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The role of Mal in macrophage responses to tuberculosis

Thesis submitted to the

University of Dublin

For the

Degree of Doctor of Philosophy

By

Clíona Maire Ní Cheallaigh

School of Medicine

Trinity College

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Ireland

September 2014

Declaration of Authorship

This thesis is submitted by the undersigned to the University of Dublin for the examination of Doctorate of Philosophy. The work herein is entirely my own with the exception of the following figures:

- Figure 4.3
- Figure 4.4
- Figure 4.8
- Figure 4.13
- Appendix 2
- Appendix 4

The work described in the above figures was carried out by Dr. Jim Harris and Dr Sarah Jones, Monash University, Melbourne, Australia (Figure 4.3), Dr Fred Sheedy, Trinity College Dublin (Figure 4.4, Figure 4.8, Appendix 2), Dr Michelle Coleman, Trinity College, Dublin (Figure 4.13) and Dr Brian Monks, Univeristy of Massacheusetts (Appendix 4).

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I would like to dedicate this thesis to my family and to the patients who have taught me so much.

Abstract

Heterozygotes for the common S180L polymorphism in the TLR adaptor Mal (TIRAP) are protected from a number of infectious diseases, including tuberculosis (TB), whereas homozygotes are at increased risk. We report that Mal has a hitherto unknown TLR-independent role in interferon-gamma receptor (IFNγR) signaling. This novel Mal-dependent IFNγR signalling pathway leads to MAPK p38 phosphorylation and autophagy. IFN-γ signalling via Mal is is required for phagosome maturation and killing of intracellular *M. tuberculosis.* The S180L polymorphism and its murine equivalent reduce Mal's affinity for the IFNγR thereby compromising IFNγR signaling in macrophages and impairing *in vitro* and *in vivo* responses to TB. These findings highlight a new role for Mal outside the TLR system and imply that genetic variation in Mal may be linked to other IFN-γ related diseases including autoimmunity and cancer.

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Abbreviations

BCG	Bacille Calmette-Guerin
BMDM	bone marrow derived macrophages
BSA	bovine serum albumin
CLR	C-type lectin domain-like containing receptor
CpG	methylated cytosine-guanine DNA
DAMP	Damage-associated molecular pattern
DC	dendritic cell
EDTA	Ethylenediaminetetracetic acid
EEA1	Early endsomal antigen
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal regulated kinase
FCS	Foetal calf serum
h	hour(s)
HIV	Human Immunodeficienct Virus
iBMM	Immortalised bone marrow-derived macrophages
IFN	Interferon
IFNγR	Interferon-γ receptor
Ig	Immunoglobulin
IκB	Inhibitor of NFκB
IKK	IκB kinase

IL	Interleukin
IL-1R I	IL-1 receptor type I
iNOS	inducible nitric oxide synthase
IPAF	ice protein activating factor
IRAK	Interleukin 1 receptor associated kinase
IRF	Interferon Response Factor
JNK	c-jun-N-terminal kinase
LC3	Microtubule-associated light chain protein 3
LTA4H	Leukotriene A4 hydrolase
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
M. tuberculosis	*M. tuberculosis*
Mtb	*M. tuberculosis*
Malp-2	Macrophage-activating lipoprotein-2kDa
MAPK	Mitogen activated protein kinase
Mal	MyD88 adaptor like
MARCO	Macrophage receptor with collagenous structure
MDM	monocyte-derived macrophage
MDR-TB	Multi-drug resistant tuberculosis
min	minute(s)
MR	Mannose receptor
mRNA	messenger RNA

mTOR	molecular target of rapamycin
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa B
NK	Natural killer
NLP3	Nod-like receptor protein
NLR	Nod-like receptor
NOD	Nucleotide-binding oligomerisation domain
PAGE	Polyacrylamide gel electrophoresis
pam3CSK4	$pam_3CysSerLys_4$
PAMP	Pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p(I:C)	polyinosinic:polycytidilic acid
PIP	phosphoinositol-3-phosphate
PMA	phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsuphonylflouride
PRR	Pathogen recognition receptor
PVDF	Polyvinylidene diflouride
RLR	RIG-I like receptor
SARM	SAM and ARM-containing protein
SDS	Sodium Dodecyl Sulphate

siRNA	small interfering RNA
SLE	Systemic lupus erythematosis
SNP	Single nucleotide polymorphism
SOCS	Suppressor of Cytokine Signalling
SR	Scavenger receptor
STAT-3	signal transducer and activator of transcription-3
TAK	TGFβ-activated kinase
TB	Tuberculosis
TBK-1	Tank binding kinase -1
TEMED	N, N, N', N', - Tetramethylethylenediamine
TGF-β	tumour growth factor-β
Th	T helper
TIR	Toll-IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRIF	TIR domain-containing adaptor inducing IFN-beta
TRAF	TNF-receptor associated factor
TRAM	TRIF-related adaptor protein
WT	Wild-type
XDR-TB	Extensively drug-resistant tuberculosis

Chapter 1 : General Introduction

1.1 Tuberculosis

1.1.1 The history of tuberculosis

1.1.1.The history of tuberculosis (TB) is entwined with the history of human evolution. It is thought that *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of TB, has infected humans since the expansion of "modern" human populations out of Africa 40,000 years ago (Wirth, Hildebrand et al. 2008). *M. tuberculosis* infection has been confirmed by morphological and molecular methods in a population living in one of the first villages established approximately 9,000 years ago (Hershkovitz, Donoghue et al. 2008). An epidemic of tuberculosis in Europe starting in the 17th century lasted more than 200 years, earning it the title "The Great White Plague". Until the decline in the incidence of TB in the 20th century, TB killed approximately one in seven adults in Western Europe, leaving deep traces on our culture, history and genetic makeup.

1.1.2 Tuberculosis epidemiology

Tuberculosis kills more people world-wide than any other bacterial infection. There were 8.7 million new cases of tuberculosis in 2011 (World Health Organisation 2009). The death of 1.4 million people from tuberculosis in 2011 highlights the fact that we are far from controlling the disease. Drug- resistant TB has emerged over the past twenty years and represents an extremely worrying development. The number of drug-resistant cases of TB continues to grow, with an estimated 310,000 cases of multi-drug resistant tuberculosis, i.e. tuberculosis which is resistant to the two front-line anti-tuberculous antibiotics: isoniazid

and rifampicin, in 2011. In some parts of Eastern Europe, up to 50% of TB cases are multi-drug resistant (MDR-TB) (World Health Organisation 2010). World-wide, almost one in four cases of MDR-TB results in death (World Health Organisation 2010). Patients with multi-drug resistant tuberculosis are more costly to treat, remain infectious for longer, and have a greatly increased rate of mortality (World Health Organisation 2010). Recent years have seen the development of extensively drug-resistant tuberculosis (XDR-TB), a subset of multi-drug resistant tuberculosis which has acquired additional resistance to the most effective second-line TB antibiotics: fluoroquinolones and any of the three injectable agents: amikacin, capreomycin and kanamycin. Mortality rates with extensively drug-resistance tuberculosis in HIV-infected individuals have been reported to be as high as 98% (Gandhi, Moll et al. 2006).

1.1.3 Tuberculosis pathophysiology

Tuberculosis infections are acquired when an individual inhales droplets containing *M. tuberculosis*, which have been coughed up by someone with active pulmonary TB disease. Three outcomes of infection are possible: 1) elimination of infection, 2) containment of the infection as latent tuberculosis infection (LTBI) or 3) development of active tuberculosis disease (Figure 1). It is estimated that 10-20% of exposed individuals eliminate the infection (Morrison, Pai et al. 2008). The majority of those who do not eliminate the infection will develop latent tuberculosis: these individuals are asymptomatic and non-infectious, carrying about 100-1000 bacteria which are in a semi-dormant state (Figure 2). It is estimated that one-third of the world's population has latent tuberculosis. Individuals with latent tuberculosis are estimated to have an approximately 5-10% life-time risk of losing control of the infection and developing active TB, although a recent study from the

US estimated this as a much lower risk of 0.04 cases per 100 person years (Horsburgh, O'Donnell et al. 2010).

Those with active tuberculosis have a variety of clinical presentations. Systemic symptoms common to all sites of infection include fever, weight loss and night sweats, and are consistent with the systemic effects of pro-inflammatory cytokines such as TNF-α and IL-1β. The most common site of disease is the lungs, and symptoms of pulmonary TB include persistent cough which is usually, but not always, productive of sputum, which may be blood-stained. Extensive destruction of lung tissue may result in catastrophic haemoptysis. Extra-pulmonary disease is present in 10-42% of patients (Golden and Vikram 2005). The most common site of extra-pulmonary disease is the pleura, but TB disease can also affect the meninges, the kidneys, the peritoneum and bones including the spine. The degree of inflammation in response to infection varies greatly: some patients have minimal fever and inflammation with a high bacillary burden, whereas other patients may have a profound inflammatory response to a low bacillary burden. The immunological factors that determine the outcome of infection and degree of inflammatory response in those with active infection remain to be fully determined.

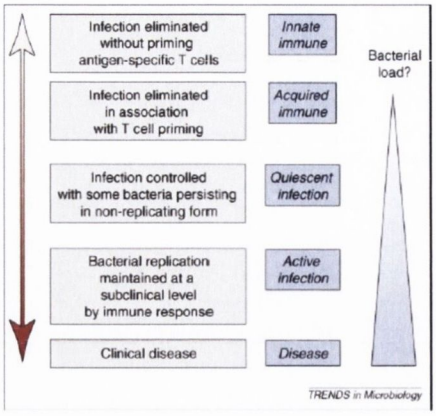


Figure 1-1 The spectrum of responses to tuberculosis infection.

Host outcomes in tuberculosis infection range from elimination of infection to clinical disease with a high bacterial burden. *Reprinted from Wilkinson, Trends in Microbiology 2008.*

1.1.4 What is needed to eliminate tuberculosis and how immunology can help

A more effective TB vaccine is needed to achieve global TB elimination. An ideal TB vaccine could be used not only to prevent infection, but also to treat latent TB. The current TB vaccine is a live attenuated stain of *M. bovis*: bacille Calmette Guerin (BCG). BCG has variable efficacy, depending on which population it is used in (Dockrell, Smith et al. 2012), and is estimated to be only 50% effective in preventing tuberculous disease (Colditz, Brewer et al. 1994). BCG is not useful as a therapeutic vaccine to promote the elimination of latent infection. Newer candidate vaccines have not, so far, been effective in preventing infection despite, in some cases, generating antigen-specific multi-functional T-cell responses (Meyer and McShane 2013, Tameris, Hatherill et al. 2013). One of the main barriers in designing an effective vaccine is the lack of predictive animal models and biomarkers of TB vaccine efficacy. This prevents rational vaccine selection at a pre-clinical stage and necessitates prolonged and expensive clinical efficacy trials in target populations. Newer models for assessing efficacy include human whole blood assays

(Fletcher, Tanner et al. 2013, Brookes, Hakimi et al. 2014) and using BCG as a measure of immunity (Harris, Meyer et al. 2013). An improved understanding of what constitutes protective immunity would facilitate vaccine development (Meyer and McShane 2013).

Current TB diagnostics also need improvement. The gold-standard method of detection of active tuberculosis remains culture of mycobacteria, which is slow and inaccurate. The recently developed GeneXpert PCR method is quicker, but also has limited sensitivity in smear-negative and extrapulmonary cases (Zeka, Tasbakan et al. 2011). Improved diagnostics would facilitate quicker identification of individuals with active and latent infection, and are needed to control the TB pandemic (Dorman 2010). Panels of biomarkers looking at immunological parameters have been found to be of promise, although trying to identify individuals with non-pulmonary or subclinical disease remains challenging (Berry, Graham et al. 2010, Hur, Gorak-Stolinska et al. 2013, Kaforou, Wright et al. 2013, Naranbhai, Hill et al. 2013, Brookes, Hakimi et al. 2014, Hur, Crampin et al. 2014). As in the case of vaccines, an improved understanding of TB immunology could lead to more accurate diagnostic markers. These markers could also be used to access the efficacy of new anti-tuberculous agents (Cliff, Lee et al. 2013).

Current antibiotic regimens for drug-sensitive tuberculosis are lengthy (6-12 months) and consist of a minimum of three antimycobacterial agents. Side effects are common, and up to half of patients fail to adhere to this protracted course of treatment. Rifampicin, which forms the backbone of TB treatment, has numerous drug interactions, and is particularly problematic when using antiretroviral agents to treat HIV (Cuneo and Snider 1989). Drugs used to treat multi-drug resistant tuberculosis are extremely toxic, costly and are usually given for up to two years with limited success. Adjunctive immunotherapy could potentially boost the compromised host immune response and shorten therapy and/or

reduce the need for drugs with side-effects (Ni Cheallaigh, Keane et al. 2011). This could also enable the use of rifamycin-sparing regimens, which would not affect HIV medications. In this context, inhaled interferon gamma (IFN-γ) has been trialled as an adjunctive immunotherapy but with limited success (Suarez-Mendez, Garcia-Garcia et al. 2004).

Paradoxically, when tuberculosis is treated, patients' symptoms may worsen, possibly due to increased pro-inflammatory responses to dead mycobacteria (Malik, Thompson et al. 2003, Sly, Hingley-Wilson et al. 2003). This "paradoxical reaction" can cause serious clinical complications, such as compression of the airways in patients with tuberculosis in neck lymph nodes. The inflammatory response to *M. tuberculosis* is particularly problematic in patients with TB meningitis, in whom it can cause stroke and death. Steroids are used to treat paradoxical reaction and TB meningitis, but are not very effective (2011). Immunomodulatory treatment could possibly promote the clearance of dead mycobacteria, and thereby reduce the overactive inflammatory response. An improved understanding of TB immunology may also enable clinicians to target treatment to host inflammatory genotype with improved clinical outcomes (Tobin, Roca et al. 2012).

Latent TB infection (LTBI) represents a reservoir from which active disease and subsequent transmission can propagate, particularly when the immune system is compromised. It is estimated that rates of LTBI in the community need to be less than 1% to allow TB elimination (Styblo 1990). At present, there are no accurate tests to predict which of the 2 billion individuals with LTBI will fail to contain the infection and progress to active tuberculosis. Improved understanding of host susceptibility factors could potentially enable individual testing for genotypes associated with a reduced risk of active tuberculosis, such as IRGM-261T (Intemann, Thye et al. 2009), LTA4H (Tobin,

Vary et al. 2010) and Mal S180L (Khor, Chapman et al. 2007) which could enable clinicians to target treatment for LTBI to patients at a higher risk of progression.

1.1.5 Bacterial factors determine the outcome of infection with M. tuberculosis

Bacterial characteristics specific to individual strains of *M. tuberculosis* have been shown to play a role in determining the outcome of infection. Extensively transmissible strains of TB such as CDC 1551 have been reported to result in an unusually high number of contacts developing latent and active infection (Valway, Sanchez et al. 1998). The Beijing strain's world-wide spread is thought to be due to increased virulence (resulting in more cases of active disease) and possibly infectivity as reviewed by Malik and colleagues (Malik and Godfrey-Faussett 2005). Certain strains of isoniazid-resistant *M. tuberculosis* also seem to be more likely to result in infection (Gagneux, Burgos et al. 2006). Other species of mycobacteria such as *M. africanum* are equally likely to transmit but less likely to cause infection (de Jong, Hill et al. 2008).

The number of bacteria or inoculum to which an individual is exposed also affects the outcome of infection, as evidenced by the fact that both close contact with the index case and a higher number of bacteria in sputum in index cases with sputum smear positive TB result in a higher proportion of contacts developing active TB (Musher 2003). This was illustrated by the case of a US sailor with active pulmonary TB on a navy vessel for six months in 1965: 6 of his berth mates developed active TB while the remainder of his berth mates developed LTBI, whereas none of those who were in different berths but shared an air system with the index case developed active TB, although some developed LTBI. None of those who did not share an air system with the index case developed LTBI (Houk, Baker et al. 1968).

1.1.6 Host factors determines the outcome of infection with M. tuberculosis

Host immune responses also determine the outcome of TB infection. A number of diseases, most particularly HIV, and medications, particularly anti-TNF agents, increase host susceptibility to TB. In addition, other, poorly understood host characteristics also affect susceptibility.

HIV increases the risk of re-activation of latent tuberculosis infection (LTBI) from approximately 0.04 cases per 100 person years (Horsburgh 2004) to more than 10 per 100 person years (Selwyn, Hartel et al. 1989, Holmes, Wood et al. 2006, Komati, Shaw et al. 2010). Tuberculosis is the most common opportunistic infection in HIV-infected individuals and is responsible for one-third of HIV-associated deaths (Raviglione, Dye et al. 1997). HIV infection has a number of effects on host immune responses to TB. Depletion of CD4+ T-cells is the hallmark of HIV infection, and HIV has a particular predilection for CD4+ T-cells specific for *M. tuberculosis* (Geldmacher, Ngwenyama et al. 2010), which are depleted rapidly after HIV infection. The importance of CD4+ T-cells to TB immunity is highlighted by the dramatic increase in active TB disease associated with reduction in CD4+ T-cell counts (Getahun, Gunneberg et al. 2010) and by the increased incidence of severe extrapulmonary TB in those with low CD4+ T-cell counts (Leeds, Magee et al. 2012). Antigen-specific CD4+ T cells respond to *M. tuberculosis* by secreting IFN-γ and TNFα, which are critical in macrophage responses to tuberculosis and to granuloma formation (Cooper, Dalton et al. 1993, Flynn, Chan et al. 1993, Keane, Gershon et al. 2001, Lin, Myers et al. 2010). HIV also depletes CD4+ T-cells in the granuloma, impairing granuloma function (Diedrich, Mattila et al. 2010). The clinical observation that HIV-infected individuals with CD4+ T-cell counts in the normal range also display markedly increased susceptibility to TB,

although not to the same degree as those with advanced CD4+ depletion, led to an assessment of the impact of HIV infection on the innate immune system. Recent work has shown that HIV-infected macrophages are impaired in a number of anti-mycobacterial responses, including apoptosis (Patel, Zhu et al. 2007) and autophagy (Van Grol, Subauste et al. 2010).

Anti-TNF agents have revolutionised the treatment of a number of inflammatory conditions, including rheumatoid arthritis and Crohn's disease. However, their use is associated with an increased risk of active tuberculosis, which is often disseminated and clinically difficult to diagnose (Keane, Gershon et al. 2001). These agents affect anti-mycobacterial immunity in a number of ways: they impair macrophage functions including phagosome maturation and apoptosis as well as cell-mediated immune functions including IFN-γ secretion by memory T-cells (Harris, Hope et al. 2008, Harris and Keane 2010).

Other host factors that increase the risk of developing active tuberculosis and/or increase disease severity include diabetes, smoking (Chan, Keane et al. 2010, Reed, Choi et al. 2013) and extremes of age. Ethnicity also affects immune responses to tuberculosis (Stead, Senner et al. 1990), with lymph node tuberculosis being more common in those from the Indian subcontinent and genitourinary tuberculosis being more common in those of Caucasian ancestry. The mechanisms underlying these ethnic differences are not yet well established.

When these known susceptibility factors are accounted for, there remains a large amount of variability in host susceptibility to tuberculosis which is not understood. This is illustrated by the tragic events which occurred in Lubeck in 1929 and 1930 in which a

single virulent viable *M. tuberculosis* strain was inadvertently used instead of BCG to immunize 252 infants. Responses to the vaccine in these age- and ethnically matched individuals who were all inoculated with the same number and strain of bacilli ranged from death in 72 infants to recovery, arguing for a genetic basis for resistance to tuberculosis .

A number of lines of evidence indicate that part of host susceptibility is inherited, as reviewed extensively by Azad and colleagues (Azad, Sadee et al. 2012). Twin studies have demonstrated a heritable element (van der Eijk, van de Vosse et al. 2007). Genome-wide linkage studies (Bellamy, Beyers et al. 2000, Miller, Jamieson et al. 2000, Mahasirimongkol, Yanai et al. 2008) and genome-wide association studies (GWAS) (Thye, Vannberg et al. 2010, Png, Alisjahbana et al. 2012) provide further evidence of the impact of host genetics on susceptibility to TB. The genetic factors involved are multiple, and likely to interact with each other and with environmental and bacterial factors, making it difficult to tease out the importance of the various genetic factors (Stein 2011). A large number of these candidate genes are associated with innate immunity.

1.2 Innate immunity and tuberculosis

1.2.1 Innate immunity

The immune systems of vertebrates provide protection against microbes including bacteria, viruses and fungi while minimising inflammatory damage to the host. To this end, the immune system possesses two arms: innate and adaptive immunity. The innate immune response is the initial response to a pathogen. It utilises germline-encoded pattern recognition receptors (PRRs) which respond to various pathognomonic components of

pathogens called pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (O'Neill, Golenbock et al. 2013). PAMPs are slowly evolving components which are usually essential for the survival of the pathogen – e.g. cell wall components, RNA. PAMPs are shared by whole classes of either bacteria, fungi or viruses. For example, lipopolysaccharide (LPS) is a cell wall component found in all Gram negative bacteria which is recognised by TLR4 (Beutler, Du et al. 2001) .

The innate immune system is multifaceted, involving components including phagocytosis, cytokine production and complement activation. It also activates the adaptive immune system via antigen presentation and cytokine production. The adaptive immune response is specific to each antigen, and includes antibody and T-cell responses (Figure 3). The adaptive immune system uses antigen-specific receptors to identify pathogens. Billions of such receptors exist, each one specific for a particular antigen. The adaptive immune system responds differently to an antigen that it has previously encountered, and is therefore said to exhibit memory.

1.2.2 Innate immunity and tuberculosis

The innate immune system has been the subject of intense study over the last 20 years. Evidence continues to emerge highlighting the importance of the initial innate response to infection with *M. tuberculosis.* Upon initial inhalation of *M. tuberculosis*, alveolar macrophages ingest the bacteria. An optimal innate response involves the macrophages killing the intracellular bacteria, presenting antigen and producing cytokines to activate the adaptive immune system. If the macrophages fail to contain the infection, mycobacteria can replicate intracellularly and eventually kill the macrophage releasing large numbers of bacteria which can infect other cells. Infected macrophages initiate the

recruitment of new monocytes from the bloodstream, which can provide a continuous source of niche cells for the bacilli (Salgame 2011). Other innate cells with important roles in tuberculosis immunity include dendritic cells, which are present in the alveolar space and have been shown to phagocytose mycobacteria. DCs containing mycobacteria then travel to local lymph nodes and present antigen to T cells (Ryan, O'Sullivan et al. 2011) . Neutrophils are also present at sites of mycobacterial infection and can phagocytose and kill mycobacteria via the generation of reactive oxygen species (Yang, Cambier et al. 2012). In established disease, neutrophils contribute to immunopathology.

Animal models have higlighted the importance of immune responses as early as 3 hours post-infection (Subbian, Bandyopadhyay et al. 2013). At this early time point, bacteria can replicate logarithmically and a high bacterial burden can be established (van Crevel, Ottenhoff et al. 2002). Human studies have shown that polymorphism in genes for proteins involved in innate responses, including Toll-like receptors (Killick, Ni Cheallaigh et al. 2013) and the Vitamin D receptor (Bornman, Campbell et al. 2004), can affect susceptibility.

1.2.3 Adaptive immunity and tuberculosis

Two to three weeks after the initial infection, the adaptive arm of the immune system is activated. Antigen-specific T lymphocytes arrive, proliferate at the site of infection and activate macrophages to kill the intracellular mycobacteria. At this point, infection may become stationary or may progress. Granuloma form which are dynamic structures consisting of a large variety of innate and adaptive immune cells (Ehlers and Schaible 2012). In cases which progress, liquefied caseous foci may form which provide excellent

conditions for extracellular growth of *M. tuberculosis*. Cavities may form and rupture into nearby bronchi, allowing the bacilli to spread through the airways to other parts of the lung and the outside environment. Primary and secondary deficiencies in adaptive immunity, such as severe combined immunodeficiency (SCID) and HIV respectively, can greatly increase susceptibility to mycobacterial infection, including avirulent mycobacteria and disseminated tuberculosis.

Immunity to *M. tuberculosis* is reliant on a predominantly Th1-biased response, characterised by the localised secretion of IFN-γ, TNF-α and IL-12 (Salgame 2005), while Th2 responses in the lungs and periphery of patients, indicated by increased secretion of IL-4 and high antibody titres, have been associated with more severe disease (van Crevel, Karyadi et al. 2000, Mazzarella, Bianco et al. 2003). Infection with *M. tuberculosis* results in increased expression of mediators which counteract Th1 responses and promote Th2 responses (Almeida, Lago et al. 2009).

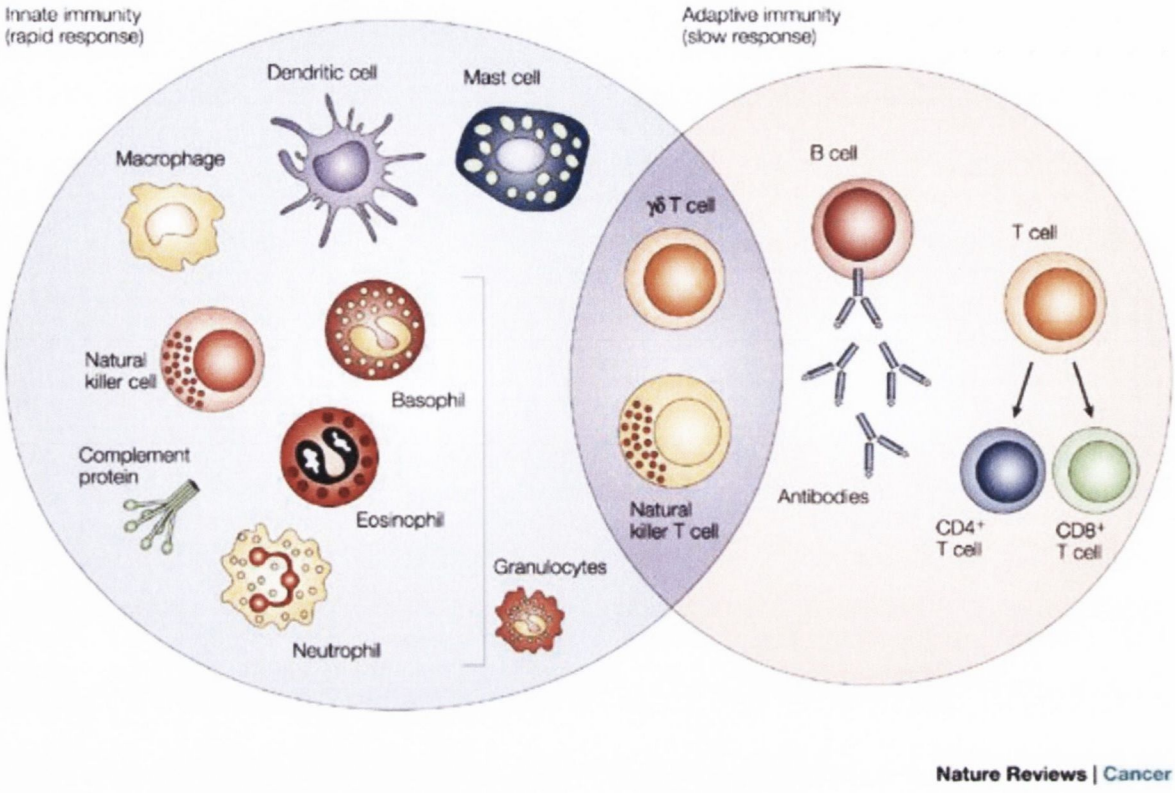


Figure 1-2: Innate and acquired immunity.

Innate immunity is a rapid, non-antigen specific response to pathogens or tissue damage involving macrophages, dendritic cells and other immune cells. Adaptive immune responses are delivered by T and B-lymphocytes and are antigen specific. *Reprinted from Dranoff,* Nature Reviews Cancer *, 2004.*

1.2.4 The granuloma

Granulomas are multi-cellular complex structures resulting from the interaction between the innate and the acquired immune system, which form in individuals with latent and active tuberculosis. Human TB granulomas are composed of a central mass of infected macrophages and neutrophils. These macrophages have responded to stimulation by differentiating into multinucleated giant cells, epithelioid cells and foamy macrophages containing lipid droplets. As the acquired immune system becomes activated, these cells are surrounded by CD4+ lymphocytes, CD8+ T cells and B cells, FoxP3+ regulatory T cells, NK cells, dendritic cells and by fibroblasts in a peripheral fibrotic capsule (O'Garra, Redford et al. 2013). The granuloma is thought to act as both an immunological and physical barrier to the spread of infection (Saunders and Britton 2007, Dockrell, Geluk et al. 2011), although it has recently been postulated, based on work in zebrafish, that the granuloma may function as a site of infection of new macrophages and dissemination of infection (Davis and Ramakrishnan 2009). The granuloma is likely a structure under delicate immunological control, requiring the localised production and secretion of pro-inflammatory cytokines to maintain macrophage activation, coupled with directed release of anti-inflammatory cytokines to prevent excessive immune responses.

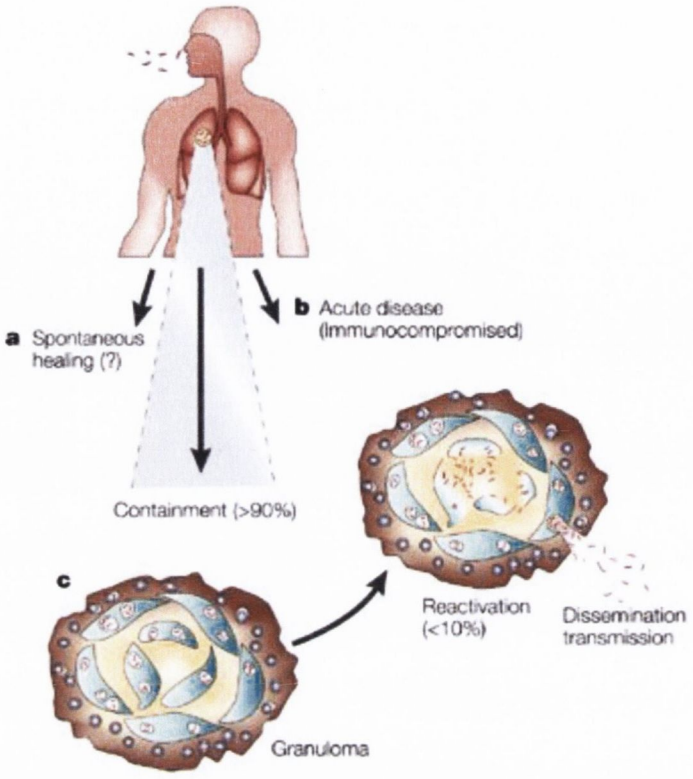


Figure 1-3: The Granuloma Contains Latent Infection.

More than 90% of individuals contain the infection, usually in a granuloma. 10% will develop acute disease. *Reprinted from Kaufmann,* Nature Reviews Immunology, 2001

1.2.5 Macrophages and tuberculosis

Alveolar macrophages (AM) are innate immune cells which comprise greater than 90% of all immune cells in a normal lung (Holt, Strickland et al. 2008) . AM are normally quiescent but can be highly phagocytic (Thepen, Claassen et al. 1993). Alveolar macrophages are the first cells to phagocytose *M. tuberculosis* after infection, and play a key role in secreting cytokines which polarise subsequent adaptive immunity to a beneficial Th1 or deleterious Th2 type response. They also carry out a number of key mycobactericidal functions including autophagy and phagosomal maturation, which if successful can kill intracellular mycobacteria (Deretic, Singh et al. 2006, Harris, Hope et al. 2009, Ni Cheallaigh, Keane et al. 2011). If these functions are unsuccessful,

macrophages can provide a niche cell in which *M. tuberculosis* can replicate. Macrophages can also contribute to immunopathology and they are pivotal in the formation of granulomas and the maintenance of latent disease (Ni Cheallaigh, Peral de Castro et al. 2013).

1.2.6 Phagosome maturation

After uptake by macrophages, *M. tuberculosis* resides in phagosomes. These may fuse with lysosomes or may remain immature. This is a highly complex process with multiple interactions with organelles, and is modulated by many mediators of the innate and acquired immune systems (Russell 2011). If phagosomes are successful in fusing with late endosomes and lysosomes (termed phagosome maturation), the mycobacteria contained in the phagosome are exposed to reactive oxygen species (ROS) and nitric oxide (NO). If phagosomes interact with endosomes but fail to fuse with lysosomes (Russell, Mwandumba et al. 2002), mycobacteria contained in the phagosome can access intracellular iron and replicate (Schaible, Collins et al. 2002). Phagosome maturation is controlled by the small GTP-binding proteins Rab5 and Rab7. Rab 5 interacts with phosphoinositol-3-phosphate (PI3P), which then recruits EEA1 to the membrane, which in turn recruits Rab7, which interacts with Rab7-interacting lysosomal protein and allows fusion with lysosomes (Ni Cheallaigh, Peral de Castro et al. 2013) (Figure 4).

The role of TLRs in phagosome maturation is controversial, with Blander and Medzhitov reporting that maturation of phagosomes containing *E. coli*, *S. typhimurium* and *S. aureus* was TLR 2 and 4 dependent (Blander and Medzhitov 2004); whereas David Russell and colleagues reported no effect of TLR stimulation on phagosome maturation, although phagosome maturation was decreased in MyD88$^{-/-}$ macrophages (Yates and Russell 2005).

TLR $2/4/9^{-/-}$ macrophages have been reported to show normal maturation of phagosomes containing *M. tuberculosis* but as in the case of the Russell study, this paper did report a defect in phagosome maturation in $MyD88^{-/-}$ macrophages (Holscher, Reiling et al. 2008).

A number of mycobacterial factors which prevent phagosome maturation have been identified, including mycobacterial cell wall components lipoarabomannan (LAM), which inhibits PI3P; secreted acid phosphatase (SapM), which dephosphorylates PIP3; and phosphatidyl myo–inositol mannoside (PIM) (Fratti, Backer et al. 2001, Vergne, Chua et al. 2005, Deretic, Singh et al. 2006) (Figure 4). IL-10, secreted in response to *M. tuberculosis* infection, also inhibits phagosome maturation (Via, Fratti et al. 1998, O'Leary, O'Sullivan et al. 2011). To counter these inhibitory factors, host cytokines including TNF-α, IL-1β and IFN-γ can activate the macrophage resulting in acidification of the phagosomes, killing of the mycobacteria and antigen presentation (Schaible, Sturgill-Koszycki et al. 1998, Via, Fratti et al. 1998, Harris, Hope et al. 2009). This occurs via a process tightly linked to phagosome maturation: autophagy.

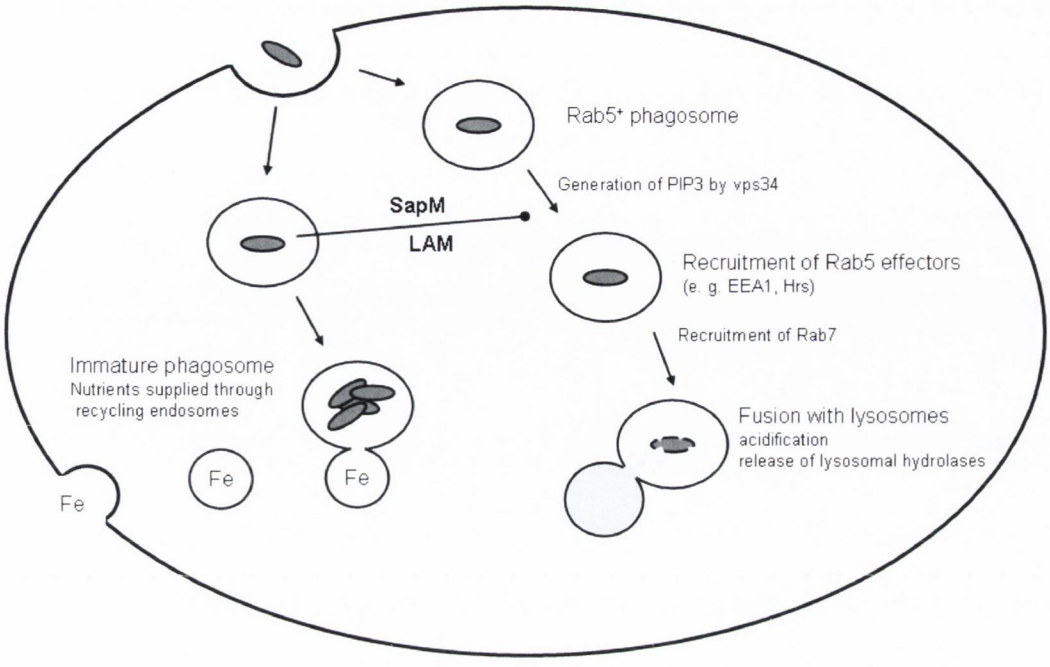


Figure 1-4: Phagosome maturation.

Normal phagosome maturation requires the recruitment of Rab5 to the phagosomal membrane, PIP3 generation, and recruitment of Rab5 effectors. Subsequent recruitment of Rab 7 allows fusion with lysosomes, acidification of the phagosome and release of lysosomal hydrolases into the lumen of the mature phagosome. *M. tuberculosis* subverts this process through the release of lipoarabinomannan (LAM) and the PI3P phosphatase secreted acid phosphatase M (SapM). *Reprinted from Harris, Clinical and Experimental Immunology, 2010.*

1.2.7 Autophagy

Autophagy is a highly conserved process for the delivery of long-lived cytosolic macromolecules and whole organelles to lysosomes for degradation. During starvation, autophagy acts as a cell survival mechanism, providing essential amino acids (Kuma, Hatano et al. 2004, Shintani and Klionsky 2004). Autophagy is also important for removing potentially harmful cellular constituents, such as damaged mitochondria, misfolded proteins or protein aggregates (Wong and Cuervo 2010) and intracellular pathogens (Deretic 2011, Levine, Mizushima et al. 2011). Three distinct types of autophagy have been described; microautophagy, in which cytosol is directly engulfed by lysosomes (Ahlberg, Marzella et al. 1982); chaperone-mediated autophagy, in which specific proteins are recognised by a cytosolic chaperone and targeted to the lysosome (Dice 1990); and macroautophagy (hereafter referred to as autophagy), in which an isolation membrane, or phagophore, fuses with itself to form an autophagosome with a distinctive double-membrane, which can then fuse with lysosomes (Shintani and Klionsky 2004).

Autophagy is regulated by a complex network of factors as detailed in a recent review by Choi and colleagues (Choi, Ryter et al. 2013) (Figure 5) Autophagosome formation happens in three stages (Harris 2011). The first stage is initiation of autosome formation, and occurs when starvation, rapamycin or other inducers of autophagy inhibit molecular target of rapamycin (mTOR), thereby inducing autophagy. Inhibition of mTOR leads to the translocation of a protein complex containing ULK1/2, Atg13, Atg101 and FIP-200 from the cytosol to the endoplasmic reticulum. This causes recruitment of a complex containing the type III phosphotidylinositol-3-kinase (PI3K) VPS34, Beclin 1 (Atg6), VPS15 and Atg14 (complex I). Initiation ends with the emergence of the isolation membrane. The second stage, or elongation, occurs when the isolation membrane

lengthens around its target. As part of this process, cytosolic LC3 I is lipidated to form LC3 II which is recruited to the autophagosome membrane. The third stage, or maturation, occurs when the autophagosome fuses with a lysosome, allowing the degradation of the contents of the autophagogosome. This process is also dependent on the complex containing PI3K VPS34 and Beclin 1. Akt signalling negatively regulates autophagy by activating mTOR in response to insulin and other growth-factor signalling. BCL-2 can inhibit Beclin-1 and therefore inhibit autophagy. Immune related GTPases (IRGMs) of which there are 20 in mice and only 1 in humans induce autophagy by translocating to mitochondria and regulating autophagy in association with mitochondrial fission (Singh, Davis et al. 2006, Singh, Ornatowski et al. 2010), although their role is humans is controversial (Bekpen, Hunn et al. 2005, Bekpen, Marques-Bonet et al. 2009). Dysfunction in autophagy can lead to cancer, neurodegenerative diseases such as Parkinsons and inflammatory bowel diseases (Mizushima, Levine et al. 2008).

Evidence is emerging that autophagy is responsible for a number of critical host immune responses to *M. tuberculosis* (Deretic 2008, Harris, Hope et al. 2009, Ni Cheallaigh, Keane et al. 2011). Autophagy acts as a mechanism of bypassing the phagosome block and killing intracellular mycobacteria(Gutierrez, Master et al. 2004). Autophagy is induced by cytokines including TNF-α and IFN-γ and vitamin D and inhibited by IL-10 and Th2 cytokines including IL-4 (Schaible, Sturgill-Koszycki et al. 1998, Via, Fratti et al. 1998, Gutierrez, Master et al. 2004, Deretic, Singh et al. 2006, Liu, Stenger et al. 2006) (Figure 6). IFN-γ induces both formation of and maturation of autophagosomes (Gutierrez, Master et al. 2004, Matsuzawa, Kim et al. 2012) Autophagy also modulates the inflammatory response to mycobacteria via its effect on cytokine secretion (Saitoh, Fujita et al. 2008, Peral de Castro, Jones et al. 2012). Further evidence that autophagy is a critical part of

immune defense against *M. tuberculosis* comes from *in vivo* infections in autophagy deficient mice which show a marked increase in TB susceptibility in the absence of autophagy (Castillo, Dekonenko et al. 2012, Watson, Manzanillo et al. 2012) and evidence of a link between IRGM polymorphisms in humans and TB susceptibility (Intemann, Thye et al. 2009, Che, Li et al. 2010, Bahari, Hashemi et al. 2012). The importance of autophagy in the host immune response against *M. tuberculosis* is further highlighted by the fact that virulent mycobacteria, unlike avirulent strains, have evolved mechanisms to inhibit autophagy (Harris and Keane 2010, Kumar, Nath et al. 2010).

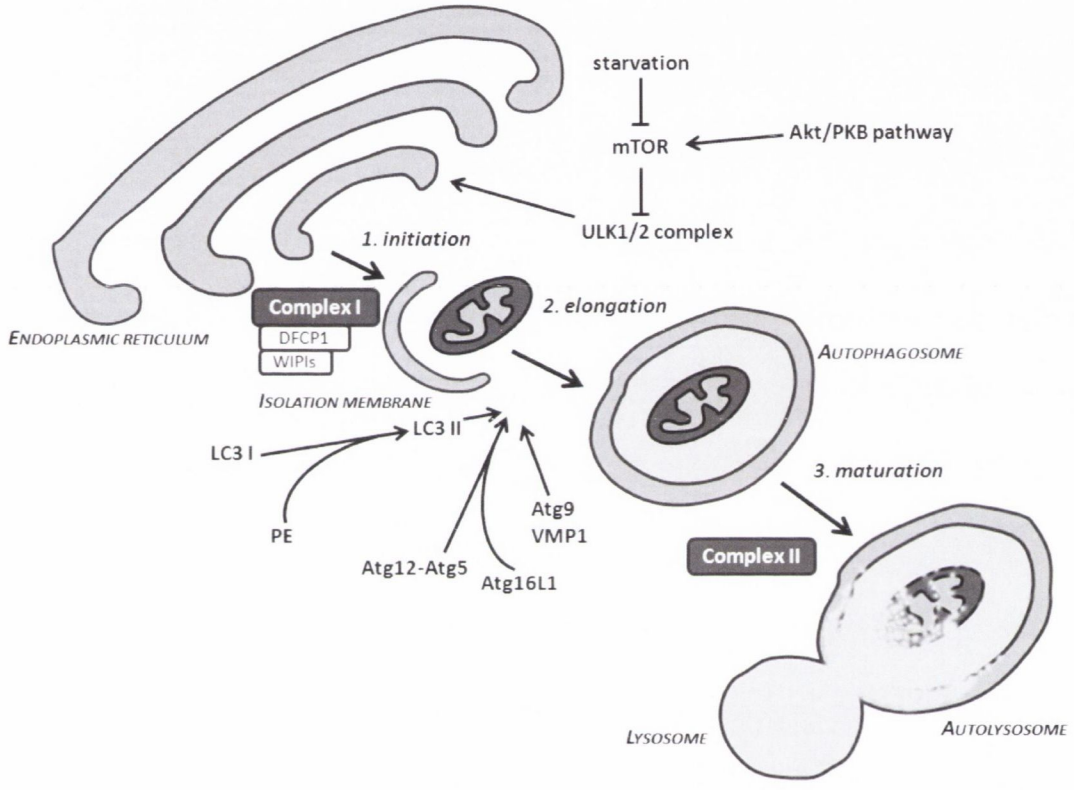


Figure 1-5: Autophagy

Autophagosome biogenesis occurs in three stages. *1. Initiation:* Inhibition of mammalian target of rapamycin (mTOR), leads to translocation of a protein complex containing ULK1/2 and Atg13,to the ER. This allows recruitment of a complex consisting of the type III PI3K, VPS34, Beclin 1 (Atg6), VPS15 and Atg14 (complex I) and leads to the formation of omegasomes, from which the isolation membrane is thought to emerge. *2. Elongation:* The isolation membrane forms an autophagosome with a double membrane. Cytosolic LC3 I is lipidated to form LC3 II, which is recruited to the autophagosomal membrane. *3. Maturation:* The outer membrane of the autophagosome is able to fuse with lysosomes, allowing the degradation of the luminal contents.. *Reprinted from Harris, Cytokine, 2011*

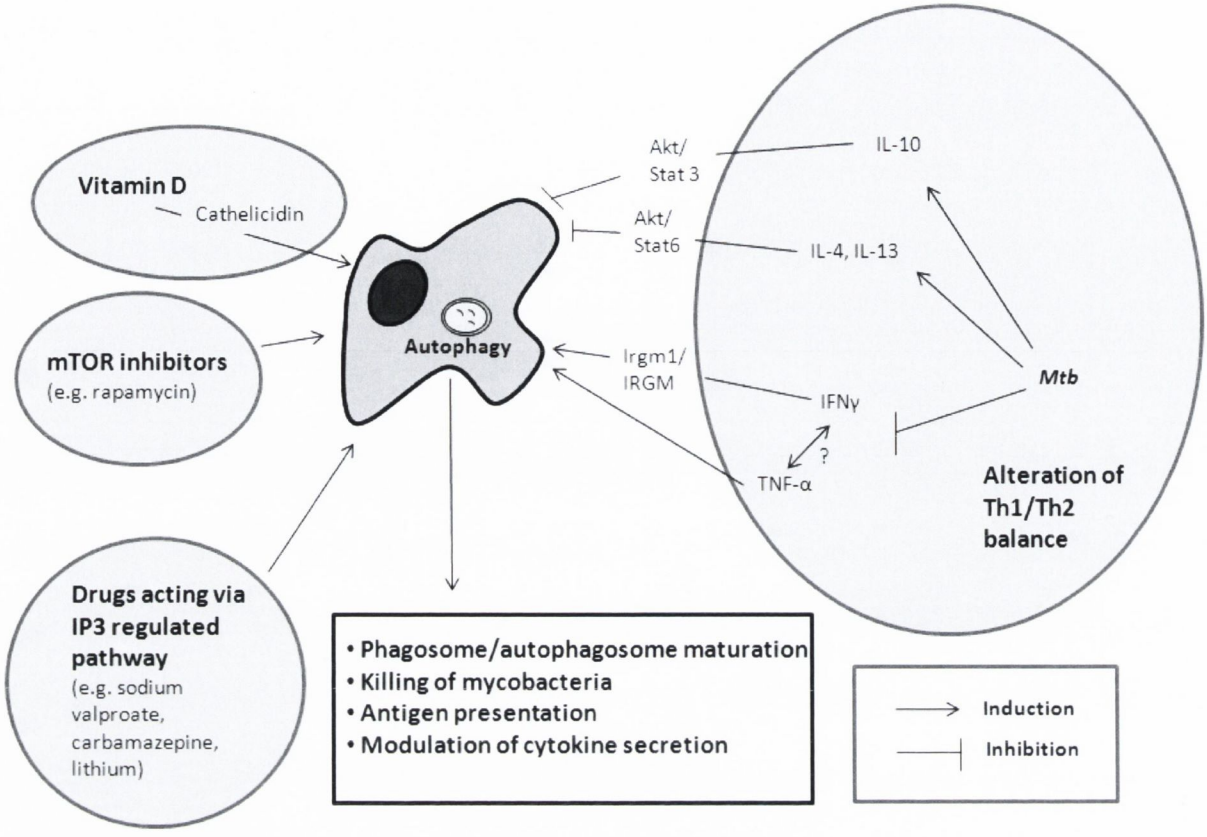


Figure 1-6: Modulation of autophagy in macrophages.

Autophagy can be induced by mammalian target of rapamycin (mTOR) inhibitors, such as rapamycin; by drugs acting via the D-myo-inostitol-1,4,5-triphosphate (IP3) pathway, such as sodium valproate; by vitamin D (through the induction of the anti-microbial peptide cathelicidin) and by cytokines, including TNF-α and IFN-γ. Other cytokines, including IL-4, IL-13 and IL-10 inhibit autophagy through protein kinase B (PKB/Akt), signal transducer and activator of transcription (STAT)6 and STAT3 signalling pathways. In cells infected with *M. tuberculosis*, induction of autophagy leads to increased acidification and maturation of mycobacteria-containing phagosomes and increased killing of intracellular bacteria. Autophagy also plays a role in antigen presentation and inhibits the processing and secretion of pro-inflammatory cytokines, including IL-1β and IL-18. *Reprinted from Ni Cheallaigh, Clinical and Experimental Immunology, 2011.*

1.2.8 Innate cytokines and their role in tuberculosis

Cytokines produced by cells of the innate immune system orchestrate the innate and adaptive immune responses to tuberculosis. They act as an intricate network in tuberculosis infection, and regulate the production and effector functions of other cytokines (Figure 7). These have been extensively reviewed in recent publications (Cooper, Mayer-Barber et al. 2011, Ni Cheallaigh, Peral de Castro et al. 2013, O'Garra, Redford et al. 2013). Cytokines studied in this thesis are discussed briefly below.

Il-1α and Il-1β are pro-inflammatory cytokines produced by macrophages and dendritic cells which signal through the IL-1 receptor (IL-1R). Mice deficient in IL-1R1 are more susceptible to pulmonary tuberculosis after infection with *M. tuberculosis* and display increased mortality, defective granuloma formation and enhanced mycobacterial growth in the lungs, spleen and liver (Fremond, Togbe et al. 2007). In humans, IL-1 receptor antagonist/IL-1β polymorphisms influence cytokine responses to *M. tuberculosis* (Wilkinson, Patel et al. 1999) and polymorphisms in the IL-1 receptor 1 (IL-1R1) are associated with increased susceptibility to TB (Motsinger-Reif, Antas et al. 2010).

IL-1β is activated by processing in the inflammasome (Schroder and Tschopp 2010). IL-1β is activated by processing, principally by caspase 1 but roles for caspase 8 and neutrophil proteases have also been reported (Netea, Simon et al. 2010, Bossaller, Chiang et al. 2012). Caspase 1 activation relies on the assembly of multicomponent cytoplasmic complexes, termed inflammasomes (Schroder and Tschopp 2010). A number of inflammasomes have been identified, including NLP3 and IPAF. It is not yet clear which inflammasomes are involved in IL-1β secretion in response to mycobacteria. One study suggests a role for IPAF in response to *M. bovis* BCG (Master, Rampini et al. 2008), while another implicates

NLRP3 in response to *M. marinum* and *M. tuberculosis* (Mishra, Moura-Alves et al. 2010) and a third demonstrates that the mycobacterial protein ESAT6 activates the NLRP3 inflammasome (Mishra, Moura-Alves et al. 2010). However, a recent study suggests that NLRP3 is not required for early protection against infection with *M. tuberculosis in vivo*, at least in mice (Walter, Holscher et al. 2010). The importance of IL-1β itself is controversial, as a recent study has demonstrated that mice treated with neutralising antibodies against IL-1α, but not IL-1 β, are more susceptible to infection with *M. tuberculosis* (Guler, Parihar et al. 2011). However, another study has suggested that both IL-1α and IL-1 β, produced by inflammatory monocyte-macrophages and dendritic cells in the lungs of mice, have critical, non-redundant, roles for host resistance to infection with *M. tuberculosis* (Mayer-Barber, Andrade et al. 2011).

IL-10 is an anti-inflammatory cytokine which is secreted by macrophages in response to mycobacterial infection (Shaw, Thomas et al. 2000). IL-10 impairs phagosome maturation(O'Leary, O'Sullivan et al. 2011) and inhibits production of TNF-a and IL-6 (Fiorentino, Zlotnik et al. 1991). Data from human studies show that polymorphisms in NRAMP, a protein which regulates IL-10 production (Fritsche, Nairz et al. 2008), are associated with TB susceptibility (Hsu, Chen et al. 2006). In animal models, IL-10 reduces the protective response to *M. tuberculosis* (Murray and Young 1999, Jacobs, Brown et al. 2000). HIV-infected macrophages secrete an increased amount of IL-10 compared to non-infected macrophages (Patel, Zhu et al. 2007).

IL-12 is a pro-inflammatory cytokine produced by macrophages and dendritic cells in response to infection with *M. tuberculosis* and other pathogens in response to TLR ligation (Bafica, Scanga et al. 2005). It is a heterodimeric cytokine comprising two subunits encoded by different genes, IL-12A (p35) and IL-12B (p40). IL12 can also exist as a

homodimer of the p40 subunit (IL-12p80). IL-12 signals through the IL-12 receptor B1 (IL12Rb1), drives dendritic cell migration and activates naive T-cells. Mice lacking IL-12p40 are susceptible to mycobacterial infection and show impairment of granuloma formation, Th1 and cytotoxic T-cell activation (Holscher, Atkinson et al. 2001). Humans with mutations in IL-12 receptors show a dramatic increase in mycobacterial susceptibility (Altare, Durandy et al. 1998, Caragol, Raspall et al. 2003, Filipe-Santos, Bustamante et al. 2006).

TNF-α is secreted by macrophages, lymphocytes, neutrophils, mast cells and endothelial cells. It exerts its pro-inflammatory functions by inducing other pro-inflammatory cytokines (IL-1 and IL-6), inducing chemokine production and up-regulating adhesion molecules, thereby recruiting other immune cells (Lopez Ramirez, Rom et al. 1994, Roach, Bean et al. 2002). Experimental models have demonstrated that TNF-α is secreted in response to mycobacterial infection (Balcewicz-Sablinska, Keane et al. 1998, Keane, Shurtleff et al. 2002). TNF-α induces macrophage apoptosis (Keane, Balcewicz-Sablinska et al. 1997, Keane, Shurtleff et al. 2002) and to kill mycobacteria (Denis 1991). TNF-α also increases autophagy, phagosome maturation and nitric oxide (NO) production (Rojas, Olivier et al. 1999, Harris, Hope et al. 2008, Harris and Keane 2010). Mice deficient in TNF-α or TNF receptor 1 are more susceptible to mycobacterial infection and display impaired granuloma formation (Flynn, Goldstein et al. 1995, Bean, Roach et al. 1999). TNF blockers are monoclonal antibodies directed against TNF-α: adalimumab (Humira) and infliximab (Remicade) bind to monomeric (membrane-bound) and trimeric (soluble) TNF-α, and etanercept (Enbrel) binds to trimeric TNF-α only. TNF blockers are extensively used to treat inflammatory diseases, particulary rheumatoid arthritis and Crohn's disease. Treatment with TNF blockers is associated with an increased risk of developing active

tuberculosis, which may be dissseminated and/or otherwise atypical in its clinical presentation (Keane, Gershon et al. 2001, Harris and Keane 2010). Co-infection with HIV decreases TNF-a secretion and bioavailability (Patel, Zhu et al. 2007).

Type 1 interferons, a group of interferons which includes Interferon-α, Interferon-β (IFNβ) but not Interferon-γ, are cytokines which play an important role in anti-viral immunity. Their role in anti-tuberculous immunity is less well understood. It has recently been reported that pulmonary tuberculosis induces a signature in PBMCs of type 1 interferon-inducible genes (Berry, Graham et al. 2010). A variety of animal models, reviewed by O'Garra and colleagues, have provided evidence that type 1 interferons impair effective immune responses to tuberculosis (O'Garra, Redford et al. 2013). Type 1 interferons induce IL-10 and suppress production of IL-1 and IL-12, as well as reducing sensitivity to IFN-γ (McNab, Ewbank et al. 2013).

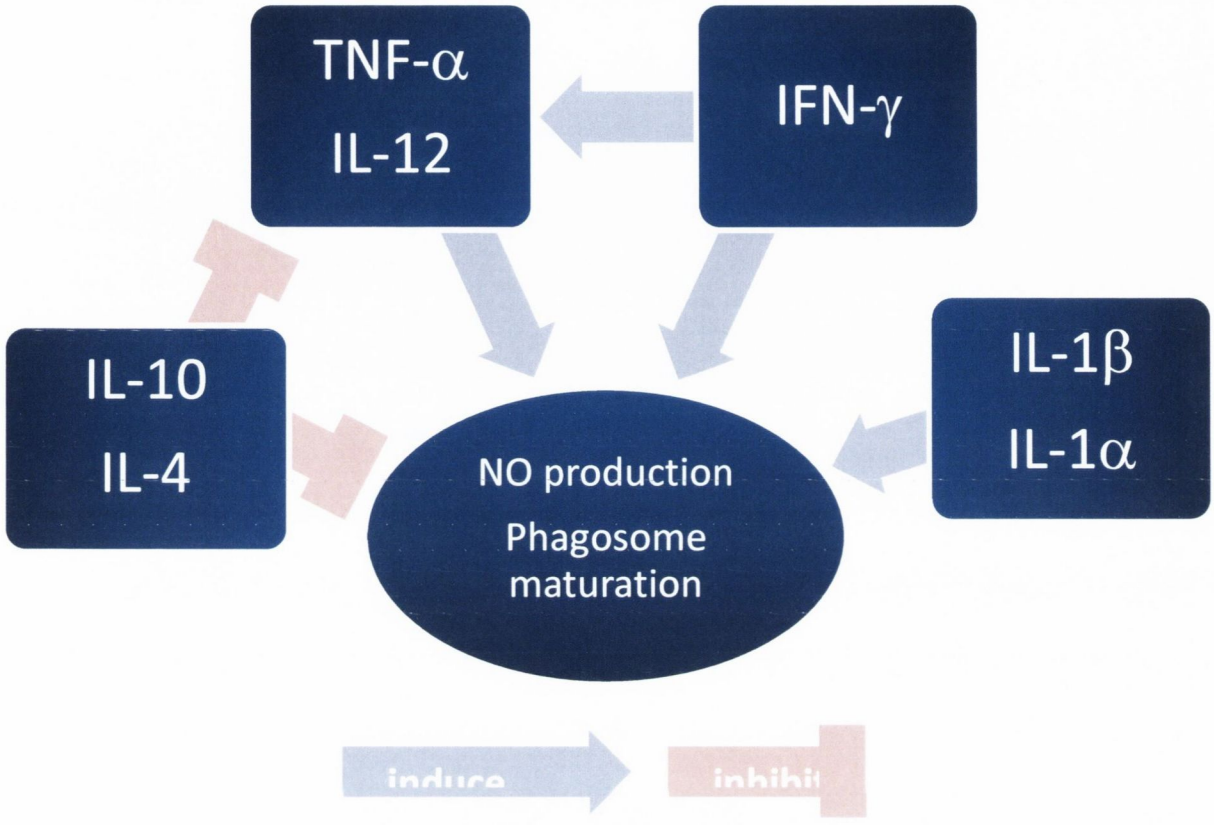


Figure 1-7: Cytokines and TB.

IFN-γ induces anti-mycobacterial responses in macrophages, including nitric oxide (NO) generation, phagosome maturation, autophagy and NO-dependent apoptosis. IFN-γ also induces the secretion of TNF-α and IL-12, which have similar effects on macrophage responses to infection with mycobacteria and are important for maintenance of the granuloma. IL-1 family cytokines (IL-1α, IL-1β and IL-18) induce phagosome maturation and autophagy and enhance intracellular elimination of mycobacteria in macrophages. IL-10, IL-4 and IL-13 have inhibitory effects on macrophages responses and also limit secretion of TNF-α. *Reprinted from Ni Cheallaigh, Clinical and Experimental Immunology, 2011.*

1.2.9 Phagocytosis

Alveolar resident macrophages are the primary cell type involved in the initial uptake of *M. tuberculosis*. After this first encounter, dendritic cells and monocyte-derived macrophages also take part in the phagocytic process (van Crevel, Ottenhoff et al. 2002). Macrophage phagocytosis of *M. tuberculosis* is mediated by a number of receptors

including scavenger receptors, the mannose receptor (MR), and complement receptors which are responsible for the uptake of approximately 80% of opsonized bacteria (Schlesinger, Bellinger-Kawahara et al. 1990, Hirsch, Ellner et al. 1994, Weikert, Edwards et al. 1997). Uptake of the bacilli can occur after opsonization with complement factors, or it can be initiated as a nonopsonic event. TLRs 2 and 4 have been reported to play a role in phagocytosis of other bacteria, including *E. coli* and *Staphylococcus aureus* (Blander and Medzhitov 2004) although others in the field disagreed with this finding (Russell and Yates 2007).

1.2.10 Reactive nitrogen and oxygen species

Nitric oxide is produced by nitric oxide synthase within 24 hours after infection, and is able to permeate the microbial coat and kill the pathogen. It is well-established that murine macrophages can kill intracellular mycobacteria by nitric oxide production (Chan, Xing et al. 1992). The importance of nitric oxide in human macrophages is more controversial, but human alveolar macrophages also have been reported to kill intracellular mycobacteria via nitric-oxide dependent mechanisms (Rich, Torres et al. 1997). Cytokines such as IFN-γ and microbial products can induce production of nitric oxide.

Superoxides are other important effector molecules used by the innate immune system to kill pathogens. Activated macrophages can also produce reactive oxygen intermediates (ROI), as reviewed by Fang and colleagues (Fang 2004). TLR2 stimulation by purified protein derivative of TB (PPD) has been reported to result in an increased ROS production (Yang, Shin et al. 2008). Macrophages from X-CGD mice with non-functional phagocyte oxidase cytochrome B do not display a defect in mycobactericidal activity, but *in vivo* experiments showed that the mice were unable to control mycobacterial infection

(Cooper, Segal et al. 2000). Chronic granulomatous disease (CGD) is a rare disorder of the phagocytic oxidative burst leading to recurrent pyogenic infections. Individuals with CGD show increased susceptibility to BCG and *M. tuberculosis* (Lee, Chan et al. 2008).

1.2.11 Antimycobacterial Peptides

An additional innate immune defense against mycobacterial infection is the production of antimicrobial peptides: defensins and cathelicidin. Defensins are small cationic peptides (3–5 kDa), which are produced abundantly by phagocytic cells and mediate oxygen-independent killing of ingested microorganisms. Defensins are divided into three subfamilies: α, β and θ defensins. α-defensins are predominantly produced by neutrophils and possess antimicrobial activity *in vitro* against mycobacteria including *M. tuberculosis*. (Ogata, Linzer et al. 1992, Miyakawa, Ratnakar et al. 1996). Macrophages predominantly produce β –defensins, which are also produced by respiratory epithelial cells. β-defensins have bactericidal activity, and also recruit acquired immune cells, thus representing a link between innate and acquired immunity (Yang, Chertov et al. 1999). β –defensins are present in human bronchoalveolar lavage fluid at bactericidal concentrations (Travis, Conway et al. 1999). Their production is upregulated by pro-inflammatory cytokines known to be involved in effective host responses to *M. tuberculosis*, such as TNF-α and IL-1β (Harder, Meyer-Hoffert et al. 2000, Mendez-Samperio, Miranda et al. 2006) in a NFκB-dependent manner (Tsutsumi-Ishii and Nagaoka 2003). The role of defensin beta 4, (DEFB4), formerly known as human β defensin 2, in infection with *M. tuberculosis* has been extensively studied. Human respiratory epithelial cells produce defensin beta 4 during infection with *M. tuberculosis*(Rivas-Santiago, Schwander et al. 2005). Liu and colleagues have shown that human monocytes produce defensin beta 4 (DEFB4), when stimulated by mycobacterial

TLR2/1 ligands in a vitamin D receptor –and IL-1β dependent manner (Liu, Schenk et al. 2009). They have also shown that DEFB4 has antimycobacterial activity *in vitro*. In contrast, Duits and colleagues report that human monocyte-derived and alveolar macrophages do not produce DEFB4 (Duits, Ravensbergen et al. 2002). Kisich also found that human macrophages did not produce DEFB4 when infected with *M. tuberculosis*, but interestingly when primary monocyte-derived macrophages were transfected with DEFB4 mRNA they produced DEFB4 which co-localised with *M. tuberculosis* and restricted the growth of intracellular *M. tuberculosis* (Kisich, Heifets et al. 2001). Whether human macrophages, particularly alveolar macrophages, produce DEFB4 when infected with *M. tuberculosis* in the presence of physiological levels of vitamin D and IL-1β has not yet been established.

Only one human cathelicidin gene, and one functional gene product, cathelicidin LL-27, have been identified. Cathelicidin has mycobactericidal activity against extracellular *M. tuberculosis* and *M. smegmatis* (Liu, Stenger et al. 2006, Liu, Schenk et al. 2009) and intracellular *M. smegmatis* (Sonawane, Santos et al. 2011). Liu and colleagues have shown that TLR2/1 stimulation resulted in upregulation of cathelicidin expression in a vitamin D receptor dependent manner (Liu, Stenger et al. 2006). Cathelicidin induces autophagy and co-localisation of mycobacteria with autophagosomes (Yuk, Shin et al. 2009) and maturation and acidification of mycobacterial phagosomes (Yuk, Shin et al. 2009, Sonawane, Santos et al. 2011).

