway have been associated with the control of macrophage viability during mycobacterial infection (Bhattacharyya et al., 2003; Wojtas et al., 2011), neither was involved in maintenance or reduction of Mtb-infected macrophage viability in the present study. This may be due to differences in experimental species: both of these studies were performed with murine macrophages, while the present work utilised human macrophages. Certain responses differ between human and murine macrophages – such as the anti-mycobacterial

activity of nitric oxide, which is necessary for immunity against Mtb in mice (Chan et al., 1992; Chan et al., 1995), but appears to be dispensable in human Mtb defence (Thoma-Uszynski et al., 2001) – and this may account for the lack of involvement of the p38 and ERK1/2 pathways in the viability of human Mtb-infected macrophages observed in this study.

Macrophage death during Mtb infection was not mediated by either the intrinsic apoptosis pathway or by parthanatos. The retention of the mitochondrial death proteins (AIF and cytochrome *c*) within the mitochondria of macrophages during Mtb infection, together with the fact that Mtb-induced macrophage death is caspase-independent (O'Sullivan et al., 2007), also indicates that this form of macrophage death is not pyronecrosis (which is dependent on the activity of caspase 1) (Cookson and Brennan, 2001). The mechanism mediating macrophage death during Mtb infection, therefore, remains to be determined. As the type of cell death undergone by macrophages plays a vital role in the immune response and mycobactericidal activity during Mtb infection (Molloy et al., 1994; Divangahi et al., 2009; Rodrigues et al., 2009), a full understanding of macrophage cell death events and mediators may be beneficial in developing new targeted treatments. Necroptosis, a caspase-independent form of programmed necrosis, remains to be investigated in the context of Mtb-induced macrophage death. Loeuillet et al. (2006) demonstrated that Mtb can inhibit caspase activity, which correlates with the findings of O'Sullivan et al. (2007) that macrophage cell death following Mtb infection is caspase-independent. These reports, and the current work demonstrating that Mtb-induced macrophage death occurred without involvement of either parthanatos or the intrinsic apoptosis pathway, suggest that necroptosis represents a potential non-apoptotic pathway mediating macrophage death during Mtb infection.

Mtb H37Ra is an attenuated laboratory strain and, as such, not one which ordinarily causes human disease. It would be advantageous to replicate the experiments reported in Chapter 3 using the related virulent H37Rv strain, or a clinical Mtb strain. Given that the genomes of H37Ra and H37Rv differ, as do those of the laboratory and clinical Mtb strains, the macrophage death induced by strains other than H37Ra may differ from that reported herein (Kinger and Tyagi, 1993; Brosch et al., 1999; Mostowy et al., 2004; Gao et al., 2005; Li et al., 2010). The genome of Mtb encodes a number of genes whose products actively suppress macrophage death and maintain bacillary intracellular survival, such as *nuoG*, *secA2* and *pknE* (Kurtz et al., 2006; Velmurugan et al., 2007; Kumar and Narayanan, 2011). The activity of these proteins is likely to have a significant effect on the cell death phenotype of Mtb-infected cells. Macrophage death in response to Mtb infection is morphologically and biochemically

distinct from many defined forms of death, perhaps due to inhibition of multiple death signalling pathways by Mtb.

Dendritic cells (DCs) can also be infected by Mtb (Henderson et al., 1997). In contrast to macrophages, there is a dearth of information regarding the viability of DCs during Mtb infection and the consequences of DC death on the viability of intracellular Mtb. DC death induced by Mtb infection could impair DC migration and T cell priming (Wolf et al., 2007). As the viability of DCs may be important during Mtb infection, it was studied in Chapter 4. The observed DC death was characterised by assessment of nuclear fragmentation, DNA fragmentation and caspase activity. The viability of Mtb within infected DCs was also investigated to assess the mycobactericidal capacity of human DCs, which is the subject of conflicting reports within the literature (Fortsch et al., 2000; Bodnar et al., 2001; Tailleux et al., 2003). Human DC infection with live Mtb led to maturation and a tendency towards increased cytokine production. DCs underwent non-apoptotic death during infection with live (but not dead) Mtb. The executioner caspases 3 and 7 did not play a role in Mtb-induced DC death. DC death during Mtb infection displayed similarities to Mtb-induced macrophage death (O'Sullivan et al., 2007), being caspase-independent, without nuclear fragmentation and featuring DNA fragmentation. DC death was not mycobactericidal. This was perhaps due to *in vitro* culture of DCs in isolation, without lymphocytes which potentiate APC anti-bacterial activity by releasing IFN-γ (Bodnar et al., 2001), or it is possible that DC function does not include bacterial killing during Mtb infection.

