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Host-directed therapy in the treatment of Tuberculosis

Sathyavani Subbarao

Centre for Molecular Bacteriology and Infection, Division of Infectious Diseases,
Department of Medicine, Imperial College London

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Declaration of Originality

I, Sathyavani Subbarao, declare that this work was composed by me and is an account of work undertaken by me. Any assistance or contributions from colleagues are acknowledged in the text, and references to published data or hypotheses of others are cited appropriately.

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Abstract

As *M. tuberculosis* continues to exert itself as the leading cause of mortality attributed to an infectious disease, novel treatment strategies are urgently needed. Host-directed therapy (HDT) is an avenue by which the host immune response can be augmented to drive pathogen clearance and perhaps lessen the tissue damage associated with chronic infection. When administered alongside standard chemotherapy, HDT has the potential to reduce treatment duration and ameliorate organ damage resulting from chronic inflammatory processes. Chapter 1 in this thesis explores innate aspects of the immune response during infection with *M. tuberculosis*. I discuss current HDT strategies, including those in clinical trials. In chapter 2, I examine the melanocortin pathway during mycobacterial infection. Melanocortin is central to many physiological pathways including food intake, obesity, pigmentation and steroid neo-genesis. It has also been implicated as an anti-inflammatory agent. Despite earlier research suggesting an association between melanocortin receptor-4 gene polymorphisms and susceptibility to *M. tuberculosis* I was unable to demonstrate any immunological or anti-microbial benefits using melanocortin receptor agonists, such as melanocyte stimulating hormone. In chapter 3, I review the role of the inflammasome during *M. tuberculosis* infection. The inflammasome is an intracellular multimeric protein comprised of an intracellular sensor (NLR), an adaptor protein called ASC, and pro-caspase-1. Assembly is induced following stimulation by a physiological and pathological stimuli. Assembly of the inflammasome leads to the processing of the pro-inflammatory cytokine, IL-1 β . In this chapter I demonstrate that clinical isolates have a differential ability to activate the inflammasome and induce IL-1 β processing. Using immortalised BMDMs carrying null mutations in individual components of the inflammasome I show that IL-1 β can be processed in the absence of what were previously thought of as critical sensors during *M. tuberculosis* infection. This led me to demonstrate that bacterial replication is reduced in conditions where IL-1 β release is low. I also show that that pharmacological blockade of the inflammasome elicits both anti-inflammatory and anti-microbial activity, and can be used in combination with rifampicin. Interestingly, the anti-microbial effect afforded by inflammasome inhibition varies with the clinical isolate used. These results show inflammasome modulation to be highly attractive for the purposes of HDT.

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Contents

Declaration of Originality	1
Copyright Declaration	1
Abstract	2
Acknowledgements	4
Abbreviations	10
Contents	13
1 Introduction	14
1.1 Introduction	14
1.2 Macrophages: the stalwart of the immune response	16
1.3 Regulators of the macrophage following recognition of <i>M. tuberculosis</i>	17
1.3.1 Toll-like receptors (TLRs)	17
1.3.2 Dectin-1	21
1.3.3 Mincle	21
1.3.4 Nucleotide-binding and oligomerisation domain (NOD) leucine-rich repeat (LRR)-containing proteins (NLRs)	21
1.4 Negative regulators of the macrophage during <i>M. tuberculosis</i> infection	22

1.4.1	C-type lectin receptors (CLRs) and mannose receptors	23
1.4.2	Interleukin-1 receptor associated kinase - M (IRAK-M)	24
1.4.3	Suppressor of cytokine signalling (SOCS) proteins	24
1.5	Host-Directed Therapy (HDT)	25
1.5.1	Enhancing macrophage anti-microbial mechanisms as a potential major target of the innate response	26
1.5.2	Reducing inflammation in order to limit tissue damage	32
1.5.3	Engaging host-directed therapies in TB treatment	36
1.5.4	The research gap - how can additional aspects of the host response to <i>M. tu-</i> <i>berculosis</i> infection be manipulated for therapeutic purposes?	37
2	The melanocortin system	39
2.1	The melanocortin system	39
2.1.1	MCR activation and the signalling response	41
2.1.2	Melanocortins and their effect on infection	44
2.1.3	Pharmacological MSH agonists	47
2.2	Methods	48
2.2.1	Ethics statement	48
2.2.2	Bacterial culture	48
2.2.3	Cell culture	48
2.2.4	Macrophage infection	48
2.2.5	Colony counting	49
2.2.6	Drug treatment	49
2.2.7	Cytokine detection by Enzyme-linked immunosorbant assay (ELISA)	50

2.2.8	<i>In vivo</i> effect of NDP- α -MSH following infection with H37Rv: Early versus Late treatment	50
2.2.9	Preparation of lung and spleen samples for bacterial load	51
2.2.10	Statistical analysis	51
2.3	Results: The melanocortin system as a potential for host-directed therapy	52
2.3.1	Aims	52
2.3.2	Identifying a role for α -MSH as an immunomodulator during <i>in vitro</i> infection with severely attenuated mycobacteria	52
2.3.3	The α -MSH analogue, NDP- α -MSH, does not confer protection to the laboratory mycobacterial strain, H37Rv, <i>in vivo</i>	57
2.4	Discussion	61
3	The inflammasome and its role regulating IL-1β processing	65
3.1	Introduction	65
3.1.1	IL-1 β and the importance of the inflammasome that governs it	66
3.1.2	The canonical activation of the inflammasomes: NLRP1, NLRP3, NLRC4, AIM2 and Pyrin	68
3.1.3	How is <i>M. tuberculosis</i> detected by the inflammasomes?	87
3.1.4	Current research on inflammasome blockers as anti-inflammatory agents . . .	96
3.2	Methods	100
3.2.1	Bacterial culture	100
3.2.2	Resazurin microtiter assay (REMA) assay for detection of the susceptibility of H37Rv to rifampicin and MCC950	100
3.2.3	Cell culture	101
3.2.4	Macrophage infection	101
3.2.5	Drug treatment	101

3.2.6	Colony counting	102
3.2.7	Cytokine detection by Enzyme-linked immunosorbant assay (ELISA)	102
3.2.8	Lactate dehydrogenase assay for cell cytotoxicity	102
3.2.9	Antibodies used for western blotting	102
3.2.10	Initial western blotting protocol	103
3.2.11	Optimised western blotting protocol	103
3.2.12	Statistical analysis	104
3.3	Results: Understanding and characterising the inflammasome during <i>M. tuberculosis</i> infection and evaluating its potential for host-directed therapy	104
3.3.1	Aims	104
3.3.2	Development of assays and determination of conditions necessary for inflammasome activation	105
3.3.3	Higher bacillary loads are able to induce IL-1 β release compared to lower bacillary loads but at the expense of increased cell death	105
3.3.4	Optimisation of Western blotting for infections with <i>M. tuberculosis</i>	108
3.3.5	Clinical isolates vary in their ability to activate the inflammasome and promote IL-1 β processing	111
3.3.6	How do individual inflammasome components contribute to IL-1 β processing?	117
3.3.7	Can the inflammasome be modulated and used as a therapeutic adjunct during infection with <i>M. tuberculosis</i> ?	126
3.4	Discussion	139
3.4.1	Determining the conditions necessary for inflammasome activation	139
3.4.2	Determining phenotypic differences between clinical isolates: bacterial uptake and TNF- α release	142
3.4.3	Strain differences in inflammasome activation and IL-1 β processing and release	144
3.4.4	H37RV, 212, 411 and their differential effect on the inflammasome	146

3.4.5	Inflammasome activation is partially dependent on NLRP3 and AIM2 suggesting the involvement of an additional sensor	147
3.4.6	Genetic and pharmacological inhibition of the inflammasome is associated with reduced bacterial replication	149
3.5	Summary	152
3.6	Future work	154
3.7	Potential clinical applications	156
3.7.1	Inflammasome modulation and the potential role in precision medicine	156

Abbreviations

AAM	Alternatively activated macrophage
ALR	absent in melanoma 2-like receptors
Arg	Arginase
BAL	Bronchoalveolar lavage
Bcl10	B-cell lymphoma 10
BMDM	Bone marrow derived macrophages
CAM	Classically activated macrophage
cAMP	cyclic adenosine monophosphate
CARD	Caspase activation and recruitment domain
CD	Cluster differentiation
CFU	Colony forming unit
cGAS	cyclic-GMP-AMP synthase
COX2	Cyclooxygenase
CREB	cAMP response element-binding protein
DAMP	danger-associated molecular patterns
ESAT-6	early secreted antigenic target protein 6
GBP	Guanylate binding protein
HDT	Host directed therapy
HIV	Human immunodeficiency virus

HO-1	Haemoxygenase 1
I κ B	Inhibitory κ B
IFN- γ	Interferon gamma
IL-10	Interleukin-10
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinases
IRF	Interferon regulatory factors
ITAM	immunoreceptor tyrosine-based activation motif
JAK	Janus Kinases
JUN	JUN N-terminal kinase
LM	Lipomannan
LPS	Lipopolysaccharide
LRR	leucine-rich repeats
LTA ₄ h	LTA ₄ hydrolase
LTB ₄	Leukotriene B ₄
MALT1	Mucosa associated lymphoid tissue lymphoma translocation 1
ManLam	mannose-capped lipoarabinomannan
MAPK	mitogen activated protein kinases
MCR	Melanocortin receptor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MR	Mannose receptor
MSH	Melanocyte stimulating hormone

MyD88	Myeloid differentiation primary response gene 88
NBD	nucleotide-binding-and-oligomerisation domain
NFκB	nuclear factor κB
NLR	NOD-leucine-rich repeat (LRR)-containing proteins
NO	Nitric oxide
NOD2	nucleotide-binding oligomerization domain containing protein 2
PAMP	pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PET-CT	Positron emission tomography and computerised tomography
PGE	Prostaglandins
PIMs	phosphatidyl- <i>myo</i> -inositol mannosides
POMC	pro-opiomelanocortin
PPAR-γ	peroxisome proliferator-associated receptor
PRRs	pattern recognition receptors
RIP2	receptor interacting protein 2
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
SNP	single nucleotide polymorphisms
SOCS	Suppressor of cytokine signalling
STAT	signal transducer and activation of transcription
STING	Stimulator of interferon genes
TAB	TAK1-binding protein
TAK	TGF-β activated kinase
TB	Tuberculosis
TDM	trehalose dimycolate

TGF- β	Transforming growth factor β
TIR	Toll/IL-1 receptor domain
TIRAP	TIR associated protein
TLR	Toll like receptor
TNF- α	Tumour necrosis factor-alpha
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain containing adaptor protein inducing interferons

Chapter 1

Introduction

1.1 Introduction

Although significant strides have been made in reducing the incidence of infection due to *Mycobacterium tuberculosis*, it still remains a considerable cause of mortality, with the developing world in particular being a victim of its destruction. The oft-quoted statistic that one third of the world's population is latently infected with *M. tuberculosis* (WHO 2016) is likely to be an underestimate due to the difficulty in formal quantification (Esmail et al. 2014). Current figures estimate that the incidence of disease in 2015 was 10.4 million and the annual global mortality was 1.8 million, enabling *M. tuberculosis* to be ranked alongside Human Immunodeficiency Virus (HIV) as the leading cause of mortality attributable to a single infectious disease (WHO 2016).

Progress has been impeded by the HIV epidemic and the dramatic rise of drug-resistant *M. tuberculosis* (Maartens et al. 2015), but there are several other factors that contribute to the development of disease. To some extent, the inability to curtail tuberculosis (TB) and anti-microbial resistance is a man-made phenomenon due to lack of infrastructure so desperately needed for effective control (Yates et al. 2016). However, this does not address the fact that *M. tuberculosis* is an extraordinarily successful pathogen. Firstly, it emerged prior to the neolithic period and flourished as population increase provided an expanded reservoir for transmission (Comas et al. 2013). Secondly, it has an intrinsic capacity to use the host environment to alter its metabolic state and modulate the host immune response (Novikov et al. 2011) allowing it to reside within antigen-presenting cells where it can bide its time until an opportunistic moment arises. Thus, most notified cases are as a result of reactivation rather than new infection itself (Gengenbacher et al. 2012).

Treatment for *M. tuberculosis* disease is given for a minimum of 6 months (Swaminathan et al. 2010),

with longer therapies potentially employed for extra-pulmonary TB, and even longer regimens required for drug-resistant cases. The prolonged duration is largely believed to be due to mycobacterial metabolism (Connolly et al. 2007). Anti-microbials target actively replicating bacteria, but the intracellular population is composed of a mixed phenotype (Connolly et al. 2007). Therefore, prolonged therapy is needed to eradicate the bacteria that transiently switch from a slowly replicating state into an actively replicating state (Gomez et al. 2004). However, this lengthy duration lends itself to non-compliance, not least because the regime is cumbersome, has side effects and is associated with drug interactions, most of which are exacerbated in vulnerable individuals (Khan et al. 2010). Also, there is no current accurate and widely available readout that completion of treatment correlates with total eradication of bacteria — as in a recent study which showed that patients who were culture negative after treatment for fully sensitive TB still exhibited bacterial mRNA in their bronchioalveolar lavage (BAL) sample and displayed inflammatory changes as shown by positron emission tomography and computerised tomography (PET-CT) (Malherbe et al. 2016). Moreover, the micro-environment within which the bacilli reside (induced by the host and the bacteria), is highly variable and may shield bacteria from the circulation making access difficult for drugs (Dartois 2014).

Thus, the complex interaction between the host and the bacteria necessitates prolonged therapy, which increases the risk of bacteria acquiring drug resistance (Connolly et al. 2007) and drug tolerance (Gomez et al. 2004). The host immune response also favours an environment which allows bacteria to survive and is arguably not only counterproductive to bacterial killing but may also permit excessive tissue damage (Wallis et al. 2015).

Thus, the concept of host-directed therapy (HDT) — where the host immune response to *M. tuberculosis* may be augmented, impairing bacterial survival — is becoming increasingly attractive as an adjunct to standard *M. tuberculosis* treatment. My PhD has explored various aspects of HDT, initially examining the role of the melanocortin system and thereafter looking at the inflammasome during *M. tuberculosis* infection.

In this chapter, I review macrophage phenotypes and some of the main pattern recognition receptors involved during *M. tuberculosis* recognition and I present a brief overview of current HDTs. In the following chapters, I introduce the melanocortin system and the inflammasome with some quite varying results between the two in my experimental system.

1.2 Macrophages: the stalwart of the immune response

Macrophages are one of the principle lines of defence following *M. tuberculosis* invasion; they are responsible for the acquired and innate immune response yet they also provide the environment for bacterial replication and persistence during all stages of *M. tuberculosis* infection (Rajaram et al. 2014). Macrophages derive from peripheral blood mononuclear cells (PBMCs) and can migrate into the tissue to replace long-lived resident macrophages or arrive in response to a stimulus. They represent the most common cell type within the granuloma, which forms shortly after the initial entry, and since *M. tuberculosis* is contained within the granulomatous structure, it has the consequence of being the reservoir of 'latent *M. tuberculosis*' in some 2 billion individuals world-wide (Dorhoi et al. 2016).

Macrophages have traditionally been classified into a pro- ('classically activated') or anti-inflammatory phenotype ('alternatively' activated), which aims to control infection without damaging the alveolar environment (Hussell et al. 2014). However, it is likely that macrophage function exists as a spectrum rather than a binary phenotype (O'Garra et al. 2013). An appropriate balance of the two responses helps to result in swift bacterial eradication - for example, ineffective inflammation may impair macrophage activation and microbiocidal reactions. Equally, excessive inflammation may recruit cells and cell death that might propagate infection (Dorhoi et al. 2016). Ultimately, an imbalance in the immune responses that are primarily aimed at pathogen eradication can lead to bacterial persistence and continuous activation of pathways that result in tissue damage, fibrosis and impaired lung function in the long term (Hawn et al. 2013).

Whilst human studies represent the ideal in terms of the pathological processes encompassing *M. tuberculosis*, the use of monocyte/macrophage cell lines have contributed to our knowledge of intracellular disease. Often the results from studies using macrophage cells lines (or for that matter any cell line) are extrapolated to the source of interest; this is necessary in situations where lack of expertise prevents using the relevant cells, and the convenience and limited expense of using immortalised cells often outweighs the downsides. Thus it is important to understand how the phenotypic characteristics of the cells used may vary. However, there are few studies comparing the phenotype of different primary cells and immortalised cell lines in their resting and activated states. In experimental systems, resting murine primary bone marrow-derived macrophages (BMDMs) expressed higher levels of IL-10 and TGF- β compared to peritoneal macrophages and splenic macrophages, suggesting a tilt towards the alternatively activated state (Wang et al. 2013). However, peritoneal macrophages are themselves obtained by injecting thioglycolate into the peritoneum, which acts as an irritant to induce the inflammatory response; therefore these macrophages can be similar to classically activated types (Layoun et al. 2015). In a different study, Berghaus et al. 2010 aimed to look specifically at the

characteristics of primary BMDMs and the immortalised cell line RAW 264.7 following activation. Cells were treated with LPS, Pam3CSK4 and poly I:C (microbial ligands for Toll-like receptors 4, 1/2 and 3 respectively). Both cell lines exhibited similar expression of cell surface markers and both responded to LPS incubation with equivalent levels of TNF- α suggesting that the TLR4–ligand interaction is similar. Moreover, both cell types expressed similarly high levels of the cell surface marker CD11b, which has also been reported in peritoneal macrophages (Zhang et al. 2008). CD11b pairs with CD18 to form a transmembrane receptor (CD11b/CD18) known as complement receptor 3 (CR3) (Ling et al. 2014). The CD11b–ligand interaction mediates complement-coated particle uptake and removal of cellular debris without triggering other inflammatory pathways (Fossati-Jimack et al. 2013). However, CD11b has frequently been reported at low levels in naïve alveolar macrophages (Hussell et al. 2014; Zhang et al. 2008) suggesting functional differences between inactivated alveolar macrophages and BMDMs/RAW 264.7 cells and thus experiments using these cell lines to ascertain alveolar macrophage function should be interpreted with caution.

1.3 Regulators of the macrophage following recognition of *M. tuberculosis*

Recognition of mycobacterial lipoproteins, glycolipids and carbohydrates is mediated by a number of cell surface receptor families situated on the alveolar macrophage surface or inside the cytosol. Ligand recognition usually leads to a pro-inflammatory response (see figure 1.2). In this section, I particularly focus on TLRs and NLRs as these are primarily involved in signalling during the inflammasome activation, which is the focus of my third chapter.

1.3.1 Toll-like receptors (TLRs)

TLRs are a conserved group of pattern recognition receptors, of which 12 members have been identified in mammalian (10 human and 12 murine) species (Kleinnijenhuis et al. 2011). TLR2 and TLR4 (cell surface receptors) and TLR9 (intra-vacuolar) are well known to be involved during *M. tuberculosis* infection. However, single-nucleotide polymorphisms (SNPs) within the *TLR8* gene that confer susceptibility to *M. tuberculosis* infection as well as raised levels of the gene transcript during active disease provide some evidence that TLR8 plays an additional role in *M. tuberculosis* infection (Davila et al. 2008). Mycobacterial cell wall glycolipids such as mannose-capped lipoarabinomannan (LAM), lipomannan (LM), phosphatidyl-*myo*-inositol mannosides (PIMs), trehalose dimycolate (TDM), the 19kDa mycobacterial lipoprotein (LpqH) and the 38kDa lipoprotein (LprG) are TLR2 agonists (Kleinnijenhuis et al. 2011; Basu et al. 2012). TLR4 binds the mycobacterial heat shock protein

(HSP) 60/65 which is a protein secreted by a variety of *M. tuberculosis* isolates. TLR9 is able to recognise unmethylated CpG motifs within bacterial DNA (Kleinnijenhuis et al. 2011, see figure 1.1).

To a large degree, the vast repertoire of ligands that the TLRs are able to bind is enabled by their ability to heterodimerise with each other; for example, the cytoplasmic tail of TLR2 is able to form a functional pair with either TLR6 or with TLR1 (Basu et al. 2012). TLR2–TLR6 dimers are required for responses to diacylated lipoproteins while TLR2–TLR1 dimers recognise triacylated lipoproteins (Ozinsky et al. 2000). Upon ligand–TLR interactions, the formation of the TLR dimeric complex allows a conformational change that is needed in order to recruit a variety of adaptor proteins via interaction with the cytoplasmic TIR domain. This leads to the activation of signalling pathways that ultimately activate transcription factors and gene expression. Several adaptor proteins exist and include the Toll/interleukin-1 receptor (TIR) domain-containing proteins, myeloid differentiation primary response 88 (MyD88), Toll/interleukin-1 receptor domain adaptor protein (TIRAP), MyD88 adaptor-like protein (Mal), TIR domain-containing adapter-inducing interferon- β (TRIF) and TRIF-associated membrane protein (TRAM) (Kawasaki et al. 2014). However, since most TLRs, apart from TLR3, and IL-1 cytokines elicit a signal via MyD88, and especially with regards to *M. tuberculosis* infection, downstream TLR signalling can be broadly divided into MyD88-dependent and MyD88-independent pathways.

After the TLR–ligand interaction occurs, MyD88 forms a complex with a family of kinases known as the IL-1R–associated kinase (IRAK) family (Deguine et al. 2014), primarily with IRAK1 and IRAK4. IRAK4 activates IRAK1 through phosphorylation and both kinases leave the complex (Kawasaki et al. 2014). IRAK1 activation induces the activation of TNF receptor-associated factor (TRAF) 6 (Kawasaki et al. 2014). TRAF6 acts as a ubiquitin protein ligase (E3) which, together with the ubiquitination enzyme complex (also known as E2), catalyses the polyubiquitination of TRAF6 itself and another family, TGF- β -activated protein kinase (TAK), which ultimately drives the activation of TAK1 (Mogensen 2009). TAK1 activation enables signalling through two different pathways. Firstly, TAK1 couples with the IKK complex (composed of the catalytic subunits, IKK- α and IKK- β) and the regulatory subunit NEMO, leading to the activation and nuclear translocation of NF- κ B (Mogensen 2009). In addition, TAK1 also activates members of the MAPK family which induce the activation of another transcription factor, activator protein 1 (AP-1) (Mogensen 2009). The mycobacterial ligand determines whether the pathways are primarily pro-inflammatory or not. For example, the LpqH–TL2 interaction leads to the transcription of TNF- α , IL-1 β and IL-12 (Kleinnijenhuis et al. 2011; Mortaz et al. 2015) and induces the up-regulation of the vitamin D receptor which contributes to microbial killing (Liu et al. 2006). However, LprA, LprG, LAM, LM and PIMs which also bind to TLR2 lead to the production of IL-10, IL-4 and TGF- β (Rajaram et al. 2014) which dampen inflammation and may alter the replication of *M. tuberculosis*. Thus in some senses, with its repertoire of ligands and

modulation of receptors, *M. tuberculosis* has the ability to induce either a classical or alternative macrophage response, or even both, which may help its survival capabilities.

TLR signalling can also serve to dampen inflammation. Aside from the MyD88 pathway, the TLR4–ligand interaction may mediate a MyD88-independent signalling cascade, through the interaction between another adaptor protein, TRIF, and either TRAF6 or TRAF3. Recruitment of either TRAF3 or TRAF6 leads to contrasting downstream cascades; the interaction with TRAF6 activates TAK1 and thus the induction of the MAPK and NF- κ B pathways, whereas the interaction with TRAF3 recruits the IKK-related kinases, TBK1 and IKKi, which phosphorylate and activate IRF3, which induces the expression of type I IFN genes. However TRAM and TRIF may induce the transcription of type I IFNs in the TLR4 signaling pathway (Kawai et al. 2007). Therefore, MyD88 is responsible for induction of pro-inflammatory cytokines and the TRIF/TRAM pathways induce the type 1 interferons. Furthermore, ESAT-6 expressed by *M. tuberculosis* can bind TLR2 and inhibit the formation of the complex between MyD88 and IRAK, therefore preventing NF- κ B activation (Pathak et al. 2007). There are other ways in which the pro-inflammatory pathways are controlled: previous studies have shown that Toll/IL-1 receptor 8 (SIGIRR/TIR8), which is a member of the TIR family, can negatively modulate TLR4-mediated signalling through inhibition of the NF- κ B pathway and that SIGIRR gene silencing leads to a heightened inflammatory response (Li et al. 2015). Dampening excessive inflammation may favour host survival; in a study by Garlanda et al. 2007, the authors showed that *Tir8*^{-/-} mice showed increased numbers of neutrophils and macrophages following *M. tuberculosis* infection, and whilst the numbers of bacteria were relatively well controlled, there was an increased mortality in these mice and exaggerated hepatic necrosis.

However, how critical the individual TLRs are in *M. tuberculosis* host defense remains uncertain. Previous studies have shown conflicting responses in the roles attributed to the TLRs. Some reports have shown that, following infection with low dose aerosolised bacteria, TLR2 KO mice (Drennan et al. 2004) and TLR4 KO mice (Abel et al. 2002) have defective bacterial clearance, chronic pneumonia and succumb to infection, although TLR2 KO mice do survive for 120 days post-infection. Similarly, mycobacterial DNA can induce pro-inflammatory cytokines via TLR9 and — in line with studies showing synergy between different TLRs (Kleinnijenhuis et al. 2011) — a previous study has shown cooperation between TLR2 and TLR9 to enhance phagocytosis and bacterial killing (Bafica et al. 2005). In contrast, bacteria recovered from the lungs of TLR2 KO mice showed no major differences colony-forming units (CFUs) at 12 weeks compared to wild-type (Reiling et al. 2002; Sugawara et al. 2003). Moreover, the numbers of CD4⁺, CD8⁺, activated CD4⁺ and compact granulomas were similar between wild-type, TLR2 KO and TLR4 KO mice suggesting that adaptive immunity is not significantly altered in the absence of the key TLRs implicated in *M. tuberculosis* infection (Reiling et al. 2002). Similarly, the absence of TLR6 did not affect bacterial counts at any time-point after

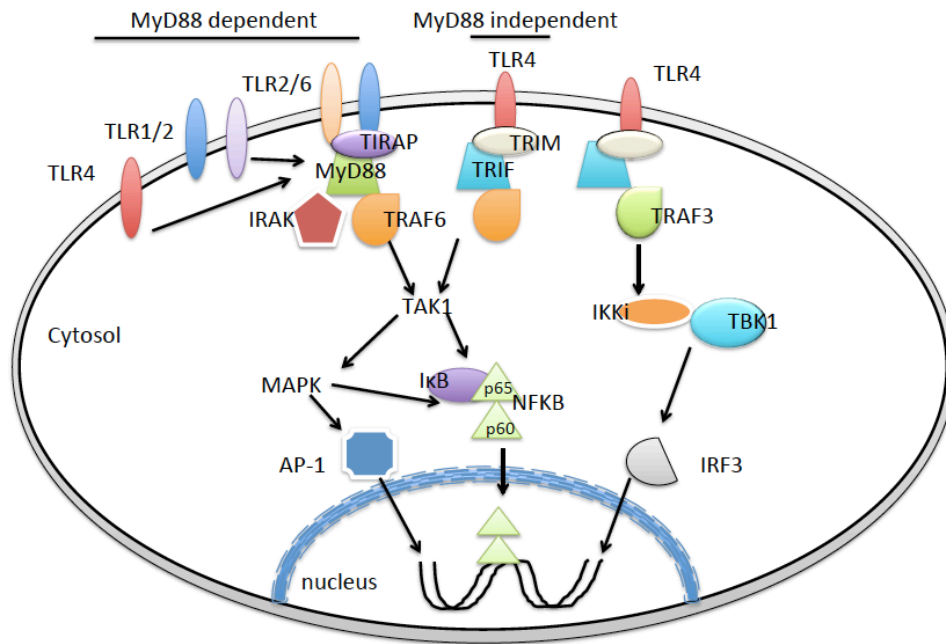


Figure 1.1: Overview of TLR 2 and 4 signalling. TLRs mostly form homodimers but TLR2 can also form heterodimers with TLR6 or TLR1. These initiate signalling to the 2 major downstream adaptor proteins, MyD88 and TRIF (MyD88-independent). TIRAP conducts the signal from TLR2 and TLR4 to MyD88 and TRAM conducts the signal from TLR4 to TRIF. TLR-MyD88 engagement forms the myddosome which contains MyD88, IRAK1 and IRAK4. IRAK1 induces activation of TRAF6 and TAK1. TAK1 leads to activation of IKK which induces the phosphorylation of IκB. IκB lies in a complex with NF-κB and when phosphorylated allows its release from the complex and subsequent translocation to the nucleus. It also activates the MAPKs which lead to the activation of AP-1. TLR4-TRIF engagement may also lead to the activation of TAK1 and subsequently NF-κB and MAPK through interaction with TRAF6. In contrast, interaction with TRAF3 recruits the IKK-related kinases, TBK1 and IKKi, which phosphorylate and activate IRF3 which induces the expression of type 1 IFN genes.

infection (Sugawara et al. 2003). In contrast to the findings by Bafica et al. 2005, TLR2/4/9-deficient mice demonstrated no changes in pro-inflammatory cytokines such as TNF-α and IL-12; additionally, they were also able to control replication following bacterial replication in a similar manner to wild-type mice (Hölscher et al. 2008). It is difficult to reconcile these vast differences in phenotypes following *M. tuberculosis* infection but there certainly appears to be some degree of receptor cooperation as well as redundancy, but ultimately, the discrepancies seen might be due to differences in the experimental design, the species used, the strain of *M. tuberculosis* and the inoculum administered. The ability of *M. tuberculosis* to interact with a multitude of receptors helps to explain the redundancy seen and there may be some cross-talk between TLRs and non-TLR PRRs (Rajaram et al. 2014). Perhaps even more significantly, studies have shown that *M. tuberculosis* H37Rv strains from different laboratories display unique single-nucleotide polymorphisms (SNPs) (Ioerger et al. 2010) which may provide an explanation for the phenotypic differences seen. What does not seem to be in dispute is the fact that MyD88 does have a definitive role in mycobacterial containment (Fremond et al. 2007; Hölscher et al. 2008), although this may be in part due to the signalling downstream of MyD88.

1.3.2 Dectin-1

Dectin-1 is a fungal pattern recognition receptor that binds β -glucans, but can also bind *M. tuberculosis* antigens, although its ligand in this case is not yet known (Rajaram et al. 2014). It is one of several types of C-type lectin receptor (CLR) and is highly expressed in immune cells (Rajaram et al. 2014) and it can contribute to pathogen containment (Brown et al. 2001).

Dectin-1 has been implicated during *M. tuberculosis* infection *in vitro* (Rothfuchs et al. 2007). However, Dectin-1 may not be required for protection against *M. tuberculosis in vivo*; with *Dectin-1*^{-/-} mice exhibiting reduced bacillary load compared to wild-type but no difference in pulmonary pathology or mortality (Marakalala et al. 2010).

1.3.3 Mincle

Mincle is another CLR family member which is expressed in abundance in mouse macrophages (Rajaram et al. 2014) and it can be upregulated following exposure to glycolipids, such as TDM (Ishikawa et al. 2009), however its essentiality in combatting *M. tuberculosis* infection is equivocal (Ishikawa et al. 2009).

1.3.4 Nucleotide-binding and oligomerisation domain (NOD) leucine-rich repeat (LRR)-containing proteins (NLRs)

The NLRs are a family of intracellular receptors which are able to recognise pathogen-associated molecular patterns and danger associated molecular patterns. To date, 22 human and 34 mouse NLR members have been identified (He et al. 2016). They share a common domain organisation, which tends to consist of a tripartite structure. They are composed of a central core known as the nucleotide-binding and oligomerisation domain (also known as NACHT or NBD domain), a carboxy-terminal leucine-rich repeat (LRR) domain (He et al. 2016) and an amino-terminal variable interaction domain. The LRR motif often plays a role in pathogen recognition, ligand binding and initiates activation of the molecule (Kleinnijenhuis et al. 2011). The amino-terminal domain contains an effector function and on the basis of this is divided into four subfamilies (He et al. 2016): the acidic trans-activation domain (NLRA), the baculoviral inhibitory repeat-like domain (NLRB), the caspase activation and recruitment domain (CARD; NLRC), and the pyrin domain (NLRP).

The NLRA subfamily has just one member; the NLRB subfamily consists of one member in humans (NAIP) and 7 members in mice; the NLRC subfamily has six members: NLRC1 (NOD1), NLRC2

(NOD2), NLRC3, NLRC4, NLRC5 and NLRX1; and finally, the NLRP subfamily consists of 14 members, NLRP1–14.

Activation of the NLRs leads to a number of functions including inflammasome formation, transcriptional activation of the MHC Class I and II genes, induction of autophagy and signal transduction (Motta et al. 2014). I shall be discussing the role of the inflammasome and autophagy in greater detail later on.

Both NOD1 and NOD2 are able to detect cytosolic peptidoglycan fragments — for example, NOD1 detects D-glutamyl-*meso*-diaminopimelic acid found in gram-negative bacteria and NOD2 detects muramyl dipeptide (MDP) found in both gram-positive and gram-negative bacteria. Receptor–ligand interactions recruit an adaptor protein, receptor-interacting protein 2 (RIP2), a serine/threonine kinase that can activate NF- κ B (Kim et al. 2016). NOD2 has been previously demonstrated as a receptor for *M. tuberculosis* antigens (Ferwerda et al. 2005). In fact, the mycobacterial enzyme NamH is responsible for producing a glycosylated form of maltose dimycolate, which is a potent activator of NOD2 and leads to heightened production of TNF- α . However the necessity of NOD2 is unclear in terms of mycobacterial control, whilst it is clear that optimal TNF- α and IL-12 release require NOD2, Gandotra et al. 2007 showed no difference in bacterial replication between wild-type and NOD2 KO mice *in vivo* whilst Divangahi et al. 2008 demonstrated a statistically significant difference. However, these studies had a crucial difference in the time-points at which the organs were harvested. Both studies showed comparative bacterial burden at 4 and 8 weeks post infection. However, after 24 weeks, NOD2 KO mice had impaired resistance to chronic infection and lower survival rates and this result tied in with a reduction in T-cell responses suggesting that NOD2 has a significant role to play driving in adaptive immunity and recognition of *M. tuberculosis* long after the initial infection.

1.4 Negative regulators of the macrophage during *M. tuberculosis* infection

Ligand recognition may be counteracted by the presence of receptors which are able to negatively regulate the pro-inflammatory process. This is especially important for alveolar macrophages, where a heightened pro-inflammatory response can increase tissue damage (see figure 1.3).

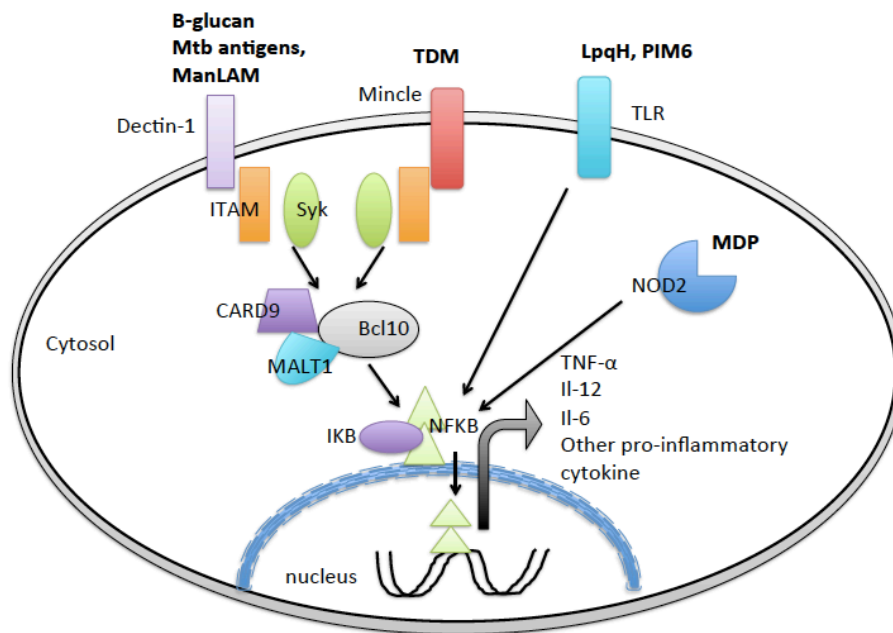


Figure 1.2: Some aspects of how different *M. tuberculosis* ligands induce a pro-inflammatory response. Indicated *M. tuberculosis* ligands (in bold) bind various receptors to induce a pro-inflammatory cytokine response, through differing signalling pathways. NF- κ B is the principal pro-inflammatory transcription factor featured here but others may be activated, as discussed briefly in section 1.3.1: Toll-like receptors (TLRs).

1.4.1 C-type lectin receptors (CLRs) and mannose receptors

The mannose receptor (MR), CD206, is another member of the C-type lectin family that can bind the polysaccharide components of the mycobacterial cell wall. The MR mediates phagocytosis of *M. tuberculosis* and is highly abundant on alveolar macrophages (Rajaram et al. 2014). *M. tuberculosis* has evolved to interact with these receptors through mannosylation of LAM (ManLAM); in doing this they render themselves antigenically similar to mammalian mannose and thus trick mammalian receptors into happily engaging the ManLAM, where it can subsequently inhibit phagosomal maturation and phagolysosomal fusion (Stamm et al. 2015). Furthermore, variations in virulence between strains could be due to the varying expression levels of ManLAM (Torrelles et al. 2010). MRs can also bind other structures within the cell wall such as PIMs (Kleinnijenhuis et al. 2011) which in pathogenic mycobacteria are also heavily mannosylated and preferentially bind the MR. Engagement of the MR down-regulates the induction of nitric oxide, reactive oxygen species and pro-inflammatory cytokines whilst also limiting phagolysosomal fusion (Kang et al. 2005), thereby reducing the microbicidal responses against mycobacteria. Some mycobacteria, which contain higher numbers of mannosylated PIMs and ManLAM compared to mycobacteria such as *Mycobacterium smegmatis*, bind the MR and use this to restrict phagolysosomal fusion thereby enhancing their own survival (Torrelles et al. 2006). MR engagement also leads to the activation of the peroxisome proliferator-associated receptor- γ (PPAR γ), which enhances IL-8, cyclooxygenase expression and *M. tuberculosis* survival (Rajaram et al. 2010).

1.4.2 Interleukin-1 receptor associated kinase - M (IRAK-M)

IRAK-M is a negative regulator of MyD88-dependent TLR signalling and may help to ameliorate tissue damage (Ballinger et al. 2015). However, in murine models of sepsis, *IRAK-M*^{-/-} mice have increased levels of pro-inflammatory cytokines, increased neutrophil recruitment and higher survival rates compared to wild-type mice (Deng et al. 2006). IRAK-M skews macrophages towards an alternatively activated cell type in a murine model of pulmonary fibrosis (Ballinger et al. 2015). However, in a contrasting clinical scenario, IRAK-M attenuates post-myocardial infarction remodelling and prevents the activation of destructive proteases (Chen et al. 2012). Thus beneficial activation may depend on the clinical insult. During *M. tuberculosis* infection, ManLAM induces the expression of IRAK-M and, in doing so, reduces the interaction between IRAK and TRAF6 and decreases activation of NF-κB pro-inflammatory signalling (Pathak et al. 2005). More recently it has been shown that *M. tuberculosis* delays the onset of TH-1 cell-mediated immunity and pro-inflammatory responses via the activation of IRAK-M and IL-10 and deactivation of NF-κB (Jeyanathan et al. 2014).

1.4.3 Suppressor of cytokine signalling (SOCS) proteins

SOCS are a family of eight intracellular proteins that operate in a feedback loop to negatively regulate cytokine signalling through inhibition of the Janus kinases (JAK) and signal transducers and activators of transcription (STAT). Inhibition occurs through direct binding, or targeting the protein complex for ubiquitination therefore enabling proteasomal degradation (Yoshimura et al. 2007). SOCS1 inhibits STAT1 responses, which help to negatively regulate the activity of IFN-γ (Carow et al. 2011). Mice deficient in SOCS1 have a heightened immune response to *Leishmaniasis major* including increased IFN-γ which can be deleterious rather than helpful (Alexander et al. 1999). SOCS1 also regulates IL-12; in the absence of SOCS1, there is increased T-cell proliferation and natural killer cell hyper-toxicity (Eyles et al. 2002). *M. tuberculosis* infection increases the transcription of SOCS1 in macrophages (Carow et al. 2011) and this is mediated by TLR2, NOD2 and MyD88 signalling. Whilst SOCS can inhibit growth control through reducing IFN-γ secretion in response to IL-12, SOCS expression in non-macrophage cells helps to reduce inflammation and acts as 'damage-control'. Additionally, SOCS1 mRNA was observed to be upregulated in patients with extra-pulmonary TB, although in this cohort there were also elevated levels of pro-inflammatory cytokines, which suggests that in response to infection, cells naturally attempt to rein in excessive inflammatory activity (Ashenafi et al. 2014).

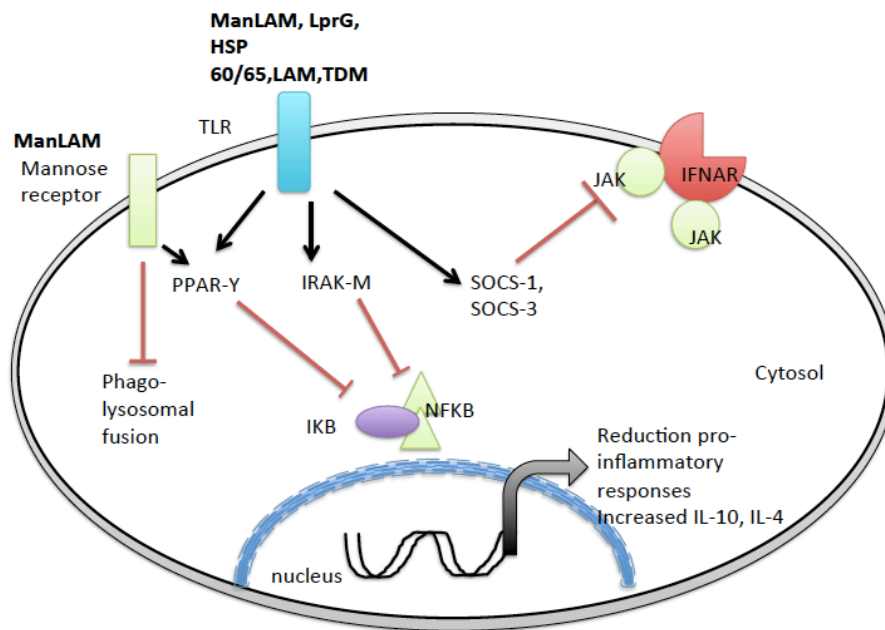


Figure 1.3: Negative regulators of macrophage response to *M. tuberculosis* and its cell wall components. Stimulation leads to enhancement of various negative regulators, such as PPAR-γ, IRAK-M and SOCS which help suppress pro-inflammatory activity and induce alternative macrophage activation.

1.5 Host-Directed Therapy (HDT)

Host-directed therapy (HDT) aims to modulate the immune system, in order to augment the efficacy of macrophage effector mechanisms or reduce inflammation and prevent tissue damage mediated by the host–pathogen interaction (Zumla et al. 2016). HDT may also serve to rebalance the immune response that *M. tuberculosis* has so cleverly manipulated. It is an attractive prospect as it tends not to have a specific target as with anti-microbials and therefore the risk of resistance is minimal (Hawn et al. 2013), although optimisation of such therapies may require intrinsic knowledge of the host genotype.

In many instances HDT has been repurposed from treatment of other diseases such as cancer — this is often because *M. tuberculosis* has learned to hijack key signalling mediators such as tyrosine kinases, which are also dysregulated in certain cancers and whose inhibition has been evaluated previously (Hawn et al. 2013). As such, utilising such drugs shortens the length of the drug development pipeline. In the following sections, I discuss some of the various approaches employed for host-directed therapy and in most parts the results of how they fared if entered into clinical trials. For reasons of ease I split HDT into two groups — those that augment the host response and those that modulate tissue damage and tissue homeostasis. I also largely concentrate on inflammatory modulation that can be reduced rather than enhanced, as this is where most current strategies are targeted.

1.5.1 Enhancing macrophage anti-microbial mechanisms as a potential major target of the innate response

In the following, I look at potential avenues by which the host response can be utilised for therapy, although since many signalling molecules and drugs have pleiotropic effects, they may engage more than one mechanism.

Phagocytosis and phagolysosomal fusion

Binding of mycobacterial ligands to their respective receptors leads to induction of actin polymerisation and formation of the phagosome (Weiss et al. 2015). Phagocytosis may occur after opsonisation with complement factors or it may occur in the absence of opsonisation (Kleinnijenhuis et al. 2011). In the alveolar space, phagocytosis is largely undertaken in the absence of opsonins (Kleinnijenhuis et al. 2011). The phagosomal stages are divided into early (10 minutes after uptake), late (10–30 minutes after uptake) and the phagosomal phase (later than 30 minutes) but the length of these stages may be determined by the receptors involved in uptake — for example engagement by the MR may avoid the final phagosomal stage (Torrelles et al. 2008). The early phagosome does not contain membrane proton-pumping vesicular ATPases, therefore the intraluminal pH is only mildly acidic (Weiss et al. 2015). However, following macrophage activation by IFN- γ , the pH drops to less than 5.0 which is associated with increased accumulation of proton ATPases (Schaible et al. 1998). Phagosomal maturation is associated with this drop in pH and the generation of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) where the phagosomal cargo is degraded. Proteases, such as cathepsins and lipases are also delivered into the late endosome and are likely to act with other endogenous molecules such as defensins, in order to evoke anti-bacterial activity (Weiss et al. 2015). The later stages of the phagosome (late endosome) also link with a motorised complex that facilitates the fusion between the late endosome and the lysosome (Jordens et al. 2001). The late phagosome is defined by the low intraluminal pH (5.0), facilitated by the expression of proton-pump vacuolar ATPases (vATPases).

As well as altering the receptors through which it is phagocytosed — as discussed in the previous section — *M. tuberculosis* also has evolved strategies by which it manipulates the phagosomal response. *M. tuberculosis* is reported to enhance actin nucleation around the mycobacterial vacuole and depolymerisation of the actin scaffold leads to subsequent phagosome maturation (Kolonko et al. 2014). *M. tuberculosis* may also modify acidification of the late phagosome by activating Abl tyrosine kinase, which negatively regulates the expression of vATPases (Bruns et al. 2012). Even when these innate defence mechanisms manage to overwhelm the resilience of the pathogen, *M. tuberculosis* can

still survive at a pH of 4.5 with bacterial metabolism drastically reduced resulting in growth arrest and non-replicating persistence but not eradication (Prosser et al. [2017](#)).

1. Metformin

The current trend of increasing type 2 diabetes mellitus (T2DM) prevalence will make an important contribution to the TB epidemic since diabetes increases the risk of developing active TB (Riza et al. [2014](#)). Monocytes from patients with T2DM have reduced association with serum-opsonised *M. tuberculosis* and exhibit reduced phagocytosis via complement receptors (Restrepo et al. [2014](#)).

Metformin, a biguanide traditionally used as an oral diabetic agent, is increasingly taking on additional roles in HDT. Metformin increases the expression of an energy-sensing kinase, AMP-activated protein kinase (AMPK; Zhou et al. [2001](#)). AMPK can respond to low intracellular ATP states and can alter the macrophage phenotype, from M1 which requires glycolysis for energy, to the anti-inflammatory M2 phenotype, which uses fatty acids for energy, with an associated increase in the production of anti-inflammatory cytokines (Zhou et al. [2016b](#)). Recent *in vitro* studies have shown that metformin promotes phagocytosis and phagolysosomal fusion following *M. bovis* BCG infection and leads to a reduction in *M. tuberculosis* bacillary replication. *In vivo* however, combination with isoniazid only elicited a modest reduction in bacillary load compared to isoniazid alone; similarly lung pathology did not differ much between the two treatments (Singhal et al. [2014](#)). However, immune responses — in particular IFN- γ -releasing cells — improved in the metformin group, and a retrospective study which examined patients with TB and T2DM showed that those who were taking metformin had fewer pulmonary cavities compared to those who were not taking the drug (Singhal et al. [2014](#)). This suggests that there may be some value in pursuing this as a line of treatment, and although metformin treatment is associated with lactic acidosis in high-risk patients (those with renal failure for example) it is generally well-tolerated (Restrepo [2016](#)).

2. Tyrosine kinase inhibitors

As discussed above, Abl tyrosine kinase activation dampens the level of phagosomal acidification (Hawn et al. [2013](#)). Targeted inhibition of Abl tyrosine kinase with imatinib increases trafficking of bacteria into acidified pockets, whereas non-selective inhibitors had no effect (Bruns et al. [2012](#)). Furthermore, monocytes from healthy donors that were incubated with sera from patients with chronic myeloid leukaemia (CML) who were taking imatinib displayed increased phagolysosomal fusion and enhanced bacterial killing following infection with *M. tuberculosis* compared to controls (Bruns et al. [2012](#)). Additionally, recent studies have shown that imatinib increases clearance of other pathogenic

bacteria such as *Francisella* spp. However, imatinib has been associated with increased mobilisation of neutrophils into the infected area (Napier et al. 2015) and may be of some concern in established TB disease, where neutrophils have been reported to contribute to enhanced tissue pathology (Lowe et al. 2012).

Autophagy

The term autophagy is derived from the ancient Greek meaning of self-devouring (Glick et al. 2010). It is an evolutionarily conserved process in response to starvation, which aids the rapid turnover of damaged cellular contents or organelles through the lysosomal machinery and does so with minimum inflammatory damage (Choi et al. 2013). By clearing away protein aggregates that may accumulate with stress, or sequestering damaged mitochondria (mitophagy) or other organelles, it becomes a mechanism by which cell death is avoided. It is also responsible for the clearance of intracellular pathogens (xenophagy) which are trafficked to autophagosomal vesicles (Deretic et al. 2013) and is therefore a way by which pathogen dissemination is prevented.