1.2.12 Vitamin D

Prior to the discovery of antibiotics active against *M. tuberculosis*, tuberculosis patients were treated with cod-liver oil and exposure to sunlight (Martineau, Honecker et al. 2007).

A number of recent papers have shown that vitamin D may be the mechanism through which these treatments acted. Several papers have reported an association between low vitamin D levels and active tuberculosis, as reviewed by Nnoaham et al (Nnoaham and Clarke 2008); however, these studies were unable to confirm the direction of the association. Studies showing an association between vitamin D receptor polymorphisms, as reviewed by Lewis et al (Lewis, Baker et al. 2005), indicate that vitamin D may play a determining role in tuberculosis infection, although further studies are needed to confirm this.

The active metabolite of vitamin D, $1,25(OH)_2D_3$, has been shown to be involved in a number of key antimycobacterial macrophage functions. $1,25(OH)_2D_3$ plays a key role in the TLR2/1 induced killing of intracellular mycobacteria and is required for induction of cathelicidin(Liu, Stenger et al. 2006), and, in conjunction with IL-1β, $1,25(OH)_2D_3$ also induces defensin beta 4 (Liu, Schenk et al. 2009) as described above. Yuk and colleagues reported that $1,25(OH)_2D_3$ induces autophagy via cathelicidin and transcription of Beclin1 and ATG5. $1,25(OH)_2D_3$ also induces recruitment of cathelicidin to autophagosomes and co-localisation of autophagosomes with mycobacteria (Yuk, Shin et al. 2009). Interferon gamma-induced autophagy and phagolysosomal maturation are also Vitamin D-dependent (Fabri, Stenger et al. 2011).

Given the anti-mycobacterial activity *in vitro* of vitamin D, there was hope that it would be an effective adjunctive treatment for active tuberculosis. Unfortunately, clinical trials have been disappointing. A trial in Guinea-Bissau failed to show any statistically significant benefit in clinical outcome in HIV-negative and –positive TB patients (Wejse, Gomes et al. 2009), and another study found that benefit was limited to individuals with VDR polymorphism *Taq1 tt* (Martineau, Leandro et al. 2010). Studies to look at the benefit of

vitamin D in treating latent tuberculosis are under consideration, but would require large numbers of patients to show a significant effect.

1.3 Pattern recognition receptors and tuberculosis

1.3.1 Pattern recognition receptors

As discussed above, a characteristic of the innate immune system is that innate sentinel cells recognise conserved pathogenic motifs using pattern recognition receptors (PRRs). PRRs can be found on the surface or intracellularly in numerous cells including macrophages and dendritic cells. PRRs can also be secreted into the bloodstream. Many different types of PRR exist, and each is specific for a certain PAMP or DAMP. This enables the innate response to be tailored to a certain type of pathogen, as PRRs activate particular signal transduction pathways culminating in the activation of a set of genes or gene products. 5 major classes of PRRs exist: CLRs (C-type lectin receptors); NLRs (nucleotide-binding domain-, leucine-rich repeat-containing receptors; RLRs (RNA helicase RIG-I–like receptors); ALRs (cytoplasmic DNA receptor AIM2–like receptors) and TLRs (Toll-like receptors) (Netea, Wijmenga et al. 2012).

1.3.2 TLRs

TLRs were the first family of PRRs to be discovered in the 1990s, and have been the subject of intense investigation since then. TLRs are located on plasma or endosomal membranes and contain an extracellular N-terminal leucine rich repeat domain, a transmembrane domain and an intracytoplasmic C-terminal Toll/interleukin-1 receptor (TIR) domain which bears homology to the type I interleukin-1 receptor (IL-1RI) intracellular domain and is required for signalling. Each TLR is specific to a type of

PAMP: for example TLR4 recognises LPS from Gram negative bacteria, TLR2/1 recognises triacetylated lipoporoteins, TLR2/6 recognises diactylated lipoproteins and TLR3 recognises RNA from ssRNA viruses. PAMPs engage with TLRs, cause homo- or heterodimerisation and activate an acute inflammatory reaction via pathways which include a number of adaptor proteins (Figure 8) and culminate in the nuclear translocation of NF-κB and the subsequent modulation of adaptive cellular and humoral immune responses (Medzhitov and Janeway 1999, O'Neill, Golenbock et al. 2013).

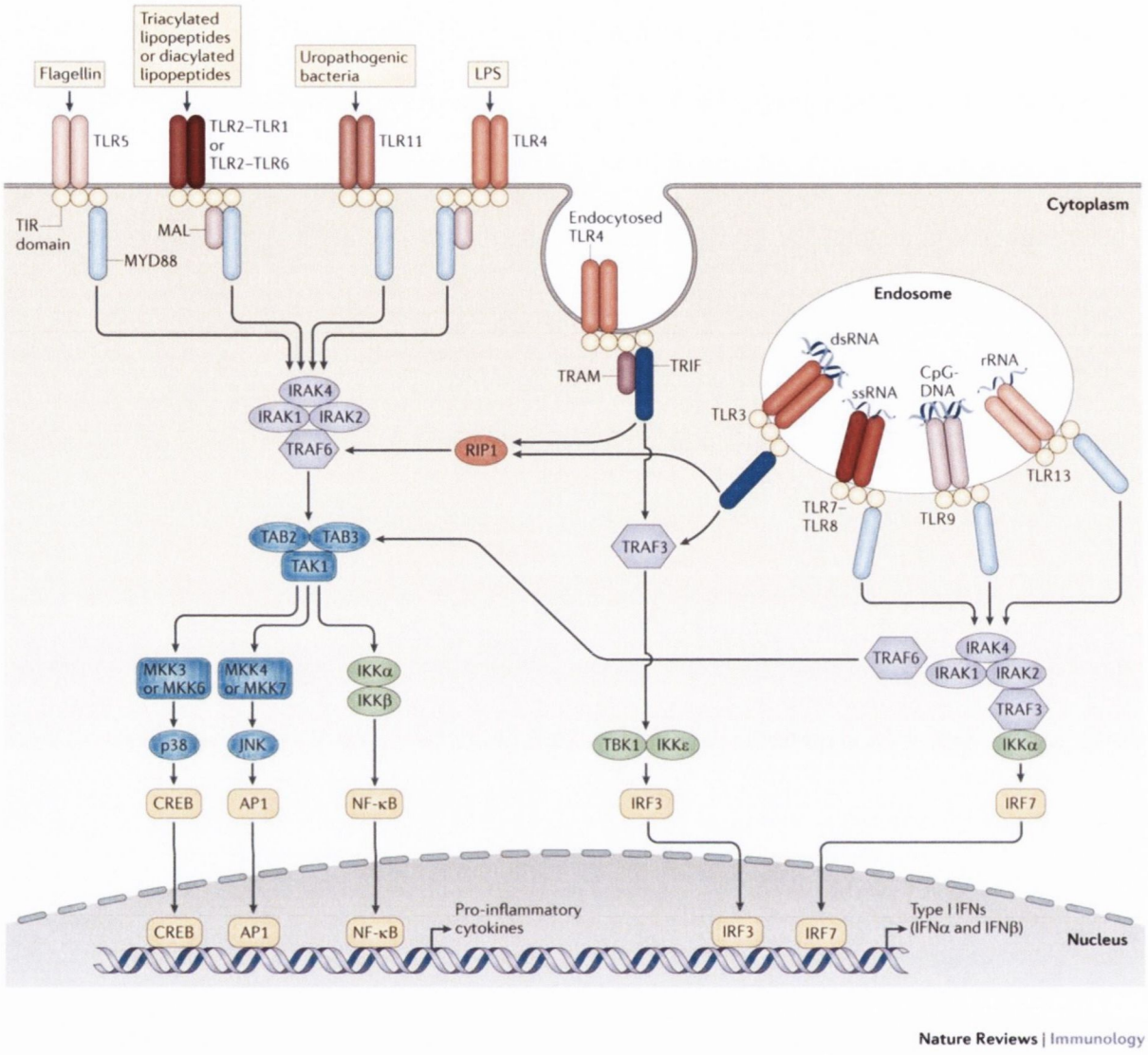


Figure 1-8: Toll-like receptors (TLRs) and their pathways.

TLR5, TLR11 and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface. TLR3, TLR7–TLR8, TLR9 and TLR13 localize to the endosomes, where they sense microbial and host-derived nucleic acids. TLR4 localizes at both the plasma membrane and the endosomes. TLR signalling is initiated by ligand-induced dimerization of receptors. Following this, the TIR domains of TLRs engage TIR domain-containing adaptor proteins MYD88 and Mal or TRIF and TRAM. Engagement of the signalling adaptor molecules stimulates downstream signalling pathways that involve interactions between IRAKs and the adaptor molecules TRAFs, and that leads to the activation of the MAPKs JNK and p38, and to the activation of transcription factors such as nuclear factor-κB (NF-κB) and the interferon-regulatory factors (IRFs). A major consequence of TLR signalling is the induction of pro-inflammatory cytokines. *Reprinted from O'Neill, Nature Reviews Immunology, 2013.*

1.3.3 MyD88

MyD88 is known as the universal TLR adaptor protein, as it interacts with all TLRs except TLR3. MyD88 has a TIR domain which interacts with the TIR domain present in TLRs. MyD88 also has a death domain, which recruits members of the IRAK (IL-1 receptor–associated kinase) family of serine-threonine kinases in a structure termed a Myddosome(Lin, Lo et al. 2010). Recruitment of IRAKs results in activation of signalling pathways that culminate in the activation of transcription factors, most notably NF-κB (O'Neill and Bowie 2007). IL-1, IL-18 and IL-33 also signal through the Myddosome (Adachi, Kawai et al. 1998).

MyD88 is also involved in IFN-γ signalling, as first reported by Shi and colleagues. They found that, although the canonical IFN- γ signalling pathway did not involve MyD88, the expression of many genes in response to IFN- γ is MyD88-dependent (Shi, Nathan et al. 2003). Sun and Ding reported that MyD88 transduced signals from IFNGR to MLK3, which in turn interacted with JIP-1 and resulted in p38 phosphorylation. This resulted in stabilisation of IFN- γ -induced transcripts, such as IP-10 mRNA (Sun and Ding 2006).

Autosomal recessive MyD88 deficiency was first reported in 2008 (von Bernuth, Picard et al. 2008). Approximately 22 individuals with this disorder have been identified (Picard, Casanova et al. 2011). Leukocytes from these patients do not produce IL-6 when stimulated with IL-1 or TLR ligands, except for TLR3 ligands. Adaptive immune responses are relatively well-preserved, with normal antigen-specific T- and B-cell responses, with two notable exceptions: impairment of IgG and IgM antibody responses to pneumococcal and AB glycans in up to one-third of patients and elevation of serum IgE and IgG4 concentrations in up to two-thirds and one-third, respectively, of patients tested. Patients

with MyD88 deficiencies have a greatly increased risk of invasive pyogenic bacterial infections (meningitis, sepsis, arthritis, osteomyelitis, and abscesses) often caused by *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* but appear to have normal resistance to common fungi, parasites, viruses, and many bacteria. Patients with MyD88 deficiencies also have an increased risk of necrotising superficial bacterial infections. The risk of invasive bacterial infections declines sharply once these patients reach adulthood, but they continue to be at increased risk of superficial bacterial infections. A mutation in MyD88, L265P, which leads to activation of NF-κB has been reported to cause Waldenstrom's macroglobulinaemia, a IgM secreting lymphoma (Treon, Xu et al. 2012).

In addition to MyD88, four additional TIR-domain containing signalling adaptor proteins involved in TLR signalling have been identified. TRAM and TRIF signal downstream of TLR4 and lead to activation of IRF3. TRIF is also used by TLR3. SARM, is inhibitory for TRIF-dependent signalling and limits signalling by TLR3 and TLR4 (Carty, Goodbody et al. 2006).

1.3.4 Mal

MyD88 adapter-like (Mal), also termed Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP), is the fourth TLR signalling adaptor protein. Mal was initially described as a signalling adaptor protein leading to NF-κB activation downstream of TLR 4 (Fitzgerald, Palsson-McDermott et al. 2001, Horng, Barton et al. 2001) and TLR 2 (Horng, Barton et al. 2002, Yamamoto, Sato et al. 2002). Mal contains a TIR domain which differs from the TIR domain of other TLR signalling molecules: it has a long, protruding AB loop instead of a BB loop (Lin, Lu et al. 2012) (Figure 9b). Mal is localised

at the plasma membrane and on endocytic vesicles, where it is binds to phosphatidylinositol 4,5-bisphosphate (PIP2) (Kagan and Medzhitov 2006). The PIP2 binding domain and membrane localisation is required for TLR4 signalling via Mal. MyD88, TLR2 and TLR4 are largely electronegative, and positively-charged Mal recruits MyD88 to the plasma membrane (Kagan and Medzhitov 2006) and enables it to bind to TLRs 2 and 4 in the Myddosome (Bonham, Orzalli et al. 2014), thereby facilitating signalling (Dunne, Ejdeback et al. 2003).

More recently, Mal has also been reported to have a number of functions in addition to that of bridging between MyD88 and TLRs 2 and 4. Downstream of TLR2, but independently of MyD88, it interacts with phosphoinositol-3 kinases (PI3Ks), thereby converting PIP2 to PIP3. resulting in Akt phosphoryation, cytoskeletal remodeling and macrophage polarization (Santos-Sierra, Deshmukh et al. 2009). A number of reports have suggested that Mal has an additional MyD88-independent function in which it facilitates TLR 2 and 4 signalling by recruiting TRAF6, resulting in p65 phosphorylation and NF-κB transactivation (Mansell, Brint et al. 2004, Verstak, Nagpal et al. 2009). Mal can also interact with TRAF6, p38 MAPK and MK2 to enhance CREB activation, leading to IL-10 production (Mellett, Atzei et al. 2011). Mal E190A mutants are unable to activate NF-κB but retain their ability to activate CREB [185]. In association with TLR2, Mal has also been reported to be required for maintenance of the barrier function of epithelial surfaces, via an interaction with PKC (Corr, Palsson-McDermott et al. 2013). With MyD88, Mal has also been reported to function as a negative regulator of TLR3 signalling including IFN-β gene induction(Siednienko, Halle et al. 2010) (Johnson, Li et al. 2008, Kenny, Talbot et al. 2009)- the mechanism of this is unclear but may involve sequestration of IRAK-2. Natural ligands of endosomal TLRs 8 and 9 also

require Mal for signalling (Bonham, Orzalli et al. 2014). A TLR-independent role for Mal has also been reported as a signalling adaptor downstream of the receptor for advanced glycation end products (RAGE) (Sakaguchi, Murata et al. 2011). RAGE responds to advanced glycation end products (Kislinger, Fu et al. 1999), DNA, RNA and amyloid fibrils. RAGE signalling induces a number of responses via pathways including ERK1/2, p38 MAP kinase, and NF-κB.

Signalling via Mal is controlled by a number of mechanisms. The ARF6 GTPase regulates the PIP2-dependent localisation of Mal on the plasma membrane and therefore TLR signalling via Mal (Kagan and Medzhitov 2006). Caspase-1 cleaves Mal, and this cleavage is required for Mal- dependent TLR 2 and 4 signalling via Mal (Miggin, Palsson-McDermott et al. 2007). As caspase-1 is activated by inflammasomes such as NLRP3, this is a potential point of cross-talk between pathways, as activation of NLRs such as NLRP3 will activate caspase 1, thereby potentiating signalling via Mal. In addition, the phosphorylation of Mal on tyrosine 86, 106 and 159 by Bruton's tyrosine kinase (Btk) is also required for Mal activation (Gray, Dunne et al. 2006, Piao, Song et al. 2008). Phosphorylation by Btk activates Mal, but also leads to Mal polyubiquitination and degradation by suppressor of cytokine signalling 1 (SOCS1), thereby limiting signalling (Mansell, Smith et al. 2006). IRAK-1 and IRAK-4 also promote degradation of Mal (Dunne, Carpenter et al. 2010). Bacteria may target immune responses by inducing degradation of Mal, as has been reported with *Brucella sp* and *E. coli*, which produce a Mal-mimic called TcpB which enhances polyubiquitination and degradation of Mal, thereby subverting TLR signaling (Newman, Salunkhe et al. 2006{Cirl, 2008 #2496, Radhakrishnan, Yu et al. 2009, Sengupta, Koblansky et al. 2010).

The crystal structure of Mal has recently shown it to be a single molecule composed of a TIR domain fold containing a five-stranded parallel β-sheet surrounded by four α-helices (Valkov, Stamp et al. 2011). It displays a low level of amino acid similarity to other human TIR domains, with the closest homologue being MyD88 (24% identity). A prominent acidic patch is the proposed interaction site with MyD88 and/or TLRs 2 and 4. This patch is the region in which the polymorphism, D96N, occurs.

Two non-synonymous single nucleotide polymorphisms (SNPs) in Mal with functional consequences have been identified to date: D96N and S180L. D96N is a rare SNP which changes the distribution of negative charge at a prominent binding site (Valkov, Stamp et al. 2011) resulting in an inability to interact with MyD88 and a loss of TLR 2 and TLR4 stimulated NF-κB activation and cytokine production. S180L is common in European and Indian populations, with approximately 15-20% of European populations carrying the L allele and 2-3% of the population being homozygotes for the L allele (Ferwerda, Alonso et al. 2009). The S180L SNP results in steric occlusion of a cavity close to the acidic binding site where the D96N mutation occurs (Figure 9) (Valkov, Stamp et al. 2011). It was initially reported that the S180L mutation led to a loss of binding affinity for TLR2 and a loss in NFκB activation in response to TLR2 and 4 ligands (Khor, Chapman et al. 2007). A subsequent study, involving LPS injection into human volunteers with the polymorphism showed an increased inflammatory cytokine response (Ferwerda, Alonso et al. 2009). A recently published study showed decreased cytokine responses to heat-killed Mtb in PBMCs from individuals with the SNP (Capparelli, De Chiara et al. 2013).

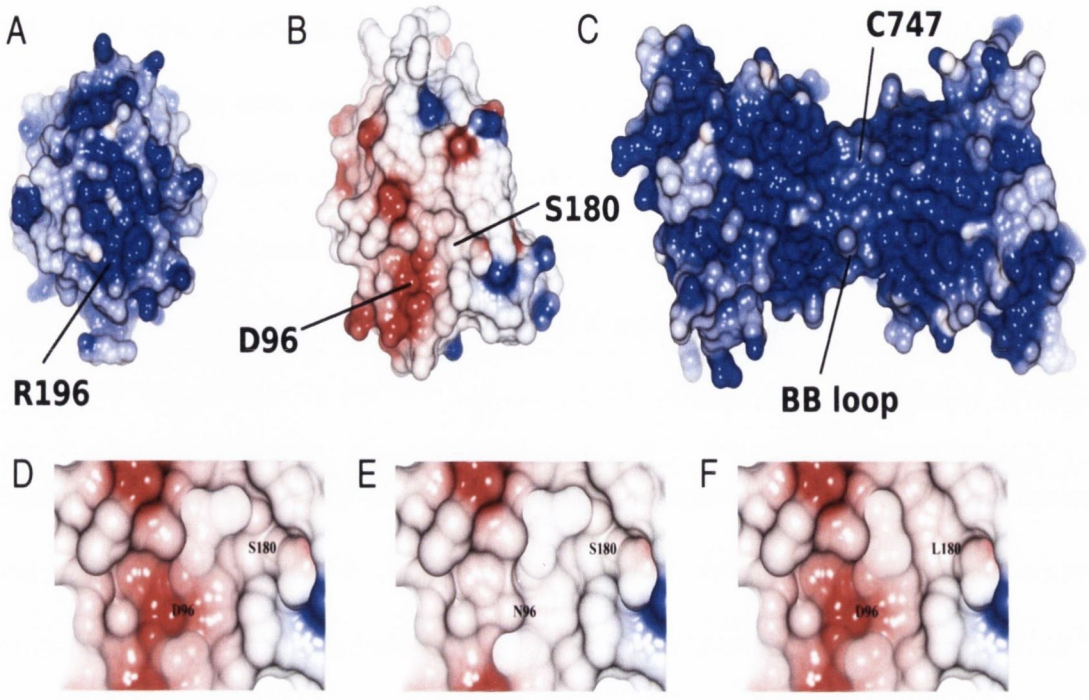


Figure 1-9: The structure of Mal and associated SNPs.

(***A***) The surface of MyD88-TIR consists of significant patches of basic residues (blue). (***B***) The surface of Mal is more hydrophobic with acidic patches in several locations, one of which is shown (red). (***C***) The surface of the homology model of the TLR4-TIR dimer . (***D-F)***) Close-up of the Mal surface containing the D96 and S180 residues. The surface cavity flanked by S180 can be seen in the center. (***E***) A model of the D96N variant showing the loss of the acidic patch in the region predicted to interact with MyD88-TIR. (***F***) A model of the S180L variant showing the physical occlusion of the hydrophobic surface cavity by the bulkier leucine side chain. *Reprinted from Valkov et al, PNAS, 2011.*

Heterozygosity for the S180L SNP has been associated with protection from a number of infectious diseases including pneumococcal disease and malaria{Khor, 2007 #842}. Homozygosity for the S180L SNP has been associated with severe sepsis (Ferwerda, Alonso et al. 2009, Hamann, Kumpf et al. 2009, Song, Tong et al. 2010) and severe pneumococcal disease (Khor, Chapman et al. 2007, Zakeri, Pirahmadi et al. 2011). These associations had been attributed to the effect of S180L on TLR2 and TLR4 signalling. The mechanism underlying the heterozygote advantage and homozygote disadvantage is unknown. Mutations with a similar pattern including that underlying sickle

cell anaemia, persist in the population as heterozygosity presents an evolutionary advantage. Interestingly, invasive *Haemophilus influenzae* infection post-vaccine (Ladhani, Davila et al. 2010) shows a similar pattern with heterozygote protection and homozygote susceptibiity, indicating that acquired immunity may also affected in individuals with the SNP. Whether this is as a result of innate immune or acquired immune defects is not clear. Also intriguing is the association of systemic lupus erythematosis (SLE) with the S180L SNP (Castiblanco, Varela et al. 2008), as the role of TLRs 2 and 4 in the pathogensis of SLE is not thought to be significant (Kim, Sreih et al. 2009).

1.3.5 PRRs and tuberculosis

M. tuberculosis is recognised by numerous PRRs, including TLRs, NLRs and CLRs (Fenton, Riley et al. 2005, Killick, Ni Cheallaigh et al. 2013) (Figure 10). PRRs mediate the uptake of *M. tuberculosis* into host cells and also are responsible for activation of the innate immune response, including secretion of key pro-inflammatory cytokines; antimicrobial peptides, such as cathelicidin; and activation of anti-mycobacterial effectors, such as autophagy and generation of reactive oxygen and reactive nitrogen species.

1.3.5.1 Mycobacterial PAMPs

M. tuberculosis contains a large variety of PAMPs. TLR2 ligands present in mycobacteria include triacylated lipoprotein TLR2/1 ligands, such as mycobacterial p19 lipoprotein, diacylated lipoprotein TLR2/6 ligands, including lipoarabinomannan (LAM), lipomannan (LM) and phosphatidyl-*myo*-inositol (PIM) (Quesniaux, Fremond et al. 2004) . Alternatively, whole viable mycobacteria and heat-labile mycobacterial soluble factor can act as TLR4 ligands (Tsuji, Matsumoto et al. 2000, Means, Jones et al. 2001) and CpG-

containing mycobacterial DNA can engage TLR9 (Quesniaux, Fremond et al. 2004). Mycobacteria also contain the NOD2 ligand muramyl dipeptide (MDP) and carbohydrates such as Mannose-capped LAM (ManLAM), which are recognised by C-type lectins (particularly the MR).

1.3.5.2 Cellular biology of PRRs and TB

Uptake of *M. tuberculosis* is mediated via a variety of host cell receptors, including complement receptor C3 (Schlesinger, Bellinger-Kawahara et al. 1990), mannose-binding receptors, such as mannose-binding lectin, surfactant protein A and D, the membrane-bound macrophage mannose receptor (MR), and dendritic cell-specific intercellular-adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN) (Korbel, Schneider et al. 2008). NF-κB nuclear translocation is a key step in activation of a large number of innate immune responses. Work on CHO/CD14 cells showed that stimulation of CHO/CD1 cells expressing TLR2 or TLR4 constructs respond to infection with *M. tuberculosis* with activation of NFκB, unlike cells without TLR2 or 4 (Means, Wang et al. 1999). Interestingly, whereas live *M. tuberculosis* activated NF-κB via both TLR2 and TLR4, heat-killed bacteria only activated TLR2. Live *M. tuberculosis* predominantly engaged TLR2, as expression of a dominant-negative TLR2 construct in RAW 264.7 cells blocked live *M. tuberculosis*-induced NFκB activation by around 80%, whereas expression of a dominant-negative TLR4 construct blocked by around 40%. Co-expression of both TLR2 and TLR4 dominant negative constructs resulted in an almost complete absence of NFκB activation (Means, Jones et al. 2001).

Given that stimulation of TLRs 2 and 4 by *M. tuberculosis* lead to NF-κB activation, it is not surprising that they play a role in secretion of pro-inflammatory cytokines known to

be downstream of NF-κB. Murine macrophages transfected with a dominant/negative TLR2 construct failed to produce TNF-α in response to stimulation with *M. tuberculosis* and a number of mycobacterial ligands (Underhill, Ozinsky et al. 1999). Primary human alveolar macrophages also showed TLR4 dependent TNF-α production (Means, Jones et al. 2001). *M. tuberculosis* induced production of IL-1β is also TLR2 dependent in macrophages. (Holscher, Reiling et al. 2008, Kleinnijenhuis, Joosten et al. 2009) NOD2 may also play an additional role, as monocytes from individuals with a functionally defective mutation in NOD2 fail to produce TNFα and IL-10 in response to stimulation with *M. tuberculosis* (Ferwerda, Girardin et al. 2005). Human monocyte-derived macrophages (MDM) and alveolar macrophages treated with siRNA against NOD2 show a reduction in production of TNF-α and IL-1β in response to infection with *M. tuberculosis* (Brooks, Rajaram et al. 2011).

Apoptosis has also been shown to be TLR2 and/or 4 dependant (Means, Jones et al. 2001, Lopez, Sly et al. 2003, Sánchez, Rojas et al. 2010) as has production of beta-defensin (Birchler, Seibl et al. 2001) and cathelicidin (Liu, Stenger et al. 2006). A mycobacterial lipoprotein, 19 kDa, was shown to activate macrophages, resulting in the control of intracellular bacterial growth, in a TLR2-dependent manner (Thoma-Uszynski, Stenger et al. 2001). This pathway was NO-dependent in murine cells, but NO-independent in human macrophages. In human cells, stimulating TLR2 results in a pathway which involves the up-regulation of the vitamin D receptor and the vitamin D activating enzyme, CYP2R1, transcription of cathelicidin and beta 4 defensin and culminated in the colocalisation of cathelicidin and mycobacteria and killing of intracellular mycobacteria (Liu, Stenger et al. 2006). Similarly, autophagy in response to the mycobacterial lipoprotein LpqH is TLR2/1 and vitamin D receptor-dependent

(Shin, Yuk et al. 2010). Interestingly, though, TLR2/4$^{-/-}$ macrophages show no defect in killing intracellular mycobacteria *in vitro* (Holscher, Reiling et al. 2008). TLRs 2 and 4 have also been shown to play a role in responses to *M. tuberculosis* in cells other than macrophages. DC maturation in response to BCG is dependent on TLRs 2 and 4 (Tsuji, Matsumoto et al. 2000), as is IFN-γ production by CD4 and CD8 lymphocytes stimulated *in vitro* with mycobacteria (Heldwein, Liang et al. 2003).

Mycobacteria actively inhibit host immune responses and TLR2 may also be involved in these pathways, to the benefit of the bacillus. Noss and colleagues reported that inhibition of MHC-II Ag processing by 19kDA mycobacterial lipoprotein was TLR2 dependent (Noss, Pai et al. 2001) and further work by Gehring and co-workers demonstrated that this was mediated via TLR2-dependent inhibition of IFN-γ-regulated HLA-DR protein and mRNA expression (Gehring, Rojas et al. 2003). It has subsequently been shown that exposure of the murine J774 macrophage cell line to the TLR2 ligands 19 kDa or zymosan, but not the TLR4 ligand LPS, inhibits IFN-γ-induced killing of *M. bovis* Bacillus Calmette–Guérin (BCG) in a TLR2-dependent manner (Arko-Mensah, Julián et al. 2007).

The mannose receptor (MR) is highly expressed on macrophages and its ligation by mycobacterial polysaccharides, such as ManLAM, has an anti-inflammatory effect, characterised by the production of IL-4, IL-13 and inhibition of bactericidal oxidative responses (Nigou, Zelle-Rieser et al. 2001). Moreover, engagement of the MR with ManLAM results in impaired phagosome maturation (Kang, Azad et al. 2005), suggesting that there may be an important role for such mannosylated polysaccharides in the evasion of host immune responses by mycobacteria.

The scavenger receptor A (SRA) and macrophage receptor with collagenous structure (MARCO) have also been shown to play a role in immunoregulatory responses to *M. tuberculosis in vitro.* SRA is up-regulated in macrophages after exposure to BCG and signalling from SRA has an immunoregulatory effect and has been shown to protect against endotoxin shock in BCG-primed mice (Haworth, Platt et al. 1997). MARCO has been shown to bind to trehalose 6,6′-dimycolate, also known as cord factor, a mycobacterial cell wall lipid which is reported to be the key mycobacterial factor in granuloma formation (Bekierkunst, Levij et al. 1969). MARCO tethers cord factor to the cell membrane and enables inflammatory responses in co-operation with TLR2 and CD14 (Bowdish, Sakamoto et al. 2009).

1.3.5.3 PRRs and tuberculosis: In vivo infection models

Studies on susceptibility to tuberculosis infection in mice deficient in various receptors have been informative on the role of PRRS *in vivo*, although results have varied between studies, depending on dose and route of infection and the length of time for which the mice were observed. TLR2-deficient mice can survive low-dose infection, but show an increased susceptibility to high-dose aerosol infection at later time points, with increased lung inflammation, decreased granuloma formation and increased bacterial burden. TLR2 deficient mice succumbed to infection around 150 days post-infection (Drennan, Nicolle et al. 2004). Other groups (Reiling, Holscher et al. 2002, Bafica, Scanga et al. 2005) also found some degree of increased susceptibility in TLR2 knockout mice with high doses of *M. tuberculosis*. Whether TLR4 knockout mice show increased susceptibility is more controversial, with initial work on TLR4 deficient C3H/HeN mice showing increased susceptibility and bacterial burden and dissemination when

compared to HeJ mice (Abel, Thieblemont et al. 2002). However, another group failed to replicate these results, even with high dose infection (Reiling, Holscher et al. 2002). Bafica and co-workers also reported increased susceptibility associated with impaired production of IL-12p40 and interferon gamma in TLR9 deficient mice (Bafica, Scanga et al. 2005) and TLR2/9 double knockout mice have been shown to be particularly susceptible (Bafica, Scanga et al. 2005, Mayer-Barber, Barber et al. 2010). TLR2, TLR4 and TLR9 seem to be less important. at earlier time points, as TLR2, TLR4 and TLR9 triple deficient mice show no increase in bacterial burden or death at 21 and 82 days following a low dose infection [80].

PRRs other than TLRs may also play a role *in vivo*. NOD2 deficiency resulted in decreased immunopathology and T cell recruitment to the lungs at early time points after infection with BCG (Divangahi, Mostowy et al. 2008). Moreover, NOD2 deficient mice showed increased susceptibility to chronic infection with *M. tuberculosis,* with a higher bacterial burden in the lungs six months after infection. These mice succumbed to infection sooner than the corresponding wild-type controls (Divangahi, Mostowy et al. 2008).

Scavenger receptors and C-type lectins, such as the MR and specific ICAM-3 grabbing nonintegrin-related receptors (SIGNR and DC-SIGN), have been shown to recognise *M. tuberculosis in vitro.* However, murine models have shown that these may be less important *in vivo*, as mice deficient in Class A SRs (SRAI and SRAII), Class B SR (CD36), MR, specific ICAM-3 grabbing nonintegrin-related (SIGNR) do not show an impaired response to acute or chronic infection with *M. tuberculosis*. Moreover, double deficiency in SRA (I and II) and CD36 or in MR and SIGNR has only a limited effect on

the long term response to *M. tuberculosis* infection, as well as on uptake of mycobacteria by macrophages and the TNF response (Court, Vasseur et al. 2010)

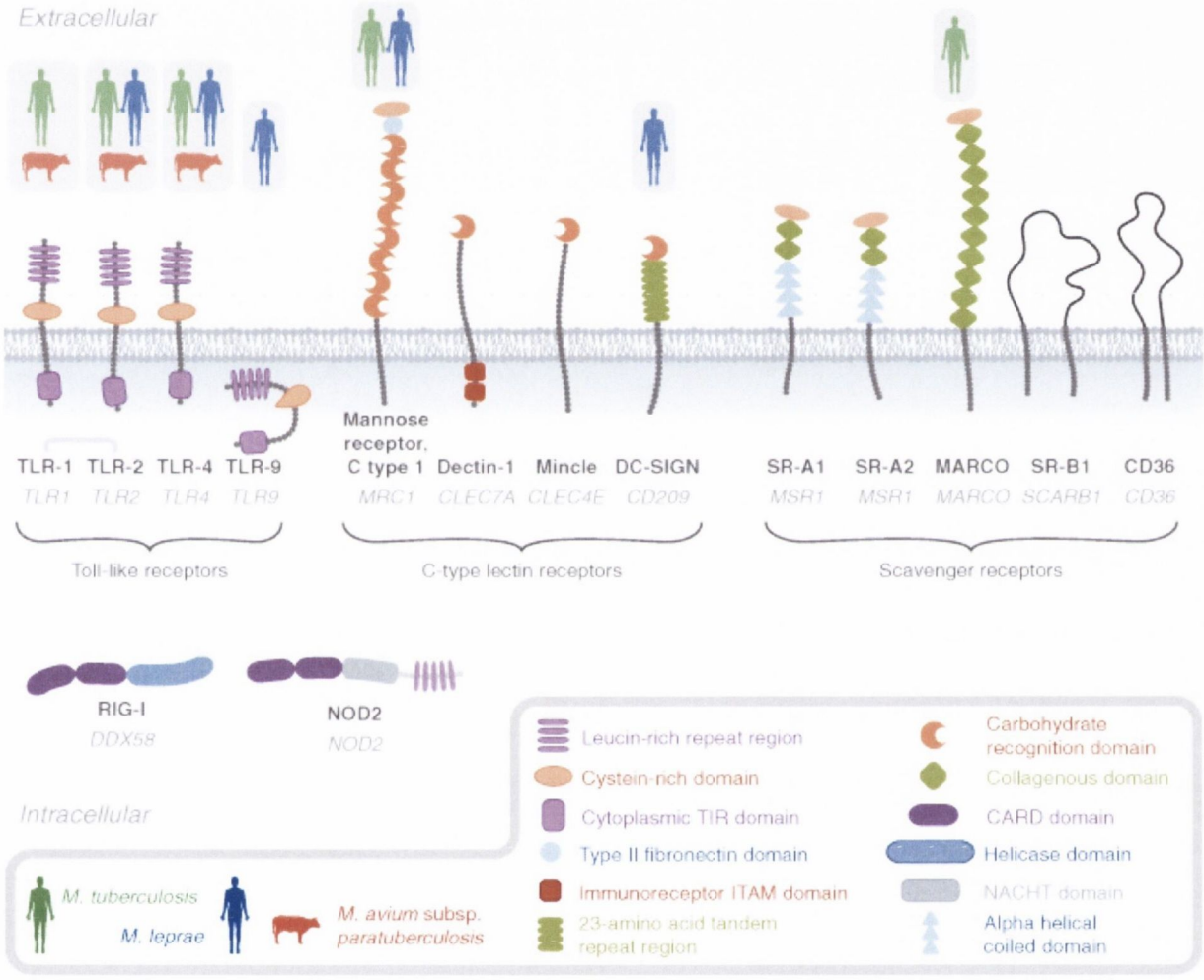


Figure 1-10: Pattern recognition receptors (PRRs) and TB.

PRRs involved in the non-opsonic recognition of mycobacterial pathogens. PRRs and their cellular locations are shown. Gene symbols are indicated in grey text and PRR genes with sequence variants associated with resistance to various mycobacterial infections are also indicated. These infections are tuberculosis caused by ***M. tuberculosis***, leprosy caused by ***M. leprae*** . *Reprinted from Killick et al, Cellular Microbiology, 2013.*

1.3.5.4 PRRs and tuberculosis: Human genetic studies

Several studies have demonstrated associations between single nucleotide polymorphisms (SNP) in PRRs and susceptibility to tuberculosis (Yim and Selvaraj 2010). An association between TLR2 polymorphisms and tuberculosis, particularly disseminated tuberculosis, has been seen in a number of studies carried out in various ethnic populations in Africa and Asia (Ben-Ali, Barbouche et al. 2004, Ogus, Yoldas et al. 2004, Thuong, Hawn et al. 2007, Caws, Thwaites et al. 2008, Chen, Hsiao et al. 2010, Motsinger-Reif, Antas et al. 2010, Velez, Wejse et al. 2010). No studies have yet showed an association between TLR 4 polymorphisms and tuberculosis in HIV-negative patients, but two studies have reported a link between a TLR4 SNP and tuberculosis susceptibility in HIV-infected individuals (Ferwerda, Kibiki et al. 2007, Pulido, Leal et al. 2010). Another study has also reported an association between a TLR9 polymorphism and tuberculosis (Velez, Wejse et al. 2010). Two studies have reported an association between NOD2 polymorphisms and susceptiblity to mycobacterial infection (Austin, Ma et al. 2008, Zhang, Huang et al. 2009, Shah, Vary et al. 2012).

1.3.6 MyD88 and tuberculosis

As discussed above, MyD88 is a signalling adaptor protein for all TLRs except TLR3 and also is required for IL-1, IL-18 and IL-33 signalling as well as playing a role in some IFN-γ signalling pathways. Unsurprisingly, given the importance of TLRs, IL-1 and IFN-γ in TB immunity, MyD88 plays a critical role in many immune responses to tuberculosis. *In vitro* studies have shown that MyD88$^{-/-}$ macrophages show impaired phagosome maturation and impaired killing of intracellular mycobacteria (Holscher, Reiling et al. 2008). Shi and colleagues reported a defect in iNOS production in MyD88^{-}

/cells infected with *M. tuberculosis,* although they did not see a decrease in mycobactericidal activity in unstimulated or IFN-γ stimulated MyD88$^{-/-}$ macrophages (Shi, Nathan et al. 2003). *In vivo* studies have shown that MyD88$^{-/-}$ mice are extremely susceptible to infection with *M. tuberculosis*, and have a much more profound defect in immunity than TLR2/4/9$^{-/-}$ mice The role of MyD88 in induction of adaptive immune responses *in vivo* is controversial; one study reported normal CD4 and CD8 T cell responses in the absence of MyD88 following infection with *M. tuberculosis* (although the mice did succumb to infection) (Holscher, Reiling et al. 2008). However, another study found a compromised adaptive response in MyD88-deficient mice infected with the mycobacterial pathogen *M. avium* (Feng, Scanga et al. 2003). The profound increase in susceptibility to mycobacterial infection in MyD88$^{-/-}$ mice has been attributed to the role of MyD88 in IL-1 signalling, as IL-1-deficient mice are also profoundly susceptible (Mayer-Barber, Barber et al. 2010), and the increased susceptibility seen in TLR2/4/9$^{-/-}$ mice is much less marked than that of MyD88-/- mice.

Intriguingly, tuberculosis has not been reported in individuals with MyD88 loss-of-function mutations (von Bernuth, Picard et al. 2008, Picard, Casanova et al. 2011). As described previously, these individuals are susceptible to pyogenic infections, particularly in childhood. It is plausible that the small sample size described has not been sufficient to contain individuals exposed to tuberculosis, although disseminated *M. avium* infection has been described in an individual with a deficiency in IRAK-4, downstream of MyD88. A recent publication described a SNP in the 3' UTR of MyD88, rs 6853, which associated with altered responses to TB *in vitro* and susceptibility *in vivo* when individuals are stratified by the Mal S180L SNP (Capparelli, De Chiara et al. 2013).

1.3.7 Mal and tuberculosis

To date, there has been no studies published on *in vitro* responses in Mal-deficient macrophages. A single paper has reported on tuberculosis infection in $Mal^{-/-}$ mice. The authors found no difference in bacterial burden and lung inflammation at an early time point of 28 days (well in advance of where differences between WT and $TLR2^{-/-}$ mice emerge). Further time points were not assessed for bacterial burden, but the authors but did show a drop-off in weight in the TB-infected $Mal^{-/-}$ mice at later time points (Fremond, Togbe et al. 2007).

Both single nucleotide polymorphisms in Mal have been associated with tuberculosis susceptibility. The D96N polymorphism has been associated with tuberculosis (TB) susceptibility in a Chinese population (Zhang, Xue et al. 2011). Heterozygosity for the S180L SNP has been reported to be protective against tuberculosis (Khor, Chapman et al. 2007, Castiblanco, Varela et al. 2008, Capparelli, De Chiara et al. 2013) and homozygosity has been reported to increase susceptibility (Selvaraj, Harishankar et al. 2010, Capparelli, De Chiara et al. 2013). Other studies have failed to replicate these findings (Nejentsev, Thye et al. 2008). Replication of genetic associations with TB susceptibility has proved notoriously difficult (Stein 2011). Of note, a recent metanalysis concluded that S180L heterozygotes are protected from tuberculosis, particularly in European populations (Liu, Li et al. 2014).

1.4 IFN-γ

1.4.1 IFN-γ

IFN-γ is the principal host-derived signal for macrophage activation (Nathan, Prendergast et al. 1984). Macrophage activation results in improved microbicidal function (Dalton, Pitts-Meek et al. 1993), production of reactive oxygen and nitrogen species, secretion of pro-inflammatory cytokines, and enhanced antigen processing and presentation (Dalton, Pitts-Meek et al. 1993). IFN-γ -activated macrophages are often referred to as being polarised to a classically activated or "M1" phenotype (Martinez and Gordon 2014, Murray, Allen et al. 2014). IFN-γ also acts as a chemokine and promotes growth and differentiation of certain cell types. IFN-γ promotes the development of Th1 immune response via a number of mechanisms, and in its absence the immune response to infection is skewed to Th2 (Wang, Reiner et al. 1994). IFN-γ also acts to limit inflammationon by causing apoptosis of CD4+ T-cells (Dalton, Haynes et al. 2000) and by inhibiting $CD4^+$ T cell production of IL-17, which regulates neutrophil recruitment (Nandi and Behar 2011). In addition, IFN-γ directly inhibits pathogenic neutrophil accumulation in the infected lung and impairs neutrophil survival (Nandi and Behar 2011). The immunoregulatory functions of IFN-γ are also illustrated by the increased susceptibility and severity of experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) in mice genetically deficient in IFN-γ or the IFN-γ receptor (Ferber, Brocke et al. 1996, Manoury-Schwartz, Chiocchia et al. 1997). It is required for immunity against intracellular pathogens including Leishmania major (Wang, Reiner et al. 1994), Salmonella (Bao, Beagley et al. 2000), Listeria monocytogenes and vaccinia virus (Huang, Hendriks et al. 1993, Dorman and Holland 2000) and *M. tuberculosis*.

1.4.2 Production of IFN-γ

IFN-γ is secreted in large quantities by activated Th1 cells (Mosmann and Coffman 1989), activated CD8+ cytotoxic cells (Sad, Marcotte et al. 1995) and NK cells (Perussia 1991). Cross-linkage of the T-cell receptor is the main stimulus for interferon γ production in T-cells. In NK cells, IFN-γ production is induced by innate macrophage-derived cytokines, particularly IL-12 (Trinchieri 1995), IL-18 (Dinarello 1999) and TNF-α (Tripp, Wolf et al. 1993), and also by IFN-γ itself (Hardy and Sawada 1989). There are also numerous reports of macrophages secreting IFN-γ (Fultz, Barber et al. 1993, Di Marzio, Puddu et al. 1994, Fenton, Vermeulen et al. 1997, Darwich, Coma et al. 2009), albeit in smaller quantities, although some authors have highlighted the role of contaminating cells in producing IFN-γ (Schleicher, Hesse et al. 2005).

1.4.3 IFN-γ Receptors

The IFN-γ receptor (IFNGR) is expressed on the surface of most nucleated cells, including macrophages, B-lymphocytes, activated T-lymphocytes and epithelial cells(Valente, Ozmen et al. 1992). It is made up of two components: two ligand-binding IFNGR1 chains associated with two signal-transducing IFNGR2 chains. The IFGR signals through the Jak-Stat pathway (Figure 11), as do many other cytokines, growth factors and hormones, including the type 1 interferons IFN-α and IFN-β (Ihle 1996). The pathway involves sequential recruitment and activation of Janus family kinases (Jaks 1-3 and Tyk) and signal transducers and activators of transcription (Stats 1-6) and culminates in phosphorylation of Stat1 and the Y701 site and phosphorylated dissociated Stat1 entering the nucleus, and binding to promoter elements to initiate or suppress the transcription of IFN-γ-regulated genes (Darnell, Kerr et al. 1994) (Figure 11). Many of the induced genes are themselves

transcription factors, e.g. IRF-1, IRF- 2 and IRF-9, which drive further alterations in gene expression.

In addition, ligand binding to the IFNGR complex also induces signalling via Stat1-independent pathways. These include the activation of other Stat complexes such as Stat1 heterodimers (e.g., Stat1:Stat2) and heterotrimers (e.g., Stat1:Stat1:IRF-9, Stat1:Stat2:IRF-9). These complexes also act as transcription factors (Ramana, Gil et al. 2001). Other signalling cascades modulated by IFN-γ include p38 MAPK (Valledor, Sanchez-Tillo et al. 2008, Matsuzawa, Kim et al. 2012), PI3K (Choudhury 2004) and protein kinase C(Ivaska, Bosca et al. 2003, Hardy, Diallo et al. 2009) (Figure 12) These Stat1-independent pathways may play a critical role in TB immunity, as IFN-γ has been reported to induce the formation and maturation of autophagosomes via an IRGM-independent pathway which used JAK1/2 and p38 MAPK but not STAT1 signalling (Matsuzawa, Kim et al. 2012).

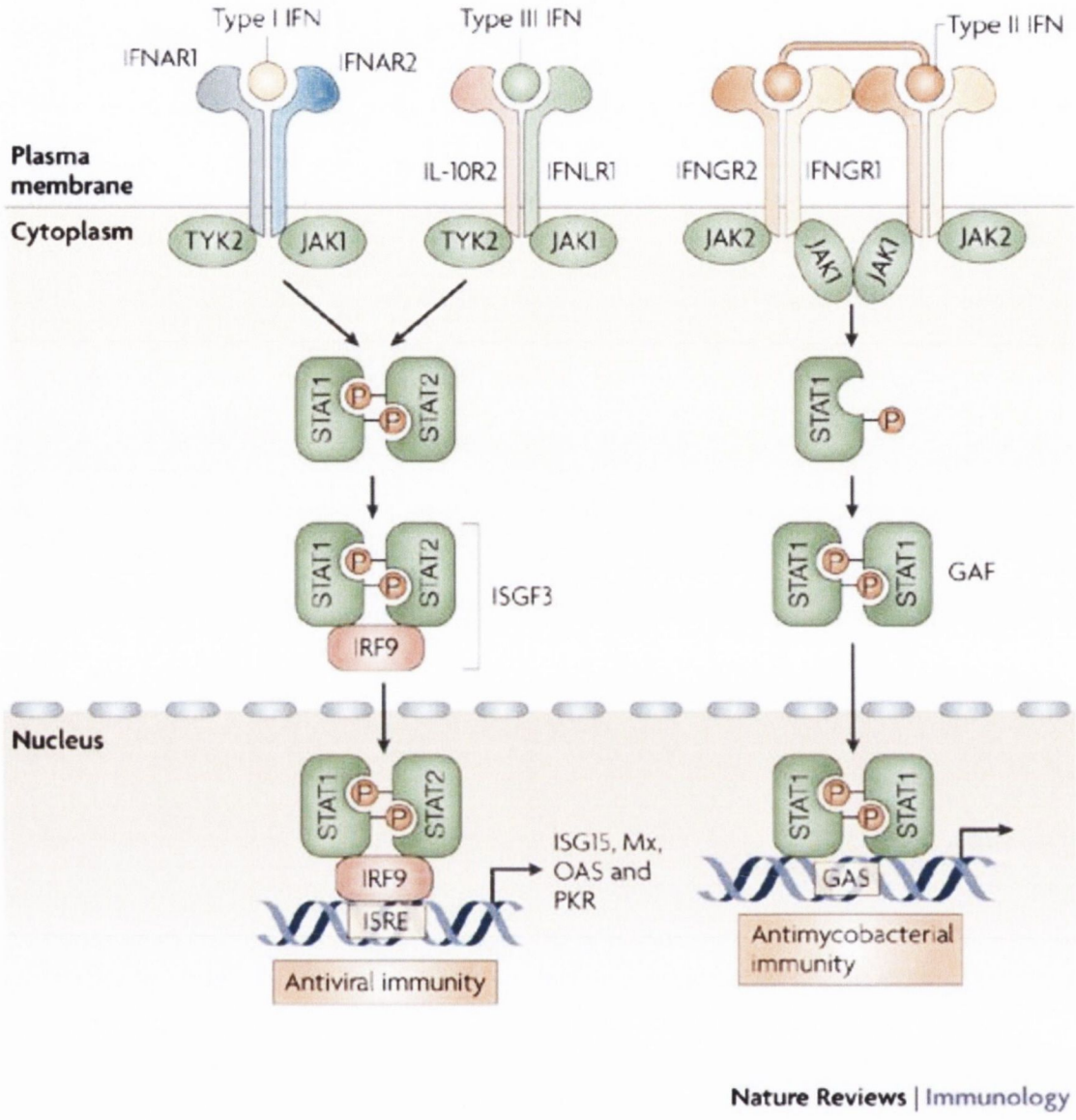


Figure 1-11: IFNγR-Stat1 signalling.

The canonical Jak-Stat signalling pathway (right) is activated by IFN-γ binding to IFNγR causing dimerisation. This leads to phosphorylation and nuclear translocation of a complex consisting of Stat-1, Stat-2 and IRF9. *Reprinted from Sadler, Nature Reviews Immunology, 2008*

1.4.4 p38 MAP kinases

MAP kinases are members of numerous diverse signaling cascades which are activated in response to a variety of extracellular stimuli. There are four subgroups of MAP kinases; (1) extracellular signal-regulated kinases (ERKs), (2) c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), (3) ERK/ big MAP kinase 1 (BMK1), and (4) the p38 group of protein kinases. p38 MAP kinases may be activated in response to UV light, heat, osmotic shock, inflammatory cytokines (TNF-α, IL-1 and IFN-γ), and growth factors (CSF-1). p38 MAP kinases regulates gene expression and post-translational modification of genes(Platanias 2003, Valledor, Sanchez-Tillo et al. 2008).

1.4.5 Regulation of IFN-γ signalling

IFN-γ signalling is regulated at a number of points. Signalling can be amplified by phosphorylation of Stat1 at S727 (in addition to its phosphorylation at Y701) thereby enhancing its ability to activate transcription of target genes. LPS, TNFα, IL-12, IL-2, type 1 interferons and IFN-γ itself all have the ability to phosphorylate Stat1 at this site and thereby increase cellular responses to IFN-γ. Interestingly, IFN-γ-induced maturation of *M. tuberculosis*-containing phagosomes is abrogated by the TNF blockers adalimumab, infliximab and etanercept (Harris, Hope et al. 2008), suggesting that TNF-α regulation of the IFN-γ signalling pathway is involved in TB immunity. Protein kinases including mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K)/AKT also have the ability to phosphorylate Stat1 at this site.

In addition, a number of negative regulatory mechanisms act to limit IFN-γ signalling. These include regulation of the expression of IFNGR2, control of the entry of Stat1 to the

nucleus and internalisation and degradation of the IFN-γ:IFNGR1 complex. An additional important mode of negative regulation of IFN-γ signalling occurs via suppressor of cytokine signalling-1 (SOCS-1), which is induced by IFN-γ signalling and promotes degradation of IFN-γ signalling machinery (Schroder, Hertzog et al. 2004).

1.4.5 IFN-γ, autophagy and phagosome maturation

Numerous studies have demonstrated that activating macrophages with IFN-γ increases acidification and maturation of mycobacteria-containing phagosomes and, in turn, leads to decreased intracellular survival of the bacilli (Schaible, Sturgill-Koszycki et al. 1998, Via, Fratti et al. 1998). The mechanism through which IFN-γ exerts this effect is not understood, although it is known to stimulate the recruitment of Irgm1 (formerly LRG-47, IRGM in humans) to the mycobacteria-containing phagosome. Recruitment of Irgm1 which may facilitate fusion with late endosomes or recruitment of NOS2, a process that is normally inhibited by pathogenic mycobacteria (Miller, Fratti et al. 2004, MacMicking 2005). IFN-γ may act through the induction of autophagy (Gutierrez, Master et al. 2004, Singh, Davis et al. 2006, Singh, Ornatowski et al. 2010). In addition, IFN-γ has been reported to induce the formation and maturation of autophagosomes independently of IRGM via a pathway which uses JAK1/2 and p38 MAPK but not STAT1 signalling (Matsuzawa, Kim et al. 2012). TNF and IFN- γ have a synergistic role in phagosome maturation, as evidenced by the finding that TNF blocking drugs inhibit IFN-γ-dependent phagosome maturation, while pre-treatment of macrophages with IFN-γ greatly enhances TNF-α release in response to infection with *M. tuberculosis* (Harris, Hope et al. 2008).

1.4.7 IFN-γ and disease

Given its pleiotropic role in innate and acquired immunity, it is not surprising IFN-γ is required for immune responses to a number of different infectious pathogens. Evidence from murine models and/or reports of humans with defects in the IFN-γ signalling pathway showing increased susceptibility to *Pneumococcus*, *Salmonella, Legionella, Listeria, Toxoplasma, Herpes simplex,* vaccinia virus and malaria (Huang, Hendriks et al. 1993, Bouley, Kanangat et al. 1995, Harty and Bevan 1995, Gazzinelli, Amichay et al. 1996, Heath, Chrisp et al. 1996, Hess, Ladel et al. 1996, Favre, Ryffel et al. 1997, Rubins and Pomeroy 1997, Pomeroy, Delong et al. 1998, Dorman and Holland 2000, McCall and Sauerwein 2010). IFN-γ signalling also plays a role in autoimmune diseases, including systemic lupus erythematosus, SLE (Peng, Moslehi et al. 1997, Csiszár, Nagy et al. 2000) and auto-immune nephritis. In addition, IFN-γ has been reported to be involved in atherogenesis (Schroecksnadel, Frick et al. 2006) and cancer (Brandacher, Winkler et al. 2006).

1.4.8 IFN-γ and TB

IFN-γ is known to play a critical role in immune responses to *M. tuberculosis*. It is produced in vitro and in vivo in response to infection with *M. tuberculosis* (O'Garra, Redford et al. 2013). In TB infection it activates macrophages and promotes nitric oxide production, autophagy, phagosome maturation and killing of intracellular mycobacteria (Denis 1991, Flesch and Kaufmann 1991, Chan, Xing et al. 1992, Schaible, Sturgill-Koszycki et al. 1998, Via, Fratti et al. 1998, Gutierrez, Master et al. 2004). Mice deficient in IFN-γ fail to control infection with *M. tuberculosis*, and display an absence of granulomas and increased tissue necrosis (Cooper, Dalton et al. 1993, Flynn, Chan et al.

1993). $IFN^{-/-}$ mice infected with Mtb show a marked increase in lung inflammation (Pearl, Saunders et al. 2001). Individuals with complete deficiency in IFN-γ signalling show increased susceptibility to all mycobacteria, including avirulent environmental mycobacteria, and poor formation of granulomas; whereas individuals with partial defects in IFN-γ signalling show an increased susceptibility to virulent mycobacteria only, and formation of paucibacillary granulomas (Dorman and Holland 1998, Dorman and Holland 2000, Filipe-Santos, Bustamante et al. 2006, Bogunovic, Byun et al. 2012).

1.5 Aims and objectives

The hypothesis of this project was that Mal plays a role in macrophage responses to tuberculosis, and that the S180L polymorphism affects these responses. The aims are as follows:

1) To determine which macrophage responses to *M. tuberculosis* are Mal-dependent

2) To determine the effect of Mal S180L on Mal-dependent macrophage responses.

Chapter 2 : METHODS

2.1 Cell culture

2.1.1 General cell culture

All cells except human monocyte-derived macrophages and HEK 293 cells were grown in RPMI 1640 medium (Biosera), which was supplemented with 2 mM L-Glutamine (Gibco) and 8% (v/v) heat-inactivated (56 C for 30 minutes) foetal calf serum (Biosera). Foetal calf serum was filter-sterilised with a 0.22μm syringe-driven filter (Millipore) before addition to medium. 50 units/ml of penicillin (Gibco) and 50μg/ml streptomycin (Gibco) was added to medium unless cells were being prepared for *Mtb* infection, in which case antibiotic-free medium was used. All macrophages were grown in in sterile plastic tissue culture flasks (Greiner Bio-one) amd maintained in a humidified incubator at 37°C and 5% v/v CO_2.

2.1.2 Isolation and culture of murine bone marrow-derived macrophages.

Mice were sacrificed by cervical dislocation and bone marrow was isolated from the femurs and tibiae of mice using sterile dissection tools. The tissue was flushed out of the bones using a 27 gauge needle. Cell aggregates were disrupted by repeated passage through a 19 gauge needle. The cell suspension was centrifuged at 500 g at room temperature for 5 min and the cell pellet was resuspended in 0.16M NH_4Cl solution for 2 minutes to lyse red blood cells. The suspension was centrifuged at 500 g at room temperature for 5 min, the pellet was resuspended in 5 ml of medium and the cells were counted. Cells were seeded at 1 x 10^6 cells/ml in sterile plastic tissue culture flasks (Greiner Bio-one) in medium (see above) supplemented with 15% (v/v) L929-conditioned medium (M-CSF).

Three days after seeding, the cells were fed by addition of a further 15% v/v of L929-conditioned medium . On day 6, medium and non-adherent cells were discarded. Cells were detached in fresh medium by scraping. Cell suspensions were spun at 500 g for 5 min and cell pellets were resuspended in 10 ml of medium. Cells were counted using the trypan blue exclusion method and cells were plated at the appropriate concentration in medium supplemented with 15% v/v of L929 conditioned medium.

On day 7, cells were treated as required.

2.1.3 Immortalisation of bone-marrow derived macrophages

Immortalised bone marrow-derived WT, $Mal^{-/-}$, $MyD88^{-/-}$, $TLR2^{-/-}$,$TLR4^{-/-}$,$TLR2/4^{-/-}$ macrophages were provided by Prof Doug Golenbock and Prof Kate Fitzgerald. These were generated from primary bone marrow-derived macrophages using J2 transforming retroviruses (Roberson and Walker 1988, Hornung, Bauernfeind et al. 2008). J2 retroviruses express v-Raf and v-Myc oncogenes. V-raf acts as a MAP3 kinase and overdrives the MEK1/2 and ERK 1/2 pathways. Myc is a transcription factor which drives cell proliferation by upregulating cyclins and downregulating p21, regulates cell growth by upregulating ribosomal RNA and proteins, downregulates apoptosis by downregulating Bcl-2. To immortalise macrophages, primary bone marrow cells were incubated in L929 mouse fibroblast–conditioned medium for 3-4 d for the induction of macrophage differentiation. Subsequently, cells were infected with J2 recombinant retrovirus. Cells were maintained in culture for 3–6 months and were slowly 'weaned off' L929 supernatant until they were growing in the absence of conditioned medium. Macrophage phenotype was verified by surface expression of the markers CD11b (M1/70; BD Pharmingen) and F4/80 (BM8; eBiosciences) as well as a range of functional parameters, including responsiveness to Toll-

like receptor ligands and bacterial uptake. Macrophage cell lines were also generated from S180L heterozygote and S180L homozygote mice, but were still undergoing 'weaning' from L929 medium at the time of writing this thesis.

2.1.4 Culture of L929 cell line.

The L929 cell line is a murine connective tissue cell line which secretes large amounts of macrophage-colony stimulating factor (mCSF or CSF-1) into their growth medium, which can be used to expand macrophages from bone marrow cultures (Murray, Allen et al. 2014).

Cells were thawed from frozen stocks (liquid nitrogen) and cultured in complete RPMI. Cells were cultured until confluent, then harvested by scraping, centrifuged and re-seeded in T175 cell culture flasks at 5 x 10^5 cells/ml and cultured for 7 days. At this point, cell medium was harvested, filter sterilized using a 0.22□m syringe filter and frozen. Cells were re-seeded at 5 x 10^5 cells/ml for future passages.

2.1.5 THP-1 cells

THP-1 cells are an acute monocytic leukaemia cell line. They were obtained from ATCC (ATCC-TIB-202, ATCC, Manassas, Virginia, US). Cell stocks were maintained in liquid nitrogen. Once thawed, cells were maintained at concentrations between 1 x 10^5 and 1 x 10^6/ml. Cells were passaged a maximum of 8 times prior to new stocks being used. Cells were differentiated into macrophage-like cells by treatment with phorbol myristate acetate (100 nmol/L) for 24 h and then cultured in normal medium for 3 days.

2.1.6 Isolation of primary human monocyte-derived macrophages

Venesection was carried out on healthy volunteers with ethical approval from the School of Medicine, Trinity College, Dublin. Blood was diluted at a ratio of 1:1 with sterile PBS (Sigma). Peripheral blood mononuclear cells were isolated by density gradient centrifugation on Lymphoprep. Mononuclear cells were washed to remove platelets. Red blood cells were lysed in red cell lysis buffer. Cell counts were adjusted to 1 x 10^6/ml and cultured in plastic dishes in RPMI 1640 with 10% human AB serum (Sigma). Non-adherent cells were removed 3 days post isolation, and medium was replaced by fresh medium. 7 days post isolation, non-adherent cells were discarded and adherent cells were removed by scraping, re-counted, and plated at 2 x 10^5/ml in 96-well plated for stimulation.

2.1.7 HEK 293 cells

Human embryonic kidney (HEK293) cells were purchased from European Collection of Animal Cell Cultures (Salisbury, UK). Cells were grown in Dulbecco's Modified Essential Medium (DMEM), supplemented with 10% foetal calf serum, 50 units/ml of penicillin, 50µg/ml streptomycin.

2.1.8 GFP-LC3 expressing macrophages

Immortalised bone marrow macrophages expressing GFP-LC3 were a kind gift from Hardy Kornfeld, Univeristy of Massachusetts. Cells were grown in medium containg 10µg/ml puromcyin (Sigma) to maintain selective pressure.

2.1.9 Cell counts.

Cell viability was determined by trypan blue exclusion. A 10µl sample of the cell suspension was diluted in 90µl of trypan blue, then 10 µl of the cell/trypan blue suspension

was loaded into a Hycor Glasstic counting slide (Hycor) and the number of viable cells was determined using a light microscope. The cell concentration was calculated using the following formula:

Number of cells/ml = viable cell number x 10^4 x dilution factor (10).

2.1.10 Transfection

Cells were transfected by nucleoporation with an Amaxa Nucleofector Device (Lonza, Wokingham, UK). THP-1 cells were cultured to a density of 1 x 10^6/ml and resuspended in 100µl of the appropriate electroporation buffer (Solution V, Lonza AG) with 40 nM siRNA against human Mal or nontargetting control (40nM, Dharmacon siGENOME SMARTpool, Thermo Scientific). The THP-1 high efficacy programme was used for electroporation. After transfection, cells were plated in antibiotic-free medium with 100nM PMA. Medium was replaced 24 hours later, and cells were stimulated 48-72 hours post-transfection.

Immortalised bone marrow-derived macrophages were harvested after 2-3 days in culture, transfected with siRNA against Beclin-1, Atg7, or non-targetting control (40nM, Dharmacon, siGENOME SMARTpool, Thermo Scientific, Lafayette, CO). iBMM were incubated overnight before use. Targetting efficienct was analyzed by Western blot for Beclin-1 and ATG-7 or q-PCR for Mal mRNA (this was carried out by Elizabeth Carroll).

2.1.11 Mal inhibitory peptides

Mal inhibitory peptide (Cat no. 613570) with the sequence RQIKIWFNRRMKWKKLQLRDAAPGGAIVS and matching control peptide (Cat no. 613571) were purchased from Calbiochem (Merck Millipore, Darmstadt, Germany). The Mal inhibitor is a synthetic peptide corresponding to the P125H region of Mal with an

antennapedia region to enable it to cross the cell membrane. It functions as a decoy peptide by binding to the TIR domain of TLRs 2 and 4(Horng, Barton et al. 2001). The control peptide consists of the reverse sequence. After an initial dose-finding experiment, the peptide was used at a concentration of 10μg/ml. Cells were pre-incubated with the peptide for 30 minutes prior to stimulation.

We also used viral inhibitory protein of TLR 4 (VIPER), a kind gift of Prof Andrew Bowie, Dept of Biochemistry and Immunology, Trinity College. This is an 11 amino acid long peptide with 9R delivery sequence which is derived from an immunomodulatory protein, A46, produced by Vaccinia virus. It masks TIR domains (critical binding) sites on Mal and TRAM and prevents TLR 4 interaction, thereby inhibiting TLR 4 signalling via Mal and TRAM (Lysakova-Devine, Keogh et al. 2010). The control peptide is an inert peptide of the same length. We carried out a dose-response experiment and subsequently used the peptide at a concentration of 10μM. Cells were pre-incubated with the peptide for 30 minutes prior to stimulation.