The mode of human DC death during Mtb infection requires further characterisation. While the present work identified Mtb-induced DC death as caspase-independent, the initiating and effector molecules involved in mediating this death remain unknown. It is unlikely that death of DCs during Mtb infection is mediated by caspase 1-dependent pyroptosis (as pan-caspase inhibition during the present study failed to reduce cell death), or by pyronecrosis (as DNA fragmentation, observed in the current work, is absent from this form of cell death). A number of other caspase-independent death pathways remain to be investigated in Mtb-induced DC death. Although there is currently insufficient data to indicate that Mtb-induced DC death occurs via a mitochondrial pathway, parthanatos could be assessed (as performed in macrophages in Chapter 3) by identifying the sub-cellular localisation of cytochrome *c* and AIF in Mtb-infected DCs. Release of mitochondrial death proteins, together with an absence of PARP-1 cleavage (detected by Western blotting) during death of Mtb-infected DCs would confirm a role for parthanatos. Additionally, RIP1 and RIP3 activity could be assessed during Mtb-induced DC death; their activity would indicate necroptosis as the mode of cell death. It also remains to be seen whether DC infection with the virulent Mtb H37Rv strain, which caused human DC death at a faster rate than H37Ra, leads to

death in a similar caspase-independent manner and this should be addressed in future work. Although further work is required to clarify the biochemical pathways involved in (or excluded from) Mtb-induced DC death, it is possible that this non-apoptotic form of cell death is influenced, in part, by the manipulation of host death signalling by Mtb.

Mtb infection poses a significant threat to individuals with HIV (World Health Organisation, 2011). HIV infection has been shown to facilitate increased growth of *Mycobacterium avium* in human DCs, compared with DCs unexposed to HIV (Salte et al., 2011). The disruption of immune function associated with HIV appears to lead to altered DC responses to mycobacterial infection. As HIV-infected DCs may respond differently to Mtb infection compared with non-HIV-infected DCs, it may be advantageous to assess the mechanism of cell death and mycobactericidal capacity during Mtb infection of DCs exposed to HIV, or DCs derived from HIV-positive donors.

Cell death also has a downstream effect on the activation and immune response of bystander cells, such as DCs and T cells. Death under physiological homoeostatic conditions tends to promote a tolerant, non-reactive response in bystander immune cells (Liu et al., 2002). In contrast, death of infected cells, which contain bacterial PAMPs, can elicit an immunogenic reaction in neighbouring DCs and the T cells they subsequently encounter (Gallucci et al., 1999; Salio et al., 2000; Torchinsky et al., 2009). Mtb itself is known to promote immune tolerance, most likely in order to inhibit host-protective inflammatory immune responses (Korf et al., 2006; Liu et al., 2010). It is known that macrophage death occurs within the tuberculosis granuloma (Keane et al., 1997; Seimon et al., 2010), but the DC and T cell activation and polarisation responses to macrophage death in the setting of Mtb have not previously been reported. Experiments detailed in Chapter 5 aimed to assess the contribution of human macrophage death to the T cell responses to Mtb infection. The cytokine release of co-cultures of bystander DCs with Mtb-infected, dying macrophages was assessed; together with the cytokine secretion from subsequent co-cultures of these DCs with T cells. The activation and proliferation of the T cells was investigated in parallel experiments. Similar to death of DCs directly infected with Mtb in the previous chapter, live Mtb bacilli were required to cause macrophage death. Exposure to dying Mtb-infected macrophages, but not sterile apoptotic macrophages, led to DC maturation and cytokine production within co-cultures. Co-culture of the DCs exposed to live Mtb-infected macrophages with allogeneic T cells resulted in T cell activation, proliferation and secretion of T_H1 and T_H17 cytokines. In contrast, DCs exposed to apoptotic macrophages did not induce proliferation or cytokine secretion above baseline levels. Killed Mtb did not induce macrophage death and DCs exposed to macrophages infected with killed Mtb induced a lower activation, proliferation and secretion of a number of inflammatory cytokines from T cells than DCs cultured with

live Mtb-infected macrophages. This study represents the first assessment, both in human cells and in Mtb infection, of the contribution of macrophage death to the maturation of DCs and cytokine release profile of innate immune cells, and the consequences of the interaction of DCs with dying, Mtb-infected macrophages on subsequent T cell activation, proliferation and polarisation. There are limitations to the current work, however, and some of these could be addressed in future studies.

It was not possible in the present study to separate the contributions of macrophage death vs. the presence of live, replicating Mtb bacilli to the T cell responses to Mtb infection, as macrophage death could not be inhibited. This issue could potentially be addressed through the generation of a non-replicating Mtb mutant; initially assessing whether it was capable of inducing macrophage death in a manner similar to wild-type Mtb and, if so, its subsequent use as a control for macrophage death in the absence of Mtb proliferation. A similar limitation was presented by the use of streptomycin-killed Mtb as a control for the presence of mycobacterial PAMPs in the absence of macrophage death. As killed bacilli did not replicate, the level of PAMPs within macrophages infected with killed Mtb remained stable throughout the duration of infection. However, macrophages infected with live Mtb were likely to contain comparatively higher concentrations of PAMPs than killed Mtb-infected macrophages, due to active replication of the live Mtb bacilli. Therefore, it is possible that variation in the concentration of PAMPs could account for differences in the DC and T cell responses to live and killed Mtb-infected macrophages. As above, the inclusion of an engineered non-replicating strain of Mtb could potentially control for this variation.