Autophagy is a highly regulated process mediated by several autophagy-related proteins (ATG) which are broadly divided into several units according to how they effect their function. The mammalian target of rapamycin complex 1 (mTORC1) is one of the key signalling pathways that responds to changes in nutrient availability (Choi et al. 2013). mTORC1 negatively regulates the ATG1/Unc-51-like kinase 1 (ULK1) complex (Choi et al. 2013). During periods of nutrient starvation, mTORC1 is inhibited leading to the derepression and assembly of the ATG1/ULK1 protein complex. ATG1/ULK1 is the key initiator of the autophagic process since it recruits other ATGs to the phagophore assembly site (PAS). Following initiation, another complex called the class III PtdIns3K complex I is recruited to the PAS (Choi et al. 2013), which is responsible for the formation of phosphatidylinositol-3-phosphate (PtdIns3P) from phosphatidylinositol and is derived from the neighbouring endoplasmic reticulum (Deretic et al. 2013). Thus, together ATG6/Beclin-1 and ATG1/ULK1 drive the formation of the isolation membrane (Russell et al. 2013). Following this, ATG12 and ATG8 (or Light Chain 3; LC3) are recruited to the membrane. However, in order for autophagosomal elongation to occur, two conjugation reactions must take place which are catalysed by ATG7. ATG12 is conjugated to ATG5 and this complex binds to ATG16 which subsequently associates with the extending phagophore (Glick et al. 2010). ATG8 is cleaved by the protease Atg4 to generate LC3B1 and in a series of steps this covalently binds to the lipid phosphatidylethanolamine (PE) to become activated LC3B2, enabling its anchoring at the autophagosomal membrane (Glick et al. 2010). At this stage, a hallmark of the process is the double-membrane structure (Espert et al. 2015). Cytoplasmic structures are shuttled to the autophagic machinery through autophagic adaptors that are able to bind LC3 (Espert et al.

2015). Autophagosomes then fuse with lysosomes and then undergo proteolysis (Hawn et al. 2013). Autophagy is metabolically regulated by the serine/threonine kinase mammalian target of rapamycin (mTOR) which inhibits and AMPK which activates autophagy. In its resting state, Beclin-1 is bound by TAB2 and TAB3 (both being upstream regulators of I κ K), but following induction of autophagy, TAB2/TAB3 dissociates from Beclin-1 and then binds TAK1, which then phosphorylates and activates AMPK (Deretic et al. 2013). Autophagy may be a consequence of cytokine-mediated mechanisms, such as IFN- γ , but equally it can regulate and sequester pro-inflammatory cytokine secretion.

Since *M. tuberculosis* has formulated ways by which it can avoid phagolysosomal fusion, targeting autophagy could help overcome this. Certainly, physiological stimulation of autophagy increases phagosome co-localisation with Beclin-1 and reduced *M. tuberculosis* intracellular survival (Gutierrez et al. 2004). Furthermore, both IFN- γ and transfection of LRG-47 (a GTPase that operates downstream of IFN- γ activation) increase bacterial killing (Gutierrez et al. 2004). These results provide the basis of the evidence of the role of cytokines in modulation of autophagy. For example, autophagy cross-talks with the inflammatory process, such that certain cytokines such as IFN- γ (Gutierrez et al. 2004) and TNF- α (Harris et al. 2010) enhance autophagy whereas IL-4 and IL-13 inhibit it (Harris et al. 2007b). There are additional mechanisms by which autophagy is initiated during *M. tuberculosis* infection. ESX-1-mediated permeabilisation of the phagosome leads to cytosolic escape of bacterial cyclic-di-adenosine monophosphate (c-di-AMP), which activates the stimulator of interferon genes (STING) pathway which leads to increased levels of type 1 IFN and increased autophagy (Dey et al. 2015). Permeabilisation also releases bacterial DNA which is sensed by the cyclic GMP-AMP synthase (cGAS)-STING pathway, which also triggers autophagy as well as activation of type 1 IFN (Watson et al. 2015). The cytosolically exposed *M. tuberculosis* is tagged with ubiquitin, partly mediated by the ubiquitin ligase PARKIN and this promotes the recruitment of autophagic adaptors such as p62, subsequent binding by LC3 and leads to controlled infection *in vitro* (Watson et al. 2015).

Isoniazid and pyrazinamide have both been demonstrated to promote autophagy (Kim et al. 2012), thus there have been some efforts to look at other potential compounds that might enhance the autophagy pathway. High throughput automated cell-based assays that can detect autophagosome content have previously identified three drugs that are approved for use in humans (including amiodarone) (Baldi et al. 2009). Other repurposed compounds that have recently been identified that increase autophagy *in vitro* are carbamazepine and valproic acid; carbamazepine was demonstrated to reduce bacterial burden and reduce lung pathology in a zebrafish model (Schiebler et al. 2015). In the next few paragraphs I shall detail how two major influences on autophagy — vitamin D and IFN- γ — have progressed from *in vitro* results to clinical trials.

3. Vitamin D

There has been a resurgence in the interest over the past few years in the use of vitamin D as a potential HDT in TB, but also in other infectious diseases. There are two forms of active vitamin D — vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol) — and both can be obtained from the diet, but sun exposure also converts 7-dehydrocholesterol to D₃ (Hawn et al. 2013). D₂ and D₃ are converted to 25-OH-D₃ which is taken up by macrophages where it is subsequently converted to 1,25-(OH)₂-D₃ by 1 α -hydroxylase (Gombart 2009). Both 1 α -hydroxylase and the vitamin D receptor are produced following TLR-*M. tuberculosis* ligand interactions (Hawn et al. 2013). The production of 1,25-(OH)₂-D₃ mediates the activation of the anti-microbial peptide cathelicidin (LL37). 1,25-(OH)₂-D₃ and LL37 regulate autophagy by inhibiting the expression of mTOR and increasing Beclin-1 transcription (Wu et al. 2011) and this leads to enhanced antimycobacterial activity (Yuk et al. 2009). 1,25-(OH)₂-D₃ also reduces the secretion of matrix metalloproteases induced by *M. tuberculosis* (Coussens et al. 2009) and T-cells are able to induce the production of this form of D₃ via secretion of IFN- γ (Fabri et al. 2011). Moreover, IFN- γ -induced autophagy was found to be dependent on 1,25-(OH)₂-D₃ (Fabri et al. 2011). However, this effect was lost when monocytes were cultured with sera obtained from African-American populations which have lower 1,25-(OH)₂-D₃ levels (Fabri et al. 2011). LL37 can act on its own and is constitutively produced but increases during inflammatory processes (Torres-Juarez et al. 2015). Following *M. tuberculosis* infection, LL37 reduces TNF- α but increases TGF- β and IL-10 and reduces bacillary growth *in vitro* (Torres-Juarez et al. 2015). A recent study has also shown that vitamin D opposes *M. tuberculosis*-induced LL37 down-regulation and in doing so, also enhances TH-1 differentiation and IFN- γ secretion (Rode et al. 2017).

How does this translate clinically? A case-control study of a London Gujarati population showed an increased deficiency of 25-OH-D₃ in patients with TB (Wilkinson et al. 2000). Similar results showing an association between serum 25-OH-D₃ and TB disease were replicated in Sub-Saharan African populations (Wejse et al. 2007; Gibney et al. 2008). In contrast, a recent study has shown that significantly higher levels of 25-OH-D₃ were present in active TB cases in The Gambia (Owolabi et al. 2016) compared to healthy controls, suggesting that in Gambian adults, a higher level of 25-OH-D₃ may increase TB susceptibility. Thus the benefit of vitamin D supplementation might not be universal. In the UK, a randomised controlled trial showed that a single dose of 2.5 mg oral vitamin D enhanced the ability of whole blood taken from TB contacts to reduce *M. bovis* bacterial numbers *in vitro* at 24 hours (Martineau et al. 2007). Further, a detailed study examining the effect of 4 doses of 2.5 mg vitamin D₃ in patients with active TB demonstrated that supplementation increased the serum levels of 25-OH-D₃ when levels were low at baseline and this resulted in faster sputum culture conversion in patients with the tt genotype of the *TaqI* vitamin D receptor polymorphism (Martineau et al. 2011). 2 doses of 15 mg vitamin D₃ administered to patients with active TB in Pakistan improved clinical and radiological recovery with associated increased IFN- γ activity in whole blood assays which was

observed in patients with 25-OH-D₃ deficiency although there was no difference in sputum conversion (Salahuddin et al. 2013). Other studies have not demonstrated any change in culture conversion (Wejse et al. 2009; Ralph et al. 2013) although the doses administered were not as frequent or large compared to Martineau et al. 2011. The lack of conclusiveness in vitamin D supplementation warrants further investigation with less heterogeneity between study designs but high-dose 25-OH-D₃ in patients with a deficient baseline (so to avoid toxic side effects) may be of value in improving clinical outcomes.

4. IFN- γ

IFN- γ is produced primarily by T-cells and natural killer cells and has a central role in the host immune response, in part because of its ability to activate macrophages following infection and to increase the capacity of the macrophage to kill intracellular pathogens (Rode et al. 2017). As discussed previously, the potency of IFN- γ is largely due to induction of autophagy (Gutierrez et al. 2004). This is mediated through GTPases — induced downstream of IFN- γ activation — which promote oxidative killing and increased targeted delivery of ubiquitinated proteins to LC3 and thus enhanced autophagic engulfment and bacterial eradication (Kim et al. 2011). The importance of *in vitro* IFN- γ during autophagy is also highlighted by a recent study that which showed that activation of autophagy was increased following *M. tuberculosis* infection in PBMCs taken from patients with TB who had high levels of T-cell responses and IFN- γ secretion (Pasquinelli et al. 2004) in comparison to PBMCs taken from patients with low T-cell and IFN- γ responses ('low responders') (Rovetta et al. 2014). Moreover, in this experimental set-up, induction of autophagy was reduced by addition of monoclonal antibodies against IFN- γ and enhanced when recombinant IFN- γ was added to infected cells taken from low responders (Rovetta et al. 2014). From a clinical perspective, a mixture of randomised and non-randomised trials have been conducted (9 in total) though the numbers enrolled have been small in comparison to the vitamin D trials. However, a meta-analysis of adjunctive aerosolised IFN- γ administration demonstrated an increased rate of sputum negative conversion and radiographic improvement (Gao et al. 2011). The largest trial, consisting of 96 patients compared adjunctive nebulised or subcutaneous recombinant IFN- γ to standard TB therapy alone and showed statistically increased rates of smear negativity and a trend to culture conversion at 4 weeks and fever reduction in the treatment arm compared with the control (Dawson et al. 2009). The findings suggest some promise with IFN- γ but, aside from the fact that delivery modality in the form of inhalation or subcutaneous application would be challenging and expensive, in order to make any clear conclusions about adjunctive IFN- γ treatment, larger trials would need to be performed. Given that IFN- γ induces nearly 2000 host genes (Kim et al. 2011), it might be worth pursuing small molecules downstream of IFN- γ signalling as potential targets in order to circumvent these challenges. Further work might also

consider dual administration of IFN- γ and vitamin D in target populations for optimal treatment.

1.5.2 Reducing inflammation in order to limit tissue damage

It is no longer believed that patients with active TB have a poor pro-inflammatory signature and an over-zealous inflammatory reaction may have deleterious consequences. Progressive pulmonary TB associated with perpetual inflammation leads to varying degrees of tissue necrosis and caseating states that can increase *M. tuberculosis* dissemination and impair tissue resolution (Kaufmann et al. 2013; Subbian et al. 2016). Whilst critical to the initial host defence, disease pathology may also be driven by TNF- α , IFN- γ and IL-1 β (Kaufmann et al. 2013; Mishra et al. 2013). Certainly, impaired lung function is a well known consequence of pulmonary TB (Hnizdo et al. 2000) and previous infection with TB can increase the risk of further re-infection (Verver et al. 2005), although whether this increased risk can be directly attributed to damage to the lung tissue has not been explored. This imbalanced environment leads to the emergence of sub-populations of TB bacilli — so-called ‘persisters’ that down-regulate their own metabolism and replicative ability but are refractory to anti-microbial therapy and those which continue to exhibit rapid replication (Chao et al. 2010). Rifampicin is the major drug targeting persisters, however there may be a proportion of bacilli in varying metabolic states that are not killed by current therapy (Mitchison 2000). Thus, in addition to enhancing the capability of the host to fight infection, an alternative approach would be to dampen the inflammation that might be causing excessive immunopathology. In doing so, the modification of the environment might also lend itself to increasing the metabolic activity of the bacilliary population and therefore enhance anti-microbial killing.

1. Corticosteroids

Corticosteroids are perhaps one of the earliest immunomodulatory interventions that have been explored with 41 trials over nearly 60 years that have endeavoured to investigate their worth during TB disease (Dooley et al. 1997; Critchley et al. 2013). However, most of these trials pre-date the advent of the modern anti-tuberculosis regimen, therefore their efficacy maybe difficult to interpret (Critchley et al. 2013). A meta-analysis examining these trials demonstrated that steroids were associated with a reduction in death for all organ systems affected, though on further analysis of organ-specific sub-groups, whilst the inclusion of steroids in TB meningitis led to a statistically significant overall survival advantage, such a statistical effect fell short in pulmonary TB (Critchley et al. 2013). The fact that the vast majority of data in TB meningitis and TB pericarditis favours the use of steroids may skew the overall survival benefit (Thwaites 2013) thus the blanket use of steroids for all TB may not be warranted.

The greatest benefit of steroids in TB meningitis may occur in patients with a specific genetic polymorphisms. Leukotriene A4 hydrolase (LTA4H) is an enzyme that catalyses the production of the pro-inflammatory eicosanoid LTB₄ and controls the balance between pro- and anti-inflammatory eicosanoids (Tobin et al. 2012). Mutations in *Ita4h* increase susceptibility to *M. marinum* in zebrafish (Tobin et al. 2010) through an interesting but divergent mechanism. Both highly active LTA4H, which drives excess pro-inflammatory eicosanoid LTB₄, and reduced LTA4H activity, which drives the anti-inflammatory eicosanoid LXA₄, increase bacterial growth. Intriguingly, zebrafish with a high LTB₄ level had increased TNF- α activity, whilst increased LXA₄ was associated with TNF- α deficiency (Tobin et al. 2012). Blocking LTA4H activity with dexamethasone lowered bacterial burden in zebrafish with an increased LTA4H baseline; conversely dexamethasone increased bacterial replication in those with reduced LTA4H activity (Tobin et al. 2012). In the same study, single-nucleotide polymorphisms in *Ita4h* with the TT genotype increased the inflammatory phenotype whereas the CC genotype resulted in a low inflammatory state. Moreover, a recent study has shown that LTA4H polymorphisms may also predict survival from TB meningitis. Thuong et al. 2017 showed that dexamethasone increased the survival of HIV-negative adults with TB meningitis who had the *Ita4h* TT genotype. In this study, the baseline CSF levels of IL-1 β , IL-2 and IL-6 were markedly raised in the TT genotype and lowered in those with the CC genotype. Moreover, in those that died, there were much lower levels of inflammatory cytokines. This suggests dexamethasone may confer survival in susceptible patients whereas it may be detrimental in those that do not have a pro-inflammatory baseline at clinical presentation; thus a highly tailored HDT using steroids or indeed specific modulators of the LTA4H axis may be necessary.

Given that the mortality rates from pulmonary TB are low (0.5 per 100,000 in 2013 in the UK; Public Health England 2013), it may be more worthwhile ascertaining sputum conversion as the primary outcome following oral steroids. A meta-regression analysis of controlled clinical trials showed that oral steroids may reduce the proportion of sputum positive culture at 2 months from 15 % to 2 % (Wallis 2014). In order to achieve this, 134 mg per dose would be required which is unacceptably high for prolonged use with the benefits potentially offset by undesirable side effects. However, the premise that a potent anti-inflammatory can accelerate sputum conversion is hugely exciting but the mechanism underlying this is uncertain. This maybe due to increased anti-microbial access and a change in the metabolic profile of the bacteria that renders heightened susceptibility to anti-microbial therapy (Wallis et al. 2015) as it seems unlikely that steroids themselves can directly increase host processes such as autophagy (Kyrmizi et al. 2013). Despite the undesirable side effects, the finding that high-dose steroids can alter sputum conversion clearly has implications for other potential anti-inflammatory therapies that are able to enhance the efficacy of current chemotherapy.

2. TNF- α blockers

TNF- α is produced by cells of both the innate and adaptive immune response (Hawn et al. 2013). It is a potent pro-inflammatory cytokine which promotes the secretion of chemokines and adhesion molecules and therefore a focused accumulation of inflammatory mediators aimed at pathogen eradication (Harris et al. 2010). TNF- α enhances the effect of IFN- γ -induced phagosome maturation (Harris et al. 2008) and has a critical role in controlling *M. tuberculosis* replication in addition to granuloma formation *in vivo* (Flynn et al. 1995). *Tnf*^{-/-} mice succumb early to *M. tuberculosis* infection compared to wild-type mice and have poorly organised granulomas with extensive necrosis (Bean et al. 1999). In addition, in the absence of TNF signalling, T-cells localise to the peri-bronchial areas rather than the granuloma itself (Bean et al. 1999). Clinically, the importance of this cytokine on other aspects of the immune response is demonstrated by the use of anti-TNF agents, such as infliximab, which is a chimeric (75 % human and 25 % mouse) monoclonal antibody against TNF- α (Jacobs et al. 2000). Infliximab down-regulates IFN- γ production in colonic T-cell cultures in patients undergoing treatment for Crohn's disease (Agnholt et al. 2001). Furthermore, the use of infliximab in patients with latent TB has previously been shown to increase progression to active TB (Keane et al. 2001). By contrast, soluble TNF- α receptors (such as etanercept) which are a fusion of the extracellular ligand portion of the TNF receptor combined with the Fc moiety of human IgG are relatively inefficient in their ability to reactivate latent TB (Wallis et al. 2015). By comparison with infliximab, etanercept is less potent at blocking transmembrane TNF receptor-induced signalling and has a markedly reduced half-life (Jacobs et al. 2000).

However, TNF- α may lead to excessive immunopathology and TB persistence, since mycobacteria may utilise the early granuloma as a place of sanctuary (Davis et al. 2009). Additionally whilst TNF- α -mediated enhancement of apoptosis is traditionally thought to inhibit *M. tuberculosis* replication (Keane et al. 2000), there is equal concern that apoptosis may facilitate its dissemination (Davis et al. 2009). Mice infected with mycobacteria that elicited high circulating levels of TNF- α exhibited an overwhelming inflammatory response that also compromised lung function (Bekker et al. 2000). Moreover, infection of human alveolar macrophages with *M. tuberculosis* induced higher levels of TNF- α compared to attenuated strains and this correlated with increased bacterial replication (Engele et al. 2002). Surprisingly, neutralising anti-TNF- α antibodies reduced bacterial growth and addition of exogenous cytokine increased bacterial growth (Engele et al. 2002). A similar picture is seen during clinical infection: following initiation of anti-TB treatment, raised plasma TNF- α was associated with clinical deterioration (Bekker et al. 1998). Such results suggest that anti-TNF- α treatment could prove promising. A phase one safety study showed that etanercept treatment in HIV-infected patients who were co-infected with TB tended to improve radiological responses and time to culture

conversion compared to the control arm (Wallis et al. 2004). There have even been reports of TNF- α blockers being used in steroid-refractory paradoxical TB reactions (Blackmore et al. 2008). This suggests that whilst TNF-blockade maybe deleterious in latent TB, it maybe adapted to clinical effect during active infection. However, etanercept is expensive and unlikely to be rolled out to larger trials, thus alternatives have been sought.

In the past 20 years, the anti-tumour drug, thalidomide, has been explored as a potential immunomodulatory agent. Whilst the mechanism of action is unknown, its anti-tumour activity is believed to be due to it binding a ubiquitin ligase leading to proteasomal degradation of B-cell transcription factors (Lu et al. 2014). Initial reports showed that thalidomide reduced TNF- α at the protein and transcriptional levels in human alveolar macrophages obtained from patients with lung disease, including active TB (Tavares et al. 1997). In mice infected with *M. tuberculosis*, thalidomide reduced blood TNF- α levels and necrotic granuloma (Moreira et al. 1997). Administration of adjunctive thalidomide to patients with active TB with or without concurrent HIV infection, reduced TNF- α release and IL-1 β transcription and promoted an early increase in IFN- γ levels — thalidomide also enhanced weight gain which was reversed upon thalidomide cessation (Tramontana et al. 1995). Children with intractable intracranial tuberculosis demonstrated neuroradiological improvement with thalidomide (Schoeman et al. 2006) although a randomised study assessing the effects of high-dose thalidomide against standard TB therapy and steroids had to be terminated early due to increased adverse allergic side effects in the treatment arm (Schoeman et al. 2004). In order to circumvent these side effects, thalidomide analogues and other potential TNF- α blockers are currently being developed.

3. Metalloprotease inhibitors

One of the principal causes for cavitary breakdown during lung disease is the destruction of the extracellular matrix (Hawn et al. 2013). The matrix metalloproteases (MMPs) are hydrolytic enzymes that are largely responsible for this degradation (Salgame 2011), however they also play a role as anti-microbials and modulate the activity of cytokines such as IL-1 β (Salgame 2011). There are 24 members, grouped by collagenases, gelatinases and elastases (Ong et al. 2014) in mammals (Hawn et al. 2013) and they have specific inhibitors, the tissue inhibitors of metalloproteases (TIMPs) that bind the proteases and inactivate them (Ong et al. 2014). During *M. tuberculosis* infection, MMPs are up-regulated and drive the breakdown of their respective tissue groups but evidence suggests that there is no parallel increase in TIMPs (Price et al. 2003) meaning that they may drive pathology without opposition. In particular, *M. tuberculosis* induces the expression of MMP-9 (Price et al. 2003), MMP-1, MMP-7 and MMP-10 in human monocytes (Coussens et al. 2009) which are interestingly attenuated by 1,25-(OH) $_2$ -D $_3$ (Coussens et al. 2009). In addition, up-regulation of MMP-9 is also

observed in the cerebrospinal (CSF) fluid of TB-meningitis patients at the start of treatment with an associated correlation in CSF neutrophils and MMP-9 was observed to be reduced by dexamethasone at day 5 compared to placebo (Green et al. 2009). Similarly, MMP-1 and products of collagen and elastin degradation were also increased in sputum from patients with active TB (Seddon et al. 2013). Thus, there has been some success in evaluating potential inhibitors of MMP in an attempt to reduce matrix degradation (Izzo et al. 2004) suggesting that MMPs play a large role in cavitary destruction and may increase bacillary dissemination. MMP inhibition might prove useful adjuncts to standard chemotherapy.

1.5.3 Engaging host-directed therapies in TB treatment

In the above sections, I have reviewed some of the mechanisms that might be targeted for HDT, although this is not exhaustive. Some of these have gone on to clinical trials but the gap between *in vitro*, *in vivo* and clinical trials remains large. Future work examining HDT in clinical trials needs to focus on the target audience (for example, drug-resistant populations, HIV-TB co-infected individuals, extra-pulmonary cases rather than pulmonary TB patients). I would also argue that those with extensive cavitary disease (Zumla et al. 2015) or those patients with the potential to develop worsening disease, for example HIV patients recently starting combined anti-retroviral therapy (Lai et al. 2015), would also be candidates for treatment. The use of repurposed medications which have already established safety profiles will hopefully reduce the length of pre-clinical timelines, although they will still require extensive dose-response optimisation, safety tests and trials with current chemotherapy.

Other considerations need to be taken into account. Important work by Martineau et al. 2011, Tobin et al. 2012 and Thuong et al. 2017 has demonstrated that the clinical efficacy of such treatments may be largely attributed to the genotype of receiving individuals making a general roll-out challenging. Additionally, we often look at culture conversion rates and culture negativity, which is an often used marker of bacterial clearance. However, as shown by Malherbe et al. 2016, patients deemed to be culture negative and 'cured' following treatment of fully sensitive TB still had bacterial mRNA in their sputum. Even if culture conversion rates with adjunctive therapy were only to prove non-inferior rather than demonstrate any increased conversion rates (compared to standard chemotherapy) other markers, such as changes in inflammatory activity (radiographic PET scanning or transcriptomic and proteomic profiling are both increasingly being used) and improvement in lung function would provide useful knowledge as well as an additional surrogate on the effect imposed by HDTs. Since there may be variability in the efficacy of HDT, as we have seen from the vitamin D studies, multi-national trials would need to be employed in order to determine whether geographical differences

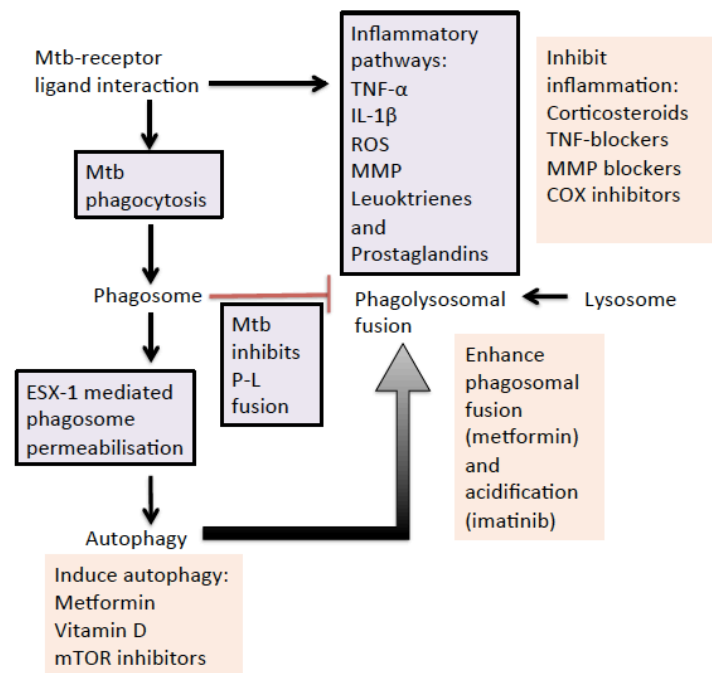


Figure 1.4: Aspects by which HDT can be used as therapeutic adjuncts. *M. tuberculosis* can suppress phagocytosis and phagolysosomal fusion, and escape into the cytosol via the ESX secretion system and induce a potent pro-inflammatory response (highlighted in purple boxes). This might be overcome by agents that promote phagolysosomal fusion and acidification and induce autophagy. Inflammatory pathways may also be dampened in order to readdress the inflammatory balance (highlighted in pink boxes). Adapted from Wallis et al. 2015.

might render a treatment successful or not as HDTs are unlikely to lend themselves to a uniform application. However, successful scanning in trialled populations would at least demonstrate improved or worsening inflammatory foci that might not be presented clinically. Future advances will also utilise bacterial radiotracers that will help to detect residual bacterial metabolic activity throughout treatment (Bunschoten et al. 2013).

In summary, the use of HDT is a potentially exciting avenue but requires intricate knowledge of the host in which it is trialled, and a spectrum of radiological, biochemical and functional tools will need to be employed in several settings to identify markers and parameters of efficacy.

1.5.4 The research gap - how can additional aspects of the host response to *M. tuberculosis* infection be manipulated for therapeutic purposes?

Given that one of the major aims of HDT is to reduce tissue damage at the point of infection, I pursued this angle in my own work. In the following two chapters, I discuss and evaluate in mixed *in vitro* and *in vivo* settings two aspects of immunotherapy that have the potential to dampen the inflammatory response.

In chapter 1, I sought to examine the role of melanocyte stimulating hormone (MSH) which is derived from adrenocorticotrophic hormone (ACTH) (Catania et al. 2010). MSH has been shown to exhibit

anti-inflammatory properties and has previously been implicated in *M. tuberculosis* susceptibility (Cooke et al. 2008). MSH is attractive as it does not elicit the side effects imposed by steroid use. Chapter 1 provides the background on the melanocortin system and using *in vitro* and *in vivo* models, I test whether synthetic analogues of MSH are able to reduce the pro-inflammatory cytokines, IL-6 and TNF- α and bacterial replication following infection with mycobacteria.

In chapter 2, I evaluated how the inflammasome is activated during *M. tuberculosis* infection. There has been recent evidence for inflammasome hyper-activity during *M. tuberculosis* infection and immune reconstitution syndrome following anti-retroviral treatment initiation (Tadokera et al. 2011; Lai et al. 2015) therefore using *in vitro* systems, I tested whether inflammasome blockade using both genetic and pharmacological inhibition would dampen IL-1 β activity and also have any influence on bacterial replication.

Examining the melanocortin system and the inflammasome during mycobacterial infection yielded very different consequences, with the latter providing an exciting platform for the future of HDT (see figure 1.4).

Chapter 2

The melanocortin system

The first approach to investigating HDT was conceived on the back of genomic studies suggesting an association between melanocortins and TB disease susceptibility (discussed later in this chapter; Cooke et al. [2008](#)).

2.1 The melanocortin system

The melanocortin system has a role in a large number of physiological pathways, such as food intake, obesity, pigmentation and steroid neo-genesis (Todorovic et al. [2005](#)). It consists of the precursor molecule, pro-opiomelanocortin (POMC) and its derivatives, adrenocorticotrophic hormone (ACTH), the MSH family (α -, β -, γ -), the melanocortin receptors and their natural antagonists (the Agouti and the Agouti-related peptide, AGRP; Catania et al. [2010](#)).

Whilst the original school of thought believed that POMC synthesis was firmly within the domain of neurobiology we now know that POMC mRNA is expressed in immune cells and production is stimulated through endogenous mediators such as the cytokines (IL-1 β , IL-6, TNF- α) and bacterial LPS (Kariagina et al. [2004](#)). Whilst IL-6 signals via the Janus kinase family and transcription factors of the STAT family (Heinrich et al. [1998](#)), TNF- α signals via activation of the IKK/NF- κ B pathways (Scarlett et al. [2007](#); Purkayastha et al. [2011](#)). Signalling either way leads to the transcription of POMC. POMC contains 3 domains — γ -MSH at the amino-terminal end, the central ACTH₁₋₃₉ and β -lipotropin at the carboxy-terminus (Getting et al. [2006](#)). The structure lends itself to extensive post-translational modifications where differential expression of serine proteases called pro-hormone convertases (PC) mediate the production of specific melanocortin proteins (Catania et al. [2010](#)), the nature of which depends on the tissue. PC1 cleaves POMC into ACTH₁₋₃₉, γ -MSH and β -lipotropin.

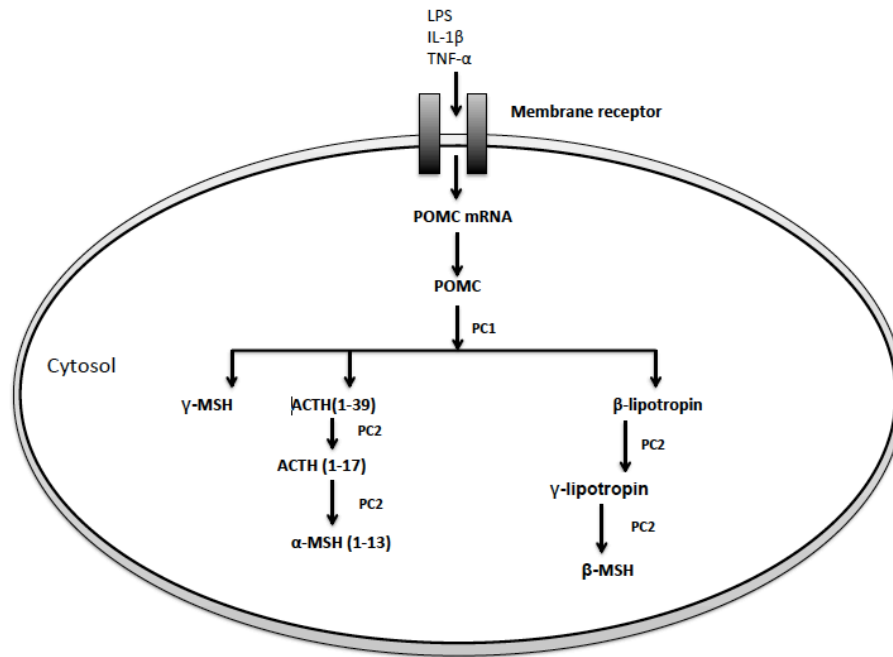


Figure 2.1: Receptor-ligand interaction is negatively regulated by the transcription and translation of POMC
 Production of POMC is stimulated via a G protein-coupled mechanism, in response to bacterial LPS and pro-inflammatory cytokines, such as IL-1 β and TNF- α . The precursor POMC protein undergoes extensive post-translational modifications, which yield several active peptides. The relative predominance of these peptides depends on the intracellular expression of precursor convertases (PC1 and PC2). PC1 cleaves POMC into γ -MSH, adrenocorticotropin (ACTH₁₋₃₉) and β -lipotropin. PC2 cleaves ACTH₁₋₃₉ into ACTH₁₋₁₇ and β -lipoprotein into β -MSH. The roles of β -lipotropin and β -MSH are not discussed here.

MCR subtype	Ligand affinity	Immune tissue expression
MC1R	α -MSH $\geq$ ACTH $\gg$ γ -MSH	Macrophage/monocyte, peripheral-blood, derived dendritic cells, lymphocytes, neutrophils
MC2R	ACTH	Lymphocytes
MC3R	γ -MSH = ACTH $\geq$ α -MSH	Macrophages, B-lymphocytes
MC4R	α -MSH = ACTH $>$ γ -MSH	Neurons, glial cells, astrocyte (phagocytic capable) glial cells
MC5R	α -MSH = ACTH $>$ γ -MSH	Thymus, B-lymphocytes and T-lymphocytes

Table 2.1: Biological distribution and agonist profiles of the MCRs in immune tissue cells. Adapted from (Getting 2006)

Since α -MSH comprises the first 13 amino acids of ACTH, PC2 cleaves ACTH into α -MSH; PC2 also cleaves β -lipotropin into β -MSH (Getting et al. 2006, see figure 2.1). MSH peptides all have in common a core four amino acid sequence — His-Phe-Arg-Trp (HFRW), which is contained in the 6-9 sequence of α -MSH (Singh et al. 2014).

The melanocortins bind to their cognate receptors; five melanocortin receptors (MC1R–MC5R) have thus far been cloned. The MCRs belong to the family of G protein-coupled, seven-transmembrane receptors (GPCR) and their function depends largely on their tissue distribution (table 2.1). The N-terminal extracellular loops confer ligand affinity (Chhajlani et al. 1996). The C-terminal tail is responsible for its translocation from the endoplasmic reticulum to the plasma membrane (Wolf Horrell et al. 2016). All receptors aside from MC2R are expressed in brain and immune cells and bind MSH and ACTH (Singh et al. 2014).

2.1.1 MCR activation and the signalling response

Despite their close relationship with corticosteroids, melanocortins exert a potent inhibition on pyresis and inflammatory signalling independent of steroids (Huang et al. 1998). *In vivo* evidence shows this is mediated by central expression of melanocortin receptors which synapse peripherally and extra-neural release of α -MSH that serves to down-regulate the pro-inflammatory response (Lipton et al. 1991; Huang et al. 1998).

Several receptor subtypes may be present in varying concentrations in individual cells and tissues (Catania et al. 2004) but basal levels tend to be quite low. The expression of MCRs is up-regulated in response to pro-inflammatory stimuli, such as LPS and pro-inflammatory cytokines (Bhardwaj et al. 1997). Furthermore, TNF- α can act on macrophages to induce synthesis of α -MSH which can in turn act on MC1R in an autocrine manner to modulate the production of nitric oxide (Star et al. 1995) and TNF- α (Taherzadeh et al. 1999). MC1R and MC3R are both expressed on macrophages (Li et al. 2008) and MC1R in particular is widely distributed and has been identified as a potent immunomodulator when activated. Human MC1R binds α -MSH and ACTH with relatively equal affinity; the mouse MC1R recognises only α -MSH as its agonist, thus α -MSH and ACTH are deemed the natural agonists for MC1R (Suzuki et al. 1996).

As the MCRs are up-regulated rapidly, GPCRs must be kept at acceptable levels to prevent over-stimulation. GPCR signalling itself is regulated by a process called desensitisation, where they may be internalised for lysosomal degradation to avoid prolonged activation (Pierce et al. 2001). Whilst this has not been investigated in other MCRs, MC4R undergoes such a phenomenon — following brief agonist exposure, phosphorylation of the C-terminal tail leads to internalisation facilitated by β -arrestins (Shinyama et al. 2003).

α -MSH has a high affinity for MC1R and as such most of the immune studies regarding MSH are in the context of α -MSH–MC1R signalling. Since MC1R and MC3R are well distributed on peripheral immune cells and hence arguably the most significant in deciphering the role of melanocortins in peripheral immune signalling, I shall be concentrating on these two receptors. Alternatively spliced variants of the *MC1R* gene may occur in response to different environmental stimuli, although the physiological consequences of these various transcripts have yet to be determined (García-Borrón et al. 2014). MC1R expression in immune cells requires picomolar concentrations of α -MSH to be activated compared to nanomolar concentrations in other tissues (Suzuki et al. 1996; Kalden et al. 1999; Scholzen et al. 2000) suggesting a strong role in immune regulation. For example, colonic inflammation secondary to infection with *Citrobacter rodentium* was eventually fatal in *MC1R*^{-/-} compared to wild-type mice (Maaser et al. 2006). Furthermore, antibodies against MC1R reduced

the inhibition of TNF- α by α -MSH in LPS-treated macrophages (Taherzadeh et al. 1999).

Haemoxygenase-1 (HO-1) is an enzyme that catabolises haem and, providing that the catabolites are rapidly sequestered, has a potential cytoprotective effect during the massive waves of cellular inflammation (Wagener et al. 2003). *In vitro* and *in vivo* treatment with the MC3R agonist, MT11, leads to a temporal induction of HO-1 and *in vivo* leads to a reduction in polymorphonuclear neutrophil migration following monosodium urate treatment (Lam et al. 2005). ICAM-1, VCAM-1 and E-selectin are responsible for cell recruitment but can also mediate vessel damage when activated in excess (Scholzen et al. 2003). α -MSH acting on Human microvascular endothelial cells expressing MC1R also reduces LPS-induced transcription of the adhesion molecules and *in vivo* reduces vascular oedema and haemorrhage by down-regulating the expression of adhesion molecules (Scholzen et al. 2003). In addition to the *in vivo* work, an *ex vivo* study of plasma comparing patients admitted with sepsis syndrome and septic shock to controls showed that plasma concentrations of α -MSH were reduced in the early phases of sepsis and addition of α -MSH to LPS-stimulated whole blood inhibited TNF- α and IL-1 β , suggesting that α -MSH might make a reasonable biomarker for disease severity (Catania et al. 2000).

In addition to the full length peptide, truncated forms — the C- (MSH₁₁₋₁₃; consisting of lysine, proline, valine; KPV) and the N-terminal (MSH₄₋₁₀) domains — of β -MSH have been identified as being able to interfere with MCR signalling, although they require higher concentrations for efficacy, compared to the full length version (Haycock et al. 2000). MSH₄₋₁₀ is identical to ACTH₄₋₁₀ and can reduce neutrophil migration and accumulation in murine experimental gout (indeed, abrogation of neutrophil recruitment is a principal feature of melanocortin agonists; Getting et al. 1999). KPV reduced IL-8 and attenuated weight loss during experimental colitis *in vivo* (Kannengiesser et al. 2008) and reduced release of IL-8, TNF- α and IL-1 β (in a caspase-independent manner) following stimulation by monosodium urate crystals in human macrophages (Capsoni et al. 2009). It has been suggested that the anti-inflammatory effects of KPV are not due to a direct interaction with the MCR as it does not contain the core HFRW domain — this might explain the need for higher concentrations in the Kannengiesser et al. 2008 study and the fact that whilst it can modulate immune cells *in vivo*, *in vitro* there are some reports proposing that it does not alter pro-inflammatory signalling mediated by MCR, but rather an unidentified mechanism (Wikberg et al. 2000; Getting et al. 2003).

NF- κ B signalling

The effects of α -MSH are mostly through the regulation of the transcription factor NF- κ B response (see figure 2.2). α -MSH reduces NF- κ B activity in a dose-dependent manner (Chen et al. 2013). As with all GPCRs, ligand binding results in the dissociation of the G $_{\alpha}$ subunit from the stable $\beta\gamma$

complex; G_{α} binds to adenylyl cyclase, to catalyse the formation of cyclic adenosine monophosphate (cAMP) from ATP (Serezani et al. 2008). cAMP is degraded by phosphodiesterases to keep the activity of the secondary messenger in check (Serezani et al. 2008). It is the potent pleiotropic effects induced downstream of cAMP activation that are largely responsible for the immunomodulatory effects. For example, cAMP binds and activates protein kinase A (PKA). PKA can directly inhibit the activation of NF- κ B, by preventing its translocation to the nucleus (Minguet et al. 2005) or it may phosphorylate target proteins such as the transcription factor cAMP response element-binding protein (CREB; Serezani et al. 2008). CREB binds its co-factor, CBP (CREB-binding protein), which can then stimulate transcription through chromatin modification (Wen et al. 2010). However, CPB also acts as a co-activator of the NF- κ B subunit, p65 (Gerritsen et al. 1997). Thus, activation of the PKA pathway can indirectly inhibit NF- κ B by activation of CREB which competes with p65 for limited quantities of CPB (Parry et al. 1997) and pharmacological blockade of either PKA or NF- κ B can reverse this inhibitory effect (Manna et al. 1998). The downstream result of both pathways is an inhibition of pro-inflammatory cytokine production and enhanced anti-inflammatory responses (Getting et al. 2006). In the context of melanocortin signalling, α -MSH was observed to inhibit nitric oxide (NO) production following LPS stimulation, through a NF- κ B nuclear cAMP/PKA-dependent and -independent manner (Mandrika et al. 2001) α -MSH also reduces LPS-induced IL-6 and TNF- α release in RAW 264.7 macrophages and is associated with increased intracellular cAMP (Chen et al. 2013).

MAPK signalling pathways

The mitogen-activated protein kinase (MAPK) pathways play a crucial role in generating an immune response. They consist of a core of least three protein kinases, where kinases downstream are phosphorylated by upstream kinases — a MAPK kinase kinase (MAP3K) is at the start of the pathway and is activated further upstream by TLR or IL-1R ligation (Arthur et al. 2013). MAP3K activates a MAPK kinase (MAP2K) and this in turn activates the MAPK (Arthur et al. 2013). The classical MAPKs in the third tier are divided into 'ERK1 and ERK2', the 'p38 MAPK' and the Jun N-terminal family of kinases (JNK1,2,3 Arthur et al. 2013). Activation of the MAPKs, including p38, leads to enhanced pro-inflammatory cytokine signalling and an increased M1 macrophage phenotype (Arthur et al. 2013). However, phosphorylation of p38 α , one of the p38 family, is paradoxically able to limit the inflammatory responses in addition to activating them, through activation of the mitogen-and stress-activated kinases, which leads to enhanced IL-10 and IL-1R antagonism (Risco et al. 2012). This is interesting, because MCR signalling can occur through the MAPKs. MC3R activation can also lead to the rapid phosphorylation of the p38 MAPKs and is mediated by PKA, without affecting ERK1/2 or JNK signalling (Lam et al. 2006). Similarly, α -MSH can also up-regulate

the phosphorylation of JAK2 and ERK1/2 leading to STAT3 activation following cardiac ischaemia which results in a reduction of TNF- α and HO-1 (Ottani et al. 2013, see figure 2.2). Conversely, other studies examining the effect of α -MSH show that it can suppress LPS-induced phosphorylation of p38 MAPK (Yoon et al. 2003; Li et al. 2008). Quite why these differences are seen is unknown, given that the studies by Lam et al. 2006 and Li et al. 2008 were performed in the same cell lines, and has not been addressed. It is possible that a temporal effect may be seen, where following the initial stimulus, phosphorylation of p38 is induced, which may serve to partially regulate inflammatory pathways, but following chronic stimulation, p38 is not activated at all in order to attenuate the inflammation further. Deactivation of p38 is seen *in vivo*, where treatment with α -MSH rapidly reduced kidney and lung NF- κ B, p38 and JUN MAPK activation induced by renal ischaemia (Deng et al. 2004), also suggesting that melanocortins can operate via a number of signalling pathways.

In addition to anti-inflammatory signalling, there are reports that MC3R mediates inflammatory resolution as a whole. Agonists to MC3R have been observed to enhance phagocytosis of apoptotic neutrophils (efferocytosis) *in vivo* (Montero-Melendez et al. 2011) and zymosan particulates (Montero-Melendez et al. 2015) and augmented intake of apoptotic neutrophils was absent in mice lacking either MC1R or MC3R (Montero-Melendez et al. 2011; Montero-Melendez et al. 2015). In the latter study, MC3R agonists were shown to induce ERK1/2 activation in preference to cAMP increase and promotion of efferocytosis was mediated by ERK1/2 not cAMP (Montero-Melendez et al. 2015). This is in contrast to studies showing that synthetic melanocortin peptides can reduce phagocytosis (Ji et al. 2013), in which melanocortin treatment was associated with a rise in intracellular cAMP. The reasons for these differences have not been explored, but it is interesting to note the signalling pathway that is utilised in both outcomes: cAMP, is a known negative regulator of phagocytosis (Peters-Golden 2009), so perhaps the coordinated increase of cAMP by melanocortin peptides in specific cell lines, following varying stimuli may oppose phagocytosis. A holistic approach looking at the varying stimuli, the intracellular pathways undertaken and the presence of phagocytosis is needed in order to learn more about the clinical applicability of melanocortin peptides.

2.1.2 Melanocortins and their effect on infection

As a relatively small, 13-amino acid peptide, α -MSH has gained attention as a potential anti-microbial peptide (AMP). Cathelicidin LL-37 is a well known AMP, and its expression is enhanced by vitamin D(3) during infection with *M. tuberculosis* which is associated with increased phagocytic killing (Gombart 2009). The additional anti-inflammatory effects exerted *in vitro* and *in vivo* by melanocortins has led researchers to examine whether they are able to act in a similar manner to AMPs and harbour direct anti-microbial activity. Indeed, there are a few reports suggesting it does have an effect.

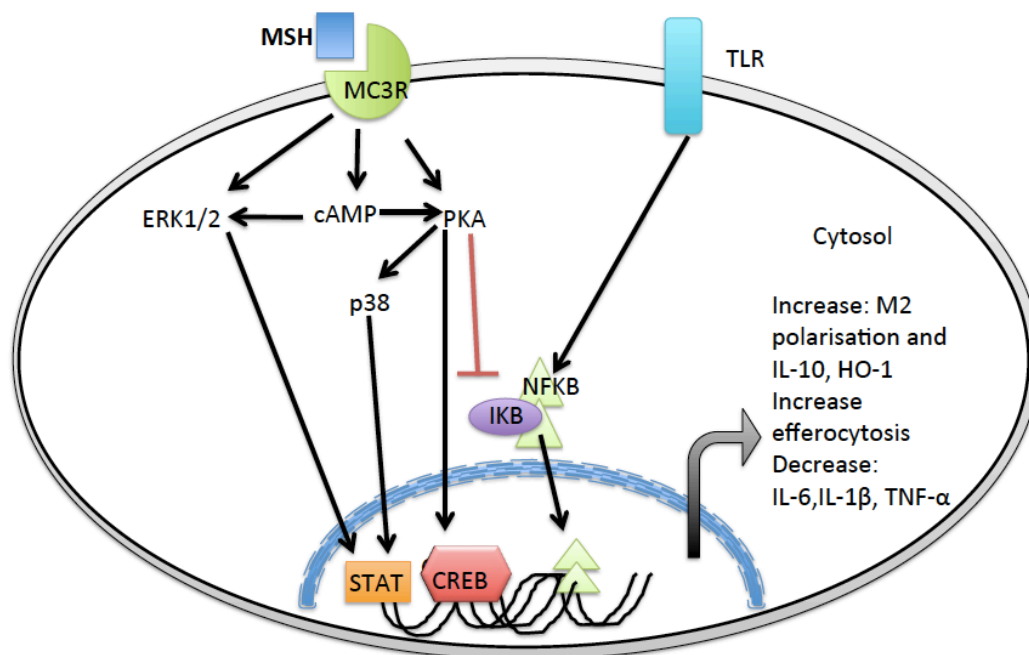


Figure 2.2: Known signalling pathways mediated by MSH-MCR interaction during immunomodulatory activity MSH binds to the G protein-coupled receptor, MCR. MCR transmits the signal to adenylyl cyclase to catalyse the conversion of ATP to cAMP. This activates PKA which translocates to the nucleus where it phosphorylates CREB to initiate gene transcription. PKA may also inhibit the translocation of NF-κB to the nucleus. Whilst cAMP-PKA is the most well observed pathway during MCR activation, ERK1/2 has also been reported following MC3R activation in a manner that is both dependent and independent on cAMP. Another MAPK, p38 is may also activated following PKA phosphorylation. Both p38 and ERK1/2 activate the transcription factor, STAT3. Activation of both CREB and STAT3 following ligand interaction leads to down-regulation of pro-inflammatory signals and increases anti-inflammatory responses.

Madhuri et al. 2009 showed that 2-hour treatments with α -MSH exhibited anti-bacterial activity against *Staphylococcus aureus* at nanomolar concentrations; this was associated with membrane permeabilisation, which suggests membrane damage. A similar dose-dependent reduction in *S. aureus* and *Escherichia coli* bacterial survival was observed (Charnley et al. 2008), although this study used a modified version of α -MSH and the effect on *S. aureus* bacterial numbers remained low 6 hours after incubation. α -MSH also acted synergistically with gentamicin and ciprofloxacin to reduce minimum bactericidal concentrations following incubation with methicillin-resistant *S. aureus*; this was associated with increased membrane permeabilisation, and a reduction in DNA and protein synthesis with little effect on RNA synthesis (Singh et al. 2013). Unfortunately, there was no synergism with rifampicin, which makes the clinical significance of this questionable, given that few rigorous high-powered trials have been undertaken looking at the efficacy of synergistic gentamicin in relevant infections (such as infective endocarditis) (Sexton et al. 1998). Nonetheless, the *in vitro* effect is there.