2.2 Bacterial culture

2.2.1 Culture of M. tuberculosis *H37Ra*

M. tuberculosis H37Ra, obtained from ATCC, was grown to log phase at 37°C in 5% CO_2 in Middlebrook 7H9 broth (Difco), supplemented with albumin-dextrose-catalase (Becton Dickinson) and 0.05% Tween-80 (Difco), and made up in endotoxin-free water.

2.2.2 Culture of M. tuberculosis *H37Ra*

M. tuberculosis H37Rv was grown to log phase at 37°C in 5% CO_2 in Middlebrook 7H9 broth (Difco), supplemented with albumin-dextrose-catalase (Becton Dickinson), and made up in endotoxin-free water. Tween was not included in the broth used to grow *M. tuberculosis* H37Rv to avoid the stripping of surface lipids (Ortalo-Magne, Lemassu et al. 1996). Prior to use a single cell suspension of *Mtb* H37Rv was obtained by pelleting bacteria by centrifugation at 4000 rpm, vortexing pellet with sterile glass beads (Sigma) for 60 seconds, reuspending bacteria in RPMU and centrifuging bacterial suspension at 400 rpm to remove clumps. Supernatants were then sonicated for 60 seconds prior to determining optical density. An OD reading of 0.1 was taken to be equivalent to a bacterial density of 2×10^7 bacteria/ml. Bacterial density was then adjusted by adding antibiotic-free complete medium until the desired density was obtained. The correlation between OD reading and multiplicity of infection was confirmed by fixing infected cells at 3 hours, staining with Auramine O and assessing intracellular bacterial load by fluorescence microscopy.

2.2.3 Preparation of irradiated M. tuberculosis *for in vitro use*

Irradiated *M. tuberculosis* H37Rv was obtained from Colorado Bio-resources. Prior to use a single cell suspension of irradiated *Mtb* H37Rv was obtained by pelleting bacteria by centrifugation at 4000 rpm, vortexing pellet with sterile glass beads (Sigma) for 60 seconds, reuspending bacteria in RPMU and centrifuging bacterial suspension at 400 rpm to remove clumps. Supernatants were then sonicated for 60 seconds prior to determining optical density. An OD reading of 0.1 was taken to be equivalent to a bacterial density of 2×10^7

bacteria/ml. Bacterial density was then adjusted by adding antibiotic-free complete medium until the desired density was obtained.

2.2.4 Assessment of uptake of M. tuberculosis *by fluorescence microscopy*

Macrophages were plated at 5 x 10^5/ml on glass coverslips and infected with *M. tuberculosis* H37Rv. Cells were fixed at 3 hours with 4% paraformaldehyde for 16 hours. Mycobacteria were stained with Rapid Modified Auramine O Stain Set (Scientific Device Laboratory Inc.) The number of mycobacteria/cell was quantified using a fluorescent microscope.

2.2.5 Quantification of intracellular M. tuberculosis

Macrophages were plated at 1 x 10^5/ml in antibiotic-free medium in 12-well plate. All conditions were done in duplicate or triplicate. Macrophages were infected with a suspension of *M. tuberculosis* H37Rv at a ratio of 10 bacteria/macrophage. Extracellular bacteria were washed off and medium was replaced at 3 hours.

Cell were lysed at 3, 24 or 72 hours by removing medium (this was added to cell lysate for the 24 and 72 hour time points), adding 0.5 ml of sterile filtered 0.1% Triton X, incubating for 5 minutes and washing with 1 ml PBS. Cell medium, Triton-X and PBS were pooled for each well and centrifuged at 3800 rpm for 10 minutes to harvest bacteria. The pellet was resuspended in 800µl of Middlebrook medium. Ten-fold serial dilutions were plated on Middlebrook agar plates in triplicate. Colonies were counted approximately 21 days later.

2.3 ELISA

2.3.1 Harvesting of cell supernatants for analysis by ELISA

Cells were plated at 1 x 10^6/ml and stimulated for 16 hours. Supernatants from cells stimulated with live virulent *M. tuberculosis* were syringe filtered through low protein-binding 0.22um filters (Millex filter unit, Merck Millipore)

2.3.2 Detection of cytokine production by ELISA (Enzyme-linked Immunosorbent Assay).

Purified anti-cytokine antibody was added to the wells of 96 well high binding ELISA plates (Greiner Bio-One) and incubated at 4°C overnight. Plates were washed 4 times with wash buffer (see Appendix I A7.3). After this, non specific antibody interactions were blocked by incubating the plates for 2hrs at room temperature with blocking solution (see Appendix I A7.3). After incubation with blocking solution, plates were washed 4 times and cell supernatant, medium or cytokine standard was added to each well. Medium alone was added to 3 wells in each plate to act as a negative control for the assay. In order to generate a standard curve for cytokine concentrations, recombinant mouse cytokines were serially diluted in doubling dilutions across 12 wells in triplicate. Once the plates were loaded with samples and standard, plates were incubated at 4°C overnight. The next day, plates were washed 4 times and incubated with detection antibody at room temperature for the indicated length of time. The plates were washed 4 times and Streptavidin Horseradish Peroxidase solution (see Appendix I A7.3) was added to the plate and incubated in the dark at room temperature for 20 min. Plates were washed 4 times and incubated with developing solution (see Appendix I A7.3). The enzymatic reaction was stopped by addition of Stop solution (see Appendix I A7.3). Finally absorbance was measured at 492nm or 550 nm in a microplate reader (Multiskan FC, Thermo Scientific) and cytokine concentration was calculated by interpolation of absorbance values from the cytokine standard curve.

2.3.3 IFN-β ELISA.

Purified anti-IFN-β antibody (IFN-β Coating Antibody, rat, #sc-57201, Santa Cruz Biotechnologies) was diluted in carbonate buffer (1/1000 dilution, see Appendix I A7.3) was added (40 □l/well) to the wells of 96 well high binding ELISA plates (Greiner Bio-One) and incubated at 4°C overnight. Plates were washed 4 times with wash buffer. After this, non specific antibody interactions were blocked by incubating the plates for 2hrs at 37°C with100 µl/well of 10% FCS/PBS. After the incubation with blocking solution, plates were washed 4 times and 40 µl of cell supernatant, medium or cytokine standard was added to each well. Medium alone was added to 3 wells in each plate to act as a negative control for the assay. In order to generate a standard curve for IFN-β concentrations, recombinant IFN-β (Murine recombinant IFN-β, Alpha Technologies) were serially diluted in doubling dilutions across 12 wells in triplicate starting from 5 U/ml of IFN-β. Once the plates were loaded with samples and standard, plates were incubated at 4°C overnight. The next day, plates were washed 4 times and incubated with 40 µl/well of diluted (1/2000 dilution in 10% FCS/PBS) IFN-β detection antibody (Murine IFN-β Detection Antibody, rabbit, #32400-1, PBL Interferon Source) and incubated at 4°C overnight. The plates were washed 4 times and 40 µl/well of diluted (1/2000 in 10% FCS/PBS) was added to the plate and incubated in the dark at room temperature for 20 min. Plates were washed 4 times and incubated with 40 µl/well of TMB developing solution (see Appendix I A7.3). The enzymatic reaction was stopped by addition of 20 µl/well of Stop solution. Finally absorbance was measured at 450nm in a microplate reader (Multiskan FC, Thermo Scientific) and IFN-β concentration was calculated by interpolation of absorbance values from the IFN-β standard curve.

2.4 Other Macrophage Functional Assays

2.4.1 Quantifying cell death

Cells were stained simultaneously with 5 μg/ml Propidium Iodide (PI), 20 μg/ml Hoechst 33342 and 50 μg/ml Hoechst 33258. For analysis of cells infected with live virulent *M. tuberculosis* H37Rv, cells were then washed four times with PBS prior to fixation with 4% paraformaldehyde overnight and assessement with a fluorescent microscope. For live cell analysis of cells infected with avirulent *M. tuberculosis* H37Ra, plates were imaged using an INCell Analyzer 1000 cellular imaging system (GE Healthcare) and images were analyzed using GE INCell Analyzer 1000 Workstation software version 3.6 (GE Healthcare) as previously published (Ryan, O'Sullivan et al. 2011) . Total cell numbers were detected via Hoechst staining of nuclei, and the dying/dead cells were identified via positivity for PI staining and/or nuclear condensation, which was characterised by elevated intensity of Hoechst staining within the nuclei. 12–18 fields of view per well were acquired.

2.4.2 Nitrite assay

Immortalised murine macrophages were plated at 3 x 10 5/ml in a 96 well plate and stimulated overnight with IFN-γ and irradiated *M. tuberculosis.* All conditions were assayed in triplicate using Griess reagent, which measures nitric oxide levels, using sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions.. The following day, a nitrite standard curve was prepared with 100μM nitrite as the the top standard and serial dilutions of 1:2 in a 96 well plate. 50μl/well of cell supernatant from the stimulated cells was also transferred to the 96 well plate. 50μl/well of the sulfanilamide solution to all experimental samples and wells containing the dilution series for the nitrite standard reference curve. Samples were incubated for 10

minutes at room temperature, protected from light. 50µl of the NED Solution was added to all wells. Samples were incubated at room temperature for 10 minutes, protected from light. Nitric oxide results in a colour change in the substrate from colourless to purple. Absorbance was measured by a plate reader with a filter at 550nm. Nitric concentration was calculated by interpolation of absorbance values from the standard curve.

2.4.3 Assessment of reactive oxygen species

The Amplex® Red Assay (Invitrogen) measures the presence of hydrogen peroxide produced by cells using an Amplex® Red reagent which reacts with H_2O_2 in the presence of peroxidase to produce resorufin, a red-fluorescent oxidation product. Macrophages were plated the previous day at 5 x 10^5/ml in a 96 well plate. A reaction mixture was prepared, containing 50 µM Amplex® Red reagent and 0.1 U/mL HRP in Krebs–Ringer phosphate. Medium was removed from the cells and they were washed twice with Krebs-Ringer phosphate. 100ul of reaction mixture was added to each well and cells were stimulated. 30 minutes later, fluorescence was measured using a microplate reader at an absorbance at ~560 nm.

2.4.4 Assessment of autophagy by confocal microscopy

GFP-LC3 expressing macrophages were plated at 5 x 10^5/ml on glass coverslips in 12 well tissue culture plates. Cells were stimulated for sixteen hours prior to fixation with 4% paraformaldehyde overnight. Alternatively, primary bone-marrow derived macrophages were plated at at 5 x 10^5/ml on glass coverslips in 12 well tissue culture plates. Cells were stimulated for sixteen hours prior to fixation with 4% paraformaldehyde overnight. Cells were washed with PBS, permeabilised with 0.1% Triton X for 5 minutes and blocked with

1% BSA in PBS with 5% goat serum. Cells were incubated with primary antibody (anti-LC3 antibody, Invitrogen) for 1 hr and then with secondary anti-rabbit antibody (Alexafluor) for an additional 1 hr. Coverslips were mounted onto glass slides with Dako mounting medium (Thermo Scientific, Waltham, MA, USA) and analyzed on a Olympus FV 1000 laser-scanning confocal microscope. Fluorescent puncta per cell were quantified for a minimum of 100 cells per condition.

2.4.5 Assessment of phagosome maturation by confocal microscopy

Cells were cultured on glass coverslips at 5 x 10^5/ml. Cells were infected with FITC-labelled *M. tuberculosis* H37Rv at a multiplicity of infection of 10 bacteria/macrophage. Cells were washed off at one hour, and medium was replaced. Cells were fixed in 4% paraformaldehyde overnight at room temperature, permeabilized with 0.1% Triton-X, and blocked in PBS with 1% BSA, and 5% goat serum for 30 min at room temperature. Cells were incubated with primary antibody (anti-CD63, Santa Cruz, H-193) for 1 hr and then with secondary antibody for an additional 1 hr. Alternatively, cells were incubated with Lysotracker Red DND-99 (LT, Invitrogen) for 1 hr during incubation with mycobacteria. Coverslips were mounted onto glass slides with Dako mounting medium (Thermo Scientific, Waltham, MA, USA) and analyzed on a Olympus FV100 laser-scanning confocal microscope.

2.5 *In vivo* techniques

2.5.1 Mice

For in vivo experiments carried out in the US (Chapters 3 and 5), pathogen-free male C57/BL6 (Jackson Laboratory) were maintained under barrier conditions in an animal room

at University of Massachusetts Medical School. Mal S200L heterozygote and homozygote mice (C57/BL6 background) were a kind gift of Douglas Golenbock. Animals were fed a sterile commercial mouse diet and water ad libitum. The University of Massachusetts Medical School Institutional Animal Care and Use Committee approved these experiments.

2.5.2 Infection of mice with M. tuberculosis

Aliquots of frozen *M. tuberculosis* H37Rv were thawed and then sonicated for 5 minutes. A volume of sonicated stock previously titrated to deliver approximately 500 cfu per mouse was added to the nebulizer of a Glas-Col Inhalation Exposure System (Glas-Col, LLC, Terre Haute, IN) and mice were exposed to the infectious aerosol for 30 minutes. Mice were infected at approximately 8 weeks of age, and were kept in a pathogen-free BSL3 facility for a further 8 weeks. Two mice were killed 24 hours after infection to confirm the actual delivered dose.

2.5.3 Assessment of Bacterial Load

At 8 weeks, mice were sacrificed. Lung homogenates from six mice were plated to measure bacterial burden. Lungs were homogenised in PBS-Tween, diluted serially 10-fold over 4 logs, and plated in duplicate on Middlebrook 7H11 agar (DIFCO, Becton Dickinson, Sparks, MD). Plates were cultured at 37°C for 3 weeks and then counted using a dissecting microscope to confirm colony morphology.

2.5.4 Lung Histology

Lungs were inflated and fixed with 10% buffered formalin for 24 hours and then processed for staining. Tissue sections were stained with hematoxylin and eosin (H&E). Sections were

made at intervals spanning the whole lung, and the left cranial lobe was examined using light microscopy. Lung surface area of inflammation was measured with a Nikon Eclipse E400 microscope (Nikon Instruments, Melville, NY) at x 20 magnification using Spot Insight v3.5 software (Diagnostic Instruments Inc, Sterling Heights, MN). Percent total lung area involved with inflammation was calculated by dividing the cumulative area of inflammation by the total lung surface area examined for each lung studied.

2.5.5 Lung cytokine expression

Lungs were homogenized in PBS-T, an equal volume of cell lysis buffer (0.5% Triton X-100, 150 nM NaCl, 15 mM Tris, 1 mM $CaCl_2$ and 1mM $MgCl_2$, pH 7.4) was added and the mixture was vortexed, incubates (20 min, 4°C), vortexed again, centrifuged (10 min, 12,000-14,000 x *g*) and the supernatant was filter-sterilised. Lung lysates were assayed for TNFα by ELISA (R&D Systems).

2.5.6 Mouse intraperitoneal injection model and tissue isolation.

C57BL/6 or Mal $^{-/-}$ mice were injected intraperitoneally with interferon gamma (Immunotools, Germany) at the indicated dose. Tail bleeds were carried out 6 hours after injection. Mice were sacrificed at 24 hours, and blood and peritoneal lavage were retrieved. Peritoneal cells were analysed using flow cytometry. Peritoneal cells were also plated at 1 x 106/ml, stimulated with irradiated Mtb or other TLR ligands, and supernatants harvested after 16 hours for analysis by ELISA. Serum was recovered from blood by centrifugation at 10,000 rpm for 10 minutes. Peritoneal lavage fluid was also analysed by ELISA.

2.6 Flow cytometry.

Fluorochrome staining compensations were prepared using Anti-Rat and Anti-Hamster Igκ Negative Control Compensation Particles (BD). To distinguish auto-fluorescent cells from cells expressing low levels of individual surface markers, thresholds for auto fluoresce were established by staining samples with fluorescence-minus-one (FMO) control stain sets in which a fluorochrome for a channel of interest was omitted.

Single cell suspensions were analyzed using a BD Canto II flow cytometer. In all prepared samples debris was excluded by analyzing FSC-A vs. SSC-A plots and all debris were excluded from further analysis. To exclude artifacts caused by cell doublets, cells were analyzed on FSC-A vs. FSC-H plot. Samples were acquired using FACSCanto II (BD Biosciences) and data were analyzed using FlowJo software.

2.6.1 Flow cytometric analysis of intracellular cytokine staining after immunization.

Cells were isolated from peritoneal exudates of immunized mice. The cell suspension was centrifuged at 1200rpm at room temperature for 5 min and cell pellet was resuspended in 0.16M NH_4Cl solution for 2 minutes to lyse red blood cells. After determination of cell numbers via trypan blue exclusion, 1 x 10^6 cells/ml were plated in 96 well plates. Cell suspensions were centrifuged at 1200 rpm for 5 min and washed with PBS buffer. Cells were then incubated with Aqua Live/Dead stain (Invitrogen) at 4°C for 30 minutes. After washing again, cells were resuspended in 100µl of FACS buffer and incubated at 4°C for 10 minutes with anti-CD16/CD32 monoclonal antibodies to block FcγRII/III. Cells were then stained with fluorochrome-labelled antibodies against surface markers (Appendix 1 A8.2) diluted in 50µl of FACS buffer, at at 4°C for 30 min in the dark. After 30 minutes, cells

were washed twice with 200µl per well of FACS buffer and resuspended in 200 µl of FACS buffer before being analyzed by flow cytometry.

2. 6.2 Flow cytometric analysis of intracellular staining in cultured cells

Cells were infected with *M. tuberculosis* for 3-72 hours. Cells were then washed with PBS prior to incubation with Aqua Live/Dead stain at (Invitrogen) at 4°C for 30 minutes. After washing again, cells were resuspended in FACS buffer with anti- IFN-γ antibodies and incubated at 4°C for 30 minutes.

2.6.3 Flow cytometric analysis of mitchondrial superoxide

Cells were treated with a variety of ligands for 16 hours. Cells were then washed with PBS prior to incubation with Aqua Live/Dead stain (Invitrogen) as above and MitoSox Red (Molecular Probes, Invitrogen). After washing cells were suspended in FACS buffer and analyzed by flow cytometry.

2.7 Generating plasmids

2.7.1 Site-directed mutagenesis

HA-Mal has been previously described (Valkov, Stamp et al. 2011). Primers were designed for site-directed mutagenesis: forward primer sequence caccatcccccctgctgttgggcctca, reverse primer sequence ctgaggcccaacagcaggggggatg. Site-directed mutagenesis was carried out to generate HA-tagged S180L variant Mal which was amplified using DH5α competent cells and isolated with Miniprep (Promega) and Maxiprep (High Pure RNA Isolation Kit) The sequence of both HA-Mal and HA-S180L Mal were confirmed by seqencing (Eurofins)

2.8 Immunoblotting

2.8.1 Western blotting

Cells were plated at 5 x 10^5/ml in 12 well tissue culture plates (Grenier) prior to stimulation for six hours. Cell lysates were obtained by adding 75 µl of lysis buffer (Appendix 1 A5.3) to cells and incubating at 4C for 15 minutes prior to scraping cells. Lysates were heated for 10 minutes at 90C. Samples were then mixed with Laemmli sample buffer.

15% acrylamide gels were prepared according to standard protocol (Appendix 1 A5.1/5.2). 12-30µl of sample were loaded/well. 5µl/well of protein ladder (Bio-rad). Gels were run at 100V. Samples were transferred to PVDF membrane (kj) using the semidry transfer method. Briefly 75 mA/gel was applied for 45-60 minutes.

Membranes were blocked by incubating with 5% skimmed milk in PBS for one hour. Membranes were then incubated with primary antibody (anti-LC3, Sigma; anti-β-actin, Sigma, anti-Beclin 1, Santa Cruz H-300; anti-ATG7, Santa Cruz; anti-BCl-1 Santa Cruz (100)) at the appropriate concentration overnight. Membranes were washed in washing buffer (appendix 1 A5.5) four times prior to incubation with Licor antibodies (anti-mouse and anti-rabbit secondary antibodies, Licor) for one hour. Membranes were washed four times prior to scanning on Odyssey.

2.8.2 Co-immunoprecipitation

HEK293 were incubated for 24 h with DNA encoding various proteins in the presence of Genejuice. Cell lysates were prepared by lysis on ice for 10 min in 50 mM HEPES, pH 7.5, 250 mM NaCl, 20 mM β-glycerophosphate, 1% Nonidet-P40, 2 mM dithiothreitol and a protease inhibitor 'cocktail' (Sigma) at a dilution of 1:500 for immunoprecipitation or by

being boiled directly in Laemmli sample buffer for immunoblot. Immunoprecipitation was initiated by incubation of lysates for 2 h with protein A/G sepharose beads (Amersham) plus control antibodies. Precleared lysates were then incubated at 4 °C for at least 2 h with various antibodies and protein G beads (Amersham). Washed beads were boiled in sample buffer; proteins were separated by SDS-PAGE and were transferred onto nitrocellulose membrane. Membranes were blotted with the various antibodies and samples were visualized with an enhanced chemiluminescence system (Licor).

2.9 Genotyping

Mal genotype was determined on DNA extracted from buccal swabs (Isohelix, Cell Products) using a DNA isolation kit (Isohelix, Cell Products) according to the manufacturer's instructions. Ethical perimission was granted by the Ethics Committee of the School of Medicine, Trinity College, Dublin. Informed consent was obtained. Genotyping of the Mal S180L and polymorphism was performed using the Kapa Probe Fast PCR kit and the TaqMan Allelic Discrimination System (PE Biosystems, Foster City, California, USA). Genotyping results were verified using positive sequenced controls. PCR reactions were set up according to the manufacturer's instructions and thermal cycling was performed on 384-well reaction plates on PTC-225 DNA engine Tetrad (MJ Research, San Francisco, California, USA) as follows: initial denaturation and enzyme activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Genotypes were determined using an ABI Prism 7900HT (PE Biosystems).

2.10 Statistics.

Mann-Whitney was performed to assess for statistically significant difference of the means between groups. Chi-squared analysis was used to assess statistically signficant proportions of co-localisation between groups. P values <0.05 were considered significant. Error bars represent standard deviations of the mean.

Chapter 3 : The role of Mal in immune responses to *M. tuberculosis*

3.1 Introduction

As discussed in the introductory chapter, MyD88 adaptor-like (Mal), also known as Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) is a signalling adaptor protein which was first described by Fitzgerald and colleagues (Fitzgerald, Palsson-McDermott et al. 2001) and by Horng and colleagues (Horng, Barton et al. 2001). Mal was initially reported to be involved in signalling cascades initiated by TLR2 or TLR4 ligation and leading to NF-κB activation (Fitzgerald, Palsson-McDermott et al. 2001, Horng, Barton et al. 2001, Horng, Barton et al. 2002, Yamamoto, Sato et al. 2002). It is proposed to recruit cytosolic MyD88 to TLRs at the cell membrane in a structure called the Myddosome (Gay, Gangloff et al. 2011). Subsequent work showed that high doses of TLR2 ligands may be able to overcome the requirement for Mal (Kenny, Talbot et al. 2009) and that intraphagosomal TLR2 signalling may not require Mal (Cole, Laird et al. 2010). Additional roles identified for Mal include inhibition of TLR3 signalling, similar to that seen with MyD88 (Kenny, Talbot et al. 2009) and signalling from the receptor for advanced glycation endproducts (RAGE).

A number of studies have found an association between the S180L and D96N polymorphisms in Mal and tuberculosis immunity (Khor, Chapman et al. 2007, Castiblanco, Varela et al. 2008, Selvaraj, Harishankar et al. 2010, Zhang, Xue et al. 2011, Capparelli, De Chiara et al. 2013, Liu, Li et al. 2014). However, $Mal^{-/-}$ mice have been reported to show no increase in susceptibility to tuberculosis at early stages of infection (Fremond, Togbe et al. 2007). Also, no *in vitro* characterisation of the $Mal^{-/-}$ macrophage reponse to *Mtb* had been reported.

We set out to identify Mal-dependent macrophage responses to *M. tuberculosis*. We hypothesised that responses previously reported as being TLR2-dependent, such as cytokine secretion (Means, Jones et al. 2001, Kleinnijenhuis, Joosten et al. 2009) and apoptosis (Means, Jones et al. 2001), would be Mal-dependent; whereas those previously reported as TLR2-independent, such as bactericidal activity (Holscher, Reiling et al. 2008) and phagosome maturation (Holscher, Reiling et al. 2008), would be independent of Mal.

3.2 Results

3.2.1 Pro-inflammatory cytokine responses to M. tuberculosis *are Mal-dependent*

Innate cytokines including IL-1α, IL-1β, TNF-α and IL-12 are known to be required for effective antituberculous immunity (Cooper, Mayer-Barber et al. 2011). I sought to determine the role of Mal in the secretion of these cytokines by macrophages.

3.2.1.1 Verification of Mal$^{-/-}$

I started by confirming that the murine immortalised Mal$^{-/-}$ bone marrow-derived macrophage (iBMM) cell line behaved as previously reported. WT and Mal$^{-/-}$ macrophages were stimulated overnight with LPS (TLR4 ligand), Pam_3CysK_4 (TLR2 ligand), Poly I:C (TLR3 ligand) or R848 (TLR7/8 ligand). As expected, given Mal's documented role as a signalling adaptor protein for TLRs 2 and 4 (Horng, Barton et al. 2002), Mal$^{-/-}$ macrophages were hyporesponsive to TLR4, and to a lesser extent to TLR2 ligands. Responses to TLR3 and TLR7/8 ligands were increased (Figure 3.1), as previously described (Kenny, Talbot et al. 2009).

3.2.1.2 Pro-inflammatory cytokine secretion by immortalised murine macrophages in response to MT is Mal-dependent

Next, the role of Mal in macrophage secretion of key pro-inflammatory cytokines in response to *M. tuberculosis* was assessed, focusing on cytokines known to be important in TB immunity, as discussed in the introductory chapter. Murine immortalised WT and $Mal^{-/-}$ macrophages (iBMM) were infected with virulent *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) ranging from 2.5 to 10 bacteria/macrophage. Supernatants were removed at 16 hours post-infection, and analysed by ELISA. IL-1α and IL-1β secretion was greatly reduced in the absence of Mal, as was IL-6 and IL-12p40 secretion. TNF-α secretion was reduced by approximately half. TGF-β and IL-27 secretion was not Mal-dependent. IFN-β secretion was increased in the absence of Mal. iBMM did not produce detectable IL-10 after infection with *M. tuberculosis* (Figure 3.2)

3.2.1.3 Pro-inflammatory cytokine secretion by primary murine macrophages in response to M. tuberculosis is Mal-dependent

Given concerns that the immortalisation process may result in additional mutations affecting macrophage function, experiments were conducted to confirm results in primary bone-marrow derived macrophages from WT and $Mal^{-/-}$ mice. Macrophages were derived from progenitor bone marrow using mCSF (see Methods chapter for further details). Primary macrophages were infected with virulent *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) ranging from 2.5 to 10 bacteria/macrophage. Supernatants were removed at 16 hours post-infection, and analysed by ELISA. Similarly to results seen in immortalised macrophages, IL-1β secretion was almost completely absent in the $Mal^{-/-}$ macrophages, whereas TNF-α secretion was reduced by approximately 50% . IL-10 secretion was reduced

at lower multiplicity of infection in the absence of Mal, but not at higher multiplicity of infection. IL-27 secretion was unaffected by the absence of Mal (Figure 3.3).

3.2.1.4 Pro-inflammatory cytokine secretion by human macrophage-like cell line THP-1 cells in response to is Mal-dependent

To address whether these findings were relevant to human tuberculosis, the role of Mal in human macrophage responses to *M. tuberculosis* was assessed. THP-1 cells, a human monocytic acute leukaemia cell line (Tsuchiya, Yamabe et al. 1980), were differentiated to a macrophage-like mature form by treating with phorbol 2-myristate 13-acetate (PMA) (Kohro, Tanaka et al. 2004) for 24 hours. Cells were transfected with small interfering RNA (siRNA) against Mal or scrambled control siRNA prior to differentiation. Knockdown was confirmed by rtPCR (Figure 3.4). Cells were infected with virulent *M.tuberculosis* H37Rv 48 hours post transfection and differentiation at a multiplicity of infection (MOI) ranging from 2.5 to 10 bacteria/macrophage. Supernatants were removed at 16 hours post-infection and analysed by ELISA. Results were similar to those seen in murine macrophages. IL-1β, IL-6, IL-10 and TNF-α secretion were all reduced in the absence of Mal. TNF-α responses to Poly I:C, a TLR3 ligand, were increased in the absence of Mal (Fig. 3.5) as previously reported(Kenny, Talbot et al. 2009).

These findings were also confirmed in human macrophages by treating differentiated THP-1 cells with Mal inhibitors. The first inhibitor we used was a commercially available Mal inhibitory peptide (Calbiochem, Millipore). This is a synthetic peptide corresponding to the P125H region of Mal with an antennapedia region to enable it to cross the cell membrane. It functions as a decoy peptide by binding to the TIR domain of TLRs 2 and 4 (Horng, Barton et al. 2001). The control peptide consists of the reverse sequence. The peptide was initially used at a concentration of 1 μg/ml, as had been reported in the literature (Kenny, Talbot et

al. 2009). At this concentration, we did not see an effect on TNF-α secretion in response to LPS or *M. tuberculosis*. However, when the peptide was used at 10 μg/ml, TNF-α responses to Mtb were attenuated, consistent with the results to in other models of Mal deficiency (Figure 3.6 A and C). The reduction in IL-1β was minimal (Figure 3.6b), suggesting that this peptide may not be fully effective in blocking Mal signalling.

The impact of another Mal inhibitory peptide, viral inhibitory protein of TLR 4 (VIPER), was also assessed. This is an 11 amino acid long peptide with 9R delivery sequence which is derived from an immunomodulatory protein, A46, produced by *Vaccinia* virus. It masks TIR domains (critical binding) sites on Mal and TRAM and prevents TLR4 interaction, thereby inhibiting TLR4 signalling via Mal and TRAM (Lysakova-Devine, Keogh et al. 2010). The control peptide is an inert peptide of the same length. A dose-response experiment in differentiated THP-1 cells was carried out to determine the optimal concentration of VIPER for inhibiting *M. tuberculosis* induced TNF-α secretion (Figure 3.7). Based on this, 10 μM was chosen as the optimal concentration for subsequent experiments. At this concentration, a reduction in IL-1β, IL-6 and TNF-α secretion in THP-1 cells infected with *M. tuberculosis* (Figure 3.8) was observed.

3.2.1.5 Pro-inflammatory cytokine secretion in macrophages infected with M. tuberculosis is also dependent TLRs 2 and 4.

The role of other components of the TLR2/4 – Mal – MyD88 signalling pathway on cytokine secretion by macrophages in response to infection with *M. tuberculosis* was assessed. Specifically, the relative roles of TLR2 and TLR4 signalling in the defects in cytokine secretion seen in the Mal-/- cells were investigated. Responses of TLR2 and TLR4 single knockout immortalised macrophages to Pam_3CysK_4 (TLR2 ligand) and LPS (TLR4 ligand) were measured. The $TLR2^{-/-}$ iBMM had a somewhat reduced TNF-α response to

TLR4 ligand LPS, perhaps due to TLR2 ligand contamination of the LPS or perhaps due to a difference in seeding density or viability of the cells, but displayed a dramatic reduction in TNF-α secretion in response to stimulation with to Pam_3CysK_4. $TLR4^{-/-}$ iBMM had preserved TNF-α secretion in response to Pam_3CysK_4, but a dramatic reduction in TNF-α response to LPS (Figure 3.9). Previous publications in different cell types had shown a role for TLR2, and a lesser role for TLR4, in pro-inflammatory cytokine responses to *M. tuberculosis* in macrophages (Means, Wang et al. 1999, Means, Jones et al. 2001, Kleinnijenhuis, Joosten et al. 2009). In the current model, we found that IL-1β and IL-12/23p40 secretion was greatly reduced in the absence of either TLR2 or TLR4; whereas TNF-α was reduced in the absence of TLR2 but less so in the absence of TLR4 (Figure 3.10).

In the absence of one pattern recognition receptor, another is often able to compensate. As Mal is involved in both TLR2 and TLR4 signalling, we measured cytokine secretion in TLR2/4 double knockout iBMM to eliminate the effect of such compensation . Interestingly, IFN-β was increased in the absence of TLRs 2 and 4, in a similar manner to that seen in $Mal^{-/-}$ iBMM. Secretion of IL-1β, IL-12p40 and TNF-α was almost abolished in the absence of TLRs 2 and 4 (Figure 3.11).

3.2.1.6 Pro-inflammatory cytokine secretion in response to is also dependent on MyD88

Mal functions as a bridging adaptor protein between TLRs 2 and 4 and MyD88. I hypothesised that pro-inflammatory cytokine responses to *M. tuberculosis* would be impaired in $MyD88^{-/-}$ iBMM in a similar manner to that seen in $Mal^{-/-}$ iBMM. IFN-β secretion was unaffected, unlike in $Mal^{-/-}$ and $TLR2/4^{-/-}$ iBMM. IL-1β, IL-12p40 and TNF-

α secretion were greatly reduced in the absence of MyD88 (Figure 3.12). These results were consistent with those seen in the absence of Mal.

In summary, secretion of pro-inflammatory cytokines IL-1β, IL-12p40, TNF-α are dependent on all the components of the TLR2/4 signalling pathway: TLRs 2 and 4, Mal and MyD88.

3.2.2 Macrophage killing of virulent Mtb

The role of the TLR2/4-Mal-MyD88 pathway in killing of intracellular *Mtb* was determined. Intracellular killing of Mtb is the endpoint for numerous effector mechanisms, with clear *in vivo* relevance.

3.2.2.1 Mal is required for bactericidal activity

Bactericial activity was initially measured in immortalised BMMs. WT and $Mal^{-/-}$ iBMM were seeded at 1 x 10^5/ml and infected with *M. tuberculosis* H37Rv at an MOI of 10 bacteria/macrophage for 3 hours, at which point extracellular bacteria were washed off. Macrophages were lysed at 3, 24 and 72 hours, and lysates were plated out for assessment of bacterial count. Colonies were counted manually when they reached an appropriate size for counting at approximately 21 days. $Mal^{-/-}$ iBMM showed a marked inability to kill intracellular Mtb, with 2 to 4-fold higher bacterial loads in the Mal-deficient cells at 72 hours (Figure 3.13). Results were confirmed in primary murine macrophages (Figure 3.14). PMA-differentiated THP-1 cells transfected with siRNA against Mal were used to assess whether Mal was required for bactericidal acivity in human cells, and displayed a similar deficit in killing of bacteria in Mal-deficient cells (Figure 3.15). A similar impairment of

bactericidal activity was seen in PMA-differentiated THP-1 cells treated with the Calbiochem Mal inhibitor (Figure 3.16).

3.2.2.2 TLRs 2 and 4 are not required for macrophage bactericidal activity, although MyD88 is.

The role of TLRs 2 and 4 in killing of intracellular mycobacteria was evaluated. Surprisingly, TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR 2/4$^{-/-}$ (Fig 3.17) murine macrophages and THP-1 macrophages treated with a TLR2 inhibitor previously shown to inhibit TLR2 signalling in human cells *ex vivo* (Nic An Ultaigh, Saber et al. 2011) (Fig 3.18) did not show a defect in killing. MyD88$^{-/-}$ macrophages showed a similar defect to Mal$^{-/-}$ cells (Fig 3.17). These data indicated that Mal and MyD88 had a function in killing of *M. tuberculosis* that was additional to its known role downstream of TLRs 2 and 4.

I then sought to identify the mechanism of killing of intracellular *M. tuberculosis* which was Mal- and MyD88-, but not TLR 2 and 4 dependent.

3.2.3 Uptake of M. tuberculosis *is not Mal-dependent.*

Bacterial counts in immortalised and primary Mal$^{-/-}$ BMM and Mal siRNA transfected THP-1 at 3 hours post-infection carried out in the assays looking at bactericidal activity demonstrated that uptake of *M. tuberculosis* did not differ significantly between WT and Mal-deficient macrophages (Figs 3.13-3.16). We confirmed this by fixing infected macrophages 3 hours post-infection, staining *M. tuberculosis* with Auramine O, a diarylmethine fluorescent dye which binds to the mycolic acid in mycobacterial cell walls. The number of intracellular mycobacteria was quantified by fluorescence microscopy for a minimum of one hundred cells per condition. We saw no difference in uptake of

mycobacteria between WT and Mal$^{-/-}$ immortalised and primary murine macrophages or THP-1 cells transfected with siRNA against Mal or scrambled control (Figure 3.19).

3.2.4 Cell death in macrophages infected with M. tuberculosis *is not Mal-dependent.*

Apoptosis of infected macrophages can result in killing of intracellular bacteria (Keane, Balcewicz-Sablinska et al. 1997, Keane, Remold et al. 2000, Arcila, Sánchez et al. 2007), whereas necrosis of infected macrophages is associated with unrestricted bacterial replication (Arcila, Sánchez et al. 2007). The role of Mal in apoptosis and necrosis after infection with *M. tuberculosis* H37Rv was evaluated.

3.2.4.1 Cell death in immortalised macrophages infected with M. tuberculosis is not Mal-dependent

Initially, cell death in immortalised WT and Mal$^{-/-}$ iBMM at 3 and 72 hours was assessed with dual propidium iodide/hoescht staining. Propidium iodide stains the nuclei of necrotic cells, whereas cells undergoing apoptosis have condensed nuclei on Hoescht staining. Cells were stained and washed prior to fixation with paraformaldehyde. Levels of cell death in immortalised macrophages were too low to draw any meaningful conclusions (Figure 3.20). The cells have been immortalised using J2 transforming retroviruses (expressing Raf and Myc oncogenes) as previously described (Roberson and Walker 1988). V-raf acts as a MAP3 kinase and overdrives the MEK1/2 and ERK 1/2 pathways. Myc is a transcription factor which drives cell proliferation by upregulating cyclins and downregulating p21, regulates cell growth by upregulating ribosomal RNA and proteins, and downregulates apoptosis by downregulating Bcl-2. Expression of these oncogenes may prevent the immortalised macrophages from undergoing cell death.

3.2.4.2 Cell death in THP-1 cells is not Mal-dependent

Cell death in THP-1 cells transfected with siRNA against Mal or scrambled control was also assessed. These cells were differentiated with PMA and infected with *M. tuberculosis* H37Rv at a MOI of 10:1. Dual propidium iodide/Hoescht staining was carried out, but on this occasion propidium iodide staining did not work well. Cell death was assessed by counting condensed nuclei, representing apoptotic and necrotic cells. Cell death was assessed at 3 and 48 hours post-infection. No difference was seen in cell death between THP-1 cells transfected with siRNA against Mal or scrambled control (Figure 3.21).

3.2.4.3 Cell death in primary murine macrophages is not Mal-dependent

Finally, cell death was assessed in non-immortalised cells by infecting primary bone-marrow derived macrophages from WT and $Mal^{-/-}$ mice. *M. tuberculosis* H37Ra was used to enable an assessment of cell death by a high-throughput method in non-fixed cells. Cells were stained with dual propidium iodide/Hoescht and assessed using the In Cell high-throughput machine at 3, 24 and 48 hours .There were more apoptotic $Mal^{-/-}$ cells at 3 and 24 hours (Figure 3.22 and 3.23). However, 48 hour measurements of apoptosis appear unreliable, and may reflect the fact that the cells were reaching the end of viability (not shown). Interestingly, $Mal^{-/-}$ macrophages were more sensitive to cycloheximide-induced cell death than WT controls, which may be due to impairment of mitophagy as described later in this chapter.

In summary, there was no difference in cell death between WT and Mal-deficient cells that would account for the marked defect in mycobactericidal activity seen in the Mal-deficient cells. Therefore, other mechanisms of killing intracellular mycobacteria were investigated.

3.2.5 Nitric oxide production in macrophages infected with M. tuberculosis *is not Mal-dependent.*

It is well-established that murine macrophages can kill intracellular mycobacteria by nitric oxide production (Chan, Xing et al. 1992). The importance of nitric oxide in human macrophages is more controversial, but human alveolar macrophages also have been reported to kill intracellular mycobacteria via nitric-oxide dependent mechanisms (Rich, Torres et al. 1997). Nitric oxide synthase 2 has been reported to be markedly decreased in $MyD88^{-/-}$ mice infected with *M. tuberculosis* (Scanga, Bafica et al. 2004). The role of Mal in macrophage production of nitric oxide was assessed.

As this was a live-cell assay carried out in the non-BSL3 lab, irradiated *M. tuberculosis* H37Rv and IFN-γ were used to stimulate WT, $Mal^{-/-}$ and $MyD88^{-/-}$ immortalised macrophages for 16 hours. Nitric oxide production was measured using Griess reagent, which uses sulfanilamide and ***N***-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Nitric oxide results in a colour change in the substrate, which is measured by a plate reader.

Induction of nitric oxide was detected in WT cells at lower concentrations of IFN-γ (20 iu/ml) in combination with higher doses of irradiated *Mtb* (100 bacteria/cell) and with higher concentrations of IFN-γ (20 iu/ml) with lower doses of irradiated *Mtb* (100 bacteria/cell). Nitric oxide production was not reduced in the absence of Mal- or MyD88 (Figure 3.24) .

3.2.6 Reactive oxygen species production in macrophages infected with M. tuberculosis *is increased in Mal-deficient cells.*

Activated macrophages can produce reactive oxygen intermediates (ROI), as reviewed by Fang and colleagues (Fang 2004). Although a defect in mycobactericidal activity was not seen in macrophages from X-CGD mice with a nonfunctional allele for the $gp91^{phox}$ subunit of the phagocyte oxidase cytochrome b, the mice were unable to control mycobacterial infection *in vivo* (Cooper, Segal et al. 2000). TLR2 stimulation by purified protein derivative of TB (PPD) has been reported to result in an increase in ROS production (Yang, Shin et al. 2008).

The role of Mal in ROS production in macrophages infected with irradiated *M. tuberculosis* was assessed. Irradiated bacteria were used as this is a live cell assay. The Amplex Red assay uses a colourless substrate that reacts with hydrogen peroxide (H_2O_2) with a 1:1 stoichiometry to produce highly fluorescent resorufin which can be measured with a plate reader. Interestingly, elevated levels of reactive oxygen intermediates were detected basally in Mal-deficient immortalised macrophages and in THP-1 cells transfected with siRNA against Mal prior to differentiation (Figure 3.25). This may be related to defects in mitophagy in these cells.

3.2.7 Autophagy

As discussed in the introductory chapter, autophagy contributes to the innate immune response to a number of intracellular pathogens and parasites (Levine, Mizushima et al. 2011), including *M. tuberculosis* (Harris, Hope et al. 2009, Deretic 2011). Autophagy may play multiple roles, including facilitating the maturation of mycobacteria-containing phagosomes and increasing intracellular killing of the bacilli (Gutierrez, Master et al. 2004,

Singh, Davis et al. 2006, Singh, Ornatowski et al. 2010). Studies have shown that autophagy in response to the mycobacterial lipoprotein LpqH is TLR1/2- and vitamin D receptor-dependent (Shin, Yuk et al. 2010).

3.2.7.1 Mal is required for autophagy in response to TLR 2 and 4 ligands

To determine whether autophagy induced by TLR 2 and 4 ligands was Mal-dependent, immortalised murine bone marrow-derived macrophages stably expressing GFP-LC3 were utilised. Microtubule-associated protein light chain 3 (LC3) exists in two different forms, an unprocessed cytosolic protein (LC3-I) and, following the induction of autophagy, a lipidated membrane-bound protein (LC3-II). When autophagy is induced, lipidated LC3 is recruited to autophagosomes, forming characteristic puncta which can be quantified by fluorescence microscopy (Figure 3.26). GFP-LC3 expressing iBMM were pre-treated with Mal inhibitors, stimulated with TLR2 agonist Pam_3CysK_4, TLR 4 agonist LPS and TLR3 agonist Poly I:C for 16 hours and fixed and analysed. The Calbiochem Mal inhibitor, which blocks TLR2 and TLR4 interaction with Mal, inhibited autophagosome formation in response to Pam_3CysK_4 and LPS but not Poly I:C; whereas VIPER, which blocks TLR 4 but not TLR 2 interaction with Mal, inhibited autophagosome formation in response to LPS but not Pam_3CysK_4 (Figure 3.27).

3.2.7.2 Mal is required for autophagy in response to M. tuberculosis in immortalised BMM

Next, the role of Mal in autophagy in response to *M. tuberculosis* was evalated. GFP-LC3 expressing iBMM were pre-treated with Mal inhibitors and then infected with *M. tuberculosis* H37Rv. Both inhibitors inhibited autophagosome formation in response to *M. tuberculosis* (Figure 3.28).

3.2.7.2 Mal is required for autophagy in response to M. tuberculosis in primary BMM

These findings were confirmed in primary $Mal^{-/-}$ cells. Autophagy is a dynamic process, ending with fusion of the autophagosome with the lysosome, and degradation and recycling of LC3-II (Kabeya, Mizushima et al. 2004). Therefore, autophagic flux may be determined by comparing LC3-II levels among samples in the presence and absence of a lysosomal protease inhibitor (e.g. bafilomycin A1, E64d, and pepstatin A) (Mizushima 2007, Klionsky, Abeliovich et al. 2008). In the absence of a lysosomal protease inhibitor, elevated levels of LC3-II detection may indicate either an increase in autophagosome formation or a blocking of autophagic degradation (Mizushima 2007). In the presence of protease inhibitors, further accumulation of LC3-II indicates autophagic flux.

Autophagic flux in primary WT and $Mal^{-/-}$ bone marrow-derived macrophages was measured using an anti-LC3 antibody to label autophagosomes (Invitrogen). The antibody co-localised with GFP-LC3 labelled puncta (Figure 3.29). Macrophages were pre-treated with bafilomycin A1 prior to infection with *M. tuberculosis* H37Rv for 16 hours. Starvation medium was added for the last 2 hours only. Cells were fixed, stained and analysed by confocal microscopy. WT cells infected with *M. tuberculosis* exhibited numerous $LC3^{+}$ autophagosomes, and this increased in the presence of bafilomycin, indicating active autophagic turnover. $Mal^{-/-}$ cells had signficantly fewer autophagosomes than WT cells on infection with *M. tuberculosis*, and this number did not increase in the presence of bafilomycin, indicating an impairment in initiation and turnover of autophagy. Starvation-induced autophagy was unaffected (Figure 3.30)

3.2.8 Mitochondrial reactive oxygen species accumulate in the absence of Mal

Autophagy is one of the major pathways by which damaged mitochondria are cleared from the cells through a process known as mitophagy (Kim, Rodriguez-Enriquez et al. 2007). In the absence of autophagy, dysfunctional mitochondria accumulate and cells display elevated levels of mitochondrial reactive oxygen species (ROS) (Zhang, Qi et al. 2007). ATG-5 (autophagy protein-5) deficient macrophages show an accumulation of mitochondrial ROS as measured using a Mitosox Red assay (Tal, Sasai et al. 2009). MitoSOX™ Red reagent permeates live cells where it selectively targets mitochondria. It is rapidly oxidized by superoxide but not by other reactive oxygen species (ROS) and reactive nitrogen species (RNS). The oxidized product is highly fluorescent upon binding to nucleic acid.

Given the impairment of autophagy in $Mal^{-/-}$ macrophages, a question arose as to whether mitophagy was also impaired. Basal levels of mitochondrial ROS were elevated in $Mal^{-/-}$ immortalised and primary macrophages (Figures 3.31 and 3.32). 3-methyladenine (3MA) is a type III PI3 kinase inhibitor which is used experimentally to inhibit autophagy. When autophagy was inhibited by 3MA in WT cells, an accumulation of mitochondrial ROS was observed. In $Mal^{-/-}$ macrophages basal mitochondrial ROS was elevated, indicating a defect in basal mitophagy. 3MA did not increase mitochondrial ROS to the same extent in the $Mal^{-/-}$ cells as in WT cells, possibly because autophagy was already impaired in these cells.

3.2.9 Autophagy is required for phagosomal maturation and killing of intracellular virulent M. tuberculosis

Autophagy has been reported to be required for phagosome maturation and killing of *M. bovis* BCG in a number of cell types and *M. tuberculosis* H37Rv in RAW 264.7 cells (Gutierrez, Master et al. 2004, Singh, Davis et al. 2006), but had not been shown to be required for phagosome maturation and killing of *M. tuberculosis* in macrophage types in

this model. Two proteins involved in the initiation of autophagy, Beclin 1 and ATG7,were knocked down using siRNA in immortalised mouse macrophages. Knockdown was confirmed by immunoblotting (Figure 3.33d). Phagosome maturation and killing of *M. tuberculosis* H37Rv was then evaluated in these cells. In findings similar to those in $Mal^{-/-}$ macrophages, phagosome maturation was reduced at 2 hours and intracellular bacterial burden was elevated at 72 hours (Figure 3.33) in cells deficient in Beclin-1. The ATG7 knockdown was not complete (Fig 3-33d) and may not have been sufficient to fully inhibit autophagy and killing of mycobacteria.

3.2.10 Mal is required for interferon gamma induced trafficking of M. tuberculosis to mature phagosomes

Phagosomal maturation is a process which is tightly linked to autophagy. *M. tuberculosis* can survive in macrophages by inhibiting phagolysosome biogenesis. Induction of autophagy overcomes the mycobacterial phagosome maturation block, and delivers the tubercle bacilli to degradative compartments, where they are eliminated (Deretic 2008). We assessed the role of Mal in maturation of phagosomal compartments containing *M. tuberculosis* by assessing the co-localisation of FITC-labelled *M. tuberculosis* with phagolysosomes labelled with anti-CD63 antibody or with the acidotropic dye Lysotracker Red. Mal was required for the increase in phagosome maturation seen in IFN-γ treated immortalised murine (CD 63 staining: Figure 3.34, Lysotracker: Figure 3.35) and differentiated THP-1 cells (Figure 3.36). Unstimulated $Mal^{-/-}$ iBMM also showed a not statistically signficant reduced rate of phagosome maturation compared to unstimulated WT iBMM when assessed with Lysotracker; however, there was no difference in unstimulated THP-1 transfected with siRNA against Mal, although there was a wide margin of error which may mean that a small difference would not be detected.

The role of TLRs 2 and 4 in phagosome maturation was determined. Whereas IFN-γ treated MyD88$^{-/-}$ iBMM showed an impairment in phagosome maturation, IFN-γ treated TLR 2$^{-/-}$, TLR4$^{-/-}$ and TLR2/4$^{-/-}$ iBMM showed an increase in phagosome maturation similar to that seen in WT iBMM (Figure 3.37).

3.2.11 Mal does not co-immunoprecipitate with Beclin-1 or BCL-2

A mechanism by which Mal was involved in autophagy was sought by looking for interactions between Mal and proteins involved in autophagy. It is not possible to immunoblot for endogenous Mal, so HEK-293 cells were transfected with HA-tagged wild-type Mal or HA-tagged empty vector and an immunoprecipitation was performed with antibodies to HA and Beclin 1 or BCL-2 on cell lysates as indicated. Lysates were then blotted with anti-interferon gamma receptor antibody (Figure 3.38). No interaction between Mal and Beclin-1 or BCL-2 was seen.

3.2.12 Mal$^{-/-}$ mice do not display increased susceptibility to tuberculosis infection.

Finally the role of Mal in a murine model of *in vivo* tuberculosis infection was assessed. WT and Mal$^{-/-}$ mice were bred on a BL6/C57 background. 8 age and sex-matched mice of each type were infected with 500 cfu of live virulent *M. tuberculosis* H37Rv via aerosol. Weights were measured weekly. There was no significant difference in weight gain between the groups (Figure 3.39). Mice were sacrificed at 8 weeks of age and serial dilutions of lung homogenates were plated out for determination of bacterial burden. Again, no significant difference was seen between the groups (Figure 3.39). Lung homogenates were also assayed with ELISA for IL-1β and TNF-α (Figure 3.41). There was no significant difference in IL-1β or TNF-α production. Alternatively, lungs were fixed in formaldehyde and then

stained with H&E. The percentage of inflamed lung tissue was assessed by microscopy. Again, there was no significant difference between the groups (Figure 3.40).

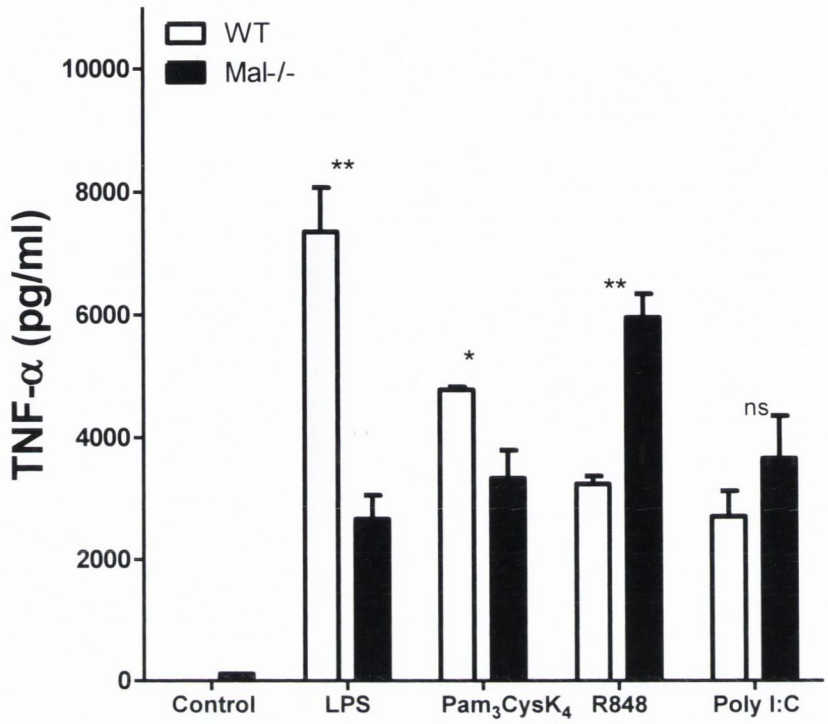


Figure 3-1: TNF-α secretion in response to TLR 2 and TLR 4 ligands, but not TLR3 and TLR7/8 ligands, is Mal-dependent.

Murine iBMM (1 x 10^6 cells/ml) were stimulated with LPS (100 ng/ml), Pam_3CysK_4 (50nM), R848 (100 ng/ml) or Poly I:C (50μg/ml). Supernatants were harvested after 16 hours and TNF-α concentrations determined by ELISA. Data are means +/- S.D from one experiment representative of at least 3 separate experiments. WT vs $Mal^{-/-}$ for each condition, * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney)

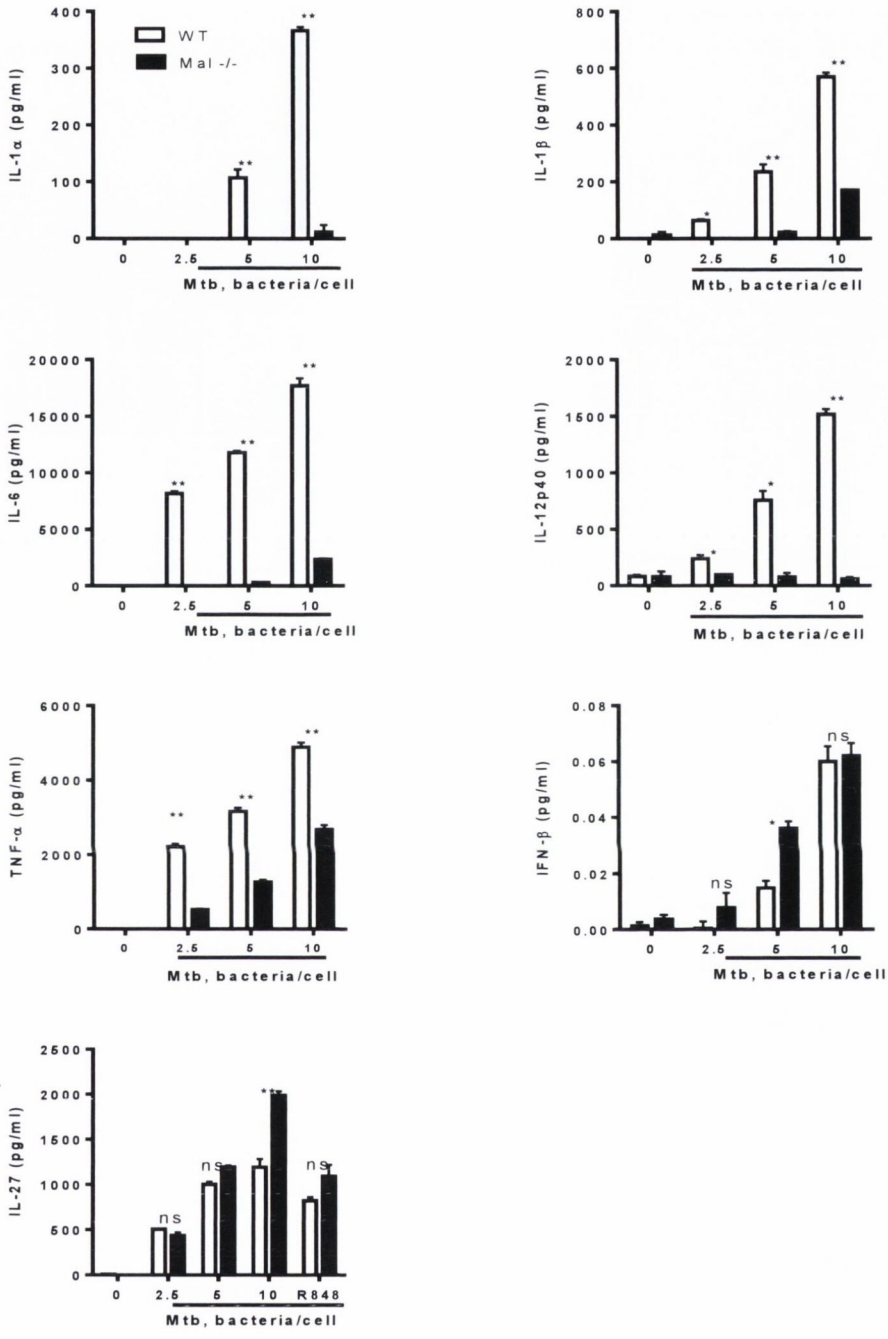


Figure 3-2: Pro-inflammatory cytokine secretion in response to *M. tuberculosis* is Mal-dependent.

WT and Mal$^{-/-}$ murine iBMM (1 x 10^6 cells/ml) were infected with *M. tuberculosis* H37Rv at a range of multiplicity of infection (MOI) as indicated. Supernatants were harvested after 16 hours and cytokine concentrations determined by ELISA. Data shown as mean +/- SEM for triplicate samples. Data representative of 3 independent experiments. WT vs Mal$^{-/-}$ for each condition, * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney)

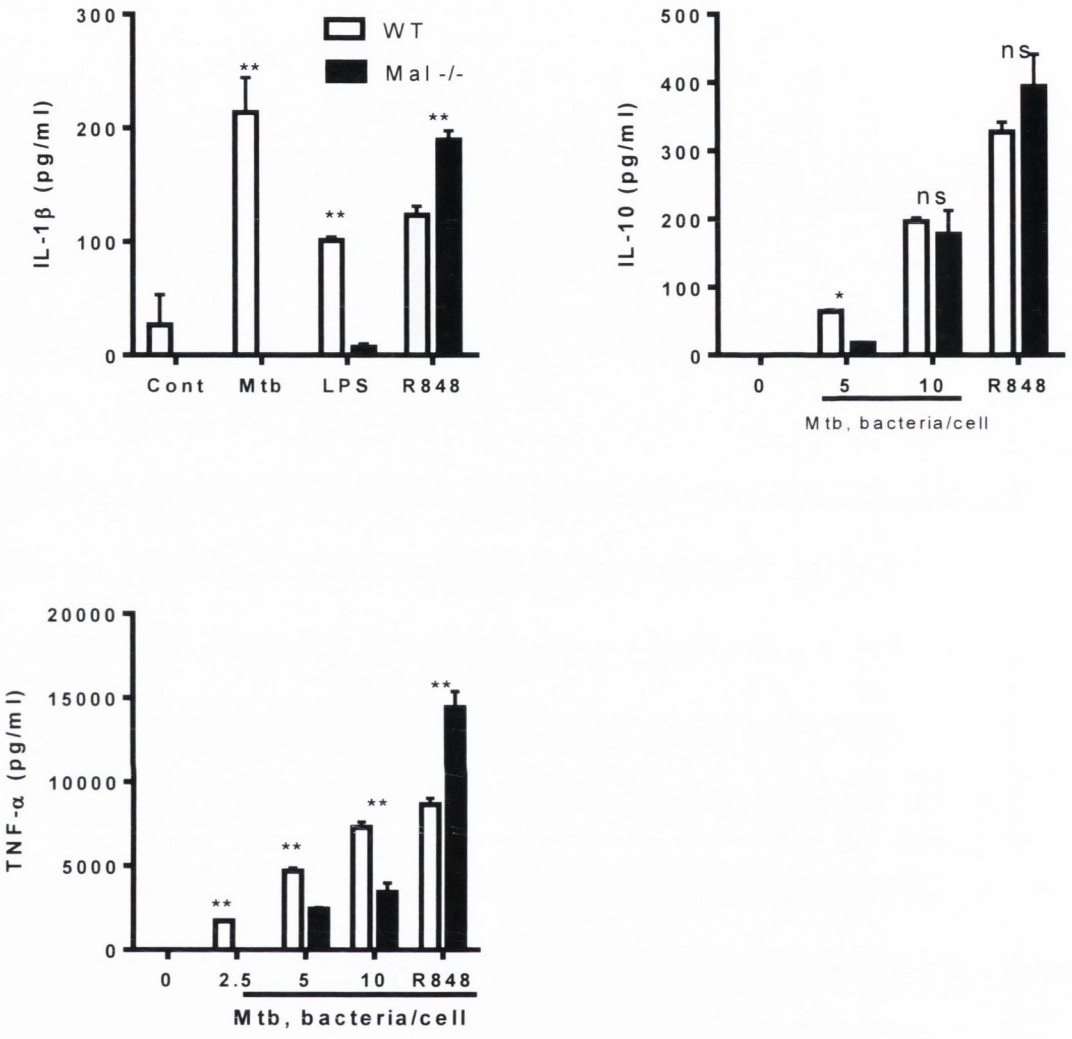
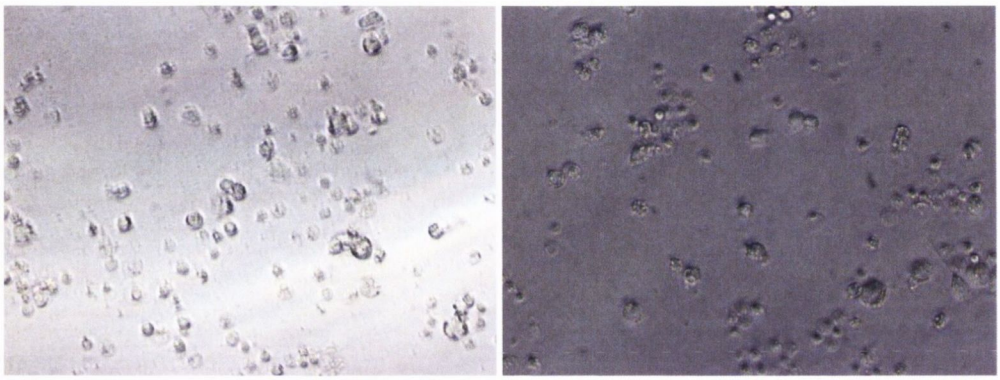


Figure 3-3: IL--1β and TNF-α, but not IL-10 and IL-27. Secretion by primary macrophages in response to *M. tuberculosis* is Mal-dependent.

WT and Mal$^{-/-}$ murine primary BMM (1 x 10^6 cells/ml) were infected with *M. tuberculosis* H37Rv at a range of multiplicity of infection as indicated or treated with R848 100 ng/ml. Supernatants were harvested after 16 hours and analysed by ELISA. Data are means +/- S.D from one experiment representative of at least 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney), WT vs Mal$^{-/-}$.