Although the current work identified the cytokines released from DC-T cell co-cultures, T cell polarisation could be more comprehensively investigated by specific flow cytometric detection of intracellular T cell cytokines. The experiments detailed in Chapter 5 could also be performed using the virulent H37Rv strain of Mtb. Rivera-Ordaz and colleagues recently identified differences in the cytokine secretion profiles of human macrophages in response to infection with virulent laboratory and clinical Mtb strains, revealing variation in cytokine responses dependent on the strain of Mtb involved in infection (Rivera-Ordaz et al., 2011). It is possible that the T cell responses observed in the present study would also vary according to the strain of Mtb. These experiments were carried out using total $CD2^+$ T cells. The activation, proliferation and cytokine release from $CD4^+$ and $CD8^+$ T cell populations could also be assessed, in addition to the total T cell responses reported here. As the human alveolar macrophage (AM) is the most representative *in vitro* model of Mtb macrophage infection, it would be of interest to replicate the experiments detailed in Chapter 5 using human AMs. In spite of these limitations, the demonstration in this study that maximum immunogenic T cell responses to Mtb infection were induced by a

combination of live Mtb infection and macrophage death supports research into vaccines that induce cell death for maximally efficient immune responses.

6.1 Translational benefits of the current work

Tuberculosis represents a major global public health burden. There is currently no completely effective vaccine against the disease, and drug-resistant Mtb strains are emerging. A greater understanding of the mechanisms by which this organism establishes chronic infection is vital to the development of improved treatments and vaccines. The present study contributes to the knowledge of the interaction between Mtb and the immune system (specifically, the mediators and mechanisms involved in macrophage and DC death induced by Mtb, and the contribution of macrophage death to the T cell response to Mtb infection) and could potentially lead to more targeted therapies for tuberculosis treatment. DCs are being investigated as vaccine adjuvants and clarification of both the role of DCs during Mtb infection, and of the effect of Mtb on DCs, may result in the development of DC-based vaccines for the prevention of tuberculosis. Similarly, the identification in the current work of the enhanced immunogenicity of macrophages infected with live Mtb, which causes macrophage death, indicates that research into the development of a TB vaccine capable of causing macrophage death may prove fruitful.

6.2 Research studies in the societal context

The overarching aim of translational research is ultimately to benefit society. It is therefore important for funding agencies and research groups to seek input from regions and groups affected by a disease under study (in this instance tuberculosis) and to allow their requirements to exert some influence on the direction and focus of the research. In order to maximise the impact of research and lead to its translation to improved treatments and vaccines, communication should be fostered between scientists, patient groups, medical professionals, research groups and funding agencies. Research findings must be communicated appropriately to all interested parties and in this regard, a portion of the current work (Chapter 4) has been published and is available online as a peer-reviewed, open-access article. Novel findings in tuberculosis research are reported in the media, reflecting a growing public realisation that tuberculosis remains a global health concern (British Broadcasting Corporation, 2010a; English, 2011; Houston, 2011). The results of tuberculosis vaccine research, in particular, are frequently reported (British Broadcasting Corporation, 2010b, 2011b, 2011a; Gallagher, 2011).

Targeted research can be used to further TB prevention programmes. Inappropriate use of drug regimens can lead to the development of drug-resistant Mtb strains (World Health Organisation, 2007). Monitoring of the prescribed drug combinations and publication of best practice regimens is advisable to maintain the efficacy of the drugs currently available to treat Mtb infection and prevent escalation of Mtb drug resistance. Advances in TB vaccines and treatments must be targeted to both HIV-negative and HIV-positive populations, as Mtb infection poses a particular risk to those living with HIV, and Mtb-HIV co-infection comprises a large proportion of Mtb-induced morbidity and mortality (World Health Organisation, 2011). The altered and compromised immune system associated with HIV infection must be taken into account during the research and development of new TB vaccines and treatments, and these should be tailored to specifically address the response of the immune system to Mtb infection during HIV co-infection. It may be beneficial to assess the responses of HIV-infected macrophages, DCs and T cells in experiments similar to those reported in the present work. Treatments and vaccines should also be affordable to those communities most affected by tuberculosis, the greatest proportion of which is in developing countries.

6.3 Intellectual property

There is no intellectual property currently arising from this work.

6.4 Concluding remarks

This study has furthered the characterisation of Mtb-induced macrophage death and has been the first to describe both the viability of DCs during Mtb infection and some morphological and biochemical features of Mtb-induced DC death. It has also revealed the immunogenicity of macrophage death during live Mtb infection in the context of its downstream effect on DC and T cell responses to Mtb infection. Future studies will aim to determine whether these findings, observed (in the main) during attenuated Mtb infection, also apply to infection with virulent Mtb. The observation that macrophage infection with live Mtb and consequent cell death may contribute to T cell responses via DCs could have important implications for the development of future tuberculosis vaccines and treatments.

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