Rather strikingly, full length and KPV α -MSH were able to reduce *Candida albicans* growth and germ tube formation with similar inhibitory activity to equimolar fluconazole and this was associated with increased cAMP formation in the yeast (Cutuli et al. 2000). I find how the increased cAMP relates to

increased killing curious, given that loss-of-function *Candida PDE2* mutants lead to an increase in cAMP and subsequent increased filament formation (Inglis et al. 2013) and disruption of the cAMP signalling pathway can increase the virulence of *C. neoformans* (Bahn et al. 2007), and thus the presence of cAMP maybe an unrelated phenomenon. Cutuli et al. 2000 also examined whether α -MSH enhanced killing of *C. albicans* by neutrophils and an effect was observed but at larger millimolar concentrations of the peptide. In a different study, testing out variants of the α -MSH structure, the peptide bearing the HFRW motif displayed moderate anti-fungal activity against *Cryptococcus neoformans* (Masman et al. 2006). *In vivo*, a modified version of KPV inhibited *Candida albicans* in a rat vaginitis model, induced M2 polarisation and arginase-1 release whilst reducing TNF- α and IL-1 β release/production. It also inhibited phagocytosis induced by IFN- γ , an effect which was reduced in MC1R siRNA knock-down *in vitro* (Ji et al. 2013). Therefore α -MSH, when called upon, may promote tissue reconstruction, dampens the early infection response and microbicidal activity is reported to be mediated by increasing cAMP (Cutuli et al. 2000; Ji et al. 2013) although it is difficult to reconcile how this might be the case. The effects on *Candida* are not equivocal however; Rauch et al. 2009 examined the direct effect of α -MSH over a range of concentrations and *C. albicans* did not exhibit any growth defect.

Given, some degree of capability of α -MSH and its variants against the aforementioned infectious diseases, does the melanocortin system have a role to play in *M. tuberculosis*? Genome-wide linkage studies have been employed to identify a susceptibility locus for a disease, in which genes have not been previously implicated. To examine for a putative disease susceptibility locus, Cooke et al. 2008 used a two-staged approach. Firstly, genome-wide screens were performed to identify putative markers from sibling-pairs from families with *M. tuberculosis* in South Africa. Secondly, these markers were then mapped onto the genomes of a Malawian population, which indicated one genomic region 20q13.31–33, which contains the MC3R gene, as being linked to TB susceptibility. A screen of 40 single-nucleotide polymorphisms (SNPs) in an independent Gambian population showed disease association conferred by rs3827103 (MC3R) and the AA genotype provided some protection against TB disease in this population. An independent case-control study in South Africa found the same SNP implicated with the SNP close to the N-terminal end of the protein (Adams et al. 2011). A different study looking at the a population in Uganda replicated this finding (Stein et al. 2008). Whilst the SNPs did not provide any insight into whether they conferred a functional change, it provided the background for the first project looking at whether melanocortins could modulate mycobacteria.

Peptide	MC1R	MC3R	MC4R	MC5R
ACTH (1-39)	2.5	87	690	17,000
α -MSH	0.12	31	660	5700
[Nle4,D-Phe7] α -MSH	0.085	0.40	3.8	5.1
γ -MSH	2.7	7.1	29,000	43,000
Melanotan II	0.67	34	6.6	64

Table 2.2: K_i values in nM of natural and synthetic agonists for the indicated melanocortin receptors expressed in COS cells were determined by direct competitive inhibition with [^{125} I]NDP-MSH using a radioligand binding assay. Adapted from Wikberg 1999.

2.1.3 Pharmacological MSH agonists

The natural α -MSH sequence (Ac-Ser-Try-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂) contains some important points concerning potency. Firstly, the N-acetyl and carboxamide groups are important for potency; the core peptide sequence, HFRW, contained within α -MSH has been identified as the pharmacophore responsible for binding affinity and its potency (Hruby et al. 1993). α -MSH is highly potent (see table 2.2) against MC3R and in particular MC1R, however the practical use of α -MSH is limited because its plasma half-life is very short (only a few minutes) *in vivo* (Lipton 1990). This may be due to the fact that the 4th Met residue (Met4) may be oxidised and confer reduced potency (Hruby et al. 1993) and is also likely to be due to proteolytic degradation, by chymotrypsin and endopeptidases (Deschodt-Lanckman et al. 1990). Stability issues have been addressed by changing the amino-acid sequence and introducing unnatural amino acids into the structure. One example of modification is replacement of Met4 in the original sequence with norleucine (Nle) and substituting Phe-7 with its D-isomer, providing the generic name, [Nle4,D-Phe7]- α -MSH (NDP- α -MSH) (Grieco et al. 2003). Making this modification increases potency compared to α -MSH (see 2.2) and the D-Phe substitution seems to increase resistance to proteolytic degradation (Hruby et al. 1993). As such, this compound has been used in research in both *in vitro* and *in vivo* models for nearly 3 decades. However, the length of these peptides was deemed difficult to market for clinical use and [Nle4,D-Phe7] α -MSH underwent further modification — truncating the compound to a heptapeptide, but keeping the core HFRW and the Nle4, D-Phe7 residue substitutions (Getting 2006). This was found to have equal efficacy and was under development as a candidate for female sexual dysfunction but marketing and development completely ceased by 2017 due to the non-selectivity and unacceptable side effects of the agonist (Nelson et al. 2012).

The C-terminal peptide, KPV is also able to induce anti-inflammatory activity, which is surprising given that it does not contain the core motif so it has been suggested that it may signal via an alternative route rather than the MCR (Catania et al. 2006).

Since, as listed above, there was evidence that α -MSH can induce anti-microbial activity (Cutuli et al. 2000; Madhuri et al. 2009) and exhibits anti-inflammatory tendencies at high potency, I chose this

peptide along with [Nle4,D-Phe7] α -MSH to examine whether these had any effect on mycobacterial growth and any associated changes in cytokine release.

2.2 Methods

2.2.1 Ethics statement

All experimental protocols complied with Home Office regulations (guidance on the operation of Animals, Scientific Procedures Act, 1986) and passed the Imperial College Ethics committee.

2.2.2 Bacterial culture

$\Delta leuD \Delta panCD$ Severely Attenuated *M. tuberculosis* (denoted as SAM Mtb from here on in; donated by S. Sampson (Sampson et al. 2004)) and the laboratory mycobacterial reference strain, H37Rv, were used in this work. Liquid cultures were grown in Middlebrook 7H9 media supplemented with 10 % oleic acid-albumin-dextrose-catalase (OADC, BD), 0.05 % Tween-80 (Sigma), 0.2 % glycerol (VWR) at 37 °C on a rotating incubator to mid-log phase, harvested and frozen in aliquots at –80 °C. Determination of colony forming units per ml (CFU/ml) for growth curve measurement were obtained by plating the bacteria at serial dilutions on Middlebrook 7H11-agar plates supplemented with 10 % OADC and 0.5 % glycerol. Estimated CFU/ml from the growth curves were subsequently used to estimate the inoculum for both *in vitro* and *in vivo* experiments. Solid and liquid cultures for SAM Mtb were supplemented with 24 $\mu\text{g ml}^{-1}$ pantothenate (Sigma), 50 $\mu\text{g ml}^{-1}$ hygromycin (Invitrogen) and 20 $\mu\text{g ml}^{-1}$ leucine (Sigma).

2.2.3 Cell culture

RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Sigma), supplemented in 10 % heat-inactivated fetal calf serum (Labtech) and 2 mM L-glutamine (Sigma) in a humidified atmosphere, supplied with 5 % CO₂ and 95 % air, until 80 % confluency was attained for use. Cells were seeded in 24-well plates at 3×10^5 cells/well for all experiments.

2.2.4 Macrophage infection

Bacteria were grown to mid-late log phase and spun at 3000 rpm for 10 minutes. The supernatant was removed and the pellet washed in PBS–Tween-80 0.05 %; the cells were spun and washed twice

more and finally re-suspended in PBS. The OD was taken and used to calculate the estimated CFU/ml. Following overnight drug treatment, cells were washed with pre-warmed DMEM in order to remove drug remnants and then infected with mycobacteria (SAM Mtb) at a multiplicity of infection (MOI) of 1:1 for 4 hours. Experiments were performed with cultures having an OD of 0.5-0.8 which corresponded to the mid to late log growth phase. Using an OD $0.6 \simeq 5 \times 10^7$ cells/ml, the required MOI was calculated.

After 4 hours, extracellular bacteria were removed by washing with pre-warmed DMEM. Additionally, cells were lysed and plated for CFU in order to detect the quantity of bacteria phagocytosed. Infected macrophages were maintained in cell culture medium at 37 °C and 5 % CO₂ for 3 days. At 24 and 48 hours, cell supernatants were harvested and spun at $10,000 \times g$ for 5 minutes using a 0.22 µm filter (Corning Costar Spin-X centrifuge tube filters) and frozen at -20 °C for ELISA experiments.

2.2.5 Colony counting

Following the indicated time-points for experiments, cells were lysed with 200 µl of distilled sterile water. After mixing, 20 µl was diluted with 180 µl PBS-Tween-80 0.05 % and further serially diluted by 1 in 10 dilutions. 20 µl of the serial dilutions were then plated in triplicate onto pre-made Middlebrook 7H11 agar and colony counting was performed for all dilutions between 2 and 3 weeks after infection. In the same experiment, colony counts were estimated from the same dilution where possible in order to minimise pipette variation.

2.2.6 Drug treatment

Initial experiments examined the effect of pre-treating RAW 264.7 cells with either α-MSH or NDP-α-MSH (a synthetic analogue of α-MSH) (all obtained from Tocris, 2584 and 2013 respectively) at incremental concentrations prior to mycobacteria infection. In subsequent experiments, the experimental design was modified slightly in order to determine whether MSH analogues would alter outcome in a post-infection set up. Therefore after cell seeding, the cells were infected as previously and following washing of extracellular bacteria, cells were treated with MSH at varying concentrations, which was left on during the remainder of the experiment, rather than as a pre-incubation treatment. Lipopolysaccharide, LPS (L7770, Sigma) and Pam₃CSK₄ (tlrl-pms, Invivogen) both at 10 ng ml⁻¹ were used as a positive controls for ELISA experiments.

2.2.7 Cytokine detection by Enzyme-linked immunosorbant assay (ELISA)

Mouse TNF- α and IL-6 were quantified using commercially available ELISA kits (e-Biosciences) and the method was followed according to manufacturer's instructions.

For all ELISA protocols, flat-bottomed 96-well ELISA plates (Nunc MaxiSorp 442404) were used. Wells were coated with 50 μ l capture antibody (250 $\times$) diluted in coating buffer and left overnight 4 $^{\circ}$ C. The following day, the wells were washed using a manual plate washer 5 times in 200 μ l PBS–Tween-80 0.05 % (washing buffer). Non-specific binding sites were then blocked with 200 μ l 1 $\times$ assay diluent for 2 hours then washed again in washing buffer 5 times. The standard was prepared according to the protocol and concentrations ranged from 8–1000 pg ml $^{-1}$. 2-fold serial dilutions of the top standard were performed in duplicate in order to make a standard curve of 8 points. 50 μ l of undiluted sample was added to the appropriate wells. The plate was sealed with a lid and incubated at room temperature for 2 hours after which all the samples were removed and the plate was washed in washing buffer for 5 times. 100 μ l of detection antibody (250 $\times$) diluted in 1 $\times$ assay diluent was added to the wells for 1 hour. The samples were removed and washed again in washing buffer. 100 μ l of Avidin-HRP (250 $\times$) diluted in 1 $\times$ assay diluent was added to the wells and the plate was incubated for 30 minutes at room temperature. The plate was washed again for a final rinse, 5 times and 100 μ l 1 $\times$ TMB solution was added to the wells. The plate was incubated at room temperature for 15 minutes. 50 μ l of pre-made 1M H $_3$ PO $_4$ was added to each well and the plate was read at 450 nm. Final concentrations were ascertained using the standard curve.

2.2.8 *In vivo* effect of NDP- α -MSH following infection with H37Rv: Early versus Late treatment

This was a pilot experiment which would corroborate the *in vitro* findings thus there was no negative control and the time-scale for infection and organ harvest was shorter than that traditionally seen during *in vivo* mice work (35 days). The α -MSH analogue, NDP- α -MSH, was used because of previously documented increased stability *in vivo* (Sawyer et al. 1980). 46 Female 6- to 8-week-old Balb/C mice (body weight $\approx$ 20g) were used for these experiments. The groups of animals were kept within individually ventilated cages. Each mouse was challenged intra-nasally with 1 $\times$ 10 ^{4} CFU/ml H37Rv and were allocated into 3 groups;

A: Untreated (total 23 mice)

B: Early treatment starting at day 1 post-infection (total 10 mice)

C: Late treatment starting at day 21 post-infection (total 10 mice)

At the designated time-points, the lungs and/or spleen were aseptically harvested for CFU/organ measurements. Drug treatment with NDP- α -MSH was administered in the treatment groups (B and C) at their allocated times.

Day 0; Infection with 1×10^4 CFU/ml H37Rv

Day 1; Lung harvesting of 6 mice.

EARLY TREATMENT: 10 mice in Group B were treated daily with for 6 days with intra-tracheal NDP- α -MSH (10 μ g reconstituted in 100 μ l PBS). The drug concentration was based on a previous paper by Doi et al. [2008](#).

Day 8; Finish treatment for Group B

Day 10; Harvest 5 mice each from Group A and Group B for lungs and spleens

Day 15; Harvest 5 mice each from Group A and Group B for lungs and spleens

Day 21; LATE TREATMENT: 10 mice in Group C were treated daily with for 6 days with intra-tracheal NDP- α -MSH (10 μ g reconstituted in 100 μ l PBS)

Day 27; Premature end to experiment due to profound weight loss. All mice in Group A and Group B sacrificed and harvested for lungs and spleens.

2.2.9 Preparation of lung and spleen samples for bacterial load

At the indicated time-points post-infection, bacterial burden was determined in the spleen and lungs. Lungs and spleen were aseptically removed, homogenised in PBS containing 0.05 % Tween-80 and filtered through a 100 μ m membrane. Serial dilutions of the organ homogenates were plated. The number of colony forming units (CFU) were enumerated 14–21 days later. I was kindly assisted in these experiments by Dr. Krishnan and Dr. Robertson.

2.2.10 Statistical analysis

In all cases, each biological replicate was performed with 3 technical replicates. Only independent means were analysed by statistical methods. Unless otherwise stated, 3 biological replicates were performed (denoted as 'n' in figure legends). Statistical analysis was performed using GraphPad Prism 7. Parametric data were analysed using a two-tailed unpaired *t*-test or paired when data was matched, or One Way Analysis of Variance (ANOVA) for three or more groups followed by either Tukey's post-hoc analysis or Dunnet's multiple comparison test (the choice of which depended on

whether a known control was being compared against). The results in chapters 2 and 3 state both the t -value (if a t -test is done) or the F -value (if an ANOVA is done) together with their corresponding p -values. The t - and F -value give a standardised quantification of the difference between groups without making any assumptions about the distribution of the data.

2.3 Results: The melanocortin system as a potential for host-directed therapy

2.3.1 Aims

1. To ascertain whether α -MSH and related analogues are able to reduce bacterial replication in *in vitro* models.
2. To ascertain whether α -MSH and related analogues are able to dampen pro-inflammatory activity in *in vitro* models.
3. To determine whether NDP- α -MSH, a derivative of α -MSH, is able to reduce bacterial replication *in vivo*.

2.3.2 Identifying a role for α -MSH as an immunomodulator during *in vitro* infection with severely attenuated mycobacteria

α -MSH does not affect bacterial replication following macrophage infection with SAM Mtb

Since I was using an attenuated strain of *M. tuberculosis* that was doubly auxotrophic for leucine and pantothenate whilst retaining the RD-1 regulon (Sampson et al. 2004), I wanted to ensure SAM Mtb replication had the capacity to be inhibited by rifampicin in the same manner as *M. tuberculosis*. Therefore, following infection with SAM Mtb, I treated the cells with 0.6 mg l⁻¹ rifampicin, approximately 10-fold greater than the MIC for rifampicin against H37Rv calculated previously (Barrow et al. 1998) to ensure that the strain behaved appropriately in response to treatment. Treatment with rifampicin led to a visible reduction in bacterial replication at both 24 and 48 hours following infection (see figure 2.3) and this would serve as the positive control.

α -MSH has previously been shown to exhibit anti-microbial properties against *Staphylococcus aureus* and *Candida albicans* (Catania et al. 2006; Madhuri et al. 2009; Grieco et al. 2013). To ascertain whether mycobacterial growth in macrophages is modulated by MSH treatment, I first examined

the effects of physiological stimulation of the melanocortin 3 receptor (MC3R). α -MSH is a natural pan-MCR agonist with varying degrees of potency, binding to melanocortin receptors.

Having seeded RAW 264.7 macrophages overnight at a density of 3×10^5 cells/well, cells were infected with SAM Mtb for 4 hours, washed and then treated with α -MSH at varying concentrations (see figure 2.4). Concentrations of α -MSH were based on observations by Rajora et al. 1996 showing that the α -MSH-MC3R interaction leads to a bell-shaped dose response curve following relevant stimuli.

In this model, the addition of α -MSH after infection did not make any statistically significant difference to bacterial replication either at 24 or 72 hours.

However, current literature appears to favour a pre-treatment regimen with α -MSH rather than a continuous incubation. Certainly, this has previously been demonstrated to exert physiological effects (Capsoni et al. 2015; Wan et al. 2017). Therefore having seeded RAW 264.7 cells, at 3×10^5 cells/well, I treated the cells overnight with the same range of concentrations of α -MSH and infected the cells with SAM Mtb (see figure 2.5).

However, pre-treatment with α -MSH also did not lead to any significant reduction in bacterial replication.

α -MSH does not elicit changes in TNF- α following infection with SAM Mtb

Whilst α -MSH did not induce any reduction in bacterial replication, it is quite possible that it would still have ability to modulate the immune response as noted previously (Böhm et al. 2016), which would have the potential to be beneficial in reducing the tissue damage imposed by the inflammatory process. α -MSH has previously been demonstrated to reduce TNF- α and IL-6 secretion following TLR4

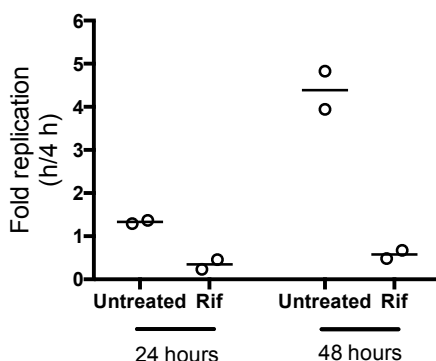


Figure 2.3: $\Delta leuD \Delta panCD$ Severely Attenuated *M. tuberculosis* is susceptible to rifampicin at 24 and 48 hours post infection. RAW 264.7 macrophages were infected with H37Rv and washed after 4 hours before being treated with equivalent DMSO concentration or 0.6 mg l^{-1} rifampicin. At 24 and 48 hours the cells were lysed and plated for CFUs. Fold replication was calculated by dividing CFUs at the given time point by CFUs at 4 hours. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 2$ biological experiments. Statistical analysis was not performed due to too few data points.

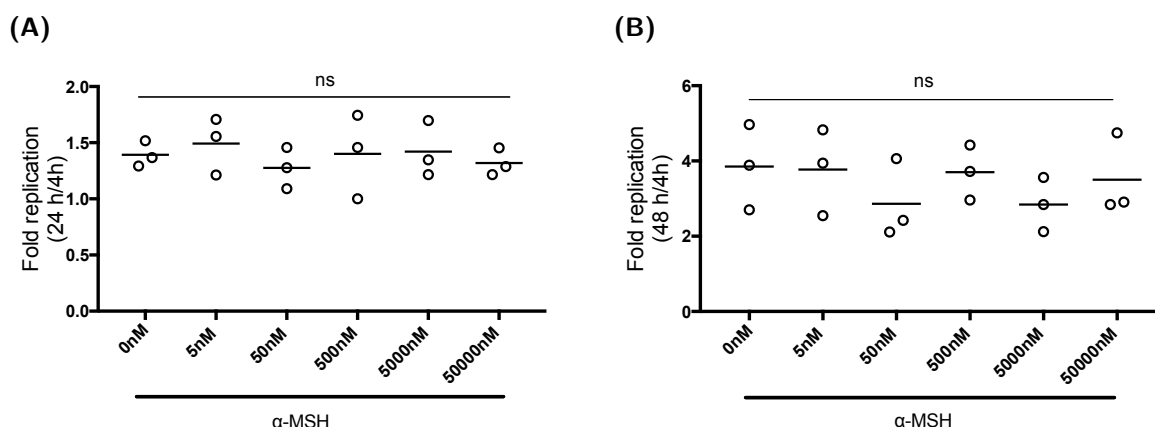


Figure 2.4: Continuous stimulation of RAW 264.7 macrophages with α -MSH does not affect bacterial replication following infection with SAM Mtb. RAW 264.7 cells were seeded overnight and infected with SAM Mtb at an MOI of 1 for 4 hours, after which cells were washed, lysed and plated for CFUs. Cells were then treated with indicated concentrations of the synthetic α -MSH. At 24 hours (A) and 48 hours (B), the cells were lysed and plated for CFUs. Fold replication was calculated by dividing CFUs at the given time point by CFUs at 4 hours. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied for the mean of each condition followed by Dunnet's multiple-comparison test to evaluate differences between the mean concentrations of the experimental drug and the untreated cells, designated as the control strain. In the graphs, significance between the experimental conditions are indicated as ns=non-significant.

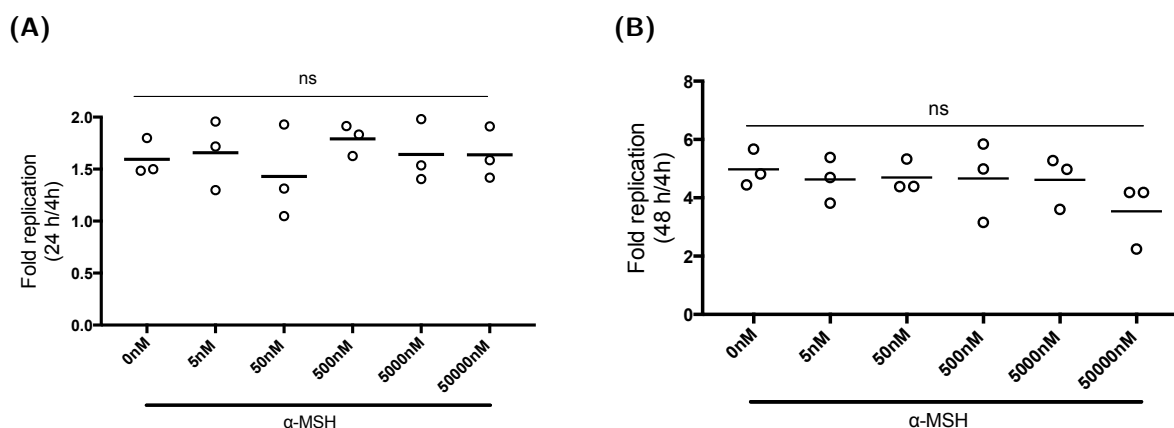


Figure 2.5: Pre-stimulation of RAW 264.7 macrophages with α -MSH does not affect bacterial replication following infection with SAM Mtb. RAW 264.7 cells were stimulated overnight with indicated concentrations of synthetic α -MSH. The following day, the cells were washed and infected with SAM Mtb at an MOI of 1 for 4 hours after which the cells were washed and left in media until plating. At 24 hours (A) and 48 hours (B), the cells were lysed and plated for CFUs. Fold replication was calculated by dividing CFU at the given time point by CFU at 4 hours. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied for the mean of each condition followed by Dunnet's multiple-comparison test to evaluate differences between the mean concentrations of the experimental drug and the untreated cells, designated as the control strain. In the graphs, significance between the experimental conditions are indicated as ns=non-significant.

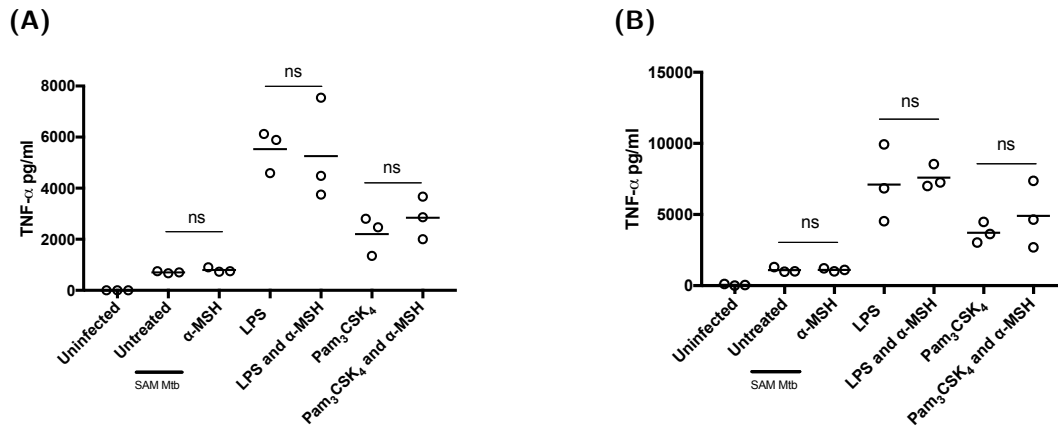


Figure 2.6: Continuous stimulation of RAW 264.7 by α -MSH does not elicit reduction in TNF- α from either SAM Mtb-infected cells or TLR4 and TLR1/2 stimulated cells. RAW 264.7 cells were seeded overnight and infected with SAM Mtb at an MOI of 1 for 4 hours after which the cells were washed. Cells were then treated with indicated concentrations of synthetic α -MSH. For the positive controls, cells were treated with either 10 ng ml^{-1} LPS or equivalent concentration of Pam₃CSK₄. At 24 hours (A) and 48 hours (B), the supernatants were removed and TNF- α concentration was determined by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. A two-tailed paired t -test was used to evaluate the difference in mean cytokine concentration between the experimental drug and the control. In the graphs, statistical significance between the experimental conditions are indicated as ns=non-significant.

activation (with LPS) and TLR1/2 activation (with Pam₃CSK₄), (Carniglia et al. 2016), therefore these were designated as the positive controls.

To ascertain whether continuous treatment with α -MSH was able to evoke any immunomodulatory change, supernatants from the cells infected with SAM Mtb or the positive controls were removed and TNF- α concentrations were determined by ELISA (see figure 2.6).

Perhaps surprisingly, continuous treatment with α -MSH did not modulate TNF- α release from any stimuli. There was no difference in the means between the LPS stimulated cells and LPS/ α -MSH treated cells at either 24 or 48 hours ($p = 0.8368$, $t = 0.218$; $p = 0.776$, $t = 0.3034$ respectively). Similarly no difference was seen in the Pam₃CSK₄ stimulated cells and Pam₃CSK₄/ α -MSH treated cells at either 24 or 48 hours ($p = 0.3841$, $t = 0.9766$; $p = 0.45$, $t = 0.8363$ respectively).

Likewise, pre-treatment with α -MSH did not lead to any reduction in TNF- α following SAM Mtb infection (see figure 2.7).

However, interestingly, pre-treatment did lead to significant reduction in TNF- α release following LPS stimulation compared to LPS stimulation alone at 24 hours ($p = 0.0005$, $t = 10.08$). At 48 hours, this effect was no longer present ($p = 0.02825$, $t = 1.24$). In a similar vein, pre-treatment also reduced TNF- α release following Pam₃CSK₄ stimulation at 24 but not at 48 hours ($p = 0.0332$, $t = 3$; $p = 0.2539$, $t = 1.331$ respectively).

The fact that the α -MSH inhibition of IL-6 and TNF- α release was induced by pre-treating the cells and was at its highest magnitude at the earlier time point (24 hours) but negated at 48 hours

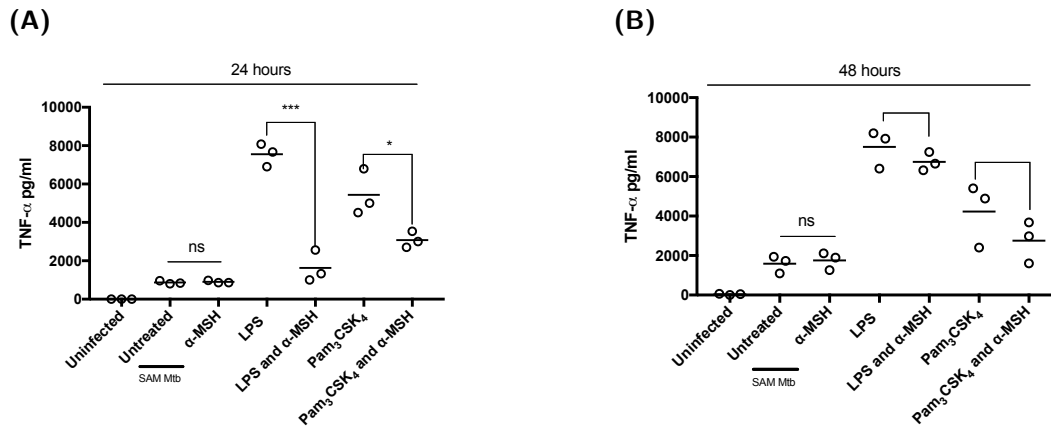


Figure 2.7: Pre-treatment of RAW 264.7 with α -MSH does not affect TNF- α release following infection with SAM Mtb. RAW 264.7 cells were pre-treated with 500 nM α -MSH overnight. Cells were then infected with SAM Mtb at an MOI of 1 for 4 hours after which the cells were washed. For the positive controls, cells were treated with either 10 ng ml⁻¹ LPS or equivalent concentration of Pam₃CSK₄. At 24 hours (A) and 48 hours (B), the supernatants were removed and TNF- α concentration was determined by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. A two-tailed paired t -test was used to evaluate the difference in mean cytokine concentration between the experimental drug and the control. In the graphs, statistical significance between the experimental conditions are indicated as * $p \leq 0.05$; *** $p \leq 0.001$; ns=non-significant.

may corroborate previous findings that the half-life of α -MSH is short (Ahmed et al. 2013). These data suggest that α -MSH does not appear to induce an effect on either bacterial replication or immunomodulatory activity following SAM Mtb infection.

Synthetic NDP- α -MSH does not affect bacterial replication or the pro-inflammatory cytokines TNF- α and IL-6 following infection with SAM Mtb

NDP- α -MSH is a semi-synthetic MSH derivative that exhibits a longer half-life and has been also previously shown to reduce TNF- α and IL-6 release following LPS stimulation (Carniglia et al. 2016). In the final set of *in vitro* experiments, I wished to eliminate short half-life as a cause for lack of efficacy by pre-treating the cells with NDP- α -MSH followed by infection with SAM Mtb (see figure 2.8). In order to determine cytokine concentrations, the experiment also included the positive controls of LPS and Pam₃CSK₄ that have previously been shown to exhibit a response following NDP- α -MSH treatment (Carniglia et al. 2016).

As previously, pre-stimulation with NDP- α -MSH did not result in reduction in bacterial replication at either 24 or 48 hours (one-way ANOVA $p = 0.6823$, $F = 0.5828$; $p = 0.8514$, $F = 0.3304$ respectively). The supernatants from this experiment were then further analysed for TNF- α and IL-6 by ELISA (see figure 2.9).

As previously, there was no difference in TNF- α ($p = 0.3798$, $t = 0.9864$ at 24 hours; $p = 0.5391$, $t = 0.6707$ at 48 hours) or IL-6 ($p = 0.8928$, $t = 0.1436$ at 24 hours; $p = 0.3299$, $t = 1.108$ at

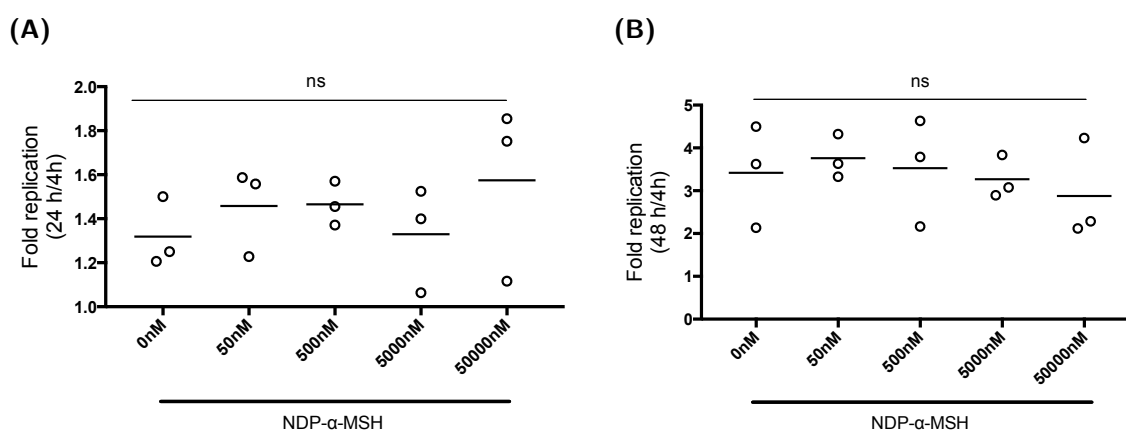


Figure 2.8: Pre-treatment of RAW 264.7 by NDP- α -MSH does not elicit reduction in bacterial replication. RAW 264.7 cells were stimulated overnight with indicated concentrations of the synthetic analogue NDP- α -MSH. The following day, the cells were washed and infected with SAM Mtb at an MOI of 1 for 4 hours after which the cells were washed and left in media until plating. At 24 hours (A) and 48 hours (B), the cells were lysed and plated for CFUs. Fold replication was calculated by dividing CFU at the given time point by CFU at 4 hours. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied for the mean of each condition followed by Dunnet's multiple-comparison test to evaluate differences between the mean concentrations of the experimental drug and the untreated cells, designated as the control strain. In the graphs, statistical significance between the experimental conditions are indicated as ns=non-significant.

48 hours) release following SAM Mtb infection. NDP- α -MSH did however lead to a statistically significant reduction of both TNF- α and IL-6 at both 24 and 48 hours suggesting that the change in efficacy could be attributed to either an increased half-life or increased pharmacological potency. The fact that the positive control responded both to α -MSH and NDP- α -MSH also indicates that ligand receptor interaction is unlikely to have been the primary issue in why there was no microbiological or pharmacological effect following treatment and infection.

2.3.3 The α -MSH analogue, NDP- α -MSH, does not confer protection to the laboratory mycobacterial strain, H37Rv, *in vivo*

In parallel with the *in vitro* experiments, I determined whether NDP- α -MSH had any impact on bacillary load *in vivo*. This was a pilot experiment; therefore no uninfected mice were used. The timing of host-directed therapy does not have any robust data behind it (Kiran et al. 2016) therefore, an early phase treatment (1 day after infection) and a late phase treatment (14 days after infection) were chosen.

Sample sizes for this were not based on a power calculation in this experiment. During *in vivo* TB studies, a difference in log CFU is often used to compare the differences between the treatment and placebo groups. Since this was a pilot study, should any differences have been established, the standard deviation and the 'effect' could have been used to ascertain power for larger scale experiments. 35 Balb/C mice in total were infected, however there were 3 unaccountable deaths in the late treatment group. The mice in the early treatment group were given 6 days of intra-tracheal injections and were

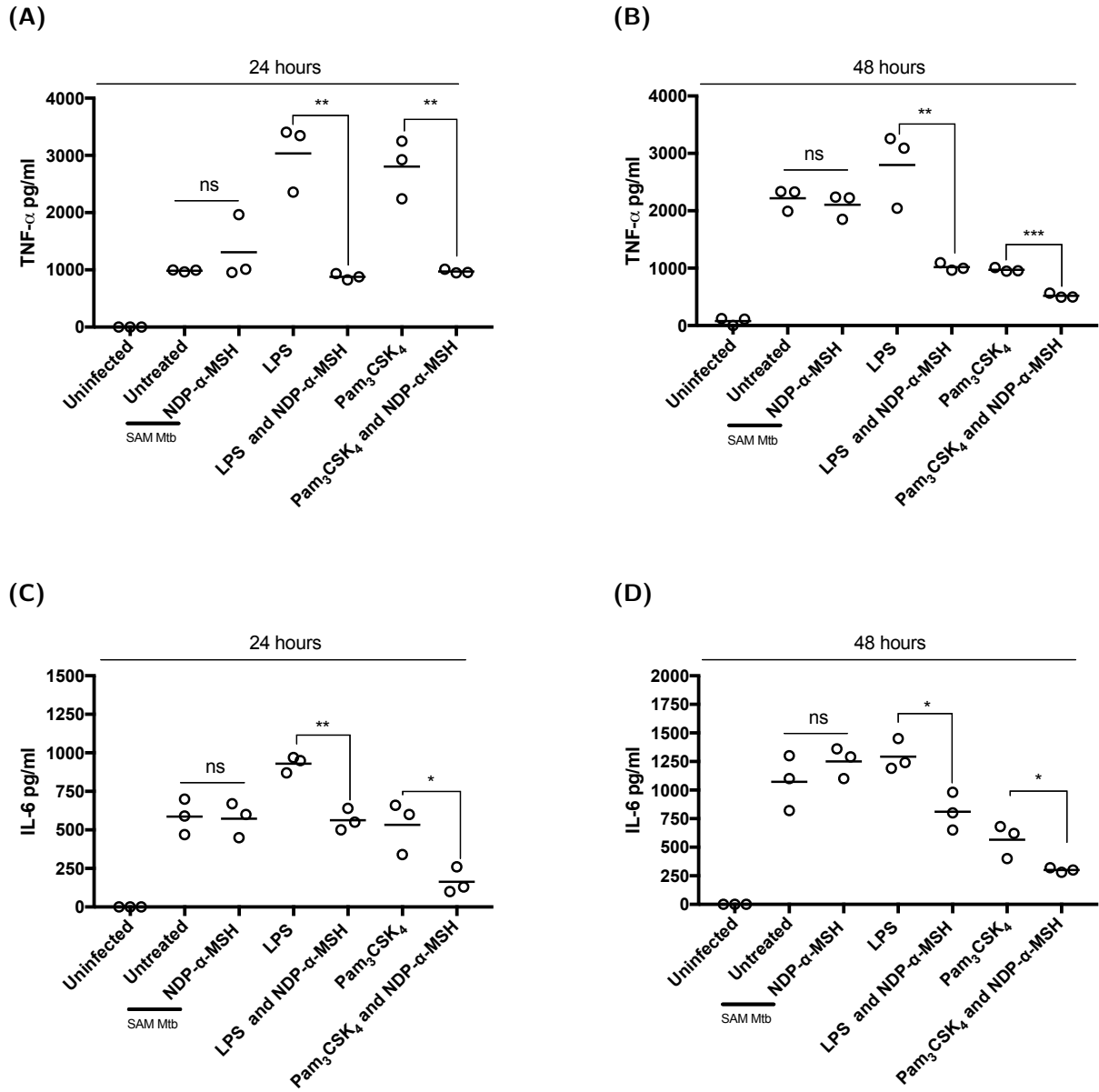


Figure 2.9: Pre-treatment of RAW 264.7 by NDP-α-MSH does not elicit reduction in TNF-α or IL-6 following SAM Mtb infection but does following TLR4 and TLR1/2 stimulation. RAW 264.7 cells were pre-treated with 500 nM NDP-α-MSH overnight. Cells were then infected with SAM Mtb at an MOI of 1 for 4 hours after which the cells were washed. For the positive controls, cells were treated with either 10 ng ml⁻¹ LPS or equivalent concentration of Pam₃CSK₄ for the duration of the experiment. At 24 hours (A;C) and 48 hours (B;D), the supernatants were removed and TNF-α and IL-6 concentrations were determined by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. A two-tailed paired t -test was used to evaluate the difference in mean cytokine concentration between the experimental drug and the control. In the graphs, statistical significance between the experimental conditions and the control are indicated as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns=non-significant.

		EARLY TREATMENT GROUP ↓		LATE TREATMENT GROUP ↓		
Day	0	1	10	15	21	28
	Intranasal H37Rv infection 1×10^4	NDP- α -MSH vs. Vehicle (PBS) (6 days)	Cull for lung CFU	Cull for lung and spleen CFU	NDP- α -MSH vs. Vehicle (PBS) (6 days)	Cull for lung and spleen CFU
Numbers	Total 43 mice	10 treated with drug and 10 treated with vehicle 3 mice culled for determination of pulmonary inoculum following infection	10 mice	10 mice	5 treated with drug and 4 treated with vehicle	8 mice

Figure 2.10: Timeline showing the treatment of *M. tuberculosis*-infected mice with NDP- α -MSH.

culled at day 10 for bacillary load quantification in lungs and at day 15 for quantification in lung and spleen. The late group were treated at day 21 for 6 days; lung and spleen CFUs were determined at day 28 (see figure 2.10).

Mice were infected intra-nasally with 1×10^4 CFU. The mean CFU, determined by plating the lungs the following day, was 747 CFU/lung.

On day 10 (see figure 2.10), 9 days after the onset of treatment, there was no difference in the lung bacillary load between the untreated and treated groups ($8.72 \pm 1.85 \times 10^4$ CFU/lung in the untreated group, $10.69 \pm 1.63 \times 10^4$ CFU/lung in the treated group; $p = 0.4469$; $t = 0.7998$). Similarly, there was no difference by day 15 in either group ($1.31 \pm 0.18 \times 10^6$, $1.30 \pm 0.20 \times 10^6$ CFU/lung; $p = 0.9713$; $t = 0.03709$). Two weeks tends to be the onset of mycobacterial dissemination following infection in mice models; however we observed no difference in splenic loads between the two groups ($3.13 \pm 1.12 \times 10^3$, $2.85 \pm 1.06 \times 10^3$ CFU/spleen; $p = 0.861$ $t = 0.182$). At day 28 the mice were culled following the second late phase treatment. Lung bacillary loads remained similar between the untreated and treated groups ($4.35 \pm 2.04 \times 10^8$, $5.45 \pm 1.60 \times 10^8$ CFU/lung; $p = 0.6868$; $t = 0.4233$). NDP- α -MSH also not have any impact on splenic dissemination at this time-point ($5.30 \pm 0.49 \times 10^5$, $5.91 \pm 0.30 \times 10^5$ CFU/spleen; $p = 0.3667$; $t = 0.9761$). These results imply that NDP- α -MSH does not have activity against *M. tuberculosis* in mice?

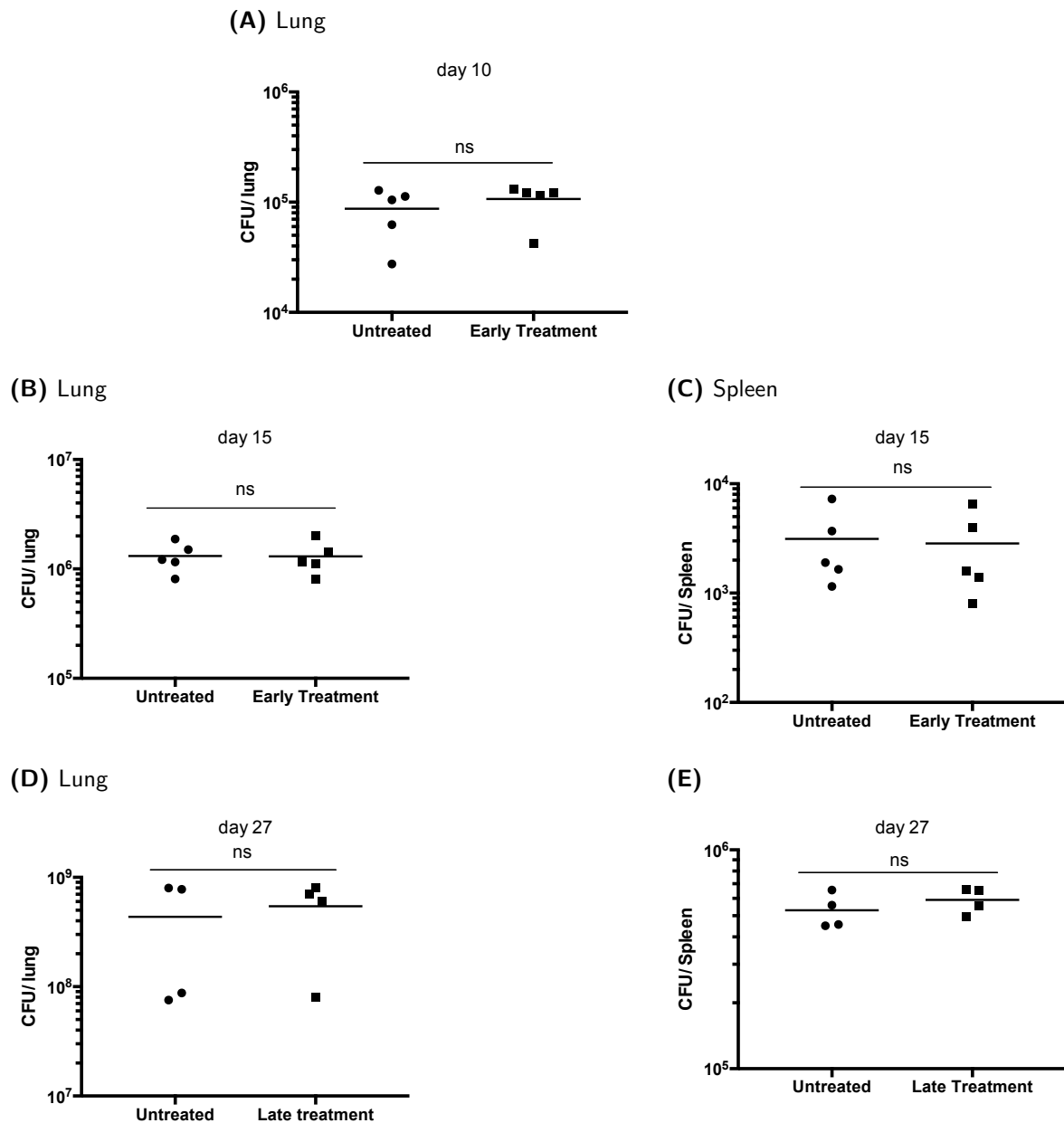


Figure 2.11: NDP- α -MSH does not reduce bacillary load following infection with H37Rv. Mice were infected intra-nasally with 1×10^4 CFU. Treated mice were divided into two groups, early phase and late phase. Early phase treated mice were administered with $50 \mu\text{l}$ of $100 \mu\text{g ml}^{-1}$ NDP- α -MSH via the intra-tracheal route for 6 days. Lung bacterial burden was determined at day 10. At day 15, lung and splenic burden were enumerated. Late phase treated mice were administered with the same drug concentration on day 21. At day 28, lung and splenic bacterial burdens were determined. Untreated mice were administered with $100 \mu\text{l}$ PBS. The number of mice in each group at each time point ranged from 4–5. A two-tailed paired t -test was used to evaluate the difference in mean bacterial burden between the untreated, designated as the control, and treated groups, ns=non-significant.

2.4 Discussion

It was quite apparent that without seeing any change in bacterial growth nor any immunomodulatory activity both *in vivo* and *in vitro*, that it was going to be difficult to carry this forward for the rest of the thesis. In the following discussion, I shall detail what I could have done to corroborate my findings and potential reasons for why we did not see any positive results using the drugs that we trialled.

In this study, treatment with α -MSH did not lead to any change in TNF- α release or bacterial numbers following infection with SAM Mtb *in vitro* nor any effect following infection with H37Rv *in vivo*. I treated the cells with concentrations ranging from 5–50 000 nM. Studies observing pro-inflammatory cytokines are able to see an effect following equivalent concentrations of α -MSH (Capsoni et al. 2015) and lower concentrations have been used effectively (Star et al. 1995). However, the most obvious question here is that α -MSH did not lead to any reduction on TNF- α following stimulation of TLR4 and TLR2 (using LPS and Pam₃CSK₄ respectively). Given that at the same concentrations, Star et al. 1995 showed a reduction in nitrite using LPS and α -MSH, in hindsight, it would have been instructive to ascertain the basal gene expression of either MC1R or MC3R on RAW 264.7 macrophages given that primers for all MCRs are readily available. However, there are a reasonable number of publications that demonstrate (Gupta et al. 2000; Sarkar et al. 2003; Taylor 2013) that these cells do have some degree of basal expression which is up-regulated following relevant stimuli. We thought instead, given that α -MSH is known to have a short half-life (Hruby et al. 1993) that the lack of efficacy might be due to the experimental set-up, that adding the peptide at a similar time as the bacteria may lend itself to increased degradation over the time-period and from external factors, such as mycobacterial proteases that may contribute to drug breakdown. So the initial set-up was changed, such that cells were treated with α -MSH for 24 hours prior to mycobacterial stimulation. This 24 hour initial pre-treatment set-up replicates most of the α -MSH experiments that are undertaken, before cells are stimulated (Gupta et al. 2000; Lam et al. 2005; Capsoni et al. 2015).

Using this method, α -MSH did not affect bacterial fold replication, but did reduce both LPS and Pam₃CSK₄ induced TNF- α release at 24 hours but not at 48 hours. Crucially, this meant that slight modifications in the experimental design led to our control working. In order to press forward further with this, we changed the synthetic α -MSH to the modified analogue, NDP- α -MSH, which has been demonstrated to exhibit increased potency compared to the natural peptide (Hruby et al. 1993). Altering the drug to pre-treatment with [Nle⁴,D-Phe⁷] α -MSH demonstrated a reduction in both LPS and Pam₃CSK₄ induced TNF- α and IL-6 release at 24 hours and at 48 hours, which suggested that peptide degradation or reduced half-life of the synthetic peptide was contributing to the lack of efficacy at both time-points. Rat microglial cells stimulated with [Nle⁴,D-Phe⁷] α -MSH followed by LPS and Pam₃CSK₄ have previously been shown to reduce TNF- α (Carniglia et al. 2016). However,

in any experimental set-up, treatment with α -MSH or its related analogues did not provoke any cytokine or bacterial change following infection with SAM Mtb.

To test the effects of [Nle4,D-Phe7] α -MSH *in vivo* we used drug concentrations based on a previous authors looking at the effect of the drug in a murine model of organ dysfunction (Bitto et al. 2011). Whilst we did not look at histology or cytokine release *in vivo*, we clearly did not see any effect on lung bacterial replication or splenic dissemination.

Other possible avenues for research could have measured additional parameters of α -MSH downstream activity, such as IL-10 (Lam et al. 2006), PGE2 and COX-2 which are implicated following mycobacterial signalling (Assis et al. 2014), and whose expression is mediated by cross-talk between C/EBP β and cAMP (Wadleigh et al. 2000) and can be reduced by α -MSH (Shiratori et al. 2004).