THP-1 transfected with scrambled Control siRNA

THP-1 transfected with siRNA against Mal

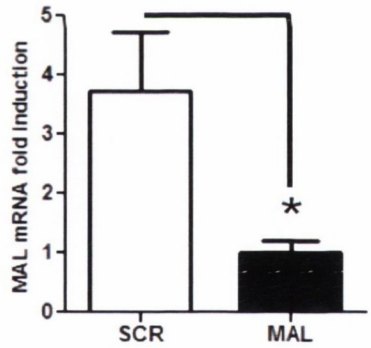


Figure 3-4: Transfection of THP-1 cells

THP-1 cells were transfected with siRNA against Mal or scrambled control (SCR) (Dharmacon, 500 nMol) prior to differentaition with PMA (100nMol) for 72 hours. Light microscopy at 40x showing showing normal morphology of transfected THP-1 cells (upper panel). qPCR was used to confirm knockdown (bottom panel)

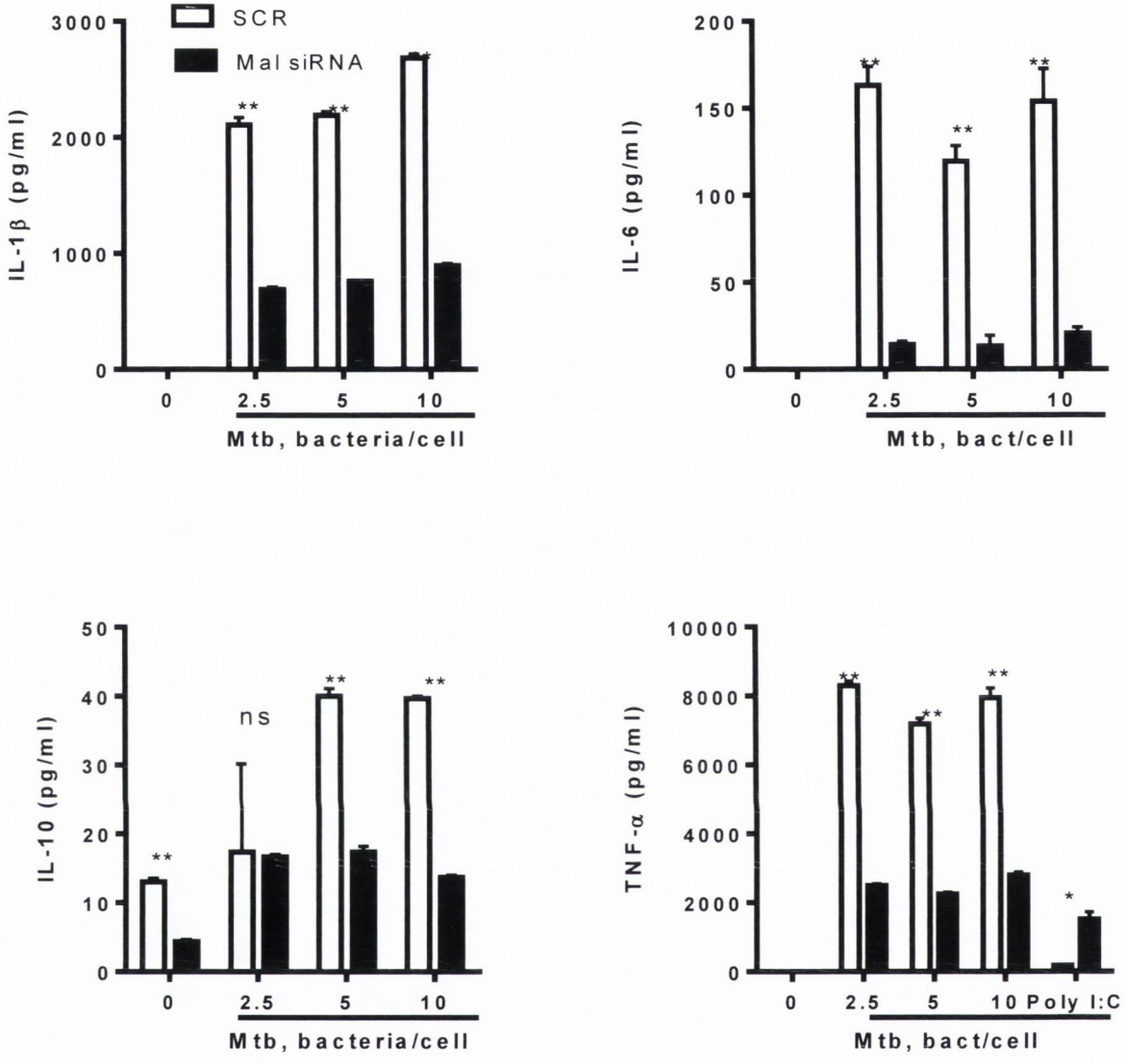


Figure 3-5 IL--1β, IL-6, IL-10 and TNF-α secretion by THP-1 cells in response to *M. tuberculosis* is Mal-dependent.

Cytokine secretion by PMA-differentiated THP-1 cells transfected with siRNA against Mal or scrambled control in response to infection with H37Rv (20 h) was measured by ELISA. . Data shown as mean +/- SD for triplicate samples. Data representative of 3 independent experiments. . * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare Mal siRNA treated cells to wild-type controls for each condition)

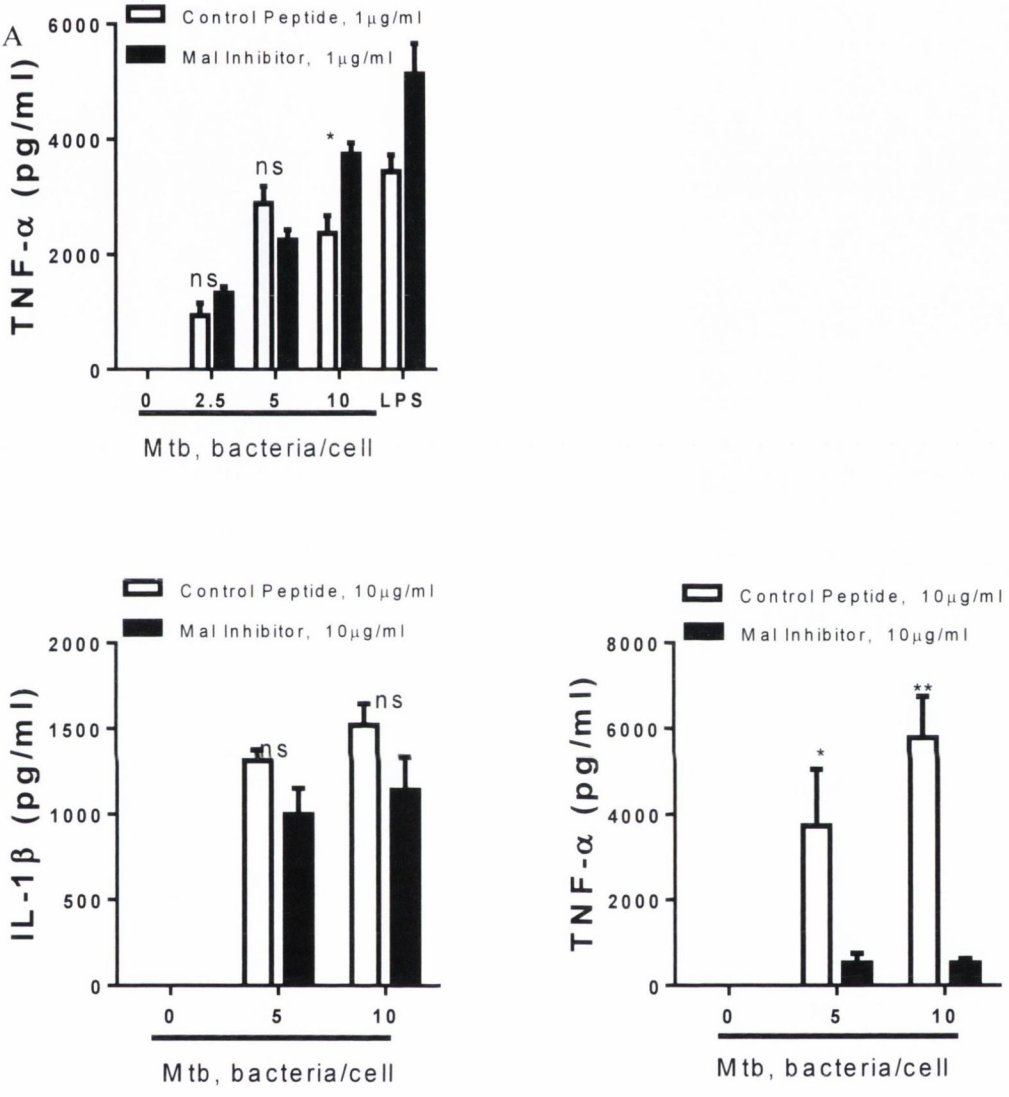


Figure 3-6: Mal inhibitory peptide inhibits cytokine responses to at 10μg/ml but not 1μg/ml.

Cytokine secretion by PMA-differentiated THP-1 cells pre-treated with Mal inhibitory peptide (Calbiochem) at indicated concentrations prior to infection with H37Rv, analysed by ELISA. Data shown as mean +/- SEM for triplicate samples. Data representative of 3 independent experiments. . * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare Mal inhibitor treated cells to controls for each condition)

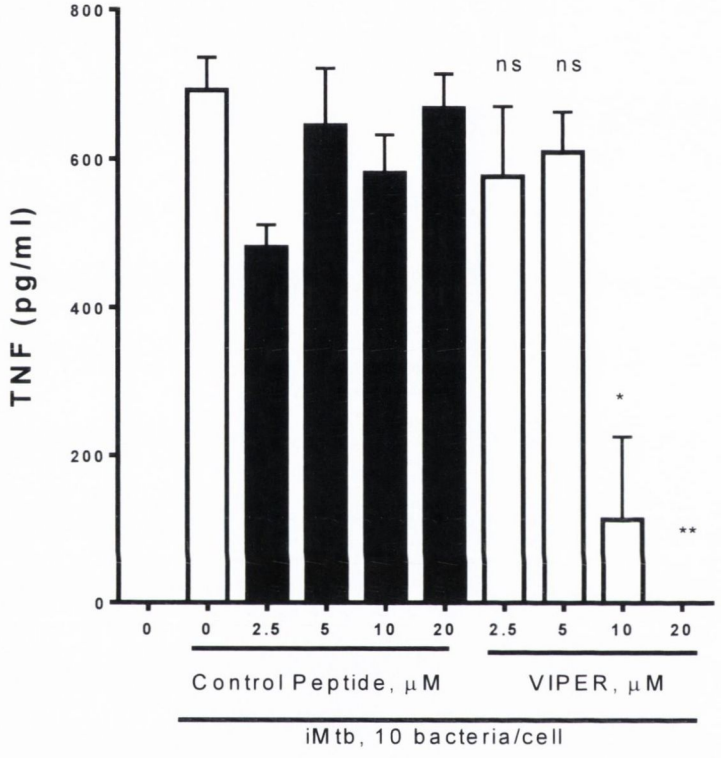


Figure 3-7 VIPER (Viral inhibitory peptide of TLR4) inhibits TNF-α responses to irradiated Mtb at 10μM.

TNF-α secretion by PMA-differentiated THP-1 cells pretreated with VIPER or control peptide at concentrations indicated above, analysed by ELISA. Data shown as mean +/- SEM for triplicate samples. Data representative of 3 independent experiments. * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare Mal inhibitor treated cells to controls for each condition)

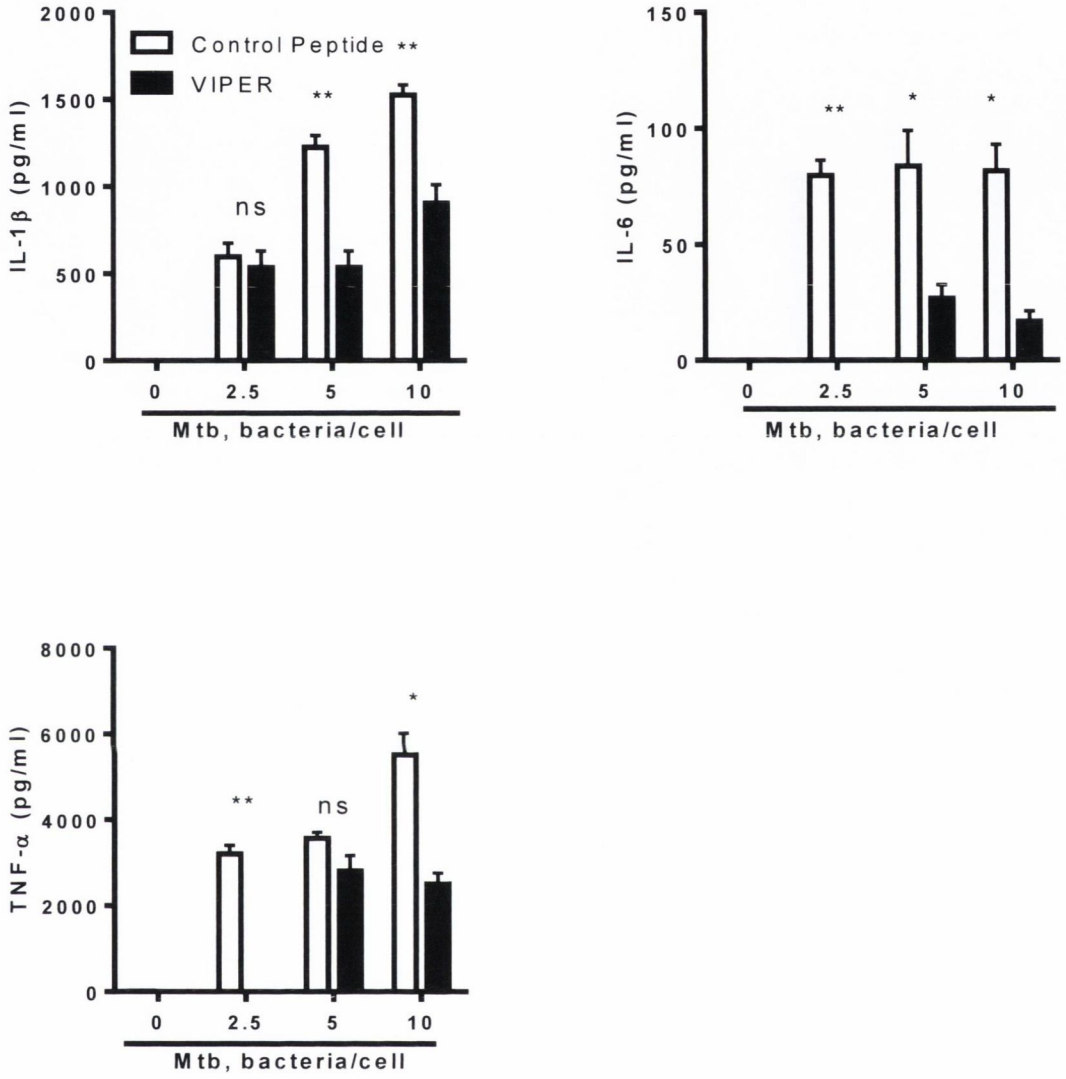


Figure 3-8 VIPER (Viral inhibitory peptide of TLR4) inhibits cytokine responses to live virulent Mtb.

TNF-α secretion by PMA-differentiated THP-1 cells pretreated with VIPER or control peptide at concentrations indicated above, analysed by ELISA. Data shown as mean +/- SEM for triplicate samples. Data representative of 3 independent experiments. * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare Mal inhibitor treated cells to controls for each condition)

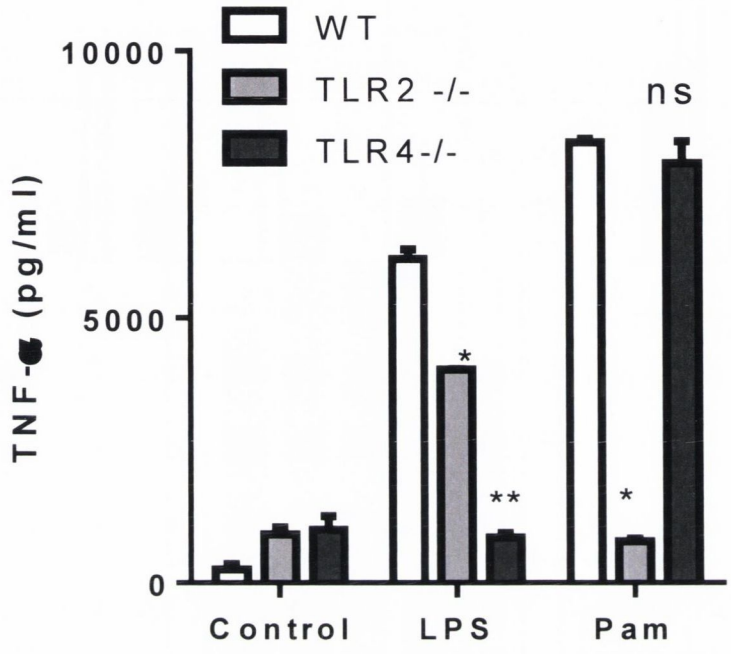


Figure 3-9: Cytokine secretion in TLR 2 and TLR4$^{-/-}$ iBMM.

iBMM (1 x 10^6/ml) were stimulated with LPS (100ng/ml) or Pam_3CysK_4 (20 nMol) for 20 hours Supernatants were analysed by ELISA. Data shown as mean +/- SEM for triplicate samples. * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for each condition)

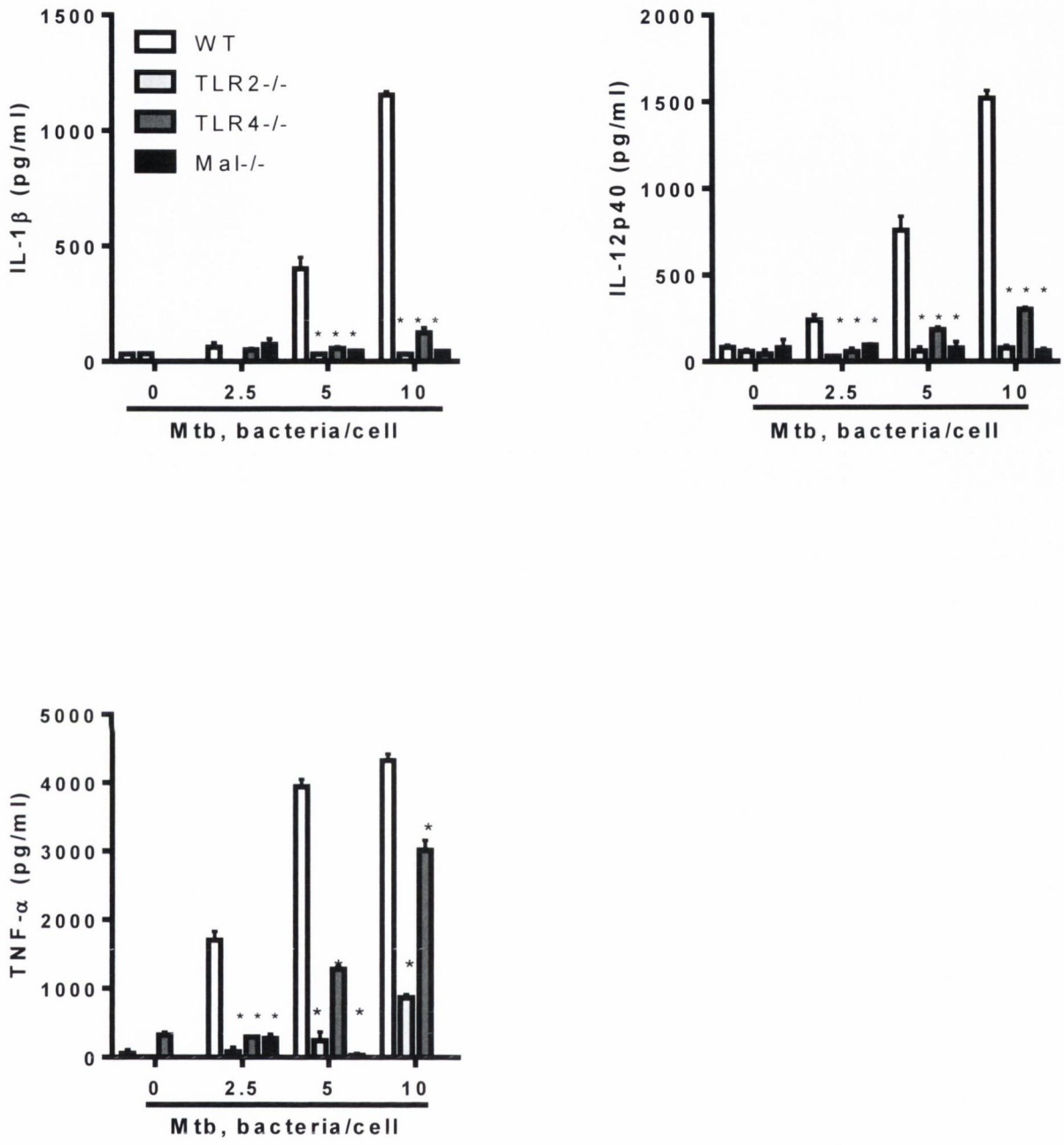


Figure 3-10: Cytokine production in response to *M. tuberculosis* is TLR2- and, to a lesser extent, TLR4- dependent.

Cytokine secretion by iBMM (1 x 10^6/ml) in response to infection with Mtb H37Rv (20 h) was measured by ELISA. Data shown as mean +/- SEM for triplicate samples. Data representative of 3 independent experiments. . * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for each condition)

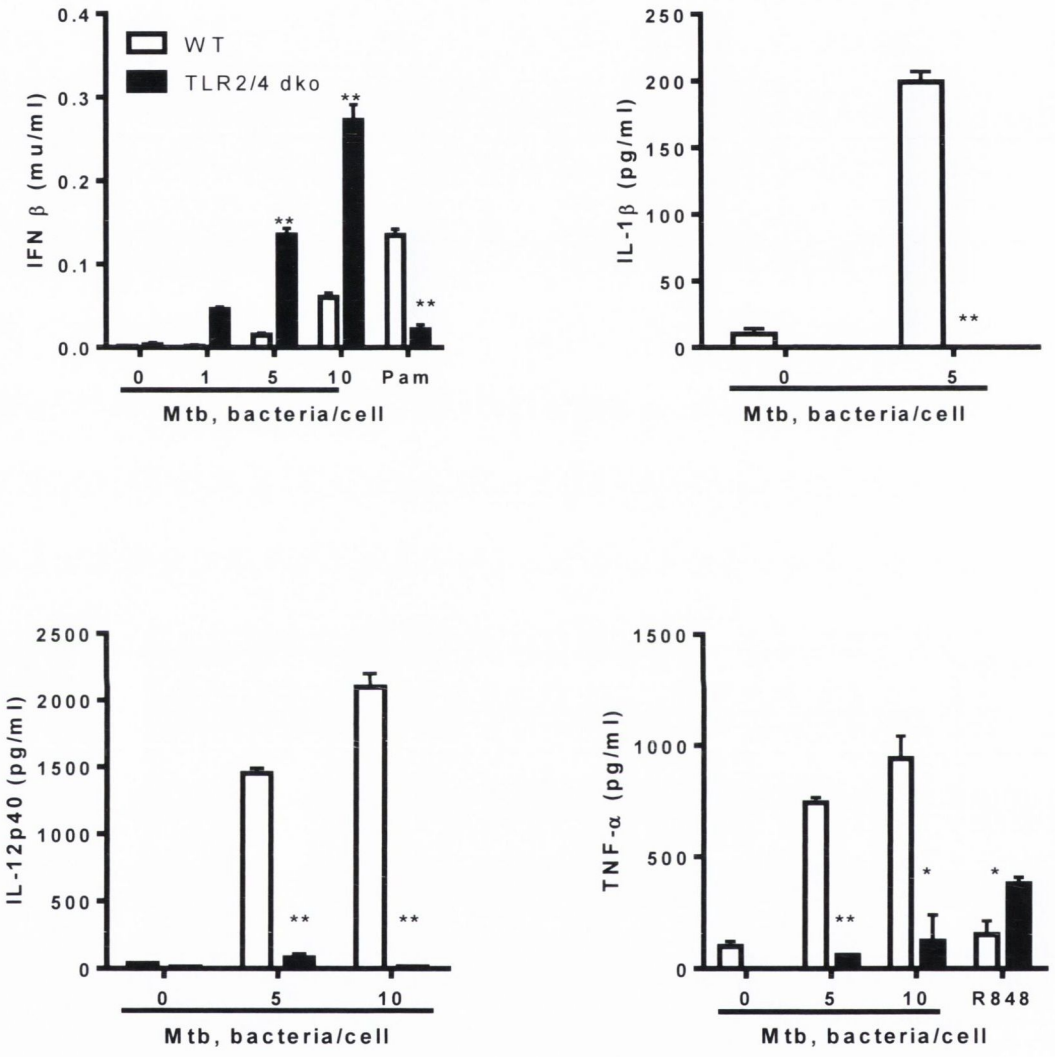


Figure 3-11 Pro-inflammatory cytokine secretion in response to *M. tuberculosis* is abrogated in TLR2/4-/- iBMM.

Cytokine secretion by iBMM (1 x 10^6/ml) in response to infection with H37Rv (20 h) was measured by ELISA. . Data shown as mean +/- SEM for triplicate samples. Data representative of 3 independent experiments. . * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for each condition)

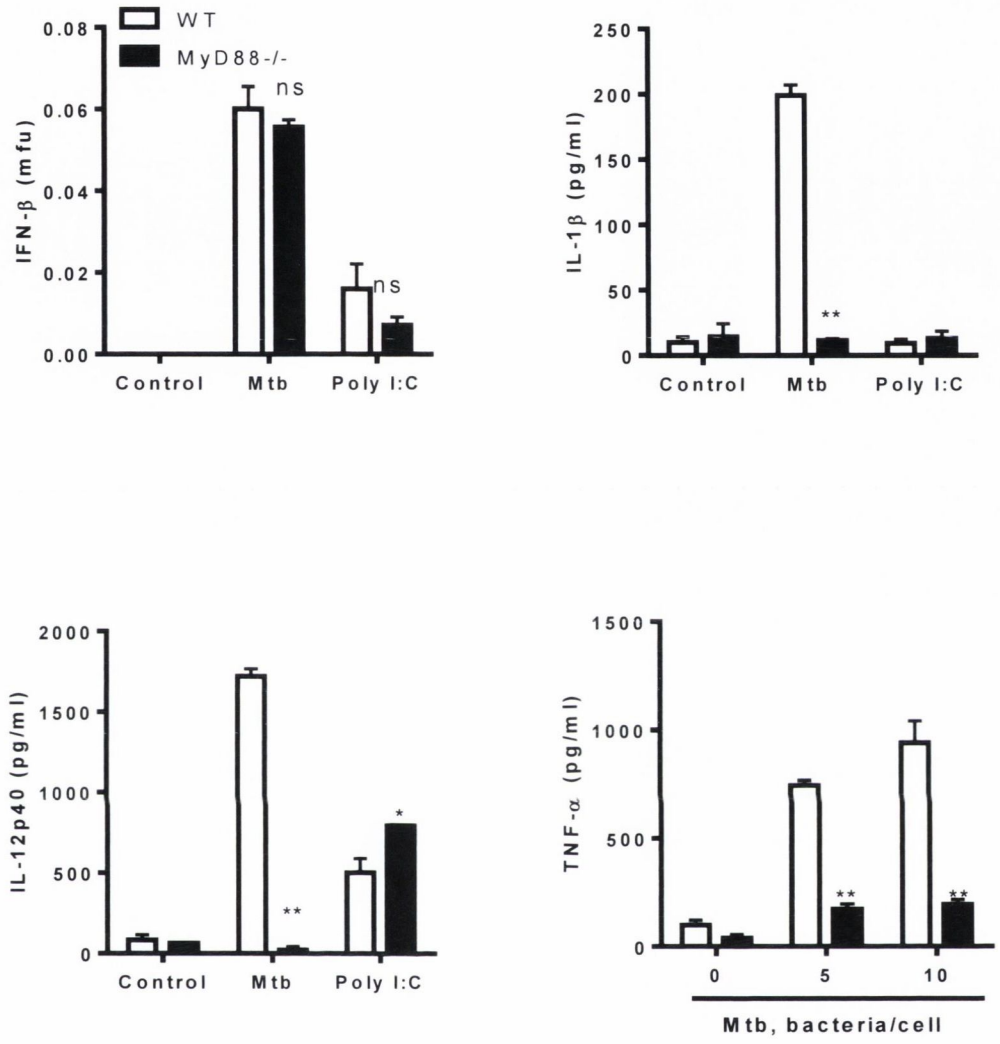


Figure 3-12: Pro-inflammatory cytokine secretion in response to *M. tuberculosis* is abrogated in MyD88$^{-/-}$ iBMM.

Cytokine secretion by iBMM (1 x 10^6/ml) in response to infection with H37Rv (20 h) was measured by ELISA. Data shown as mean +/- SEM for triplicate samples. Data representative of 3 independent experiments. . * $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for each condition)

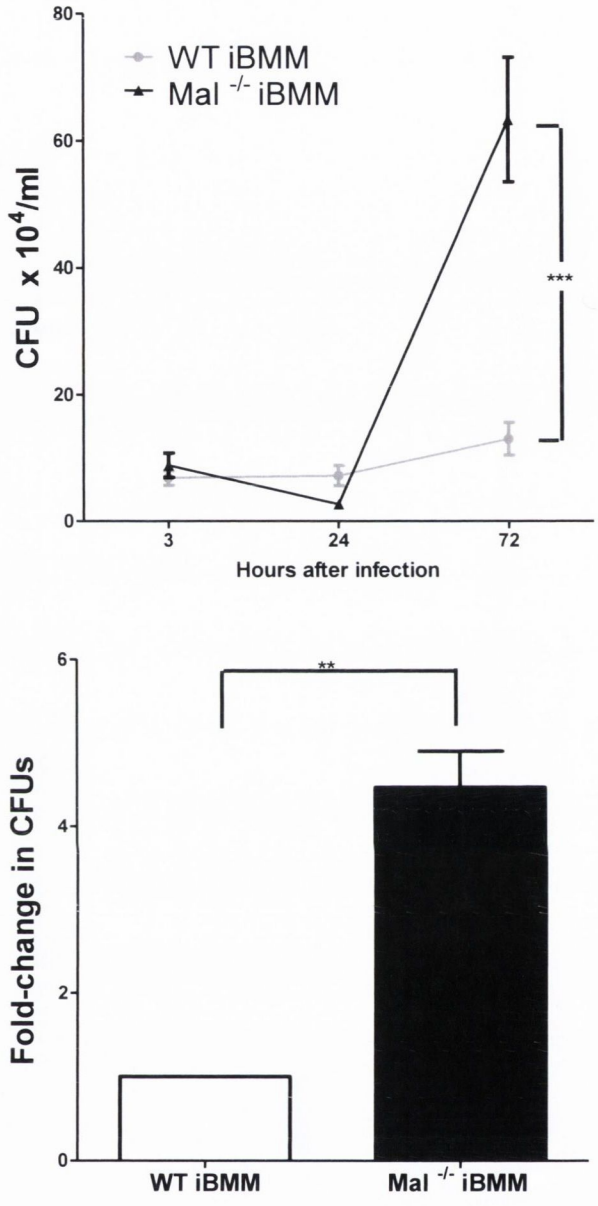


Figure 3-13: Killing of *M. tuberculosis* by iBMM is Mal- dependent.

iBMM were infected with *M. tuberculosis* (Mtb) H37Rv and lysed at 3, 24 and 72 h. Serial dilutions of lysates were plated out to determine bacterial numbers. A shows an experiment, representative of at least 3 separate experiments. B is mean +/- S.D pooled from 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls)

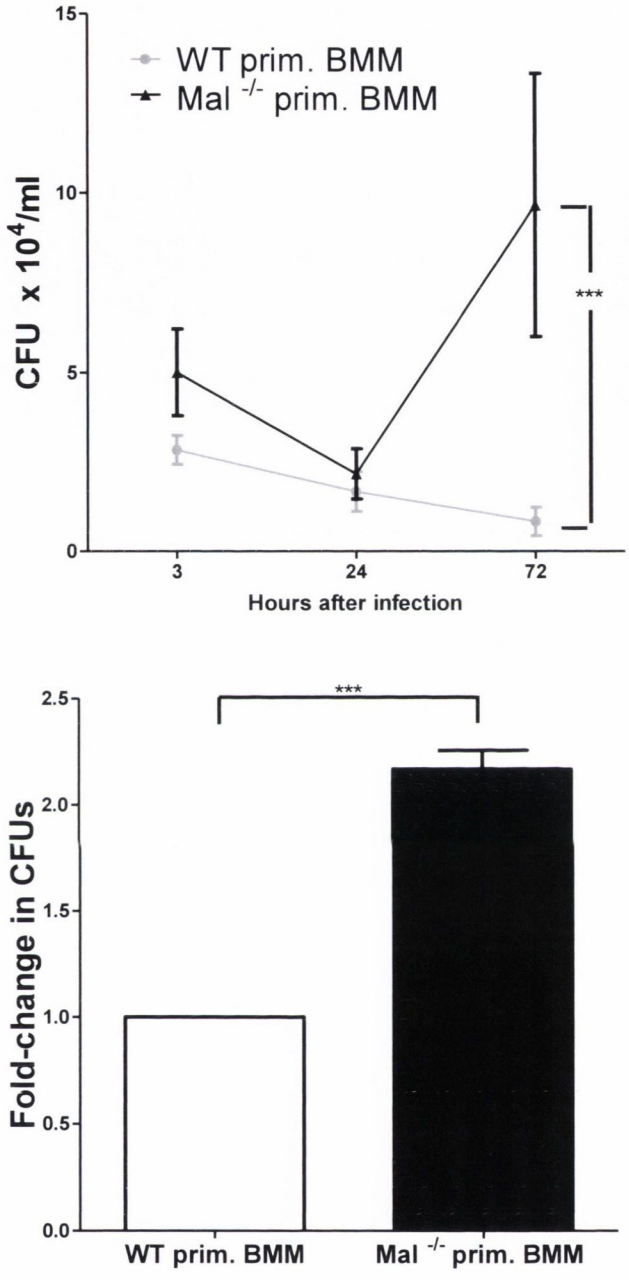


Figure 3-14 Killing of *M. tuberculosis* by primary BMM is Mal- dependent.

Primary BMM (1 x 10^5/ml) were infected with H37Rv and lysed at 3, 24 and 72 h. Serial dilutions of lysates were plated out to determine bacterial numbers. A shows an experiment, representative of at least 3 separate experiments. B is mean +/- S.D pooled from 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls)

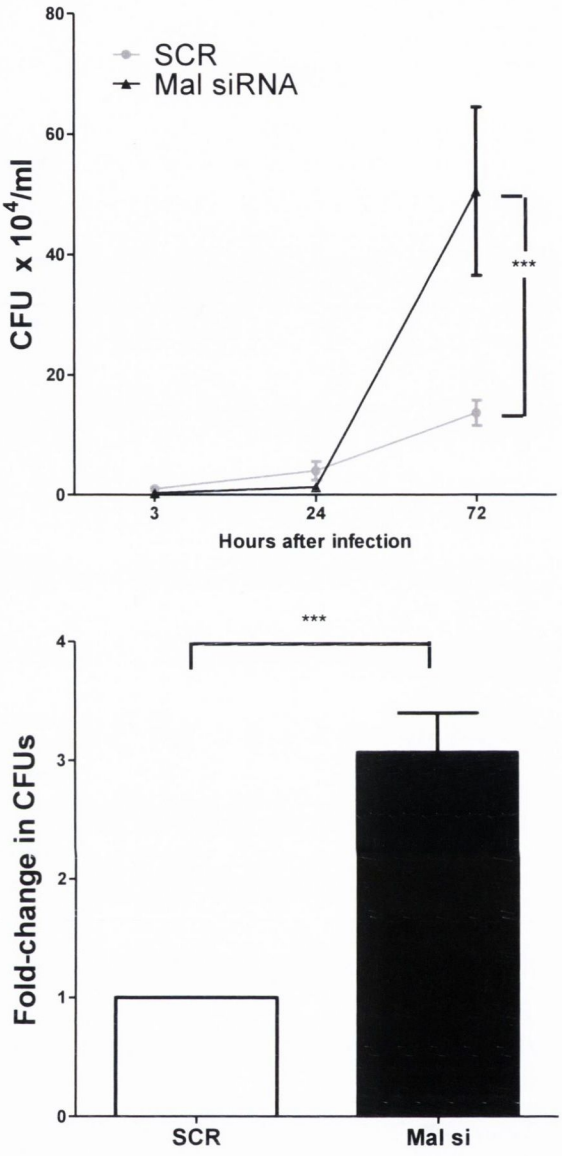


Figure 3-15 Killing of *M. tuberculosis* is Mal- dependent.

Transfected THP-1 were differentiated and infected with *M. tuberculosis* () H37Rv and lysed at 3, 24 and 72 h. Serial dilutions of lysates were plated out to determine bacterial numbers. A shows an experiment, representative of at least 3 separate experiments. B is mean +/- S.D pooled from 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls).

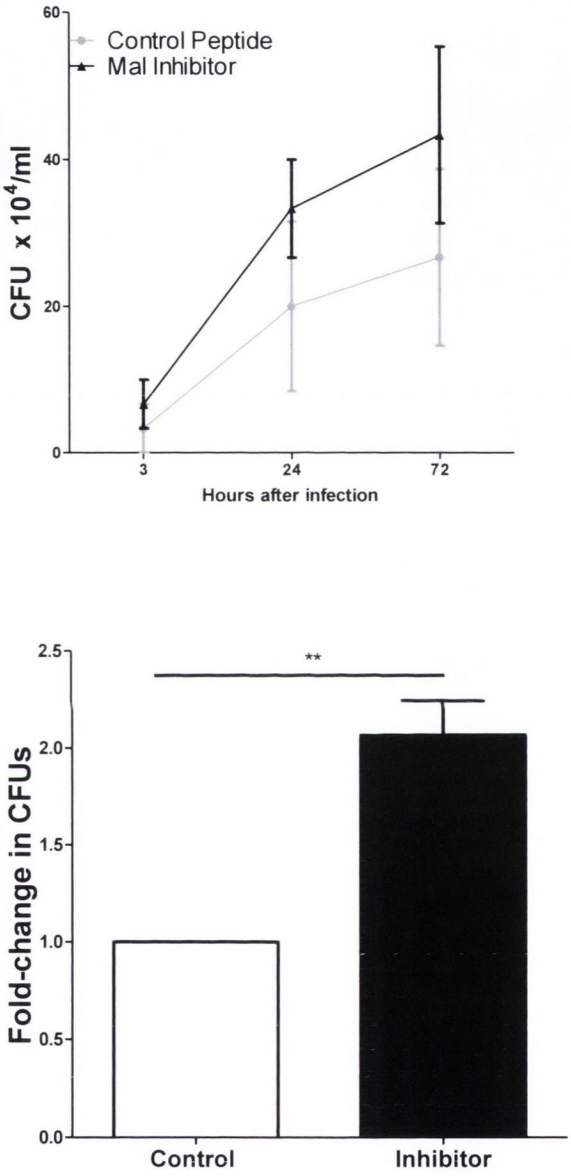


Figure 3-16 Killing of *M. tuberculosis* is Mal- dependent in human macrophages.

THP-1 were differentiated and pretreated with Mal inhibitory peptide (Calbiochem) prior to infection with *M. tuberculosis* () H37Rv and lysed at 3, 24 and 72 h. Serial dilutions of lysates were plated out to determine bacterial numbers. A shows an experiment, representative of at least 3 separate experiments. B is mean +/- S.D pooled from 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls)

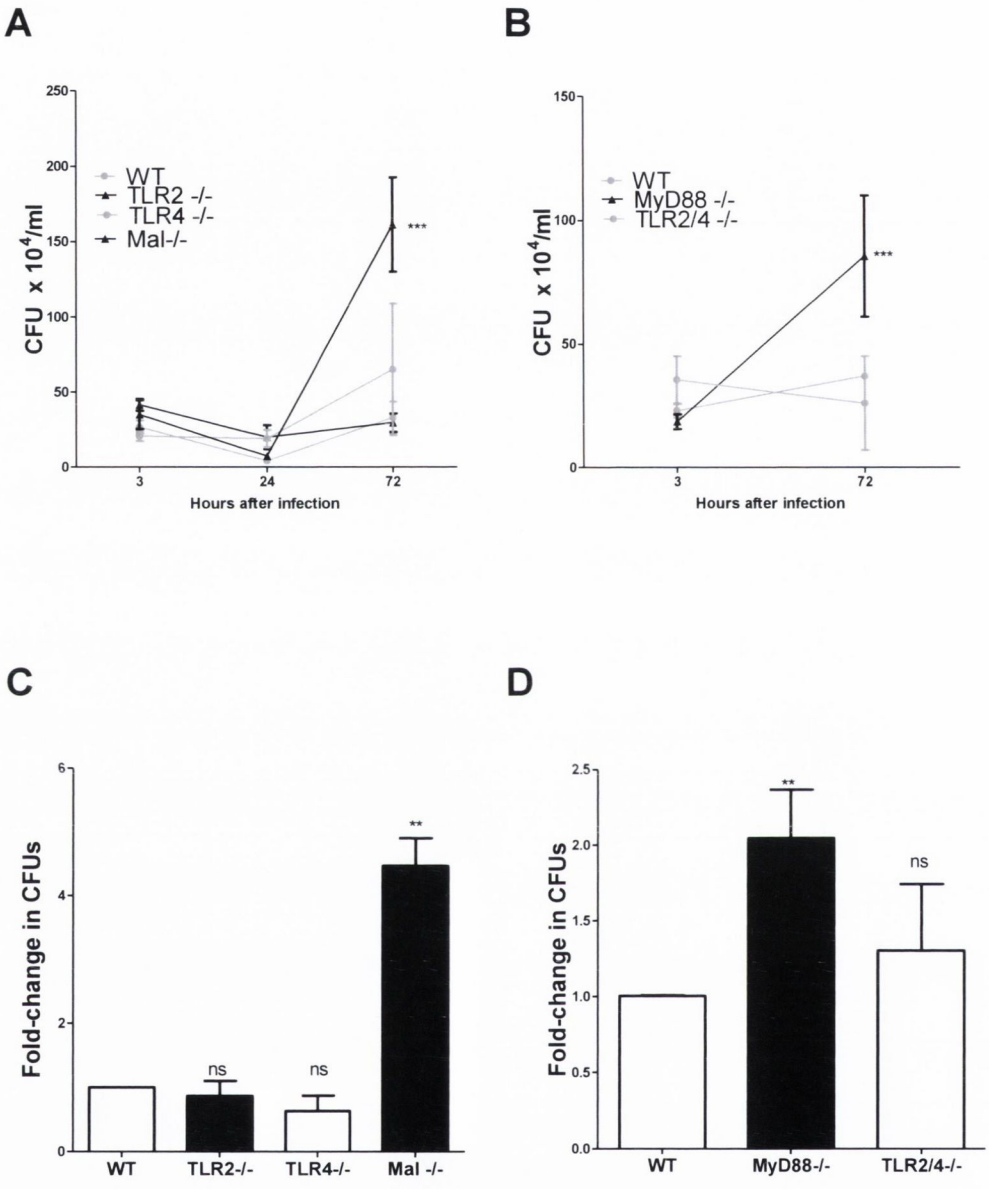
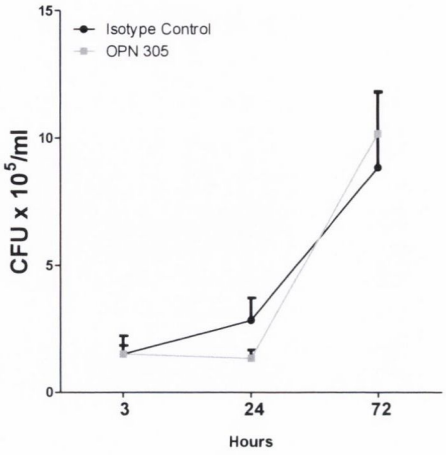


Figure 3-17: Killing of *M. tuberculosis* is MyD88- but not TLR2/4-dependent.

iBMM were infected with *M. tuberculosis* H37Rv and lysed at 3, 24 and 72 h. Serial dilutions of lysates were plated out to determine bacterial numbers. A and B show experiments, representative of at least 3 separate experiments. C and D are mean +/- S.D pooled from 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for same time point).



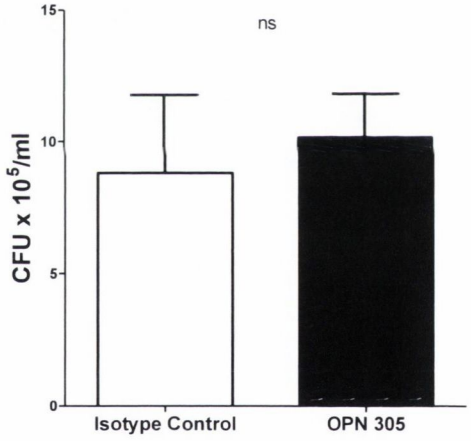
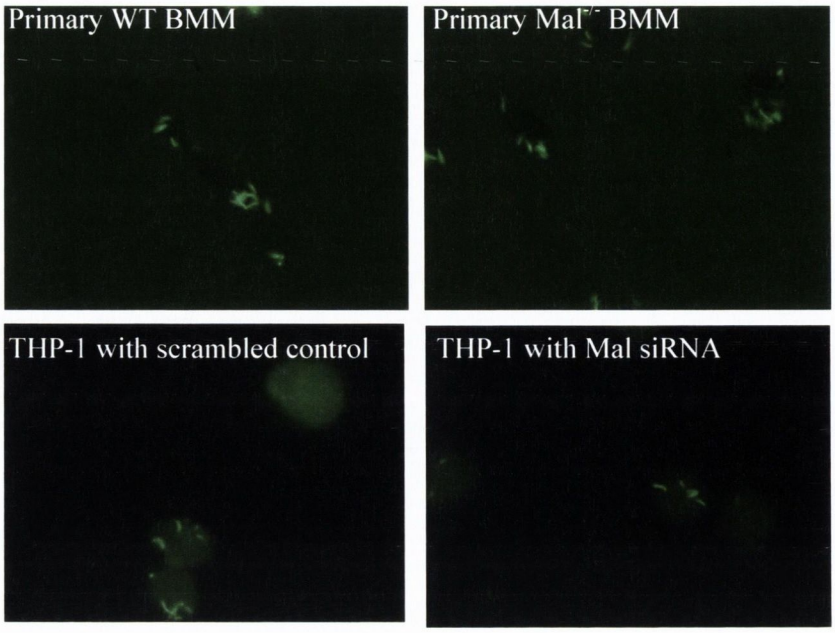


Figure 3-18: Killing of *M. tuberculosis* is not TLR2- dependent in human macrophages.

THP-1 were differentiated and pretreated with TLR2 inhibitory peptide (Opsona) prior to infection with *M. tuberculosis* H37Rv and lysed at 3, 24 and 72 h. Serial dilutions of lysates were plated out to determine bacterial numbers. A shows an experiment, representative of at least 3 separate experiments. B is mean +/- S.D pooled from 3 separate experiments. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$, ns not statistically significant (Mann-Whitney used to compare cells treated with inhibitor to cells treated with control for same time point).



A B

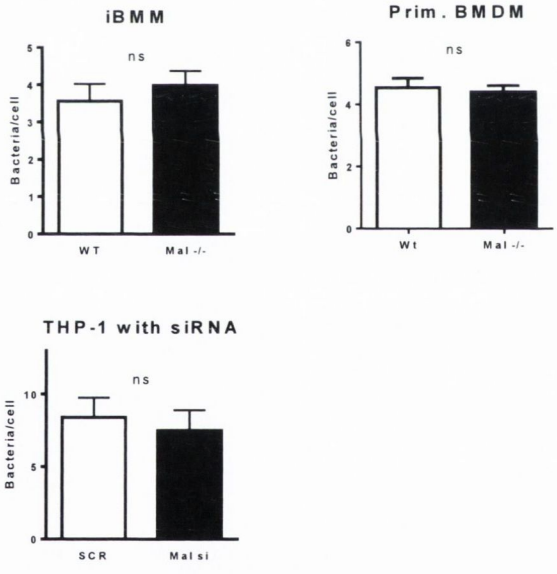


Figure 3-19: Uptake of Mtb is not Mal- dependent.

iBMM (A), primary BMM (B) and transfected differentiated THP-1 (C) were infected with *M. tuberculosis* H37Rv and fixed at 3h prior to staining with Auramine. The number of intracellular bacteria/cell was quantified for a minimum of 100 cells/condition Shown is mean +/- S.D pooled from 3 separate experiments* $p < 0.05$, **p<0.01, ***p<0.001, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls).

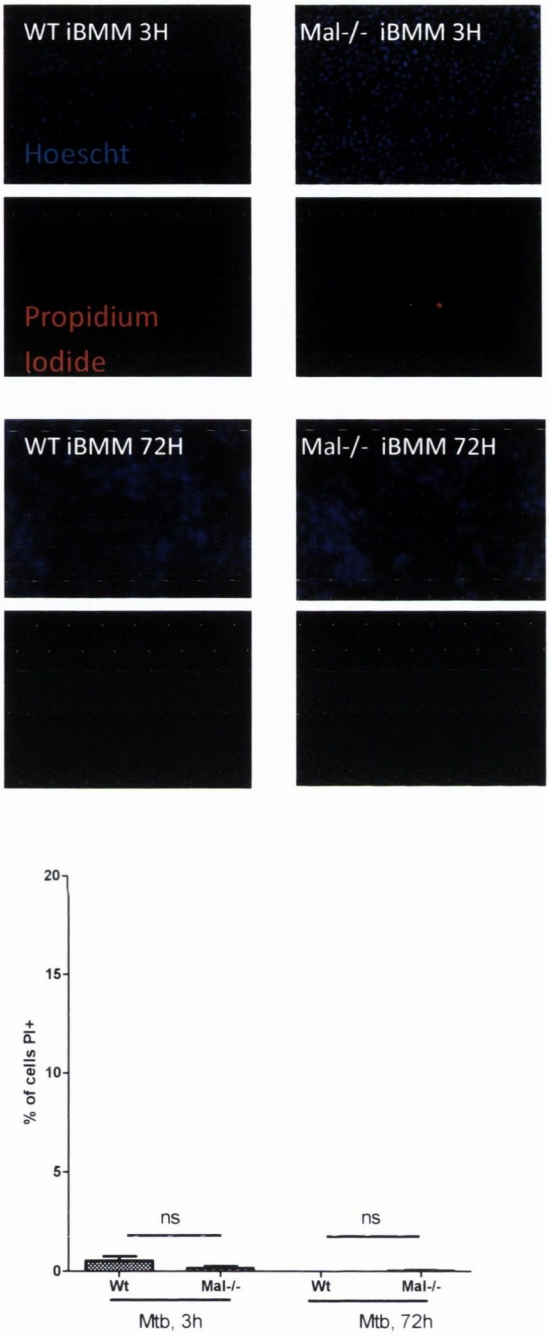
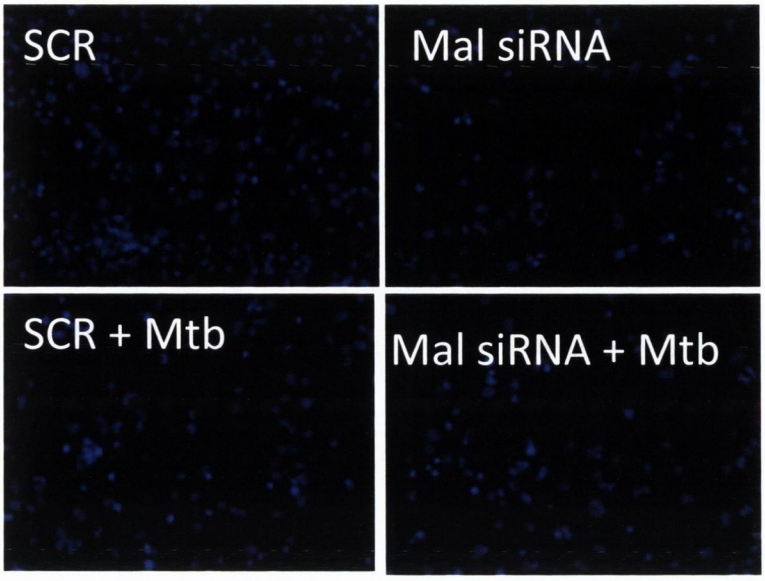


Figure 3-20: Cell death in response to Mtb infection is not Mal- dependent in iBMM.

iBMM were infected with *M. tuberculosis* H37Rv and stained with propidium iodide (necrotic cell nuclei) and Hoescht stain (all cell nuclei) and fixed at 3h and 72 h. Cells were analysed by microscopy and the number of PI^+ cells quantified for a minimum of 100 cells. Representative images and mean+/- SEM of experiment carried out in triplicate. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for same time point)



Hoescht

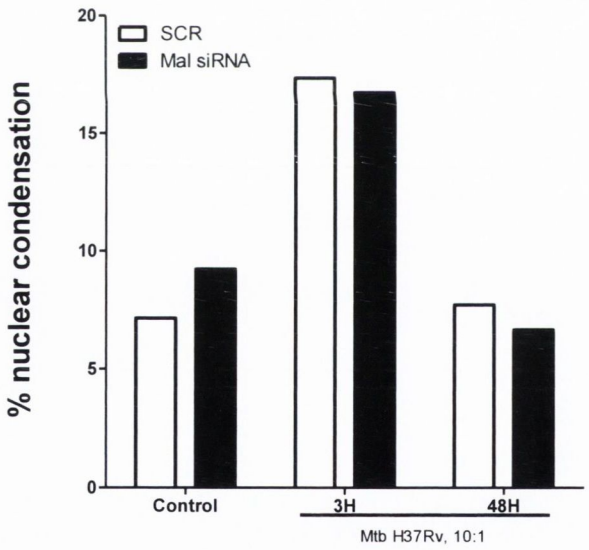


Figure 3-21: Cell death in response to Mtb infection is not Mal- dependent in human macrophages.

Transfected THP-1 were differentiated with PMA and infected with *M. tuberculosis* H37Rv for 24 h. Cells were stained with Hoescht stain and fixed. Cells were analysed by microscopy and the number of cells with nuclear condensation (apoptotic cells) quantified for a minimum of 100 cells. Representative images and mean of single experiment.

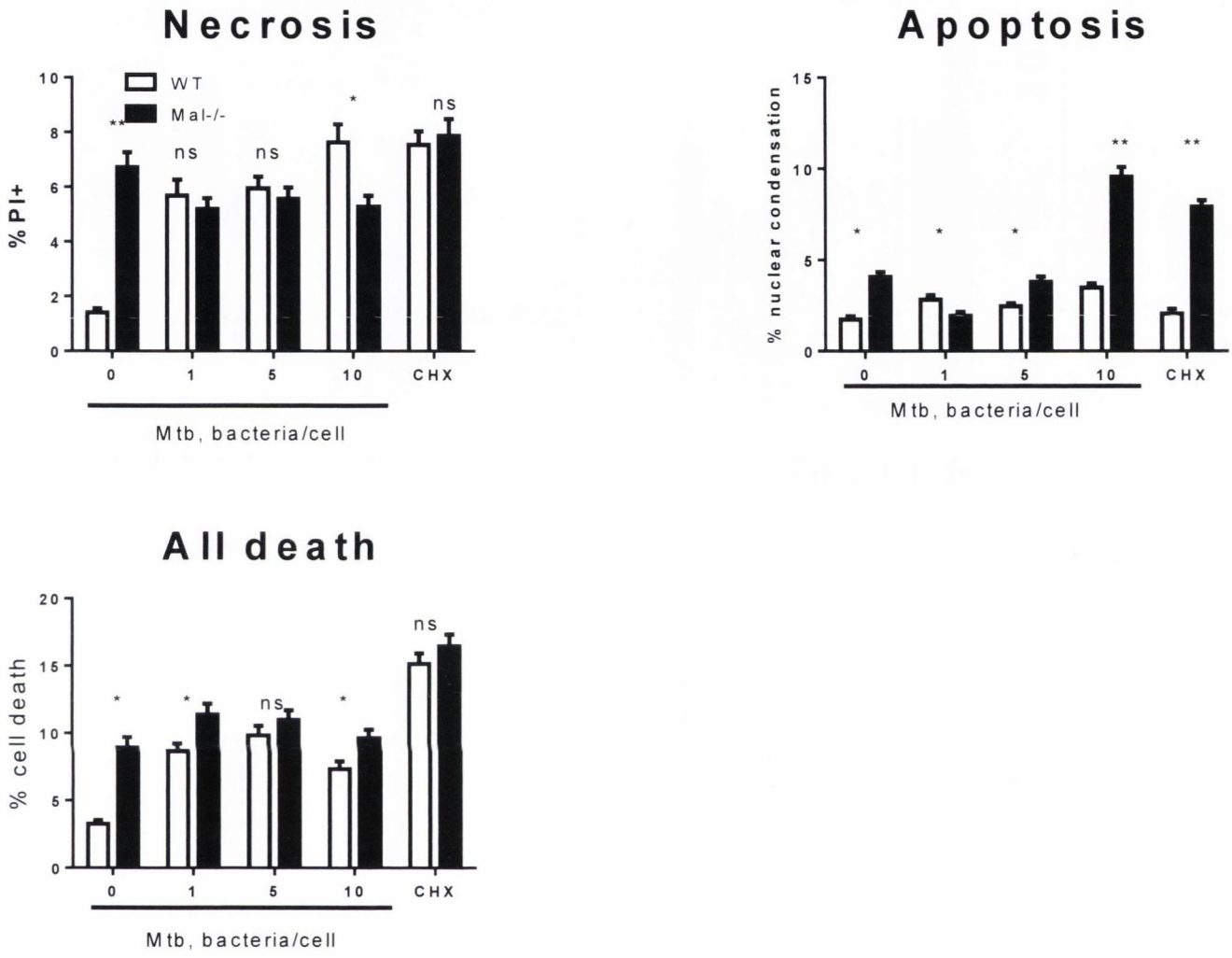


Figure 3-22: Cell death at 3h post Mtb infection is not decresased in $Mal^{-/-}$ primary BMM.

Primary BMM were infected with *M. tuberculosis* H37Ra and stained with propidium iodide and Hoescht stain. Live cells were analysed at 3 h using the InCell high-throughput microscope. Mean +/- SEM of experiment carried out in triplicate. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for same time point).

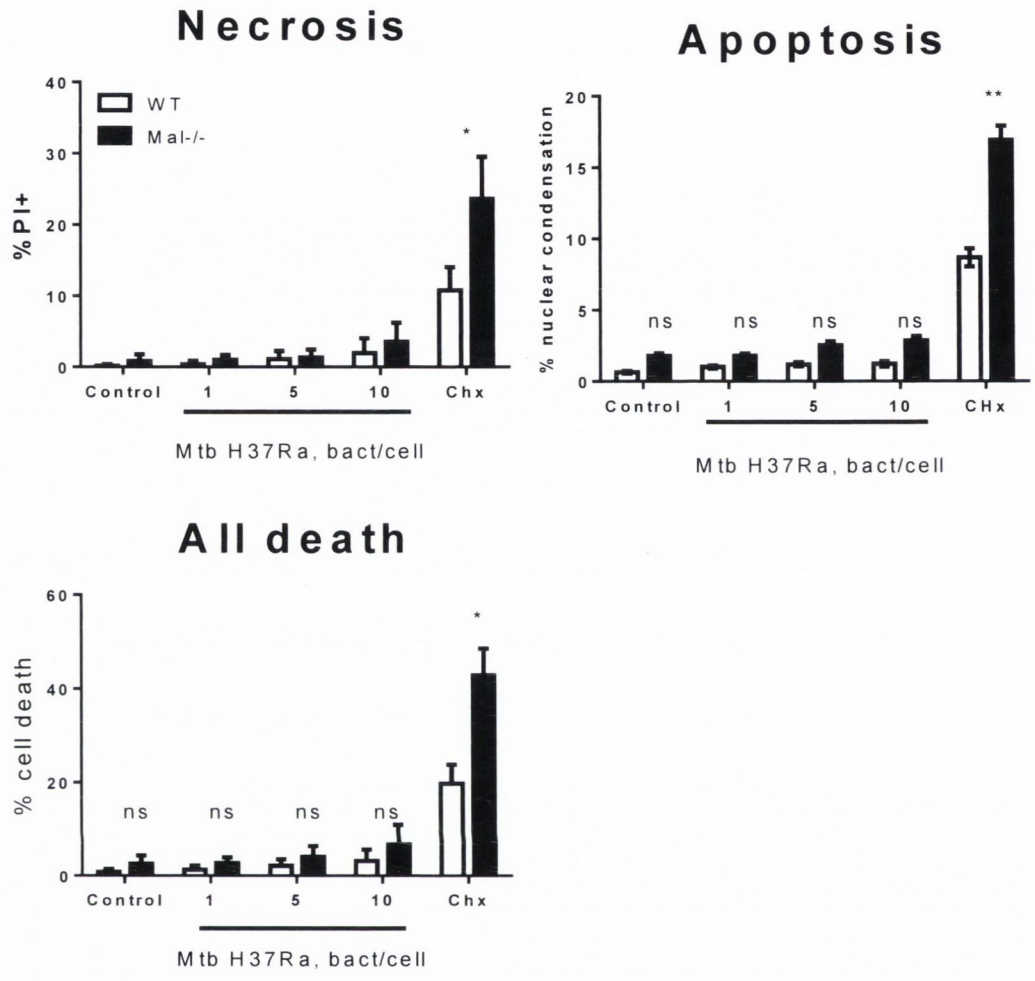


Figure 3-23: Cell death at 24 h post Mtb infection at 24 h is not reduced.

Primary BMM were infected with *M. tuberculosis* H37Ra and stained with propidium iodide and Hoescht stain. Live cells were analysed at 24 h using the InCell high-throughput microscope. Mean +/- SEM of experiment carried out in triplicate. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, ns not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for same time point).

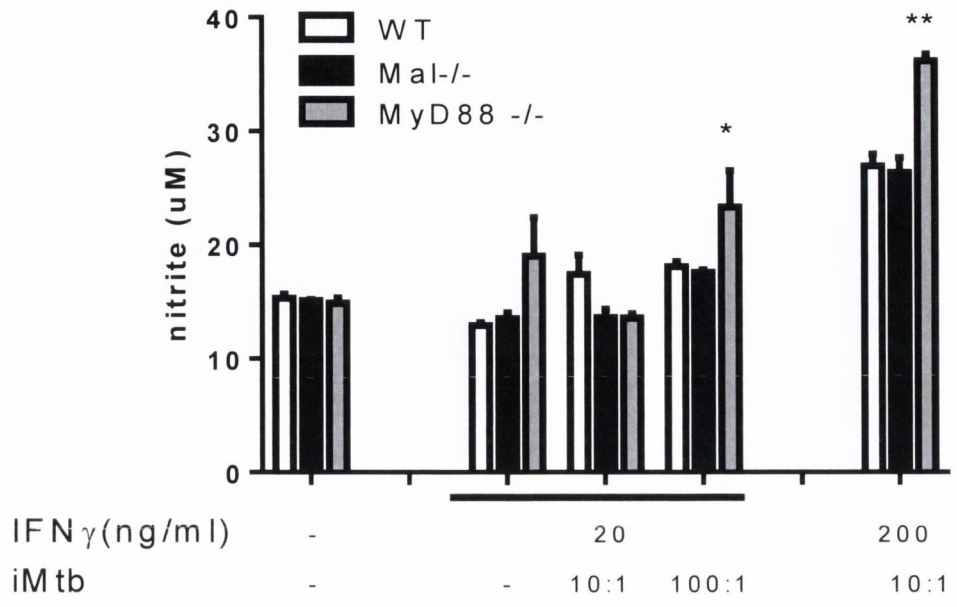
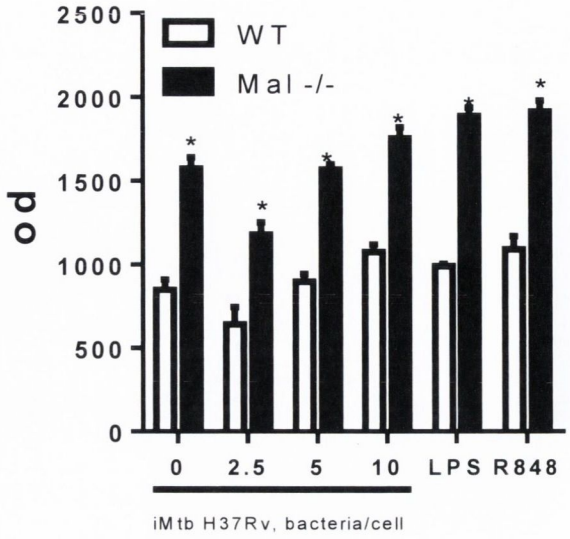


Figure 3-24: Nitric oxide production in resonse to Mtb infection is not Mal- dependent.

iBMM were pretreated with RPMI or IFN-γ and stimulated with irradiated *M. tuberculosis.* Nitric oxide production was measured were using Griess reagent. Shown is the mean +/- SEM of a single experiment carried out in triplicate. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, not statistically significant (Mann-Whitney used to compare knockout cells to WT controls for same condiyion).



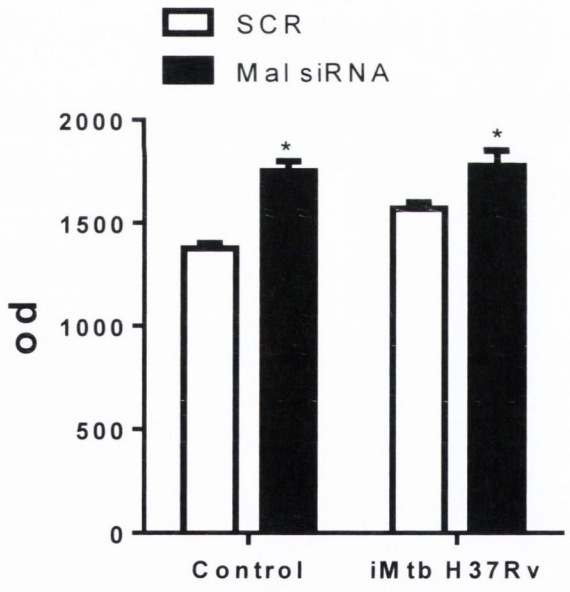


Figure 3-25: Reactive oxygen species production in response to Mtb infection is not Mal-dependent.

iBMM (upper panel) and THP-1 transfected with siRNA against Mal or scrambled control (lower panel) were stimulated with iMtb. The production of reactive oxygen species was measured using an Amplex red colorimetric assay. Shown is the mean +/- SEM of a single experiment carried out in triplicate

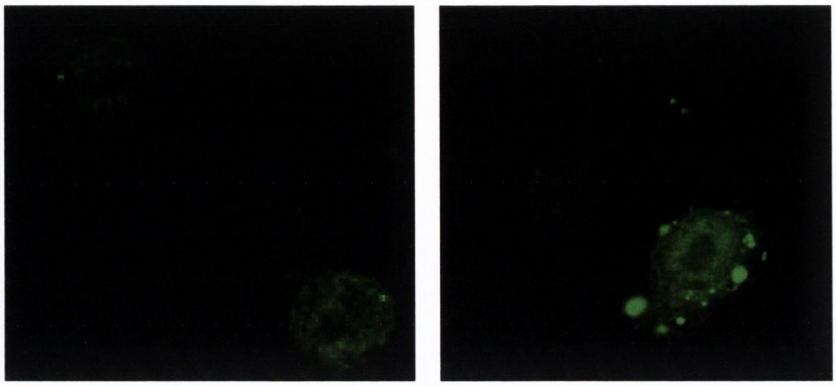
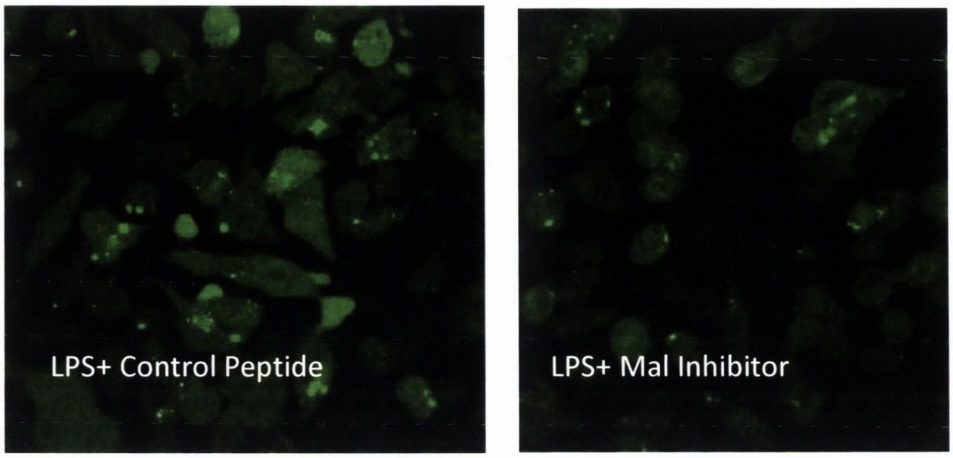


Figure 3-26: GFP-LC3 expressing iBMM develop fluorescently labelled puncta in response to LPS.

iBMM expressing GFP-labelled LC3 were treated with LPS (100ng/ml) for 6 hours, fixed and analysed by confocal fluorescent microscopy (Olympus FV100). The cells exhibit autophagosomes as characteristic puncta formation in response to stimulation.



GFP-LC3

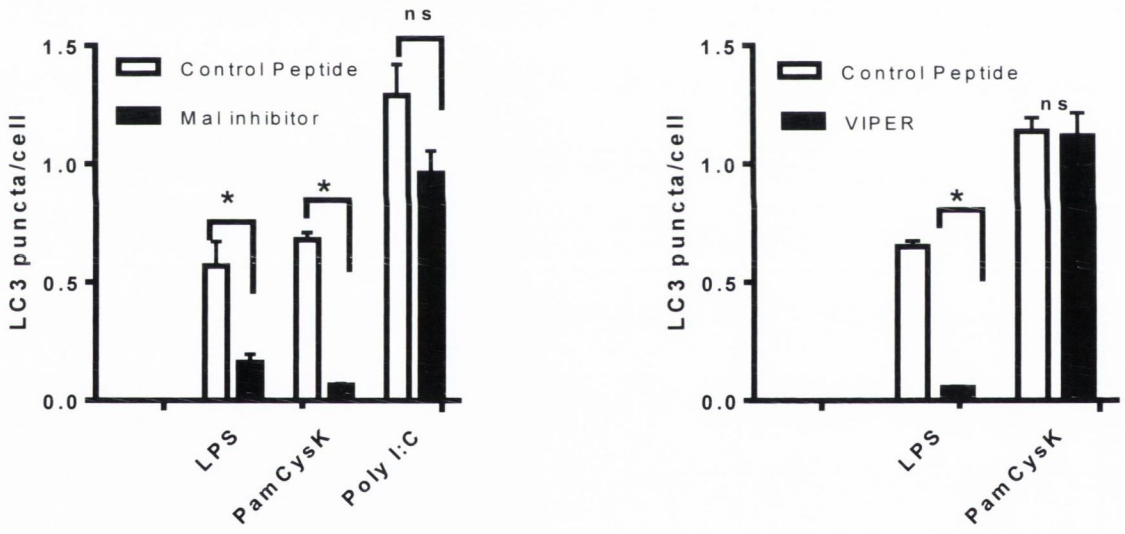


Figure 3-27: Mal is required for autophagy in response to TLR2 and TLR4 ligands.

GFP-LC3 expressing iBMM were pre-treated with Calbiochem mal inhibitor (10μg/ml) or control peptide or VIPER (10μMol) or control peptide prior to stimulation with LPS (100ng/ml). Cells were fixed at 6 hours and assessed using confocal microscopy. Puncta/cell were counted for a minimum of 100 cells/condition. Representative images and means+/- SD of experiments carried out in triplicate, representative of 3 separate experiments. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$, not statistically significant (Mann-Whitney used to compare inhibitor treated cells to control peptide for same condition).

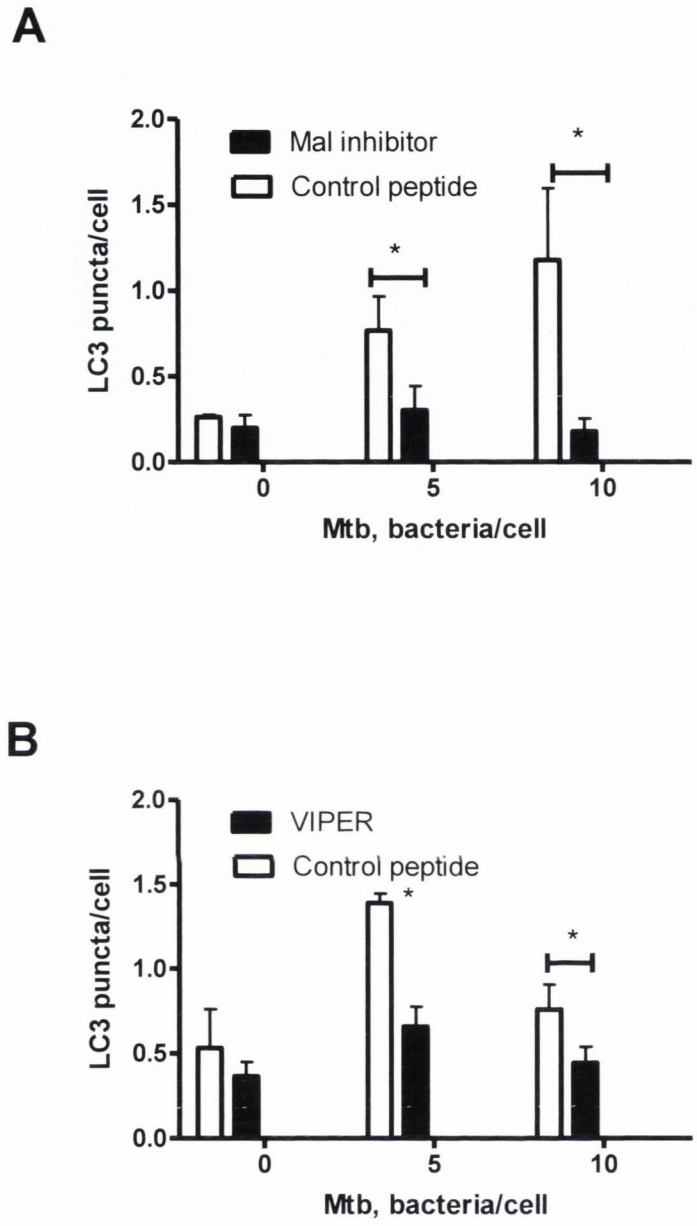


Figure 3-28: Mal is required for autophagy in response to live Mtb.

GFP-LC3 expressing iBMM were pre-treated with Calbiochem mal inhibitor (10μg/ml) or control peptide(A) or VIPER (10μM) or control peptide (B) prior to stimulation with live Mtb H37Rv. Cells were fixed at 6 hours and assessed using confocal microscopy. Puncta/cell were counted for a minimum of 100 cells/condition. Means +/- S.E.M. of single experiment representative of 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, not statistically significant (Mann-Whitney used to compare inhibitor treated cells to control peptide for same condition)

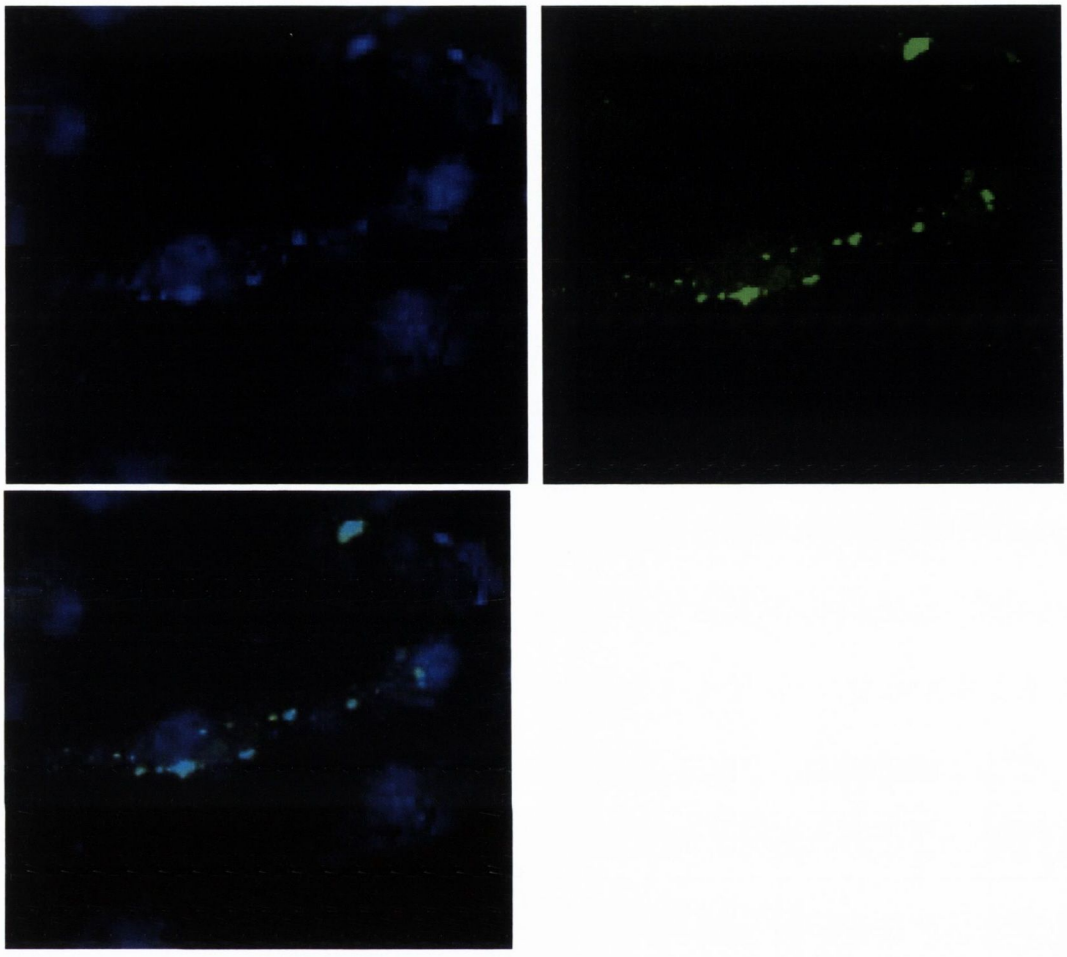


Figure 3-29: Anti-LC3 antibody (Invitrogen) co-localises with GFP-LC3 labelled puncta in LC3-GFP expressing cells.

GFP-LC3 stably expressing iBMM were treated with LPS for 6 hours prior to fixing and staining with anti-LC3 antibody (Invitrogen) and fluorescently labelled secondary antibody. Representative images.

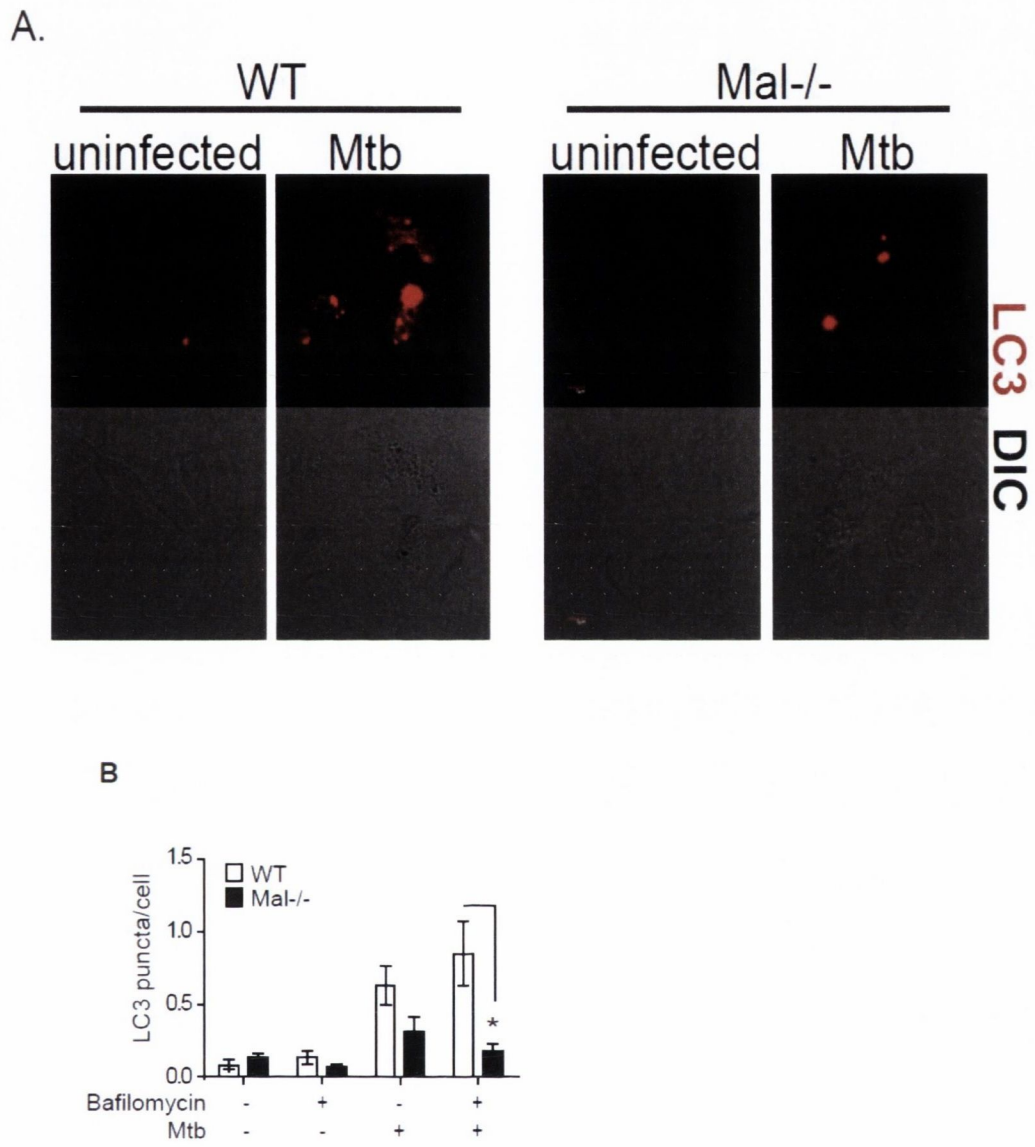
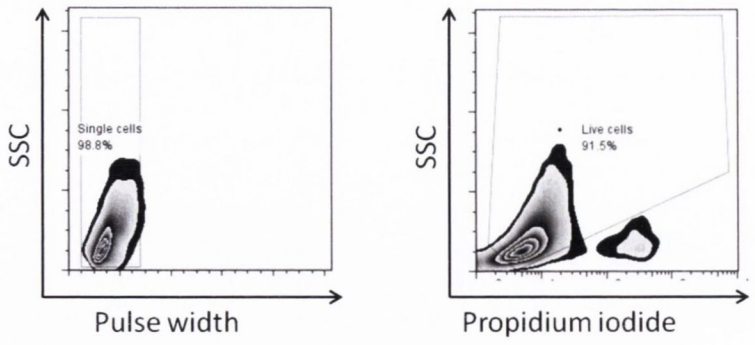


Figure 3-30: Mal is required for autophagy in response to live Mtb but not starvation in primary cells.

WT and Mal$^{-/-}$ primary BMM were pre-treated with bafilomycin and infected with live Mtb for 6 hours prior to fixation and staining with anti-LC3 antibody. Cells were assessed with fluorescent microscopy. Representative images (upper panel) and mean +/- S.E.M. for a single experiment representative of 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, not statistically significant (Mann-Whitney used to compare inhibitor treated cells to control peptide for same condition)



B

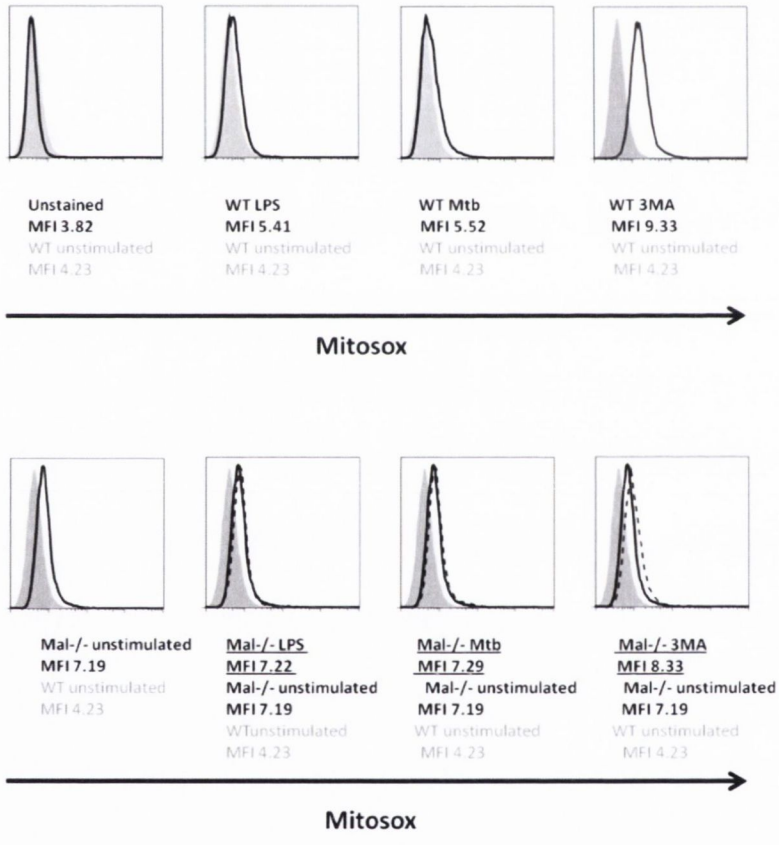


Figure 3-31: Mitochondrial ROS is increased in $Mal^{-/-}$ iBMM.

WT and $Mal^{-/-}$ iBMM (5 x 10^6/ml) were stimulated with RPMI, LPS (100 ng/ml), irradiated Mtb (10 bacteria/cell) or 3MA for 16 hours prior to staining with Mitosox and propidium iodide and analysis by FACS. MFI: Mean fluorescent intensity

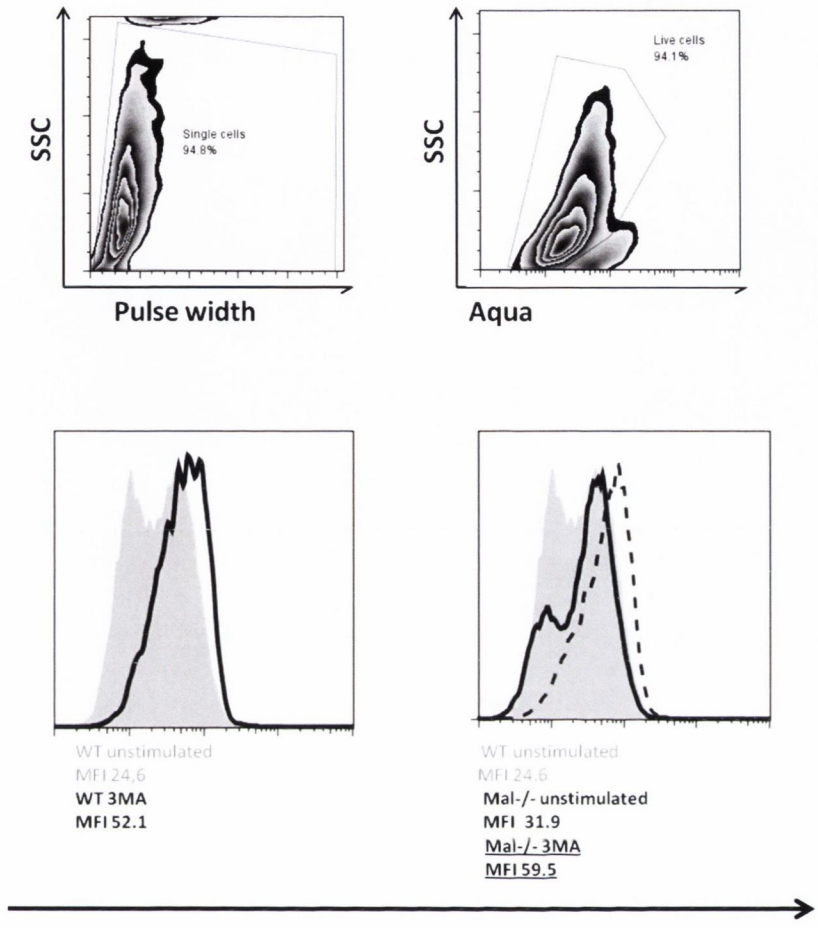


Figure 3-32: Mitochondrial ROS is increased in $Mal^{-/-}$ primary BMM.

WT and $Mal^{-/-}$ primary BMM (5 x 10^6/ml) were stimulated with RPMI, LPS (100 ng/ml), irradiated Mtb (10 bacteria/cell) or 3MA for 16 hours prior to staining with Mitosox and propidium iodide and analysis by FACS. MFI: mean fluorescent intensity

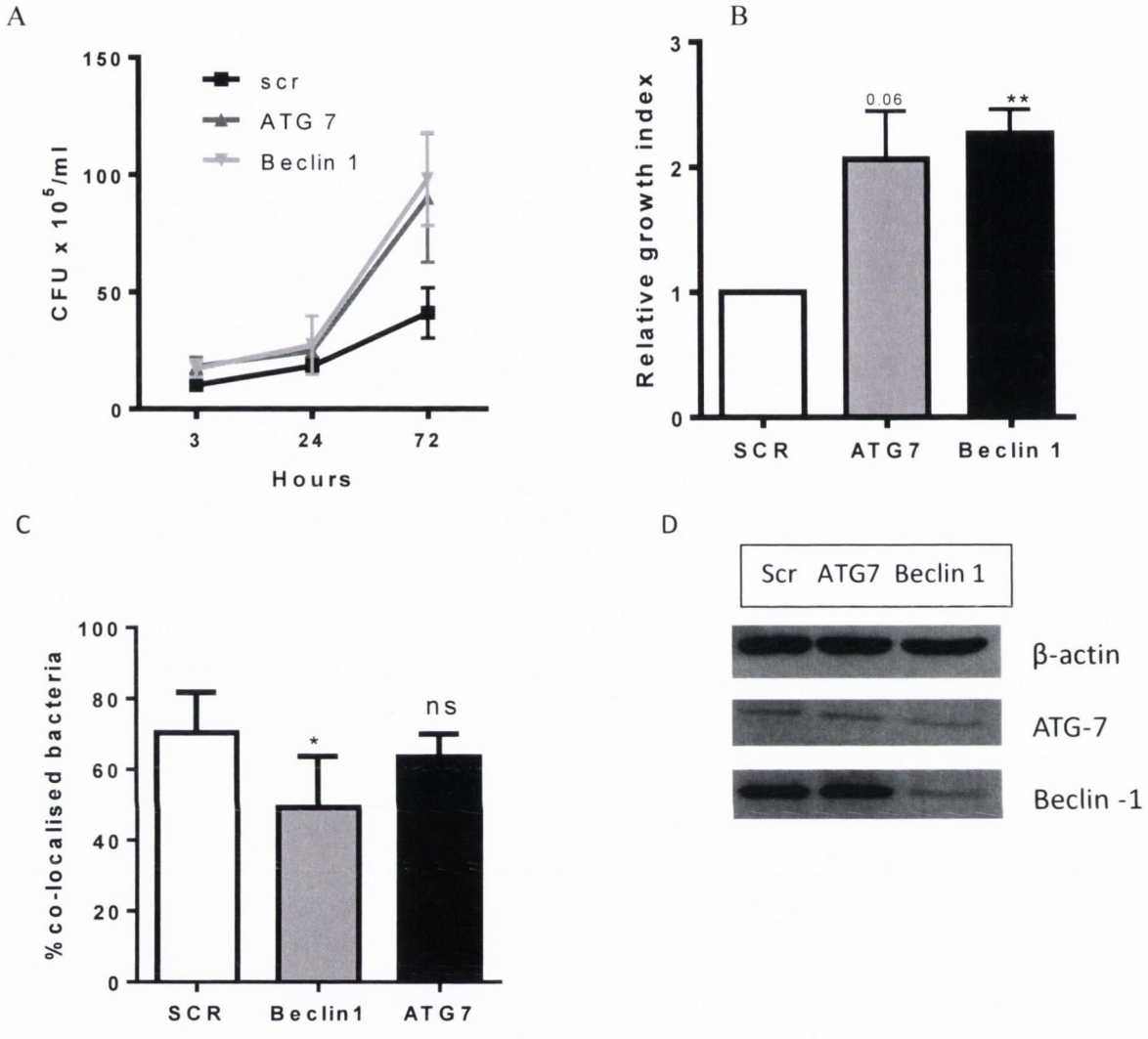
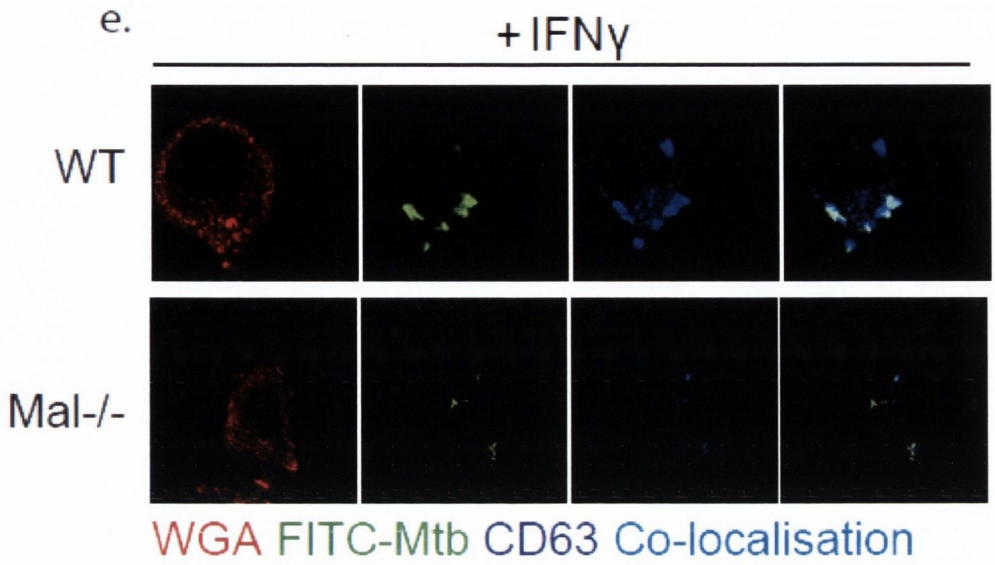


Figure 3-33: Autophagy is required for phagosomal maturation and killing.

WT iBMM were transfected with siRNA against Beclin 1, ATG7 or scrambled control. Transfected iBMM were infected with Mtb and lysed at 3, 24 and 72 hours. Lysates were plated out for quantitation of cfus. Representative experiment (A), and means +/- SD of 3 pooled experiments (B). Transfected iBMM were infected with FITC-labelled Mtb and stained with Lysotracker. Co-localisation was assessed by confocal microscopy (C). Knockdown was assessed by immunoblotting (D).



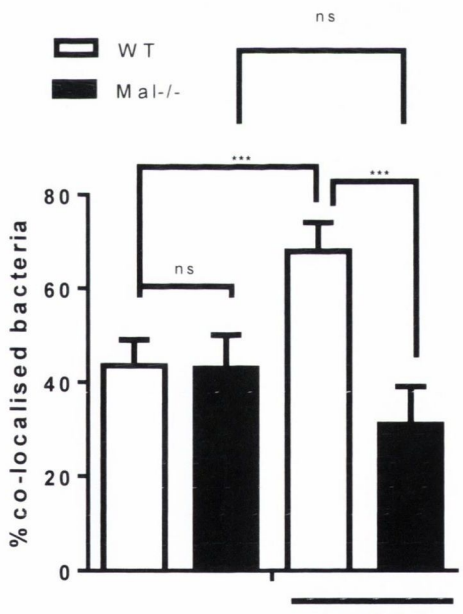
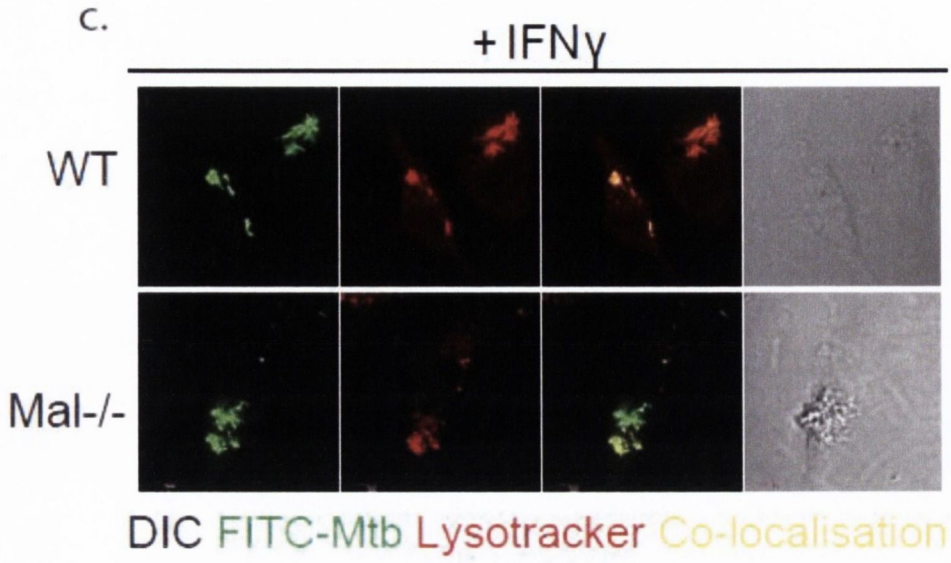


Figure 3-34: Mal is required for IFN-γ induced phagosome maturation asssesed by staining for CD63.

iBMM were pretreated with IFN-γ (20ng/ml) or RPMI prior to infection with FITC-Rv. Cells were fixed at 2 h and stained with late endosomal marker anti-CD63 and wheat germ agglutinin (WGA) to stain cell membranes. Co-localisation was assessed by confocal microscopy. Mean+/- SEM or 1 experiment in triplicate representative of 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, not statistically significant (Mann-Whitney)



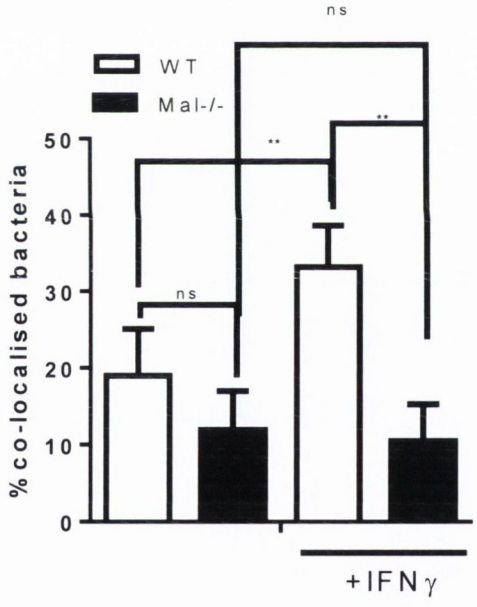


Figure 3-35: Mal is required for IFN-γ induced phagosome maturation asssesed by staining for Lysotracker

.iBMM were pretreated with IFN-γ (20ng/ml) or RPMI prior to infection with FITC-Rv. Cells were fixed at 2 h and stained with Lysotracker. Co-localisation was assessed by confocal microscopy. Mean +/- SEM or 1 experiment in triplicate represetnative of 3 separate experiments. * $p < 0.05$, **$p<0.01$, ***$p<0.001$, not statistically significant (Mann-Whitney).

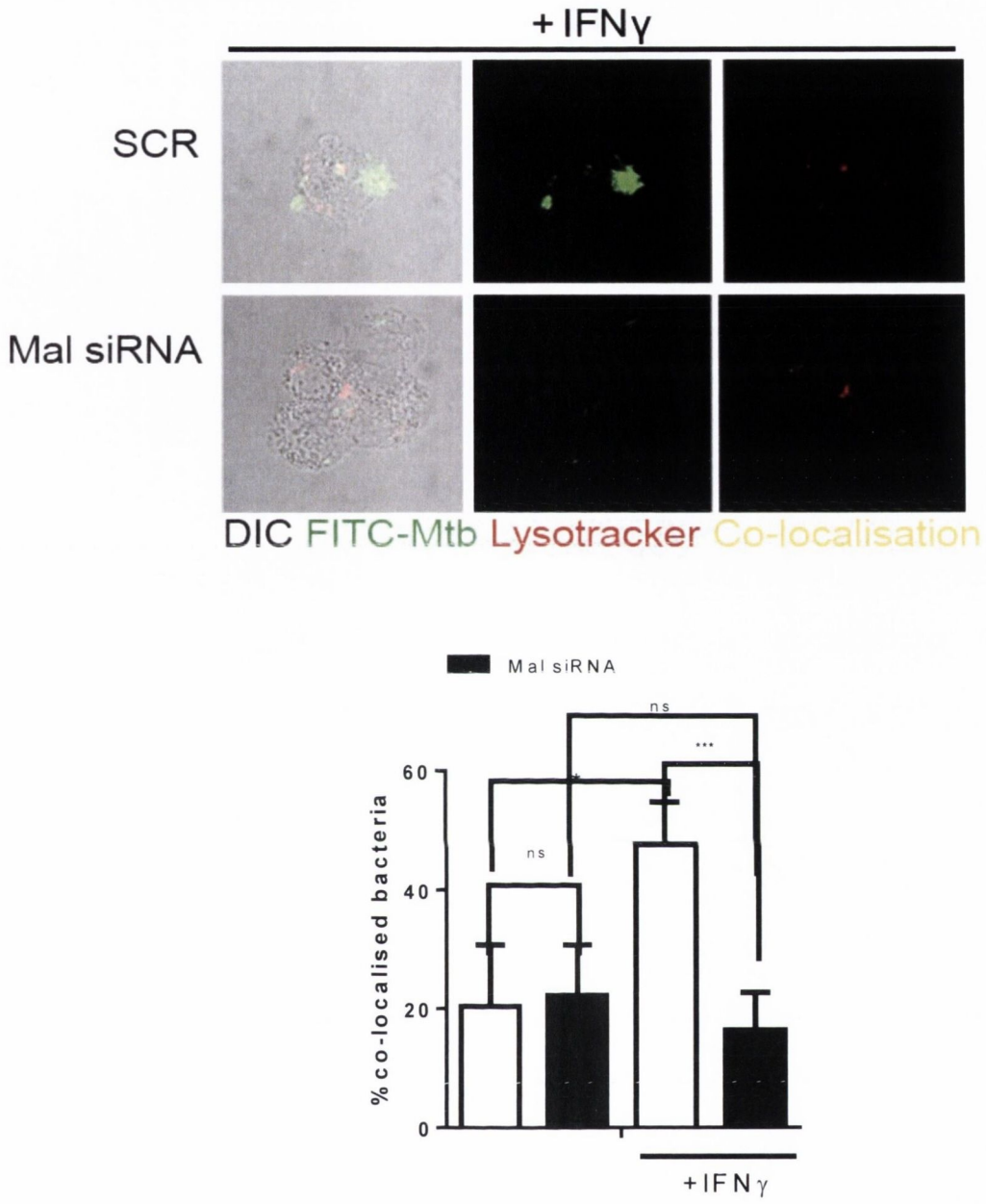


Figure 3-36: Mal is required for IFN-γ induced phagosome maturation in human macrophages

THP-1 were transfected with siRNA against Mal or scrambled control prior to differentiation. Macrophages w were pretreated with IFN-γ (20ng/ml) or RPMI prior to infection with FITC-Rv. Cells were fixed at 2 h and stained with Lysotracker. Co-localisation was assessed by confocal microscopy. Mean +/- SEM or 1 experiment in triplicate represetnative of 3 separate experiments. . * $p < 0.05$, **$p<0.01$, ***$p<0.001$, not statistically significant (Mann-Whitney used to compare Mal siRNA treated cells to controls

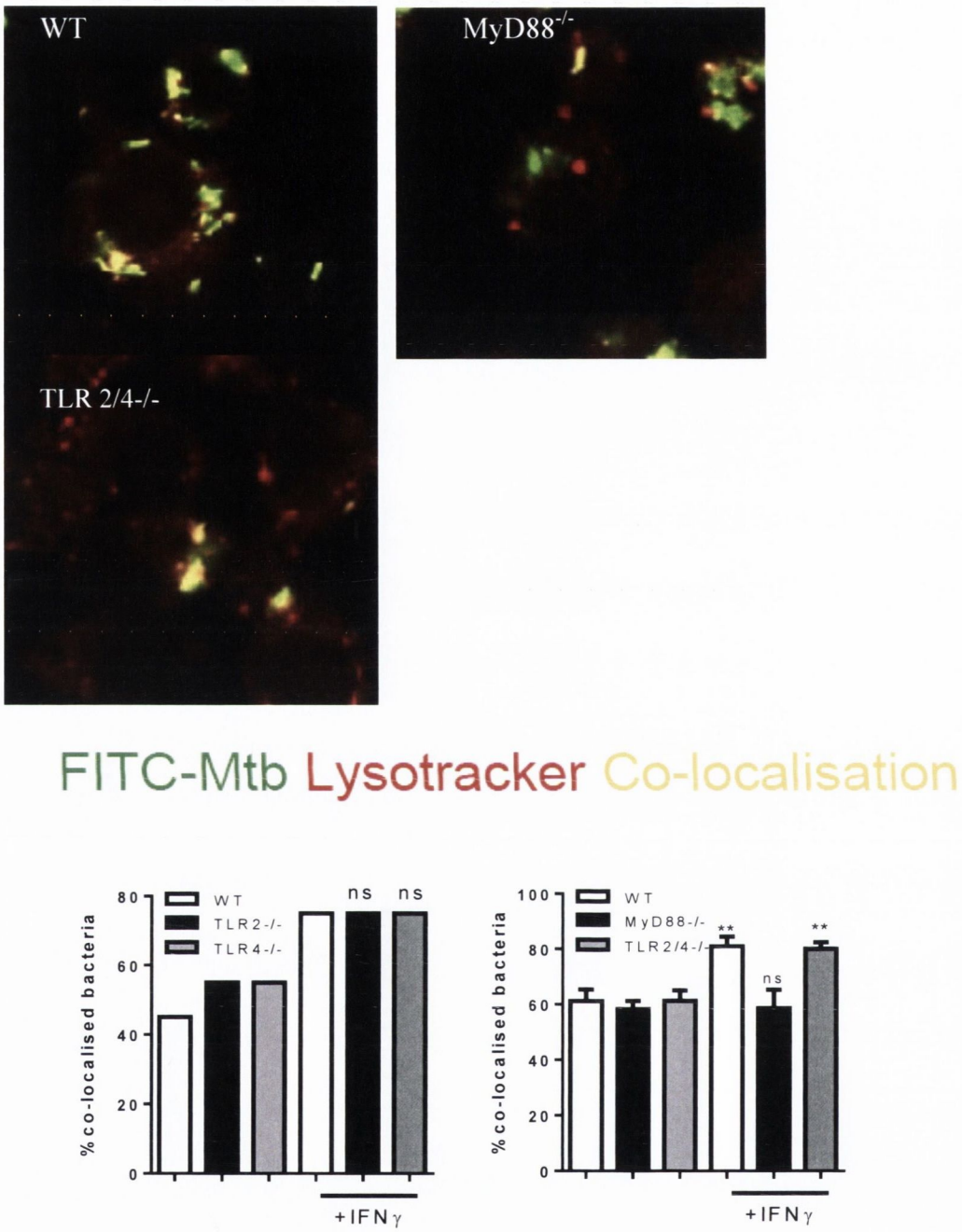


Figure 3-37: MyD88 but not TLRs 2 and 4 are required for IFN-γ induced phagosome maturation.

iBMM were pretreated with IFN-□ or RPMI prior to infection with FITC-Rv and staining with Lysotracker. Cells were fixed at 2 h. Co-localisation was assessed by confocal microscopy. Mean +/- SEM of 1 experiment in triplicate representative of 3 separate experiments. . * $p < 0.05$, **$p<0.01$, ***$p<0.001$, not statistically significant (Mann-Whitney, compared to WT controls)

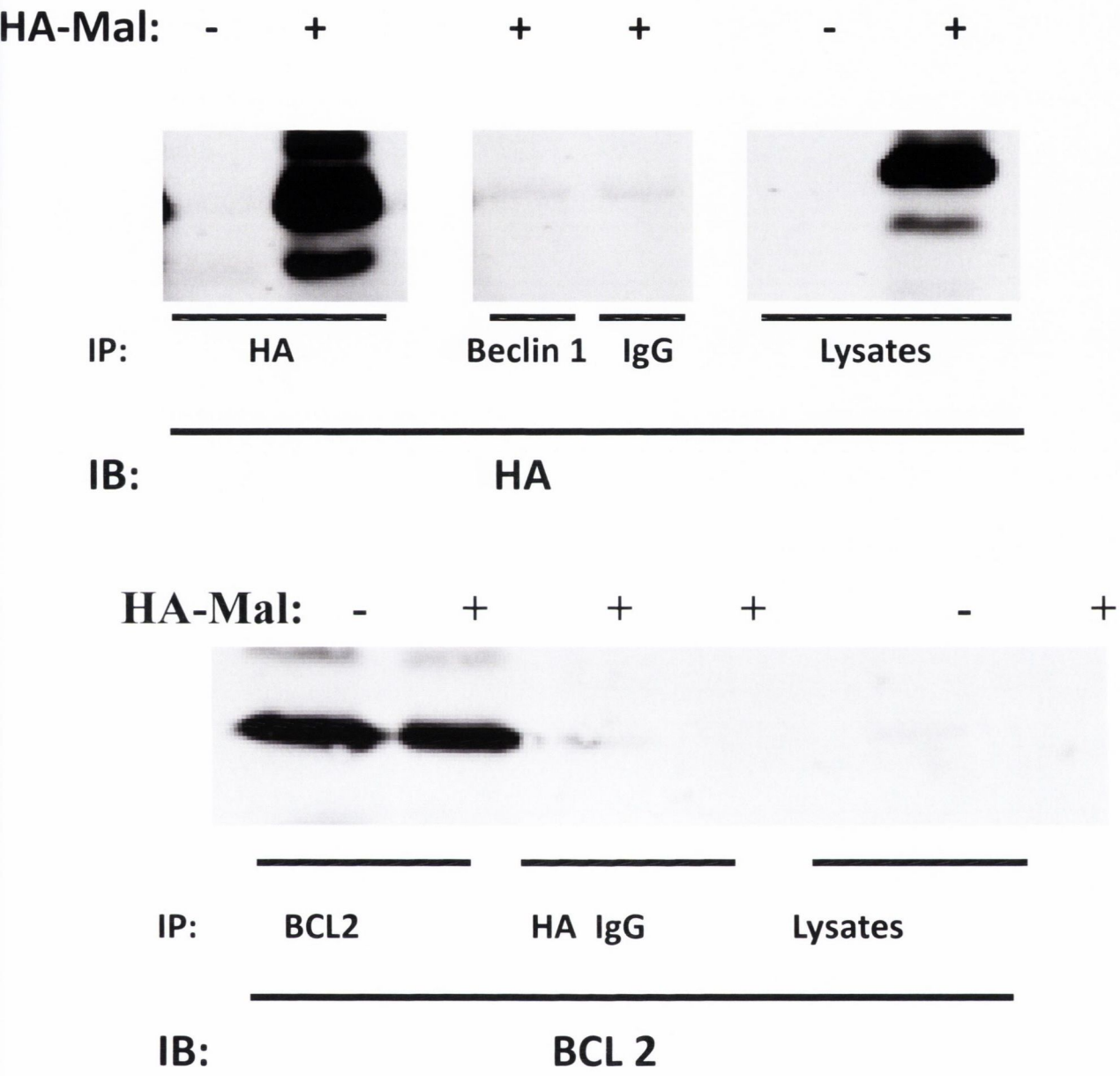


Figure 3-38: Mal does not associate with Beclin 1 or BCL2.

HEK-293 cells were transfected with HA-tagged wild-type Mal or HA-tagged empty vector and an immunoprecipitation was performed with antibodies to HA and Beclin 1 or BCL-2 on cell lysates as indicated. Lysates were then blotted with anti-interferon gamma receptor antibody.

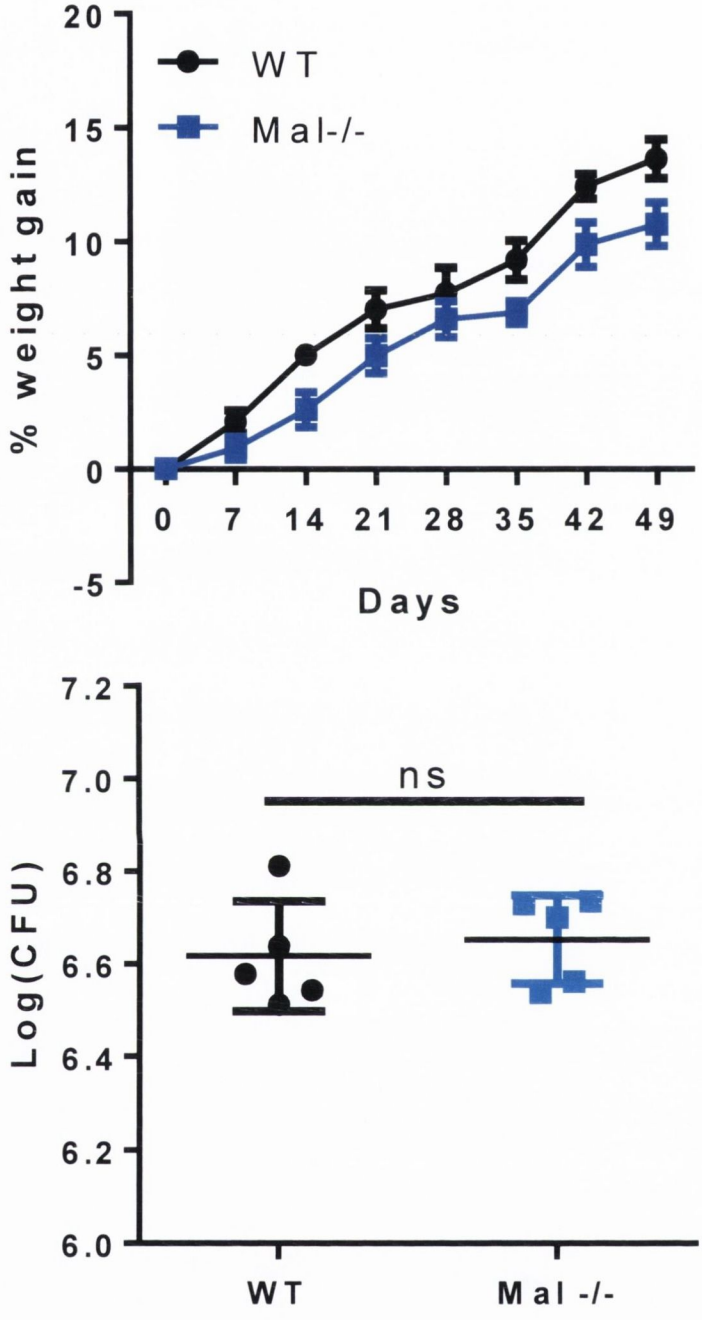


Figure 3-39: Mal$^{-/-}$ mice do not display increased susceptibility to high-dose Mtb infection.

WT and Mal$^{-/-}$ mice were infected with 500 cfu of Mtb H37Rv via aerosol. Serial weights were obtained. Mice were sacrificed at 8 weeks and lung homogenates plated for cfus.

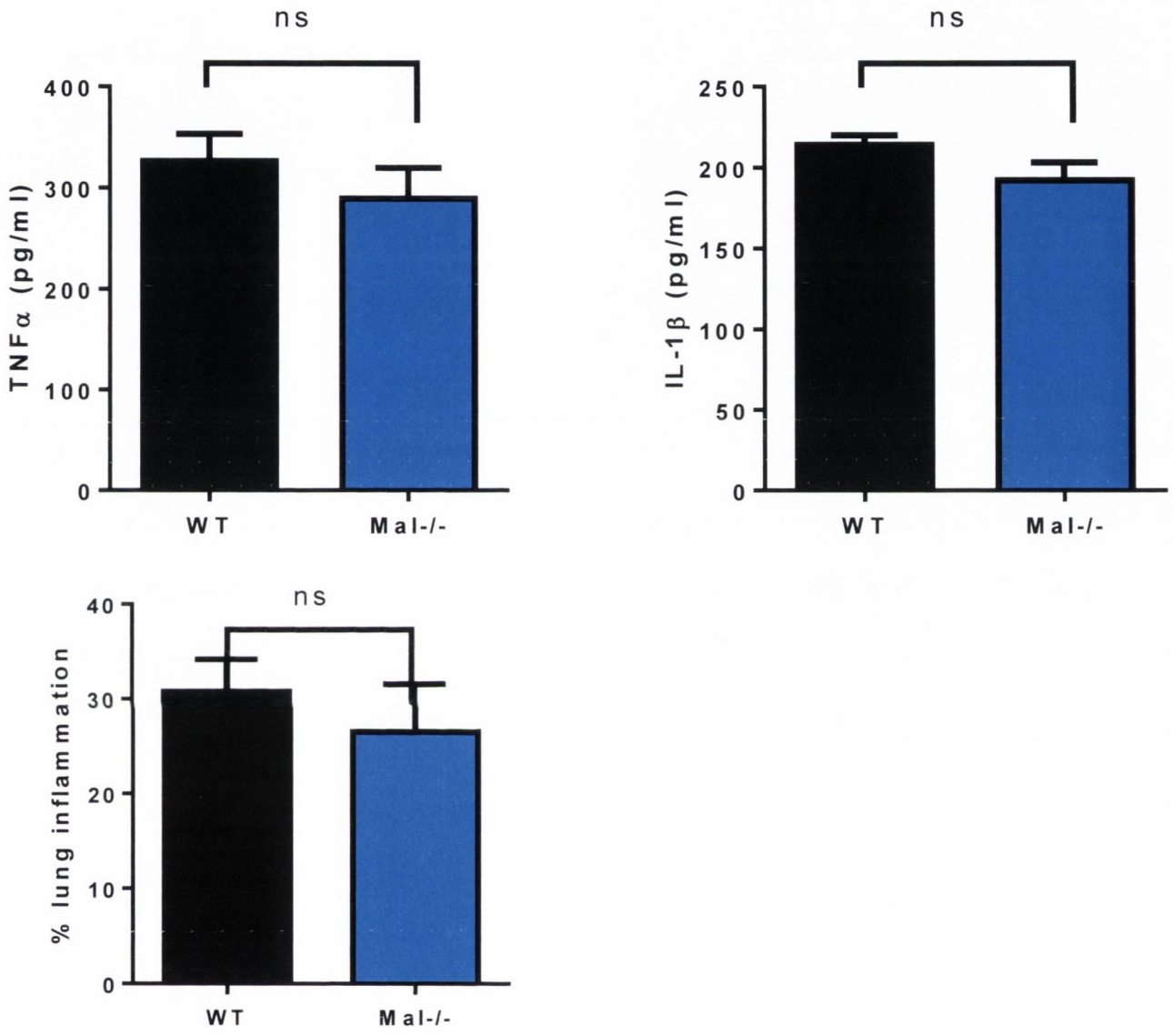


Figure 3-40: Mal$^{-/-}$ mice do not display increased inflammatory responses to high-dose Mtb infection

Lung sections were fixed and stained with H+E and assessesd by microscopy or alternatively homogenates analysed by ELISA

3.3 Discussion

In this chapter, I report a novel TLR-independent role for Mal in macrophage mycobactericidal responses to *M. tuberculosis* including autophagy and phagosome maturation in addition to a TLR 2 and 4-dependent role for Mal in macrophage release of pro-inflammatory cytokines.

Rationale *for using macrophage cell lines and primary cells*

Macrophages were the first immune cells to be described, and have a number of roles including promoting wound healing, homeostasis and defense against pathogens(Nathan 2008). Macrophages display marked heterogeneity, and environmental influences such as cytokines and pathogens can have dramatic effects on their function(Mosser and Edwards 2008). In the normal lung, alveolar macrophages comprise 90% of the phagocytic cells in the lung (Holt, Strickland et al. 2008), and infection with *M. tuberculosis* leads to an influx of macrophages into the lung (Redente, Higgins et al. 2010). As discussed in the introductory chapter, alveolar macrophages have a number of important roles in immune responses to *M. tuberculosis* including killing of intracellular bacteria, secretion of cytokines and chemokines, and antigen presentation. Alveolar macrophages present in tuberculosis infection originate from blood monocytes, which differentiate into lung parenchymal macrophages and then enter the alveolar space to become alveolar macrophages (Landsman and Jung 2007). Alveolar macrophages differ from other macrophages in a number of ways. In the quiescent state, alveolar macrophages have a number of immunosuppressive functions, including induction of immunosuppressive T regulatory cells (Coleman, Ruane et al. 2013), and suppression of dendritic cell activity (Holt, Oliver et al. 1993, Holt, Strickland et al. 2008). *In vivo* elimination of alveolar

macrophages leads to intensely inflammatory responses to harmless particulate antigens (Thepen, McMenamin et al. 1991). However, stimulation of TLRs on alveolar macrophages, as would presumably happen during *M. tuberculosis* infection, leads to temporary inhibition of their immunosuppressive functions (Takabayshi, Corr et al. 2006, Guilliams, Lambrecht et al. 2013). Infection with *M. tuberculosis in vivo* leads to polarisation of alveolar macrophages by IFN-γ to a pro-inflammatory phenotype as characterised by increased expression of iNOS (Redente, Higgins et al. 2010).

Unfortunately, alveolar macrophages require bronchoscopy to obtain them and cannot be expanded *ex vivo*. In this chapter, immortalised and primary murine bone-marrow derived macrophages and differentiated human THP-1 cells were used as a model of alveolar macrophages. This is a common approach given the relative ease of obtaining large numbers of bone-marrow-derived or immortalised macrophages. Immortalised and primary BMMs are commonly used experimentally due to their homogeneity, proliferation capacity and ability to be transfected. They are very phagocytic and have been extensively characterised (Weischenfeldt and Porse 2008, Wang, Yu et al. 2013). Immortalised and primary BMMs are available from a number of knockout mice, and were therefore used extensively in the experiments described in this chapter. They are frequently used in work looking at macrophage responses to *M. tuberculosis* (Holscher, Reiling et al. 2008, Mayer-Barber, Barber et al. 2010). THP-1 cells are a monocytic cell line, which can be differentiated into macrophages by phorbol-12-myristate-13-acetate (PMA). PMA treatment activates protein kinase C (PKC) and results in increased adherence and expression of surface markers associated with macrophage differentiation (Schwende, Fitzke et al. 1996, Daigneault, Preston et al. 2010). Results of studies using both alveolar macrophages and THP-1 cells

often show similar findings in both cell types (Keane, Balcewicz-Sablinska et al. 1997, Riendeau and Kornfeld 2003, O'Leary, O'Sullivan et al. 2011).

Rationale for using M. tuberculosis *H37Rv*

The most extensively studied strain of virulent *M. tuberculosis*, H37Rv, was used in this study. Virulent strains of tuberculosis show marked differences from avirulent strains in the amount of cell death they induce (Keane, Balcewicz-Sablinska et al. 1997, Balcewicz-Sablinska, Keane et al. 1998), mycobacterial survival in macrophages (Wei, Dahl et al. 2000), the amount of immunomodulatory proteins such as ESAT-6 that they secrete (Frigui, Bottai et al. 2008) and replication rates *in vivo*(Pascopella, Collins et al. 1994). Macrophages infected with H37Rv resembles those infected with clinical isolates of *M. tuberculosis* in a number of phenotypes including cell death and cytokine secretion (Keane, Remold et al. 2000). It is important to note, however, that clinically isolated strains of *M. tuberculosis* may differ significantly from each other and from laboratory strains (Fleischmann, Alland et al. 2002).

In this study, *M. tuberculosis* was grown in medium that did not contain Tween, a detergent commonly used to prevent clumping when *M. tuberculosis* is grown in liquid culture medium. This was due to concerns regarding the documented effect of Tween on surface lipids and proteins (Ortalo-Magne, Lemassu et al. 1996), as these lipids and proteins are TLR ligands and therefore central to this study. Instead, glass beads were used to obtain a single-cell suspension of bacteria prior to infection of macrophages.

Mal is required for cytokine production downstream of TLRs 2 and 4

Previously published data had shown a requirement for TLR2 in cytokine responses to *M. tuberculosis in vitro* (Means, Wang et al. 1999, Means, Jones et al. 2001, Kleinnijenhuis, Joosten et al. 2009). A requirement for Mal in TLR2/1 signalling in response to diacetylated liporotein Pam_3CysK_4 and TLR2/6 signalling in response to tricetylated lipoprotein Malp2 had previously been reported (Yamamoto, Sato et al. 2002, Kenny, Talbot et al. 2009), although this requirement had been shown to be dispensable at high ligand concentrations and high multiplicities of infection of *Salmonella enterica* (Kenny, Talbot et al. 2009). The role of Mal in TLR signalling in response to whole bacteria was less clear, as intraphagosomal *Francisella tularensis* had been shown not to require Mal for TLR2 signalling (Cole, Laird et al. 2010). *M. tuberculosis* had also been shown to use a number of PRRs in addition to TLR2 (Killick, Ni Cheallaigh et al. 2013).

The role of Mal in response to live virulent Mtb at multiplicities of infection similar to those seen in early stages of Mtb infection was assessed. The secretion of key anti-mycobacterial cytokines IL-1α, IL-1β, IL-6, IL-12p40 and TNF-α was dependent on Mal in a variety of cell types, whereas secretion of IL-27 and TGF-β was not affected by absence of Mal (Figures 3.2-3.8). IFN-β was the only cytokine studied which was increased in Mal-deficient cells. Previous publications have reported that increased cytosolic escape of bacteria in cells deficient in killing mechanisms leads to increased IFN-β- secretion (Manzanillo, Shiloh et al. 2012). It is possible that the impaired phagosome processing seen in Mal-deficient cells may be responsible for increased cytosolic escape and therefore increased IFN-β secretion, or alternatively the increase in IFN-β could be due to loss of Mal-dependent negative regulation of IRF-7 (Siednienko, Halle et al. 2010). Secretion of the anti-inflammatory cytokine IL-10 was Mal-dependent in human but not murine cells.

Cells deficient in TLR2 (and to a lesser extent TLR4) and MyD88 replicated the $Mal^{-/-}$ phenotype (Figures 3.10-3.12). These results indicate that the pathway required for secretion of many important innate cytokines in macrophages infected with *Mtb in vitro* involves TLR2, Mal and MyD88.

It is important to note that defects seen in cytokine secretion in macrophages *in vitro* do not necessarily correspond to defects *in vivo*. The defects in IL-12p40 and TNF-α secretion seen *in vitro* in TLR $2^{-/-}$ and TLR $2/4/9^{-/-}$ macrophages were not seen mice infected with Mtb *in vivo* (Drennan, Nicolle et al. 2004, Holscher, Reiling et al. 2008), and these cytokines were actually increased in lung homogenates in $TLR2^{-/-}$ mice. Defects in IL-1β secretion seen in $MyD88^{-/-}$ knockout macrophages were not seen in $MyD88^{-/-}$ knockout mice (Mayer-Barber, Barber et al. 2010). This may be due to partial redundancy of pattern recognition receptors *in vivo* with other PRRs compensating for those which are absent or that other cells (e.g DCs) which can secrete these cytokines may not require TLRs to respond to *M. tuberculosis*. It may also reflect the role of danger-associated molecules (DAMPs) in triggering cytokine, particularly IL-1β production, *in vivo.* Other IL-β processing pathways such as caspase 8 and neutrophil proteases may play a role *in vivo* that is not evident *in vitro.* It is also worth noting that these *in vivo* experiments only analysed cytokine production at relatively late time-points, such as 28 days. This may reflect the increased bacterial burden and pathology in the knockout mice at these time points.

Mal and MyD88, but not TLRs 2 and 4, are required for macrophage killing of virulent Mtb

Cells deficient in Mal or MyD88 showed a marked inability to kill intracellular Mtb; surprisingly, TLR2 and TLR4 deficient cells did not show a similar defect (Figures 3.13-3.18). The lack of a defect in mycobactericidal activity in TLR2/4/$9^{-/-}$ macrophages but the

presence of a defect in mycobactericidal activity in MyD88$^{-/-}$ macrophages had previously been reported by Holscher and colleagues, although this difference was only apparent when macrophages were primed with IFN-γ (Holscher, Reiling et al. 2008). A number of potential mechanisms for this had been postulated, including the role of MyD88 in IL-1 and IFN-γ signalling. The defect in killing of intracellular mycobacteria in Mal$^{-/-}$ but not TLR2/4$^{-/-}$ macrophages indicated that Mal and MyD88-dependent but TLR-independent mycobactericidal mechanisms must exist, providing a rationale to screen known macrophage mechanisms of killing intracellular *Mtb* to identify these mechanisms.

Mal is not required for uptake, apoptosis, nitric oxide synthesis or production of reactive oxygen species.

Bacterial uptake (Figure 3.19), apoptosis (Figures 3.20-3.23), NO synthesis (Figure 3.24) and ROS synthesis (Figure 3.25) were assessed and found not to require Mal. Phagocytosis of beads coated in TLR ligands had previously been reported to be TLR dependent (Blander and Medzhitov 2004), although another study did not find a difference in uptake of bacteria in TLR-deficient cells (Yates and Russell 2005). NO synthesis in response to Mtb and Mtb/IFN-γ but not Mtb/IFN-γ/TNF-α had previously been reported to be MyD88-dependent (Holscher, Reiling et al. 2008), although Sun and colleagues had found that the Stat-1 pathway resposible for NO synthesis was MyD88-independent (Sun and Ding 2006). No evidence of Mal or MyD88 being involved in this pathway was found in this model. Apoptosis in response to Mtb-derived proteins had previously been reported to be TLR2 dependent (Lopez, Sly et al. 2003), however apoptosis was not impaired in Mal-deficient immortalised and bone marrow derived macrophages and THP-1 cells. It is possible that apoptosis may be TLR2-, but not Mal-dependent, as Mal-independent TLR2 signalling has been reported (Kenny, Talbot et al. 2009, Cole, Laird et al. 2010). Unfortuately time

constraints and a lack of access to primary TLR2-/- macrophages precluded the assessment of Mtb-induced cell death in TLR2 deficient cells. Ideally, cell death would also have been assessed in primary human cells such as alveolar macrophages, however, that was not possible, again due to time constraints. The increase in cell death in $Mal^{-/-}$ macrophages treated with cycloheximide compared to WT controls was interesting, and may be related to the defects in mitophagy and autophagy in these cells described later in this chapter.

Mal is required for autophagy

The link between autophagy and TLRs is well-recognised, with a large number of PAMPs including LPS and TLR7 ligands inducing autophagy resulting in control of intracellular pathogens (Delgado, Elmaoued et al. 2008). TLR4 signalling triggers autophagy via a TRAF6 E3 ligase which ubiquitinates Beclin 1, followed by Bcl-2 dissociation from the BH3 domain of Beclin 1 (Shi and Kehrl 2010). MyD88 has been reported to be involved in LPS induced autophagy (Delgado, Elmaoued et al. 2008, Shi and Kehrl 2008), although other groups reported that autophagy in response to LPS was dependent solely on TRIF (Xu, Jagannath et al. 2007). Shi and colleagues reported an interaction between Beclin1 and the death domain of MyD88 (Shi and Kehrl 2008). The role of TLRs in autophagy in response to live virulent Mtb has not been reported, although Liu and colleagues have reported that a TLR2/1 ligand derived from Mtb, 19kDa lipoprotein can induce cathelicidin (Liu, Stenger et al. 2006), which can in turn induce autophagy (Yuk, Shin et al. 2009). Autophagy in response to Mtb has not been studied in TLR knockout cells, nor has a role for Mal in TLR-ligand induced autophagy been reported. We found evidence that TLR signalling via Mal was required for autophagy induced by TLR2 and TLR4 ligands (Figure 3.27). A requirement for TLRs in autophagy induced by live *M. tuberculosis* had also not

been reported. We found clear evidence that autophagy induced by live virulent *M. tuberculosis* was Mal-dependent (Figures 3.28 and 3.29)

Additional evidence for the role of Mal in autophagy came from findings of increased mitochondrial ROS in $Mal^{-/-}$ immortalised and primary macrophages (Figures 3.31 and 3.32). Damaged mitochondria are cleared from the cells through a type of autophagy known as mitophagy (Kim, Rodriguez-Enriquez et al. 2007). In the autophagy deficient cells, dysfunctional mitochondria accumulate and cells display elevated levels of mitochondrial reactive oxygen species (ROS) (Zhang, Qi et al. 2007, Tal, Sasai et al. 2009). 3MA is a PI3 kinase inhibitor which is used to inhibit autophagy. 3MA increased levels of mitochondrial ROS in WT, but not $Mal^{-/-}$, macrophages, suggesting that autophagy may be so impaired in the $Mal^{-/-}$ cells that additional inhibition has little effect. Elevations in intracellular ROS may have serious consequences for cells, including inflammasome activation (Heid, Keyel et al. 2013), apoptosis (Simon, Haj-Yehia et al. 2000) and carcinogenesis (Ziech, Franco et al. 2011). Interestingly, we had seen elevated levels of apoptosis in response to cycloheximide stress in $Mal^{-/-}$ primary BMMs (Figure 3.22 and 3.23). Our findings of accumulation of mitochondrial ROS in unstimulated $Mal^{-/-}$ iBMM are intriguing, as they suggest that Mal may have a role in autophagy which extends beyond TLR-ligand induced autophagy.