The experimental changes that we made, were based on common sense and previous information regarding the drug's half-life and were verified by the results of the positive controls. To further test whether the concentration of the peptide was changing during the time-course, α -MSH levels could have been detected by ELISA at 24 and 48 hours as has been done previously (Catania et al. 2000), however commercially available ELISA kits may not be a reliable way to detect synthetic peptides with the change in amino acid composition. Another, rather more extravagant way to measure α -MSH would be to use mass spectrometry-based quantitative peptidomics. This technique is increasingly being used to detect peptides and peptide processing and it can detect post-translational modifications in the peptide that ELISAs cannot detect (Gelman et al. 2012). Mass spectrometry has previously been employed to detect three forms of α -MSH; deacetylated, acetylated and α -MSH₁₋₁₂, which is the truncated form (Perroud et al. 2009), the latter of which is cleaved by prolyl-peptidases. Whilst it is possible that α -MSH₁₋₁₂ would possess activity in the same way as the tripeptide sequence, KPV, the absent valine at the C-terminus may prevent MCR binding. It is unlikely that deacylated α -MSH would harbour activity at the MCR (Hruby et al. 1993). Thus quantitative peptidomics could be used to quantify these variants over time. Such a theory does not explain why [Nle4,D-Phe7] α -MSH followed by LPS and Pam₃CSK₄ was so effective as a positive control compared to mycobacteria. One explanation is that *M. tuberculosis* has recently been shown to harbour prolyl-endopeptidases that are able to modulate the host response and induce pro-inflammatory cytokines (Portugal et al. 2017), therefore it is possible that such a prolyl-peptidase would also be able to cleave α -MSH *in vitro*.

α -MSH may lead to the inhibition of NF- κ B (Manna et al. 1998) and also inhibits the induction of C/EBP β -DNA-binding activity (Gupta et al. 2000) with a consequent reduction in nitric oxide synthase gene expression and NO production (Mandrika et al. 2001) in immune cells. The predominant mechanism by which this appears to be achieved is through G-coupling with adenylyl cyclase and

increased intracellular cAMP (Ru et al. 2016). Although, in glial cells, MC4R stimulation by [Nle4,D-Phe7] α -MSH reduced PPAR- β protein expression levels and this was partially mediated by ERK-1/2 signalling (Carniglia et al. 2013). Thus whilst other signalling pathways are implicated we could have measured the supernatants from infected cells for cAMP using ELISA as has been described previously for α -MSH treatment (Zhang et al. 2009).

However, there maybe a perhaps more intriguing and plausible reason for the apparent lack of effect by α -MSH and in particular this might explain the *in vivo* and *in vitro* findings. Quite simply, it might be that α -MSH is able to inhibit the downstream effects imposed by signalling via TLR2 and TLR4 but not able to affect other pathways that are activated. As discussed previously, mycobacteria contain ligands for many receptors, including the classical (for example, mincle) and non-classical (Dectin-1) C-type lectins, which are highly expressed on mouse macrophages (Rajaram et al. 2014). Radioprotective 105 kDa (RP105) is a TLR-like protein which seems to have cell-specific actions. RP105 appears to reduce LPS-driven cytokine responses in HEK 293 cells and dendritic cells (Divanovic et al. 2005). Although LPS-driven B-cell antibody production was reduced in mice deficient in RP105 (Nagai et al. 2005). Following *M. tuberculosis* infection, lipoproteins activate TLR2 in a RP105-dependent manner (Blumenthal et al. 2009) and the two colocalise in the cell membrane to activate phosphoinositide 3-kinase (PI3K) which induces the production of phosphatidylinositol (3,4,5) triphosphates (PIP3). Activation of PI3K leads to enhanced TNF- α and IL-6 release that is ameliorated in *RP105*^{-/-} mice in a manner that is dependent on the phosphorylation of Akt but independent of NF- κ B and p38 MAPK kinase signalling (Yu et al. 2015). Thus, even if α -MSH were inhibiting cAMP and NF- κ B, mycobacteria can still engage in other pathways that would enable the innate response.

Another possibility is as follows. If cAMP synthesis is the favoured signalling pathway induced by α -MSH, the current literature with regards to its role during *M. tuberculosis* infection is paradoxical. Mycobacteria is a potent inducer of cAMP inside macrophages with intracellular levels rising significantly during infection (Bai et al. 2009). Thus the 'effect-size' imposed by drugs such as α -MSH might not be so apparent in comparison to using drugs that directly regulate cAMP signalling — such as inhibitors of adenylyl cyclase (AC) or phosphodiesterases (PDE). For example, *M. tuberculosis* mutants lacking RV0386c which is one of the 17 ACs encoded by the *M. tuberculosis* genome, were associated with lower cAMP levels and reduced bacterial growth *in vitro* (Agarwal et al. 2009). Increasing intracellular cAMP inhibited phagosome-associated actin, phagolysosomal fusion, reduced the proportion of acidified phagosomes and increased *M. tuberculosis* growth, which was ameliorated by PKA inhibition (Kalamidas et al. 2006). *In vivo*, cAMP induces bacterial cyclic-AMP receptor proteins (CRP) which regulate mycobacterial persistence (Vashisht et al. 2014). Curiously, *in vivo* studies have demonstrated that FDA approved drugs such as PDE3 inhibitors (cilostazol) increase cAMP, reduce *M. tuberculosis* bacterial load at 28 days after infection compared to untreated mice,

reduce TNF- α and IFN- γ in infected lungs and act adjunctively with rifampicin to reduce bacterial load (Maiga et al. 2012). A similar reduction in lung pathology was seen using PDE4 inhibitors (Subbian et al. 2016). In converse, another study which used two PDE4 inhibitors actually decreased the survival time of *M. tuberculosis* infected mice. How can this explained when increasing cAMP seemingly has such pleiotropic effects that favour mycobacterial survival? A possible explanation is that elevation of cAMP and subsequent lowering of pro-inflammatory cytokines might be protective in the later stages of disease as seen by Maiga et al. 2015.

In summary, in our experimental model and in the presence of various changes to this model, I was not able to identify a role for α -MSH or its related analogues during mycobacterial infection, however it is also worth iterating that inhibition of bacterial replication is not a pre-requisite for HDT - indeed whilst steroids themselves barely have any effect on bacterial numbers, they are potent inhibitors of the inflammatory response. However, whilst previous studies have identified that MCR stimulation can have anti-microbial effects directly and following infection in cell lines (Donnarumma et al. 2004), I was not able to demonstrate this. This, in part may be attributed to the complex lipid-rich cell wall within mycobacteria compared to that of *S. aureus*. I have identified some of the avenues that could have been employed to make this a more robust finding, but *in vitro* and *in vivo* evidence shows that *M. tuberculosis* is likely signalling via another mechanism that bypasses that of α -MSH. Additionally, the induction of cAMP that has previously been identified as the main regulatory effect induced by α -MSH, may not be enough of an increase compared to *M. tuberculosis* infection and future work might be worthwhile considering not only static and dynamic measurements of cAMP but also using additional controls, that include forskolin (a cAMP inducer), and inhibitors of AC and PDE.

Chapter 3

The inflammasome and its role regulating IL-1 β processing

3.1 Introduction

With 11 members comprising the IL-1 family (Dinarello [2009](#)) and roles encompassing initiation of the pro- and anti-inflammatory responses, IL-1 is often ranked as one of the principal architects of the innate immune response. The family consists of 7 cytokine agonists with pro-inflammatory activity (IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 γ and IL-36 β), 2 receptor antagonists (IL-1RA and IL-36RA) and 2 anti-inflammatory cytokine (IL-37 and IL-38) (Garlanda et al. [2013](#)).

The IL-1 receptor family, in general, contain 3 Ig domains at the N-terminal domain and a C-terminal Toll-IL-1 resistance (TIR) domain (Garlanda et al. [2013](#)). IL-18 binding protein (IL-18BP) and Toll IL-1 receptor 8 both have a single Ig extracellular domain. Following ligand binding, there is recruitment of a second receptor subunit, the IL-1R accessory subunit (IL-1RAP) which also contains a TIR domain (Sims et al. [2010](#)). The TIR domain is essential to facilitate signalling via MyD88 and subsequent activation of IRAK and TRAF6, which leads to the activation of the NF- κ B and the MAPK pathways (Sims et al. [2010](#)).

Whilst IL-1 β , IL-1 α and IL-18 are potent pro-inflammatory cytokines that are able to bind the IL-1R (IL-1 β , IL-1 α) and IL-18R (IL-18) receptor, their actions may be offset by the inhibitory actions of IL-1RA, Toll interleukin-1 receptor 8 (TIR8), the IL-1 type II receptor — which functions as a decoy receptor to suppress the action of cytokines — and the IL-18 binding protein that can neutralise IL-18 (Dinarello [2009](#)).

IL-1 itself is ubiquitous in its influence; in addition to its role as a central pyrogen, it propagates

the acute phase response and can increase the lifespan and effector functions of neutrophils and macrophages (Mantovani et al. 2008). IL-1 β and IL-1 α induce similar biological effects but they differ in their activation and distribution. IL-1 α is constitutively active and is present in epithelial cells and in the membranes of activated monocytes (Garlanda et al. 2013). Following cell death, large apoptotic bodies containing IL-1 α can stimulate a rapid elevation of neutrophil-inducing chemokines functions (Berda-Haddad et al. 2011). Thus, IL-1 α serves as an 'alarmin' through release following cell death and therefore can rapidly mediate the initial stages of inflammation (Chen et al. 2007). In contrast, as discussed in the next section, IL-1 β is produced as an inactive precursor in haematopoietic cells following TLR stimulation; it is then processed into the bioactive form by proteolytic processing. However, mutations of the complexes that govern maturation of IL-1 β can result in spontaneous release of IL-1 β (Zhong et al. 2016). Furthermore, there is increasing information suggesting the importance and potential implications of IL-1 β during *M. tuberculosis* infection. For this reason, my thesis focuses solely on the potential maturation mechanisms of IL-1 β and inhibitors relating to this cytokine.

3.1.1 IL-1 β and the importance of the inflammasome that governs it

The inflammatory properties of IL-1 β are several fold but are pivotal orchestrators of the innate and adaptive response. The immediate non-specific responses are to mediate the fever and acute phase response (Dinarello 2009), but it also induces release of prostaglandin E2 (PGE2) by initiating early gene expression and production of cyclooxygenase type 2 (COX-2) (Molina-Holgado et al. 2000). Additionally, IL-1 β is responsible for the induction of adhesion molecules in the vascular smooth muscle and exacerbates monocyte adhesion to smooth muscle cells. Furthermore, IL-1 β is a strong inducer of T helper (TH) 17 cell differentiation (Acosta-Rodriguez et al. 2007) and increases the production of TNF- α and IL-6, which consequently act synergistically to provide a robust immune response. Thus, the overall effects of IL-1 β release are to promote a state of innate and adaptive immunity by recruiting a deluge of cytokines, chemokines and inflammatory cells into the affected area in order to fend off the insult.

The inflammasome is a large molecular weight cytosolic protein complex containing a 'sensor'. The sensor is activated through a myriad of pathological and physiological stimuli (Broz et al. 2016) and ultimately assembles to enable the activation of pro-caspase-1 into caspase-1, an enzyme required for the cleavage of pro-IL-1 β (35 kDa) into IL-1 β (17 kDa) and the processing of pro-IL-18 into IL-18 (Jo et al. 2015). However, hyperactivation of the inflammasome response is implicated in autoimmune, metabolic (Jo et al. 2015) and pathological processes related to some infectious diseases (Guo et al. 2015), suggesting that this inflammatory process is a double-edged sword. Therefore, the activation

and assembly of the inflammasome must be tightly regulated at several steps in order to prevent an over-exuberant inflammatory response.

Pro-caspase-1 is produced as a monomeric protein in the cytosol and is composed of three units: an amino-terminal pro-domain (a caspase activation and recruitment domain; CARD), a large subunit and a small subunit (termed p20 and p10 respectively) (Lamkanfi et al. 2002; Nadiri et al. 2006). A short linker peptide separates p20 and p10. Maturation of pro-caspase-1 is initiated through oligomerisation of the enzyme to the inflammasome complex. Caspase-1 possesses auto-proteolytic activity and cleaves itself to produce a catalytically active heterotetramer containing the p10 and p20 subunits (Elliott et al. 2009).

Based on the domains of the intracellular receptor, there are three types of inflammasome pattern recognition sensor (Sharma et al. 2016).

1. NOD leucine-rich repeat (LRR)–containing proteins (NLR) family members which consists of NLRP1, NLRP3 and NLRC4. The NLR family members are composed of a central nucleotide-binding domain (NBD), a C-terminal LRR domain and an N-terminal pyrin domain (PYD) or CARD.
2. Absent in melanoma 2 (AIM2) proteins, which contains a C-terminal haematopoietic interferon-inducible nuclear (HIN) domain and an amino-terminal pyrin domain.
3. The newly identified Pyrin sensor.

Activation is initiated when either the NLR, AIM2 or Pyrin sensor detects specific stimuli. This leads to the recruitment of an adaptor protein called ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) to form a bridge within the activated cell (Sharma et al. 2016). ASC is structurally important as it contains two connecting domains — the caspase activation and recruitment domain (CARD) and the pyrin domain (PYD) — which facilitate linkage between the sensor and pro-caspase-1 (Broz et al. 2016). The CARD bridges with pro-caspase-1 and the PYD associates upstream with the sensor receptor. Once the initial 'bridge' is formed, further ASC recruitment is allowed to continue. The PYD assembles into multiple helical filaments and these cross-link to the CARD leading to a dense oligomeric structure (Dick et al. 2016). This oligomerisation provides the close proximity and multiple cleavage sites needed for rapid auto-processing of pro-caspase-1 into its activated form (Yang et al. 1998, see figure 3.1).

Mature caspase-1 is then able to cleave pro-IL-1 β and pro-IL-18 into their bioactive forms, IL-1 β and IL-18 respectively, and induce pyroptosis, an inflammatory form of cell death (Sharma et al. 2016).

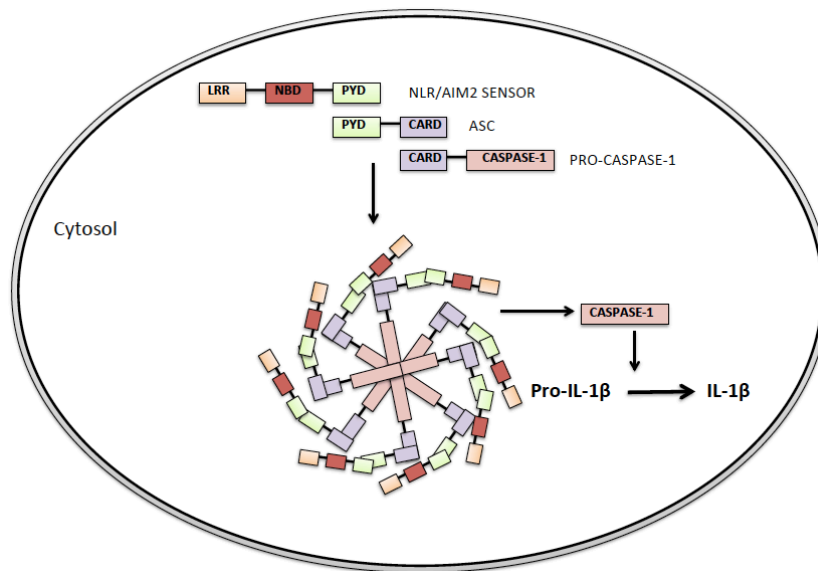


Figure 3.1: Model of inflammasome assembly and caspase-1 activation. Detection of specific stimuli by the inflammasome sensor facilitates the oligomerisation of the sensor (NLR, AIM2 or Pyrin), the adaptor protein (ASC) and pro-caspase-1. Assembly of the complex then catalyses the auto-proteolytic cleavage of pro-caspase-1 into caspase-1, which contributes to the processing of pro-IL-1 β into IL-1 β . LRR, leucine-rich repeat domain; NBD, nucleotide-binding domain; CARD, caspase activation and recruitment domain.

To date, five intracellular sensors that form the canonical inflammasome have been identified: NLRP1, NLRP3, NLRC4, AIM2 and the Pyrin sensor, which all have varying domain compositions (see figure 3.2).

NLRP6 and NLRP12 are potential inflammasome sensors (Sharma et al. 2016). Whilst I shall discuss NLRP1, NLRP3, NLRC4, AIM2 and the Pyrin sensor in this chapter, I will be expanding mostly on the activation of the NLRP3 and AIM2 inflammasome sensors as these both play a pivotal role during *M. tuberculosis* infection (Briken et al. 2013).

3.1.2 The canonical activation of the inflammasomes: NLRP1, NLRP3, NLRC4, AIM2 and Pyrin

The canonical inflammasome pathway was so named because traditional inflammasome activation was thought to require two steps for functionality — firstly, priming of the cells through transcription and translation of components of the inflammasome and pro-IL-1 β as well as oligomerisation leading to caspase-1 activation. Secondly, caspase-1 dependent catalytic processing of IL-1 β and pro-IL-18 (Mortellaro et al. 2015) as well as pyroptosis. And until recently, caspase-1 has been the only caspase identified that processes pro-IL-1 β and pro-IL-18.

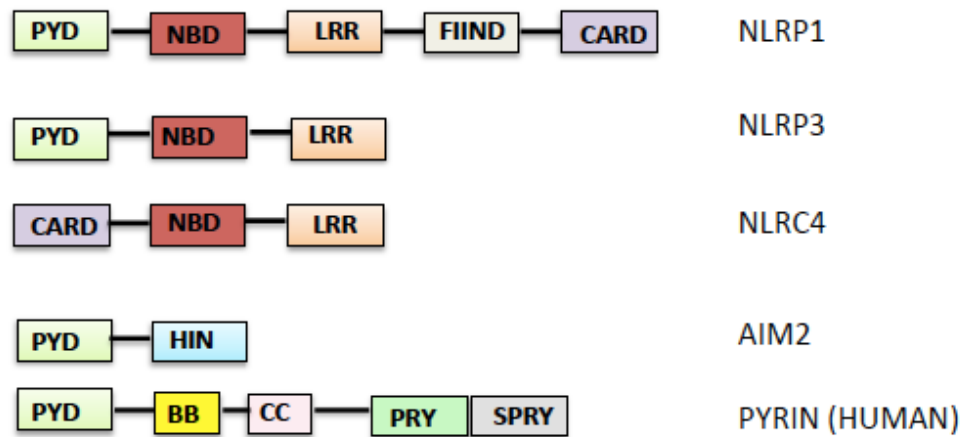


Figure 3.2: Domain compositions of the NLRP1, NLRP3, NLRC4, AIM2 and PYRIN inflammasomes. LRR, leucine-rich repeat domain; NBD, nucleotide-binding domain; CARD, caspase activation and recruitment domain; HIN, human interferon-inducible nuclear 200 protein; BB, b-box; CC, coiled-coiled; PRY and SPRY, collectively referred to as the B30.2 sub-family and not present in murine Pyrin.

NLRP1

NLRP1 was the first caspase-activating intracellular sensor identified (Martinon et al. 2002). Humans have just one NLRP1, comprised of a PYD, NBD, LRR domain, an additional ‘function-to-find’ (FIIND) domain and a CARD (Broz et al. 2016). In contrast, mice have many NLRP1 paralogues (Sastalla et al. 2013) which each have similar domain architecture to the human protein, aside from lacking the PYD (Sharma et al. 2016).

Susceptibility to the anthrax lethal toxin, the virulence factor secreted by *Bacillus anthracis*, is mapped to *Nlrp1b* and caspase-1 activation only occurs in mice homozygous for the *Nlrp1b^S* allele (*Nlrp1b^{S/S}*; termed the functional allele) where *Nlrp1b^S* confers the necessary polymorphisms needed for responsiveness to lethal toxin and inflammasome assembly (Moayeri et al. 2010). However different mouse strains harbour different alleles encoding NLRP1b. Therefore, use of different strains will impact on prognosis following infection (Sastalla et al. 2013). For example, C57/BL6 mice express a dysfunctional *Nlrp1b* allele (*Nlrp1b^{R/R}*, where R implies resistant), rendering them resistant to the lethal toxin and inflammasome activation and succumb rapidly to *B. anthracis* infection (Moayeri et al. 2010). In contrast, C3H/HeJ and Balb/C mice harbour *Nlrp1b^{S/S}* alleles and can activate inflammasome assembly following exposure to the lethal toxin (Moayeri et al. 2010). Such considerations are important when interpreting findings from mouse data.

The lethal toxin (LeTx) is a bipartite protein composed of a protective antigen and a lethal factor (a zinc metalloprotease). The protective antigen creates a channel in the host membrane through which the lethal factor is able to enter and directly cleave NLRP1b close to its N-terminus (Levinsohn

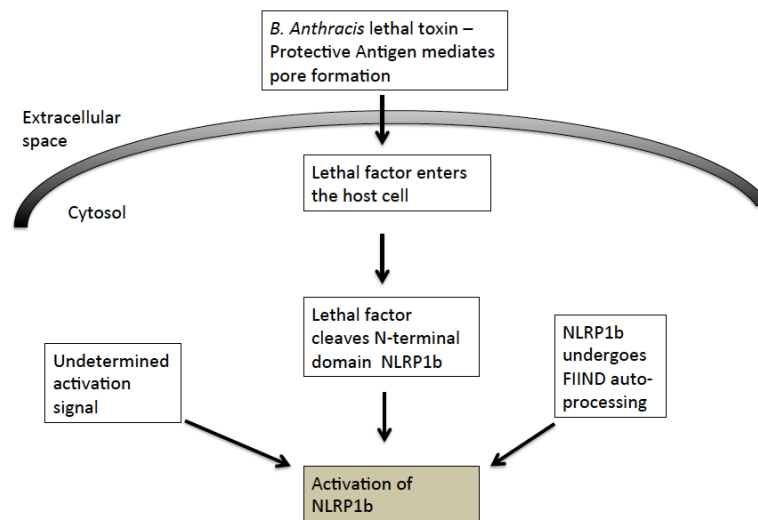


Figure 3.3: Mice NLRP1b and rat NLRP1 respond to *Bacillus anthracis* *B. anthracis* secretes a virulence factor which contains a protective antigen that mediates cell membrane pore formation and a lethal factor that cleaves NLRP1b at its N-terminal domain. However activation of NLRP1b may also require auto-proteolytical cleavage of its FIIND domain and additional signals. Adapted from Broz et al. 2016.

et al. 2012; Chavarría-Smith et al. 2013). This is the first event needed to activate NLRP1b but studies have also shown that NLRP1b is activated following intracellular depletion of ATP (Liao et al. 2013), which may be a metabolic signal that occurs alongside lethal factor cleavage. However, a further signal is likely to be required for NLRP1b assembly, since NLRP1b cleavage by the lethal factor also occurs in mice resistant to LeTx (Hellmich et al. 2012). This signal might be attributed to the FIIND domain, which undergoes auto-proteolytic processing following exposure to the lethal toxin (Chavarría-Smith et al. 2013); and alleles defective in auto-processing display impaired inflammasome assembly and are non-responsive to the LeTx and ATP depletion signal (Neiman-Zenevich et al. 2014). In addition to *B. anthracis*, NLRP1b activation also occurs following infection with the obligate intracellular parasite *Toxoplasma gondii* in mice, and siRNA against the *NLRP1a* gene promotes parasite replication (Witola et al. 2011) in human monocyte cell lines. In contrast to infection with *B. anthracis*, NLRP1b does not undergo N-terminal proteolytic cleavage in response to *T. gondii* infection (Ewald et al. 2014), which suggests that a further undetermined mechanism of NLRP1b activation is in play (see figure 3.3).

Since NLRP1 contains a C-terminal CARD, it can interact directly with the caspase-1 CARD, implying that ASC as a bridging protein may not be required during inflammasome activation. A study examining the role of ASC in inflammasome signalling showed that ASC is required for caspase-1 auto-processing and speck formation, but may be dispensable in pyroptosis and IL-1 β maturation (Broz et al. 2010; Van Opdenbosch et al. 2014) following LeTx exposure, suggesting that the functional inflammasome undergoes a different assembly pattern from the classic 'NLR–ASC–Pro-Caspase-1' complex.

The NLRP1a inflammasome has been implicated in the pyroptotic death of haematopoietic progenitor cells (Masters et al. 2012). Mice with a constitutively active *Nlrp1a* in haematopoietic progenitor cells developed a lethal multi-organ inflammatory disease which was driven by caspase-1 and IL-1 β but independent of ASC. However, mice genetically knocked out for the IL-1-Receptor (therefore abrogating the IL-1 signalling pathway) but which continued to express the constitutively active *Nlrp1a* triggered pyroptosis of the progenitor cells and active *Nlrp1a* contributed to prolonged bone marrow suppression following administration of chemotherapeutic agents. Recovery rates from cytopenias were ameliorated in the absence of NLRP1a, suggesting that this inflammasome may have an important role to play in responding to haematopoietic stress. Interestingly, in contrast to the pyroptotic promotion by NLRP1a in macrophages, NLRP1a has recently been shown to enhance tumour proliferation and inhibit caspase-2 and -9-mediated apoptosis (Zhai et al. 2017).

In humans, mutations within *NLRP1* and over-activation of this inflammasome are associated with increased risk of inflammatory disorders such as rheumatoid arthritis (Sui et al. 2012), Alzheimer's disease (Saresella et al. 2016) and more recently a novel auto-inflammatory disease (Grandemange et al. 2016). The signals required to activate NLRP1 in humans are less well understood compared to mice and rodents however, gene transcription in THP-1 cells may initially occur through the transcription factor ATF4, which is activated following stress within the endoplasmic reticulum (D'Ossualdo et al. 2015). Chavarría-Smith et al. 2016 recently showed that, similar to mouse NLRP1, proteolysis of the NLRP1 N-terminal domain is sufficient to activate the inflammasome.

NAIP–NLRC4

Assembly of the NLRC4 inflammasome occurs in response to a narrow spectrum of agonists. The stimuli activating NLRC4 encompass both bacterial flagellin and inner rods and needles of the type 3 secretion system (T3SS) (Broz et al. 2016) in mice; although in humans, the repertoire of ligands is narrower, with NLRC4 responding only to the needle proteins of the T3SS and bacterial flagellin (Eldridge et al. 2015).

NLRC4 does not directly bind to the bacterial products themselves but uses additional NLR proteins, called NAIP (NLR family apoptosis inhibitory proteins) as upstream receptors (see figure 3.4). There are seven mice NAIPs and four genes have been identified in C57BL/6 mice (NAIP1, NAIP2, NAIP5 and NAIP6) which are encoded by genes located in a cluster on chromosome 13 (Rauch et al. 2016). Only 1 NAIP has been identified in humans (hNAIP). NAIPs are the receptor molecules for bacterial molecules and the complex binds and activates NLRC4.

The T3SS — structurally related to bacterial flagellin — is often used to inject effector proteins into

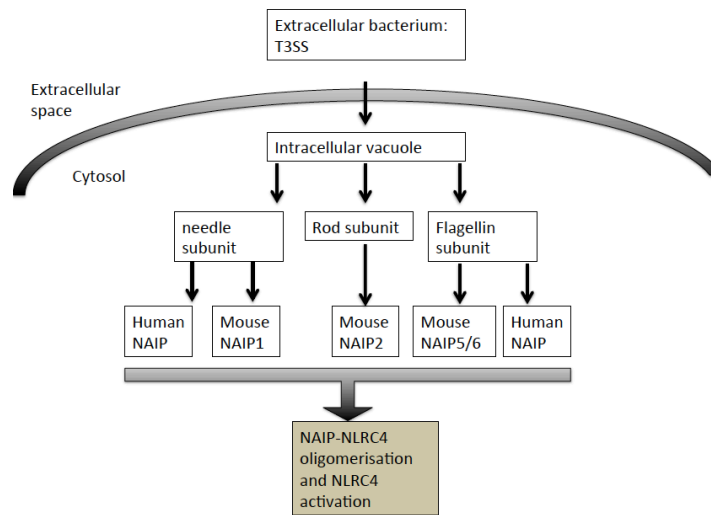


Figure 3.4: NLRC4 activation requires initial bacterial sensing by the NAIP family of proteins. NAIPs function as direct receptors for bacterial flagellin and T3SS (or T4SS) needle and rod subunits. Ligand binding leads to NAIP activation and this leads to the recruitment and activation of NLRC4. Adapted from Broz et al. 2016.

the mammalian host. They are employed by gram-negative bacteria in order to subvert the host immune response and enable their own replication (Vance 2015). However, the presumable accidental release of bacterial proteins from their surface provides a way by which the NAIP–NLRC4 system can respond to bacterial proteins and effect an immune response aimed at curtailing bacterial replication. In fact, specific NAIP proteins are critical for the recognition of bacterial flagellin and proteins of the T3SS *in vivo* (Rauch et al. 2016).

NAIP1 binds to the needle protein of the T3SS. For example, Mxi1 and Epr1 are needle proteins expressed by *Shigella flexneri* and Enterohaemorrhagic *Escherichia coli* (EHEC) respectively, which robustly induce the NLRC4 inflammasome through interaction with NAIP1 (Yang et al. 2013a). Similarly, *Nlrc4*^{-/-} and *Naip1*^{-/-} mice are protected from a pathological response following translocation of a needle protein from *Burkholderia thailandensis* (Rauch et al. 2016).

NAIP2 recognises the rod protein component of the T3SS. Both the PrgJ protein, which is secreted by *Salmonella enterica* (Zhao et al. 2015) and BsaK, which is secreted by *B. thailandensis*, induce mature IL-1 β production when introduced into NAIP2 expressing cells (Yang et al. 2013a; Rauch et al. 2016).

NAIP5 and NAIP6 bind to various bacterial flagellins (such as *Legionella pneumophila*, *S. enterica*, *Yersinia enterocolitica* and *Pseudomonas aeruginosa*), which may be translocated via their T3SS, or T4SS in the case of *L. pneumophila* (Eldridge et al. 2015).

Following ligand recognition, NAIPs assemble with NLRC4 into an oligomerized inflammasome that contains both NLRs and the ligand (Kofoed et al. 2011). The assembled inflammasome can then directly recruit and activate caspase-1 via the NLRC4 CARD (Moltke et al. 2013). Since bacteria

may harbour both T3SS structures and flagellin, any particular pathogen might induce inflammasome activation from a combination of binding to different NAIP proteins, which means that bacteria have multiple possible strategies for interfering with the innate response. For example, knocking down NAIP2 ameliorates PrgJ-induced inflammasome activation but has no effect on flagellin-induced assembly (Zhao et al. 2015).

Until recently, the human orthologue of NAIP1 (hNAIP) was only believed to detect needle proteins (Zhao et al. 2011). However a recent study has shown that intracellular delivery of flagellin from either *S. enterica* or *L. pneumophila* induces NLRC4-mediated inflammasome activation in human monocytes, which is abolished by mutations in the flagellin protein. The authors showed that siRNA-mediated knock-down of hNAIP in immortalised human cells dramatically reduces inflammasome activation following stimulation with flagellin (Kortmann et al. 2015).

How NAIP proteins sense and bind the bacterial protein and activate NLRC4 is poorly understood (Vanaja et al. 2015). There are reports that in response to cytosolic flagellin, phosphorylation of NLRC4 residue Ser533 occurs, which prepares the inflammasome for activation by the NAIP sensor (Matusiak et al. 2015). However, given that the crystal structure of inactive NLRC4 reveals Ser533 in its phosphorylated state (Hu et al. 2013), it is hard to fathom whether phosphorylation is really necessary, a point underpinned by mutations in the Ser533 only having a mild defect in caspase-1 activation following infection compared to wild-type (Qu et al. 2016). It is believed that ligand binding occurs at several NBD-associated α -helical domains within the NAIP receptor (Tenthorey et al. 2014). Ligand binding is needed for NAIPs to form an oligomeric complex with NLRC4 and to subsequently recruit and activate pro-caspase-1. Interestingly, there is evidence to suggest that individual inflammasomes act in concert — a recent study showed that LPS priming of NLRP3 aids NLRC4 activation of pro-caspase-1 following flagellin transfection compared to non-primed cells (Qu et al. 2016). Similarly to NLRP1, NLRC4 has a differential requirement for ASC, which is needed for IL-1 β processing and caspase-1 auto-proteolysis but is dispensable for pyroptosis (Van Opdenbosch et al. 2014).

The effector functions of the NAIP–NLRC4 inflammasome may vary between cell types — a recent study demonstrated that following inflammasome activation by flagellin, there is profound loss of vascular fluid in the peritoneal cavity that is characterised by a pathological release of inflammatory lipid mediators such as eicosanoids and prostaglandins, which is dependent on caspase-1 activation but independent from IL-1 β production (Moltke et al. 2012). This finding of eicosanoid biosynthesis was specific to peritoneal macrophages and not observed in BMDMs.

As with NLRP1, NLRC4 has recently been found to be associated with an inflammatory phenotype — gain-of-function mutations have been described that may disrupt how NLRC4 maintains an auto-

inhibitory state, leading to increased activation of caspase-1 in these patients (Canna et al. 2014). Although rare, patients may display features of auto-inflammatory periodic fever syndromes and haemophagocytosis that are similar to those seen in NLRP3 mutations (Hoffman et al. 2009) however mutations may be fatal (Liang et al. 2017) and these patients also presented with gastro-intestinal inflammation (Romberg et al. 2014). Whether there is complete causality between the mutation and their symptoms is not known. However, the fact that independent reports have shown *de novo* mutations in close proximity within the NLRC4 gene in affected patients who had a similar clinical phenotype (Canna et al. 2014; Romberg et al. 2014) supports the idea that NLRC4 mutations may cause a novel disease pattern.

NLRP3

The NLRP3 (also known as cryopyrin) inflammasome is the best characterised of all the inflammasomes as it is activated by a wide range of bacterial, fungal and viral pathogens, including RNA and RNA:DNA hybrids. It also responds to pathological sterile signals such as ATP and uric acid (Broz et al. 2016) and is implicated in several auto-inflammatory diseases such as rheumatoid arthritis and diabetes (Sharma et al. 2016). However, there is still much uncertainty with regards to the specific signals required for activation.

Activation of NLRP3 requires two steps in most cell types, aside from human monocytes (Gaidt et al. 2016). The first signal is denoted as the 'priming' step, where transcription of NLRP3 itself and pro-IL-1 β occurs through stimulation of NF- κ B. The second signal is called the 'activation' signal and triggers the assembly of NLRP3 into its oligomeric state, which allows the recruitment and cleavage of pro-caspase-1 (He et al. 2016). The priming step is unique to the NLRP3 inflammasome and may serve to safeguard the host from heightened inflammation given the vast number of mediators that might otherwise activate it.

Priming of the NLRP3 inflammasome

Priming occurs by extracellular inflammatory stimuli such as microbial ligands; commonly, this pertains to LPS from gram-negative species binding to TLR4 (Broz et al. 2016). However, TNF- α binding TNF receptor 1 and 2 (Broz et al. 2016), and IL-1 β (Franchi et al. 2009) have also been shown as priming signals (Bauernfeind et al. 2009) prior to NLRP3 activation by ATP. Previously, it was believed that increased NLRP3 transcription was a pre-requisite of the priming signal and NLRP3 activation. Indeed, caspase-8 and its adaptor protein, Fas-associated protein with death domain (FADD), are upstream regulators of NLRP3 and induce caspase-1 cleavage through transcriptional

up-regulation of NLRP3 (Gurung et al. 2014).

However, these *in vitro* experiments were performed during prolonged priming with LPS (beyond 4 hours). The time-scale appears important, as there is strong evidence to suggest that post-translational modifications are also an important part of the priming step independent of NLRP3 transcription during short infections and stimulation (≤ 30 minutes). TLR4 binding to LPS can rapidly non-transcriptionally prime NLRP3 by inducing its deubiquitination (Juliana et al. 2012). This is a step that is separate from new protein synthesis and can occur rapidly after the initial TLR4 stimulation, a process which is reduced by deubiquitinating enzyme (DUBs) inhibitors. Furthermore, when there is constitutive expression of NLRP3, ATP alone may be enough to activate the inflammasome, which may be attributed to the fact that ATP itself induces the deubiquitination of NLRP3 (Juliana et al. 2012). Moreover recently, screening a library of DUBs identified Lysine-63-specific deubiquitinase (known as BRCC3) as an enzyme which is able to deubiquitinate the LRR domain of NLRP3 and regulate its function (Py et al. 2013). Further to this, it was found that early phase inflammasome activation (within 20 minutes) depends on co-stimulation of both the TLRs and NLRP3 (with LPS and ATP respectively). When constitutively expressed, early phase activation is independent of new NLRP3 protein synthesis and NF- κ B activation and is mediated by IRAK1 and IRAK4 (Fernandes-Alnemri et al. 2013; Lin et al. 2014). Therefore, constitutive expression of NLRP3 may be enough to activate the inflammasome when both TLR and NLRP3 are stimulated together without the need for additional up-regulation. The rapid NLRP3 activation appears to be a way by which pre-synthesised IL-18 can be released early from the cell and pyroptosis can occur in order to quickly eliminate infected cells. The pathophysiological relevance of this has been shown by the observation that acute infection with *Listeria monocytogenes* induces an NLRP3-dependent TLR/MyD88/IRAK1-dependent activation of the inflammasome (Lin et al. 2014). In contrast, prolonged infection activates AIM2-dependent caspase-1 cleavage (Kim et al. 2010).

Thus the priming signal of NLRP3 depends on multiple signalling pathways. Short infections may stimulate NLRP3 activation without requiring priming due to the presence of constitutive expression of NLRP3. However longer infections may require NLRP3 gene transcription to replenish NLRP3 levels. These diverse pathways may indicate that the innate response is rapidly recruited following exposure to danger signals, but new protein synthesis is triggered when a more robust response to prolonged infection is required.

Activation of the NLRP3 inflammasome

The second signal is instigated by many activators (see figure 3.5). These are traditionally termed 'DAMPs' (danger associated molecular patterns) — endogenous and exogenous particulates such as

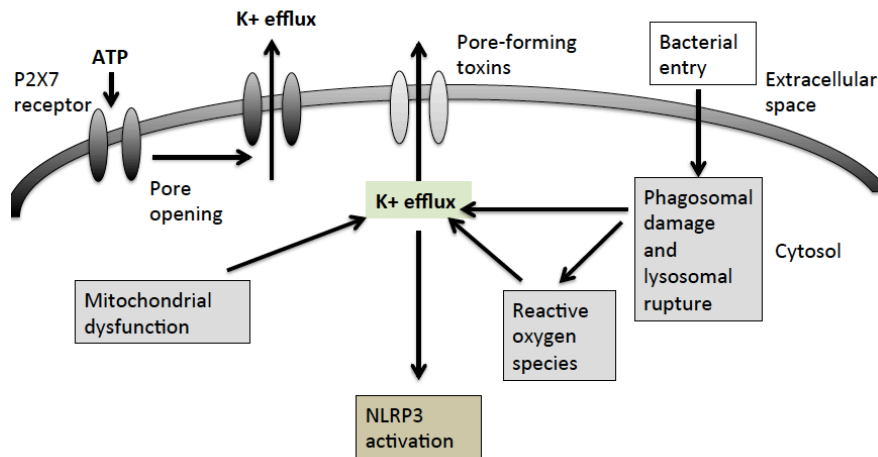


Figure 3.5: Many signals are able to activate the NLRP3 inflammasome. Phagosomal rupture, mitochondrial damage, and reactive oxygen species may all lead to NLRP3 activation. However, the unifying signal is believed to be driven by K⁺ efflux. Adapted from Broz et al. 2016.

ATP, uric acid, amyloid β and cholesterol aggregates — and ‘PAMPs’ (pathogen associated molecular patterns) such as viral and bacterial pathogens and pore-forming toxins (Vanaja et al. 2015).

Microbial activators of the NLRP3 inflammasome that have been implicated include pore-forming toxin bacteria such as *Streptococcus pneumoniae*, *L. monocytogenes*, EHEC, *Vibrio cholera*, *S. aureus*, *Burkholderia* sp., *Haemophilus* sp. and *Proteus* sp. (Eldridge et al. 2015).

In addition to bacteria, some viruses such as adenovirus and influenza virus are also able to activate NLRP3 (Ichinohe et al. 2009; Rajan et al. 2011). Viruses have been reported to depend on different signalling pathways in order to induce inflammasome activation. For example, a recent study showed that RNA viruses induce the formation of the serine/threonine kinase complex RIPK1–RIPK3 (Wang et al. 2014). The RIPK1–RIPK3 complex phosphorylates the GTPase, DRP1, and promotes its translocation to the mitochondria where DRP1 induces mitochondrial damage and subsequent NLRP3 activation (Wang et al. 2014). HIV-1 infection also induces IL-1 β production which is mediated by TLR-8 (Guo et al. 2014). Recent studies have shown that NLRP3 activation initiated by HIV-1 infection mediates neuroinflammation (Chivero et al. 2017) and contributes to HIV-associated nephropathy (Haque et al. 2016). Similarly, fungal infections such as *Cryptococcus neoformans* activate NLRP3 (Chen et al. 2015). In mice strains inherently resistant to *Paracoccidioides brasiliensis*, there is significantly higher Syk kinase phosphorylation (mediated by the surface PRR, Dectin-1) which activates NLRP3; in contrast, susceptible mice strains express lower levels of inflammasome transcripts following infection (Feriotti et al. 2015).

Nucleic acids have recently emerged as players in IL-1 β processing. Bacterial nucleic acids such as RNA:DNA hybrids are intermediates produced during the process of DNA replication (Vanaja

et al. 2015). Following phagocytosis of EHEC, bacteria are killed in the phagolysosome but bacterial RNA:DNA hybrids are able to enter the cytosol and stimulate inflammasome formation and caspase-1 processing (Kailasan Vanaja et al. 2014).

Cytosolic sensors may act upstream of NLRP3 to link the activator and the inflammasome. Kailasan Vanaja et al. 2014 demonstrated co-localisation of RNA:DNA hybrids with the inflammasome speck, although whether there was direct binding between the two remains to be seen. However, other binding partners of the NLRP3 inflammasome have been identified.

Guanylate-binding protein 5 (GBP5), a member of the interferon-inducible GBP family, has been shown to be required for inflammasome activation and assembly in response to *S. typhimurium* in IFN- γ -activated macrophages but not to crystalline agents (Shenoy et al. 2012).

Thioredoxin-interacting protein (TXNIP) is also recognised as an NLRP3 binding partner. Thioredoxin (TRX) is an endogenous antioxidant but is negatively regulated by TXNIP and during resting state, TXNIP and TRX are bound as one (Jo et al. 2015). During oxidative stress, TRX is oxidised and released from TXNIP, allowing TXNIP to bind NLRP3 and activate it (Zhou et al. 2010). Enhanced TXNIP concentrations can be seen in hyperglycaemic states (Schulze et al. 2004) and increased expression was recently seen in renal tissue under diabetic ischaemic stress with induced acute kidney injury, which was associated with heightened NLRP3 activation, which was ameliorated by TXNIP inhibition (Xiao et al. 2016).

Another NLRP3 binding partner is DHX33, a member of the DExD/H-box helicase family, a family whose members have previously been identified as DNA and RNA sensors that induce type 1 interferon (Zhang et al. 2011). DHX33 was found to bind double-stranded RNA (dsRNA) and form a complex with NLRP3 resulting in its activation (Mitoma et al. 2013).

Protein Kinase R (PKR) is reported as another binding protein. PKR phosphorylates and inhibits the stress response protein, eukaryotic initiation factor 2 α (Yim et al. 2016). PKR was reported to enhance and bind NLRP3 in response to several factors including *E. coli*, anthrax lethal toxin, ATP and uric acid (Lu et al. 2012). More recently, transfection of synthetic dsRNA into keratinocytes was shown to activate NLRP3 in a PKR-dependent fashion (Dai et al. 2017). However, given that the role of PKR is to inhibit the stress response, it does seem a little counter-intuitive that up-regulation of this should heighten a pro-inflammatory response. Indeed, Yim et al. 2016 showed that PKR represses rather than enhances inflammasome assembly and IL-1 β maturation, a finding that is in agreement with He et al. 2013 who showed that PKR was dispensable for inflammasome activity.

Because of the structural and chemical diversity of the molecules involved in NLRP3 activation, it is unlikely that NLRP3 is able to interact with them all, but rather that these activators induce a

common cellular signal which culminates in NLRP3 activation (Vanaja et al. 2015). This unifying signal is under debate, however one of the following is believed to be the proposed trigger for inflammasome assembly: (1) K^+ efflux; (2) mitochondrial dysfunction and reactive oxygen species (ROS); (3) lysosomal rupture; or (4) lysosomal rupture.

1. K^+ efflux

One of the problems in elucidating the exact mechanism of NLRP3 activation is that many known triggers, such as ATP, have pleomorphic effects and induce several of the above mechanisms (Shimada et al. 2012). In an attempt to decouple these, Muñoz-Planillo et al. 2013 have demonstrated that K^+ efflux precedes other potential activators of the inflammasome. Furthermore, NLRP3 activation is inhibited by high extracellular concentrations of K^+ (Petrilli et al. 2007). Notably, haemolysins produced by *Staphylococcus aureus* induce caspase-1 activation, which is inhibited by extracellular K^+ (Muñoz-Planillo et al. 2009). How K^+ efflux is sensed by NLRP3 is uncertain; however, recent reports suggest that K^+ efflux leads to a conformational change in NLRP3 and its subsequent activation (He et al. 2016).

2. Mitochondrial dysfunction and Reactive oxygen species (ROS)

The electron transport chain is a highly efficient process that generates 32 ATP molecules from 1 glucose molecule (Gurung et al. 2015). However, ROS may be generated as by-products of electron transport via the NADPH oxidases (the NOX proteins) situated on the cell membrane as well as on phagolysosomal membranes (Harjith et al. 2014). Following ATP stimulation or particle phagocytosis, NOX generates ROS. Initial studies suggested that ROS generated from NOX following particle phagocytosis may trigger the NLRP3 inflammasome (Dostert et al. 2008). However, peripheral blood monocytes from patients with asymptomatic chronic granulomatous disease who have defective NADPH oxidases exhibit high levels of caspase-1 activation and IL-1 β processing (Meissner et al. 2010). Additionally, inhibition of lysosomal ROS in *Nox1-4^{-/-}* mice did not reduce NLRP3 activation (Heid et al. 2013) suggesting that phagosomal ROS are dispensable in ROS-mediated inflammasome activity. Thus, this leads to the suggestion that mitochondria may be implicated in inflammasome activation since mitochondria are the greatest contributor to cellular ROS production (Vanaja et al. 2015). Blocking key enzymes in the respiratory chain leads to a loss of mitochondrial membrane potential and induction of ROS, which in turn leads to activation of the inflammasome (Zhou et al. 2011) and inhibition of voltage-dependent anion channels (VDAC), which regulate mitochondrial metabolic activity, reduce ROS production and subsequent NLRP3 activation. From a therapeutic perspective, a recent study demonstrated that the anti-viral drug, imiquimod, inhibited complex 1 of

the respiratory chain leading to the generation of ROS and activation of the NLRP3 inflammasome in a manner that was independent of K⁺ efflux (Groß et al. 2016). Similarly, liposomes are increasingly being used as efficient drug delivery conduits (Al-Jamal et al. 2011) and Zhong et al. 2013 have shown that positively charged (but not neutrally charged) liposomes activate NLRP3 in a mitochondrial ROS-dependent manner. This is interesting and may be attributed to the fact that charged liposomes are able to alter membrane potential as opposed to neutral liposomes which leads to an induction of oxidative stress. Moreover, LPS/ATP stimulation rapidly generates mitochondrial ROS which results in oxidised mitochondrial DNA that is released into the cell during apoptosis and co-localises with NLRP3 (Shimada et al. 2012). However, mitochondrial stimulation of the inflammasome may not be limited to ROS but rather a structural change may serve as an innate immune sensor. Whilst establishing the mitotoxicity profile of the antibiotic linezolid, Iyer et al. 2013 found that the mitochondrial lipid, cardiolipin — usually found on the inner membrane — externalises to the outer membrane permitting binding and activation of the inflammasome. It is interesting to note that cardiolipin redistribution following mitochondrial injury may also mediate interaction with LC-3 in order to facilitate mitophagy and rapid elimination of damaged mitochondria (Chu et al. 2013).

Despite these studies, it is difficult to conclusively say that mitochondrial damage and ROS is an absolute requirement for NLRP3 activation. Certainly Muñoz-Planillo et al. 2013 did not detect mitochondrial damage following treatment with the ionophore nigericin or ATP compared to Shimada et al. 2012. However, this discrepancy may well be due to the duration of the stimuli cells were exposed to; Shimada et al. 2012 showed increased damage after a 30–60 minute stimulation, but given that inflammasome activation occurs earlier, the mitochondrial damage seen may be due to cytotoxic effects (Muñoz-Planillo et al. 2013). It is likely that mitochondria have a hugely important role to play in terms of inflammatory regulation but that the response may depend on the stimulus and various differences in the experimental system. A recent study has shown that agonists of the NLRP3 inflammasome have differential requirements for functional mitochondria; CCCP, which disrupts the mitochondrial membrane potential, inhibited IL-1β release mediated by ATP suggesting that induction of the inflammasome by ATP needs intact mitochondria (Sadatomi et al. 2017), although there was no effect on ROS production. In contrast, CCCP permitted the release of IL-1β following other stimuli, such as nigericin treatment. This implies that mitochondrial integrity and structure depends on the stimuli and that this, rather than ROS, may play a bigger role in inflammasome activity.