Autophagy is well recognised to have a critical role in immune responses to *Mtb*, and induction of autophagy has been reported to induce phagosome maturation and killing of *M. bovis* BCG and *M. tuberculosis* (Gutierrez, Master et al. 2004). I assessed the phenotype of autophagy-deficient macrophages, to see if it corresponded to Mal-deficient macrophages in our model. A decrease in phagosome maturation and bactericidal activity similar to that seen in $Mal^{-/-}$ cells was seen (Figure 3.33).

The role of TLRs 2 and 4 in autophagy in Mtb infection

It proved technically challenging to assess the roles of TLRs 2 and 4 and MyD88 in autophagy, as measuring autophagic flux in immortalised BMDM was prone to artefacts due to high basal levels of autophagy in these cells and we did not have access to primary macrophages from mice deficient in these proteins.

The role of Mal and TLRs 2 and 4 in phagosome maturation

The role of TLRs in phagosome matuation is controversial, with Blander and Medzhitov reporting that maturation of phagosomes containing *E. coli*, *S. typhimurium* and *S. aureus* was TLR 2 and 4 dependent (Blander and Medzhitov 2004); whereas David Russell and colleagues used coated particles and *Staphylococcus aureus* and reported no effect of TLR stimulation on phagosome maturation, although phagosome maturation was decreased in MyD88$^{-/-}$ macrophages (Yates and Russell 2005). IFN-γ stimulated TLR 2/4/9$^{-/-}$ macrophages have been reported to show normal maturation of phagosomes containing *Mb*, in a study which also reported a defect in phagosome maturation in IFN-γ stimulated MyD88$^{-/-}$ macrophages (Holscher, Reiling et al. 2008). No defect in basal or IFN-γ induced maturation of Mtb containing phagosomes was identified in the TLR2,4 and 2/4$^{-/-}$ immortalised macrophages used in this study. I identified a defect in IFN-γ induced phagosome maturation in Mal-deficient cells (Figures 3.34-3.36), but no defect in phagosome maturation in the absence of IFN-γ was detected in Mal-deficient cells at this time point. Interestingly, TLR2 and TLR4-deficient cells did not display impairment in IFN-γ induced phagosome maturation, whereas MyD88-deficient cells replicated the defect in IFN-γ induced phagosome maturation seen in Mal-deficient cells (Figure 3.37). An explanation for this observation forms the basis of Chapter 4.

What is the role of Mal in autophagy?

An interesting question then arose: what was the role of Mal in the induction of autophagy? A number of possibilities existed: MyD88 had been reported to be required for autophagy, and the death domain of MyD88 had been reported to bind directly in a complex with Beclin1 with TRAF6, also known to interact with Mal (Shi and Kehrl 2008). Could Mal be involved in this interaction? I assessed this by carrying out an immunoprecipitation looking for an interaction between Beclin1 and Mal. There was no evidence of an interaction (Figure 3.38).

The vaccinia produced inhibitory peptide A46 binds to and inhibits Mal (Lysakova-Devine, Keogh et al. 2010). Interestingly, A46 contains a Bcl-2 like fold (Graham, Bahar et al. 2008), and Bcl-2 inhibits autophagy. I hypothesised that Mal may bind to Bcl-2, thereby causing dissociation of Beclin-1 and inducing autophagy. An immunoprecipitation looking for an interaction between Bcl-2 and Mal did not find any evidence of an interaction (Figure 3.38). A role for Mal in the induction of autophagy was identified and this forms the basis of Chapter 4.

$Mal^{-/-}$ mice do not display increased susceptibility to Mtb infection

Given our *in vitro* findings and the weight loss at an early stage in the paper published by Fremond and colleagues (Fremond, Togbe et al. 2007), I hypothesised that $Mal^{-/-}$ mice would display increased susceptibility to *Mtb*. I designed an experiment using high-dose aerosol infection and 100 days of infection, as the increased susceptiblity present in $TLR2^{-/-}$ mice was only evident under these conditions (Zhang, Xue et al. 2011). No increase in susceptibility was evident in $Mal^{-/-}$ mice in this experiment.

The mouse model of tuberculosis is often used, but has important limitations. Firstly, Mtb is not a natural mouse pathogen, and therefore mice have not experienced the same long co-evolution leading to the development of human adaptations to Mtb and Mtb adaptations to the human immune system. The most common mouse model of TB infection in use is a high dose aerosolised infection which results in 100% of the mice developing clinical disease. This contrasts to the situation in humans, where 95% of infected individuals do NOT develop clinical disease. The high dose aerosolised infection model may represent an overchallenge of the animals, which is not physiological. No animal model except for non-human primates shows the variety of immune responses seen in human infection: i.e. latent and active infection, and even in the non-human primate model the ratio of latent to active disease is 1:1, unlike humans where it is 19:1. In addition, animal models have a very limited dynamic range of outcomes, and may not reflect the heterogeneity of human TB. Animal models, have, to date, been quite poorly predictive of success for treatment and diagnostic strategies. For example, MV85A used to boost BCG was effective in numerous animal models (Tchilian, Desel et al. 2009, Verreck, Vervenne et al. 2009, White, Sibley et al. 2013), but failed to show protection in a clinical trial (Tameris, Hatherill et al. 2013). Linezolid failed to show much efficacy in animal models (Williams, Stover et al. 2009), but is very effective in treating humans with XDR-TB (Lee, Lee et al. 2012).

There are numerous fundamental immunological differences between mice and humans, as reviewed by Mestas and colleagues (Mestas and Hughes 2004). A number of these immunological differences may be particularly relevant to a TB *in vivo* challenge model. Expression of pattern recognition receptors varies between species: PRR genes are controlled by complex, highly conserved promoters that facilitate dynamic regulation which are extremely susceptible to evolutionary change (Schroder, Irvine et al. 2012).

Specifically, basal TLR2 expression is much lower in mice than humans (Rehli 2002); TLR9 is expressed on all myeloid cells in mice but only on B cells, pDCs and NK cells in humans; and TLR10 is not expressed in mice. TLR4 expression is divergently regulated in mouse versus human macrophages (Schroder, Irvine et al. 2012). Neutrophils have recently been reported to play a key role in the initial innate immune response to tuberculosis by phagocytosing and killing mycobacteria (Yang, Cambier et al. 2012), yet mouse blood contains dramatically fewer neutrophils than human blood (Mestas and Hughes 2004). Defensins are peptides which have have anti-mycobacterial activity which are secreted by human but not murine neutrophils (Risso 2000). Nitric oxide is a key antimycobacterial effector in mice: it can deaminate and damage bacterial DNA, induce apoptosis and kill Mtb (Chan, Xing et al. 1992, Chan, Chan et al. 2001). Mice deficient in nitric oxide production show increased susceptibility to infection with Mtb (Adams, Dinauer et al. 1997, MacMicking, North et al. 1997). The role of nitric oxide in human responses to TB infection is more controversial. Early work showed that mycobacterial killing by human alveolar macrophages is not NO-dependent (ASTON, ROM et al. 1998).

It is also worth noting that findings with *in vivo* mouse models of infection may be difficult to replicate. Studies on $TLR2^{-/-}$ and $TLR4^{-/-}$ mice have shown various degrees of increased suceptiblity (Reiling, Holscher et al. 2002, Drennan, Nicolle et al. 2004, Bafica, Scanga et al. 2005). Factors that may differ between experiments and may affect immune responses include mouse microbiota (Cebra 1999); genetic background of mice (Pan, Yan et al. 2005, Nandi and Behar 2011) and feed. In addition, the maintenace of *M. tuberculosis* in liquid culture over a prolonged period of time may lead to the emergence of mutations which may also affect experimental results (Domenech and Reed 2009)

3.4 Conclusion

In summary, in this chapter data is presented to show that the majority of pro-inflammatory cytokines by macrophages in response to Mtb infection *in vitro* is dependent on a signalling pathway involving TLR2/4-Mal-MyD88. In contrast, autophagy, and consequently killing of intracellular Mtb, is dependent on a signalling pathway including Mal and MyD88 but not TLRs 2 and 4. This pathway will be further elucidated in the following chapter. *In vivo* infection of $Mal^{-/-}$ mice with Mtb did not show increased susceptibility: this may reflect a lack of sensitivity in this model to detect impaired early innate immune responses or redundancy of this pathway *in vivo*.

Chapter 4 : The Role of Mal in IFN-γ signalling

4.1 Introduction

In the previous chapter, a requirement for Mal and MyD88, but not for TLR2 and TLR4, in killing of *M. tuberculosis* was demonstrated. Experiments were carried out to identify a function for Mal independent of TLR2 and TLR4 signalling which was required for macrophage killing of *M. tuberculosis.* The experiments described in Chapter 3 demonstrated that IFN-γ-induced phagosome maturation was reduced in the absence of Mal and MyD88 but not TLR2 and TLR4. IFN-γ is known to play a critical role in activating macrophage responses to *M. tuberculosis* (Via, Fratti et al. 1998, Gutierrez, Master et al. 2004). MyD88 has been reported to be required for IFN-γ signalling (Shi, Nathan et al. 2003, Sun and Ding 2006). The objective of the work described in this chapter was to test the hypothesis that Mal acts as a bridging adaptor between MyD88 and the IFN-γ receptor (IFN-γR) and was therefore required for MyD88-dependent IFN-γ signalling.

4.2 Results

4.2.1 Mal and MyD88, but not TLR2 and TLR4, are required for IP-10 production in response to IFN-γ stimulation.

Sun and Ding reported that MyD88 is involved in IFN-γR signalling culminating in MAPK p38 phosphorylation and IP-10 production, but is not involved in the canonical IFN-γR signalling pathway leading to STAT-1 phosphorylation (Sun and Ding 2006). Therefore, IP-10 secretion was chosen as an initial endpoint to assess whether Mal was involved in the MyD88 dependent IFN-γR signalling pathway.

Immortalised bone-marrow derived macrophages (iBMM) were stimulated overnight with IFN-γ, and supernatants analysed by ELISA. It was found that iBMM deficient in MyD88 or Mal, but not in TLR2 and/or TLR4 or the signalling adaptor protein TRAM, displayed impaired IP-10 production in response to IFN-γ (Figure 4.1A-C). TNF-α can also stimulate IP-10 production (Ohmori and Hamilton 1995), so experiments were carried out to determine whether IFN-γ-induced TNF-α secretion was Mal-dependent. TNF-α responses to IFN-γ were increased rather than decreased in the absence of Mal or MyD88, indicating that IFN-γR induced secretion of IP-10 is not TNF-α dependant in murine macrophages (Figure 4.1D). Results were confirmed in human cells using differentiated THP-1 cells transfected with siRNA against Mal, and which revealed a small but statistically significant decrease in IP-10, but not in TNF-α production (Figure 4.2). Transfection efficacy with siRNA against Mal was less than optimal for this experiment, so the decrease in IP-10 responses might be greater with improved knockdown of Mal.

4.2.2 STAT-1 phosphorylation does not require Mal

The JAK-STAT pathway is activated in response to IFN-γ binding to its receptors and culminates in the phosphorylation of STAT-1 at Y701, leading to its dissociation and entry to the nucleus where it acts as a transcription factor. Our collaborators, Dr Jim Harris and Dr Sarah Jones (Monash University, Australia), measured STAT-1 phosphorylation in response to IFN-γ treatment using FACS in WT and $Mal^{-/-}$ immortalised macrophages. No change was seen in STAT-1 phosphorylation in the absence of Mal (Figure 4.3).

4.2.3 MAPK p38 phosphorylation is impaired in the absence of Mal

P38 mitogen-activated protein kinases (MAPK) are a class of MAPK that respond to stress stimuli, including cytokines, and are involved in apoptosis and autophagy (Moruno-Manchón, Pérez-Jiménez et al. 2013, Wang, Wang et al. 2013). In addition to the JAK-STAT pathway, IFN-γ also signals via a pathway including MLK3 and p38 MAPK phosphorylation (Roy, Shuman et al. 2005, Sun and Ding 2006). This pathway had been reported to be dependent on MyD88 (Sun and Ding 2006). It was hypothesised that p38 MAPK phosphorylation might be impaired in the absence of Mal. Dr Fred Sheedy (TCD) carried out a series of experiments on primary WT and $Mal^{-/-}$ bone-marrow derived marophages (BMM), in which they were stimulated with IFN-γ and lysed at a number of time points. Immunoblotting for phosphorylated p38 was carried out on the lysates. There was a marked decrease in p38 MAPK phosphorylation in response to IFN-γ stimulation in $Mal^{-/-}$ BMM compared to WT BMM at various time points (Fig 4.4 A) and at a range of concentrations of IFN-γ (Figure 4.4 B).

4.2.4 IFN-γ -induced phagosome maturation is impaired in the absence of Mal

IFN-γ is known to promote maturation of *M. tuberculosis*-containing phagosomes (Schaible, Sturgill-Koszycki et al. 1998, Via, Fratti et al. 1998). As already shown in Chapter 3, IFN-γ-induced maturation of phagosomes containing live *M. tuberculosis* is impaired in the absence of Mal and MyD88, but not TLR2 or TLR4 (Figures 3.35-3.37).

4.2.5 IFN-γ -induced autophagy is impaired in the absence of Mal

We proceeded to evaluate the role of Mal in other related IFN-γ induced macrophage functions that are known to be important in killing of intracellular mycobacteria. Primary

BMM were treated for 16 h with IFN-γ or for 2 h with starvation medium. Macrophages were then fixed and stained with anti-LC3 antibody and the number of puncta per cell quantified by confocal microscopy (Figure 4.5)(Harris, Hanrahan et al. 2001). IFN-γ-induced autophagy was greatly reduced in the absence of Mal, whereas starvation-induced autophagy was unaffected.

These results were confirmed using Western blotting to assess the amounts of LC3 I and II present in cell lysates. Primary BMM were stimulated for 6 h with a number of stimuli. Again, IFN-γ induced autophagy, as measured by the presence of LC3II, was greatly reduced in the absence of Mal (Figure 4.6).

4.2.6 Mal physically associates with the IFN-γ receptor

The findings above suggested that Mal transduced signals from the IFN-γR resulting in p38 phosphorylation, autophagy and phagosome maturation. MyD88 had been reported to physically associate with IFN-γR1 (Sun and Ding 2006). To test whether Mal could associate with IFN-γR1, a co-immunoprecipation experiment was carried out. It is not possible to detect endogenous Mal in cell lysates using currently available antibodies. To overcome this obstacle, HA-tagged full-length human Mal was transfected into HEK-293 cells and anti-HA antibodies were used to probe for Mal. When immunoprecipation was carried out using antibodies to endogenous IFN-γR1, HA-tagged Mal was detected suggesting a physical interaction (Figure 4.7).

4.27 MyD88 fails to associate with IFN- γR1in the absence of Mal

It was hypothesised that Mal may function as a bridging adaptor between IFN-γR1 and MyD88. To confirm this, Dr Fred Sheedy (TCD) carried out a co-immunoprecipitation

experiment looking at the interaction between MyD88 and IFN-γR1 in the presence and absence of Mal (Figure 4.8). In $Mal^{-/-}$ BMM, the interaction between IFN-γR1 and MyD88 was absent, indicating that Mal is required as a bridging adaptor protein to facilitate this interaction. This experiment was carried out in cells pre-stimulated with IFN-γ or RPMI, as Sun and Ding have previously demonstrated an association between MyD88 and IFN-γR1 in cells pre-stimulated with IFN-γ but not unstimulated controls. In contrast, we did not observe a difference in the association between MyD88 and IFN-γR1 in WT cells in the presence or absence of IFN-γ.

4.2.7 Macrophages secrete small, but detectable, amounts of IFN-γ

It has long been a matter of debate whether macrophages produce IFN-γ (Fenton, Vermeulen et al. 1997, Schleicher, Hesse et al. 2005). Data described in Chapter 3 indicated a role for Mal-dependent IFN-γR signalling in *M. tuberculosis*-induced autophagy and killing of mycobacteria *in vitro* in macrophages without the addition of exogenous IFN-γ. Therefore, it was determined whether the macrophages used in the current model produced IFN-γ that could stimulate bactericidal activity. iBMM were infected with live *M. tuberculosis* and IFN-γ secretion was measured after 72 h using ELISA. WT and $Mal^{-/-}$ iBMM secreted IFN-γ in small but detectable amounts in a dose-dependent manner. $Mal^{-/-}$ iBMM secreted less IFN-γ, this may be due to impaired TLR2/4 signalling or may be due to impaired IFN-γ signalling, as IFN-γ can upregulate its own production via in a positive feedback loop (Hardy and Sawada 1989, Di Marzio, Puddu et al. 1994) (Figure 4.9 A). Primary WT BMM produced IFN-γ in response to *M. tuberculosis*, whereas BMM from IFN-$\gamma^{-/-}$ mice did not, thereby validating the ELISA results (Figure 4.9 B). Intracellular IFN-γ was also detected in iBMM stimulated with *M. tuberculosis* using FACS (Figure 4.10).

4.2.8 IFN-γ secreted by macrophages is functionally relevant

As macrophages secrete only small amounts of IFN-γ, experiments were conducted to assess whether these levels were functionally relevant in our model. Initially maturation of *M. tuberculosis*–containing phagosomes was assessed in iBMM treated with a blocking antibody to IFN-γ. Phagosome maturation was impaired in the presence of the blocking antibody (Figure 4.11). Phagosome maturation was also assessed in primary BMM from WT and IFN-$\gamma^{-/-}$ mice. The IFN-$\gamma^{-/-}$ mice do not produce IFN-γ, but do express receptors for IFN-γ. Phagosome maturation was reduced in BMM from IFN-$\gamma^{-/-}$ mice, but was restored when the BMM were treated with exogenous IFN-γ (Figure 4.12). These data indicated that endogenous IFN-γ produced by macrophages can induce phagosome maturation.

The role of IFN-γ in autophagy induction in response to *M. tuberculosis* was also assessed. Primary WT and IFN-$\gamma^{-/-}$ macrophages were infected with *M. tuberculosis* for 16 h, prior to fixation and staining with anti-LC3 antibody. Puncta were quantified by confocal microscopy. IFN-$\gamma^{-/-}$ macrophages showed reduced autophagy in response to *M. tuberculosis* (Figure 4.13). This replicated the defect in autophagy in response to *M. tuberculosis* seen in Mal$^{-/-}$ cells (Figure 3.30)

Finally, it was determined whether a lack of macrophage-secreted IFN-γ replicated the defect in bactericidal activity seen in Mal$^{-/-}$ macrophages. Cells deficient in IFN-γ showed normal bactericidal activity, but cells deficient in both IFN-γ and TLR2/4 signalling showed an impairment of killing intracellular mycobacteria which replicated that seen in Mal$^{-/-}$ cells, in which there is a defect in both IFN-γ and TLR2/4 signalling (Figure 4.14) in an experiment carrier out by Dr Michelle Coleman (TCD).

4.2.9 Mal$^{-/-}$ mice display increased pro-inflammatory cell recruitment and cytokine secretion in response to intra-peritoneal IFN-γ

Finally, a study was conducted to assess whether Mal$^{-/-}$ mice showed impaired responses to IFN-γ *in vivo*. IFN-γ was injected in to the peritoneal cavity of C57BL/6 mice, and cell infiltration and cytokine secretion in the peritoneal cavity and blood were measured. As there are few reports on short-term cytokine and cell infiltration responses to intraperitoneal IFN-γ injection *in vivo*, an initial experiment was conducted in WT mice to determine an appropriate dose and endpoints. As discussed in the introductory chapter, IFN-γ induces differentiation of macrophages into a "classically-activated" or "M1-like" phenotype (Murray, Allen et al. 2014). These were characterised using flow cytometry as CD11b^{high}, F4/80^{high}, Gr1^{high} cells. IFN-γ injected intra-peritoneally caused dose-dependent infiltration of M1-like macrophages at 24 h (Figure 4.15). Cytokine secretion in blood and peritoneal fluid was also measured. IFN-γ injected intra-peritoneally induced secretion of IL-12p40 at 24 h in blood and there was also a trend towards increased IP-10 in blood at 6 and 24 h, although this was not statistically significant (Figure 4.16). There was a non-statistically significant trend towards induction of IL-12p40 in peritoneal fluid (Figure 4.17). Peritoneal cells were re-stimulated with irradiated *M. tuberculosis* to assess their activation. Peritoneal cells from animals treated with IFN-γ produced more IL-12p40 (Figure 4.18), reflecting a change in peritoneal cell populations to a more pro-inflammatory population.

This was followed by comparing responses to intra-peritoneal IFN-γ in WT and Mal$^{-/-}$ C57BL/6 mice. Peritoneal exudate cells were analysed by flow cytometry as described above. Of note, basal levels of eosinophils were higher in Mal$^{-/-}$ mice and overall peritoneal cell numbers were lower in Mal$^{-/-}$ mice. I hypothesised that the IFN-γ response seen above (M1-like macrophage infiltration with concomitant increase in IL-12p40 secretion in blood,

peritoneal fluid and in restimulated PECS) would be reduced in $Mal^{-/-}$ mice. In contradiction of the hypothesis, $Mal^{-/-}$ mice had more recruitment of pro-inflammatory M1-like macrophages in response to IFN-γ with concomitant reduction in numbers of anti-inflammatory M2-like macrophages than WT cells (% of total cells in Figure 4.19 and absolute numbers of cells in Figure 4.20). Levels of IL-12p40 in blood increased in response to intra-peritoneal IFN-γ in both WT and $Mal^{-/-}$ mice (Figure 4.21A), with a much more marked increase in $Mal^{-/-}$ mice. Intriguingly, despite the fact that there were increased M1-like macrophages in the $Mal^{-/-}$ mice, there was a failure of production of IL-12p40 in the peritoneum in 2 of 3 of the $Mal^{-/-}$ mice compared to WT mice (Figure 4.21B). The activation of peritoneal cells and splenocytes in response to intra-peritoneal IFN-γ was assessed by isolation and re-stimulation of these cells with the TLR7/8 ligand R848, as the TLR7/8 signalling pathway in this restimulation is independent of Mal. Unlike results seen in the dose-finding experiment described above, in this experiment intraperitoneal IFN-γ did not cause much change in the IL-12p40 responses of WT peritoneal cells to restimulation, although it is important to note that R848 replaced irradiated Mtb as the restimulation stimulus in this experiment. IL-10 was decreased basally and on stimulation in PECs from $Mal^{-/-}$ mice, possibly reflecting the reduction in M2 macrophage numbers in the peritoneal cavity or the established role of Mal in IL-10 responses (Mellett, Atzei et al. 2011). IL-12p40 production was also reduced in restimulated peritoneal cells from IFN-γ treated $Mal^{-/-}$ mice compared to IFN-γ treated WT mice, which was surprising given the increased proportion of M1 cells present in the peritoneal cavity (Figure 4.22) and correlates with the results seen in blood. Intraperitoneal IFN-γ stimulated splenocytes to a higher degree in $Mal^{-/-}$ mice, as evidenced by the increased production of IL-12p40 and TNF-α in restimulated splenocytes from $Mal^{-/-}$ mice compared to those from WT mice.(Figure 4.23).

The increased IL-10 production in response to stimulation of splenocytes from PBS-treated $Mal^{-/-}$ mice as compared to PBS-treated WT mice is also notable.

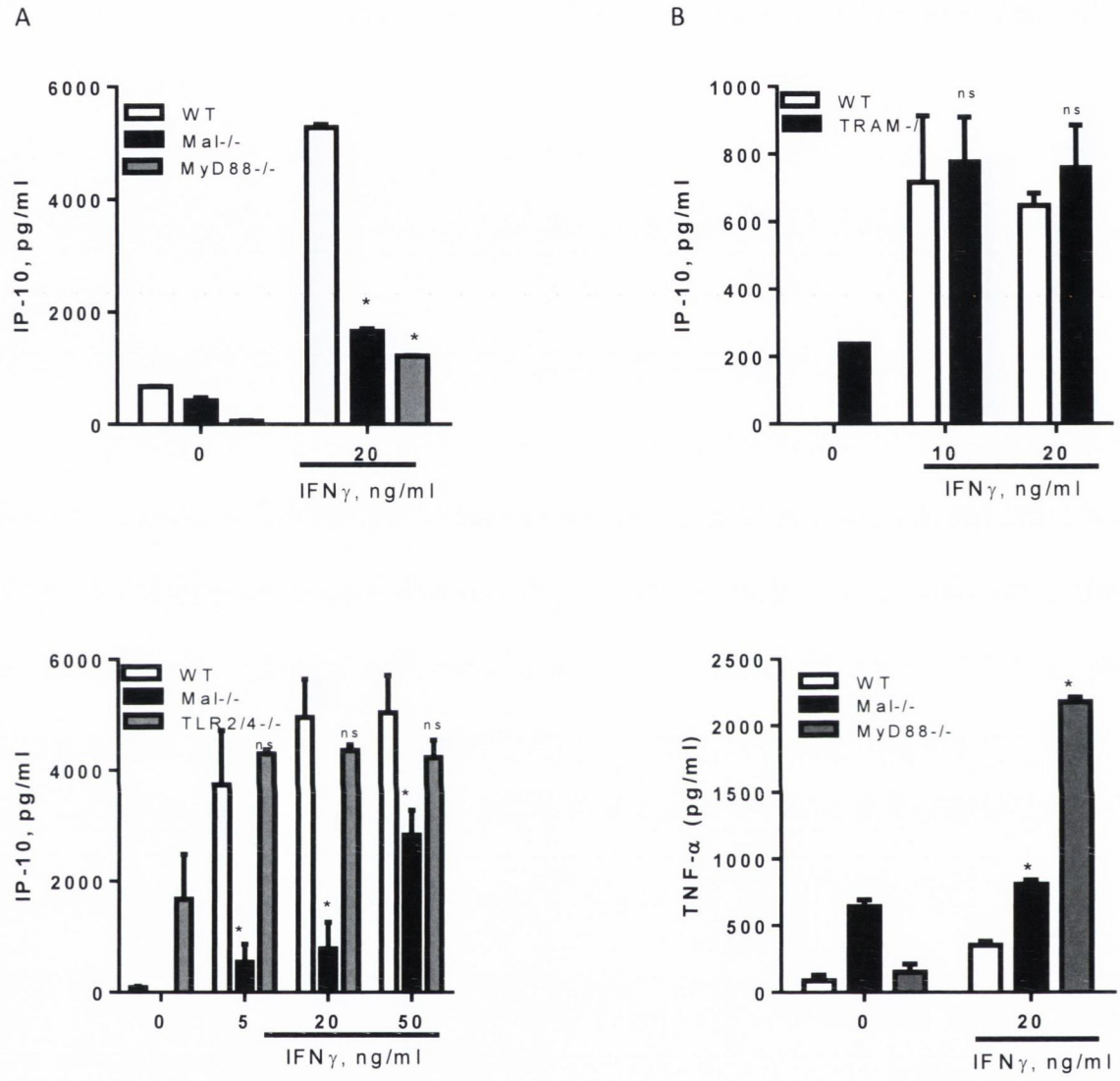


Figure 4-1: Mal and MyD88, but not TLR2, TLR4 or TRAM, are required for IP-10 responses to IFN-γ. TNFα responses are not Mal or MyD88 dependent.

Immortalised BMDM were stimulated with IFN-γ at the concentrations indicated. Supernatants were harvested at 16 hours and analysed by ELISA. Data are means +/- S.E.M. from one experiment representative of at least 3 separate experiments. * $p < 0.05$ (Mann-Whitney, comparison to WT with same stimulus)

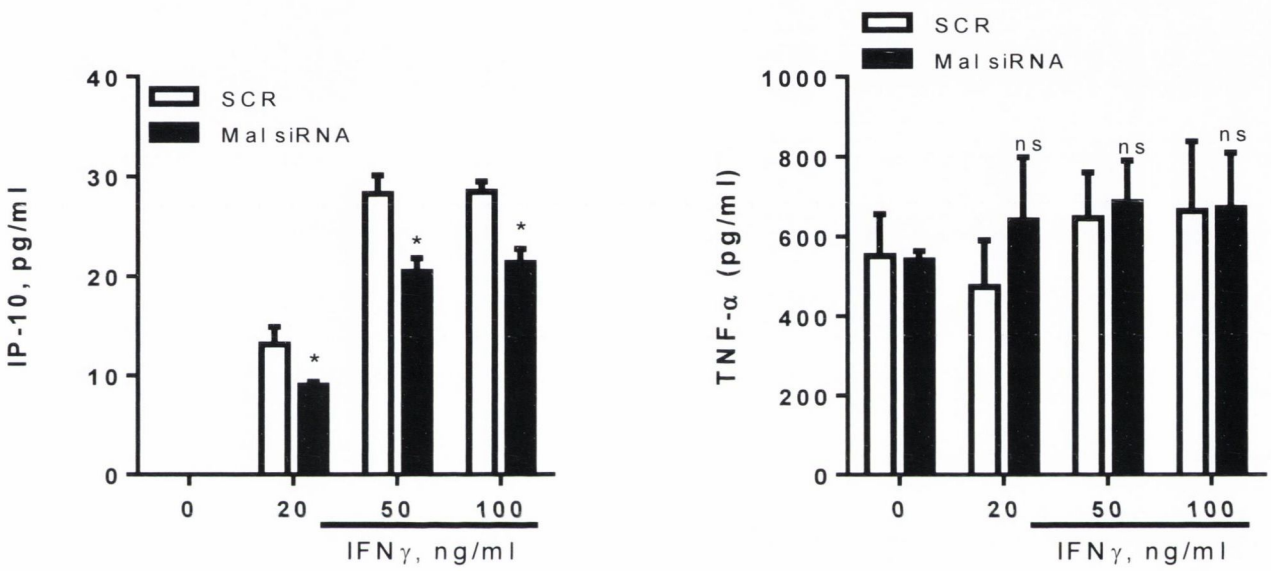


Figure 4-2: Mal and MyD88, but not TLR2, TLR4 or TRAM are required for IP-10 responses to IFN-γ.TNFα responses are not Mal or MyD88 dependent.

THP-1 cells were transfected with siRNA against Mal, differentiated with PMA and stimulated with IFN-γ at the concentrations indicated. Supernatants were harvested at 16 hours and analysed by ELISA. Data are means +/- S.E.M. from one experiment representative of at least 3 separate experiments. * $p < 0.05$ (Mann-Whitney, comparison to scrambled control for each stimulus)

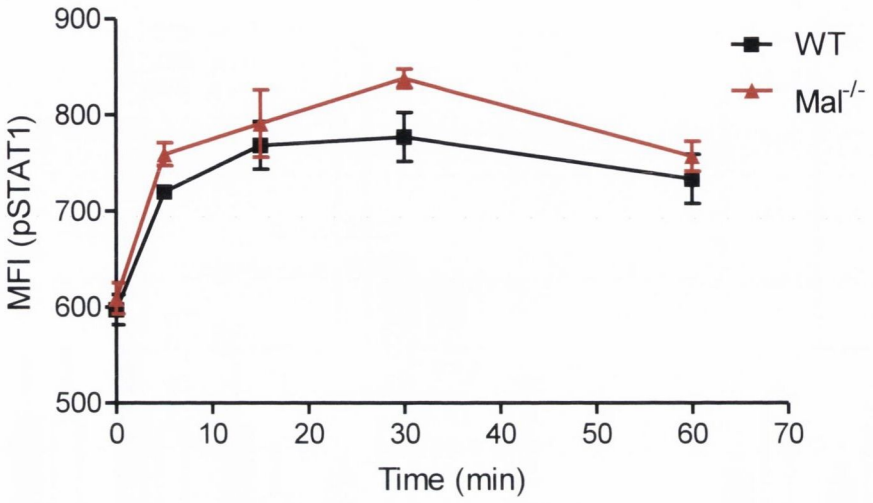
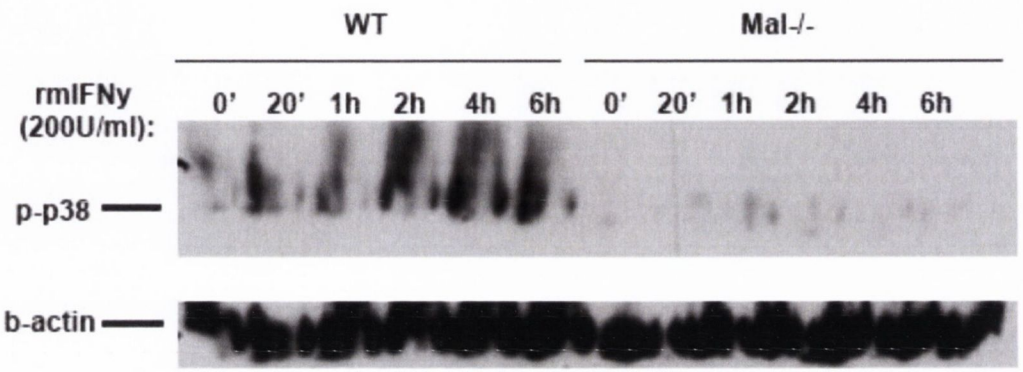


Figure 4-3: Mal is not required for IFNγ-induced STAT-1 phosphorylation.

iBMM were stimulated for the indicated times (0, 5, 15, 30, 60 min) with rmIFN-γ (10 ng/mL) before fixation and staining with anti-pSTAT1 (Y701) antibody conjugated to AlexaFluor 488 before analysis on a BD FACSCanto II analyser. **Experiment performed by Dr Jim Harris and Dr Sarah Jones, Monash University**.

A



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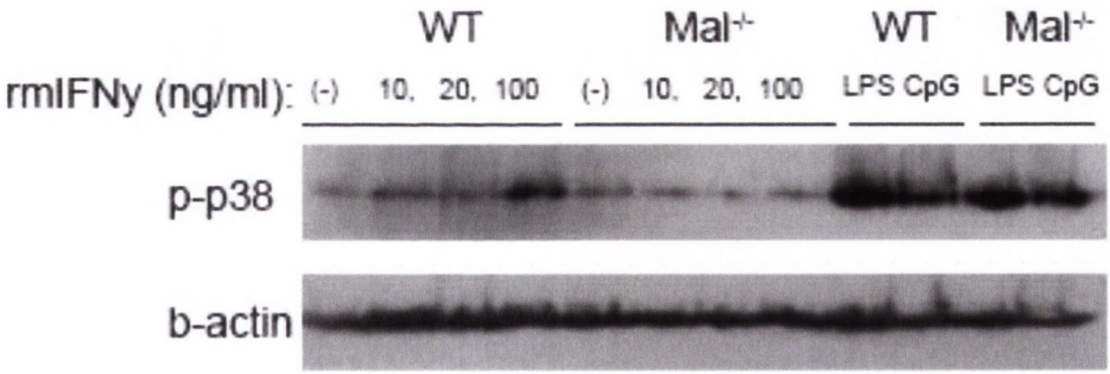


Figure 4-4: Mal is required for phosphorylation of p38 MAPK in response to IFN-γ.

Primary BMDMs were stimulated with 20 ng/ml of IFN-γ (A) or a range of IFN-γ concentrations (B). Cells were lysed at timepoints as indicated (A) or at 20 minutes (B) and lysates immunoblotted for phospho P38. **Experiments performed by Dr Fred Sheedy (IMM, TCD).**

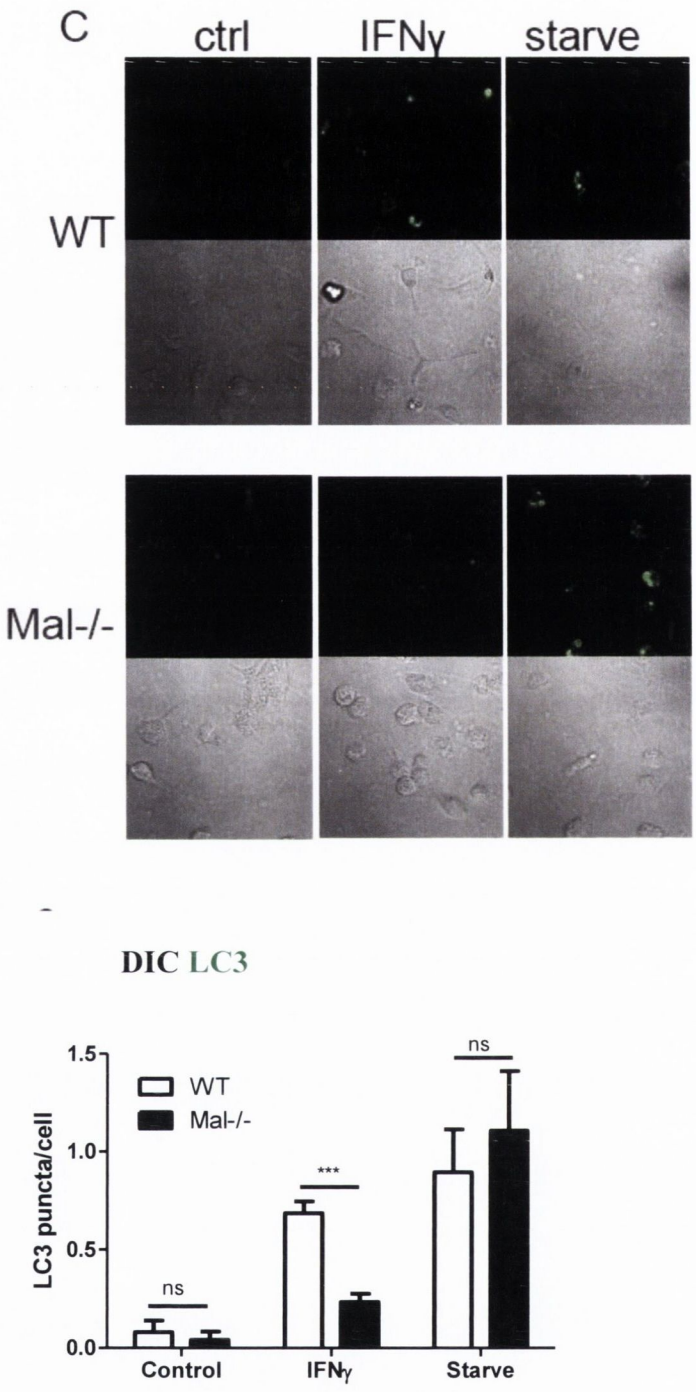


Figure 4-5: IFN-γ-induced autophagy is impaired in the absence of Mal.

Primary BMDM were stimulated for 16 hours with IFN-γ (20ng/ml) or for 2 hours with starvation medium. Cells were fixed and stained for LC3 and visualised with confocal microscopy. A minimum of 100 cells/condition were assessed in triplicate wells. Data shown are mean +/- S.E.M. from a single experiment representative of two separate experiments.* $p<0.05$, **$p<0.01$, ***$p<0.005$ (Mann-Whitney, WT vs $Mal^{-/-}$ for same condition).

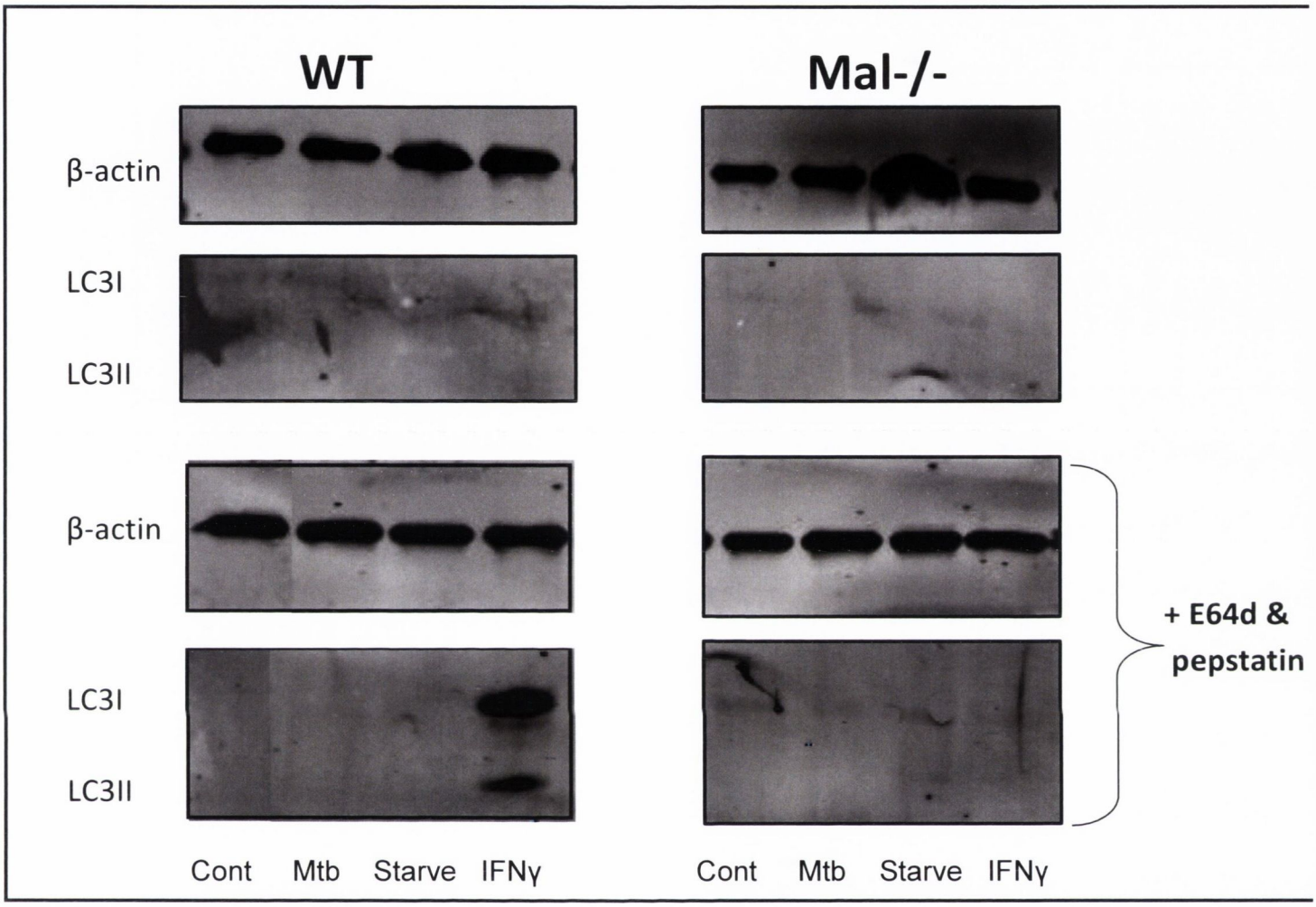


Figure 4-6: IFN-γ -induced autophagy is impaired in the absence of Mal.

Primary BMDM were stimulated for 6 hours with rmIFN-γ (20ng/ml) in the presence or absence of protease inhibitors E64d and pepstatin. Cells were lysed and lysates blotted with anti-LC3 antibody (Invitrogen). Data shown are representative of 3 separate experiments.

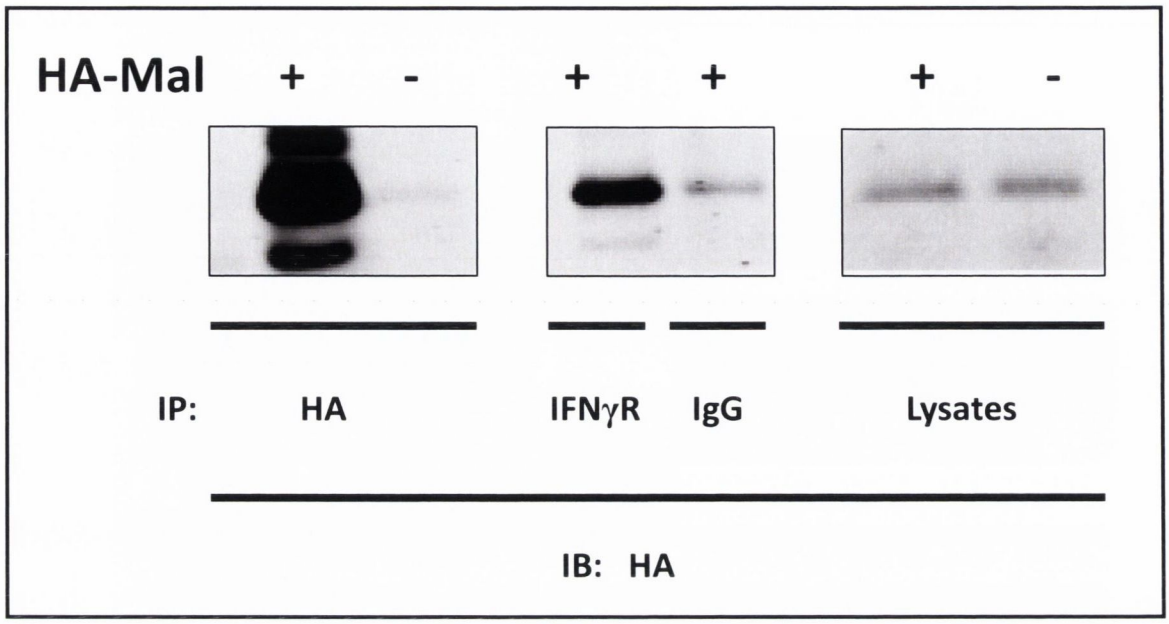


Figure 4-7: Mal associates with the IFN-γ receptor.

HEK-293 cells were transfected with HA-tagged Mal and immunoprecipitation was performed with antibodies to HA and IFN-γR1, along with an IgG control, on cell lysates as indicated. Lysates were then blotted with anti-HA antibody. Data shown are representative of 3 separate experiments.

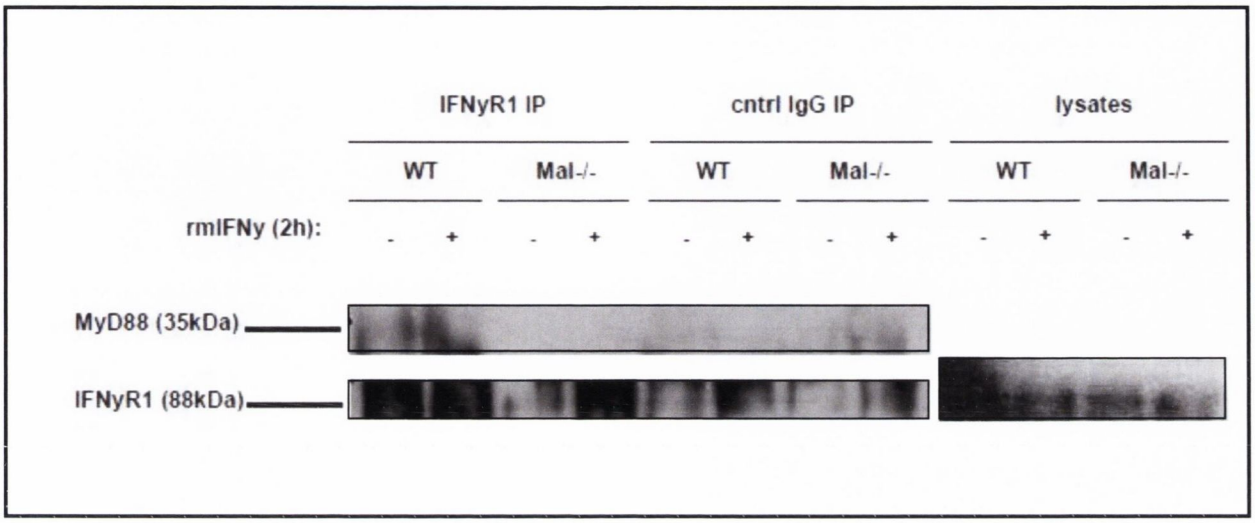
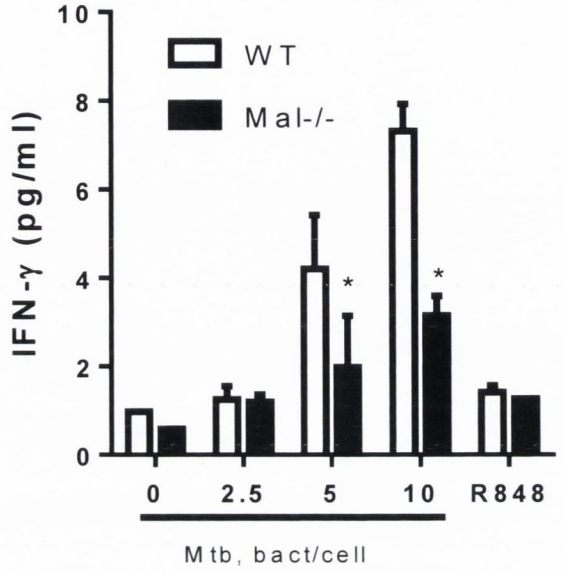


Figure 4-8: MyD88/IFNγR1 interaction requires Mal.

HEK-293 cells were stimulated with IFN-γ (20ng/ml) or RPMI and immunoprecipitation was performed with antibodies to MyD88 and IFNγR1, along with an IgG control, on cell lysates as indicated. Lysates were then blotted with anti-MyD88 and anti IFNγR1 antibody. Data shown are representative of 3 separate experiments. **Experiment carried out by Dr Fred Sheedy.**

A



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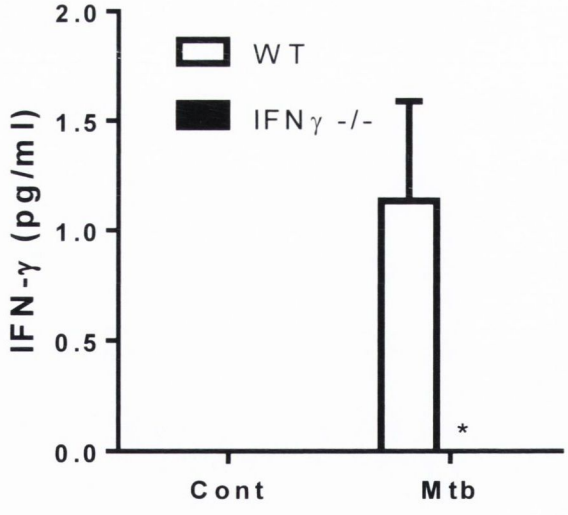

Figure 4-9: Immortalised and primary murine macrophages secrete IFN-γ in response to Mtb infection

iBMM (A) and primary BMDM (B) were infected with Mtb at the MOI indicated. Supernatants were harvested at 72 hours and analysed by ELISA. Data are means +/- S.D from one experiment representative of at least 3 separate experiments. * $p < 0.05$ (Mann-Whitney, compared to WT cells).

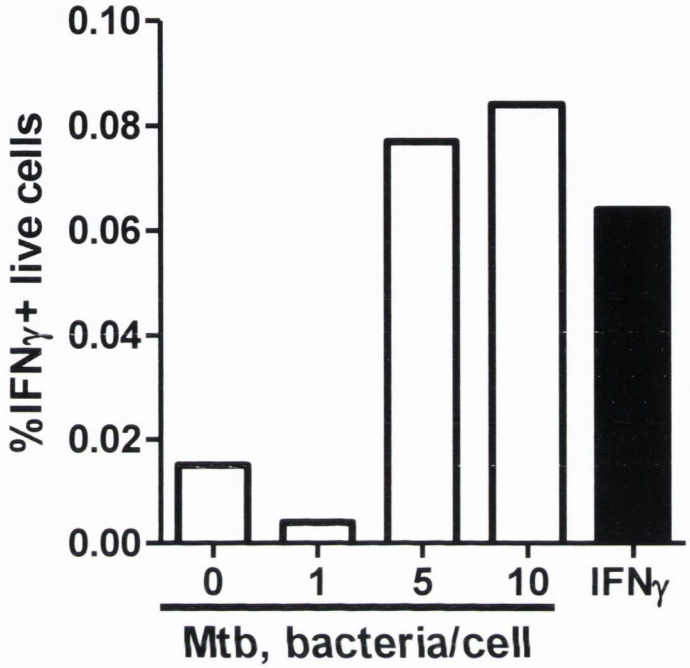

Figure 4-10: Immortalised BMDM produce IFNγ in response to Mtb infection.

Immortalised WT BMDM were infected with Mtb at the MOI indicated or stimulated with IFN-γ (20ng/ml). Cells were treated with Brefeldin A and fixed at 72 hours. Cells were then permeabilised and stained with anti-IFN-γ antibody and analysed by flow cytometry. Data are from one experiment representative of 3 separate experiments.

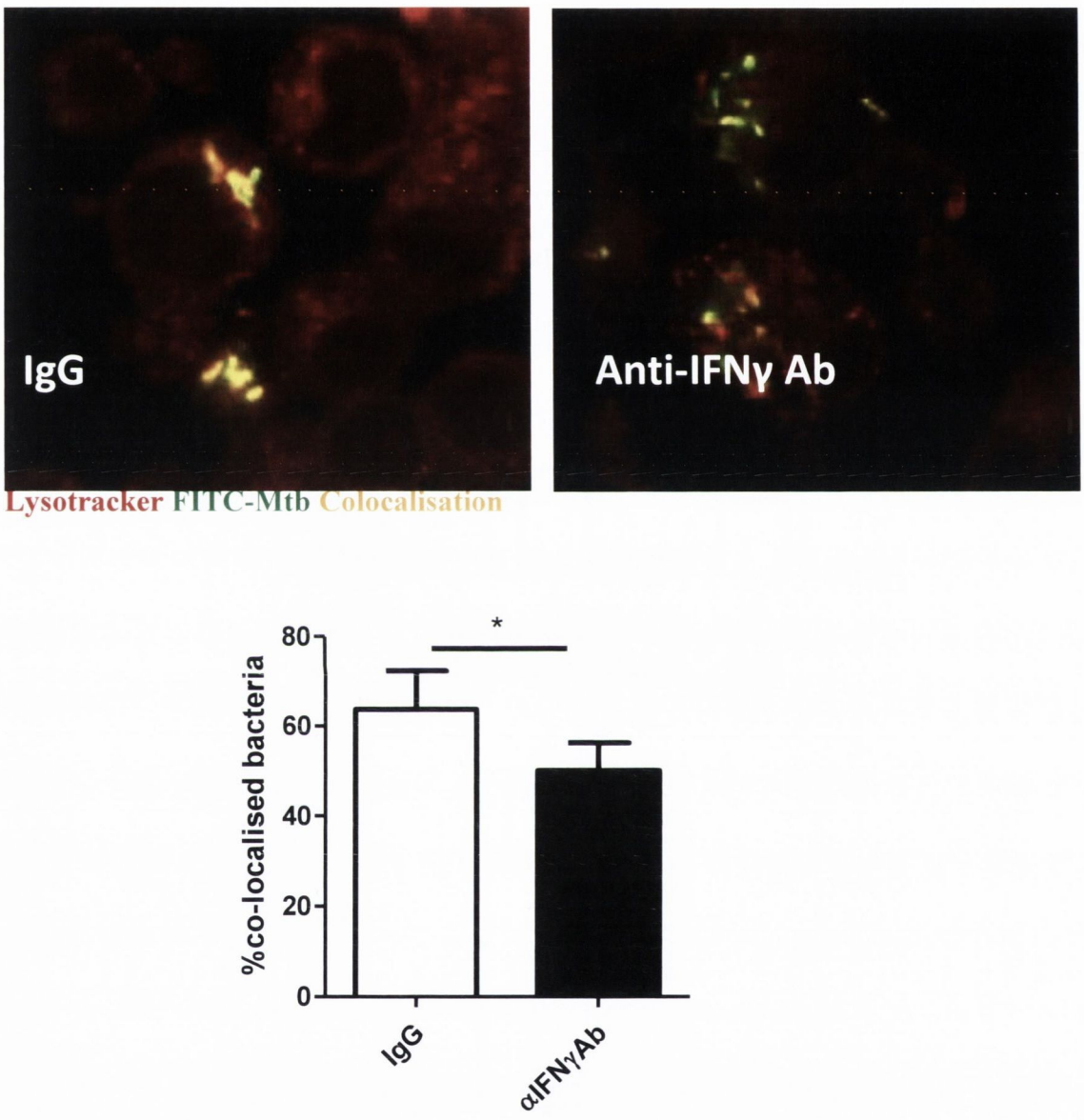

Figure 4-11: Macrophage-secreted IFN-γ is required for phagosome maturation.

Immortalised WT BMDM were pre-treated with an anti-IFN-γ antibody (10 ng/ml) or RPMI prior to infection with FITC-labelled. Cells were stained with Lysotracker and co-localisation of with phagolysosomes was assessed with confocal microscopy. Data shown are means +/- S.D. from a single experiment done in triplicate representative of two separate experiments, * $p < 0.05$, (Mann-Whitney).

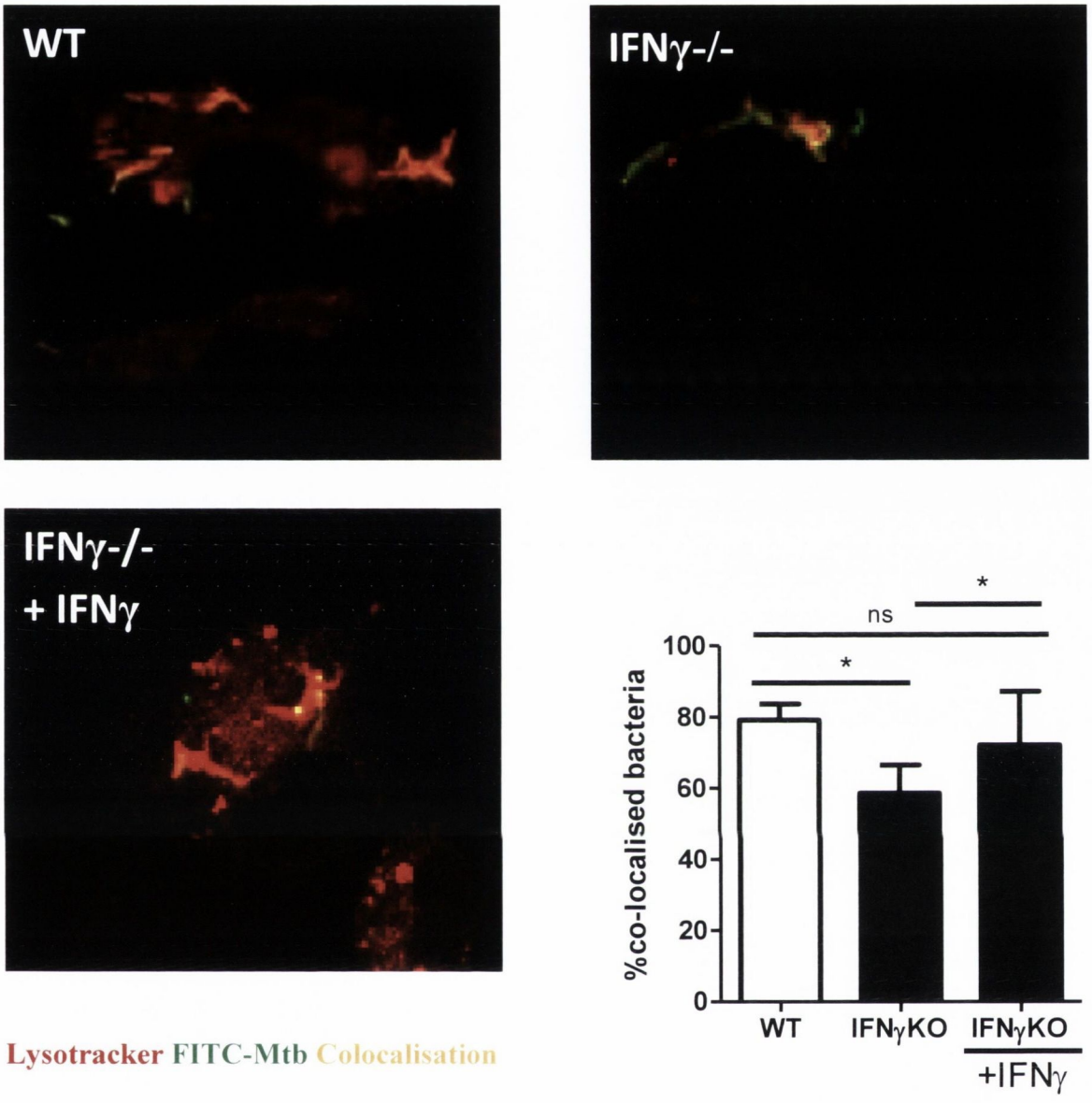

Figure 4-12: Macrophage-secreted IFN-γ is required for phagosome maturation.

Primary WT and IFN-$\gamma^{-/-}$ BMDM were treated with RPMI or IFN-γ (20 ng/ml) prior to infection with FITC-labelled. Cells were stained with Lysotracker and co-localisation of with phagolysosomes was assessed with confocal microscopy. Data shown are means +/- S.D. from a single experiment done in triplicate representative of two separate experiments, * $p < 0.05$, (Mann-Whitney, knockout vs WT)

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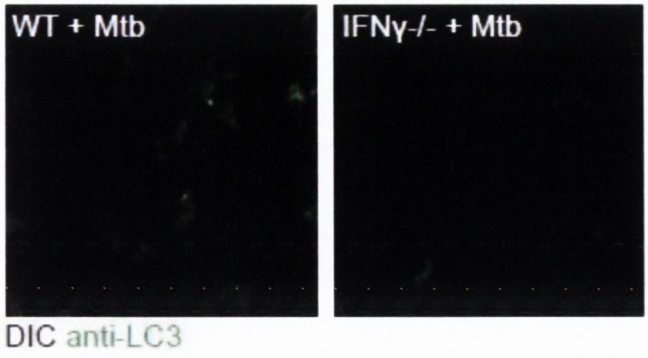


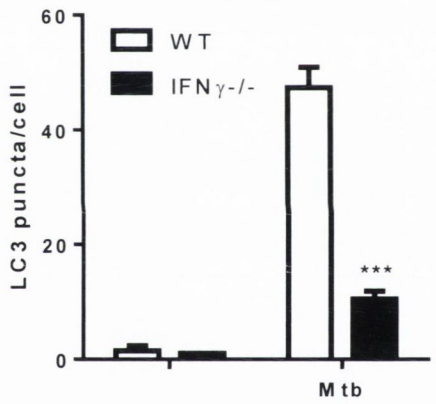


Figure 4-13: Macrophage-produced IFN-γ is required for autophagy in response to Mtb infection.

Primary WT and IFN-γ-/- BMM were infected for 16 hours with MT prior to fixation. Cells were stained with anti-LC3 antibody (Invitrogen) and LC3 puncta/cell were quantified by confocal microscopy. Data shown are means +/- S.D. from a single experiment done in triplicate representative of two separate experiments, * $p < 0.05$, (Mann-Whitney, WT vs IFNγ-/- for each condition)

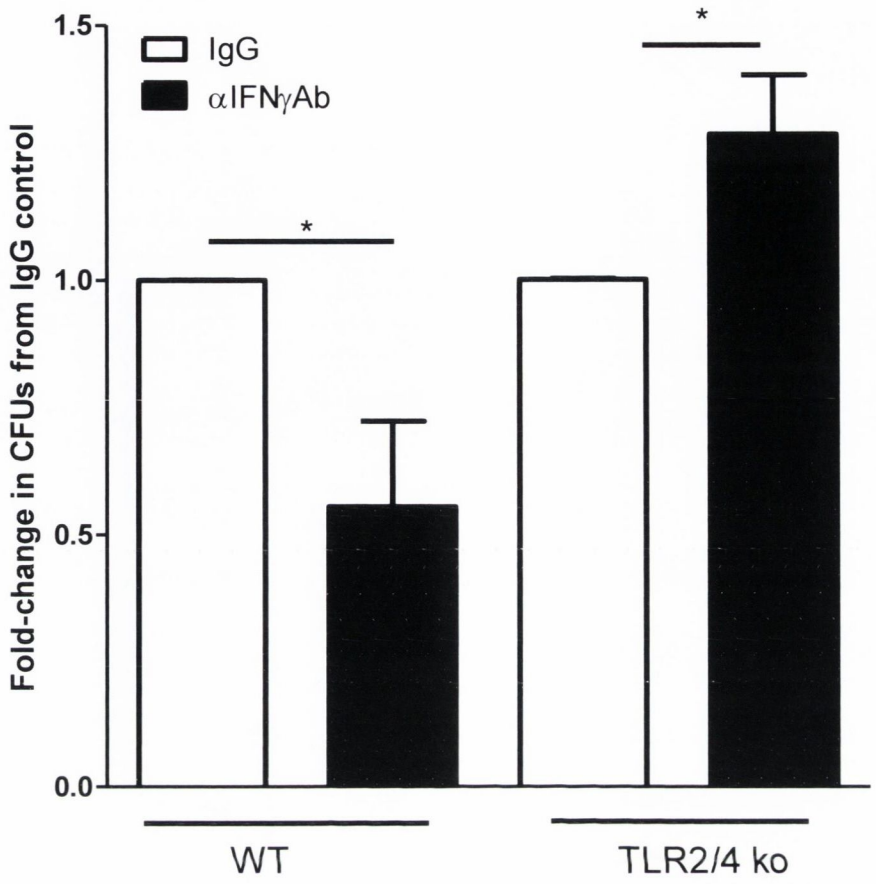


Figure 4-14: IFN-γ is required for bactericidal activity in TLR2/4$^{-/-}$ macrophages.

WT and TLR2/4$^{-/-}$ iBMM were pretreated with RPMI or IFN-γ blocking antibody and WT and IFN-γ primary macrophages were infected with H37Rv and lysed at 72 h. Serial dilutions of lysates were plated out to determine bacterial numbers. Data shown are mean +/- S.D, pooled from 2 separate experiments. (Mann-Whitney, IgG vs anti-IFN-γ antibody for each type of cell). Experiment carried out by Dr Michelle Coleman.

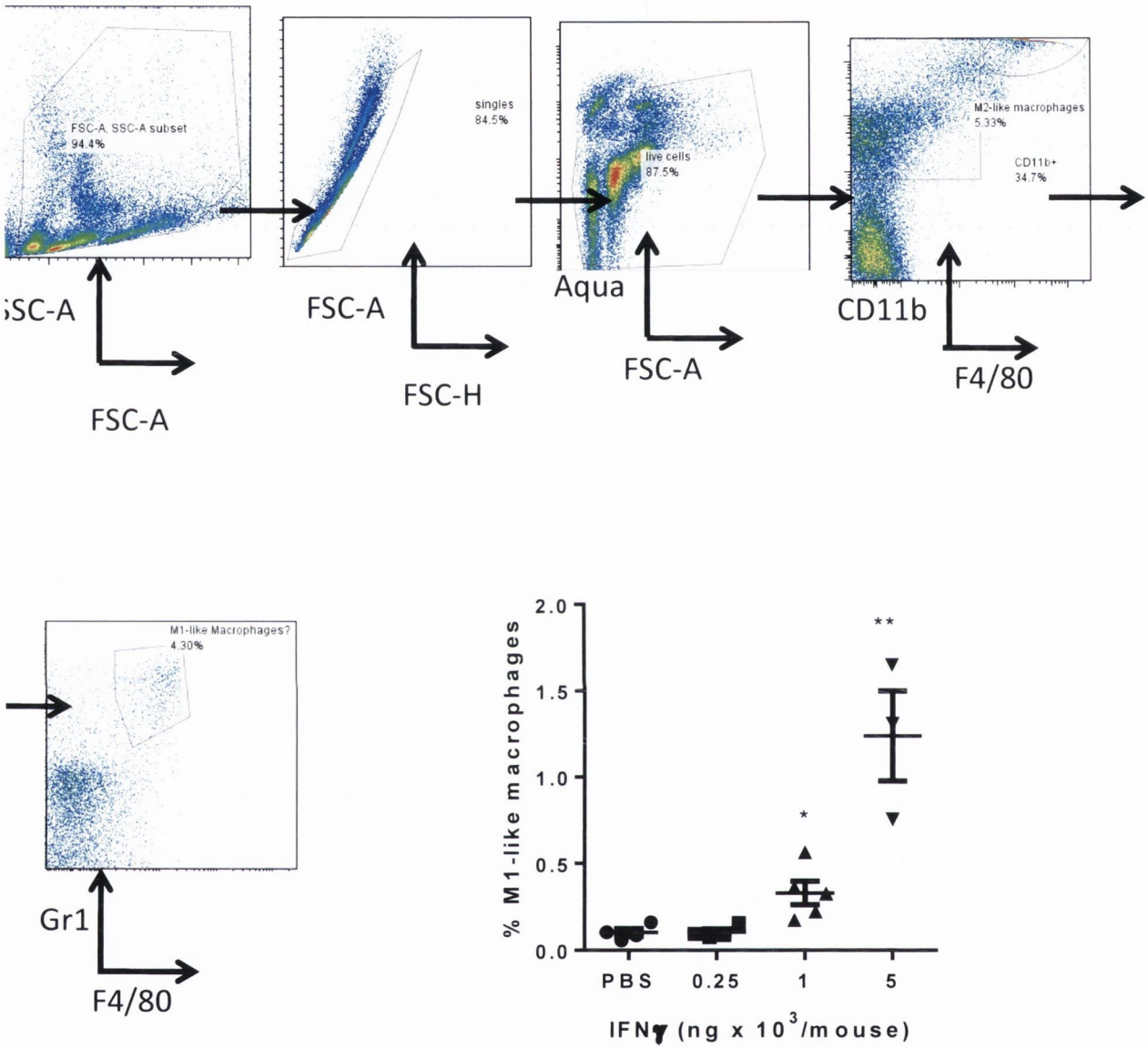


Figure 4-15: Intraperitoneal IFN-γ induces recruitment of M1-like macrophages

Female C57BL/6 mice (3 mice/group) were immunised intraperitoneally (i.p.) with rmIFN-γ at concentrations as indicated. The mice were sacrificed 24 hours post-injection. Peritoneal exudate cells (PECs) were collected and analysed by flow cytometry. M1-like macrophages were identified on the basis of their $CD11b^{+,}$ $Gr1^{high}$ and $F4/80^{high}$ profile (M1-like macrophages in representative dot plots). Percentage of M1-like macrophages per live cells for each group is shown in the scatter plot. Versus PBS, n.s., not significant, * $p<0.01$, ** $p<0.001$, Mann-Whitney

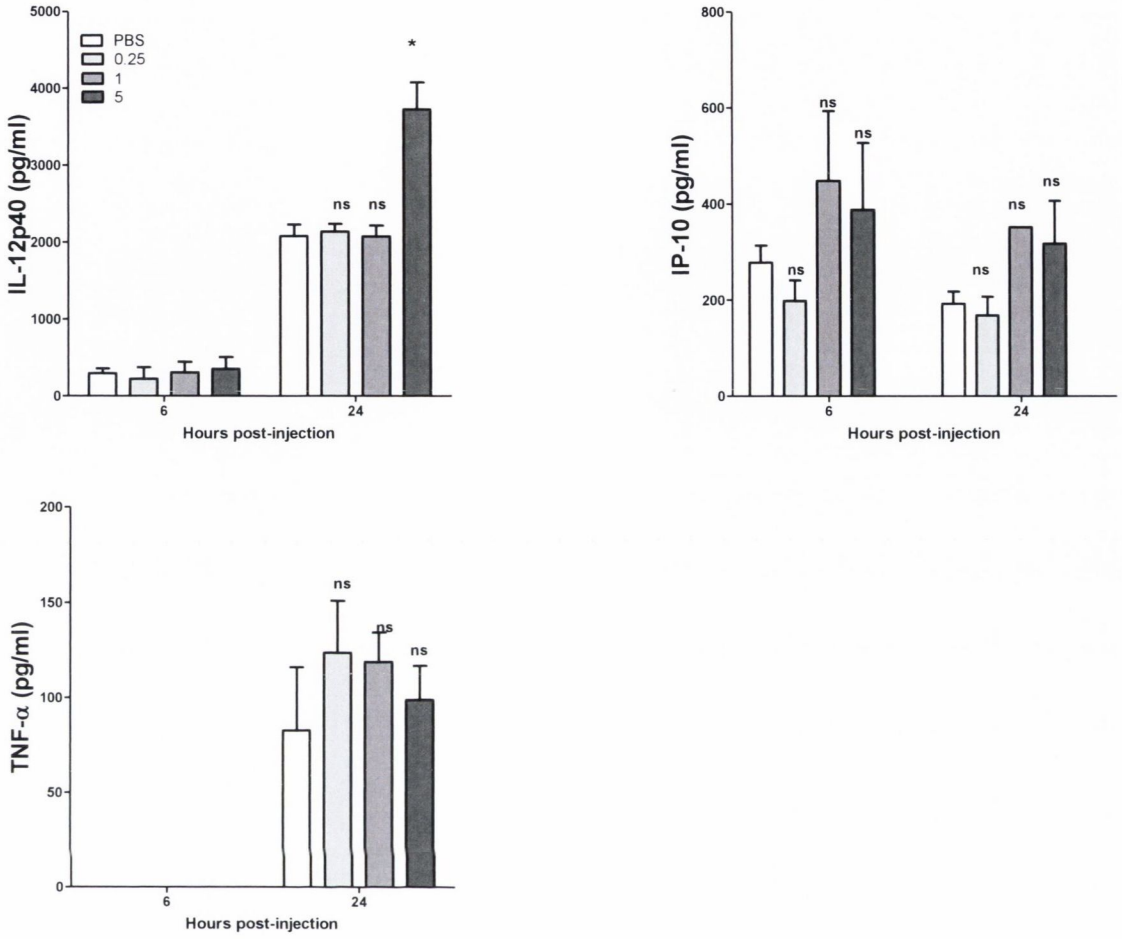


Figure 4-16: Intraperioneal IFN-γ induces secretion of IL-12p40 in blood at 24 h.

Mice were injected with intraperitoneal IFN-γ as described in Figure 4.15. Mice were bled by tail bleed at 6 hours and sacrificed at 24 hours. Blood was analysed for the cytokines IL-12p40, IP-10 and TNF-α by ELISA. Results are mean cytokine concentrations (+ SEM) for 3 mice per experimental group tested individually in triplicate. .Mann-Whitney, compared to PBS control, * $p<0.05$, ns non-significant.

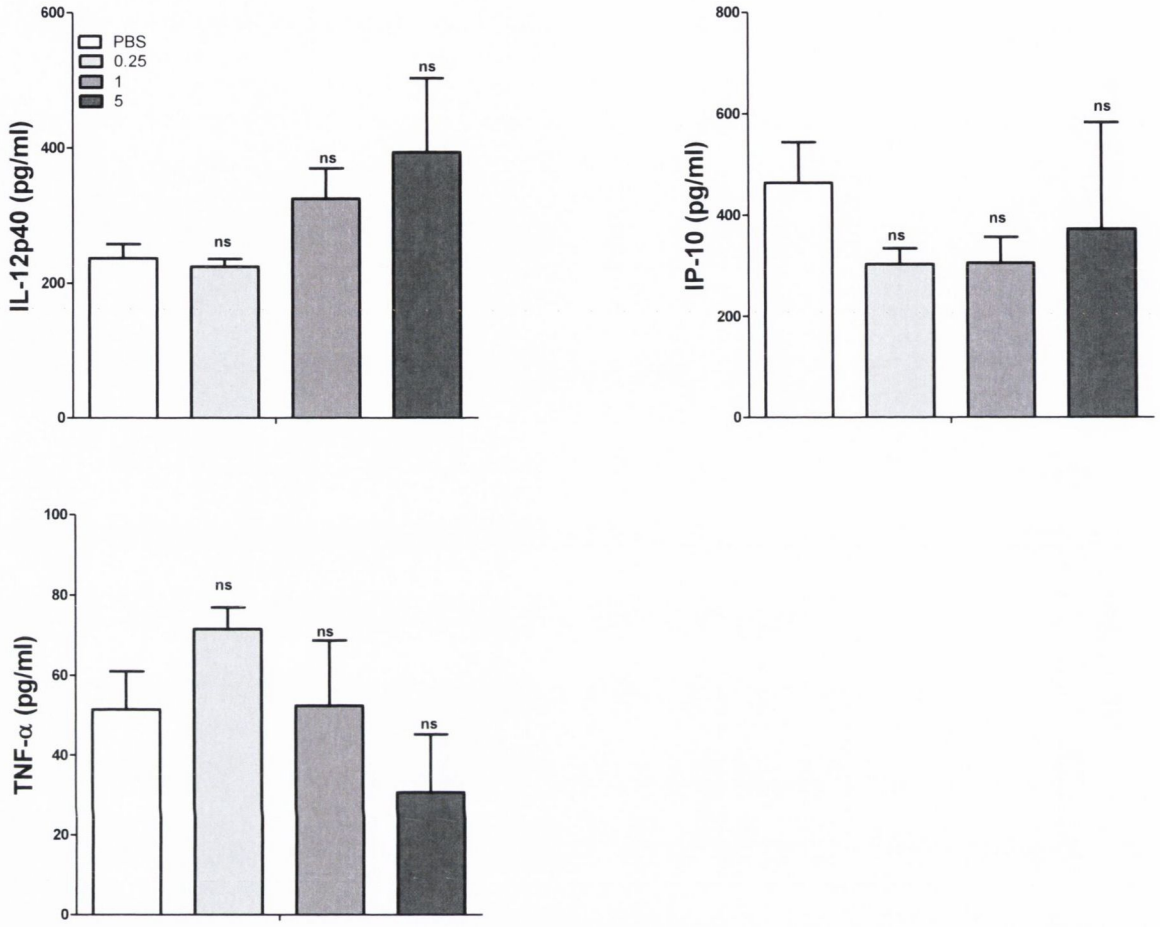


Figure 4-17: Intraperitoneal IFN-γ induces peritoneal secretion of IL-12p40.

Mice were injected with intraperitoneal IFN-γ as described in Figure 4.15. Mice were sacrificed at 24 hours, and peritoneal cavities washed with 2 ml of sterile PBS. Peritoneal washes were collected and analysed for the cytokines IL-12p40, IP-10 and TNF-α by ELISA. Results are mean cytokine concentrations (+/- SEM) for 3 mice per experimental group tested individually in triplicate. .Mann-Whitney, compared to PBS control, * $p<0.05$, ns non-significant.

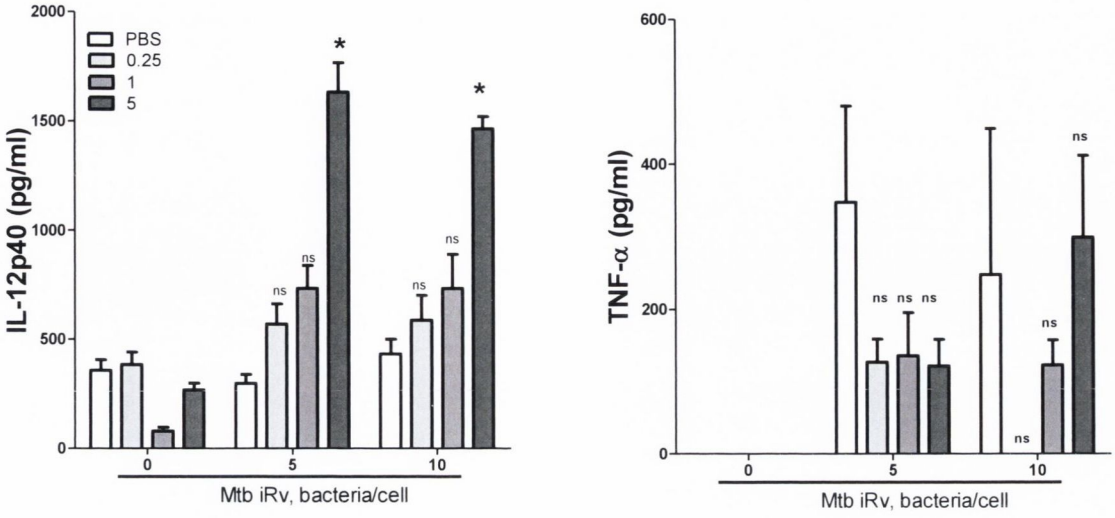


Figure 4-18: Intraperitoneal IFN-γ primes PECs for enhanced IL-12p40 secretion following restimulation.

Mice were injected with intraperitoneal IFN-γ as described in Figure 4.15. Peritoneal cells were recovered and restimulated *ex vivo* with RPMI or irradiated Mtb (MOI as indicated). After 16 hours, supernatants were collected and analysed for the cytokines IL-12p40 and TNF-α by ELISA. Results are mean cytokine concentrations (+/- SEM) for 3 mice per experimental group tested individually in triplicate. Mann-Whitney, compared to PBS control, * $p<0.05$, ns non-significant

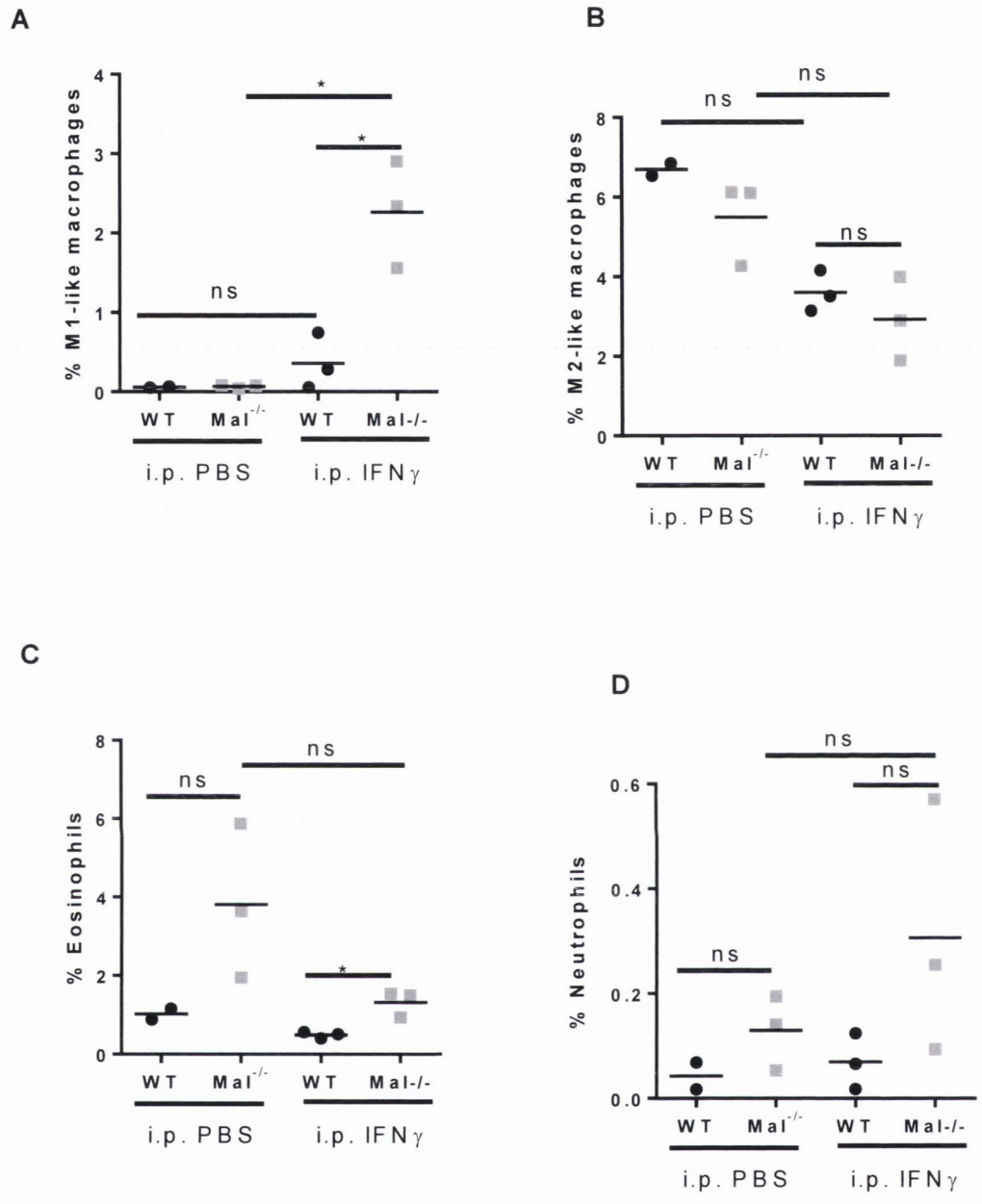


Figure 4-19: Mal -/- mice display increased recruitment of M1-like macrophages in response to intraperitoneal IFN-γ.

Male C57BL/6 WT and Mal$^{-/-}$ mice (3 mice/group) were immunised intraperitoneally (i.p.) with 5 μg of rmIFN-γ/mouse or PBS control. The mice were sacrificed 24 hours post injection. PECs were collected and analysed by flow cytometry. M1-like macrophages were identified on the basis of their CD11b^{+}, Gr1^{high} and F4/80^{high} profile (A), M2-like macrophages as CD11b^{inter} F4/80^{inter} Gr-1$^{low/inter}$ SiglecF- (B), eosinophils as SiglecF+ ckit- (C) and neutrophils were identified as CD11b^{inter} F4/80- Gr-1^{high} (D). Mann-Whitney test, n.s., not significant, * $p<0.01$, ** $p<0.001$

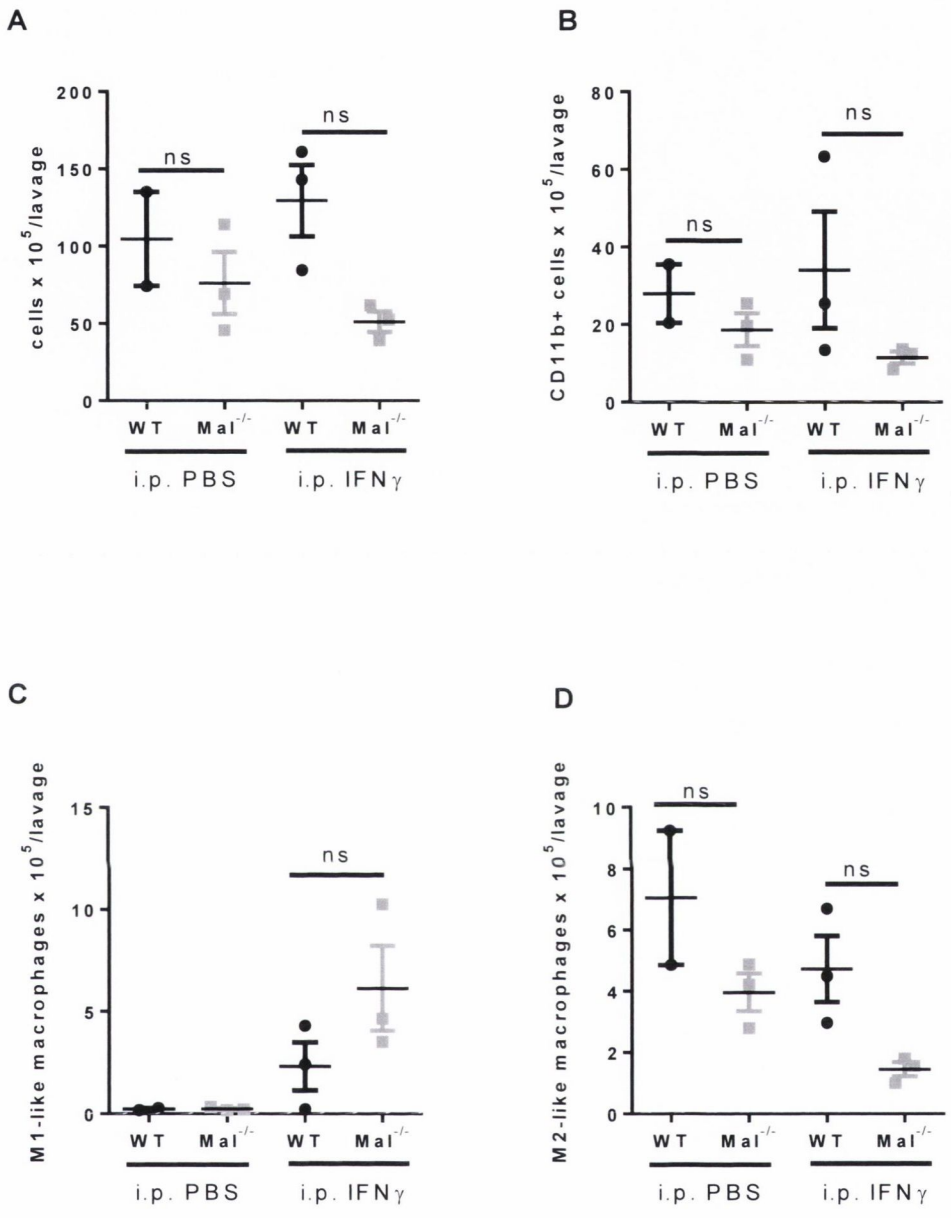


Figure 4-20: Intraperitoneal IFN-γ induces enhanced recruitment of pro-inflammatory cells in $Mal^{-/-}$ mice (absolute numbers).

Male C57BL/6 WT and $Mal^{-/-}$ mice (3 mice/group) were immunised intraperitoneally (i.p.) with rmIFN-γ as described in Figure 4.19. The mice were sacrificed 24 hours post injection. PECs were collected and analysed by flow cytometry. M1-like macrophages were identified on the basis of their $CD11b^{+}$, $Gr1^{high}$ and $F4/80^{high}$ profile, M2-like macrophages as $CD11b^{inter}$ $F4/80^{inter}$ $Gr\text{-}1^{low/inter}$ $SiglecF^{-}$). Mann-Whitney test, n.s., not significant, * $p<0.01$, ** $p<0.001$

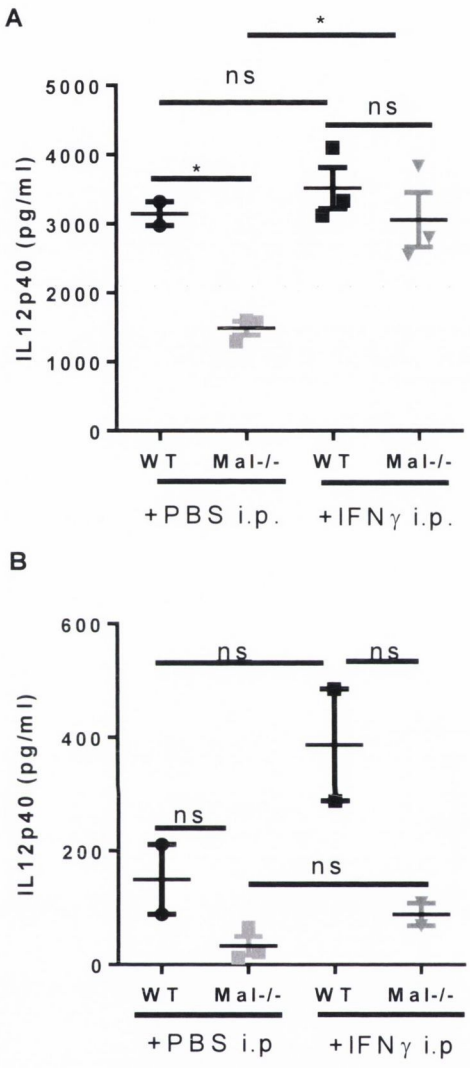


Figure 4-21: Intraperitoneal IFN-γ induces IL12p40 secretion in blood and peritoneal fluid in $Mal^{-/-}$ mice.

Male C57BL/6 WT and $Mal^{-/-}$ mice (3 mice/group) were immunised intraperitoneally (i.p.) with rmIFN-γ as described in Figure 4.19. Mice were sacrificed 24 hours post injection and blood harvested. Peritoneal cavities were washed with 2 ml of sterile PBS. Blood (A) and peritoneal lavage fluid (B) were analysed for the cytokine IL-12p40 by ELISA. Results are mean cytokine concentrations (+/- SEM) for 3 mice per experimental group tested individually in triplicate. Mann-Whitney, * $p<0.05$, ns non-significant.

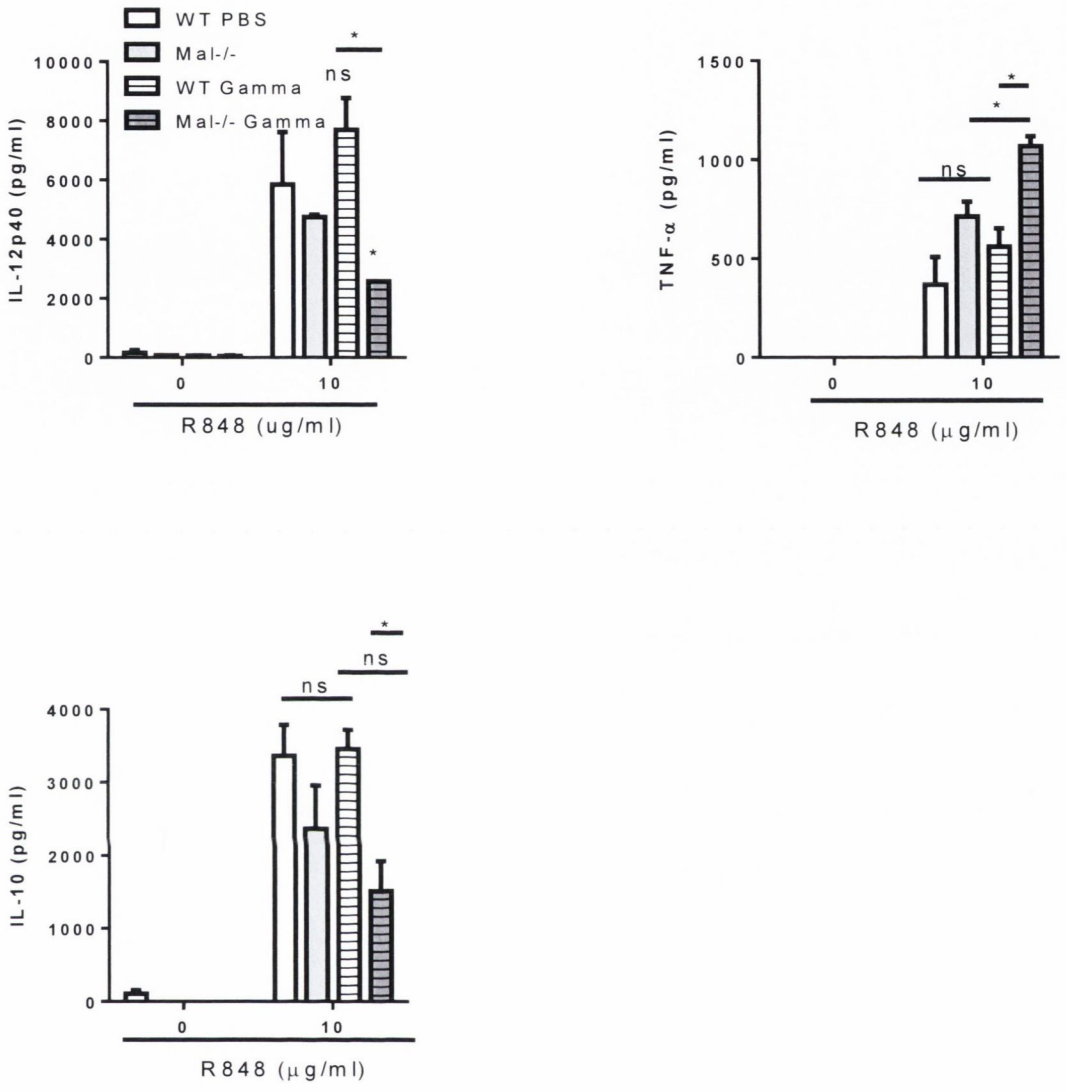


Figure 4-22: Intraperitoneal IFN-γ induces increased TNF-α secretion in PECs restimulated with R848.

Mice were injected with intraperitoneal IFN-γ as described in Figure 4.19. Peritoneal cells were recovered and restimulated *ex vivo* with RPMI or R848 at concentrations as indicated. After 16 hours, supernatants were collected and analysed for the cytokine by ELISA. Results are mean cytokine concentrations (+ SEM) for 3 mice per experimental group tested individually in triplicate. Mann-Whitney, * $p<0.05$, ns non-significant.

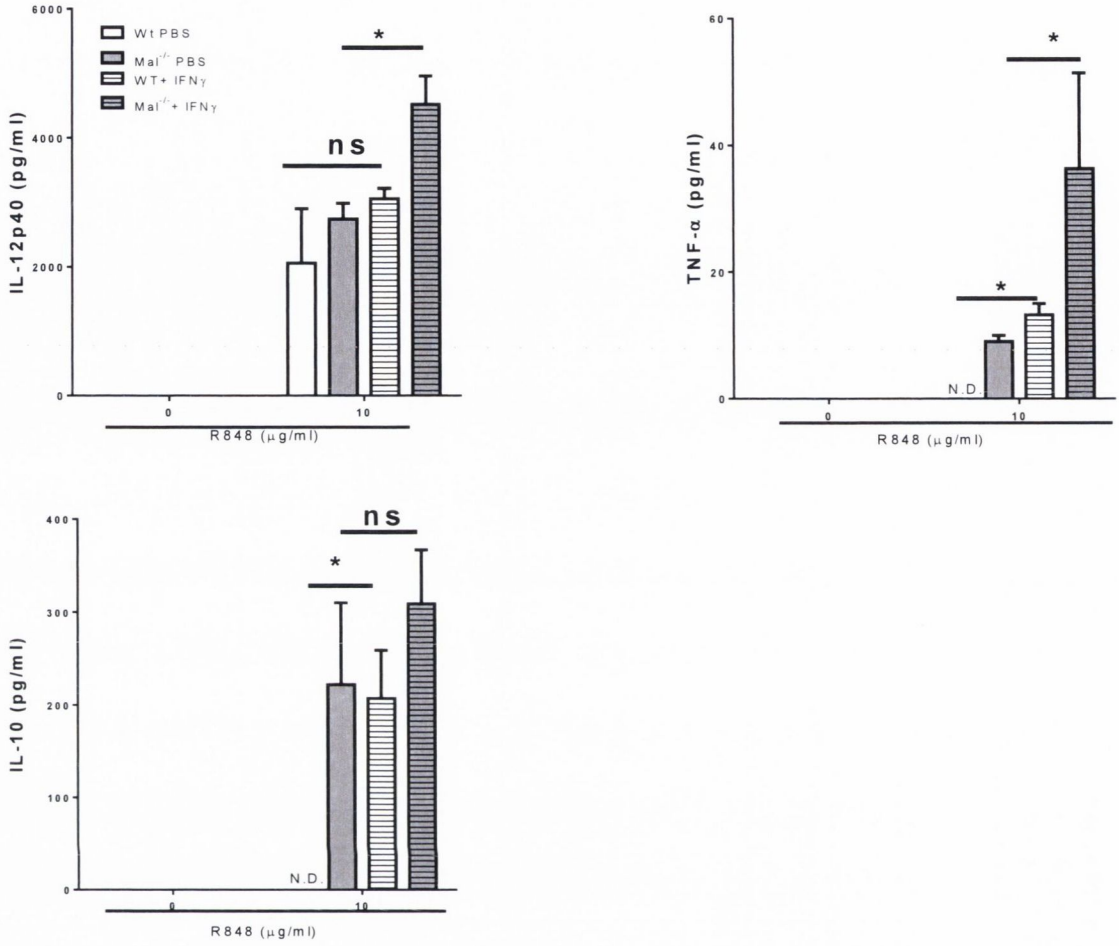


Figure 4-23: Intraperitoneal IFN-γ primes spleen cells from $Mal^{-/-}$ mice for increased IL-12p40 production in response to restimulation.

Mice were injected with rmIFN-γ as described in Figure 4.19. Spleens were harvested and plated at 2 x 10^6/ml in RPMI prior to stimulation with R848 as indicated. Supernatants were harvested at 16 hours and analysed by ELISA. Mann-Whitney, * $p<0.05$, ns non-significant.

4.3 Discussion

Mal bridges the association between MyD88 and IFN-γR1 and is required for p38 MAPK phosphorylation

In 2006, Sun and Ding reported that IFN-γ triggered a physical association between IFN-γR1 and MyD88, which activated a 'signalosome' that included mixed-lineage kinase 3 (MLK3) and mitogen-activated protein kinase kinase 3 (MKK3), resulting in activation of p38 MAPK. This signalling stabilised mRNA transcripts, including TNF-α and IP-10. This was supported by a prior report by Shi and colleagues demonstrating that IFN-γ induction of genes involved in macrophage activation was impaired in the absence of MyD88 (Shi, Nathan et al. 2003) and subsequent work by Holscher and colleagues showing that IFN-γ-induced phagosome maturation was impaired in MyD88$^{-/-}$ macrophages (Holscher, Reiling et al. 2008).

Data presented here shows that Mal bridges the association between MyD88 and IFN-γR1 and is required for IFN-γ signalling, resulting in p38 activation, IP-10 secretion, phagosome maturation and autophagy (Figures 4.1-4.9). This represents a novel role for Mal, which hitherto had been thought of primarily as a signalling adaptor protein for TLR2 and TLR4.

Not all IFN-γ signalling pathways are Mal-dependent. The IFN-γ signalling pathway including MLK3 and p38 MAPK is distinct from that involving STAT-1 (Platanias 2003, Parmar, Katsoulidis et al. 2004). Data published by Sun and Ding have shown that MyD88 was redundant in STAT-1 phosphorylation and downstream γ-activated site nucleotide-binding activity (Sun and Ding 2006). STAT-1 phosphorylation was unimpaired in the absence of Mal (Figure 4.3). IFN-γ signalling via STAT-1 is known to result in NO and ROS production (Stempelj, Kedinger et al. 2007). There was no defect in NO production in

response to IFN-γ in $Mal^{-/-}$ or $MyD88^{-/-}$ cells (Figure 3.25), providing further evidence that this IFN-γ signalling pathway is MyD88- and Mal-independent.

Therefore, Mal can now be added to the MyD88-dependent IFNγR signalling pathway culminating in p38 phosphorylation. It is unclear what site in IFN-γR1 interacts with MyD88 and Mal, as IFN-γR1, unlike TLRs and IL-1R, lacks a homophilic TIR or death domain.

The IFN-γR1-Mal-MyD88-p38 MAPK signalling pathway is required for M. tuberculosis *and IFN-γ induced autophagy and phagosome maturation.*

This study demonstrated that the IFN-γR1-Mal-MyD88-p38 MAPK signalling pathway was required for *M. tuberculosis* and IFN-γ-induced autophagy and IFN-γ-induced maturation of phagosomes containing *M. tuberculosis*. The involvement of p38 MAPK in autophagy had been reported by Matsuzawa and colleagues, who used shRNA to identify a requirement for JAK 1/2, PI3K, and p38 MAPK but not STAT1 or Irgm in IFN-γ induced autophagy (Matsuzawa, Kim et al. 2012). More recently, Matsuzawa and colleagues have reported that this STAT-1 and iNOS-independent, p38 MAPK-dependent autophagy, can kill intracellular bacteria, including *Salmonella* and *Listeria* (Matsuzawa, Fujiwara et al. 2014). A recent report also shows a requirement for p38 MAPK in starvation-induced autophagy(Moruno-Manchón, Pérez-Jiménez et al. 2013), although we did not see a defect in starvation-induced autophagy in $Mal^{-/-}$ cells. Interestingly, autophagy deficient cells have been reported to be deficient in p38 inactivation, showing a possible regulatory negative feed-back loop between autophagy and p38 (Qiang, Wu et al. 2013).

As discussed in the introductory chapter, IFN-γ is of critical importance in immune responses to TB. IFN-γ activates macrophages, leading to production of NO and ROS,

phagosome maturation, autophagy and bactericidal activity (Darnell, Kerr et al. 1994, Boehm, Klamp et al. 1997, Stark, Kerr et al. 1998, MacMicking 2012). Individuals with partial or complete defects in the IFN-γ signalling pathway have increased susceptibility to *M. tuberculosis*, as well as to other mycobacterial species (Filipe-Santos, Bustamante et al. 2006, Bogunovic, Byun et al. 2012). Vitamin D has been reported to be required for IFN-γ activation of macrophages (Fabri, Stenger et al. 2011), including expression of cathelicidin, phagosome maturation and autophagy (Yuk, Shin et al. 2009). It is important to note that the RPMI medium supplemented with foetal bovine serum used in our experiments contains Vitamin D at approximately physiological levels (1 -10M of $1,25(OH)_2D_3$) (Haverty, Haddad et al. 1987).

Interferon-inducible protein 10

Interferon-inducible protein 10 (IP-10), also known as CXCL10, is a cytokine/chemokine secreted by several cell types, including monocytes, neutrophils and fibroblasts, in response to IFN-γ and type 1 interferons (Luster, Unkeless et al. 1985, Cassatella, Gasperini et al. 1997). Several functions have been ascribed to IP-10, including chemoattraction for neutrophils and T cells, inhibition of neovascularization, and anti-tumor effects. (Farber 1997, Shen, Wang et al. 2013). IP-10 is also produced in response to stimulation with other cytokines, such as TNF-α (Ohmori and Hamilton 1995), and in response to LPS. IP-10 may play a role in tuberculosis. IP-10 is produced by macrophages stimulated *in vitro* with *M. tuberculosis* (Shi, Nathan et al. 2003). High levels of IP-10 have been found in lung and lymph node tissue infected with tuberculosis and in plasma of TB-infected patients (Ferrero, Biswas et al. 2003, Azzurri, Sow et al. 2005, Okamoto, Kawabe et al. 2005). IP-10 levels may be reflective of the intensity of the inflammatory response in TB,

as levels of IP-10 are higher in those patients with systemic symptoms such as fever and anorexia (Azzurri, Sow et al. 2005). The use of IP-10 as a biomarker for tuberculosis infection has been explored (Whittaker, Gordon et al. 2008, Ruhwald, Aabye et al. 2012).

Macrophages secrete functionally relevant quantities of IFN-γ

Given that $Mal^{-/-}$ macrophages demonstrated a defect in autophagy and bactericidal activity in the absence of exogenous IFN-γ, a question arose as to whether any endogenous IFN-γ was present to cause activation of WT, but not $Mal^{-/-}$ macrophages. IFN-γ is primarily secreted by activated Th1 cells (Mosmann and Coffman 1989), activated $CD8^{+}$ cytotoxic cells (Sad, Marcotte et al. 1995) and NK cells (Perussia 1991). Macrophages are not well-recognised as producers of IFN-γ despite numerous reports of macrophages secreting IFN-γ (Fultz, Barber et al. 1993, Di Marzio, Puddu et al. 1994, Fenton, Vermeulen et al. 1997, Darwich, Coma et al. 2009), albeit in limited quantities. The murine iBMM and primary BMM used in the *M. tuberculosis* infection model in this study do secrete small quantities of IFN-γ in response to infection with *M. tuberculosis* (Figures 4.9-4.10). These low levels of IFN-γ have functional relevance in macrophage infections with *M. tuberculosis*, as phagosomal maturation and autophagy in response to *M. tuberculosis* are reduced in macrophages deficient in IFN-γ, and mycobactericidal activity is reduced when IFN-γ signalling is blocked in TLR2/4 deficient macrophages (Figures 4.11-4.14). It can therefore be hypothesised that the defect in killing of intracellular mycobacteria seen in the $Mal^{-/-}$ and $MyD88^{-/-}$, but not in the $TLR2/4^{-/-}$ macrophages, is due to impaired signalling downstream of IFN-γ produced by the macrophages themselves. Macrophages need to be deficient in TLR2, TLR4 and IFN-γ signalling for the deficit in bactericidal activiy to become apparent (Figure 4.14)

Mal$^{-/-}$ mice display increased recruitment of inflammatory cells in response to intra-peritoneal IFN-γ injection

Finally, it was hypothesised that IFN-γ responses *in vivo* would be impaired in Mal$^{-/-}$ mice. This was tested using a model of intra-peritoneal injection of immune modulators (Tynan, Hearnden et al. 2014). A dose-finding experiment identified that pro-inflammatory M1-like macrophages were recruited to the peritoneal cavity in response to 5 μg of rmIFN-γ injected i.p. Unfortunately, this dose was not as effective at inducing pro-inflammatory cell recruitment in WT mice in the subsequent experiment comparing WT and Mal$^{-/-}$ mice. This may be due to variation in strength of the rmIFN-γ between batches. However, in contradiction to the hypothesis that Mal$^{-/-}$ mice would show reduced responses to IFN-γ, increased pro-inflammatory cell recruitment and cytokine production in response to intraperitoneal IFN-γ injection was seen in Mal$^{-/-}$ compared to WT mice. The exceptions to this pattern of increased responses to i.p. IFN-γ in Mal$^{-/-}$ compared to WT mice are 1) peritoneal IL-12p40 secretion in response to i.p IFN-γ and 2) secretion of IL-12p40 by peritoneal exudate cells primed with i.p IFN-γ and restimulated *in vitro*.

It is possible that IFN-γ induced signalling pathways have different kinetics in the absence of Mal, and that a time-course would reveal additional differences. As discussed previously, IFN-γ signals though a large number of pathways including the canonical JAK-STAT pathway. It is difficult to know which signalling pathway (or, more likely, pathways) are responsible for inflammatory cell recruitment and cytokine production and whether these pathways are p38 MAPK dependent. The endpoints selected may not test whether the Mal-dependent p38 MAPK pathway is functional. In the context of increased basal IL-10 in Mal$^{-/-}$ mice, it is intriguing to note that IFN-γ suppresses IL-10 secretion in a p38 MAPK dependent fashion (Hu, Paik et al.): this pathway could potentially be Mal-dependent

explaining the increased levels of IL-10 in the $Mal^{-/-}$ mice It is also interesting to note that pro-inflammatory cell recruitment was actually increased in the $Mal^{-/-}$ mice – this may reflect loss of regulatory negative feedback to IFN-γ signalling in the $Mal^{-/-}$ mice (Cooper, Adams et al. 2002). An additional possible confounder is that microbiota may differ between the $Mal^{-/-}$ and WT mice, particularly as they had been kept in different facilities prior to the experiment (Hooper, Littman et al. 2012).

In summary, results in this chapter demonstrate that Mal participates in IFN-γ signalling via a pathway including IFN-γR1-Mal-MyD88-p38 MAPK and culminating in autophagy and IP-10 , amongst other possible endpoints. The absence of signalling via this pathway, in addition to the TLR2/4 pathways, renders $Mal^{-/-}$ macrophages unable to effectively kill *M. tuberculosis*. The *in vivo* consequences of the defect in this pathway are difficult to evaluate, given the myriad signalling pathways used by IFN-γ, some of which are not Mal-dependent.

Chapter 5 : Mal S180L attenuates IFN-γ signalling

5.1 Introduction

As discussed in the introductory chapter, S180L, a non-synonymous single nucleotide polymorphism (SNP) in Mal with functional consequences, is common in European and Indian populations. Approximately 15-20% of European populations carry the L allele and 2-3% of the population are homozygotes for the L allele (Ferwerda, Alonso et al. 2009). It is less frequent in South East Asian and African populations. S180L results in alteration of a potential binding site leading to steric occlusion (Valkov, Stamp et al. 2011). The S180L SNP has been reported to be associated with altered susceptibility to a number of infectious diseases including invasive *Haemophilus influenzae* infection after vaccination (Ladhani, Davila et al. 2010), severe sepsis (Ferwerda, Alonso et al. 2009, Hamann, Kumpf et al. 2009, Song, Tong et al. 2010), severe pneumococcal disease (Khor, Chapman et al. 2007) and malaria (Khor, Chapman et al. 2007, Zakeri, Pirahmadi et al. 2011). A number of studies have reported an association between the S180L Mal polymorphism and TB susceptibility (Khor, Chapman et al. 2007, Castiblanco, Varela et al. 2008, Selvaraj, Harishankar et al. 2010, Capparelli, De Chiara et al. 2013), although other studies have failed to replicate these findings (Nejentsev, Thye et al. 2008, Dissanayeke, Levin et al. 2009).

D96 is another functional SNP in Mal, which causes reduced binding to MyD88 and marked reduction in TLR4 and TLR2 signalling (Nagpal, Plantinga et al. 2009). About 1% of European populations are heterozygotes for D96N. This SNP is associated with a change in conformation of a putative bindng site (Valkov, Stamp et al. 2011), and may also affect post-translational modification of Mal (Nagpal, Plantinga et al. 2009). It is present at low

frequencies in the populations studied to date, with a heterozygote prevalence of approximately 1% (George, Kubarenko et al. 2010). An association between D96N and TB susceptibility has been reported from China (Zhang, Xue et al. 2011).

Chapters 3 and 4 presented data showing that Mal is required for TLR2 signalling leading to cytokine production but also for IFN-γ signalling leading to phagosome maturation and autophagy in macrophages infected with *M. tuberculosis*. Consequently, the effect of S180L on these pathways was determined. It was hypothesised that the S180L mutation affects IFN-γ signalling, and therefore results in impaired responses to *M. tuberculosis* in macrophages. This chapter describes the effect of the S180L mutation in murine and human macrophages on 1) TLR2 and TLR4 signalling, 2) IFN-γ signalling, 3) antimycobacterial effector functions and 4) *in vivo* responses to *M. tuberculosis* infection.

5.2 Results

5.2.1 The D96N mutation, but not the S180L mutation, results in impaired TNF-α responses to TLR2 and TLR4 ligands in mouse macrophages

Mice with the equivalent of the S180L mutation, S200L, were generated (See Appendix 2). These mice were used for *in vivo* experiments, and macrophages from these mice were also studied. Intially, the effects of the S200L SNP on TLR 2 and TLR 4 signalling were characterised. (Figure 5.1). Macrophages were derived from bone marrow of WT (SS) mice and mice that were homozygous (LL) as well as $Mal^{-/-}$ mice as a control. Macrophages were stimulated with LPS (TLR4 ligand), Pam_3CysK_4 (TLR2/6 ligand), Malp-2 (TLR2/1 ligand) and R848 (TLR7/8 ligand). Supernatants were analysed by ELISA. There was no impairment in TNF-α responses to TLR2 or TLR4 ligands in macrophages from LL mice, in contrast to those from $Mal^{-/-}$ mice, where the expected reduction in responses to LPS, Malp-

2 and to Pam_3CysK_4 at lower concentrations was seen (Figure 5.1A and B). An additional experiment characterised macrophages from heterozygote (SL) mice, in addition to SS and LL mice. Again, no effect of the SNP on responses to LPS was seen (Figure 5.1C).

As an additional control. macrophages from mice with the D96N mutation were studied. As predicted, NN macrophages showed a marked impairment of TNF-α responses to LPS (Figure 5.2) .

5.2.2 The S180L mutation results in impaired TNF-α responses to TLR2 and TLR4 ligands in human macrophages

We then sought to confirm these findings in human macrophages. Genotyping was carried out on buccal swab samples from 75 volunteers using allellic discrimination. Peripheral blood monocyte-derived macrophages were obtained from 3 volunteers of each genotype. These macrophages were stimulated with TLR ligands and supernatants analysed by ELISA. In contrast to the results seen in murine macrophages, monocyte-derived macrophages from human subjects who were heterozygotes and homozygotes for the S180L mutation showed markedly impaired responses to the TLR2 ligand Pam_3CysK_4 and the TLR4 ligand LPS (Figure 5.3).

5.2.3 The S180L mutation results in minimal impairment of IL-1β and TNF-α responses to M. tuberculosis

Next, experiments were carried out to determine the effect of the S200L mutation on cytokine responses to *M. tuberculosis.* In Chapter 3, data were presented showing that this pathway involves TLR2/4-Mal-MyD88. Primary macrophages from the SS, SL and LL mice were infected with *M. tuberculosis* and supernatants analysed by ELISA (Figure 5.4).

Macrophages from LL mice showed a minimal impairment of IL-1β and TNF-α responses to *M. tuberculosis*. IFN-β levels were increased, as seen in $Mal^{-/-}$ macrophages, and this may reflect increased cytosolic escape of the bacteria in cells that have defects in phagosomal processing and killing or a loss of Mal-mediated control of IFN-β secretion (Siednienko, Halle et al. 2010) .

5.2.4 The S200L and D96N mutations result in impaired responses to IFN-γ in murine macrophages.

Given the results shown in Chapter 4 which showed a role for Mal in IFN-γ signalling, further experiments were carried out to determine whether the S200L and S180L mutations affected this pathway. Macrophages from SS, SL and LL mice and homozygotes for the D96N mutation (NN) were stimulated with IFN-γ and analysed by ELISA. IP-10 production was reduced in SL and more markedly in LL macrophages. NN macrophages showed a similar impairment (Figure 5.5).

5.2.5 The S180L and D96N mutations result in impaired responses to IFN-γ in human macrophages.

We again sought to confirm these findings from murine cells in human macrophages. Peripheral blood monocyte-derived macrophages were obtained from 3 volunteers of each S180L genotype. These macrophages were stimulated with IFN-γ and supernatants analysed by ELISA. Heterozygotes and homozygotes for the S180L mutation showed impaired responses to IFN-γ, although small numbers of subjects and a large amount of inter-individual variation meant that this was only statistically significant for individuals homozygous for the mutation (Figure 5.6).

5.2.7 L-variant Mal shows reduced affinity for IFN-γR1

It was hypothesised that the reduced IFN-γ signalling seen with L-variant Mal may result from a decreased affinity for IFN-γR1. Site-directed mutagenesis was used to derive HA-tagged L-variant Mal and this was confirmed by sequencing. A co-immunoprecipitation experiment was then carried out to look at the interaction between IFN-γR1 and S180 or L180 Mal. L180 Mal showed a decreased affinity for IFN-γR1 (Figure 5.7).

5.2.8 Macrophages from SL and LL mice show impaired phagosome maturation and bactericidal activity

It was hypothesised that the impaired IFN-γ signalling seen in macrophages with the S180L mutation would result in impaired phagosome maturation and killing of *M. tuberculosis*, similar to what had been seen in $Mal^{-/-}$ macrophages. Primary murine macrophages from SS, SL and LL mice were infected with FITC-labelled *M. tuberculosis*, stained with Lysotracker and assessed by confocal microscopy (Fig 5.8). Phagosome maturation was impaired in SL and LL macrophages. Macrophages were infected with *M. tuberculosis* and lysed at 3 and 72 hours for determination of bacillary load. SL and LL macrophages showed impaired killing of intracellular bacteria (Figure 5.9).

5.2.9 S180L heterozygotes are protected from weight loss while homozygotes show increased severity of tuberculosis disease

Given the impaired *in vitro* responses to *M. tuberculosis* in LL macrophages, it was hypothesised that LL mice would show increased susceptibility. Human association studies had reported protection for individuals who were heterozygotes for the mutation (SL), and it was also assessed whether this would hold true in a mouse model of infection.

Groups of 8 mice were infected with 500 cfu of aerosolised *M. tuberculosis* H37Rv. Serial weights were measured weekly for each mouse by Kim West. SL mice were protected from weight loss (Figure 5.10).

Mice were sacrificed at 8 weeks and lung homogenates plated out for determination of bacillary burden. There was a small but statistically significant increase in bacterial burden in the LL mice (Figure 5.11). Lungs were also fixed and stained with H&E for microscopy. Lungs from LL mice showed a marked increase in the proportion of lung that was inflamed (Figure 5.12). Finally, lung homogenates were analysed by ELISA. LL mice had increased amounts of TNF-α in lung homogenates (5.13).

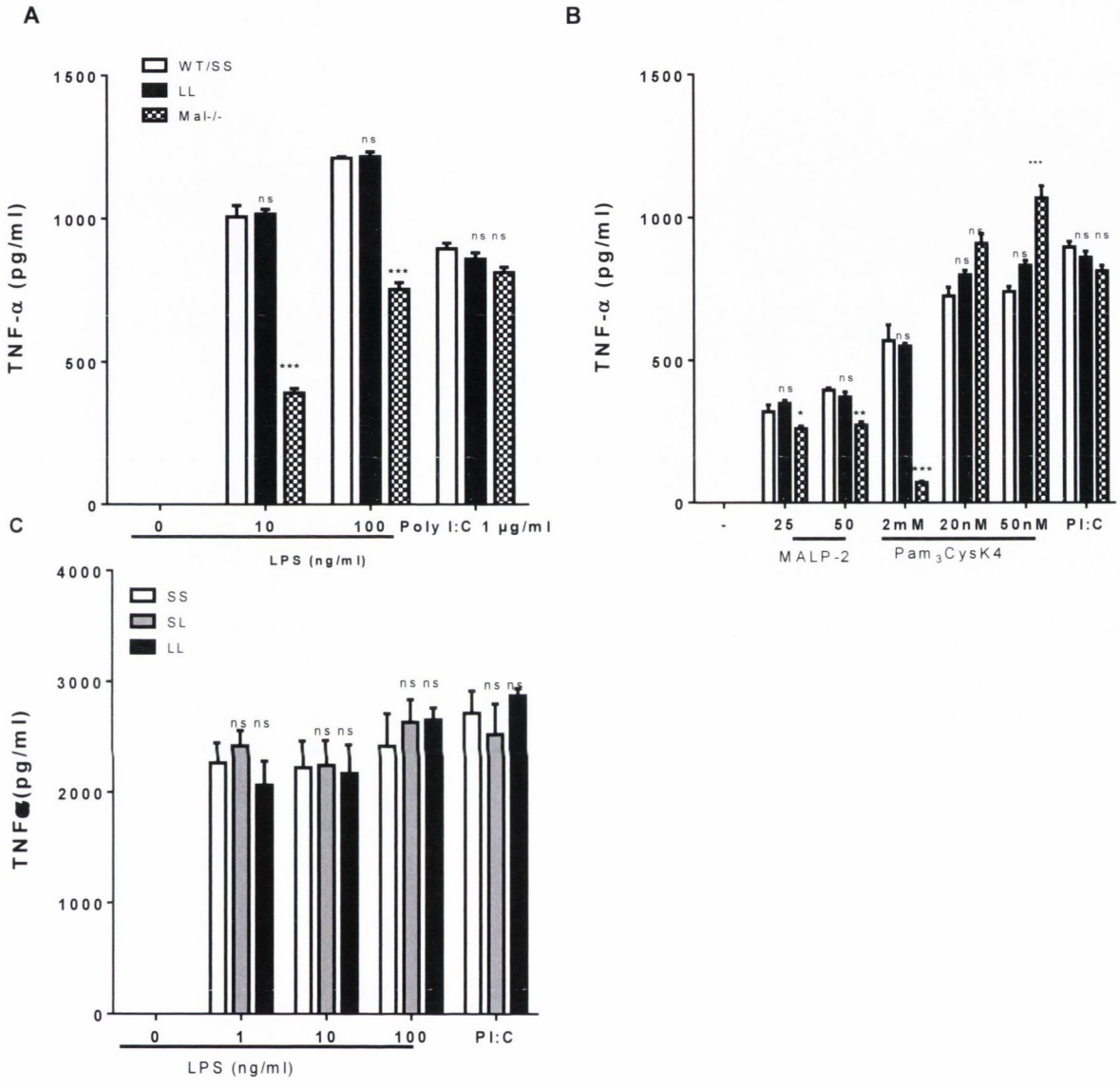


Figure 5-1: TLR2 and TLR4 signalling is not attenuated in murine macrophages with the S200L mutation.

Primary BMM (1 x 10^6 cells/ml) were stimulated with LPS, Pam_3CysK_4, Malp-2 or Poly I:C (1µg/ml) as indicated. Supernatants were harvested after 16 hours and TNF-α concentrations determined by ELISA. Data are means +/- S.D from one experiment representative of at least 3 separate experiments. * $p < 0.05$, **$p<0.01$, *** $p<0.001$, ns not statistically significant (Mann-Whitney)

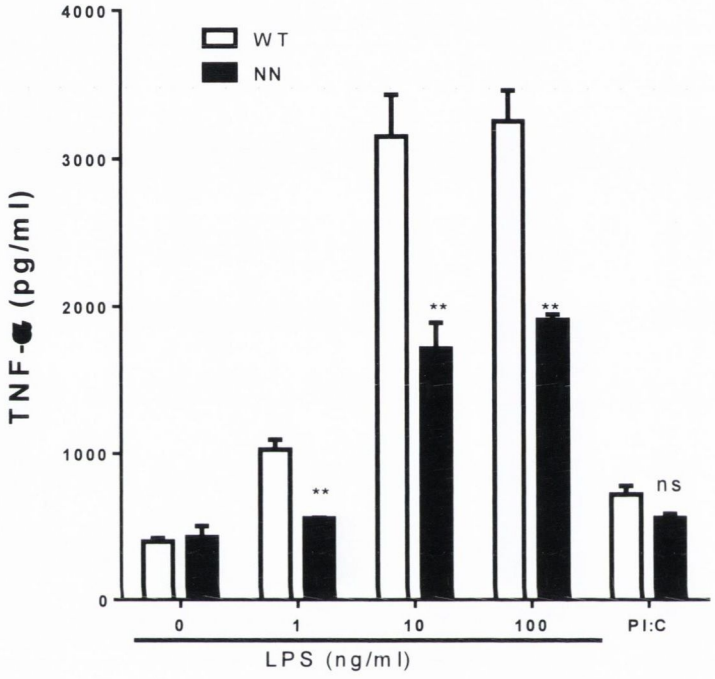


Figure 5-2: TLR signalling is attenuated in NN macrophages

Primary BMM (1 x 10^6 cells/ml) were stimulated with LPS as indicated or Poly I:C (1μg/ml). Supernatants were harvested after 16 hours and TNF-α concentrations determined by ELISA. Data are means +/- S.D from one experiment representative of at least 3 separate experiments. * $p < 0.05$, **$p<0.01$, ns not statistically significant (Mann-Whitney)

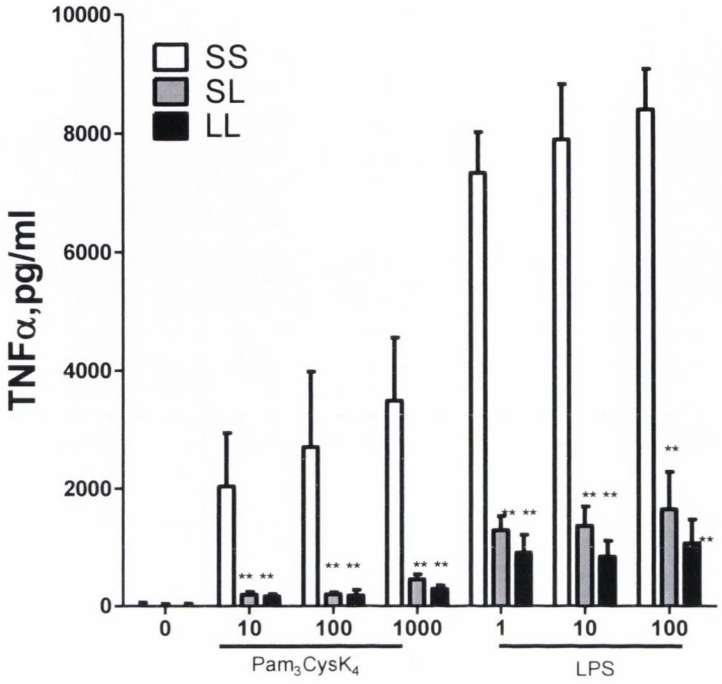


Figure 5-3: TLR 2 and 4 signalling is attenuated in human SL and LL macrophages.