3. Lysosomal rupture

Phagocytosis of particulate matter or live pathogens may disrupt the lysosomal membrane and this concept was originally proposed to activate the NLRP3 inflammasome (Jo et al. 2015). Hornung et al. 2008 showed that uptake of crystals and other particulates leads to lysosomal acidification,

swelling and leakage and subsequent activation of the inflammasome. Evidence supporting this comes from studies that show Group B *Streptococcus* (GBS)–induced IL-1 β production is dependent on phagosomal uptake and lysosomal damage, mediated by the pore-forming toxin haemolysin (Gupta et al. 2014). Lysosomal damage releases cathepsin B, a cysteine protease, which has been linked to NLRP3 activation. Pharmacological inhibition of cathepsin B, using Ca074Me, appears to block activation of NLRP3 (Dostert et al. 2008; Chu et al. 2009). Crucially however, cathepsin B–deficient macrophages only show a partial impairment of inflammasome activation (Dostert et al. 2009) suggesting that the inhibitor has off-target effects. More recently, studies have shown that at the higher concentrations used in varying studies, Ca074Me inhibits multiple cathepsins, although at lower concentrations it is specific for cathepsin B (Orlowski et al. 2015). Consistent with off-target effects, the same study showed that Ca074Me interferes with both NLRP3 priming and activation. Moreover, the range of concentrations required to inhibit inflammasome activation exceeded that in which Ca074Me works selectively against cathepsin B, as well as observed compensatory up-regulation of cathepsin activity during Ca074Me treatment, which together suggests that multiple cathepsins play a role in IL-1 β processing (Orlowski et al. 2015). In addition to cathepsin B release, lysosomal rupture leads to calcium efflux from the lysosome to the cytosol and activates one of the stress response MAP kinase pathways, TAK1–JNK, which has been implicated in aiding ASC oligomerisation and NLRP3 activation (Okada et al. 2014).

It is difficult to isolate a specific molecular event that leads to NLRP3 activation, but in general NLRP3 activators appear to trigger multiple events that include K⁺ efflux, ROS release and mitochondrial changes and lysosomal damage. Many activators have pleiotropic effects however, and whilst K⁺ efflux has been signalled as the unifying factor, this has yet to be proven in the vast array of infectious stimuli.

Cell intrinsic broad negative regulators of the NLRP3 inflammasome

In order to minimise excessive damage, steps are taken at each stage of inflammasome activity. Throughout this section, various proteins are discussed that play a negative regulatory role, but in the following, I briefly discuss the broad ways by which NLRP3 activity within the cell is kept at the minimum necessary for physiological function including: (1) autophagy and mitophagy; (2) type 1 interferons; (3) the tripartite motif–containing (TRIM) protein family; and (4) microRNA regulation of inflammasome activity.

1. Autophagy and mitophagy

Nakahira et al. [2011](#) demonstrated that deficiency in the autophagic proteins, LC3B and Beclin-1 increases the activation of caspase-1 and the processing of IL-1 β and leads to an enhanced pool of defective mitochondria following LPS treatment. The same authors also showed that induction of polymicrobial sepsis by cecal ligation and puncture led to increased mortality in mice deficient for LC3B compared to wild-type with associated higher serum IL-1 β concentrations. In a different study, the TLR–ligand interaction led to pro-IL-1 β sequestration into autophagosomes; furthermore rapamycin treatment led induction of autophagy and the degradation of the autophagosomes (Harris et al. [2011](#)). In contrast, inhibition of autophagy led to the processing and secretion of IL-1 β and IL-18. Interestingly, mice lacking Atg16L1, another autophagy-related protein, produce high amounts of IL-1 β and die following induction of colitis compared to wild-type, which is alleviated by neutralising antibodies against both cytokines (Saitoh et al. [2008](#)). Autophagy also helps protect against excessive non-canonical activation of the inflammasome as it aids in the clearance of bacteria-containing phagosomes which may be ruptured by GBPs (Meunier et al. [2015](#)).

2. Type 1 interferons

Engagement of type 1 interferons (IFN) with the type 1 IFN signalling receptor activates STAT1, which suppresses activation of NLRP1 and NLRP3 through caspase-1 inhibition by an as yet unidentified mechanism (Guarda et al. [2011](#)), and induces IL-10 production (Guarda et al. [2011](#)). IL-10 subsequently acts in an autocrine manner to reduce pro-IL-1 β expression via STAT3. Furthermore, following *S. pyogenes* infection, type 1 IFN signalling restricts inflammatory damage and helps drive pathogen clearance (Castiglia et al. [2016](#)). There is likely an optimal balance between the two cytokines with some degree of cross-talk, rather than one cytokine completely antagonising the other, that helps eliminate bacteria. For example, type 1 IFN increases AIM2 activation following infection with *F. tularensis*, even though AIM2 is not needed for type 1 IFN responses (Jones et al. [2010](#)).

3. The tripartite motif-containing (TRIM) protein family

Recent studies suggest that members of the TRIM family have increasingly important roles as inflammasome regulators. Over 60 TRIM proteins are encoded by the human genome and many are induced in response to IFNs (Zurek et al. [2012](#)). They are responsible for a number of biological processes. One member, TRIM27, harbours ubiquitin ligase activity and has been shown to inhibit NF- κ B signalling pathways through ubiquitination and proteasomal degradation of NOD2 (Zurek et al. [2012](#)). Previous studies have also shown that over-expression of another family member, TRIM30, increases the survival of mice following LPS challenge by promoting the degradation of the TAB2–TAB3–TAK1 complex, which is involved in the transcription of NF- κ B (Shi et al. [2008](#)).

In addition, siRNA knock-down of TRIM30 has been shown to increase ATP-induced caspase-1 activation and IL-1 β activation but not to interfere directly with inflammasome assembly (Hu et al. 2010). Similar reports have recently identified TRIM31 as a physiological suppressor of NLRP3, again through proteosomal degradation of the inflammasome, although in these studies co-precipitation of NLRP3 and TRIM31 in addition to co-localisation of these two proteins suggests that TRIM31 is able to directly interact with the inflammasome (Song et al. 2016). This implies that whilst members of the TRIM family have regulatory properties, there may be some mechanistic differences between them.

4. MicroRNA regulation of inflammasome activity

MicroRNAs (miRNA) are non-coding oligonucleotides that are 22–23 nucleotides in length which post-transcriptionally regulate gene expression (Qazi et al. 2013) through complementary base pairing to the 3' untranslated regions (3'UTR). A transcribed gene may be regulated by several miRNAs and similarly, a miRNA may have several targets (Qazi et al. 2013). miR-223 has been shown to decrease NLRP3 transcription and IL-1 β maturation (Bauernfeind et al. 2012). miR-223 expression itself is driven by the myeloid transcription factors, PU.1 and C/EBPs (Fukao et al. 2007). miR-7 (Tannous et al. 2009) and miR-9 (Wang et al. 2017) have recently been reported to inhibit the expression of NLRP3 and enhance M2 activation markers. On the contrary, over-expression of miR-133a-1 enhances inflammasome activation and IL-1 β release and does this without modifying basal levels of NLRP3, ASC, IL-1 β or pro-caspase-1 (Bandyopadhyay et al. 2013).

Thus, in order to keep the host inflammasome in check, several host mechanisms are employed and likely work in concert, and thus in cases of heightened inflammasome response, this may be attributed not only to the inflammasome complex but also to the factors that help regulate its activation.

NLRP3 and disease

As with all inflammatory pathways, unchecked over-activation of the NLRP3 inflammasome has been implicated in a number of diseases. Metabolic disorders have been strongly linked with aberrant NLRP3 activity, with studies demonstrating that an overload of free fatty acids increases NLRP3 activation and insulin resistance (Legrand-Poels et al. 2014). Diabetic mice exhibit increased IL-1 β in pancreatic lymph nodes compared to wild-type mice, which is driven by an increase in mitochondrial DNA (Carlos et al. 2017). Similarly, NLRP3 activation contributes to the inflammatory damage seen in cardiac (Mastrocola et al. 2016) and neural (Yu et al. 2017) ischaemic/reperfusion injury in animal models, and pharmacological inhibition of NLRP3 induces a cardio and neuro-protective effect

respectively (Mastrocola et al. 2016; Yu et al. 2017). Moreover, in a study comparing humans with atherosclerotic plaques to subjects with non-atherosclerotic vessels, there was a significant increase in NLRP3 mRNA in the symptomatic cohort and an associated increased sensitivity when exposed to TLR4 agonists and ATP (Paramel Varghese et al. 2016), suggesting that NLRP3 may contribute to accelerated cardiovascular disease.

Increased NLRP3 activation has also been linked to auto-inflammatory disorders. Gain-of-function mutations in *Nlrp3* are associated with the development of the cryopyrin-associated periodic syndromes (CAPS) which are characterised by chronic inflammation which is manifested by episodic fevers, neutrophilia and localised inflammation in the skin, joints, muscle and cerebrospinal fluid (Zhong et al. 2016). Patients with active rheumatoid arthritis have higher intracellular expression of NLRP3 compared to controls (Choulaki et al. 2015).

Disorders associated with active haemolysis may also be driven by NLRP3 activation. Certainly, evidence suggests that the release of haem during intravascular haemolysis associated with sickle cell disease drives TLR4 signalling and vaso-occlusion in transgenic sickle cell mice (Belcher et al. 2014). Further dissection of this pathway has demonstrated that haem mediates NLRP3 activation and contributes to increased lethality compared to *Nlrp3*^{-/-} mice (Dutra et al. 2014).

While excessive NLRP3 activation may be causal in inflammatory disorders, a pathogenic role for NLRP3 during infectious diseases may only be speculative. However, free haem can initiate the onset of severe sepsis, and by definition, multi-organ failure, in mice models subjected to relatively low bacterial loads (Larsen et al. 2010), suggesting that over-activation of the inflammasome may increase susceptibility to bacterial infection. In rodent malaria, symptomatic infection has been observed to be mediated by NLRP3 and NLRP12 producing low levels of IL-1 β ; however malaria-primed monocytes become hyper-responsive, producing excessive IL-1 β when challenged with low levels of TLR ligands such as LPS, suggesting that bacterial super-infection that is often described with malaria may be attributed to heightened inflammasome activity (Ataide et al. 2014). In a different study that examined host defences against vaginal candidiasis in mice, whilst NLRP3 was systemically protective, it was found to mobilise vast numbers of inflammatory cells and generated vaginal immunopathology (Borghi et al. 2015). This was surprisingly counteracted by IL-22 which leads to the subsequent activation of the NLRC4 inflammasome and the sustained production of IL-1 receptor antagonist (IL-1RA) (Borghi et al. 2015). Thus, rather than NLRP3 and NLRC4 acting cooperatively together to enhance IL-1 β maturation as reported previously (Qu et al. 2016), NLRC4 may have an additional role in negatively regulating NLRP3 signalling. Similarly, immune cell expression of MUC1 has recently been identified as a negative regulator of NLRP3 and was shown to protect against hyper-elevated IL-1 β , gastric dysplasia and death propagated by NLRP3 activation in mice following chronic *Helicobacter*

pylori infection (Ng et al. 2016). Similarly, CARD9, which has previously been implicated in driving inflammasome formation following fungal infection (Gringhuis et al. 2012), has recently been shown to be an important negative regulator of NLRP3 in BMDMs following infection with *S. typhimurium* through a combination of reducing the expression of pro-IL-1 β and suppressing Syk phosphorylation which in turn reduces activation of ASC and inflammasome assembly (Pereira et al. 2016). No such effect was seen in dendritic cells, which is likely due to the fact that Syk has no effect on NLRP3–caspase-1 activation in these cells (Hara et al. 2013) suggesting that negative regulatory functions may be cell specific. Thus, deleterious effects that may be seen with continued NLRP3 activation are perhaps due to a disturbances in the mechanisms that usually counteract this.

The non-canonical activation of the NLRP3 inflammasome

The non-canonical activation of the inflammasome involves caspase-11, rather than caspase-1, and is activated in response to gram-negative bacteria (Vanaja et al. 2015). Humans lack caspase-11, but express the homologues caspase-4 and -5 (Vanaja et al. 2015). Kayagaki et al. 2011 first observed that the mouse strain, 129, which lacks a functional caspase-11, is not able to activate NLRP3/ASC-dependent processing of IL-1 β following stimulation by gram-negative bacteria, such as *E. coli*, *Citrobacter rodentium* and *V. cholera*. Subsequently it was shown that it is cytosolic LPS that is the trigger for caspase-11 activation (Hagar et al. 2013). Bacteria that are able to escape their vacuolar niche can induce cytosolic delivery and caspase-11 activation (Kayagaki et al. 2013). The CARD of caspase-11 is able to directly bind the lipid moiety of LPS which induces oligomerisation and activation. Similarly, the CARDS of caspase-4 and -5 can bind LPS (Shi et al. 2014).

Immune recognition of LPS is independent of TLR4 signalling (Aachoui et al. 2013; Kayagaki et al. 2013) and transfection of LPS has been reported to activate caspase-11 (Kayagaki et al. 2013). However-given the low basal expression of caspase-11, a transcriptional increase may be necessary in order for it to sense cytosolic bacteria (see figure 3.6). This is mediated by an earlier signal where bacterial LPS interacts with the TLR4/MyD88 pathway or the TRIF pathway, leading to the activation of the transcription factors NF- κ B and interferon regulatory factors 3 and 7 (IRF-3, IRF-7). IRF-3 and -7 act together to induce the transcription, translation and secretion of type 1 interferons (IFN- α/β) (Lazear et al. 2013). Extracellular IFN- α/β bind to their cognate receptor to trigger the activation of the JAK–STAT signalling pathway, which in turn leads to the transcription of caspase-11 (Pellegrini et al. 2017). Interestingly, type 1 IFNs are also able to induce the family of guanylate binding proteins (GBP), in particular GBPs on chromosome 3 of the mouse genome, which have recently been shown to induce host destruction of phagosomes or vacuoles, such that the bacterial contents are recognised by caspase-11 in the cytosol (Pilla et al. 2014; Meunier et al. 2014). Once

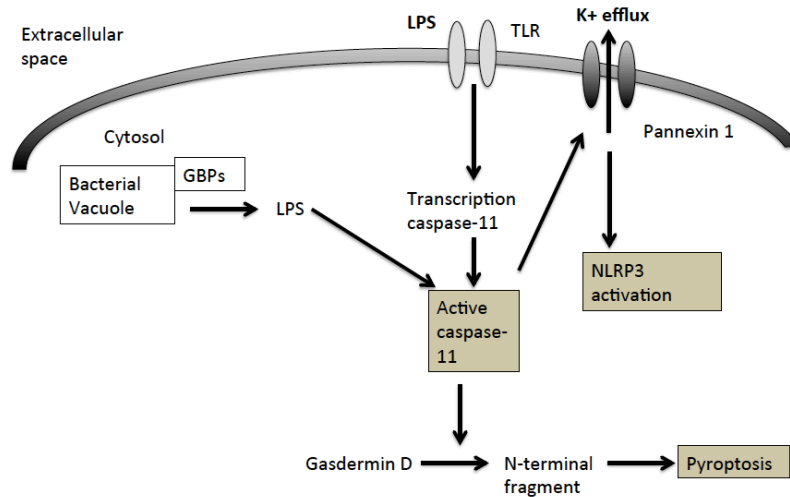


Figure 3.6: The non-canonical inflammasome. TLR–ligand interactions lead to the downstream transcription of type 1 IFNs and caspase-11. Type 1 IFNs induce GBPs which can facilitate bacterial escape from the vacuole. Cytosolic LPS then binds caspase-11 (or caspase-4 and -5 in humans) leading to their activation. Active caspase-11 cleaves Gasdermin D into an N-terminal fragment to induce pyroptotic cell death. Caspase-11 can also process pannexin-1, stimulating K^+ efflux and thus activating the NLRP3 inflammasome. Adapted from Broz et al. 2016.

caspase-11 is activated, it cleaves the protein gasdermin D into two fragments, the amino terminus and carboxyl domain. The amino terminus is able to oligomerise in cell membranes to form pores and induce pyroptosis (Aglietti et al. 2016; Liu et al. 2016b). Additionally, whilst caspase-11 does not directly lead to maturation of IL-1 β , it cleaves the cytosolic region of pannexin-1, an ATP channel, which leads to the release of ATP and subsequent rapid activation of P2X7 (Yang et al. 2015).

The alternative inflammasome pathway

Much of our understanding of activation of the NLRP3 inflammasome is based on the murine model where requirement for two signals is necessary for IL-1 β maturation. However, LPS alone is able to lead to maturation of IL-1 β in human monocytes (Schumann et al. 1998). Recently, Gaidt et al. 2016 have demonstrated that IL-1 β maturation can occur following LPS interaction with the TLR4 receptor to engage the TRIF–FADD–RIPK1–Caspase-8 pathway, which requires NLRP3 and ASC, but occurs independently of K^+ efflux, pyroptosis and speck formation. Whilst caspase-8 has previously been shown to induce priming of the inflammasome (Gurung et al. 2014) and drive direct processing of IL-1 β (Moriwaki et al. 2015), the alternative inflammasome complex does not affect priming of IL-1 β . It also does not appear allow direct cleavage of IL-1 β by caspase-8 in the absence of NLRP3, ASC or caspase-1.

AIM2

AIM2 is part of the PYHIN (pyrin domain (PYD) and HIN domain-containing) members of which are able to sense DNA (Morrone et al. 2015). Unlike other inflammasomes, AIM2 is able to bind directly to its ligand, double-stranded DNA (dsDNA), via its HIN domain and form an oligomeric structure which permits its activity. As with NLR activation, AIM2 oligomers recruit ASC through their pyrin domain and initiate its polymerisation and subsequent interaction with and processing of pro-caspase-1 (Morrone et al. 2015). A minimum sequence length of 80 base pairs of dsDNA is required for the HIN domain to bind its ligand and cause optimal IL-1 β maturation (Jin et al. 2012; Morrone et al. 2014). Since an electrostatic attraction mediates the interaction between the HIN domain and dsDNA, Jin et al. 2013 have proposed that an equivalent interaction might exist between the HIN and pyrin domain in order to maintain AIM2 in an auto-inhibitory state; indeed crystal studies have shown that their opposing charged surfaces promote a strong attraction to each other, therefore acting as a safeguard to ensure that AIM2 signals downstream only following activation by ligand interaction. The presence of the negatively charged dsDNA sugar-phosphate backbone displaces the pyrin domain and interacts with the positively charged HIN domain, liberating the AIM2 pyrin domain to bind to the ASC pyrin domain (Jin et al. 2012). However, the auto-inhibitory purpose of the pyrin domain has been put into doubt with recent work, suggesting that the AIM2 pyrin domain actually oligomerises, drives filament assembly and is essential for dsDNA binding leading to ASC recruitment (Morrone et al. 2015). AIM2 is able to bind DNA from several microbes such as *Francisella novicida*, *L. monocytogenes* and *S. pneumoniae* (Tsuchiya et al. 2010; Belhocine et al. 2012; Fang et al. 2014), which may have in common the ability for cytosolic DNA to escape into the vacuole and activate the DNA sensors cGAS and STING, which induce the transcription of type 1 interferons. During infection with *F. novicida*, the type 1 interferons are secreted from the cell and, in an autocrine manner, bind their receptors and induce the expression of the interferon regulatory factor-1 (IRF-1). This leads to the up-regulation of GTPases, including GBP2, which have been described as a key activator of AIM2 through promotion of bacterial lysis, which allows the presentation of bacterial DNA to AIM2 (Meunier et al. 2015). Whilst transfection of dsDNA directly into the cell can activate AIM2 independently of the STING pathway, vacuolar release of DNA by these bacteria has thus far not been shown to bind and activate AIM2 — why this is the case is as yet unclear and has been attributed to the fact that the cytosolic concentration may not be high enough to activate the inflammasome (Man et al. 2015). However, transfection of small DNA concentrations is still able to drive AIM2 activation, independent of STING (Meunier et al. 2015) suggesting that priming of AIM2 and DNA concentration are not the most important reasons for activation.

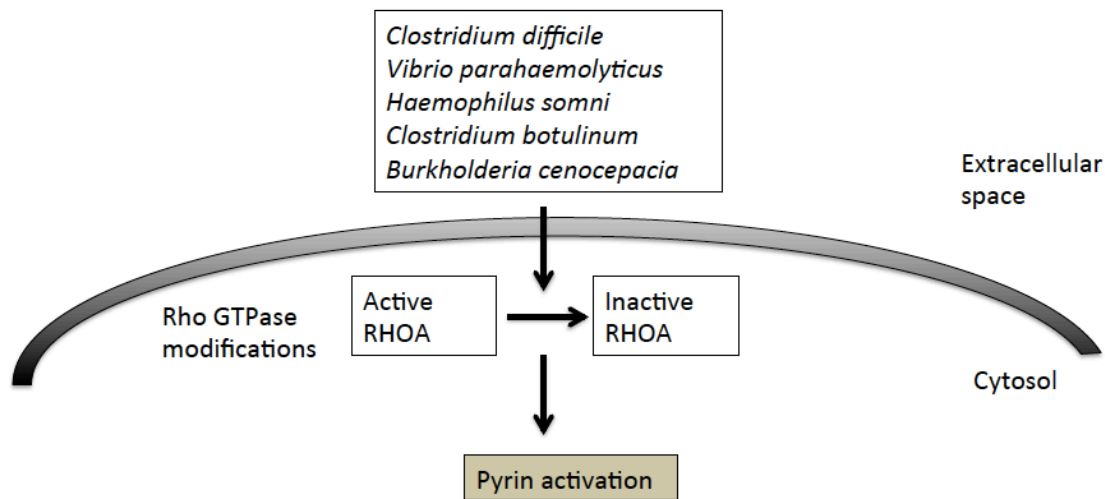


Figure 3.7: The Pyrin inflammasome senses changes in intracellular biological processes. Bacterial effectors lead to the deamidation of the RhoA GTPases leading to their deactivation. Deactivation of RhoA induces the activation of the Pyrin inflammasome. Adapted from Broz et al. 2016.

The Pyrin inflammasome

Pyrin is a non-ALR/NLR-containing inflammasome and belongs to the TRIM protein family (Broz et al. 2016). It contains an N-terminal PYD that binds ASC, a central coiled-coiled domain and a C-terminal B30.2/SPYR domain that is only present in the human protein (Torre-Minguella et al. 2017). Pyrin is activated in a rather indirect manner. Pyrin becomes activated by pathogen modulation of host proteins (Vanaja et al. 2015). Examples include bacterial effectors, such as TecA, secreted by *Burkholderia cenocepacia* type 6 secretion systems (T6SS) which can lead to the deamidation of RhoA GTPases causing their inactivation and disruption of the host cytoskeletal system (Aubert et al. 2016). Infection with *L. monocytogenes* leads to deactivation of RhoA, which induces activation of Pyrin and subsequent co-localisation of Pyrin–ASC with actin filaments, possibly implying that Pyrin may act as a specific sensor for actin (Waite et al. 2009, see figure 3.7). Other species of bacteria, such as *Clostridium botulinum* and *Vibrio parahaemolyticus*, as well as the pertussis toxin can all modify the Rho GTPases and these changes are also sensed by Pyrin (Vanaja et al. 2015). The biological relevance of this response to bacteria is uncertain, however, mutations in the B30.2/SPYR domain of human Pyrin are associated with familial mediterranean fever and heightened IL-1 β release (Hesker et al. 2012).

3.1.3 How is *M. tuberculosis* detected by the inflammasomes?

Many studies have used knock-out models in order to establish the contribution of the individual components of the inflammasome during *M. tuberculosis* infection. However the literature regarding the necessity or redundancy of various components has been rather equivocal, especially with *in vitro*

work. One of the reasons for this is possibly the variation in experimental design. Firstly, the common strain that is used is H37Rv which, as an adapted laboratory strain, may elicit functional differences between research groups. Secondly, differences in the multiplicity of infection (MOI), the choice to wash the bacteria after a set period (with or without using antibiotics to remove extracellular bacteria), and length of the incubation time before harvesting the samples for analysis will almost certainly exert their own influence on inflammasome activation. Moreover, studies vary in their reporting of IL-1 β release; whilst protein detection in modern ELISAs are more sensitive to mature IL-1 β , they will invariably include both pro- and cleaved forms of the protein. For detection of mature IL-1 β , western blots may be employed but this requires the protein in the supernatant fraction to be concentrated and again, this has not been universally performed. Additionally, a recent study by Leisching et al. [2016](#) has shown that BMDMs infected with multi-drug-resistant clinical mycobacterial strains cultured in detergent-free medium elicited a robust pro-inflammatory response compared to *M. tuberculosis* cultured in a medium containing Tween-80. Thus, variations in design provide some difficulties in interpretation. In the next few paragraphs I will detail the *in vitro*, *in vivo* and human studies that have examined the inflammasome during *M. tuberculosis* infection.

***In vitro* studies examining mycobacteria and inflammasome activation**

Macrophages and dendritic cells are both capable of inflammasome-dependent IL-1 β production during *M. tuberculosis* infection (Abdalla et al. [2012](#); Briken et al. [2013](#)). More recently, this has been shown in part to be mediated by a shift from oxidative phosphorylation to aerobic glycolysis (Gleeson et al. [2016](#)). However, an early study performed in murine macrophages suggested that inflammasome activity is in fact inhibited by mycobacteria, a mechanism which was proposed to be mediated by the secretion of the zinc metalloprotease Zmp1 (Master et al. [2008](#)). Most experiments in this study generated *zmp1* mutants in the vaccine strain *Mycobacterium bovis* BCG (Master et al. [2008](#)). However, BCG does not express the ESX-1 secretion system (encoded in part by the Region of Difference 1, RD-1). We now know that activation of the inflammasome and release of IL-1 β by *M. tuberculosis* is dependent on ESX-1 and the secreted protein ESAT-6 which promotes lysosomal membrane damage (Koo et al. [2008](#); Mishra et al. [2010](#); Dorhoi et al. [2012](#)) and necrotic cell death (Wong et al. [2011](#)). In determining the potential role for Zmp1 in virulent bacteria, deletion of *zmp1* in H37Rv and H37Rv Δ RD1 (which would therefore partially mimic BCG) does not affect IL-1 β production (Wong et al. [2011](#)), suggesting that Zmp1 does not play a direct role in inflammasome activation.

While the ESX secretion system has been demonstrated to be critical for inflammasome activity, there is some evidence showing that heat-killed mycobacteria, which are not able to secrete proteins, are

able to stimulate the inflammasome via the peptidoglycan cell wall in a CARD9-dependent manner (Shenderov et al. 2013). However, Krishnan et al. 2013 and Koo et al. 2008 did not observe any effect induced by heat-killed.

Induction of inflammasome activity requires internalisation of the bacteria since pharmacological disruption of phagocytosis with cytochalasin D reduces IL-1 β detection (Mishra et al. 2010; Dorhoi et al. 2012). Studies investigating the specific inflammasome that facilitates IL-1 β processing *in vitro* have reported conflicting results, and the *in vitro* importance of IL-1 signalling has not been universally corroborated with bacterial counts or other measures of immune protection. As such, the major studies, pertinent experimental details, outcomes and whether bacterial counts were performed are shown in table 3.1. The involvement of NLRC4 has been tested in BMDMs during infection, but appears not to play much of a role (Koo et al. 2008; Abdalla et al. 2012). Most studies identify NLRP3 as the major sensor, which is scaffolded by ASC and caspase-1 (Walter et al. 2010; McElvania Tekippe et al. 2010; Wong et al. 2011; Dorhoi et al. 2012). Certainly, inhibition of K⁺ efflux with the oral anti-diabetic agent, glibenclamide also reduces IL-1 β release (Wong et al. 2011; Dorhoi et al. 2012) suggesting a critical role for NLRP3 activation and K⁺ efflux as the common activator. Moreover, Syk kinase, which has previously been shown to activate the NLRP3 inflammasome during fungal infection (Poeck et al. 2010), has been found to exhibit a similar activating role during *M. tuberculosis* infection, without affecting phagosomal uptake (Wong et al. 2011). A similar result was seen by Lee et al. 2012 who showed that NLRP3 dependent Dectin-1/Syk-kinase signalling was required for caspase-1 activation following infection with *Mycobacterium abscessus*.

What are the other regulators of NLRP3 during *M. tuberculosis* infection? While ROS may be involved in non-mycobacterial inflammasome activation (Zhou et al. 2011), their role in mycobacterial infection has been equivocal with Wong et al. 2011; Dorhoi et al. 2012 demonstrating no effect imposed by ROS inhibition, whereas a reduction in IL-1 β processing was seen following ROS inhibition and *M. bovis* infection (Zhou et al. 2015). It is worth noting that in the latter study, the authors removed the supernatants after a longer infection time compared with Dorhoi et al. 2012, by which time the effects of ROS inhibition might be more prominent. Nitric oxide, whose levels are increased by IFN- γ , was found to post-translationally modify the inflammasome through directly S-nitrosylating NLRP3 (Mishra et al. 2013). Epigenetic modification, through the addition of methyl groups to the promoter region of the NLRP3 gene, appears to be a further negative regulatory mechanism and *M. tuberculosis* infection can demethylate the promoter region resulting in the activation of the inflammasome (Wei et al. 2016).

Since, in general, IL-1 β is only partially abrogated in some studies (Koo et al. 2008; Wong et al. 2011; Mishra et al. 2010) it has been suggested that another sensor is at play (see figure 3.8). Indeed,

studies using *M. tuberculosis*, *M. bovis* and other mycobacteria species show that the AIM2 DNA sensor is an important participant in inflammasome assembly and requires a functional ESX-1 secretion system for it to be activated (Saiga et al. 2012; Shah et al. 2013; Yang et al. 2013b; Zhou et al. 2015; Liu et al. 2016a, see table 3.1). Given that *M. tuberculosis* is highly capable of preventing phagosomal acidification in order to freely translocate into the cytosol (Simeone et al. 2015) it is perhaps not surprising that the AIM2 sensor is involved. The fact that AIM2 and NLRP3 produce such a potent pro-inflammatory response that requires the ESX-1 secretion system has recently been harnessed to enhance the efficacy of the BCG vaccine. The BCG vaccine is well known to protect against *M. tuberculosis* infection in addition to protecting against disseminated TB (Roy et al. 2014), however the efficacy wanes after 10 years (Abubakar et al. 2013). In order to design a vaccine that has the potential to increase immunogenicity, potentially by enabling increased bacterial translocation into the cytosol, but without the harmful profile of *M. tuberculosis*, a recombinant BCG candidate was created. The BCG Δ ureC::hly (rBCG) vaccine expresses the membrane-perforating listeriolysin gene (*hly*) and is depleted of the enzyme urease C (*ureC*), preventing phagosomal acidification (Grobe et al. 2005). Compared to the standard BCG vaccine, rBCG was found to induce AIM2 activation, caspase-1 activity, IL-1 β processing and increase autophagy by increasing mycobacterial DNA release into the cytosol (Saiga et al. 2015).

However, in the last few years there has been increasing interest in the role of other DNA sensors with a special focus on cGAS (cyclic GMP-AMP synthase). Disruption of phagosomal membranes via the *M. tuberculosis* ESX-1 secretion system allows the escape of DNA into the cytosol. Cytosolic DNA binds to cGAS which catalyses the synthesis of the secondary messenger cGAMP. cGAMP engages the nucleotide sensor STING (Wassermann et al. 2015) leading to the activation of the kinase TBK1 and subsequent phosphorylation of the transcription factor IRF3 to promote the type 1 IFN responses (Collins et al. 2015; Watson et al. 2015). Of note, cGAS was found to co-localise with the autophagy protein Beclin-1 (Wassermann et al. 2015) and to help ferry *M. tuberculosis* to ubiquitin-mediated autophagy pathways (Watson et al. 2015). Wassermann et al. 2015 also showed that whilst cGAS-deficient cells are not able to secrete IFN- β , there is no effect on IL-1 β release, suggesting that cGAS initiates an IFN-specific pathway. However, silencing AIM2 reduces IL-1 β release by 50 %, whereas abolishing ASC completely eliminates IL-1 β release, affirming that there is more than one inflammasome sensor and that there are likely two major DNA sensors, AIM2 and cGAS, which drive different pathways and are activated following infection.

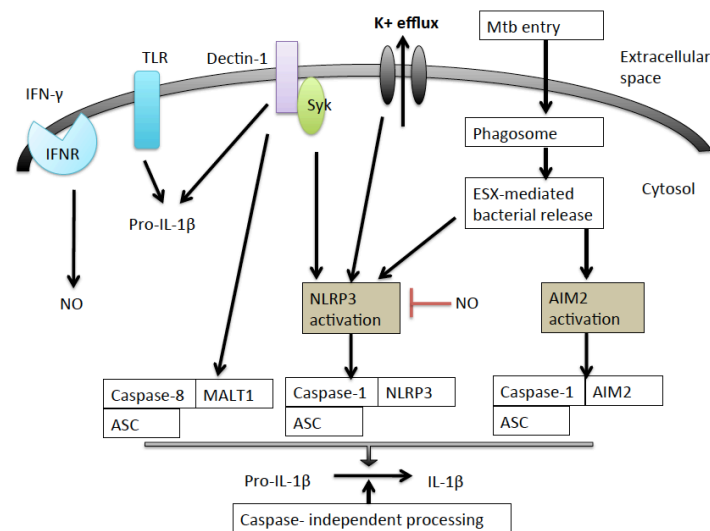


Figure 3.8: Current model of inflammasome activation during *M. tuberculosis* infection. The interaction of TLR and Dectin-1 with their cognate ligands leads to the transcription of pro-IL-1 β . Binding of Dectin-1 to its ligand can result in the canonical and non-canonical processing of IL-1 β through activation of caspase-8. This forms a complex with MALT1 and ASC to process the cytokine. The two inflammasome sensors that have thus far been identified are NLRP3 and AIM2. NLRP3 appears to be activated by K⁺ efflux and phagosomal damage and is dependent on the ESX-1 secretion system. NLRP3 activation during *M. tuberculosis* infection is known to be negatively regulated by nitric oxide. The ESX-1 system is also responsible for the activation of AIM2. Activation of the inflammasome sensor leads to formation of the sensor–ASC–caspase-1 complex and thus IL-1 β processing, however caspase-1-independent processing of IL-1 β has been identified in some studies during infection with *M. tuberculosis*.

***In vivo* studies examining mycobacteria and inflammasome activation**

Knock-outs of inflammasome genes appear to have variable outcomes in terms of bacterial load, survival and IL-1 β production. I have displayed the relevant studies in the table below (see 3.2). Overall, *IL-1R*^{-/-} and *MyD88*^{-/-} knock-out mice have greatly reduced survival (<40 days) compared to wild-type mice (Sugawara et al. 2001; Fremond et al. 2007; Mayer-Barber et al. 2010; Mayer-Barber et al. 2014). Similarly, mice deficient in IL-1 β also rapidly succumb to *M. tuberculosis* infection (Mayer-Barber et al. 2010) suggesting that IL-1 signalling, in particular IL-1 β , is crucial for defence against *M. tuberculosis*. In contrast, the necessary components upstream of the IL-1 β receptor are poorly understood. One study examining the role of NLRC4 has shown that it is redundant for IL-1 β release *in vivo* (McElvania Tekippe et al. 2010). Similarly, NLRP3 does not seem to be required for IL-1 β processing, bacterial containment or survival *in vivo* (McElvania Tekippe et al. 2010; Dorhoi et al. 2012) which contrasts with the *in vitro* data. Saiga et al. 2012 have demonstrated that AIM2 has an important role to play in IL-1 β processing and survival, but this study only compared *Aim2*^{-/-} to wild-type mice and did not look at other inflammasome components. Mice vaccinated with the rBCG vaccine, which activates the AIM2 inflammasome (Saiga et al. 2015), displayed a more robust TH-1 inflammatory response (Grode et al. 2005; Rao et al. 2013) and increased numbers of antigen-specific memory CD4⁺ (including Ag85B-specific) T-cells to the lung compared to BCG alone which contributed to increased protection against *M. tuberculosis* (Vogelzang et al. 2014).

Model	Genotype	Treatment	Infection length	MOI	Outcome	Reference
J774A macrophages	wild-type	<i>M. bovis</i> BCG	4 hours	—	<i>M. bovis</i> BCG inhibits IL-1 β release	Master et al. 2008*
Primary BMDMs C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-} , <i>Asc</i> ^{-/-}	<i>M. marinum</i> (Mm), <i>Mm</i> Δ RD1, Erdman <i>M. tuberculosis</i> and heat-killed <i>M. tuberculosis</i>	24 hours	10	Wild-type Mm and <i>M. tuberculosis</i> induce high IL-1 β release that is dependent on the RD1 regulon (ESX-1) and live bacteria. Partial reduction in IL-1 β in <i>Nlrp3</i> ^{-/-} and <i>Asc</i> ^{-/-}	Koo et al. 2008*
THP-1 macrophages	wild-type, shRNA against NLRP3 and ASC	H37Rv, H37Rv Δ RD1, H37Rv Δ ESAT-6	18 hours	10	IL-1 β release is dependent on the RD1 regulon (ESX-1) and functional ESAT-6. Partial reduction in IL-1 β in knock-downs. Transfection of active caspase-1 reduced bacterial CFU after 3 days	Mishra et al. 2010
Primary BMDMs C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-} , <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-}	H37Rv	8 hours	10	Almost full dependence on ASC, NLRP3 and caspase-1 for IL-1 β	McElvania Tekippe et al. 2010*
Primary BMDCs C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-}	H37Rv	24 hours	3	IL-1 β fully dependent on NLRP3	Walter et al. 2010*
THP-1 macrophages	wild-type, shRNA against NLRP3	H37Rv, H37Rv Δ esxA	4 hours infection then washed and incubated for 1–3 days	5–10	ESAT-6 causes phagosomal damage and necrosis mediated by NLRP3 that is blocked by glibenclamide and IL-1 β partially inhibited by shRNA against NLRP3	Wong et al. 2011*
Primary BMDMs C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-} , <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-}	H37Rv, <i>M. bovis</i> BCG, <i>M. bovis</i> BCG::RD1	6 hours	5	IL-1 β is dependent on the RD-1 regulon. IL-1 β fully dependent on NLRP3, ASC, Caspase-1	Dorhoi et al. 2012*
Primary BMDCs C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-} , <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-}	H37Rv (ATCC 25618), H37Rv Δ esxA	4 hours infection then washed and incubated for 1 day	10	IL-1 β is partially dependent on ESX-1. IL-1 β is partially dependent on Caspase-1/11 but fully dependent on NLRP3, ASC. Host cell death not affected in <i>Nlrp3</i> ^{-/-} , <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-}	Abdalla et al. 2012*
Primary BMDMs BALB/c	wild-type	H37Rv, Clinical <i>M. tuberculosis</i> isolates	1–2 days	5	Differential strain induction IL-1 β independent of caspase-1 and cell death	Krishnan et al. 2013
Primary thioglycolate-stimulated peritoneal macrophages C57BL/6	wild-type, <i>Aim2</i> ^{-/-}	H37Rv (ATCC 358121)	6 hours infection, washed and incubated for 1 day	3	IL-1 β is partially dependent on AIM2. Co-localisation of <i>M. tuberculosis</i> DNA with AIM2	Saiga et al. 2012*
Primary BMDMs C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-} , <i>Aim2</i> ^{-/-} , <i>Nlrp3</i> ^{-/-} / <i>Aim2</i> ^{-/-}	<i>M. smegmatis</i> , <i>M. smegmatis</i> Δ esx1, H37Rv	2 hours infection, washed and incubated for 16 hours	10	IL-1 β is partially dependent on ESX-1. IL-1 β is partially dependent on either NLRP3 or Aim2. IL-1 β is still released in <i>Nlrp3</i> ^{-/-} / <i>Aim2</i> ^{-/-} . IL-1 β release by H37Rv was AIM2-independent	Shah et al. 2013*
J774A.1 macrophages and primary BMDMs C57BL/6	wild-type, shRNA against AIM2 in J774A.1	<i>M. bovis</i>	2 hours then washed and incubated between 6–48 hours	10–100	<i>M. bovis</i> induces IL-1 β processing in both J774A.1 macrophages and primary BMDMs; higher level seen at 24 hours. Increasing MOI correlates with increased AIM2 and ASC mRNA. LDH release and IL-1 β were partially dependent on AIM2. IFN- β inhibits AIM2 mRNA. Cytosolic detection of <i>M. bovis</i>	Yang et al. 2013b*
THP-1	wild-type	<i>M. bovis</i>	2 hours then washed and incubated for 16 hours	10	<i>M. bovis</i> induces IL-1 β but was inhibited by extracellular K ⁺ , prevention of new protein synthesis and ROS and deubiquitination	Zhou et al. 2015*
Primary BMDMs C57BL/6	wild-type	<i>M. bovis</i>	2 hours then washed and incubated for 16 hours	10	<i>M. bovis</i> -induced AIM2 activation decreases autophagy due to inhibition of the STING-IFN- β pathway	Liu et al. 2016a

Table 3.1: *In vitro* studies following *M. tuberculosis* infection and inflammasome contribution. * indicates study made no comment on CFUs.

ASC may not be needed early after the initial infection but does confer increased survival in the late stages of infection, however, IL-1 β and overall bacterial load surprisingly remains unaffected in ASC knock-out mice (McElvania Tekippe et al. 2010; Mayer-Barber et al. 2010). Caspase-1 may afford some protection against *M. tuberculosis*, with a trend to reduced survival in the late stages

of infection (Mayer-Barber et al. 2010) although McElvania Tekippe et al. 2010 did not detect any survival differences over the same time-frame. In both studies, however, IL-1 β and bacterial load remained the same in *Caspase-1*^{-/-} mice.

Whilst in general the production of pro-inflammatory cytokines and activation of the inflammasome clearly play a clear role in preventing the spread of *M. tuberculosis*, which is more apparent *in vivo*, perpetual activation of the inflammasome by *M. tuberculosis* promotes multiple rounds of pyroptosis and an increased influx of neutrophils which can cause devastating damage to the tissues (Zhang et al. 2014) and drive MMP recruitment in the central nervous system (Harris et al. 2007a) propagating the inflammatory damage. *M. marinum* activates the NLRP3 inflammasome in zebrafish models exacerbating inflammatory pathology with no effect on bacterial growth (Carlsson et al. 2010). In F2 mice, which are highly susceptible to TB, progressive disease can be predicted by high lung expression of IL-1 β (Lyadova et al. 2010). Thus, there must be mechanisms by which NLRP3 is not left unchecked. Through modifying and reducing activation of NLRP3, as stated above, nitric oxide reduces IL-1 β and tissue damage (Mishra et al. 2013). As identified in the previous section, autophagy is another negative regulator of the inflammasome that suppresses *M. tuberculosis* growth and reduces pulmonary inflammation, necrosis and neutrophil infiltration in mouse models (Castillo et al. 2012). TIR-8 inhibits NF- κ B activation by IL-1 and related members (Wald et al. 2003), therefore has diametrically opposing actions to IL-1 β . In a result that parallels the importance of IL-1R in bacterial containment and mortality in mice, *Tir8*^{-/-} mice succumb rapidly (within 56 days) of infection (Garlanda et al. 2007) with extensive liver necrosis but mortality can be reversed in mice treated with an IL-1 β neutralising antibody two weeks after *M. tuberculosis* infection. The granuloma poses somewhat of a conundrum in *M. tuberculosis* infection, since its formation and maintenance is needed to contain the infection, but it also provides an environmental niche for mycobacteria and can impede access for standard chemotherapy (Dartois 2014). One could play devil's advocate in the study by McElvania Tekippe et al. 2010 by suggesting that uncontained bacteria and reduced granulomas seen in *Asc*^{-/-} mice may make it an easier target for anti-microbial therapy.

In summary, there is a large discrepancy between the outcomes obtained using cell models and *in vivo* study designs. During *in vivo* infection with *M. tuberculosis*, IL-1 receptor and MyD88 are required for immediate bacterial containment. Whilst NLRP3 does process IL-1 β , AIM2 rather than NLRP3 may be the critical sensor activated during *M. tuberculosis* infection. ASC affords a late-phase survival advantage that is not mediated by IL-1 β and may be due to aiding long-term granuloma formation (McElvania Tekippe et al. 2010). Since IL-1 β may still be produced in the absence of caspase-1 and ASC, this may suggest that IL-1 β may be processed via a caspase-1-independent pathway. Moreover, as both IL-1 β and IL-1R-deficient mice succumb rapidly to *M. tuberculosis*, whilst caspase-1, NLRP3 and ASC-deficient mice do not, an alternative *in vivo* mechanism for IL-1 β processing is strongly

suggested to occur in a compensatory manner . However, whilst IL-1 β signalling forms a critical aspect of the innate defense, it must be counter-regulated in order to prevent excessive tissue damage and even death.

Mice strain	Genotype	Treatment	Infection length	Outcome of knock-out	Reference
C57BL/6	wild-type, <i>IL-1R</i> ^{-/-} , <i>MyD88</i> ^{-/-}	H37Rv	Up to 90 days	Early (<40 days) lethality	Fremond et al. 2007
C57BL/6	wild-type, <i>IL-1R</i> ^{-/-}	H37Rv	Up to 49 days	Early lethality, increased granuloma	Sugawara et al. 2001
129	wild-type, <i>IL-1α</i> / β ^{-/-}	H37Rv	Up to 49 days	reduced bacterial control	Yamada et al. 2000
C57BL/6	wild-type, <i>IL-1R</i> ^{-/-} , <i>IL-1β</i> ^{-/-} , <i>MyD88</i> ^{-/-} , <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-}	H37Rv	Up to 280 days	Early lethality for <i>IL-1R</i> ^{-/-} , <i>IL-1β</i> ^{-/-} , <i>MyD88</i> ^{-/-} , partial increase late phase lethality <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-} , Unimpaired IL-1 β and bacterial load in <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-}	Mayer-Barber et al. 2010
C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-}	H37Rv	Up to 125 days	Unimpaired IL-1 β survival and bacterial load between wild-type and <i>Nlrp3</i> ^{-/-}	Dorhoi et al. 2012
C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-} , <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-}	H37Rv	Up to 240 days	Increase late phase lethality and fewer granuloma in <i>Asc</i> ^{-/-} , Unimpaired IL-1 β and bacterial load in <i>Nlrp3</i> ^{-/-} , <i>Asc</i> ^{-/-} , <i>Caspase-1</i> ^{-/-}	McElvania Tekippe et al. 2010
C57BL/6	wild-type, <i>Nlrp3</i> ^{-/-}	low-dose and high-dose H37Rv	86 days	No difference in bacterial load or mortality in either infection dose, reduced IL-1 β in low-dose infection but similar IL-1 β in high-dose infection	Walter et al. 2010
C57BL/6	wild-type, <i>Aim2</i> ^{-/-}	H37Rv	Up to 56 days	Increased early phase mortality and bacterial load	Saiga et al. 2012

Table 3.2: *In vivo* studies following *M. tuberculosis* infection and inflammasome contribution

Human studies examining mycobacteria and inflammasome activation

To date, much attention has deservedly focused on type-1 IFN-inducible genes, since these genes have been shown to correlate with disease severity (Berry et al. 2010; Bloom et al. 2012), and since type-1 IFN appears to negatively regulate IL-1 β in tuberculosis pleural effusions (Ma et al. 2014). *In vitro* and *in vivo* experiments and blood transcriptomes have highlighted their detrimental response in excess (Berry et al. 2010; Mayer-Barber et al. 2014; McNab et al. 2015; Mayer-Barber et al. 2016), but in smaller amounts their role in controlling the immune response is likely to be important (Watson et al. 2015). It is only in recent studies that IL-1 β is garnering more attention, which is surprising given that it is one of the first set of cytokines that are produced following infection and is capable of rapidly differentiating monocytes to macrophages with a high phagocytic capacity and enhancing the presentation of mycobacterial antigens to T-cells (Schenk et al. 2014).

The robust nature of the IL-1 mediated inflammatory response led to a modulation in the BCG vaccine in order to accommodate this and increase the immune profile of the current vaccine. rBCG has successfully passed phase 1 clinical trials (Grode et al. 2013) and phase IIa trials are underway examining immunogenicity in newborns in high endemic areas — it is one of the few vaccine candidates that has a highly promising future (Kaufmann et al. 2014). But what about the role of IL-1 β and inflammasome in disease? It is likely that a functional inflammasome complex is certainly required for the initial ability to eradicate the infection. Macrophages obtained from individuals with gain-of-function gene variants of inflammasome components, NLRP3 and the adapter protein CARD8, which interacts with caspase-1, have superior control of *M. tuberculosis* growth *ex vivo* (Eklund et al. 2014). Thus, in *M. tuberculosis* infection, it is highly plausible that whilst IL-1 β is crucial in preventing the initial establishment of infection, its chronicity may be detrimental in the preservation of tissue homeostasis causing increased inflammatory pathology at the site of infection.

A few genetic susceptibility studies have examined IL-1 β gene polymorphisms with differing effects on TB susceptibility. A SNP at position -511 (T>C rs16944) has been associated with increased TB susceptibility and the homozygous *TT* allele exhibits a decrease in IL-1 β release *ex vivo* in a Gambian population (Awomoyi et al. 2005). In contrast, a study in a Chinese Han population looking at the same SNP did not observe any changes (Sun et al. 2007). However, a SNP at nucleotide position -31 (T<C rs1143627), which is close to the transcriptional start site, confers greater risk to *M. tuberculosis* in the same population (Sun et al. 2007), and increases IL-1 β release and heightens disease severity in a similar Chinese population, though it also demonstrates increased association with extra-pulmonary disease (Zhang et al. 2014). Interestingly, those carrying the *TT* genotype continued to display signs of lung damage as measured by high-resolution CT scanning two years after completion of anti-TB treatment (Zhang et al. 2014). rs1143627T confers a single base change in the promoter region of the IL-1 β gene leading to enhanced binding by the transcription factors, C/EBP β and PU.1 causing increased IL-1 β expression and increased TB susceptibility (Zhang et al. 2014). C/EBP β is strongly up-regulated following bacterial and IL-1 stimulation suggesting a rapid feedback loop (Poli 1998) although this was not explored in this study.

One might question why IL-1 β has not received the same attention as genes such as type 1 IFN in gene expression studies. However, IL-1 β is transcribed in a precursor form, pro-IL-1 β , and subsequently undergoes extensive translational and post-translational modifications as we have described earlier. Whilst IL-1 β can up-regulate its own transcription (Tseng et al. 1998), it is possible that the rapid conversion into the bioactive form means that it is not detected at a genetic level in such studies. Further, it may be rather more lucrative to examine inflammasome transcription and the downstream genes that are mediated by IL-1 β . And there is evidence of this in studies examining the transcriptome. A recent meta-analysis of deposited microarrays from 16 datasets examining the blood transcriptome

of active TB patients versus healthy controls, latently infected or patients post-treatment identified five genes that were upregulated in all datasets (Blankley et al. 2016). One of these identified genes was AIM2. In another recent analysis of public microarray repositories, GPB5 was identified as part of a three gene signature of active tuberculosis (Sweeney et al. 2016). As further evidence of its role, recent studies have clearly demonstrated the pathological role of the inflammasome in patients with HIV infection who developed TB-associated immune reconstitution inflammatory syndrome (IRIS) after commencing anti-retroviral therapy (ART). IRIS is the hyperinflammatory phenomenon that is characterised by rapid restoration and redistribution of CD4⁺ T-cell subtypes (Lai et al. 2013). It has long been known that TB-IRIS is associated with a hyper-cytokinaemia: a case-control study of patients with TB-IRIS demonstrated raised levels of cytokines including IL-1 β at the transcriptional level and also protein level within the culture supernatants. In order to dissect this further, the same group undertook a blind study of whole blood transcriptome and cytokine levels of patients tracked longitudinally after starting ART to detect differences in those who developed IRIS compared to those that did not (Tadokera et al. 2011). Indeed, whole blood taken from patients at the start of their symptoms found that transcripts belonging to the IL-1 signalling pathways were enriched, and higher concentrations of IL-1 β and IL-1 α were detected in tissue culture supernatants from TB-IRIS patients. In a different study, expression profiles of NLRP3 and AIM2 were significantly higher in patients who developed TB-IRIS compared to individuals with TB alone. Furthermore, the regulatory mechanisms that govern IL-1 β processing, such as NO and IFN- γ were lower in patients who developed IRIS prior to the onset of taking ART (Tan et al. 2016). These all suggest that hyper-activation of the inflammasome is involved in the pathology of TB-IRIS. This pattern of release is not just confined to the TB-IRIS sub-group. An early study using a hypothesis-driven approach selecting pertinent cytokines demonstrated that the protein levels of IL-1 β , TNF- α and IL-6 were upregulated in the bronchoalveolar lavage fluid in patients with active TB, although no increase of IL-1 β was seen in the sera (Tsao et al. 1999). Recently, Wang et al. 2015 have demonstrated that serum levels of IL-1 β are significantly higher in patients with drug-sensitive TB compared to healthy controls and that there is an association between IL-1 β and bilateral radiographic infiltrates. Furthermore those with drug resistance had higher levels of the cytokine compared to drug-sensitive subjects.

3.1.4 Current research on inflammasome blockers as anti-inflammatory agents

Inappropriate activity of the inflammasome has been implicated in a wide array of auto-immune and metabolic diseases. Thus inhibitors are a promising avenue for disease stabilisation. Whilst some therapies are novel, there is potential for inflammasome modulators that repurpose currently used medications. In the following section, I discuss methods by which the inflammasome pathway can be directly manipulated with reference to specific drugs used clinically or in pre-clinical development.

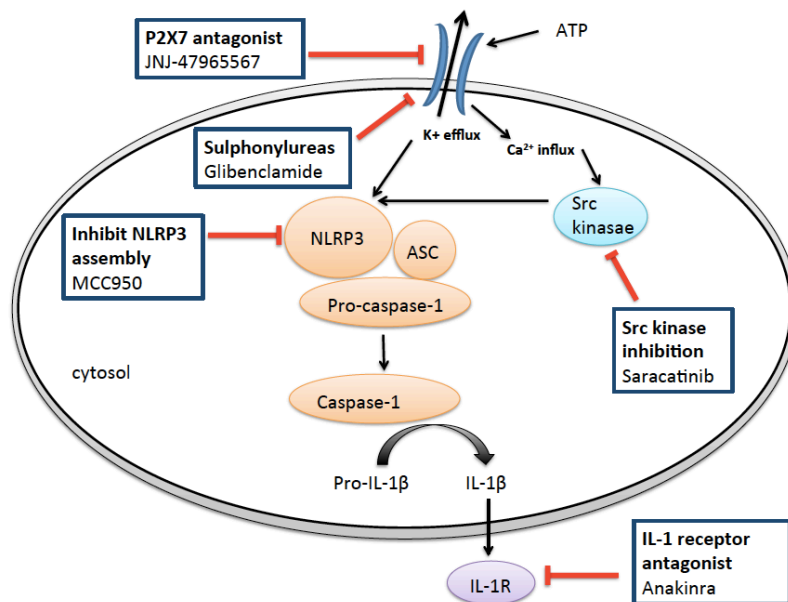


Figure 3.9: Targeting the inflammasome in *M. tuberculosis* infection. Indicated targets to reduce NLRP3 inflammasome signalling. The inflammasome pathway can be targeted at the point of inflammasome activation (PTX7 inhibition, sulphonylureas, MCC950, saracatinib) or by neutralising the actions of IL-1β (Anakinra).

The specific drugs highlighted will subsequently be evaluated and discussed in the results section of this chapter (see figure 3.9).

1. P2X7 antagonists

The inflammasome pathway begins at the purinergic receptors (specifically the P2 receptors). Extracellular ATP acts on the P2 receptors and the P2 subtype, P2X7, plays a significant role in inflammasome activation. During active inflammation, extracellular ATP concentrations are raised due to cell damage; once the concentration reaches the threshold to activate the P2X7 receptor, ATP binds to the receptor leading to pore formation and efflux of potassium ions (Arulkumaran et al. 2011), providing the signal needed for NLRP3 activation. In inflammatory diseases, blockade of P2X7 improves the outcomes of Crohn's disease and rheumatoid arthritis (López-Castejón et al. 2012). Previous studies have shown that *M. tuberculosis* infection induces less IL-1β in *P2X7*^{-/-} than wild-type BMDMs (Amaral et al. 2014). JNJ-47965567 is a P2X7 antagonist that has previously been shown to display potent inhibition of IL-1β following LPS/ATP stimulation (Bhattacharya et al. 2013). It also crosses the blood-brain barrier making it a potentially attractive target in meningeal TB where inflammatory blockade might be pertinent.

2. Sulphonylureas

Sulphonylureas, such as glibenclamide, are used in the treatment of type 2 diabetes mellitus and are inhibitors of ATP-sensitive potassium (K⁺) channels in pancreatic β cells. They also can be repurposed

to inhibit the release of IL-1 β following ATP stimulation and other stimulators of NLRP3 activation. Whilst they block ATP-sensitive K⁺ channels and the P2X7 receptor, these are not responsible for inflammasome blockade, and the mechanism of inflammasome inhibition by sulphonylureas is unknown. However, it does appear to be upstream of NLRP3 assembly (Ozaki et al. 2015). They have been shown to reduce IL-1 β release following *M. tuberculosis* infection suggesting that these channels may play a role in inflammasome activation (Dorhoi et al. 2012).

3. Src kinase inhibitors

Following ATP stimulation of the P2X7 receptor, extracellular calcium enters the cell and reactive oxygen species (ROS) are produced. Part of the mechanism underlying this has been attributed to the activation of the Pyk2/c-Src protein complex coupled to ERK1/2 activation. Certainly, *in vitro* studies have shown that inhibition of c-Src leads to inhibition of ERK1/2 and subsequent ROS production (Martel-Gallegos et al. 2013). Small molecule inhibition may be attractive as an option as their low molecular weight means that they are able to enter the cell easily, although this does mean that they do not confer the same level of specificity as compared to a monoclonal antibody. Saracatanib is a dual Abl-Src kinase inhibitor (Liu et al. 2013), which was originally developed for the treatment of a wide array of malignancies; but it did not show sufficient efficacy for this purpose. Efforts were subsequently refocused into other conditions. For example the Src kinase family is involved downstream of β amyloid signalling and may interfere with synaptic plasticity (Nygaard et al. 2015). Therefore the Src pathway has recently been heralded as a potential target in Alzheimer's disease and *in vivo* studies have shown that inhibition of Src kinase can re-establish memory loss in an Alzheimer's mouse model (Nygaard et al. 2015; Kaufman et al. 2015). Saracatanib inhibits Src kinase at nanomolar concentrations; at higher micromolar concentrations, Abl kinases are inhibited. Recently, Src kinase inhibition has been shown to reduce *M. tuberculosis* intracellular bacterial burden *in vitro* and *in vivo* (Chandra et al. 2016) as well as reducing bacterial survival of drug resistant isolates, partly through promoting macrophage phagosomal acidification (Chandra et al. 2016). This study demonstrated that saracatanib was highly efficacious at micromolar concentrations. However given many of the previous studies examining this drug looked at a nanomolar range, I initially looked at these concentrations to determine cellular viability and I examined how Src kinase inhibition influences IL-1 β production.

4. Small molecule inhibitors of NLRP3

Increasingly, specific inhibitors of NLRP3 activation and inflammasome formation have been developed. In 2001, a group of compounds named the diarylsulphonylureas were developed, which have a similar

structure to the sulphonylureas and inhibit the maturation of IL-1 β (Perregaux et al. 2001). They were termed the CRID group of compounds; and in 2015, one such compound termed MCC950 (which has gained interest largely due to its potency and seemingly few side effects) was found to specifically inhibit NLRP3 at micromolar concentrations (Coll et al. 2015). More recently, it has been shown to inhibit the pro-inflammatory response following exposure to house dust mite-induced airway inflammation in mice (Primiano et al. 2016). Furthermore, administration of MCC950 afforded some protection against influenza virus A *in vivo* (Tate et al. 2016) suggesting that inhibition of NLRP3 may have an important role in dysregulated pulmonary inflammation.

5. IL-1 receptor antagonists

IL-1 receptor antagonists such as anakinra are used for the treatment of moderate to severe rheumatoid arthritis (Mertens et al. 2009); however they are also used for a number of dermatological disorders (Pazyar et al. 2012). Recently anakinra has also been shown to reduce the severity of colitis in patients with chronic granulomatous disease (Luca et al. 2014), although long-term remission in these patients has been called into question (Hahn et al. 2015). The role of endogenous IL-1RA during *M. tuberculosis* infection is not certain. During *M. tuberculosis* infection, high ratios of IL-1 β /IL-1RA have been detected in the BAL sample of patients with cavitary *M. tuberculosis* (Tsao et al. 2000) suggesting an exuberant inflammatory response without an associated counterbalance.

6. Rifampicin as an immunomodulator

Some of the standard drugs used to treat drug-sensitive *M. tuberculosis* have immunomodulatory properties themselves; certainly pyrazinamide has been shown to be able to reduce IL-1 β and other pro-inflammatory cytokines following infection in human monocytes (Manca et al. 2013). Additionally, both isoniazid and pyrazinamide can enhance bacterial eradication through autophagy (Bradfute et al. 2013). And, whilst the central role of rifampicin is to inhibit bacterial RNA polymerase, it is also known to reduce the secretion of IL-1 β in affected macrophages (Ziglam et al. 2004). I examined the effect of rifampicin on IL-1 β following *M. tuberculosis* infection then considered it in association with potent immunomodulators of the inflammasome.

The aims of this current project were to ascertain whether differences exist in inflammasome activation following infection with a panel of *M. tuberculosis* clinical isolates and to then determine whether inflammasome modulation could be employed as a potential adjuvant to chemotherapy for *M. tuberculosis*.

3.2 Methods

3.2.1 Bacterial culture

Throughout this work, reference is made to the use of clinical isolates and their differential induction of the immune response. The clinical isolates were cultured from frozen stocks that were a kind gift from Dr. Nitya Krishnan and Dr. Guy Thwaites, using previous criteria outlined in (Krishnan et al. [2011](#)). Clinical isolates included representatives from the Beijing lineages (212, 411, 119) and Euro-American lineages (173, 639, 440, 355). The laboratory mycobacterial strain, H37Rv, was a gift from Dr. Krishnan and was used as the reference strain for the clinical isolates used. All experiments involving these strains were performed in the Biosafety laboratory Level 3, Biochemistry building, Imperial College London.

In order to grow the bacteria, 500 µl bacteria were thawed into 10 ml liquid cultures and grown over 7 days in Middlebrook 7H9 media supplemented with 10 % oleic acid-albumin-dextrose-catalase (OADC, BD), 0.05 % Tween-80 (Sigma), 0.2 % glycerol (VWR) at 37 °C on a rotating incubator.

Determination of colony forming units per ml (CFU/ml) for growth curve measurement were obtained by plating the bacteria at serial dilutions on Middlebrook 7H11 plates supplemented with 10 % OADC and 0.5 % glycerol. Estimated CFU/ml from the growth curves were subsequently used to estimate the inoculum for both *in vitro*.

3.2.2 Resazurin microtiter assay (REMA) assay for detection of the susceptibility of H37Rv to rifampicin and MCC950

The REMA assay is a well-established technique to identify the minimum inhibitory concentrations of a drug (Palomino et al. [2002](#)). Briefly, 100 µl of 7H9-broth was added to a sterile 96-well plate and serial two fold dilutions of the drug were prepared within the broth (the highest concentrations for rifampicin and MCC950 were 1.96 mg l⁻¹ and 100 mM respectively). Wells were inoculated with 100 µl of bacterial suspension such that the final OD was approximately 0.005. A growth control and sterile control was also included. The plate was covered with a lid and kept in a plastic box and kept at 37 °C incubator. After 7 days, 30 µl resazurin was added to the wells and the plate was re-incubated for 2 days. The change from blue resazurin to pink implied bacterial growth. The MIC was the lowest concentration that did not yield a change in colour (Khalifa et al. [2013](#)).

3.2.3 Cell culture

The cells used in this work were harvested from C57/BL6 mice; wild-type, *Nlrp3*^{-/-}, *Nlrp3*^{-/-}/*Aim2*^{-/-}, provided by Dr. Katherine Fitzgerald (Kalantari et al. 2014); *Caspase1/11*^{-/-}, provided by Dr. Richard Flavell (Mayer-Barber et al. 2010); and *Asc*^{-/-}, provided by Dr. Vishwa Dixit (Mariathasan et al. 2004). The BMDMs were immortalised by Dr. Avinash Shenoy and all mentions of BMDMs refers to these cells and not primary cells. BMDMs were grown in DMEM (Sigma) plus 10 % heat-inactivated fetal calf serum (Labtech), 5 mM sodium pyruvate (Life technologies) and 10 % L929 spent medium. The spent medium was obtained by growing L929 cells (a kind gift from Dr. Maximiliano Gutierrez, Francis Crick Institute) in DMEM to 100 % confluence and removing the medium after 48 hours. BMDMs were seeded when 80 % confluence was achieved, seeded in a 48-well tissue culture plate at 3.5×10^5 cells/well and kept at 37 °C and 5 % CO₂ for all experiments.

3.2.4 Macrophage infection

Macrophage experiments were performed as stated in the Melanocortin methods (section 2.2). Changes are as follows. Experiments were performed with cultures at an OD of 0.5–0.8 which corresponded to the mid to late log growth phase. Using an OD 0.6 $\simeq 5 \times 10^8$ cells/ml, the multiplicity of infection (MOI) was calculated, using Opti-MEM (Fisher scientific) as the cell culture medium rather than DMEM, in order to minimise fetal bovine serum contamination. In all initial experiments, MOI was varied in order to determine the optimum conditions. Cells incubated with LPS (L7770, Sigma) for 3 hours followed by 5 mM ATP (Sigma) stimulation were used as a positive control for IL-1 β and caspase-1 detection in the initial experiments.

At 24 and 48 hours, cell supernatants were harvested and double-filtered through 0.22 μ m filter (Corning Costar Spin-X centrifuge tube filters) at 10,000 $\times g$ for 5 minutes and frozen at –20 °C for ELISA experiments.

3.2.5 Drug treatment

The drugs used in this work are as follows: MCC950 (previously known as CRID3, Tocris Biosciences, Cat. No. 5479), anakinra (obtained as the trade name Kineret, Swedish Orphan Biovitrum AB, from St. Mary's Pharmacy, Imperial College NHS Trust), saracatinib (Selleckchem, Cat. No. S1006), glibenclamide (Tocris Biosciences, Cat. No. 0911), JNJ-47965567 (Tocris Biosciences, Cat. No. 5299) rifampicin (Sigma-Aldrich, CAS number 13292-46-1). Each pre-filled syringe of kineret contained 150 mg ml⁻¹. Stock concentrations in DMSO were made as follows: saracatinib (100 nM), JNJ-

47965567 (20 μ M), rifampicin (20 mg ml⁻¹), MCC950 (20 μ M), glibenclamide (20 μ M). Drugs were kept at -80 °C until required.

On the day of the experiment, BMDMs were infected with the bacterial strain and cells were washed after 4 hours with pre-warmed DMEM. Drugs were pre-diluted into their required working concentrations in Opti-MEM (Fisher scientific). Washed cells were then bathed in the various drugs for 24 hours.

3.2.6 Colony counting

The protocol for counting intracellular bacterial numbers is as stated in the Melanocortin methods section (section [2.2.5](#)).

3.2.7 Cytokine detection by Enzyme-linked immunosorbant assay (ELISA)

Mouse IL- β and TNF- α were quantified using commercially available ELISA kits (e-Biosciences) according to the manufacturer instructions. Please see the melanocortins methods section (section [2.2.5](#)) for further details.

3.2.8 Lactate dehydrogenase assay for cell cytotoxicity

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme released following disruption of membrane integrity and is used as an indicator of cytotoxicity. The LDH assay is therefore one determinant of membrane activity. The supernatants were removed as stated above and quantified using an LDH kit (Promega) according to the manufacturer instructions. Briefly, 50 μ l of supernatant was added to the kit reagent and incubated for 10 minutes at room temperature. After addition of stopping solution, the fluorescence intensity was measured using the microplate reader.

3.2.9 Antibodies used for western blotting

The following antibodies for western blotting were used. Anti- β -actin-HRP (sc-47778, Santa Cruz), Goat anti-mouse IL-1 β (AF401, R&D systems), Anti-mouse caspase-1 (Casper 1, AG-20B-0042-C100, Adipogen). Secondary anti-mouse and anti-goat antibodies were obtained from Santa Cruz.

3.2.10 Initial western blotting protocol

During the initial stages of this project, wild-type BMDMs were seeded in a 6-well plate at 2×10^6 cells/well. BMDMs were infected with a total bacterial suspension of 2 ml and after 24 hours, the entire supernatant was removed and concentrated overnight with ice-cold acetone at a 4:1 ratio. The solution was then centrifuged several times in 2 ml Eppendorf tubes. Essentially, 2 ml were spun, the supernatant discarded and a further 2 ml was added and respun. This was repeated until the entire volume was pelleted. The final pellet was suspended in 60 μ l loading dye. For lysate preparation, the macrophages were lysed in 200 μ l laemelli loading dye. Prior to loading on an SDS-PAGE gel, both the lysate and supernatants were boiled at 100 °C for 10 minutes. 20 μ l of the supernatant and lysate preparations were separated on a 12 % pre-cast polyacrylamide gel (NuPAGE Bis-Tris Gel) using NuPage MES running buffer (both ThermoFisher Scientific) and transferred onto a nitrocellulose membrane using NuPage transfer buffer. The membranes were then incubated overnight with the appropriate antibody and then exposed to the secondary antibody before developing. Blots were stripped using NuPage stripping buffer and re-probed with β -actin as a loading control.

3.2.11 Optimised western blotting protocol

The protocol was then adapted such that BMDMs were seeded in a 48-well plate. For western blotting experiments, the supernatants from four wells in each condition were filtered twice through 0.22 μ m filters (Corning Costar Spin-X centrifuge tube filters) and pooled. The sample was desalted using spin desalting columns (87768, Thermo-scientific). A final volume of 800 μ l was precipitated overnight with ice-cold acetone (ratio 1:4, v/v) and resuspended in 30 μ l 2 $\times$ laemelli loading buffer (4 g SDS, 20 % glycerol, 120 mM Tris pH 6.8, 0.01 % bromophenol blue and 20 mM EDTA). Cell extracts were prepared by adding 100 μ l laemelli buffer with 1 $\times$ protease inhibitor (Pierce), 1 mM PMSF and 5 % β -mercaptoethanol to each well and the duplicate samples were pooled. Prior to loading on an SDS-PAGE gel, both the lysate and supernatants were boiled at 100 °C for 10 minutes. Samples were loaded at 25 μ l on an SDS-PAGE gel. Proteins were separated by 13.5 % acrylamide SDS-PAGE using Tris-Glycine buffers, and transferred onto PVDF membranes. Membranes were blocked for 2 hours at room temperature in 10 % fat-free milk and incubated overnight at 4 °C with the appropriate antibodies. Secondary antibodies were added following washing of the membrane with PBS–Tween-80 (0.05 %). In general, the membrane was probed with antibodies against IL-1 β and developed before being stripped and re-probed with antibodies against caspase-1. Finally, blots were stripped and re-probed with β -actin as a loading control.

3.2.12 Statistical analysis

In all cases, each biological replicate was performed with 3 technical replicates. Only independent means were analysed by statistical methods. Unless otherwise stated, 3 biological replicates were performed (and denoted as 'n' in the figure legend). Statistical analysis was performed using GraphPad Prism 7. Matched parametric data was analysed using a two-tailed paired *t*-test, or One Way Analysis of Variance (ANOVA) for three or more groups followed by either Tukey's post-hoc analysis or Dunnet's multiple-comparison test (the choice of which depended on whether a known control was being compared against). Two-way ANOVA was used to determine the influence of two independent variables on a dependent variable and was followed by Tukey's post-hoc analysis to ascertain this difference. Pearson's correlation coefficient was used for assessing a correlation between IL-1 β and LDH. For all graphs shown in the following results, data points from 3 (and sometimes 4) independent experiments are shown with the mean, unless otherwise stated. The text shows the mean from stated column statistics $\pm$ the standard error of the mean in brackets. The results in chapters 2 and 3 state both the *t*-value (if a *t*-test is done) or the *F*-value (if a one-way ANOVA is done) together with their corresponding *p*-values. The *t*- and *F*-value give a standardised quantification of the difference between the groups without making any assumptions about the distribution of the data.

3.3 Results: Understanding and characterising the inflammasome during *M. tuberculosis* infection and evaluating its potential for host-directed therapy

3.3.1 Aims

1. To optimise the conditions necessary for inflammasome activation.
2. To determine whether there are any differences in inflammasome activation between a panel of clinical isolates.
3. To characterise the contribution of inflammasome components to IL-1 β release during *M. tuberculosis* infection.
4. To screen a panel of pharmacological agents and determine their role as a potential adjunct to current antimycobacterial chemotherapy.

3.3.2 Development of assays and determination of conditions necessary for inflammasome activation

While there are several reports detailing the inflammasome during infection with *M. tuberculosis*, the results themselves are quite conflicting. This may be due to many reasons — the type of cell line used, the length of the infection, and the bacterial load — all of which may change the pathological response of the macrophage to the bacteria. The ideal model should be drawn from *in vivo* work, however robust findings can be ascertained *in vitro*. When I initially began this section of my research, I followed protocols given in the literature but I soon found that I was forced to optimise many of the methods in order to draw accurate conclusions.

Optimising the conditions for inflammasome activation

The current literature examines the role of the inflammasome during *M. tuberculosis* infection using both low ($\text{MOI} \leq 10$) and high ($\text{MOI} > 10$) bacillary load and a variety of time points after the initial infection. The observation that higher mycobacterial burdens are able to induce both IL-1 β release and cell death (Welin et al. 2011; Krishnan et al. 2013) may confound the mechanisms by which mycobacteria are able to trigger inflammasome and thus IL-1 β activation. To this end, I sought to ascertain both the optimum time point post-infection as well as MOI in order to delineate inflammasome involvement.

3.3.3 Higher bacillary loads are able to induce IL-1 β release compared to lower bacillary loads but at the expense of increased cell death

To evaluate how bacterial burden affects IL-1 β release, wild-type immortalised bone marrow derived macrophages (iBMDMs) were infected with the lab-adapted virulent mycobacterial strain, H37Rv, at an MOI of between 1 and 25 for 24 hours. The MOI was verified by plating the bacterial suspension onto agar plates. After 24 hours, the supernatants were removed and analysed for IL-1 β via an ELISA. We can clearly see significant difference between MOI and IL-1 β induction (One way ANOVA, $p \leq 0.0001$; $F = 251.7$). As expected, while the induction of IL-1 β release by MOI 1 is low ($36.48 \pm 9.38 \text{ pg ml}^{-1}$), MOI of 25 is significantly greater ($2640 \pm 125 \text{ pg ml}^{-1}$), $p \leq 0.0001$.

However, higher MOIs induce significant levels of cell death compared to low bacterial loads (One-way ANOVA, $p \leq 0.0001$, $F = 110.1$), and there is no significant difference in cell death between MOI 1 and MOI 5 ($p = 0.276$, see figure 3.10A).

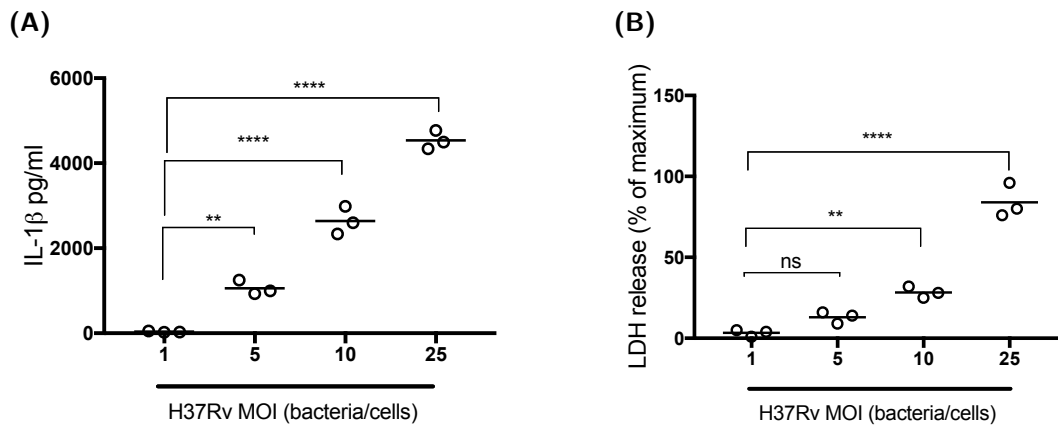


Figure 3.10: High bacterial burdens lead to increased IL-1 β release and cell death. Wild-type BMDMs were infected with H37Rv at the indicated MOIs and supernatants were removed at 24 hours and analysed for IL-1 β (A) and LDH release (B). The maximum positive control is elicited by the release of LDH induced by Triton-X detergent. Each data point shown represents the mean of 2 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied to determine a difference in means for each MOI followed by Tukey's multiple comparison test to evaluate differences between the experimental condition means and the mean of every other experimental condition. In the graphs, statistical significance between the experimental conditions is indicated as $**p \leq 0.01$, $****p \leq 0.0001$, ns=non-significant.

Taking this forward, I wished to examine the secretion of IL-1 β with minimal impact on cell death; thus whilst an MOI of 1 led to the least cell death, it did not induce sufficient IL-1 β , so I therefore chose an MOI of 5 for all future experiments.

There is a statistical increase in IL-1 β release detected by ELISA between 24 and 48 hours after H37Rv infection

The length of time that cells are infected by *M. tuberculosis* and the time point at which the supernatants are collected following infection varies in the literature. Short infection times (Master et al. 2008; Shah et al. 2013) and longer infection times (Abdalla et al. 2012) may lead to different conclusions about the contribution of *M. tuberculosis* towards inflammasome activation and IL-1 β detection. In order to decide on the optimum time frame for infection, wild-type immortalised BMDMs were infected with H37Rv. The cells were infected for 6, 24 and 48 hours (without washing off the bacteria) at an MOI of 5, and supernatants were removed at these time points and analysed for IL-1 β via an ELISA. At 6 hours, IL-1 β was not detected (data not shown) by ELISA. However, H37Rv did promote IL-1 β release at 24 hours ($911.40 \pm 50.99 \text{ pg ml}^{-1}$) and at 48 hours ($1167.00 \pm 66.01 \text{ pg ml}^{-1}$). There was a statistical increase ($p = 0.04$; $t = 4.672$) in IL-1 β release between the two time points (see figure 3.11). IL-1 β was not detected in uninfected cells at either time point.

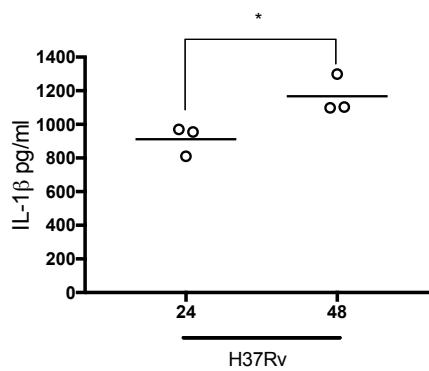


Figure 3.11: H37Rv promotes IL-1 β release at 24 and 48 hours post-infection. Wild-type BMDMs were infected with H37Rv at an MOI of 5 and the supernatants were removed at 24 and 48 hours and analysed for IL-1 β release via ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. A two-tailed paired t -test was performed to test the difference in means between each time point. In the graphs, statistical significance between the experimental conditions is indicated as $*p \leq 0.05$.

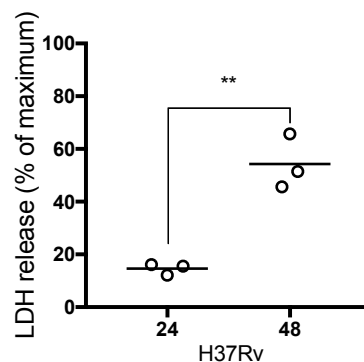


Figure 3.12: H37Rv promotes LDH release at 24 and 48 hours post-infection. Wild-type BMDMs were infected with H37Rv at an MOI of 5 and the supernatants were removed at 24 and 48 hours and analysed for LDH release. The maximum positive control is elicited by the release of LDH induced by Triton-X detergent. Each data point shown represents the mean of 2 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. A two-tailed paired t -test was performed to test the difference in means between each time point. In the graphs, statistical significance between the experimental conditions is indicated as $*p \leq 0.05$.

Increased infection duration causes a marked increase in cell death

Whilst the host uses cell death as a way to control infection, it can also play an important role in the pathogenesis of *M. tuberculosis*. Cell death dynamics vary in the literature but previous studies have shown that macrophage cell death is independent of IL-1 β and caspase (O'Sullivan et al. 2007; Krishnan et al. 2013) and also independent of the mycobacterial ESX-1 secretion system (Lee et al. 2011). In order to decide an appropriate time point for future experiments, I looked at cell death at 24 and 48 hours after infection. Cytotoxicity was monitored via LDH in the supernatant in the infected and uninfected controls. In the uninfected controls, mean LDH values were negligible at 24 hours and $4.33 \pm 1.87\%$ at 48 hours. H37Rv induced cell death at both 24 and 48 hours ($14.6 \pm 1.3\%$, $54.26 \pm 5.95\%$ respectively; difference between means $p = 0.015$; $t = 7.974$; see figure 3.12)

Since there was a large increase in cell death induced by H37Rv at 48 hours and the associated IL-1 β release was relatively small, I chose 24 hours as my optimum infection time point for infections, in

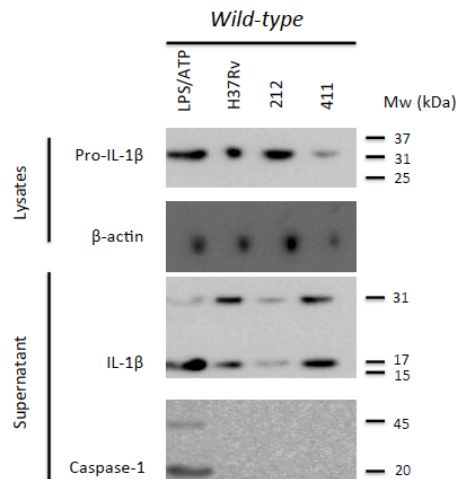


Figure 3.13: Western blot using a pre-cast gel showing the lysates and supernatants from wild-type BMDMs infected with H37Rv and the clinical isolates 212 and 411. Wild-type BMDMs were seeded in a 6-well plate at 2×10^6 cells/well. BMDMs were infected with indicated strains of *M. tuberculosis* at MOI 5 for 24 hours. Supernatants were precipitated by acetone precipitation and lysates were prepared. Proteins were separated on a 12% pre-cast (NuPAGE Bis-Tris Gel) gel using NuPAGE MES running buffer and transferred onto a nitrocellulose membrane using NuPAGE transfer buffer. Immunoblots were performed using indicated antibodies. The loading control is represented by β-actin. LPS/ATP treatment served as a positive control to activate the NLRP3–ASC–caspase-1 pathway.

order minimise the potential confounding effects of increased cell death on IL-1β release by clinical isolates.

3.3.4 Optimisation of Western blotting for infections with *M. tuberculosis*

Western blots are able to differentiate between mature and pro-IL-1β, which is not reliable in ELISAs, they can also detect pro-IL-1β in cell lysate which allows us to understand the level of priming, or signal 1 activation, through stimulation of NF-κB. Moreover, detection of mature caspase-1 in the supernatant can also convey information about caspase-1 processing.

Given the diverse outcomes seen in *M. tuberculosis* infections, the Western blotting protocol needed to be optimised in order to determine the best conditions. Initially, the western blot protocol utilised pre-cast gels and the preparation is described in the methods section (see section 3.2.10: [Initial western blotting protocol](#)).

Figure 3.13 was representative of three experiments, using freshly reconstituted buffers and shows two main observations. Firstly that some of the bands recognised by antibodies against pro-IL-1β are poorly formed and barely visible (in particular, cells infected with H37Rv); this is corroborated by the poor loading control shown by the β-actin blot. Secondly, processed caspase-1 is not seen. We know that the timing of caspase-1 activation is sufficient, having already compared infections at 24 and 48 hours and shown detectable levels of IL-1β in the supernatant. The antibody against caspase-1 is effective as we see cleaved caspase-1 in the positive control. Additionally, given that the

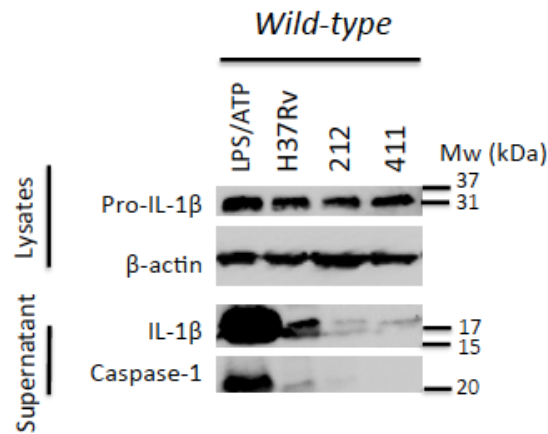


Figure 3.14: Western blot showing the lysates and supernatants from wild-type BMDMs infected with H37Rv and the clinical isolates 212 and 411 using an in-house gel. Wild-type BMDMs were seeded in a 6-well plate at 2×10^6 cells/well. BMDMs were infected with indicated strains of *M. tuberculosis* at MOI 5 for 24 hours. Supernatants were precipitated by acetone precipitation and lysates were prepared. Proteins were separated by 13.5% acrylamide SDS-PAGE using Tris-Glycine buffers, and transferred onto PVDF membranes. Immunoblots were performed using indicated antibodies. The loading control is represented by β -actin. LPS/ATP treatment served as a positive control to activate the NLRP3–ASC–caspase-1 pathway.

primary mediator of conversion of pro-IL-1 β into IL-1 β is processed caspase-1 then the fact that we both detect IL-1 β in the ELISA and see processed IL-1 β in the supernatants in response to all four stimuli (LPS/ATP, H37Rv, 212 and 411) suggests that caspase-1 is being processed but not detected under the current conditions. Given the number of gels that would be required in future experiments and the expense associated with this, I decided that the best way to optimise this technique was to prepare my own gels and buffer solutions for running SDS-PAGE and protein transfer.

The gel preparation is described above in the methods section (see section 3.2.11: [Optimised western blotting protocol](#)) and the experiment was repeated under the same conditions. In figure 3.14, we see that with the lysate preparation, actin expression is uniform between different conditions as expected.

However, the appearance of processed caspase-1 remains faint in spite of obvious pro-IL-1 β processing. Whilst previous studies, using a very low MOI and duration of infection, have suggested that IL-1 β processing and caspase-1 activation are inhibited during *M. tuberculosis* infection (Master et al. 2008), more recent studies have shown that inflammasome activation does occur and caspase-1 is detected by Western blotting in the supernatants of THP-1 cells infected with *M. tuberculosis* (Novikov et al. 2011). Furthermore, primary BMDMs infected with the virulent *M. bovis* demonstrate caspase-1 processing after 24 hours post-infection (Yang et al. 2013b). Therefore, while it is evident that caspase-1 processing can be detected by Western blotting following *M. tuberculosis* infection, the finer details regarding the optimum number of cells required for pooling for detection are absent. Given that the total volume **after** acetone precipitation is 8 ml, the multiple high-speed centrifugation steps required to elicit the final pellet may have led to protein loss along the way.

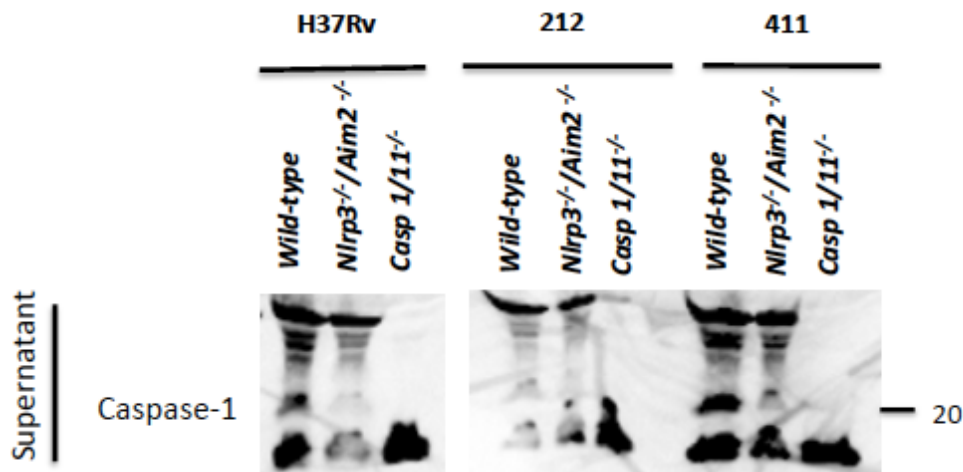


Figure 3.15: Western blot using a home-made gel from an experiment in a 48-well plate and showing the lysates and supernatants from wild-type, *Nlrp3*^{-/-} and *Caspase1/11*^{-/-}. BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well. BMDMs were infected with indicated strains of *M. tuberculosis* at MOI 5 for 24 hours. Supernatants were precipitated by acetone overnight and lysates were prepared. Proteins were separated by 13.5% acrylamide SDS-PAGE using Tris-Glycine buffers, and transferred onto PVDF membranes. Immunoblots were performed using indicated antibodies.

Therefore, I reduced the scale of the experiment such that the cells were seeded at 3.4×10^5 cells/well in a 48-well plate. The total volume that the cells were infected with totalled 200 μ l. After 24 hours, the supernatants from 4 infected wells under the same condition were removed, pooled, and precipitated overnight in acetone; the reduction in total overall volume meant that the solution could be spun fewer times in order to pellet the protein. The final pellet was resuspended in 30 μ l of loading buffer. To harvest the lysate, each well was lysed with 100 μ l of loading buffer and pooled. The samples were boiled and 30 μ l and 20 μ l of the supernatants and lysates respectively were loaded onto the gel as described in the methods section.

The rescaling of the experiment clearly presented a different problem (figure 3.15). With the pooling and concentrating of the supernatants, the bands recognised by antibodies against caspase-1 are hugely smudged and blurred with no obvious discernible protein. Fortunately, pro-IL-1 β lysate preparations ran well (not shown). This could be due to one of a few reasons: firstly too high a voltage can cause this effect, however all gels were run at the same voltage (including the lysate preparations) and the quality was not affected. Secondly, it is possible that total protein concentration was too high but this is less likely in the supernatant fraction. Another theory which was easier to verify was that the samples contained excess salt which affected the running of the gels.

In order to test this, I performed a small experiment to evaluate how Opti-MEM alone and Opti-MEM with 5 mM ATP affected migration through a gel. ATP here mimics the ATP/LPS step used as a positive control in inflammasome experiments and given its high ionic strength can effect running of a gel. 800 μ l Opti-MEM and another tube containing Opti-MEM and 5 mM ATP were precipitated

in acetone overnight. The samples were collected the following day, mixed with 4 µl of the protein molecular weight ladder (Biorad) to test whether the precipitate affected the banding pattern of the ladder and loaded onto a gel. A Coomassie blue stain was performed for ease and visualisation of the individual proteins within the ladder (see figure 3.16).

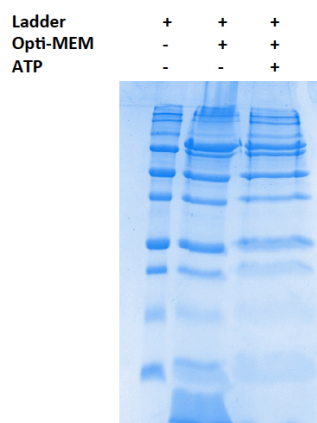


Figure 3.16: Coomassie staining showing the differences in running of the ladder when mixed with different preparations of Opti-MEM. 800 µl Opti-MEM and 800 µl Opti-MEM/5 mM ATP were precipitated with acetone overnight. A protein ladder on its own ran against a ladder mixed with Opti-MEM and Opti-MEM and 5 mM ATP and the proteins were separated by 13.5 % acrylamide SDS-PAGE using Tris-Glycine buffers. Coomassie blue stain was used to visualise the protein bands

The ATP positive control shows co-precipitation with the proteins within the ladder leading to widening and smearing; the Opti-MEM alone does not show as much a dramatic effect but the presence of smearing is still seen. To rectify this, desalting columns are often used to remove the excess salt. They use the principle of size exclusion chromatography, whereby there is a rapid separation between high molecular weight substances and low molecular weight substances. Thus, small molecules such as salt are efficiently embedded within a resin matrix without having an impact on larger molecules. Following sample collection, the samples were spun through a desalting spin columns (ThermoFisher Scientific) and the final volume (which remained unchanged at 800 µl) was precipitated overnight in acetone.

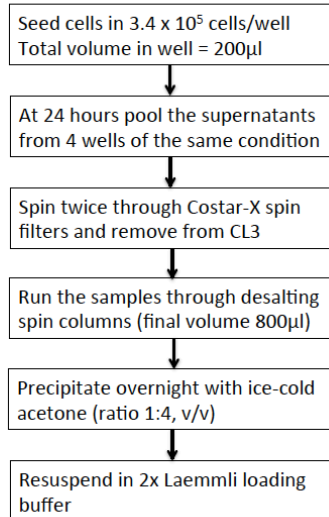
In figure 3.17B, we can see that the caspase processing is much cleaner and has less smearing; moreover the signal intensity has not been compromised. Therefore, for all Western blots shown in the subsequent sections, this protocol including the desalting technique was adopted (see figure 3.17A and section 3.2.11: Optimised western blotting protocol).

3.3.5 Clinical isolates vary in their ability to activate the inflammasome and promote IL-1 β processing

Previous studies have demonstrated distinct immunological changes following infection by various clinical isolates and lineages of *M. tuberculosis* (Portevin et al. 2011; Krishnan et al. 2011; Sarkar

(A)

Supernatant extraction protocol



(B)

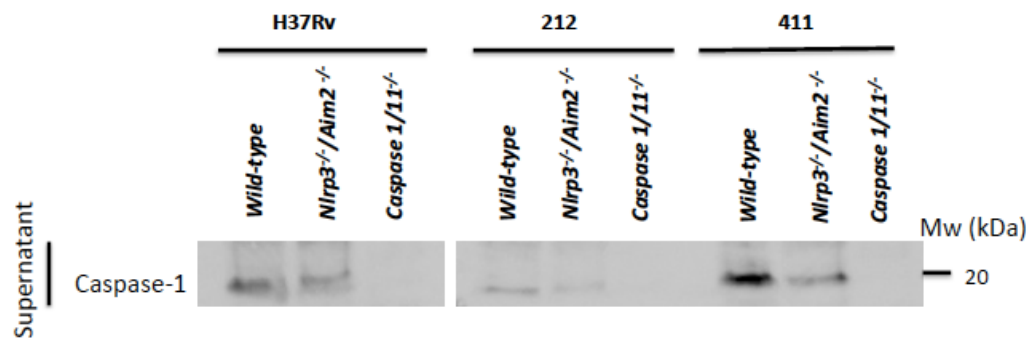


Figure 3.17: Optimised protocol for supernatant sample collection and western blot. The finalised protocol for obtaining the supernatant fraction during infection experiments (A) and western blot using a home-made gel from an experiment in a 48-well plate showing the supernatants from wild-type, *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1*/*11*^{-/-} (B). BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well. BMDMs were infected with indicated strains of *M. tuberculosis* at MOI 5 for 24 hours. Supernatants were desalted and then precipitated by acetone overnight. Proteins were separated by 13.5 % acrylamide SDS-PAGE using Tris-Glycine buffers, and transferred onto PVDF membranes. Immunoblots were performed using indicated antibodies.

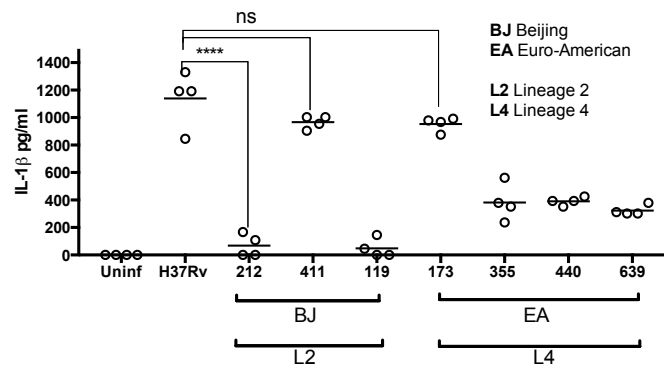


Figure 3.18: Clinical isolates promote a differential IL-1 β release at 24 hours post-infection. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and were infected with indicated isolates across 2 lineages at an MOI of 5 for 24 hours. The supernatants were removed at 24 hours and analysed for IL-1 β by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 4$ biological experiments. One-way ANOVA was applied to determine a difference in means between each strain followed by Tukey's multiple comparison test to evaluate the differences in means between each of the experimental conditions. In the graphs, statistical significance between the experimental conditions and the control is indicated as **** $p \leq 0.0001$. Only a few comparisons are represented.

et al. 2012; Krishnan et al. 2013). This project explored one aspect of this further by determining the differential induction of IL-1 β by differing clinical isolates.

Strain differences in TNF- α , IL-1 β and caspase-1 processing

I used a panel of clinical isolates taken from the Euro-American and Beijing lineages which have previously been examined for their IL-1 β induction as determined by ELISA (Krishnan et al. 2013). Cells were infected for 24 hours and the supernatants and lysates were harvested for Western blotting as described previously; additional cells were infected and supernatants removed for ELISA (for IL-1 β and TNF- α) and LDH assays. As expected, the uninfected control did not induce any IL-1 β release. As previously demonstrated by Krishnan et al. 2011, there was a differential induction in IL-1 β release ($p \leq 0.0001$; $F = 85.89$). Compared to reports by Krishnan et al. 2011 and Krishnan et al. 2013, the pattern of secretion was slightly different, in that in my experiments, 639 induced low IL-1 β ($323.4 \pm 18.6 \text{ pg ml}^{-1}$) and 173 high levels ($953.5 \pm 26.3 \text{ pg ml}^{-1}$; results by Krishnan et al. 2011 not stated since the overall magnitude of the values were much larger as the authors used primary BMDMs rather than immortalised cells). The strains, 212 ($68.59 \pm 23.77 \text{ pg ml}^{-1}$; $p \leq 0.0001$ comparing the mean against H37Rv) and 411 ($966.4 \pm 23.8 \text{ pg ml}^{-1}$; $p = 0.232$) followed the same trend as before (see figure 3.18).

I also looked at the inflammasome-independent cytokine, TNF- α ; in keeping with Krishnan et al. 2011, there was a differential induction of TNF- α ($p \leq 0.0001$; $F = 31.23$). The Euro-American strains induced less TNF- α than H37Rv; however 212 and 411, both Beijing strains, induced comparable quantities ($p = 0.473$; $p = 0.291$ respectively; see figure 3.19).

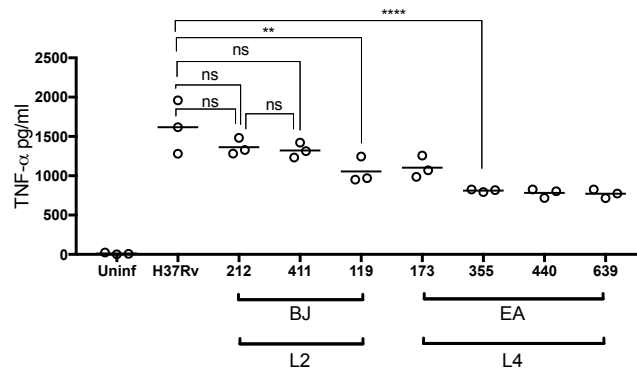


Figure 3.19: Clinical isolates promote a differential TNF- α release at 24 hours post-infection. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and infected with indicated isolates across 2 lineages at an MOI of 5 for 24 hours and the supernatants were removed at 24 hours and analysed for TNF- α by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied to determine a difference in means between strains followed by Tukey's multiple comparison test to evaluate the differences in means between each of the experimental conditions. In the graphs, statistical significance between the experimental conditions and the control is indicated as $**p \leq 0.01$, $****p \leq 0.0001$, ns=non-significant. Only a few comparisons are represented.

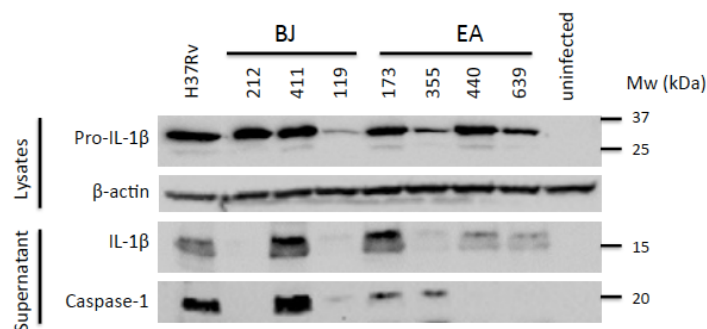


Figure 3.20: Clinical isolates promote differential IL-1 β and caspase-1 processing at 24 hours post-infection. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and infected with indicated isolates across 2 lineages at an MOI of 5 for 24 hours. Supernatants were desalted and precipitated by acetone overnight and lysates were prepared. Proteins were separated by 13.5% acrylamide SDS-PAGE using Tris-Glycine buffers, and transferred onto PVDF membranes. Immunoblots were performed using indicated antibodies. The blot is representative of $n = 2$ biological experiments.