Monocyte-derived macrophages (MDMs) (1 x 10^6 cells/ml) were stimulated with LPS or PamCysK as indicated or Poly I:C (50μg/ml). Supernatants were harvested after 16 hours and TNF-α concentrations determined by ELISA. Data are means +/- S.E.M from one experiment representative of at least 3 separate experiments. Comparisons are to SS macrophages with the same stimulus,* $p < 0.05$, ** $p<0.01$, ns not statistically significant (Mann-Whitney)

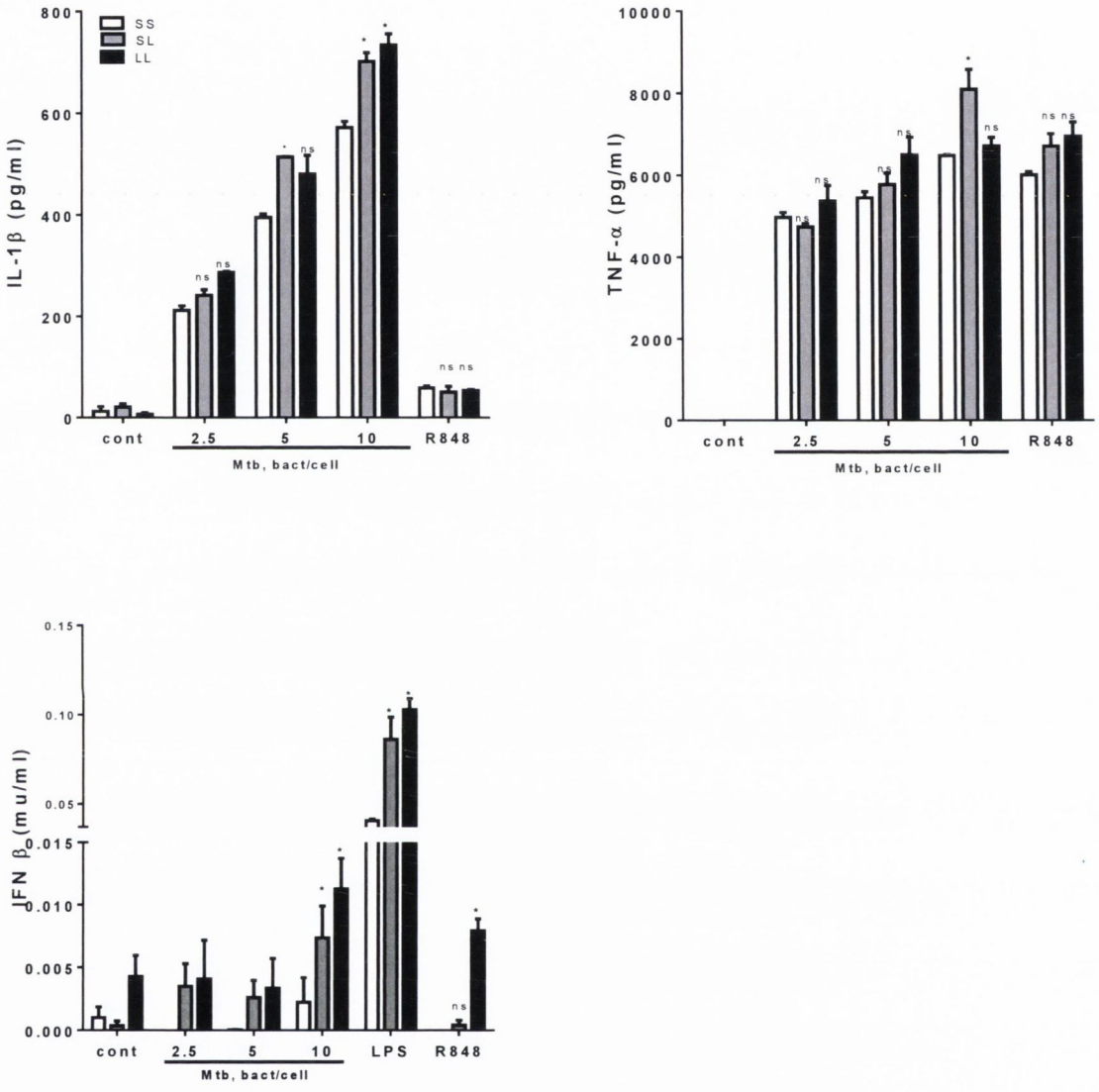


Figure 5-4The S200L SNP in murine macrophages has a minimal effect on TNF-α secretion in response to *M. tuberculosis.*

Primary BMDMs (1 x 10^6 cells/ml) were infected with Mtb H37Rv or stimulated with LPS (100 ng/ml) or R848 (10μg/ml). Supernatants were harvested after 16 hours and cytokine concentrations determined by ELISA. Data are means +/- S.D. from one experiment, representative of at least 3 separate experiments. WT/SS vs SL, WT/SS vs LL,* $p < 0.05$, **$p<0.01$, ns not statistically significant (Mann-Whitney)

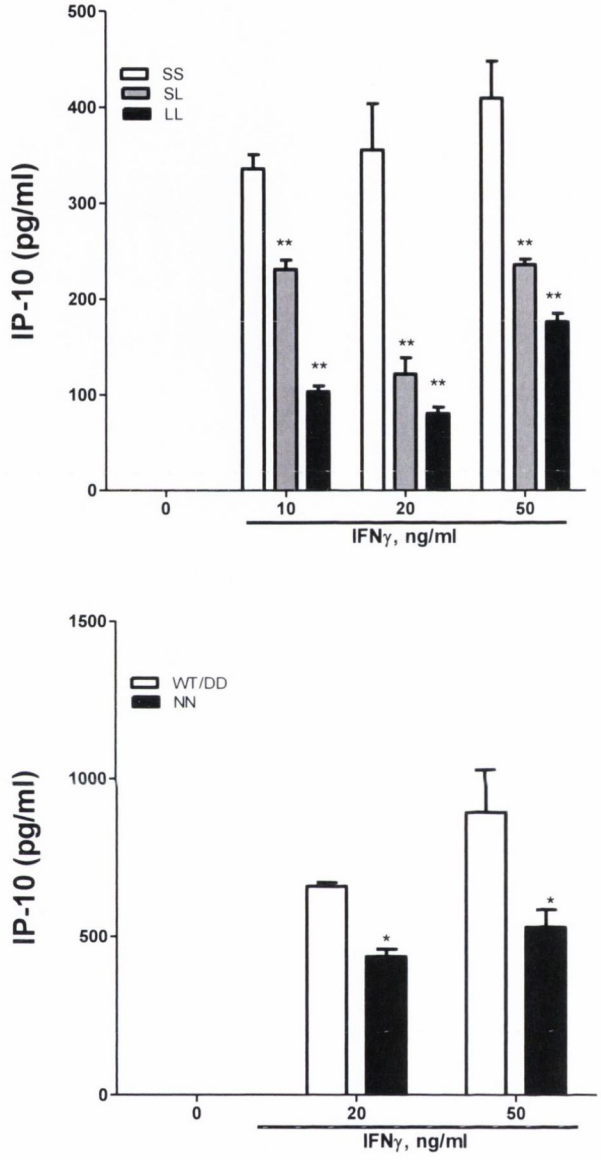


Figure 5-5: The S200L and D96N SNPs impair IP-10 responses to IFN-γ in murine macrophges.

Primary BMDMs (1 x 10^6 cells/ml) were stimulated with rmIFN-γ at concentrations as indicated. Supernatants were harvested after 16 hours and IP-10 concentrations determined by ELISA. Data are means +/- S.D. from one experiment representative of at least 3 separate experiments. * $p < 0.05$, **$p<0.01$, ns not statistically significant (Mann-Whitney)

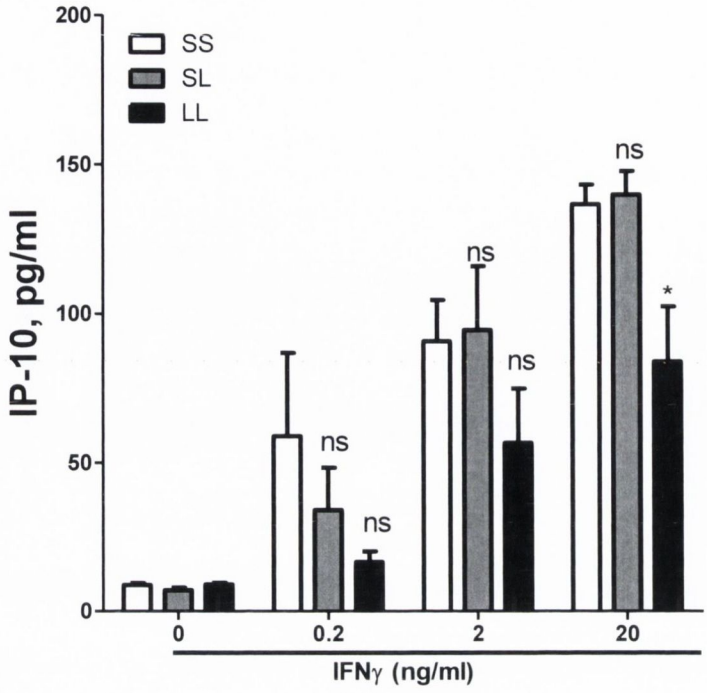


Figure 5-6: IFN-γ signalling is attenuated in human SL and LL macrophages

Monocyte-derived macrophages (MDMs) (1 x 10^6 cells/ml) were stimulated with rhIFN-γ as indicated. Supernatants were harvested after 16 hours and IP-10 concentrations determined by ELISA. Data are means +/- S.E.M from one experiment, representative of at least 3 separate experiments. Comparisons are to SS macrophages with the same stimulus,* $p < 0.05$, ns not statistically significant (Mann-Whitney)

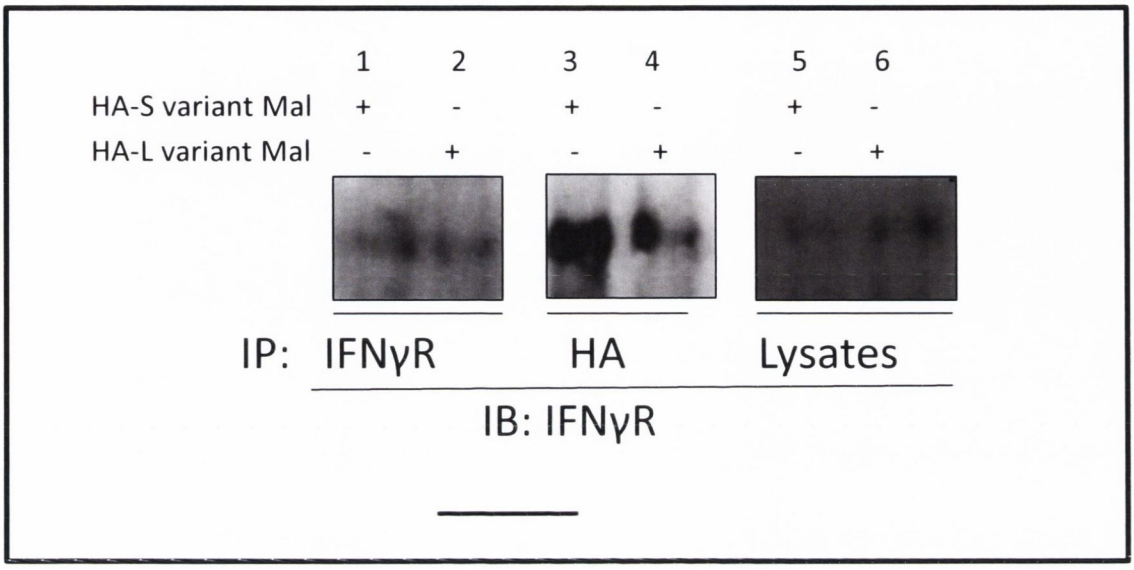
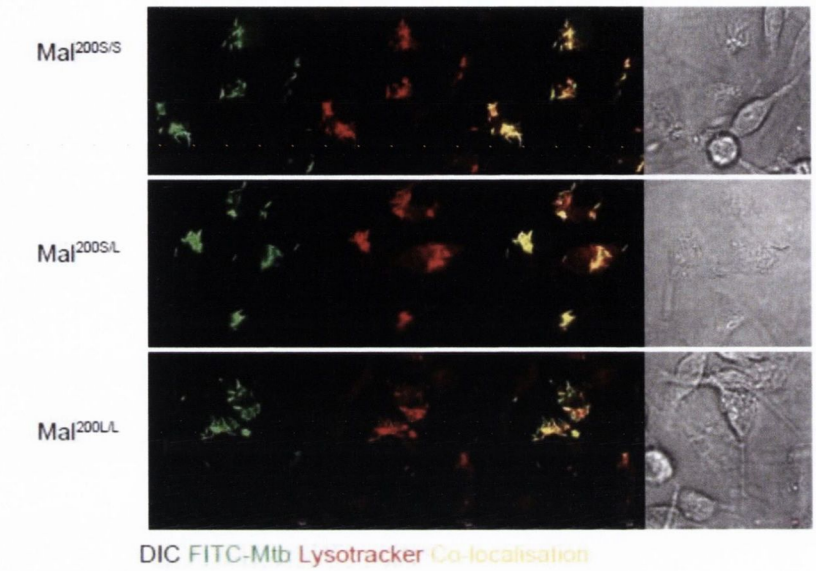


Figure 5-7: L-variant Mal has reduced affinity for IFN-γR1.

HEK-293 cells were transfected with HA-tagged wild-type, HA-tagged S180L variant Mal or HA-tagged empty vector and an immunoprecipitation was performed with antibodies to HA and IFN-γR1 on cell lysates as indicated. Lysates were then blotted with an anti-IFN-γR1 antibody.



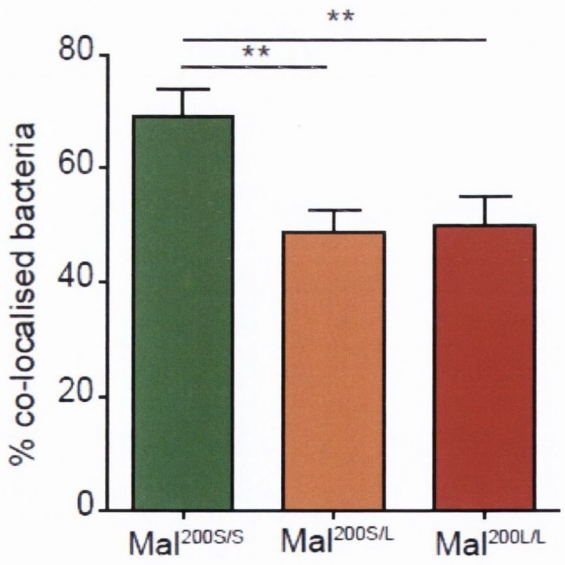


Figure 5-8: The S200L mutation causes reduced phagosome maturation.

Primary murine BMM were infected with FITC-labelled Mtb H37Rv and stained with Lysotracker. Cells were fixed after 2 hours and co-localisation assessed by confocal microscopy. Mean +/- S.D from a single experiment (representative of 3 separate experiment), Comparison to SS, ** $p<0.01$; Mann-Whitney.

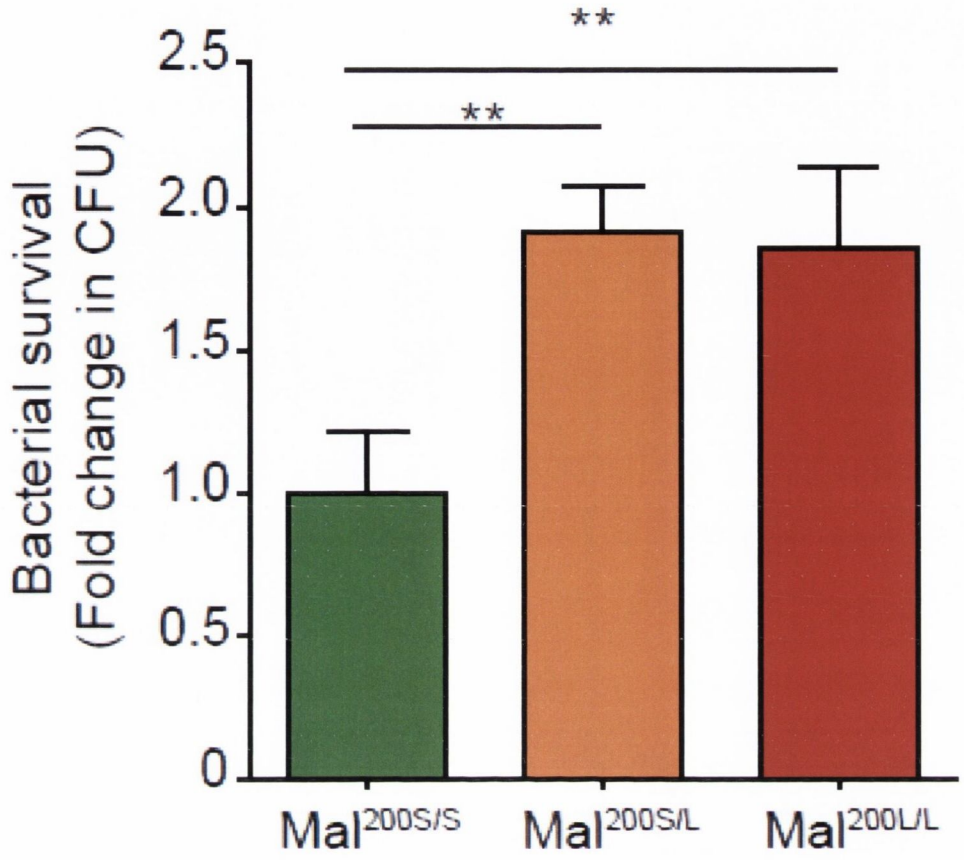


Figure 5-9: The S200L mutation causes reduced mycobactericidal activity.

Primary murine BMM were infected with *M. tuberculosis* () H37Rv and lysed at 72 h. Serial dilutions of lysates were plated out to determine bacterial numbers. (Mean +/- S.D pooled from 3 separate experiments ** $p<0.01$, Mann-Whitney).

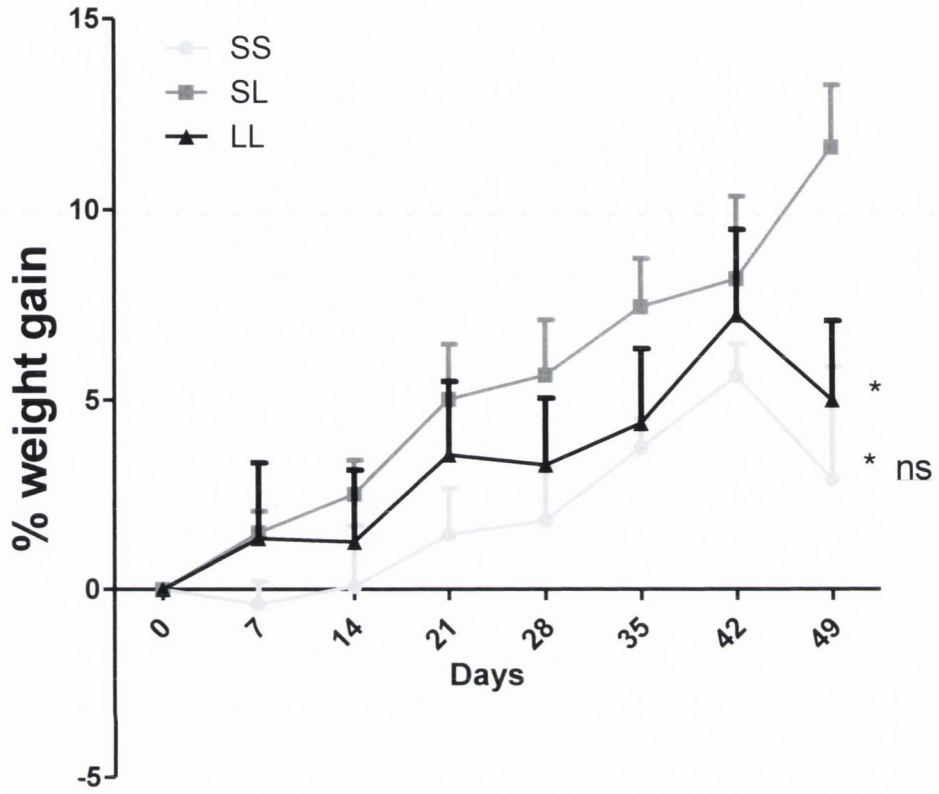


Figure 5-10: *In vivo*, the S200L heterozygote mouse is protected from weight loss

WT (SS), heterozygote (SL) and homozygote (LL) S180L mice were infected with 500 cfu of H37Rv by aerosol. Weights of 8 mice in each group were measured weekly, data shown is the mean +/- S.D. * $p<0.05$, comparison to SL group. ns, SS vs LL.

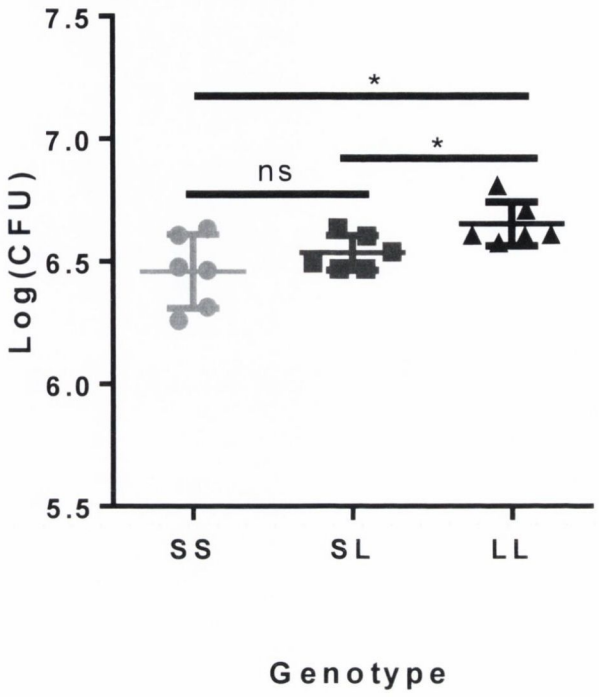
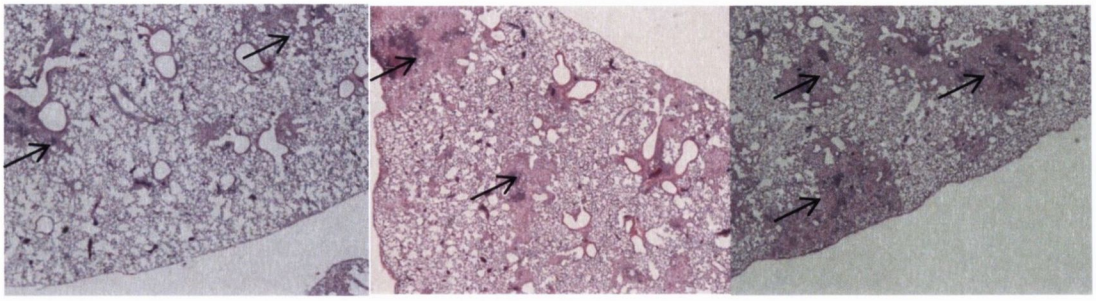


Figure 5-11: *In vivo,* the S200L homozygote mouse has an increased bacterial burden.

WT (SS), heterozygote (SL) and homozygote (LL) S180L mice were infected with 500 cfu of H37Rv by aerosol. Mice were sacrificed at 8 weeks post-infection, and lung homogenates plated for measurement of bacterial burden as described above.



SS SL LL

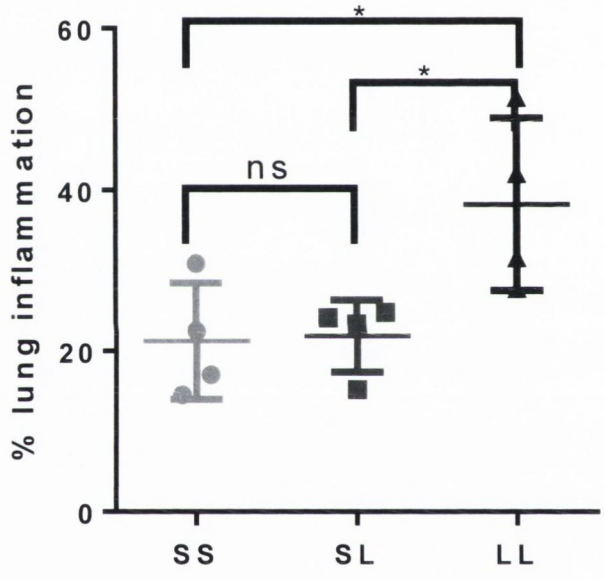


Figure 5-12: S200L homozygote mice infected with Mtb display increased lung inflammation.

WT (SS), heterozygote (SL) and homozygote (LL) S180L mice were infected with 500 cfu of H37Rv by aerosol. Mice were sacrificed at 8 weeks post-infection, and lungs were fixed in formalin, stained with haemotoxylin and eosin and area of inflammation assessed by microscopy. Representative images shown in the upper panel, bottom panel is mean +/- S.D. * $p<0.05$, ns non-significant, Mann-Whitney

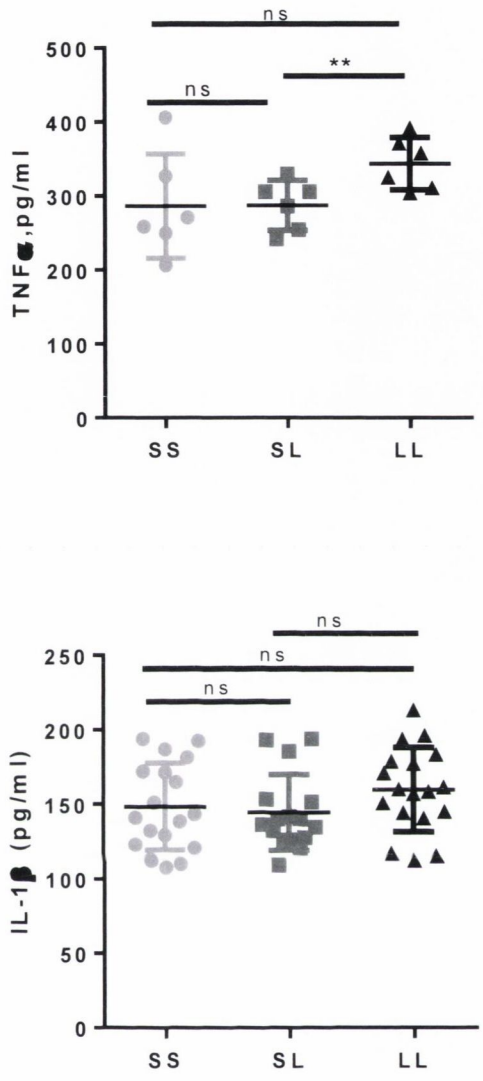


Figure 5-13: S200L homozygote mice infected with Mtb display increased lung inflammation.

WT (SS), heterozygote (SL) and homozygote (LL) S180L mice were infected with 500 cfu of H37Rv by aerosol. Mice were sacrificed at 8 weeks post-infection, and lung homogenates were evaluated by ELISA. Mean+/- S.D. * $p<0.05$, ns non-significant Mann-Whitney

5.3 Discussion

The Mal S180L SNP in humans, and the D96N SNP but not the S200L SNP in mice, affects TLR2 and TLR4 responses

A limited amount of work looking at the effect of the S180L SNP on cell signalling has been carried out, and results to date have been conflicting. Prior to our work, the role of Mal in cell signalling was thought to be solely that of a bridging adaptor between TLRs 2 and 4 and MyD88 (Fitzwater, Caviedes et al. 2010). Hence, studies on the effect of the S180L mutation focussed on TLR2 and 4 signalling. The initial paper to report on the association between the S180L polymorphism and infectious diseases included some functional work using an L180 construct transfected into $Mal^{-/-}$ murine fibroblasts (Khor, Chapman et al. 2007). This paper reported that the L180 variant of Mal was unable to interact with TLR2, and that TLR2 and TLR4 signalling as assessed by NF-κB activation and IL-6 production in response to LPS and Malp-2 was abrogated. A subsequent publication looked at TLR2 and 4 signalling in PBMCs from individuals with SS, SL and LL genotype (Ferwerda, Alonso et al. 2009) and reported no difference in TLR 2 and 4 signalling between PBMCs from individuals with the SS and SL genotypes, but did show an increased response to low doses of TLR2 ligand in PBMCs from a single individual with the LL genotype (Ferwerda, Alonso et al. 2009). Finally, a recent paper reporting an interaction between the Mal S180L SNP and a MyD88 SNP (Capparelli, De Chiara et al. 2013) showed data from *in vitro* stimulation of PBMCs from individuals with the Mal S180L SNP sorted by MyD88 genotype. The authors revealed reduced IFNγ, NO and TNFα production in response to heat-killed *M. tuberculosis* in individuals who were homozygotes for the S180L mutation in two of the MyD88 genotype groups.

In accordance with the papers by Khor and Capparelli, the current study found evidence of an decreased response to TLR2 or TLR4 ligands at a range of concentrations in monocyte-derived macrophages from humans with the SL or LL genotype (Figure 5.3). The report by Ferweda and colleagues of *increased* TLR2 signalling in PBMCs from an individual with the LL genotype is in contrast to these findings. Perhaps the single donor used in that work carried another mutation which affected their responsiveness to TLR2 ligands.

Interestingly, the mouse equivalent of the S180L, S200L, did not affect TLR signalling. Murine Mal differs from human Mal in that it has 20 more amino acids in the N terminus. This may alter the structural effect of the mutation. A recent paper by Kagan *et al* reported another mutation in Mal which selectively impaired responses to the TLR9 ligand CpG but not the TLR4 ligand LPS (Bonham, Orzalli et al. 2014)

The D96N SNP occurs in the same binding region as S180L. It has been reported to impair Mal binding to MyD88, unlike S180L, and to affect TLR2 and 4 signalling (Nagpal, Plantinga et al. 2009, Valkov, Stamp et al. 2011). As a control for S180L, the effect of the D96N SNP on TLR4 responses was assessed in murine macrophages, and, consistent with previous reports, TLR4 signalling was impaired (Fig 5.2). The effect of the D96N mutation on TLR4 signalling was more profound than the S180L mutation, consistent with its more marked impairment of binding to MyD88.

As discussed in Chapter 3, pro-inflammatory cytokine secretion by macrophages *in vitro* in response to *M. tuberculosis* is dependent on a pathway including TLRs 2 and 4 and Mal. It was hypothesised that, given its lack of effect on TLR2 and 4 signalling, the S200L mutation would not impair cytokine responses to *M. tuberculosis*. No significant difference in TNF-α secretion in response to *M. tuberculosis* was detected. However, a small but

statistically significant increase in IL-1β and IFN-β secretion was seen in macrophages with the S200L mutation which may be a consequence of impaired killing of intracellular bacteria.

Given that the effect of the S180L mutation on TLR2 and 4 signalling is seen in human but not mouse macrophages, it is reasonable to hypothesise that the S180L mutation may impair cytokine responses to *M. tuberculosis* in human but not mouse macrophages. However, this was not assessed directly in the current study due to time constaints.

As noted in the discussion in Chapter 3, impairments in cytokine secretion seen *in vitro* with purified cells may differ from responses seen *in vivo*. There is only one report on the effect of S180L on cytokine responses to TLR stimulation *in vivo*. Ferweda *et al* described increased cytokine production in response to intravenous LPS injection in humans with SL genotypes compared to those with SS genotypes (Ferwerda, Alonso et al. 2009). It is unclear why a SNP that reduces TLR4 signalling in macrophages *in vitro* would lead to increased TLR4 signalling *in vivo*. It may well be that the effect of the S180L mutation on TLR2/4 signalling varies according to cell type, and that *in vivo* responses may reflect this. However, given that this paper showed an increase in TLR2 signalling *in vitro* in direct opposition to our findings and that of other groups, there may be additional explanations for the discrepancy.

The S180L SNP in humans and the S200L SNP in mice impair IFN-γR signalling and macrophage anti-mycobacterial effector functions

Given the findings described in chapter 4 of a role for Mal in IFN-γR signalling, it was hypothesised that the S180L SNP may affect responses to IFN-γ. Murine and human macrophages with the L allele showed an impairment in IP-10 production in response to

IFN-γ stimulation which was more profound in homozygotes for the mutation (Figures 5.5 and 5.6). Co-immunoprecipitation showed that L-variant Mal displayed reduced affinity for IFN-γR1, similar to the reduced affinity for TLR2 which had previously been reported (Figure 5.7). The S180L SNP results in steric occlusion of a cavity close to the acidic binding site where the D96N mutation occurs (Valkov, Stamp et al. 2011). Occlusion of this cavity impairs binding to both TLR2 and IFN-γR1 in human macrophages and to IFN-γR1 only in murine macrophages.

The effect of Mal S200L on responses to M. tuberculosis.

Importantly, the selective impairment of IFN-γR1, but not TLR signaling, in S200L macrophages offered us an excellent opportunity to focus specifically on the functional consequences of the impairment in IFN-γR1 signaling associated with the S180L SNP. The reduction in phagosome maturation and bactericidal activity seen in the S200L heterozygote and homozygote macrophages indicate that it is the impairment of the IFN-γR1-Mal-p38 MAPK pathway that underlies the defects in phagosome maturation and bactericidal activity seen in $Mal^{-/-}$ macrophage. This is supported by data showing that phagosome maturation and killing are not TLR2/4 dependent (Chapter 3) but that IFN-γ signalling deficient cells replicate the defect in phagosome maturation seen in $Mal^{-/-}$ and S200L macrophages.

S200L heterozygotes show increased inflammation in vivo

An aerosolised H37Rv infection of C57BL/6 mice was carried out as an *in vivo* model of TB infection. As discussed in the discussion for Chapter 3, the mouse model of tuberculosis has important limitations: it is distant from human exposure in terms of dose and duration of exposure and is poorly predictive of vaccine or drug efficacy. It was hypothesised that LL

mice would show increased susceptibility to tuberculosis, and that heterozygote mice would be protected. Evidence was found for increased susceptibility in LL mice, with increased bacterial burden and an increased inflammatory response. The increased inflammation seen in the homozygote mutant mice may reflect the slight but statistically significant increase in bacterial burden in the homozygote mice, or may reflect a loss of IFN-γ-mediated regulation of neutrophil recruitment and inflammation (Nandi and Behar 2011). The increase in bacterial burden and inflammation is not as marked as the defect seen in mice entirely deficient in IFN-γ (Cooper, Dalton et al. 1993, Flynn, Chan et al. 1993), but could be consistent with a partial defect in IFN-γR signalling.

SL mice did not differ from SS/WT mice in bacterial counts or in inflammatory response in the lungs, however, their weight loss was attenuated compared to SS and LL mice. Cachexia or weight-loss is a cardinal symptom of TB disease, and this is an intriguing finding which may reflect the protection associated with heterozygosity for the SNP in humans.

Interestingly, the increased susceptibility and inflammation seen in the S200L homozygote mice was not replicated in $Mal^{-/-}$ mice (Figure 3.39-3.40). One hypothesis is that the impairment in the IFN-γR/p38MAPK signalling pathway in the absence of impairment in TLR2/4 signalling seen in the S200L mice leads to a different phenotype than that seen when both IFN-γR/p38 MAPK and TLR2/4 signalling are impaired in $Mal^{-/-}$ mice. The increase in susceptibility seen in the S200L mice may be due to increased inflammation caused by preserved function of the IFN-γR/Stat-1 signalling pathway in the absence of function of the IFN-γR/p38MAPK signalling pathway. $Mal^{-/-}$ mice may have a defect in IFN-γ production in response to *M. tuberculosis* infection, as seen in $Mal^{-/-}$ macrophages *in vitro* (Figure 4.9), due to the absence of TLRs 2 and 4 which may mask the imbalance in

IFN-γR signalling pathways. To explore this further, IFN-γ production *in vitro* and *in vivo* in $Mal^{-/-}$, $TLR2/4^{-/-}$ and S200L mice should be measured.

Chapter 6 : General Discussion

This work was commenced in an effort to understand the mechanism underlying the altered susceptibility to TB seen in heterozygotes and homozygotes for the Mal S180L polymorphism. It generated a unified model through which IFN-γ signalling via Mal and p38 MAPK is required for effective macrophage responses to *M. tuberculosis* and in which the S180L polymorphism results in reduced IFN-γ signalling and thereby impairs immunity to Mtb.

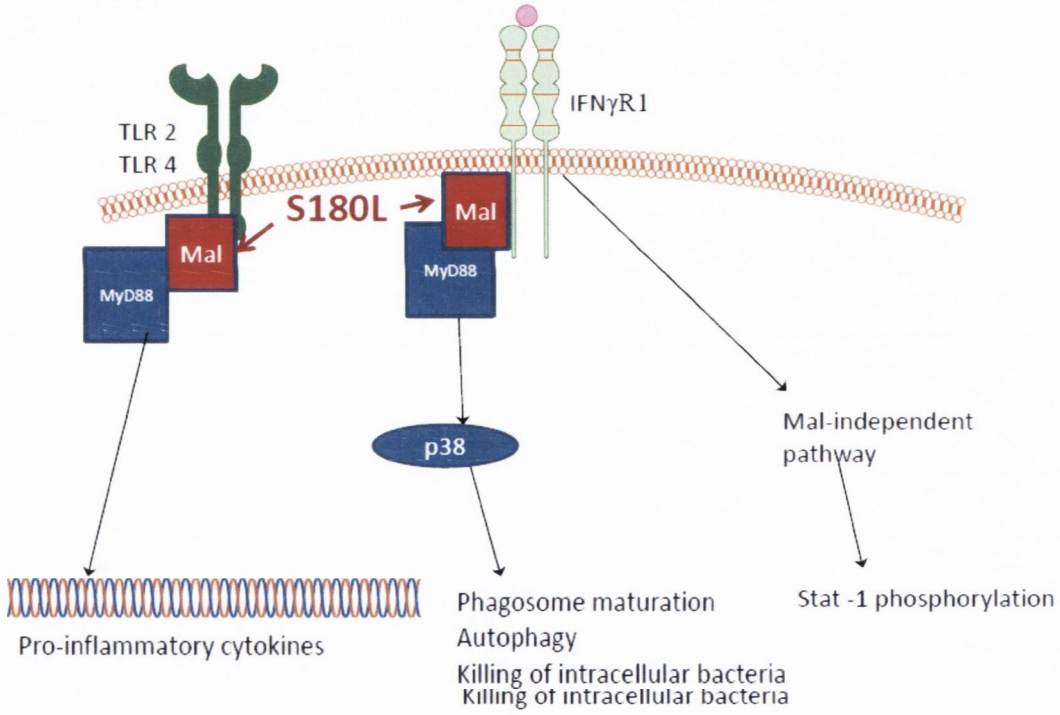


Figure 6-1: Mal is a signalling adaptor protein for TLR2, TLR4 and IFNγR. The S180L mutation in humans affects both of these pathways

Host determinants of infectious diseases

Human immunity has evolved to meet an incredible demand: to protect the individual against a vast array of pathogens while limiting unwarranted collateral damage. Infectious diseases have acted as a huge evolutionary force over millenia, creating an incredibly complex system to recognise and respond appropriately to challenges from pathogens. Recent advances in genetics have led to the theory that marked genetic heterogeneity underlies human disease (McClellan and King). Individual mutations may be incredibly rare, but collectively they may account for a large proprtion of complex human diseases. Individual mutations may provide an advantage in the heterozygote state against one disease, but be deleterious in homozygotes in the form of another disease, as in the case of sickle cell in which heterozygotes for the trait are protected from malaria, but homozygotes develop sickle cell anaemia.

How understanding host susceptibility can yield therapeutic benefits

Human genetic research has already contributed to new therapeutic strategies against infectious diseases. Identification of individuals with rare but severe monogenic disorders of susceptibility to infectious diseases such as Mendelian Susceptibility to Mycobacterial Disease (MSMD) or X-linked agammaglobulinaemia enables clinicians to initiate appropriate preventative strategies and genetic counselling. The finding that individuals with a mutation in the CCR5 receptor were less susceptible to HIV (Huang, Paxton et al. 1996) led to the development of a new class of antiretrovirals, entry inhibitors, such as maraviroc (Esté and Telenti 2007). Research showing that Duffy blood group negative individuals are protected against *P. vivax* has informed vaccine development (Hill 2012).

As described below, genetic typing of tuberculosis meningitis patients could potentially guide immunomodulatory treatment to improve outcomes (Tobin, Roca et al. 2012).

The heterozygote advantage

A number of publications on the effect of the S180L SNP on TB susceptibility in humans have reported a heterozygote advantage with increased susceptibility seen in homozygotes (Khor, Chapman et al. 2007) (Castiblanco, Varela et al. 2008, Selvaraj, Harishankar et al. 2010, Capparelli, De Chiara et al. 2013). Work described in this thesis did not show a heterozygote advantage *in vitro*, but did show heterozygote protection from weight loss in an *in vivo* model. Interestingly, a similar pattern has been reported for another SNP regulating innate immune responses. A polymorphism in leukotriene A4 hydrolase (LTA4H) affects the balance between pro-inflammatory and anti-inflammatory eicosanoids and has also been associated with an optimal outcome in tuberculosis infection for heterozygotes for the SNP with increased susceptibility and severity in homozygotes (Tobin, Vary et al. 2010). Why should heterozygosity for these immunoregulatory polymorphisms be protective whereas homozygosity increases both susceptibility and severity? This effect has been referred to as the "Goldilocks Hypothesis" and is the focus of much interest among the tuberculosis research community.

What mechanism, or more likely mechanisms, could underly the advantage seen with an intermediate inflammatory response? Lalita Ramakrishnan and collagues have looked at the downstream effects of the LTA4H polymorphism and found that it regulated the balance of lipoxin A4 and leukotriene B4, which in turn determined TNF-α production in reponse to mycobacterial infection. A deficiency of TNF-α led to increased numbers of mycobacteria within macrophages followed by necrosis of the macrophages and release of the

mycobacteria into the growth-permissive extracellular milieu.An excess of TNF-α enabled the macrophages to control mycobacterial replication at an early stage, but later they also underwent necrosis and release of mycobacteria (Tobin, Roca et al. 2012). Necrosis in the high TNF-α state was attributed to excess production of mitochondiral ROS (Roca and Ramakrishnan 2013).

Ramakrishnan and colleagues have highlighted the potential of using LTA4H host genotypes to guide choice of treatment for tuberculosis: individuals with tuberculous meningitis with a LTA4 genotype associated with an excessive inflammatory response benefited from adjunctive immunomodulatory steroid treatment, whereas this was harmful in those with a LTA4 genotype associated with an inadequate inflammatory response (Tobin, Roca et al. 2012). It is tempting to speculate that the Mal S180L genotype could also be used to inform treatment decisions for tuberculosis patients in a similar manner. Additional clinical applications of defining the Mal S180L genotype could include stratifying individuals for progression from LTBI to active TB, enabling clinicians to concentrate isoniazid preventative therapy on individuals at highest risk of progression.

Mal S180L: Implications for other infectious diseases

Heterozygosity for Mal has been reported to be associated with protection and homozygosity has been reported to be associated with increased susceptibility or severity to not only mycobacterial disease, but also to other infectious diseases including pneumococcal disease, malaria (Khor, Chapman et al. 2007, Zakeri, Pirahmadi et al. 2011) and Chaga's disease (Ramasawmy, Cunha-Neto et al. 2009). Hitherto, this has been ascribed to the effect of S180L on TLR2 and TLR4 signalling; however, given the results presented in this thesis and reports that IFN-γ signaling also plays important roles (both

protective and pathogenic) in immune responses to streptococci (Rubins and Pomeroy 1997, Rijneveld, Lauw et al. 2002, Nakamatsu, Yamamoto et al. 2007, Sun, Salmon et al. 2007, Matsumura, Ato et al. 2012, Mitchell, Yau et al. 2012), malaria (Favre, Ryffel et al. 1997, McCall and Sauerwein 2010, Hunt, Ball et al. 2014) and Chaga's disease (Laucella, Postan et al. 2004), it is tempting to speculate that the documented role of Mal S180L may be, at least in part, via its regulation of the IFN-γ-Mal-p38 MAPK pathway. Mal S180L has also been shown to offer heterozygote advantage in adaptive immune response to *Haemophilus influenzae* post vaccination (Ladhani, Davila et al. 2010), which again could be mediated by regulation of the IFN-γ-Mal-p38 MAPK pathway in addition to TLR2/4 signalling. The mechanism of intermediate inflammatory advantage in these diseases is not yet understood, and would benefit from further study.

Mal S180L: Implications for non-communicable diseases

IFN-γ has a criticial role in immunity, including promoting differentiation of macrophages to a classically activated phenotype (Martinez and Gordon 2014, Murray, Allen et al. 2014), activating neutrophils (Ellis and Beaman 2004), enhancing MHC class II expression on mononuclear phagocytes and dendritic cells, upregulating NK activity, inducing production of a wide range of cytokines including IL-12 and TNF-α, favouring Th1 over Th2 immunity and controlling cell trafficking. It also has a number of key immunoregulatory function including the induction of Treg cells and inhibition of Th17 cells and has a wide range of other bidirectional immune functions as reviewed by Billiau and Matthys (Kelchtermans, Billiau et al. 2008, Billiau and Matthys 2009). IFN-γ plays a role in a large number of non-infectious diseases ranging from atherogenesis (Schroecksnadel, Frick et al. 2006) and cancer to autoimmune diseases such as systemic lupus erythematosis (SLE).-

SLE is a multisystem autoimmune disease characterised by loss of immunological tolerance to components of the host cell nucleus (Crampton, Morawski et al. 2014). The immunological basis for the disease is not completely understood, but an abundance of type 1 interferons and other inflammatory cytokines including IFN-γ are observed. SLE, particularly severe forms involving the kidneys, is far less common in European populations with a higher prevalence of S180L than in populations from Asia and Africa (Borchers, Naguwa et al. 2010). A study from Columbia reported reduced risk of SLE in heterozygotes for S180L (Castiblanco, Varela et al. 2008), and a recent study from India showed that S180L appears to protect against psychosis associated with SLE. The association of Mal S180L with SLE had been difficult to explain, given the absence of significant evidence of a role for TLR2 or TLR4 in the disease (Kim, Sreih et al. 2009). Castiblanco and colleagues had hypothesised that MalS180L might protect against an infectious trigger of SLE. Intriguingly, IFN-γ has been observed to be involved in pathogenesis, as evidenced by an association between polymorphisms affecting IFN-γ expression and lupus susceptibility (Miyake, Nakashima et al. 2002, Tangwattanachuleeporn, Sodsai et al. 2007, Hirankarn, Tangwattanachuleeporn et al. 2009); studies showing elevated IFN-γ mRNA in SLE patients (Csiszár, Nagy et al. 2000); and mouse models reporting that increased expression of IFN-γ led to an increased risk of developing SLE (Hodge, Berthet et al. , Seery, Carroll et al. 1997, Seery 2000, Pollard, Cauvi et al. 2013) whereas mice deficient in IFN-γ were protected.(Peng, Moslehi et al. 1997). It is tempting to speculate that the protection from SLE seen in S180L heterozygotes may be related to attenuated IFN-γ signalling rather than attenuated TLR2 or TLR4 signalling. Another possibility, given the recent report of Mal involvement in TLR7 and TLR9 signalling in response to natural ligands(Bonham, Orzalli

et al. 2014), and the known role of these TLRs in sensing the nucleic acids thought to play a part in SLE pathogenesis, is that the S180L polymorphism may affect these pathways.

IFN-γ has been reported to play a number of roles in atopic dermatitis, a common chronic skin disease disease which is characterised by a Th2 skew. It has been reported that PBMCs from patients with atopic dermatitis produce reduced amounts of IFN-γ, leading to trials of recombinant IFN-γ as treatment for the disease (Chang and Stevens 2002). However; enhanced apoptosis of keratinocytes in response to IFN-γ has been reported to be a key pathogenic feature of atopic dermaitits (Rebane, Zimmermann et al. 2012), with IFN-γ knockout mice showing protection from the skin thickening characteristic of the disease(Spergel, Mizoguchi et al. 1999). IFN-γ has also been shown to be pathogenic by decreasing expression of enzymes reqiured for production of ultra long-chain ceramines which protect against the disease (Feingold 2014). A decrease in IFN-γ responsiveness could therefore be postulated to protect against atopic dermatitis. Unpublished data on an Irish cohort (Appendix 3) generated by Dr Fred Sheedy (IMM) in collaboration with Dr Alan Irvine shows that carriage of the S180L allele is protective against atopic dermatitis in an Irish cohort, with homozygotes showing greatest protection. Similar data has also been published from a Japanese cohort (An, Matsui et al. 2011).

Mal S180L and cancer

A number of observations have led to the hypothesis that IFN-γ plays a role in anti-tumour surveillance. IFN-γ has been shown to have a number of anti-tumour effects (Ikeda, Old et al. 2002, Brandacher, Winkler et al. 2006, Lippitz 2013). One key mechanism is the up-regulation of MHC Class I antigen presentation on tumour cells, which induces killing of the cancer cells by cytotoxic T-lymphocytes (Weber and Rosenberg 1988). IFN-γ also up-

regulates the expression of a number of chemokines, which attract cytotoxic lymphocytes to the site of tumours (Kunz, Toksoy et al. 1999). In addition, IFN-γ inhibits tumour angiogenesis (Beatty and Paterson 2001), tumour cell growth (Chin, Kitagawa et al. 1996) and promotes apopotic cell death at certain concentrations (reviewed in (Chawla-Sarkar, Lindner et al. 2003)). Mice with defects in the IFN-γ signalling pathway (e.g. IFNGR1 $^{-/-}$ and STAT1$^{-/-}$mice) show enhanced tumour growth in a methylcolanthrene-induced tumour model (Kaplan, Shankaran et al. 1998). Clinical studies have shown an improved prognosis for patients with ovarian tumours with higher levels of IFN-γ expression (Marth, Fiegl et al. 2004). A recent review reported that the IFN-γ-IL-12 axis was protective against many cancers (Lippitz 2013).

However, IFN-γ has also been shown to have a number of pro-tumour effects. In melanoma cells, high expression of IFN-γ was associated with a more aggressive phenotype (Brocker, Zwadlo et al. 1988). The dose of IFN-γ may also be important, with low-dose IFN-γ treatment enhancing resistance to NK cells, and human lymphocytes expressing low levels of IFGR2 showing anti-apoptotic and proliferative responses to IFN-γ, while those expressing high levels of IFNGR2 expressed a pro-apopotic phenotype (Bernabei, Coccia et al. 2001).

Clinical trials of genetically engineered IFN-γ1b (Actimmune) in cancer have had mixed success. A trial of intravesicular IFN-γ found that there was a reduction in recurrence of bladder cancer, although this failed to attain statistical significance in this small trial ($p=0.08$)(Giannopoulos, Constantinides et al. 2003). A phase III clinical trial of adjunctive IFN-γ with chemotherapeutic agents in first-line therapy in ovarian cancer found a statistically significant benefit in progression-free survival with median times to progression of 17 and 48 months in intervention and control groups respectively

(Windbichler, Hausmaninger et al. 2000). An additional non-controlled trial of IFN-γ in recurrent ovarian cancer found that 32% responded to IFN-γ mono-therapy (Pujade-Lauraine, Guastalla et al. 1996). However, a large phase III clinical trial of adjunctive IFN-γ with chemotherapy found decreased survival in those receiving IFN-γ compared to controls (Alberts, Marth et al. 2008).

Given the importance of IFN-γ in cancer pathogenesis, it is tempting to speculate that MalS180L may affect cancer susceptibility. There have been a number of reports linking Mal with cancer. A recent study reported increased incidence of colon cancer in $Mal^{-/-}$ mice in a colitis model, although this was attributed to loss of intestinal epithelial integrity in the $Mal^{-/-}$ mice(Aviello, Corr et al. 2014). Another recent paper reported an increased risk of gastric cancer in individuals with a Mal polymorphism, rs7932766, known to be in linkage disequilibruim with S180L(Hawn, Dunstan et al. 2006, Castaño-Rodríguez, Kaakoush et al. 2014). Examining the role of Mal and Mal polymorphisms in other cancers may be worthwhile.

Other non-communicable diseases in which IFN-γ is thought to play a key role include graft-vs-host host disease (as reviewed recently by Wang and Yang (Wang and Yang 2014)) and pre-eclampsia (Lockwood, Basar et al. , Murphy, Tayade et al. 2009). The role of Mal, and the effect of Mal polymorphisms on these diseases may also warrant further study.

Future Directions

As discussed in Chapter 5, IFN-γ induced p38 MAPK phosphorylation is Mal- and MyD88-dependent, whereas the canonical IFN-γ- Stat-1 signalling pathway is not. IFN-γ induced IP-10 secretion, autophagy, phagosome maturation and bactericidal activity were Mal-dependent, while IFN-γ induced TNF-α secretion, production of reactive nitrogen and oxygen species were found to be Mal-independent. It is not known which IFN-γ signalling pathways lead to endpoints such as macrophage polarisation, lymphocyte proliferation, upregulation of MHC II and induction of Tregs. It is also unclear what the role of the Mal-dependent IFN-γ signalling pathway is in the pathogenesis of SLE, cancer and other non-communicable diseases. What are the consequences of the impairment of the IFNγR-Mal-p38 MAPK pathway in the presence of a functional IFNγR- Stat-1 signalling in macrophages, other immune cells and *in vivo*? Are other IFNγR-signalling pathways Mal-dependent? Future work should include characterising macrophage polarisation, antigen presentation by macrophages and DCs, lymphocyte proliferation and type 1 interferon responses in Mal-/- and S180L cells. Other Mal polymorphisms including D96N should also be further characterised. The effect of S180L and D96N on the endosomal sorting function of Mal also warrants study(Bonham, Orzalli et al. 2014). Further *in vivo* work should be carried out to characterise Mal-dependent read-outs which could be further evaluated in S200L mice, possibly including other infection models (e.g. pneumococcus), cancer models and/or atopic dermatitis models. The findings described in this thesis also provide a rationale for looking for associations betwen Mal polymorphisms and IFN-γ- dependent diseases, including atherogenesis, graft-vs-host disease, cancer and pre-eclampsia. Finally, a deeper understanding of the heterozygote advantage in infectious diseases seen with the

intermediate level of inflammatory response in S180L heterozygotes may yield a paradigm-shift in the treatment of these diseases.

Appendix I: Materials

A1 Solutions for Cell Culture

A1.1 Complete RPMI 1640 medium

Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera) was supplemented with 2 mM L-Glutamine (Gibco) and 8% (v/v) heat-inactivated (56 C for 30 minutes) foetal calf serum (Biosera). Foetal calf serum was filter-sterilised with a 0.22μm syringe-driven filter (Millipore) before adding to medium. 50 units/ml of penicillin (Gibco) and 50μg/ml streptomycin (Gibco) were added to medium, except when cells were being prepared for infection, in which case antibiotic-free medium was used.

A1.2 0.88% Ammonium chloride (NH_4CL) red blood lysis solution

This was made by dissolving 8.8g ammonium chloride in 1 L endotoxin-free water (Baxter). This was filter-sterilised with a 0.22μm syringe-driven filter (Millipore).

A1.3 Pathogen recognition receptor (PRR) agonists used in vitro

Agonist	**Receptor**	**Supplier**
CpG ODN 1826	TLR 9	Oligos etc
Pam3CSK	TLR 1/2	Invivogen
R848	TLR 7/8	Invivogen
LPS from *E. Coli* serotype R515	TLR 4	Enzo LifeSciences
Poly I:C	TLR 3	Invivogen

A1.4: Inhibitors used in vitro

Inhibitor	**Target**	**Supplier**
Bafilomycin	Lysosome acidification	Sigma Aldrich
Mal inhibitor	Mal	Calbiochem

A1.5: Other agents used in cell culture

Inhibitor	Target	Supplier
Phorbal myristate acetate (PMA)	Differentiation of THP-1 cells	**Sigma**
Murine interferon gamma		**Immunotools**
Human interferon gamma		**Immunotools**

A2 Bacterial Culture Materials

A.2.1 Middlebrook medium

7H9 broth (Becton Dickinson) containing 0.05% 0.2% glycerol, 0.5% BSA, 0.2% glucose and 0.085% NaCl. Cultures were incubated at 37 C standing in vented 500ml erlenmeyer flasks.

A2.2 Middlebrook agar

Mycobacteria were grown on solid 7H11 media (Bectin Dickinson) containing glycerol, 0.5% BSA, 0.2% glucose and 0.085% NaCl. Agar plates were sealed in plastic bags and incubated at 37C for 3-4 weeks to obtain colonies

A2.3 Triton-X

Triton-X was diluted to 0.1% in sterile PBS and syringe filtered before use.

A3 Other solutions

A 3.1 Krebs-Ringer Phosphate

3mM Kcl, 140 mM Nacl, 25mM TRIS, 2mM $MgCl_2$, 2mM CaCl. Add 10mM glucose and pH to 7.3 before use

A4 Western blot materials

A4.1 Separating Gel (15 ml)

% Gel	10%	15%
H20	5.9	3.5
30% Acrylamide Mix	5	7.5
1.5 M Tris (pH 8.8)	3.8	3.8
10% SDS	0.15	0.15
10% APS	0.15	0.15
TEMED	0.006	0.006

A4.2 Stacking Gel (5 ml)

H20	3.4
30% Acrylamide Mix	0.83
1.5 M Tris (pH 8.8)	0.63
10% SDS	0.05
10% APS	0.05
TEMED	0.005

A4.3 Lysis buffer

2% Igepal CA630, 1:25 complete EDTA-free protease inhibitors, 2mM PMSF, 2mM EDTA, 150 mM NaCl, 10mM Tris, 0.5% Deoxycholic acid

A4.4 Transfer buffer

25mM Tris base, 192mM Glycine, 15% Methanol

45.5 5x Running Buffer

15g Tris, 72g Glycine, 5gSDS, DH_20 to make 1L

A5.6 Co-IP lysis buffer

50 mM HEPES, pH 7.5, 250 mM NaCl, 20 mM β-glycerophosphate, 1% Nonidet-P40, 2 mM dithiothreitol , protease inhibitor 'cocktail' (Sigma)

A.5.7: Primary antibodies for Western Blotting

Antibody specificity	**Supplier**
LC3	Sigma
Beclin 1	Santa Cruz
BCL 2	Santa Cruz
HA	Invivogen
IFN-γ R	Santa Cruz
B-actin	Sigma

A5.8 Secondary antibodies for Western Blotting

Antibody specificity	**Supplier**
Anti-mouse	Li-Cor

IgG	
Anti-rabbit IgG	Li-Cor

A6 Microscopy

A6.1 Primary antibodies for microscopy

Antibody specificity	**Supplier**
LC3	Invitrogen
CD63	Santa Cruz

A6.2 Secondary antibodies for microscopy

The appropriate anti-mouse, anti-rabbit or anti-goat fluorescent antibodies were used (Alexafluor).

A7 ELISAs

A7.1 : Reagents used in ELISA

Antibody specificity	**Species**	**Supplier**
IL-1α	Mouse	BioLegend
IL-1β	Mouse	R & D Systems
IL-6	Mouse	BD Pharmigen
IL-10	Mouse	BioLegend
IL-12p40	Mouse	BD Pharmigen
IL-17	Mouse	BioLegend
IL-23	Mouse	R & D Systems
IFN-γ	Mouse	BD Pharmigen
IP-10	Mouse	R & D Systems
TNF-α	Mouse	R & D Systems
IL-1β	Human	eBioscience

TNF-α	Human	eBioscience
IP-10	Human	Peprotech
IL-10	Human	BD Biosciences

A7.2 Cytokine ELISA

Antibody specificity	**Streptavidin dilution**	**Streptavidin supplier**	**Blocking solution**
IL-1α	1/1000	Biolegend	1 % BSA
IL-1β	1/180	R & D systems	1 % BSA
IL-6	1/750	Sigma Aldrich	3 % BSA
IL-10	1/1000	Biolegend	1 % BSA
IL-12p40	1/750	Sigma Aldrich	10 % skimmed milk
IL-12p70	1/180	R & D systems	1 % BSA
IL-17	1/1000	Biolegend	1 % BSA
IL-23	1/180	R & D systems	1 % BSA
IFN-γ	1/750	Sigma Aldrich	10 % skimmed milk

A7.3: Solutions for ELISA

Phosphate-buffered Saline (PBS) pH 7.2

137 mM NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 3 mM KCl

Wash Buffer

0.05% v/v Tween 20 in PBS

Blocking solutions (in PBS)

1% Bovine serum albumin (BSA, Fisher)

3% BSA

10% w/w Skimmed milk (Fluka) in PBS

Phosphate Citrate Buffer pH 5 (in dH_20)

5 mM citric acid, 260 mM Na_2HPO_4

Stop solution (in dH_20)

1M H_2SO_4

Carbonate buffer pH 9.5 (in dH_20)

0.1 M $NaHCO_3$

34 mM Na_2CO_3

Developing solutions

2.2 mM O-phenylendiamine dichloride(OPD tablets, Sigma) in phosphate citrate buffer with 0.28µl of H_2O_2 per ml of solution added just prior to use.

1X TMB (Tetramethylbenzidine) ELISA Substrate Solution (eBioscience, UK).

A8 Flow Cytometry

***A8.1:** FACS buffer*

2% v/v FCS and 15 mM sodium azide in PBS

A8.2 FACS antibodies

Marker	Label
CD11b	PECy7
F4/80	PerCP-Cy5.5

Gr-1	APC Cy7
c-kit	FITC
SiglecF	PE
Live/dead	Aqua

A9 Primers

A9.1 Primers for site directed mutagenesis S180-L180

Forward: caccatccccctgctgttgggcctca

Reverse: ctgaggcccaacagcagggggatggtg

Appendix 2: Atopic Dermatitis Data

Allele and genotype frequencies of Mal S180L SNP in Irish patients with atopic dermatitis (n = 227) and healthy Irish individuals (n = 449)

ALLELE FREQUENCIES	**Allele**	**N patients (%)**	**N controls (%)**	**OR (95% C.I.) - 180L as risk allele**	**p value**	**OR (95% C.I.) - 180S as risk allele**	**p value**
	180S	402 (88.5)	753 (83.3)	1.0		1.49 (1.05 – 2.12)	0.02
	180L	52 (11.5)	145 (16.7)	0.67 (0.47 – 0.96)	0.02	1.0	
GENOTYPE FREQUENCIES							
Model	**Genotype**	**N patients (%)**	**N controls (%)**	**OR (95% C.I.) – 180L as risk allele**		**OR (95% C.I.) – 180S as risk allele**	**p value**
Multiplicative	**SS**	179 (78.8)	321 (71.5)	1.0		2.37 (0.74 – 8.46)	0.115
	SL	44 (19.4)	111 (24.7)	0.71 (0.47 – 1.07)	0.088	17 (4.18 – 75.88)	0.000
	LL	4 (1.8)	17 (3.8)	0.42 (0.12 – 1.36)	0.115	1.0	
Dominant	**SS**	179 (78.85)	321 (71.5)	1.0		1.49 (1.0 – 2.21)	0.04
	SL/LL	48 (21.15)	128 (28.5)	0.67 (0.45 – 1.0)	0.004	1.0	
Recessive	**SS/SL**	223 (98.2)	432 (96.2)	1.0		2.19 (0.68 – 7.8)	0.15
	LL	4 (1.8)	17 (3.8)	0.46 (0.13 – 1.46)	0.15	1.0	

Heterozygote Advantage	**SS**	179 (78.8)	321 (71.5)	1.41 (0.93 – 2.13)	0.08		
	SL	44 (19.4)	111 (24.7)	1.0			
	LL	4 (1.8)	17 (3.8)	0.59 (0.16 – 2.02)	0.37		
	SS/LL	183 (80.6)	338 (75.3)	1.37 (0.91 – 2.06)	0.1	1.0	
	SL	44 (19.4)	111 (24.7)	1.0		0.73 (0.5 – 1.1)	0.1

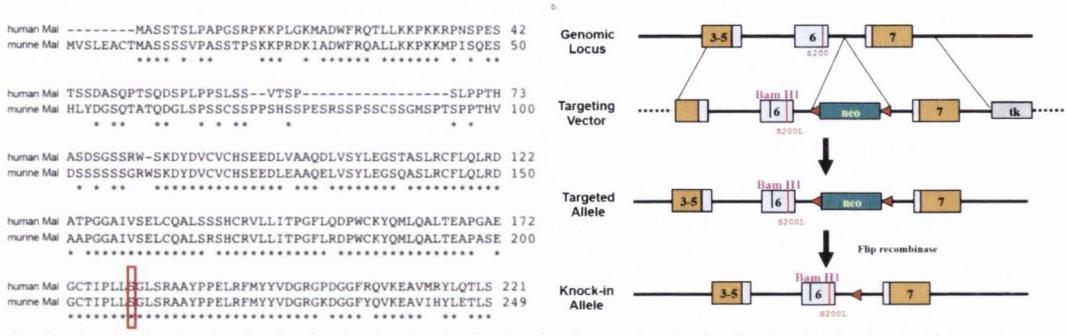
Trend test illustrating association of S allele with disease

Exposure Score	N AD patients	N healthy Controls	ODDS RATIO
0 (LL)	4	17	1.0
1 (SL)	44	111	1.68
2 (SS)	179	321	2.37
P value	0.0267		
Chi squared value for linear trend	4.938		

Appendix 3: List of SNPs in Mal and associated diseases

rs7932766	A186A (diseq with S180L)	H pylori assoc gastric ca
ns8177374	S180L/C539T	TB, malaria, SLE etc.
rs4937114	Promoter G/T	
rs10893493	Promoter A/G	
rs3802813	S55N	
rs8177376	T/G 3′-UTR	
rs625413	C/T 3′-UTR	Colon ca
rs611953	G/A 3′-UTR	Colon ca
Rs8177410	P125H D96N	Dominant negative

Appendix 4: Generation of S200L Mouse



c.

Mal-180:	human	mouse
WT allele	TCG (Ser)	TCC (Ser)
mutant allele	T*T*G (Leu)	*CT*C (Leu)

d.

E6

```
ACTGGTTCAGGCAGGCTCTGTTGAAGAAGCCCAAGAAGATGCCGATCTCCCAGGAAAGCC
ACCTCTATGATGGTTCACAGACAGCCACACAGGATGGTCTCTCACCCTCGAGCTGCAGCT
CACCCCCGAGTCACAGTTCACCGGAGAGCCGTAGCTCACCCTCGAGCTGCAGTTCAGGAA
TGTCACCTACCTCGCCACCAACACACGTGGACAGCAGCAGCAGCAGCAGTGGCCGCTGGA
GCAAAGACTACGATGTCTGCGTGTGCCACAGTGAGGAGGACTTGGAGGCGGCCCAGGAGC
TGGTCTCCTACTTGGAGGGatcCCAGGCCAGTCTACGCTGCTTCCTGCAGCTTCGGGATG
CAGCCCCGGGTGGCGCCATTGTTTCGGAGCTATGCCAGGCACTGAGTCGTAGTCACTGCC
GTGTGCTGCTCATCACTCCAGGCTTCCTTCGGGACCCCTGGTGCAAGTACCAGATGCTGC
AGGCCCTGACGGAGGCCCCGGCGTCGGAGGGTTGCACCATACCCCTGCTGctCGGCCTGT
CCAGAGCCGCCTATCCGCCGGAACTCCGATTCATGTACTATGTGGATGGCAGAGGCAAGG
ACGGAGGCTTTTACCAAGTCAAGGAGGCTGTTATACACT
```

Design and generation of mutant Mal S200L mice, the equivalent mutation of human S180L.

a) Clustal alignment of human and murine Mal protein sequences. Serine 180 in human Mal (top line) and the corresponding amino acidin the murine TIR domain, Serine 200 (bottom line), is highlighted by a red box. b) Cloning strategy for the generation of S200L knock-in mice. Targeting vector was constructed covering a sequence from exon 3-7 of murine Mal. This modified sequenced contained a mutated BamH1 site in exon 6 (for use in confirmation of recombination in ES cells by southern blotting and restriction digestion) as well as the mutated coding sequence for murine Mal at codon 200 (TCC (Serine) -> **CT**C (Leucine) (illustrated in **c.**). It also contained the neomycin resistance gene flanked by 2 Flp sites. After confirming homolgous recombination occured through southern blotting and digestion of fragments, the neomycin cassette was removed by incubation with Flp recombinase. The resulting DNA was sequenced and annotated sequence for exon 6 is shown in **d**. The resulting S200L mice generated were bred with wild-type mice to generate different combintions of the 3 genotypes annotated as $Mal_{200S/S}$ (wild-type, Serine/Serine homozygotes), $Mal_{200S/L}$ (Serine/Leucine heterozygotes) and $Mal_{200L/L}$ (knock-in, Leucine/Leucine homozygotes), for use in experiments. **Experiments performed by Dr Brian Monks, U. Mass**

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