In the Western blot, there were differences in the expression of pro-IL-1 β . H37Rv demonstrated a similar degree of priming to 212, 411, 173 and 440 but low levels were seen in 119 and 355. There is a reduced maturation of IL-1 β which matches the degree of priming induced by these two strains. The ELISA was consistent with the western blot based on the level of processed IL-1 β detected. Additionally there were similar degrees of processing between H37Rv, 411 and 173. Interestingly, whilst 440 induces a reasonable pro-IL-1 β expression, the levels of maturation seen in the supernatant are poor and there is no caspase-1 activation. Similarly, whilst the levels of pro-IL-1 β induced by 173 match with the conversion into the bioactive form, the actual degree of caspase-1 activation is less than that seen by either H37Rv or 411. In contrast, 355 induces moderate expression of pro-IL-1 β and processed caspase-1 is detected in the supernatant; however, activated IL-1 β is not seen (see figure 3.20).

Strain differences in bacterial uptake

Since engagement of the toll-like receptors and various pattern recognition receptors control expression of pro-IL-1 β primarily through the MyD88 signalling pathway, and because many of these receptors are involved in phagocytosis, I examined the differences between bacterial uptake at 4 hours after infection with the clinical isolates. Cells were infected with an estimated MOI of 5 and the cells were lysed in water after 4 hours and plated. Whilst there was an overall difference in mean bacterial uptake ($p \leq 0.0001$; $F = 26.29$) between strains, uptake of 212 and 411 did not deviate significantly from the parent strain ($p = 0.142$ and $p = 0.684$ respectively; see figure 3.21). Despite similar uptake and TNF- α production, IL-1 β and caspase-1 processing were markedly different between 212 and 411. This indicates an inflammasome-dependent differential sensing of these two strains by the host.

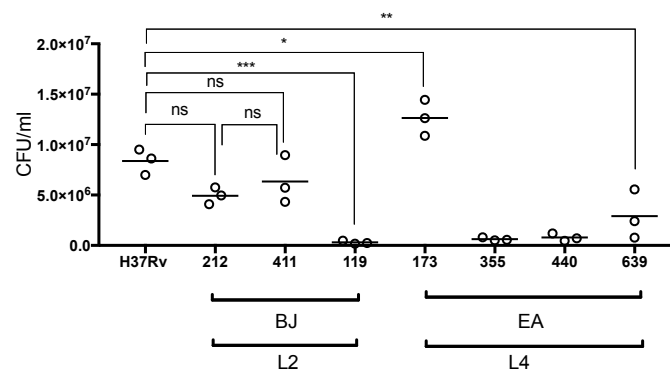


Figure 3.21: Clinical isolates show differential bacterial uptake at 4 hours post-infection. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and were infected with indicated isolates across 2 lineages at an MOI of 5. At 4 hours, the cells were lysed with sterile water and serially diluted using PBS–Tween-80 (0.05 %) and plated onto 7H11 agar plates for CFUs. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied to determine a difference between means of each strain followed by Tukey's multiple comparison test to evaluate the differences between each of the experimental condition. In the graphs, statistical significance is indicated as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns=non-significant. Only a few comparisons are represented.

Strain differences in cell death

Given how cell death can be effective in controlling *M. tuberculosis* infection but is also manipulated by the infection itself in order to facilitate its spread, I looked at LDH release in the same panel of clinical isolates. Krishnan et al. 2013 have shown previously that that whilst there was variation in cell death between the isolates, there was no correlation between IL-1 β release and cell death (examining both apoptotic and necrotic death) in infected macrophages. Similarly, I found that there was a great deal of significant variation in cell death ($p \leq 0.0001$; $F = 7.938$). 411, 119, 173 and 355 did not induce cell death significantly; however 212 induced lower cell death compared to H37Rv ($p = 0.013$; see figure 3.22).

Whilst I was not able to pursue the mechanism of cell death induced by the different strains in this

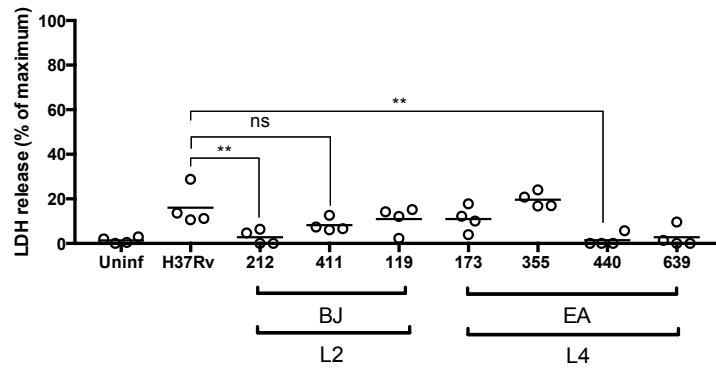


Figure 3.22: Clinical isolates promote differential LDH release at 24 hours post-infection. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and infected with indicated isolates across 2 lineages at an MOI of 5. The supernatants were removed at 24 hours and analysed for LDH release. The maximum positive control is elicited by the release of LDH induced by Triton-X detergent. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 4$ biological experiments. One-way ANOVA was applied to determine a difference between means of each strain followed by Tukey's multiple comparison test to evaluate differences the difference between each of the experimental conditions. In the graphs, statistical significance is indicated as $*p \leq 0.05$, $**p \leq 0.01$, ns=non-significant. Only a few comparisons are represented.

project, I tested whether cell death correlates with IL-1 β release (see figure 3.23). With a correlation coefficient of 0.4771 ($p = 0.1941$), we assume that at 24 hours, there is no linear dependence between cell death and IL-1 β release.

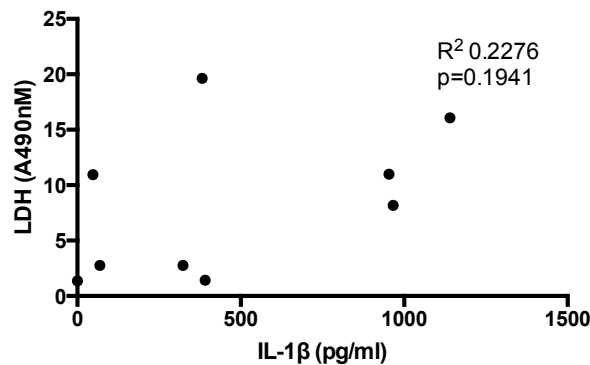


Figure 3.23: IL-1 β release does not correlate with cell death. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and were infected with indicated isolates as described previously across 2 lineages at an MOI of 5 and the supernatants were removed at 24 hours and analysed for LDH release. Each data point shown represents the mean IL-1 β from 3 biological replicates for each strain plotted against the corresponding mean LDH. Pearson's correlation coefficient was calculated to test the linear dependence of the two variables

In summary, the clinical isolates examined vary markedly in their ability to induce caspase-1 activation and IL-1 β processing. However, whilst the differences are interesting, it is difficult to draw any firm conclusions and take this forward when there is an obvious variation in the levels of priming, as shown by the pro-IL-1 β expression and the differences in initial bacterial uptake, suggesting that the differences in processing of IL-1 β can not be fully attributed to inflammasome activation. The strains 212 and 411 displayed significantly different IL-1 β phenotypes but had levels of priming and bacterial uptake that were similar to the parent strain H37Rv. Therefore in order to make robust conclusions about differential inflammasome activation, I would look at these clinical isolates only.

3.3.6 How do individual inflammasome components contribute to IL-1 β processing?

To date, several studies have examined how *M. tuberculosis* is able to induce IL-1 β processing. But as highlighted previously, there is some discrepancy with regards to the necessity of the individual components. Most studies so far have also looked at the lab-adapted strain, H37Rv in inflammasome induction, which may have limitations. In this section, I have examined how the clinical isolates 212 and 411, in addition to H37Rv vary in their ability to activate individual components of the inflammasome. Despite the lack of corroboration with *in vivo* data, this is the first time that the individual components of the inflammasome (using genetic knock-outs) have been shown side-by-side for their ability to activate IL-1 β following infection with varying isolates of *M. tuberculosis*. Caspase-11 is transcribed from the same locus as caspase-1 and in the following experiments, as explained previously, the *Caspase-1*^{-/-} knock-out cell lines were generated from mice stem cells that are also deficient in caspase-11. Caspase-11 (the equivalent of which in humans is caspase-4 and -5) has an important role to play in the non-canonical inflammasome activation pathway, whereby LPS from gram-negative bacteria is able reach the cytosol and bind and activate caspase-11, which cleaves gasdermin D leading to pyroptotic cell death (Kayagaki et al. 2015). Caspase-11 does not lead to IL-1 β processing directly, but it does facilitate the activation and assembly of the NLRP3 inflammasome leading to the indirect maturation of IL-1 β (Kayagaki et al. 2011; Broz et al. 2016). Thus far, activation of the inflammasome during *M. tuberculosis* infection is believed to be due to the canonical pathways and the activation of caspase-11 through LPS interaction is *assumed* not to play a role; therefore, for the purposes of this project, whilst I am using *Caspase-1/Caspase-11*^{-/-} double knock-outs, I am only interested in the role of caspase-1.

The protein ASC is required for IL-1 β processing during *M. tuberculosis* infection

ASC is an adaptor protein that has previously been shown *in vivo* and *in vitro* to have a fundamental role in IL-1 β processing (Mishra et al. 2010; McElvania Tekippe et al. 2010). To verify this, I infected immortalised wild-type and *Asc*^{-/-} BMDMs with the *M. tuberculosis* strains and analysed the supernatants and lysates by ELISA and Western blotting (see figure 3.24).

Indeed, in this experimental model, there was a clear difference between the means of the groups (Two-way ANOVA: between bacterial strains, $p \leq 0.0001$; between genotypes $p \leq 0.0001$; and interaction between the two $p \leq 0.0001$) and the induction of IL-1 β release was completely dependent on ASC. 212 might appear to have less dependence on ASC ($p \leq 0.01$) however this is likely due to lower IL-1 β release seen in the wild-type, therefore the magnitude of effect is not as large. The supernatants in

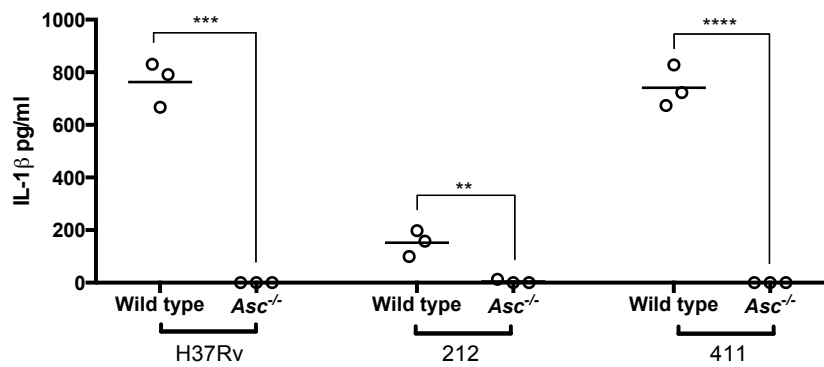


Figure 3.24: IL-1 β release induced by *M. tuberculosis* strains is completely dependent on the adaptor protein ASC. Wild-type and *Asc*^{-/-} BMDMs seeded in a 48-well plate at 3.4×10^5 cells/well and were infected with indicated isolates at an MOI of 5 for 24 hours. After 24 hours, supernatants were removed and analysed for IL-1 β release by ELISA. Two-way ANOVA was performed for each time-point to ascertain a difference in mean replication between the different groups followed by Tukey's multiple comparison test. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. Statistical significance is depicted in some of the comparisons and is indicated as * $p \leq 0.05$, **** $p \leq 0.0001$.

the Western blot reflected the ELISA results, with mature IL-1 β and activated caspase-1 bands seen in the wild-type cells and an absence of any band seen in the *Asc*^{-/-} cells. Unfortunately however the expression of pro-IL-1 β was low in the wild-type cells and completely absent in the *Asc*^{-/-} cells (see figure 3.25).

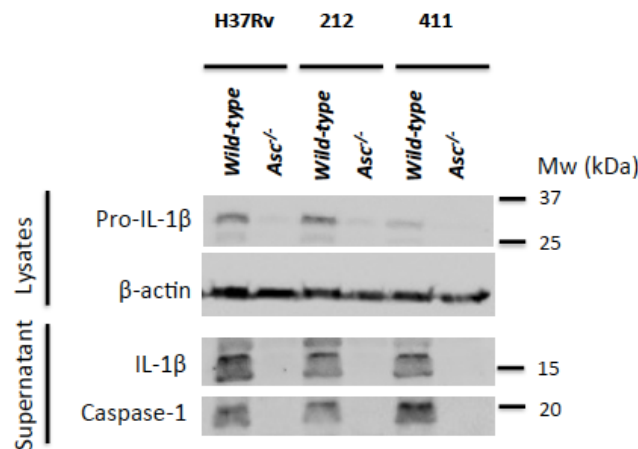


Figure 3.25: ASC knock-out cell lines lead to poor expression of pro-IL-1 β following infection with *M. tuberculosis*. Wild-type and *Asc*^{-/-} BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and infected with indicated isolates across 2 lineages at an MOI of 5 for 24 hours. Supernatants were desalted and precipitated by acetone overnight and lysates were prepared. Proteins were separated by 13.5 % acrylamide SDS-PAGE using Tris-Glycine buffers, and transferred onto PVDF membranes. Immunoblots were performed using indicated antibodies. The blot is representative of $n = 2$ biological experiments.

This lack of priming does not appear to be due to a reduction in bacterial uptake given that there was no clear difference between wild-type and *Asc*^{-/-} when infected with H37rv (statistical analysis not performed as only 2 experiments were conducted but depicted in figure 3.26).

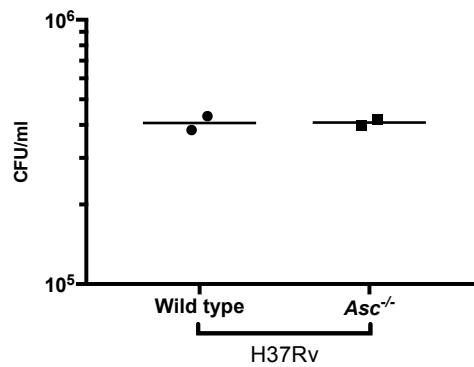


Figure 3.26: H37Rv leads to similar levels of bacterial uptake in both wild-type and *Asc*^{-/-} BMDMs. Wild-type and *Asc*^{-/-} BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and were infected with H37Rv at an MOI of 5 and cells were lysed and plated for CFUs after 4 hours. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 2$ biological experiments. No statistical analysis was performed.

Clinical isolates can induce partial IL-1 β processing in the absence of NLRP3 and AIM2 but is completely dependent on Caspase-1

Previous studies have shown some discordance regarding the necessity of individual inflammasome components. Dorhoi et al. 2012 showed that NLRP3 is required for maturation of IL-1 β *in vitro* but not *in vivo*. Other authors have since showed that AIM2 plays an important role in the IL-1 β signalling pathway both *in vitro* and *in vivo* (Saiga et al. 2012).

In order to ascertain which inflammasome components were involved in IL-1 β processing, I used immortalised cell lines from *Nlrp3*^{-/-}, *Aim2*^{-/-} double knock-out mice and a *Caspase1/11*^{-/-} cell line. In murine cells, caspase-1 knock-out mice are also deficient in caspase-11 expression (Kayagaki et al. 2011), and although a role for caspase-11 has not been identified in murine tuberculosis, *Caspase1/11*^{-/-} cell lines have been used extensively in studies previously (Abdalla et al. 2012). Using the clinical isolates, I investigated how *M. tuberculosis* can vary in its ability to activate AIM2, NLRP3 and Caspase-1/11. As previously, cell lines were infected with an MOI of 5 and supernatants and lysates were removed after 24 hours for Western blot analysis and ELISA.

Surprisingly, we see that individual strains appear to vary in their dependence on the inflammasome for IL-1 β release. At 24 hours, there was a significant difference in the mean IL-1 β release by both the host cell genotype and bacterial strain on IL-1 β release (Two-way ANOVA, $p \leq 0.0001$) and also a demonstrable interaction between the two factors ($p \leq 0.0001$).

Whilst the double knock-out of AIM2 and NLRP3 does lead to significant reduction in IL-1 β release, it does not completely abolish it. This is most prominent in infections with H37Rv (642.7 ± 79.1 pg ml⁻¹ and 173.9 ± 29.0 pg ml⁻¹ in wild-type and *Nlrp3*^{-/-}/*Aim2*^{-/-} cell lines respectively, $p \leq 0.0001$) and 411 (714.2 ± 14.8 pg ml⁻¹ and 231.7 ± 49.0 pg ml⁻¹ respectively, $p \leq 0.0001$). IL-1 β induction

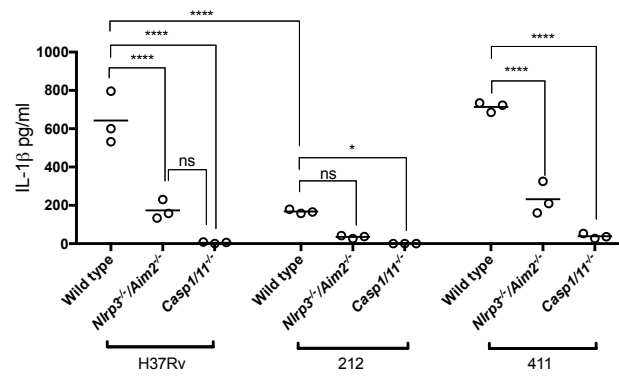


Figure 3.27: IL-1 β processing is partially dependent on AIM2 and NLRP3 and fully dependent on caspase-1/11. Wild-type, *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1/11*^{-/-} BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and infected by the indicated isolates at an MOI of 5 for 24 hours. Supernatants were removed after 24 hours and analysed for IL-1 β release by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. Two-way ANOVA was performed for each time point to ascertain a difference in mean replication between the different groups followed by Tukey's multiple comparison test. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. Statistical significance is indicated as * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$, ns=non-significant. Only a few comparisons are depicted

by 212 in wild-type cells ($167.8 \pm 6.3 \text{ pg ml}^{-1}$) does not fall much in *Nlrp3*^{-/-}/*Aim2*^{-/-} cell lines ($35.88 \pm 4.70 \text{ pg ml}^{-1}$, $p = 0.173$) although again, this may be due to a low baseline (see figure 3.27). By contrast, induction of IL-1 β by all strains relies fully on caspase-1/11.

A western blot (data not shown) confirmed the lack of NLRP3 in the *Nlrp3*^{-/-}/*Aim2*^{-/-} cell lines. All the strains shown in figure 3.28 induced a similar degree of priming in the wild-type cells and this translated into differential processing of pro-IL-1 β into IL-1 β . Thus, H37Rv and 411 induced IL-1 β processing with associated caspase-1 activation and 212 did not activate the inflammasome. In keeping with the ELISA results, we saw that there is still some processing, albeit reduced, of pro-IL-1 β and caspase-1 induced by H37Rv and 411 in *Nlrp3*^{-/-}/*Aim2*^{-/-} cells while there was no IL-1 β processing in the *Caspase1/11*^{-/-} cell line. Only a few studies have simultaneously investigated *Nlrp3*^{-/-}, *Asc*^{-/-}, *Caspase1/11*^{-/-} macrophages together with wild-type to delineate their contribution to IL-1 β processing (Dorhoi et al. 2012; Abdalla et al. 2012). Similarly Shah et al. 2013 examined *Nlrp3*^{-/-}, *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Aim2*^{-/-} against wild-type but did not additionally explore *Caspase1/11*^{-/-} and *Asc*^{-/-} macrophages.

Here, I show that in an *in vitro* experimental model, caspase-1/11 and ASC are required for IL-1 β processing although there are some defects in the initial priming. Although there is reduced priming in the *Nlrp3*^{-/-}/*Aim2*^{-/-} cells, I still see maturation of IL-1 β and associated caspase-1 processing following infection with H37Rv and 411.

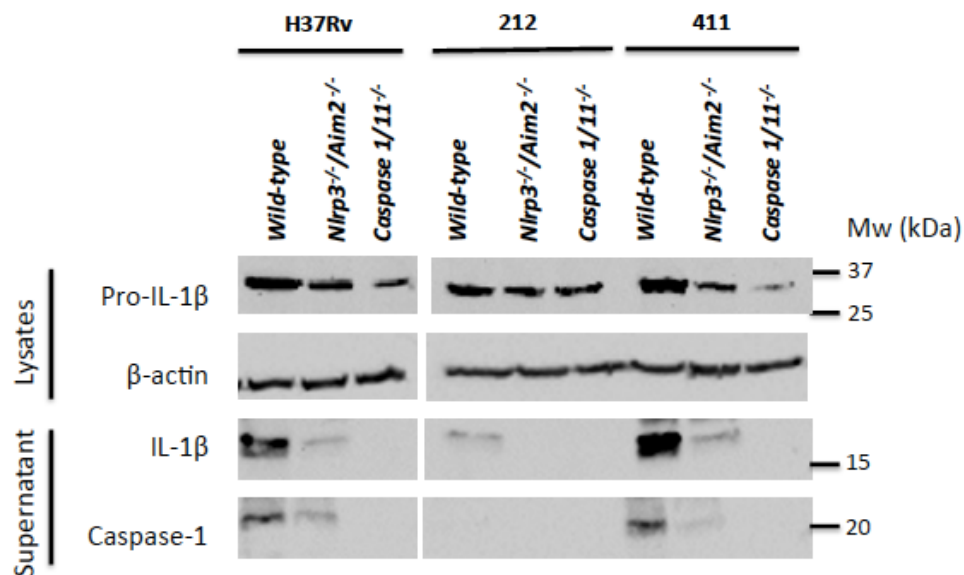


Figure 3.28: IL-1β processing is partially dependent on AIM2 and NLRP3 and fully dependent on caspase-1/11. *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1/11*^{-/-} BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and infected with indicated isolates across 2 lineages at an MOI of 5 for 24 hours. Supernatants were desalted and precipitated by acetone overnight and lysates were prepared. Proteins were separated by 13.5% acrylamide SDS-PAGE using Tris-Glycine buffers, and transferred onto PVDF membranes. Immunoblots were performed using indicated antibodies. The blot is representative of $n = 2$ biological experiments

Cell death does not depend on the inflammasome components, NLRP3, Caspase-1/11 or AIM2

Activation of caspase-1 leads to the processing of IL-1β and the induction of pyroptosis, an inflammatory form of cell death, where the membrane permeabilises and cytoplasmic contents are released leading to the propagation of the inflammatory cascade. However a wealth of other cell death mechanisms exist, such as necrosis and apoptosis which have conflicting results in their role in effective anti-microbial control (Amaral et al. 2016). Non-pyroptotic mechanisms of cell death appear to play a key role during *M. tuberculosis* infection and previous studies have shown that caspase-1 is not required for induction of cell death (Lee et al. 2011; Welin et al. 2011). Using the same experimental design as previously shown, I examined the LDH released by wild-type, *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1/11*^{-/-} BMDMs.

I tested the response of these knock-out cell lines to LPS/ATP stimulation (see figure 3.29). Given that previous reports have shown that cell death following ATP-induced membrane destabilisation is dependent on NLRP3 and caspase-1 (Yu et al. 2014), I hypothesised that LDH release following LPS/ATP stimulation would be significantly reduced in the knock-outs. As previously demonstrated, LPS/ATP-mediated cell death was reduced in *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1/11*^{-/-} BMDMs (see figure 3.29). These therefore verify the integrity of the knock-outs in response to known stimuli. Having shown this, I then examined the infected cell lines for LDH.

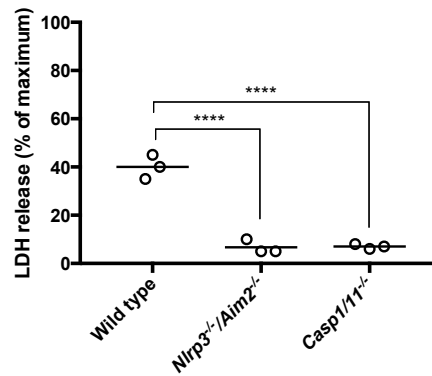


Figure 3.29: LPS/ATP-mediated cell death is dependent on NLRP3/AIM2 and caspase-1. LPS-primed BMDMs of the indicated genotype were stimulated with 5 mM ATP for 1 hour and supernatants were removed and analysed for LDH. The maximum positive control is elicited by the release of LDH induced by Triton-X detergent. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied for each MOI followed by Tukey's multiple comparison test to evaluate differences between the experimental condition means and the mean of every other experimental condition. Statistical significance is indicated as **** $p \leq 0.0001$

H37Rv and the clinical isolates, 212 and 411, induced similar levels of cell death in wild-type BMDMs as in caspase-1 knock-out BMDMs (see figure 3.30). Likewise whilst there appeared to be a slight trend to a decrease in cell death in the *Nlrp3*^{-/-}/*Aim2*^{-/-} double knock-out cells, there was no statistically significant reduction induced by any of the isolates (figure 3.30).

Similarly, I also examined the supernatants from the wild-type and *Asc*^{-/-} BMDMs infected with the clinical isolates. Short course infections have previously been shown to exhibit cell death independent of ASC and dependent on caspase-1 (Broz et al. 2010), because sensors containing a CARD, such as NLRC4 and NLRP1b, are able to directly recruit pro-caspase-1 without ASC. Different authors have shown that the differences in time frame for cell insult may lead to variability in the involvement of caspase-1 in cell death but demonstrate a complete dependence on ASC, although this study utilised AIM2 rather than NLRP activation (Sagulenko et al. 2013).

In our experimental model, there were no differences in cell death between the wild-type and *Asc*^{-/-} BMDMs when infected with the clinical isolates(see figure 3.31). In the ASC knock-out models, there was a general reduction in pro-IL-1 β expression (see figure 3.25), therefore whilst it is difficult to formally compare, it is interesting to note that cell death is occurring at an equivalent level suggesting that at 24 hours post-infection, *M. tuberculosis* is inducing cell death in a manner that does not rely on inflammasome-dependent mechanisms (Lee et al. 2011).

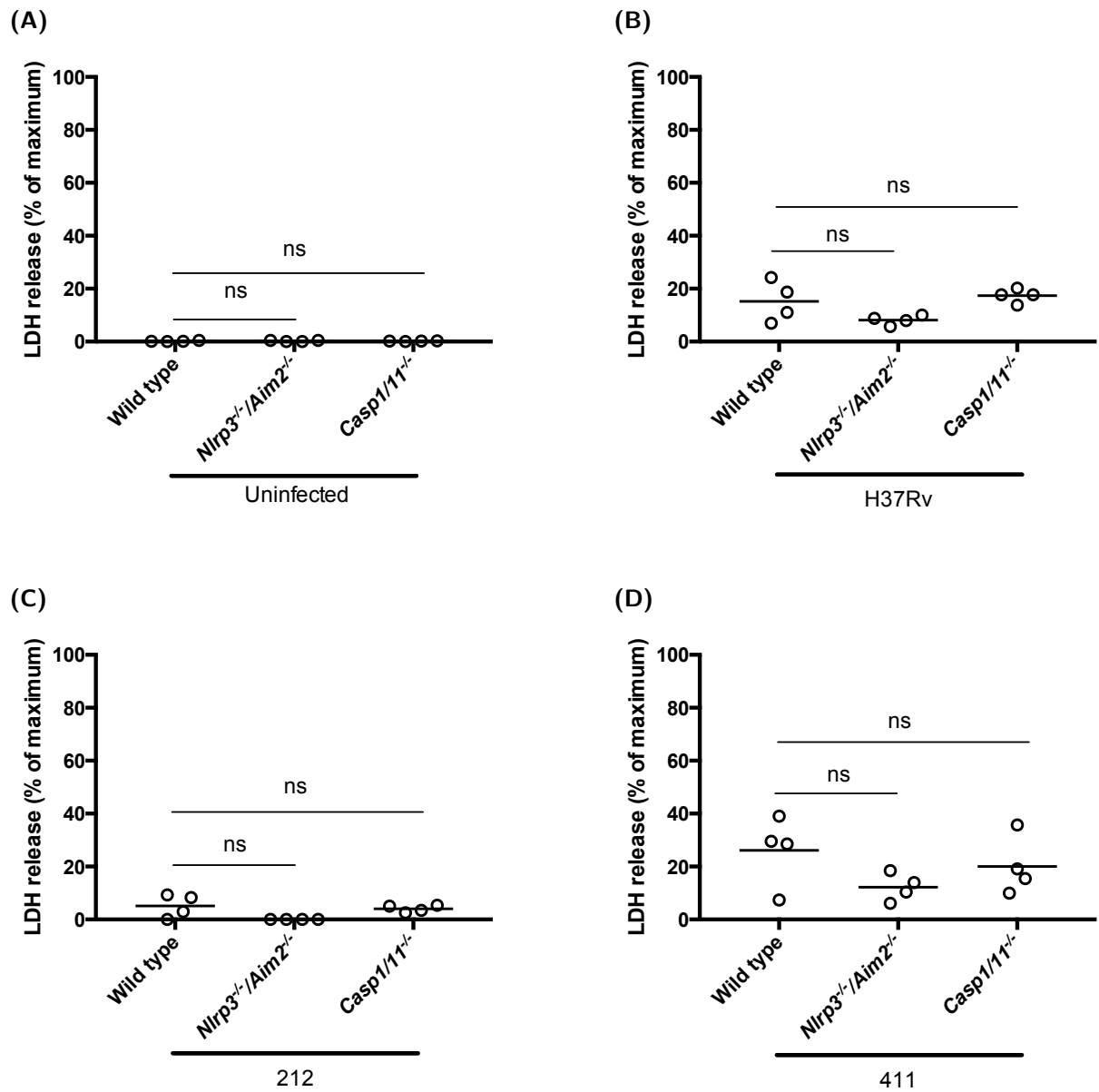


Figure 3.30: During infection with H37Rv, 212 and 411, wild-type and *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1/11*^{-/-} BMDMs display similar levels of cell death. BMDMs of the indicated genotype were seeded in a 48-well plate at 3.4×10^5 cells/well and were left uninfected (A) or infected with H37Rv (B), 212 (C) or 411 (D) at an MOI of 5 for 24 hours, the supernatants were removed and analysed for LDH release. The maximum positive control is elicited by the release of LDH induced by Triton-X detergent. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 4$ biological experiments. One-way ANOVA was applied to determine a difference in means between genotypes followed by Tukey's multiple comparison test to evaluate differences between the experimental condition means and the mean of every other experimental condition. Statistical significance is indicated as ns=non-significant.

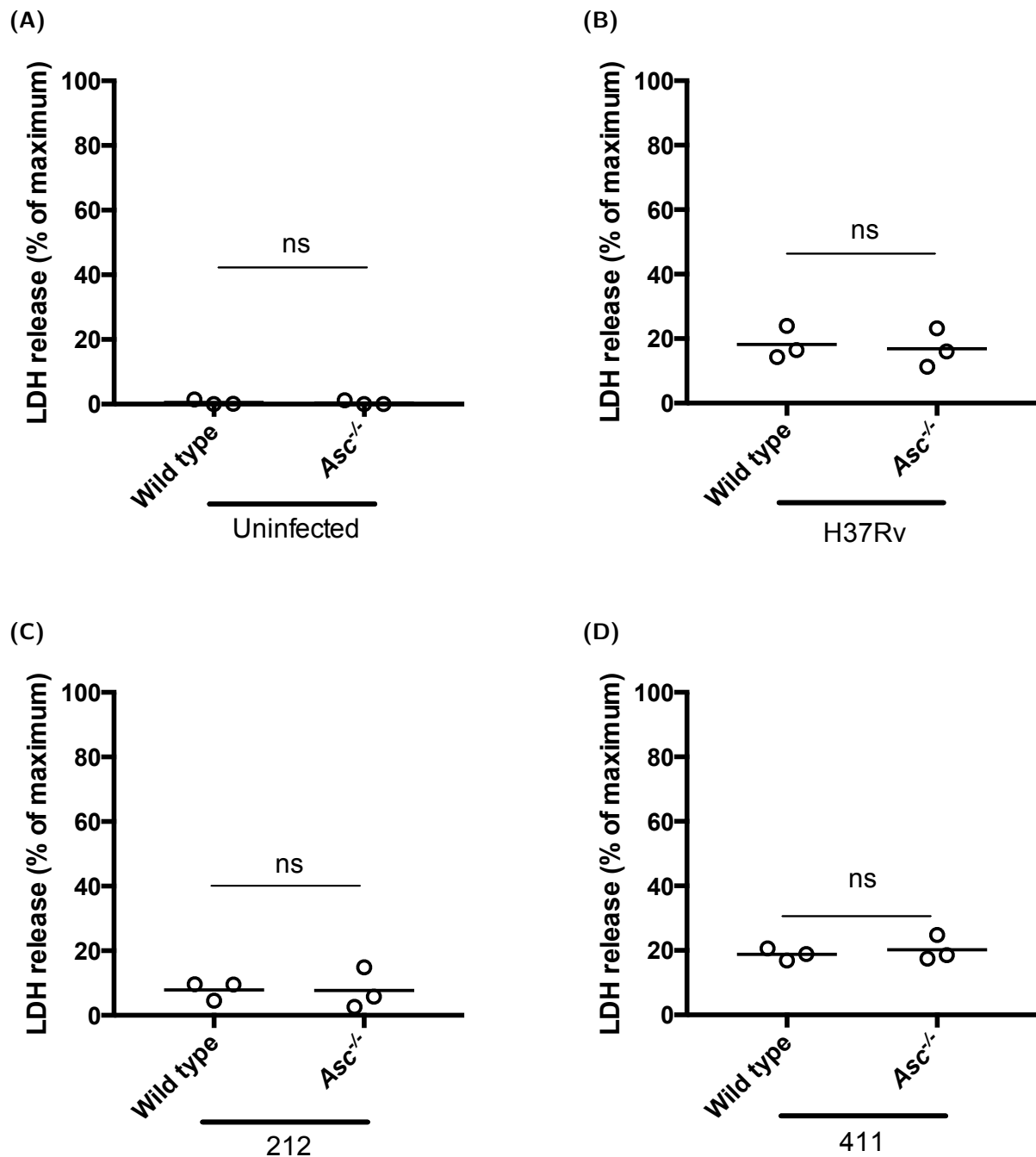


Figure 3.31: During infection with H37Rv, 212 and 411, wild-type and *Asc*^{-/-} BMDMs display similar levels of cell death. BMDMs of the indicated genotype were seeded in a 48-well plate at 3.4×10^5 cells/well and were left uninfected (A) or infected with H37Rv (B), 212 (C) or 411 (D) at an MOI of 5 for 24 hours, the supernatants were removed and analysed for LDH release. The maximum positive control is elicited by the release of LDH induced by Triton-X detergent. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 4$ biological experiments. A two-tailed paired *t*-test was used to analyse the difference in means induced by the indicated strain in each cell line compared to the wild-type. In the graphs, statistical significance between the experimental conditions is indicated as ns = non-significant.

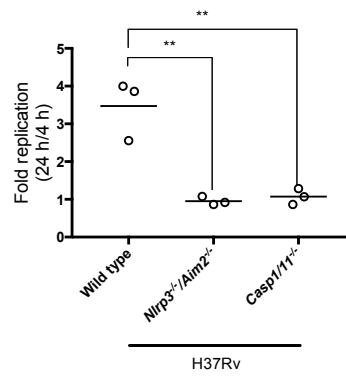


Figure 3.32: Bacterial replication is reduced following infection in *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1*/*11*^{-/-} BMDMs *in vitro*. Wild-type, *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1*/*11*^{-/-} BMDMs were infected with H37Rv at an MOI of 5. At 4 and 24 hours, the cells were lysed with sterile water and serially diluted using PBS–Tween-80 (0.05 %) and plated onto 7H11 agar plates for CFUs to ascertain bacterial uptake. Fold replication was calculated by dividing CFUs at 24 hours by CFUs at 4 hours for each genotype. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied to determine a difference in means between genotypes followed by Tukey's multiple comparison test to evaluate the differences between each of the experimental conditions. Statistical significance between the experimental conditions is indicated as $**p \leq 0.01$

Bacterial replication is reduced following infection in *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1*/*11*^{-/-} BMDMs *in vitro*

So far, our experimental system has shown that the clinical isolates can vary in their ability to induce IL-1 β , that this processing appears to be partially dependent on AIM2 and NLRP3 but fully dependent on caspase-1/11, but it is unclear how this affects bacterial replication.

Certainly, thus far, suppression of inflammasome signalling *in vitro* may not have much of a positive effect on *M. tuberculosis* bacterial replication. However, Verway et al. 2013 have shown that silencing IL-1 receptor expression leads to an increase in bacterial CFUs at 72 hours following infection with H37Rv *in vitro*. Recombinant IL-1 β added to human monocytes infected with H37Rv leads to a reduction in CFUs compared to untreated cells at longer infection time frames (6 days; Jayaraman et al. 2013). However, few other studies have looked at the impact of knocking out components of the inflammasome *in vitro*. *In vivo* is a different matter, with a range of studies suggesting that excessive inflammasome activation may be detrimental to the host (Garlanda et al. 2007).

For ease and as an initial gauge of the characteristics of *M. tuberculosis* intracellular replication, I infected *Nlrp3*^{-/-}/*Aim2*^{-/-}, *Caspase1*/*11*^{-/-} and wild-type BMDMs with H37Rv, lysed and plated for CFUs after 24 hours to determine intracellular bacterial numbers (see figure 3.32), comparing them with bacterial uptake at 4 hours in order to ascertain fold replication within the given time frame.

There was a significant difference between the CFU counts between the cell-lines ($p \leq 0.01$, $F = 26.25$) but somewhat surprisingly whilst *Nlrp3*^{-/-}/*Aim2*^{-/-} and *Caspase1*/*11*^{-/-} BMDMs displayed similar levels of fold replication (see figure 3.32; 0.9529 ± 0.0644 and 1.072 ± 0.121 respectively, $p = 0.4289$). Furthermore, bacterial replication was significantly lower in *Nlrp3*^{-/-}/*Aim2*^{-/-} cells compared to

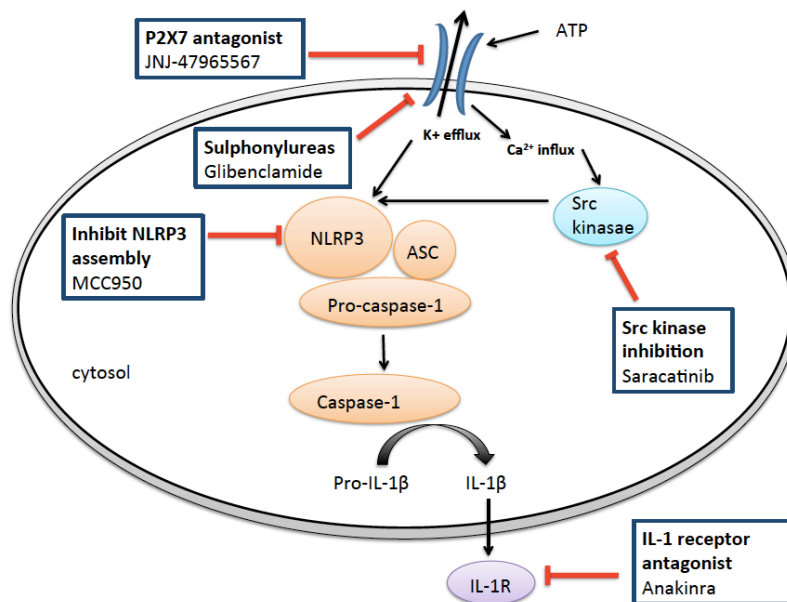


Figure 3.33: Targeting the inflammasome in *M. tuberculosis* infection. Schema showing the chosen targets to reduce NLRP3 inflammasome signalling.

wild-type BMDMs ($p \leq 0.01$).

3.3.7 Can the inflammasome be modulated and used as a therapeutic adjunct during infection with *M. tuberculosis*?

In parallel to examining infection with the knock-out cells, I also looked at a panel of pharmacological agents that are able to block aspects of the IL-1 signalling pathway. Rather than focus on aspects that modulate the transcription of pro-IL-1 β through NF- κ B activation (i.e. the priming signal), I examined agents that are able to modulate the maturation of IL-1 β (see figure 3.33).

How do different agents blocking the inflammasome pathway affect cellular viability?

I chose drugs to target specific components of the inflammasome pathway, though several are also noted to have uses where inflammasome blockade has been an additional finding. For example, since ROS serve as a signal for NLRP3 activation, I hypothesised that indirect inhibition of the inflammasome could be achieved through c-Src kinase blockade. Further information with regards to the drug and their mechanism of action is given in the introduction of this chapter (see section 3.1.4).

I will now describe the experiments I performed using the following drugs:

1. P2X7 anatagonists: JNJ-47965567
2. Sulphonylureas: glibenclamide

3. Src kinase inhibitors: saracatanib
4. Small molecule inhibitors of NLRP3: MCC950
5. IL-1 Receptor antagonists: anakinra
6. Anti-mycobacterial therapy: rifampicin

It was important that I examined the cellular toxicity of these drugs at a range of concentrations. For this I used the MTT assay with drug concentrations based on previous papers where pharmacological efficacy (efficacy defined as the outcome measured) was found.

In order to establish the efficacious doses of rifampicin, an *in vitro* REMA assay was performed to identify the minimum inhibitory concentration (MIC) required to inhibit growth. Using the technique established by Palomino et al. 2002 and elaborated in the methods section, serial two-fold dilutions in 7H9 media from the highest rifampicin concentration of 1.696 mg l^{-1} to the lowest concentration of 0.013 mg l^{-1} were prepared directly into 96 well plates. 48 hours after addition of liquid H37Rv broth, a change from blue to pink indicated bacterial growth. The MIC was defined as the lowest drug concentration that prevented this colour change (see table 3.3).

Rifampicin concentration mg l^{-1}	Colour
1.696	blue
0.848	blue
0.424	blue
0.212	blue
0.106	blue
0.053	blue
0.026	blue
0.013	pink

Table 3.3: REMA assay for determining MIC of rifampicin to H37Rv.

Thus using this assay, rifampicin exhibited an MIC of 0.026 mg l^{-1} against H37Rv. For subsequent macrophage infections, concentrations of rifampicin at $\times 2$ and $\times 20$ this MIC were used.

Within the concentration ranges examined, JNJ-47965567, glibenclamide, MCC950, anakinra, and rifampicin did not elicit toxicity (see figure 3.34). Current literature shows some quite significant discrepancies with regards to the concentrations of saracatanib tolerated by cancer cells and immortalised cell lines. As such I treated the cells with a wide range of concentrations ranging from nanomolar to micromolar. In doing this, I saw that treating the cells with up to $6.25 \mu\text{M}$ did not impact on cell death. However, treating the cells with anything higher than this led to a significant increase in cellular toxicity (see figure 3.34C). This is different to that reported by Chandra et al. 2016 who demonstrated that doses up to $20 \mu\text{M}$ were safe but $40 \mu\text{M}$ led to increased cell death.

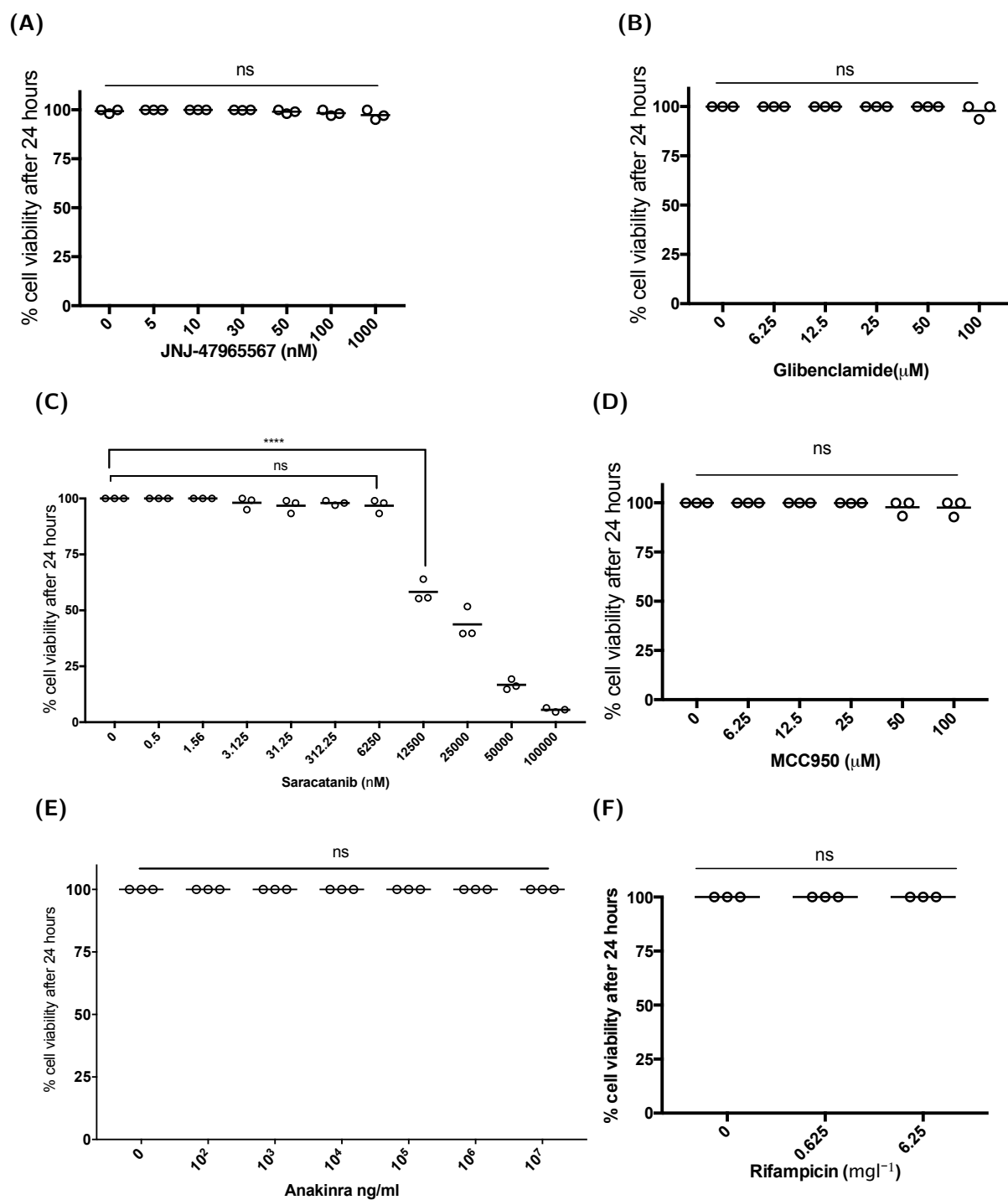


Figure 3.34: Wild-type BMDMs were treated with various concentrations of the indicated drug for 24 hours and cell viability was determined using the MTT assay. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied to determine a difference in means between experimental conditions followed by Dunnet's multiple-comparison test to evaluate differences between the experimental drug concentration and the mean of the untreated cells, designated as the control strain. In the graphs, statistical significance between the experimental conditions and the control is indicated as **** $p \leq 0.0001$, ns=non-significant

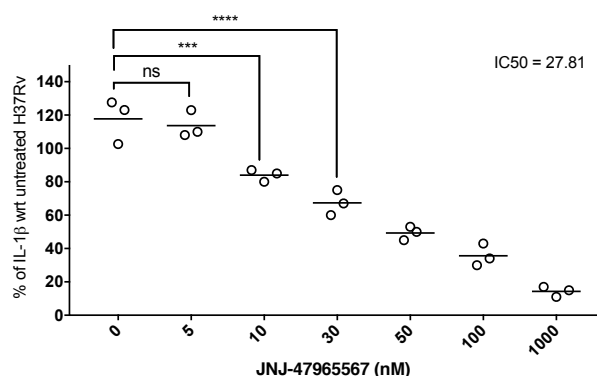


Figure 3.35: JNJ-47965567 leads to reduction in IL-1 β following infection with H37Rv. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and infected by H37Rv at an MOI of 5 for 4 hours and then washed. Cells were then treated with indicated concentrations of JNJ-47965567 for 24 hours and IL-1 β release was determined using an ELISA. In order to minimise variation between the infected control and the drug treated cells, the control was treated with the maximum concentration of solvent that the drug was dissolved in. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied to determine a difference in means between experimental conditions followed by Dunnet's multiple comparison test to evaluate differences between the experimental drug concentration and the mean of the untreated cells, designated as the control strain. In the graphs, significance between the experimental conditions and the control is indicated as: *** $p \leq 0.001$, **** $p \leq 0.0001$, ns=non-significant.

How do these different agents blocking the inflammasome pathway affect IL-1 β release following *M. tuberculosis* infection?

I then looked at how these drugs affected IL-1 β release from wild-type immortalised BMDMs following infection with H37Rv. To test the effects of saracatanib, I treated the cells with concentrations beyond 3.125 nM, the point at which there was reduced cell viability but also the potential to elicit efficacy in accordance to the data shown by Chandra et al. 2016.

IL-1 β release is expressed as a percentage of the mean maximum secreted by the wild-type BMDMs following H37Rv infection. For JNJ-47965567, glibenclamide, saracatanib and MCC950, I used the PRISM 7 software to calculate the half-maximal inhibitory concentration (IC₅₀) in relation to the maximum IL-1 β release from the untreated H37Rv infected control, by log-transforming the concentrations and fitting a non-linear regression curve to the values.

The IC₅₀ of JNJ-47965567 (figure 3.35) was 28.52 nM, although there was a statistically significant reduction (compared to the untreated control) at 10 nM ($84 \pm 2\%$) and almost complete attenuation was achieved at 1 μ M which is similar to the findings by Bhattacharya et al. 2013.

Higher concentrations of glibenclamide (figure 3.36) were required in order to elicit a reduction in IL-1 β (IC₅₀ 7.56 μ M) although doses between 0 and 6.25 μ M were not tested and it is possible a moderate reduction might have been seen in this interval.

At non-toxic concentrations of saracatanib (figure 3.37) as ascertained previously, there was no reduction in IL-1 β . The IC₅₀ of saracatanib was 43.557 μ M; a statistically significant reduction was

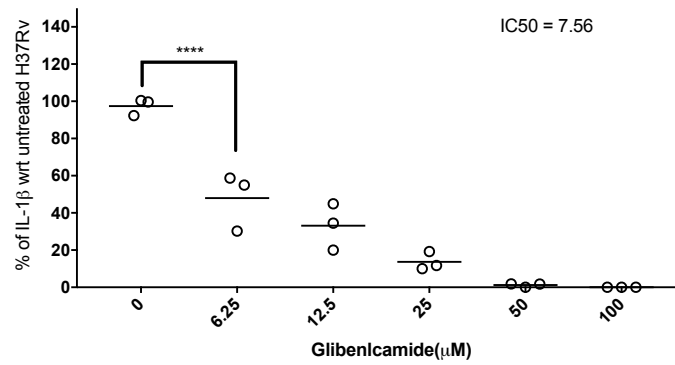


Figure 3.36: Glibenclamide leads to reduction in IL-1 β following infection with H37Rv. Method as per figure 3.35. **** $p \leq 0.0001$.

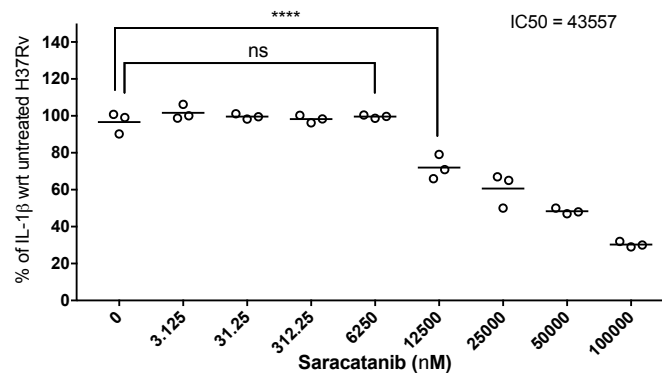


Figure 3.37: Saracatanib leads to reduction in IL-1 β at higher concentrations following infection with H37Rv. Method as per figure 3.35. **** $p \leq 0.0001$, ns=non-significant.

elicited at 12.5 μM ($p \leq 0.0001$, $71.97 \pm 3.84\%$), however this was associated with an increase in cellular toxicity as seen in figure 3.34C.

The IC₅₀ of MCC950 (figure 3.38) was 0.11 μM and complete inhibition of IL-1 β was achieved at 100 μM in line with previous observations by Coll et al. 2015 demonstrating the efficacy of MCC950 at nanomolar concentrations.

In my experimental setup, I did not see any reduction in IL-1 β release induced by H37Rv when

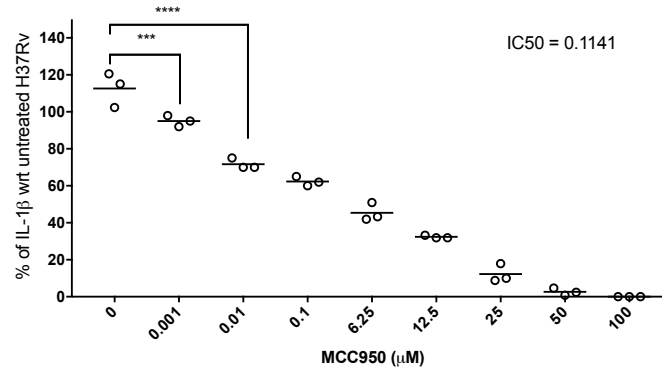


Figure 3.38: MCC950 leads to reduction in IL-1 β following infection with H37Rv. Method as per figure 3.35. *** $p \leq 0.001$, **** $p \leq 0.0001$.

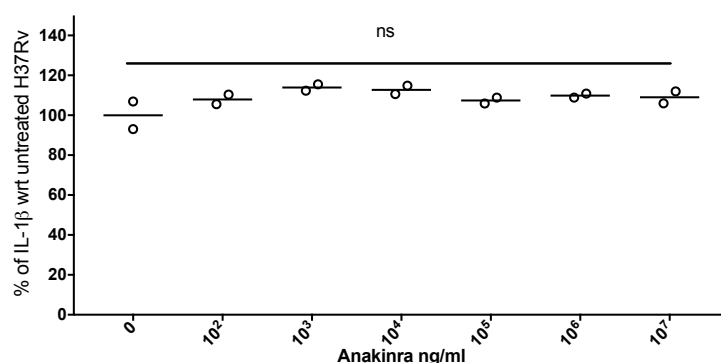


Figure 3.39: Anakinra does not lead to a reduction in IL-1 β following infection with H37Rv. Method as per figure 3.35. ns=non-significant.

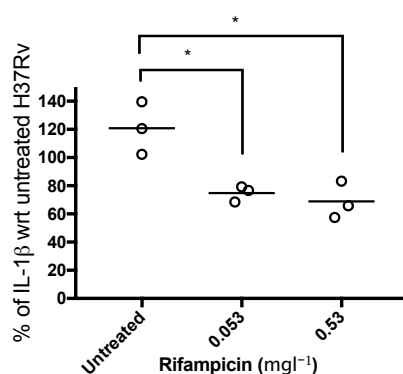


Figure 3.40: Rifampicin leads to a reduction in IL-1 β following infection with H37Rv at $\times 2$ and $\times 20$ MIC. Method as per figure 3.35. $*p \leq 0.05$.

wild-type BMDMs were treated with anakinra (figure 3.39) at concentrations up to 10 mg ml⁻¹.

At $\times 2$ and $\times 20$ the MIC to H37Rv, rifampicin (figure 3.40) led to a similar level of reduction in IL-1 β ($74.83 \pm 3.25\%$ and $68.85 \pm 7.59\%$ respectively).

In summary, aside from anakinra, all drugs tested had varying degrees of ability to reduce IL-1 β release following H37Rv infection. However, saracatanib was not pursued further at this stage due to the fact that its MIC correlated with unacceptable cellular toxicity. Whilst glibenclamide is an attractive candidate as a 'repurposed' drug, the IC₅₀ required to inhibit IL-1 β release was in the micromolar concentration range and this drug is well known to cause hypoglycaemia (Ting et al. 2010). The IC₅₀ of both JNJ-47965567 and MCC950 were in the nanomolar range demonstrating their potency; furthermore neither drug induced cellular toxicity even at the higher concentrations after 24 hours. At this stage both would have been reasonable candidates to investigate as adjunctive therapy with rifampicin. However, with increased literature available on MCC950, in both *in vitro* and *in vivo* models, I felt it appropriate to continue with this as the sole 'candidate'.

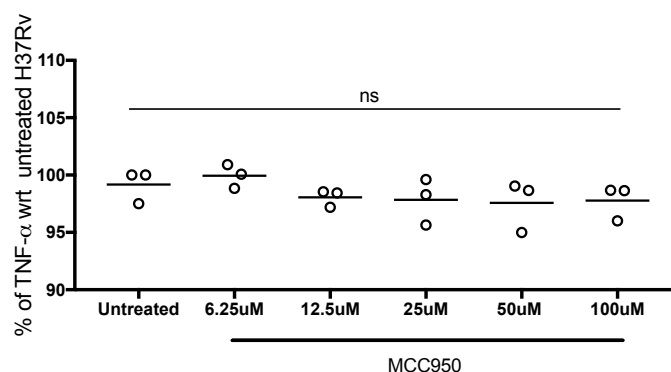


Figure 3.41: Treatment of cells with MCC950 does not affect TNF- α release. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and infected with H37Rv at an MOI of 5 and washed after 4 hours before being treated with the indicated concentrations of MCC950. After 24 hours, the supernatants were removed and TNF- α secretion was determined by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied to determine the difference in means between experimental conditions followed by Dunnet's multiple comparison test to evaluate differences between the experimental drug concentration and the mean of the untreated cells, designated as the control strain. In the graphs, significance between the experimental conditions and the control is indicated as ns=non significant.

MCC950 does not affect TNF- α release following infection with *M. tuberculosis*

Having established the potency of MCC950, I wanted to determine its specificity. Coll et al. 2015 showed that treatment with MCC950 did not affect TNF- α release following stimulation with ATP. In a similar manner, I examined the supernatants taken from wild-type BMDMs treated with varying concentrations of MCC950 following *M. tuberculosis* infection. Like Coll et al. 2015, I did not detect any statistically significant difference in TNF- α release with treatment with MCC950 following infection compared to the untreated control (One-way ANOVA, $p = 0.4272$, $F = 1.063$).

In order to gain an initial idea about the safety of MCC950 and rifampicin together, rifampicin and MCC950 were used in combination and the MTT assay was performed after 24 hours. Combining rifampicin (at 0.053 mg l^{-1} and 0.53 mg l^{-1}) with MCC950 (at concentrations up to $100 \mu\text{M}$) did not lead to any reduction in cell viability (data not shown).

The NLRP3 inhibitor, MCC950, when used as an adjunct with rifampicin reduces IL-1 β release following *M. tuberculosis* infection

Using adjunctive therapy to standard anti-microbial treatment is an attractive prospect that might shorten overall treatment duration, reduce tissue damage and improve outcomes. Recent studies have shown that the addition of anti-inflammatory host-directed therapy may satisfy all these criteria (Ivanyi et al. 2013; Singhal et al. 2014; Dutta et al. 2016; Subbian et al. 2016).

To demonstrate that MCC950 and rifampicin could act in concert to reduce IL-1 β release, I infected wild-type BMDMs with H37Rv for 4 hours and then washed the cells. The cells were then treated

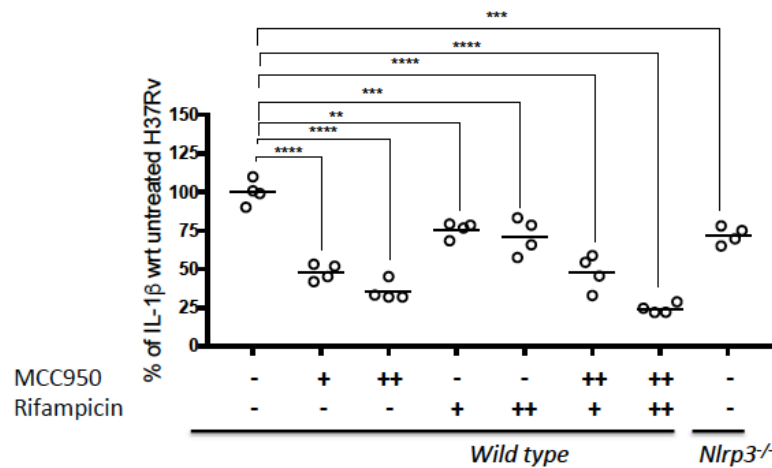


Figure 3.42: Dual treatment with rifampicin and MCC950 significantly reduces IL-1 β release compared to rifampicin alone. Wild-type BMDMs were seeded in a 48-well plate at 3.4×10^5 cells/well and were infected with H37Rv at an MOI of 5 and washed after 4 hours before being treated with equivalent DMSO concentration or 6.25 μ M (+) or 12.5 μ M MCC950 (++) and/or 0.053 mg l⁻¹ (+) or 0.53 mg l⁻¹ (++) rifampicin. *Nlrp3*^{-/-} BMDMs were used as a comparative control against MCC950. After 24 hours, the supernatants were removed and IL-1 β secretion was determined by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 4$ biological experiments. One-way ANOVA was applied to determine a difference in means between experimental conditions followed by Dunnett's multiple comparison test to evaluate differences between the experimental drug concentration and the mean of the untreated cells, designated as the control strain. In the graphs, significance between the experimental conditions and the control is indicated as ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

with 12.5 μ M MCC950, or 0.053 mg l⁻¹ or 0.53 mg l⁻¹ rifampicin, or combinations of the two drugs together for 24 hours. Supernatants were removed at 24 hours and an ELISA performed for IL-1 β . Since MCC950 was previously demonstrated to specifically inhibit the NLRP3 inflammasome, I also infected *Nlrp3*^{-/-} BMDMs as a comparative control (see figure 3.42).

As previously, the results are expressed as a percentage of the mean maximum levels of IL-1 β released induced by H37Rv. Drug treatment with all combinations induced a differential reduction in IL-1 β compared to the untreated control (One way ANOVA $F = 43.6$; $P \leq 0.0001$). In line with previous results, treatment with 12.5 μ M MCC950 lead to a significant reduction in IL-1 β release following H37Rv infection (100.00 ± 4.05 % compared with 35.56 ± 3.22 %). I expected that pharmacological inhibition of NLRP3 and *Nlrp3*^{-/-} BMDMs would lead to similar levels of inhibition of IL-1 β following *M. tuberculosis* infection. However, interestingly and unexpectedly, there was a significant difference in IL-1 β release between MCC950 treated wild-type BMDMs (35.56 ± 3.22 %) and untreated *Nlrp3*^{-/-} BMDMs (71.93 ± 2.90 %; unpaired two-tailed t -test; $t = 8.396$; $p = 0.0002$). Just treating with rifampicin alone at $\times 2$ and $\times 20$ the MIC induced similar levels of reduction for IL-1 β (75.77 ± 2.49 % and 71.29 ± 5.90 % respectively). Adding rifampicin at either concentration to MCC950 led to further reduction in IL-1 β release alone ($p \leq 0.0001$). Addition of rifampicin at 0.53 mg l⁻¹ in combination with MCC950 further inhibited IL-1 β release (24.37 ± 1.61 %) compared to MCC950 alone (35.56 ± 3.22 %), unpaired two-tailed t -test $t = 3.109$, $p = 0.02$.

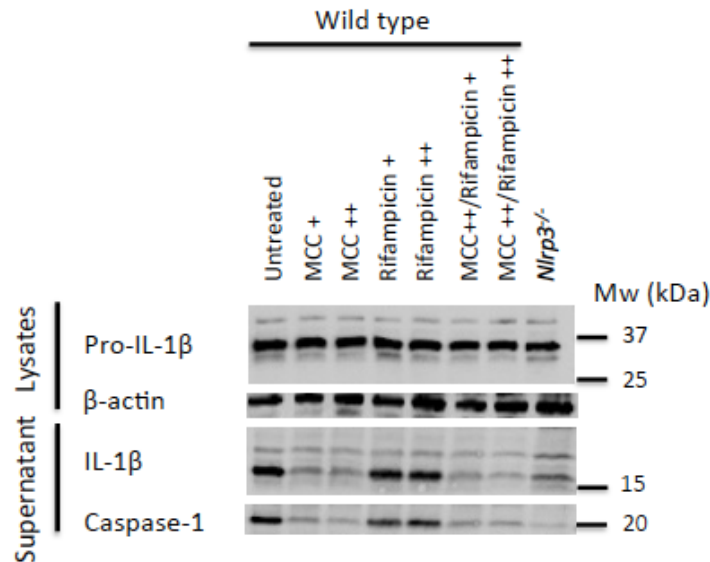


Figure 3.43: MCC950 inhibits IL-1 β processing and caspase-1 activation in wild-type BMDMs and phenocopies *Nlrp3*^{-/-} following infection with H37Rv. Wild-type and *Nlrp3*^{-/-} BMDMs were infected with H37Rv at an MOI of 5 for 4 hours and then washed. The cells were then treated with equivalent DMSO concentration or 6.25 μ M (+) or 12.5 μ M MCC950 (++) and/or 0.053 mg l⁻¹ (+) or 0.53 mg l⁻¹ rifampicin (++) . After 24 hours, the supernatants were removed, desalted and precipitated by acetone overnight and lysates were prepared. Proteins were separated by 13.5% acrylamide SDS-PAGE using Tris-Glycine buffers, and transferred onto PVDF membranes. Immunoblots were performed using indicated antibodies. The blot is representative of $n = 2$ biological experiments. MCC=MCC950.

To ensure that there was no direct activity of MCC950 against H37Rv, I performed a REMA assay; even at molar concentrations of MCC950, there was no effect on *in vitro* bacterial growth (data not shown).

To determine whether the pharmacological inhibition of IL-1 β was mediated by a reduction in caspase-1 activation, I performed a Western blot using the supernatants and lysates obtained from the experiment completed in figure 3.42.

Processing of IL-1 β appears to correlate closely with the activation of caspase-1 as expected (see figure 3.43). Treatment with MCC950 leads to a partial reduction in the processing of IL-1 β in agreement with the ELISA (see figure 3.42) and an associated reduction in caspase-1 activation. Dual treatment with MCC950 and rifampicin reduces IL-1 β processing compared to MCC950 alone. As expected, there is reduced but detectable IL-1 β processing in *Nlrp3*^{-/-} BMDMs. However, inconsistent with the ELISA (3.42) IL-1 β processing is similar between wild-type cells treated with MCC950 and *Nlrp3*^{-/-} following infection. This suggests that the ELISA is detecting pro-IL-1 β in addition to the processed form.

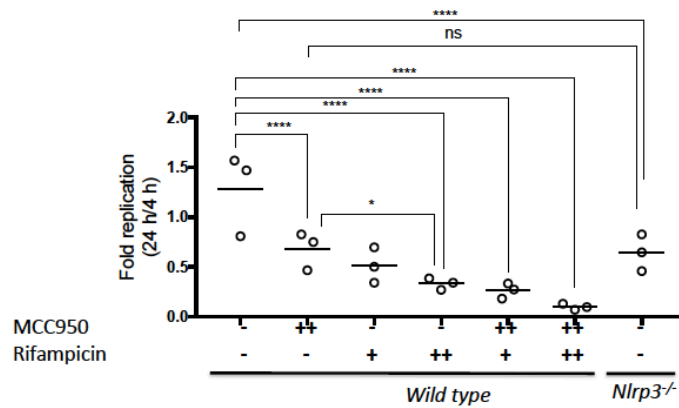


Figure 3.44: Dual therapy with rifampicin and MCC950 significantly reduces bacterial replication compared to rifampicin alone. Cells were infected with H37Rv and washed after 4 hours before being treated with equivalent DMSO concentration or 12.5 μ M MCC950 (++) and/or 0.053 mg l^{-1} (+) or 0.53 mg l^{-1} (++) rifampicin. *Nlrp3*^{-/-} BMDMs were used as a comparative control against MCC950. At 24 hours, the cells were lysed and plated for CFUs. Fold replication was calculated by dividing CFU at 24 hours by CFU of the comparative genotype at 4 hours. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. One-way ANOVA was applied to determine a difference in means between experimental conditions followed by Tukey's multiple comparison test to evaluate differences between each of the experimental drug concentrations. In the graphs, statistical significance between the experimental conditions and the control is indicated as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

The NLRP3 inhibitor, MCC950, when used as an adjunct with rifampicin reduces bacterial replication following *M. tuberculosis* infection

Given that I previously showed that the immortalised BMDMs genetically knocked-out for individual components of the inflammasome appeared to reduce bacterial replication *in vitro*, I hypothesised that pharmacological blockade would elicit a similar phenotype (see figure 3.44)

There was a significant difference in bacterial replication between the different groups (see figure 3.44; one-way ANOVA, $F = 35.82$, $p \leq 0.0001$). As shown in the previous section, knocking out the inflammasome protected against bacterial replication; bacterial growth inside *Nlrp3*^{-/-} BMDMs was significantly reduced compared to wild-type BMDMs ($p \leq 0.0001$). Similarly, treating the cells with the NLRP3 inflammasome inhibitor also reduced bacterial fold replication compared to untreated cells ($p \leq 0.0001$). Unlike the difference in IL-1 β release between *Nlrp3*^{-/-} BMDMs and MCC950 treated cells, there was no difference in bacterial replication between the two groups ($p \geq 0.99$). As expected, 0.053 mg l^{-1} rifampicin led to a reduction in bacterial replication ($p \leq 0.0001$) and this effect was similarly replicated by increasing the rifampicin concentration to 0.53 mg l^{-1} . Adding MCC950 in addition to rifampicin at 0.53 mg l^{-1} reduced bacterial replication compared to the untreated control ($p \leq 0.0001$); however most interestingly, the combination treatment provided additional inhibition of replication in comparison to 0.53 mg l^{-1} rifampicin alone ($p = 0.032$). Addition of MCC950 in addition to rifampicin at the lower dose, 0.053 mg l^{-1} , also led to a statistically significant reduction in bacterial replication compared to rifampicin alone at the same dose (0.2618 ± 0.0441 and 0.512 ± 0.103 fold

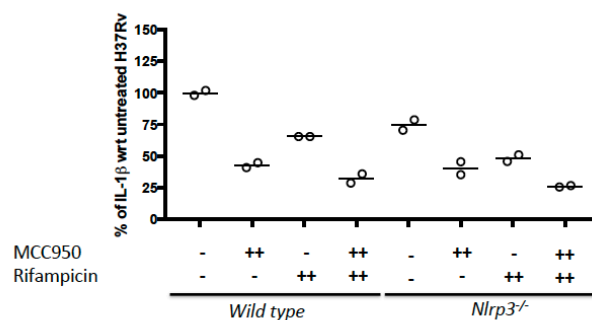


Figure 3.45: Treatment of *Nlrp3*^{-/-} BMDMs does not confer any further reduction in IL-1β compared to wild-type BMDMs. Wild-type and *Nlrp3*^{-/-} BMDMs were infected with H37Rv and washed after 4 hours before being treated with equivalent DMSO concentration or 12.5 μM MCC950 (++) and/or 0.53 mg l⁻¹ (++) rifampicin. After 24 hours, the supernatants were removed and IL-1β secretion was determined by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of *n* = 2 biological experiments. Statistical analysis was not done due to too few data points.

replication respectively; *p* = 0.041).

Whilst I did not formally determine the difference in MIC evoked by the adjunctive treatment, the addition of MCC950 in combination with rifampicin (0.053 mg l⁻¹) led to a comparable reduction in bacterial replication as compared to 0.53 mg l⁻¹ rifampicin alone (*p* = 0.672), which suggests that MCC950 might be able to reduce the effective concentration at which rifampicin is able to act. Further studies examining lower doses of rifampicin with MCC950 may help to shed more light on this.

I looked to see whether the addition of MCC950 and/or rifampicin to *Nlrp3*^{-/-} BMDMs would have a similar results to the phenotype seen in the wild-type background (see figure 3.45). IL-1β release is expressed as a percentage of the mean maximum secreted by the wild-type BMDMs following H37Rv infection. Treatment with MCC950 led to further reduction in IL-1β release by the *Nlrp3*^{-/-} BMDMs by comparison with *Nlrp3*^{-/-} BMDMs alone suggesting that there may be some off target effects induced by MCC950 other than NLRP3, although this would need to be repeated in order to determine this.

Treatment of the *Nlrp3*^{-/-} BMDMs with rifampicin elicited similar levels of IL-1β as rifampicin/MCC950 dual therapy on wild-type BMDMs. Addition of MCC950 in combination with rifampicin led to a further reduction in IL-1β in *Nlrp3*^{-/-} BMDMs by comparison to rifampicin alone (see figure 3.45). Further replications are needed to verify this.

In the same experiment, I investigated whether bacterial growth was different in *Nlrp3*^{-/-} BMDMs treated with the same drugs as their wild-type counterparts (see figure 3.46). As expected, the addition of MCC950 to *Nlrp3*^{-/-} cells did not evoke any further reduction in bacterial replication compared to the untreated cells. Dual MCC950/rifampicin therapy led to similar levels of bacterial

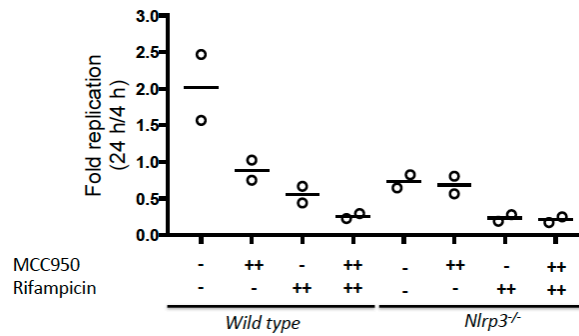


Figure 3.46: Treatment of *Nlrp3*^{-/-} BMDMs does not confer any further reduction in bacterial replication compared to wild-type BMDMs. Wild-type and *Nlrp3*^{-/-} BMDMs were infected with H37Rv and washed after 4 hours before being treated with equivalent DMSO concentration or 12.5 μ M MCC950 (++) and/or 0.53 mg l⁻¹ rifampicin (++). At 24 hours, the cells were lysed and plated for CFUs. Fold replication was calculated by dividing CFU at 24 hours by CFU of the comparative genotype at 4 hours. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 2$ biological experiments. Statistical analysis was not done due to too few data points.

reduction compared to rifampicin treated *Nlrp3*^{-/-} BMDMs which would be consistent with MCC950 as a specific NLRP3 inhibitor.

The restriction of bacterial replication by inflammasome inhibition is strain dependent

I then used *Nlrp3*^{-/-} BMDMs to evaluate the differences in the bacterial strains over time as I wanted to use this as a proxy for the effects induced by pharmacological inhibition of NLRP3, rather than examining *Nlrp3*^{-/-}/*Aim2*^{-/-} which does not have a current pharmacological blocker.

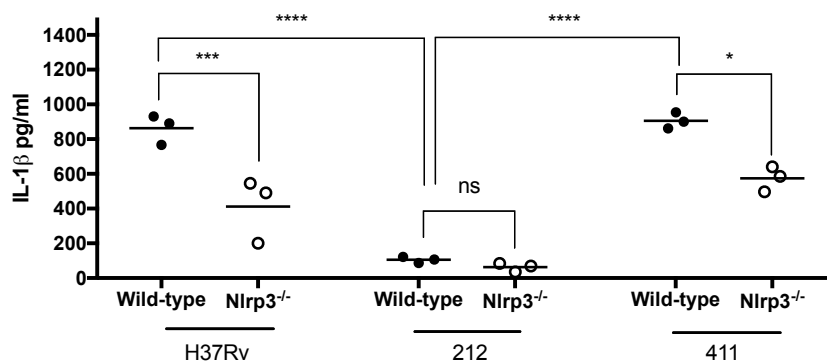


Figure 3.47: Reduction of IL-1 β in *Nlrp3*^{-/-} is strain dependent. Wild-type and *Nlrp3*^{-/-} BMDMs were infected with H37Rv, 212 and 411 and washed after 4 hours. After 24 hours, the supernatants were removed and IL-1 β secretion was determined by ELISA. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. Two-way ANOVA was performed to test for an interaction between genotype and bacterial strain on IL-1 β release and this was followed by Tukey's multiple comparison test to determine which of the means were different. In the graphs, only some of the comparisons are shown, marked * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

I infected both wild-type and *Nlrp3*^{-/-} BMDMs with H37Rv and the clinical isolates 212 and 411, and determined IL-1 β at 24 hours and bacterial fold replication at 8 and 24 hours. I hypothesised that so-called high IL- β inducers, such as H37Rv and 411 would induce less IL-1 β following infection

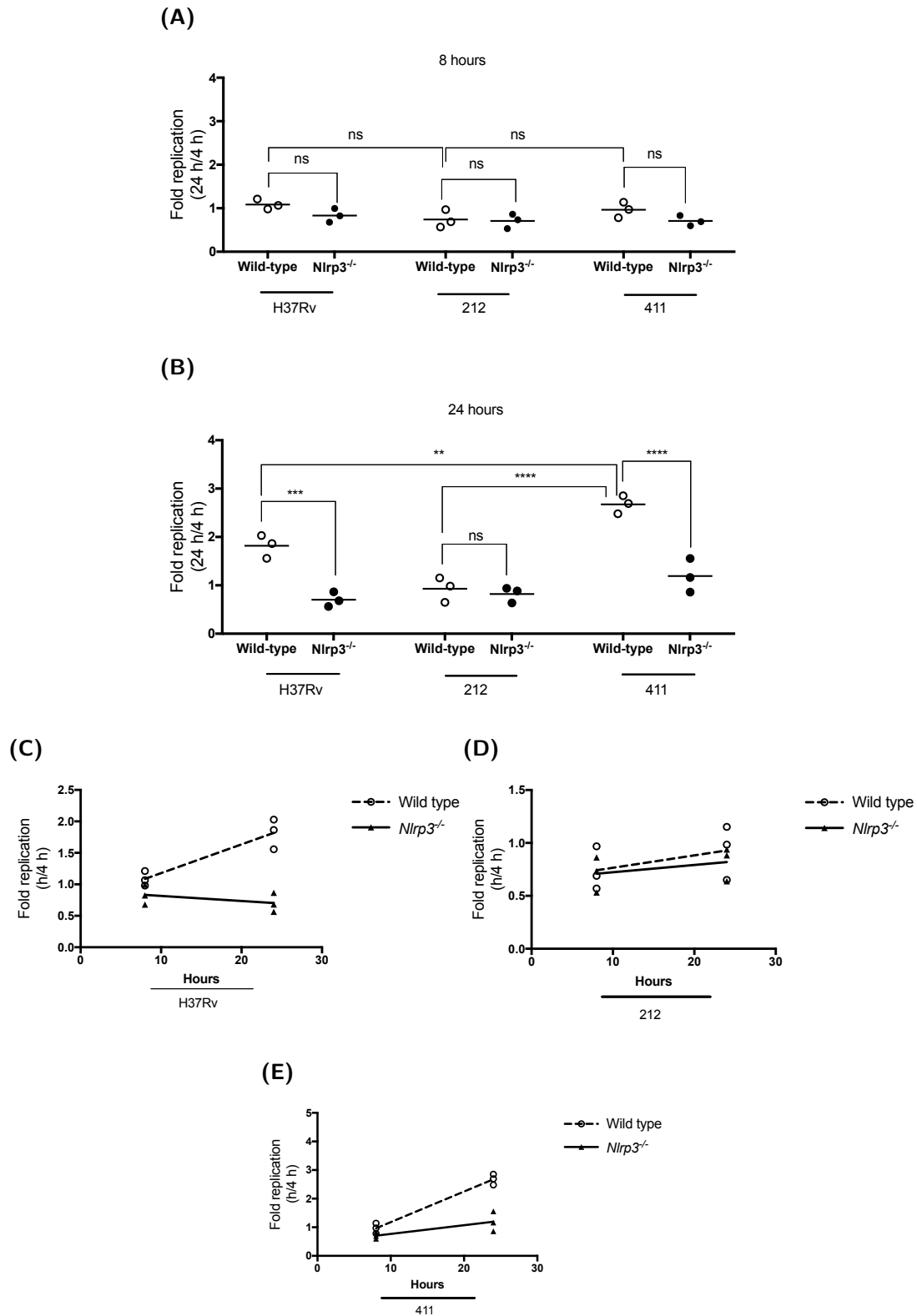


Figure 3.48: *Nlrp3*^{-/-} knock-out cells are able to restrict bacterial replication late in infection in a strain dependent manner. Wild-type and *Nlrp3*^{-/-} BMDMs were infected with the indicated clinical isolates at an MOI of 5 for 4 hours and washed. At 8 and 24 hours, the cells were lysed and plated for CFUs. Fold replication was calculated by dividing CFU at 8 (A) and 24 hours (B) by CFU at 4 hours. Two-way ANOVA was performed for each time point to ascertain a difference in mean replication between the different groups followed by Tukey's multiple comparison test. Each data point shown represents the mean of 3 technical replicates and the overall mean values (shown as a bar) of $n = 3$ biological experiments. Statistical significance is depicted in some of the comparisons and is indicated as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$, ns=non-significant. For each bacterial strain (C, D, E), the graph depicts the changes in bacterial replication between 8 and 24 hours.

in *Nlrp3*^{-/-} BMDMs, but the effect imposed by low IL-1 β inducers such as the 212 strain would not be as great (see figure 3.47). ELISA was performed at 8 hours and no IL-1 β was detected. At 24 hours, there was a significant difference in the mean IL-1 β release by both the host cell genotype and bacterial strain on IL-1 β release ($p \leq 0.0001$) and also a demonstrable interaction between the two factors ($p = 0.006$). IL-1 β release induced by H37Rv and 411 was reduced in *Nlrp3*^{-/-} BMDMs by comparison to wild-type ($p \leq 0.001$, $p \leq 0.05$ respectively). However, IL-1 β release did not change following infection with 212 in *Nlrp3*^{-/-} BMDMs ($p = 0.99$). A western blot confirmed the lack of NLRP3 in the *Nlrp3*^{-/-} cell line (data not shown).

I then examined how different bacterial strains might differ in their abilities to replicate under the conditions of inflammasome inhibition. I determined the replication of H37Rv, 212 and 411 at 8 hours in both wild-type and *Nlrp3*^{-/-} BMDMs and found that whilst there was a difference in the mean bacterial replication between the two genotypes ($p = 0.03$), there was no difference in the means between the three bacterial strains ($p = 0.781$) nor any evidence that the bacterial strain interacts with the genotype ($p = 0.407$) (see figure 3.48A). However at 24 hours (see figure 3.48B), there is a clear statistical significance in mean bacterial replication between the bacterial strains ($p \leq 0.001$), between the two cell types ($p \leq 0.0001$) and an interaction between the two factors ($p \leq 0.0001$). H37Rv and 411 replication was reduced in *Nlrp3*^{-/-} BMDMs by comparison to wild-type ($p \leq 0.001$, $p \leq 0.0001$ respectively). However, there was no reduction in bacterial replication following infection with 212 in *Nlrp3*^{-/-} BMDMs ($p = 0.99$). This suggests that the protection against bacterial replication may be a late phase event (where 24 hours is classed as late and 8 hours is classed as early) and strain dependent (figures 3.48CDE).

In summary, pharmacological inhibition of NLRP3 with MCC950 leads to a reduction in IL-1 β release that is greater than that achieved using a genetic knock-out, suggesting that MCC950 may have off-target effects that account for its enhanced efficacy. This does not translate into changes in bacterial replication, with MCC950 eliciting similar levels of bacterial reduction as that exhibited by *Nlrp3*^{-/-} BMDMs.

3.4 Discussion

3.4.1 Determining the conditions necessary for inflammasome activation

In this chapter, I established an experimental system that formed the backbone for all the future work during this second project.

I have shown that using an MOI of 5 and a time point of 24 hours is sufficient to activate the

inflammasome and release IL-1 β (see figure 3.10A). An MOI of 5 or higher is associated with increased cell death (see figure 3.10B), which is in line with previous authors (Welin et al. 2011; Lee et al. 2006). In this experiment, maximum LDH release was observed at an MOI 25 and compared against the manufacturer's positive control (cell lysis triggered by Triton X-100 detergent), however staurosporine, which triggers caspase-3 mediated cell death may additionally be employed as a positive control (Schwartz et al. 2012). Shorter time frames of infection appear to favour caspase-dependent cell death, although this changes at higher bacillary burdens (Butler et al. 2012). Higher MOIs have been reported to lead to caspase-independent cell death (O'Sullivan et al. 2007; Welin et al. 2011) and virulent mycobacteria (such as H37Rv or the clinical isolates used in this project) have been linked to necrotic cell death through the ESX secretion system (Wong et al. 2011). However, other studies have shown that it is the strain rather than the virulence *per se* that determines the method of cell death, with H37Rv favouring apoptotic death. The experimental setting can greatly influence the form of cell death, and can tip the balance between apoptosis and other modes of cell death which can favour *M. tuberculosis* survival. For example, recent time-lapse studies have shown that macrophage phagocytosis drives bacillary replication and leads to host cell death — however, replication is able to continue inside the dead cell, leading to the propagation of infection when other macrophages internalise the dead infected cell (Mahamed et al. 2017). This somewhat dispels the common conception that cell death in the form of apoptosis is favourable to the host and demonstrates that it can drive bacterial dissemination.

Pyroptosis is a process by which the infected macrophage executes its own death whilst releasing IL-1 β . This was the conventional method by which we believed IL-1 β is released from the cell. Initial research has suggested that the release of mature IL-1 β is likely to herald early cell death (Brough et al. 2007) although recent studies have highlighted the fact that membrane permeabilisation akin to necrosis is required for the release of mature IL-1 β following an array of stimuli (Cullen et al. 2015). However, IL-1 β with associated caspase-1 activation can be released without concomitant cell death (Lopez-Castejon et al. 2011) and has recently been reported in a trans-differentiated monocyte cell culture model that does not require classical inflammasome assembly in specks (Conos et al. 2016). I sought to examine the activation of the inflammasome with minimal cell death. As with previous authors, I found a threshold at which IL-1 β can be detected in response to increasing the MOI and whilst there was a trend to increase in LDH release at MOI 5 compared with an MOI 1 but this was not significantly different. In this work, I have shown that clinical isolates show similar degrees of LDH release and this does not correlate with IL-1 β release. Lee et al. 2011 have demonstrated previously that cell death was attributable to lysosomal permeabilisation and ultrastructural membrane damage that was independent of caspase-1 and the ESX secretion system. Thus certainly in my results, cell death is occurring by a mechanism other than pyroptosis (see figure 3.30). Indeed recent

studies have demonstrated that inflammasome activation and IL-1 β release is facilitated through the assembly of gasdermin D pore formation and is independent of pyroptotic cell death (Evavold et al. 2018). Additionally, in my model I utilised immortalised cell lines, which compared to primary cells display abnormal proliferative potential - previous studies have shown that immortalised cell lines can resist their entry into senescence and therefore prolong cell survival (Maya-Mendoza et al. 2014). Furthermore, immortalised cells are highly sensitive to factors that affect glycolysis compared to primary cells; therefore, given that *M. tuberculosis* leads to heightened glycolysis and IL-1 β release (Gleeson et al. 2016), it is conceivable that intrinsic changes in oxidative phosphorylation in these cell lines may also influence IL-1 β . Thus it is imperative that future work is able to replicate these findings in primary cells as well as demonstrating the role of gasdermin D in *Gsdmd*^{-/-} cells.

In these results, cell death was measured through LDH release from each cell, by which LDH release is quantified relative to the positive control cell, where the cell membrane is manually disrupted through addition of Triton-X. However, the positive control could also be represented by other methods of membranous disruption, such as staurosporin.

Higher MOIs and increased timing of infection did lead to increased IL-1 β release, as has been previously reported with mycobacteria (Welin et al. 2011; Yang et al. 2013b). However, this is at the expense of increased cell death and this is likely to be complicated by secondary necrosis, a process that occurs following the failure to clear up apoptotic debris, which may obscure the true level of IL-1 β activation (Miao et al. 2011). My group has previously looked at two time frames of infection (Krishnan et al. 2013) with clinical isolates (24 and 48 hours). As I began this project, I repeated the time frame infection experiments and included a 6 hour time point, but decided that 24 hours provides the correct balance to ascertain IL-1 β processing without inducing high levels of cell death. The literature regarding *in vitro* models is highly variable in terms of the infection time adopted. Dorhoi et al. 2012 and McElvania Tekippe et al. 2010 were both able to detect IL-1 β at short time frames (6 and 8 hours respectively); but I did not detect any release as determined by ELISA under my experimental conditions. Whilst the cells I used were originally harvested as primary cell lines, they were immortalised for this work. Although I was careful not to passage the cells too many times, it is inevitable that when performing multiple assays cells will be extensively passaged. However, serial passaging may influence immunogenic properties (Liao et al. 2014) and can influence gene expression compared to primary cell lines (Mouriaux et al. 2016). Moreover, the addition of growth factors has also been shown to modify key protein expression, such as autophagy markers (Eom et al. 2014), though since I supplemented my cells with L929 spent medium, this is unlikely to be a factor. It is hard to reconcile how the effect of changing the infection time frame might be different between these cell lines, but an interesting recent study by Andreu et al. 2017 showed that the transcriptomic profile in uninfected controls dramatically changed between 4 hours and 24 hours

post-infection in primary BMDMs compared to J7741.A macrophages; whether this impacted protein expression was not examined but this could account for why other authors are able to observe protein differences at earlier time points using primary cells. Future work might examine the protein release at the same time points, comparing immortalised BMDMs against primary BMDMs from the same mouse background to ascertain whether differences observed are due to the passaging of cells.

3.4.2 Determining phenotypic differences between clinical isolates: bacterial uptake and TNF- α release

Using clinical isolates rather than the standard laboratory strain, H37Rv, is important not least because this strain was originally derived in 1934 (from E.R. Baldwin's human-lung isolate H37 by W. Steenken in New York) (Kubica et al. 1972) but also because there are such vast immunological differences between varying lineages and even sublineages. The course of infection *in vitro* and *in vivo* is dependent on the bacterial load, virulence and host genetics (Marquina-Castillo et al. 2009).

In this study I examined *M. tuberculosis* strains belonging to the Beijing family of strains known as lineage 2 (or the East-Asian) and strains belonging to lineage 4 (Euro-American), both of which comprise the 'modern' lineage group. The reference strain, H37Rv, also belongs to lineage 4. The isolates were originally cultured from the CSF of patients with TB-meningitis (Caws et al. 2008) apart from one isolate, 639, which is of unknown origin. In the Caws et al. 2008 study, 23% (27/119) of patients infected with fully susceptible Beijing strains had died by 9 months, whereas there were no deaths amongst those infected with the Euro-American strain (0/8). However we are unable to correlate the strains used in this study with the disease phenotype and patient outcome.

I found a marked variation in bacterial uptake among the different strains. Aside from 119, infection with 212 and 411 led to similar degrees of bacterial uptake and in general bacterial uptake of the Euro-American strains was low compared to H37Rv (see figure 3.21). High bacterial uptake has previously been shown for avirulent strains (such as H37Ra) and virulent strains (such as H37Rv) but not for Beijing lineages (Chakraborty et al. 2013). The phenotypic differences seen in the clinical isolates may in part be attributed to variability in the mycobacterial cell wall. In a study by Homolka et al. 2010, examination of 17 mycobacterial strains showed an overexpression of *virS* in the Euro-American lineages which may indirectly play a role in modifying the mycolic acid components of the cell wall. Clinical isolates have previously been shown to harbour distinct structural differences in their ManLAM cell wall component, where strains with a truncated form of ManLAM and a reduction in higher order PIMs are not able to bind the mannose receptor (MR) and have a defect in MR-mediated phagocytosis (Torrelles et al. 2008). ManLAM has also been shown to prevent phagolysosomal fusion by the PI3K-dependent pathway *in vivo* (Richmond et al. 2012). Torrelles

et al. 2008 also showed increased PGL in clinical strains, which they speculated masks ManLAM and therefore further prevents MR receptor engagement; cells were still able to undergo phagocytosis as engagement with other C-type lectin receptors still occurred, but the subsequent host response was not explored. This is interesting since host-adapted strains, such as H37Rv, have been reported to have a heavily mannosylated backbone and are able to bind the MR, therefore it is somewhat of a surprise that some clinical strains have the opposite structural composition and use alternative means for their survival. The clinical isolates used in this study have not been specifically tested for ManLAM but rather total lipid content (Krishnan et al. 2011), where total lipid content from Indo-Oceanic and Beijing strains induced higher levels of TNF- α in comparison to the Euro-American lineage. Could the difference in bacterial uptake be due to variability in ManLAM composition and thus phagocytosis? This is tempting, since there are previous reports of mutations in sub-lineages that affect cell wall biosynthesis (Pérez et al. 2004). Given that strain 119 was poorly taken up compared to 212 (see figure 3.21), it is probably more complicated than this and would imply that there are inherent lipid differences within the lineages themselves. However, bacterial cell wall lipid composition has been previously shown to be highly conserved within the lineages (Krishnan et al. 2011) suggesting that there are other reasons for variable bacterial uptake.

I confirmed previous findings showing that mycobacterial strains exhibit immunological diversity. The isolates in general followed a similar pattern of TNF- α release compared to Krishnan et al. 2013. Circulating modern Beijing strains have been reported to induce lower levels of pro-inflammatory cytokines, including TNF- α (Sarkar et al. 2012; Laarhoven et al. 2013; Chen et al. 2014), compared to ancient lineages, which has been proposed as a selective advantage for the bacteria. Certainly, current literature indicates that lineage 2 induces significantly lower concentrations of TNF- α and IL-1 β than the Euro-American lineages *in vitro* (Laarhoven et al. 2013). In contrast, Chacón-Salinas et al. 2005 and Krishnan et al. 2013 both observed increased TNF- α and IL-1 β in lineage 2 compared to the Euro-American strains. I found that two of the Beijing strains (lineage 2), 212 and 411, induced similar levels of TNF- α to H37Rv and the Euro-American strains all induced significantly lower TNF- α (see figure 3.19). The differences that I see may be due to differences in cell lines used — studies in murine BMDMs show that the Beijing lineage induces higher expression of IL-1 β and TNF- α compared with other lineages or *M. canettii* (Chacón-Salinas et al. 2005; Krishnan et al. 2011). It is of interest that my IL-1 β ELISA experiments were able to detect differences between the isolates, since this indicates that the ELISA kit is relatively specific to mature IL-1 β (since there was similar expression of pro-IL-1 β in supernatants of those clinical isolates that induced similar priming).

3.4.3 Strain differences in inflammasome activation and IL-1 β processing and release

In this section I shall discuss all the strains together; I will discuss H37Rv, 212 and 411 in greater detail in the next section.

Whilst TNF- α behaved relatively uniformly between lineages, this cannot be said for caspase-1 activation and IL-1 β processing. We can dissect the findings into the initial priming step, represented by pro-IL-1 β and then inflammasome activation, represented by caspase-1 and IL-1 β processing. The strains induced differential expression of pro-IL-1 β (see figure 3.20), which, given the variability in bacterial uptake suggests that there are differential interactions with cell surface pattern recognition receptors. In a similar experiment by Krishnan et al. 2013, clinical isolates also display differences in the expression of pro-IL-1 β at 24 hours, although the expression at 6 hours was similar. I cannot say whether I would have observed similar levels of expression had I looked at priming at 6 hours. What is interesting is that the level of pro-IL-1 β does not necessarily correlate with bacterial uptake as we might expect. For example, induction of bacterial uptake during infection with 212 and 411 appears to loosely correlate with an equivalent ability to activate pro-IL-1 β ; similarly 119 and 355 were poor activators of pro-IL-1 β and induced poor uptake (see figure 3.20). However, 440 induced increased transcription of pro-IL-1 β despite inducing relatively poor bacterial uptake. This might well suggest a differential engagement of the TLRs and Dectin-1, which facilitate MyD88 signalling and NF- κ B activation independently from phagocytic capacity. TLR2 is central to the innate immune response to mycobacteria *in vitro*, although it has some redundancy with other TLRs *in vivo* (Bhatt et al. 2007). A study which examined the immunogenic differences between morphological variants (so called smooth (S) and rough (R) colonies) of *M. abscessus*, the R variant was associated with a hyper-inflammatory profile which was characterised by an increased synthesis of lipoproteins and interaction with TLR2 (Roux et al. 2011). In addition to a general activation of TLR2, a set of *M. tuberculosis* clinical isolates have been observed to differentially activate TLR4, which activates both the MyD88 and TRIF pathways (Carmona et al. 2013), producing both pro- and anti-inflammatory cytokines. A different study showed that the Latin American and Mediterranean lineages were more capable of inducing ROS and apoptosis compared to the Haarlem lineage in a manner that was attributed, but not formally investigated, to increased engagement with TLR2 (Romero et al. 2012). Therefore, it is plausible that modifications in the mycobacterial lipid cell wall might rapidly engage the MyD88 signalling pathway to induce the transcription and translation of pro-IL- β without requiring binding to the phagocytic receptors.

What is striking in the western blot, are the clear differences in inflammasome activation despite similar expression of pro-IL-1 β in some of the strains (see figure 3.20). A case in point are the strains

173 and 440 which both induce similar expression of pro-IL-1 β . However, 173 manages to activate caspase-1 and in doing so processes a large amount of IL-1 β . In contrast, 440 does not activate caspase-1 nor does it (by comparison to 173) process much IL-1 β (on the western blot and the ELISA). So what could be happening? 173 manages to be taken up by the macrophage in a robust fashion, which is the opposite to 440. This may help to explain the difference if 173, through its secretion system, is able to permeabilise the phagosome and activate NLRP3 and AIM2. A review of the bioinformatics — which have been performed previously for all the strains used in this study — identified no mutations in ESAT-6, so this hypothesis is unlikely. However, as discussed in the previous section, there are additional activators of the inflammasome. Another possibility is that there is a differential requirement for the Dectin-1 receptor. Dectin-1 has previously been shown to cooperate with TLR2 to mediate MyD88 production of TNF- α during mycobacterial infection (Yadav et al. 2006). However, Dectin-1 also signals in a Syk kinase-dependent manner, triggering the assembly of a CARD9–Bcl-10–MALT1 complex that leads to the transcription of *IL1b* and a MALT1–caspase-8–ASC complex that activates caspase-8 and processes IL-1 β (Gringhuis et al. 2012). Whilst this is mainly apparent in fungal infections, it has also been demonstrated to a lesser degree in *M. tuberculosis* (Gringhuis et al. 2012). We currently do not know the identity of the *M. tuberculosis* ligand that triggers Dectin-1, but *M. tuberculosis* does express glucans (Schwebach et al. 2002), which are also present in fungal pathogens. Thus, it is attractive to think that differential Dectin-1 signalling is responsible for the transcription and processing of pro-IL-1 β in a caspase-1-independent and caspase-8-dependent manner. Given that so little caspase-1 processing occurs (in both 173 and 440) despite the difference in IL-1 β (see figure 3.20), it does suggest that IL-1 β is being processed via other means.

In a similar vein, both 639 and 440 induce poor bacterial uptake and activate equivalent levels of pro-IL β . IL-1 β is still detected in the ELISA and Western blot for both strains, yet caspase-1 activation is absent (see figures 3.18 and 3.20). Once again, this suggests a caspase-1-independent mechanism for IL-1 β processing is at play. This is in keeping with Krishnan et al. 2013, where, using the same isolates, caspase-1 inhibition did not completely abrogate IL-1 β release, whereas inhibition of serine proteases did decrease IL-1 β secretion. Caspase-1-independent IL-1 β processing has been reported previously — other serine proteases, chymases, chymotrypsin, granzyme A and matrix metalloproteases (MMPs) have all been noted to cleave IL-1 β (Netea et al. 2010). SpeB is a cysteine protease that is secreted by Group A *Streptococcus* during infection, and is able to activate IL-1 β by mimicking caspase-1 activity (LaRock et al. 2016). Late stationary phase *C. albicans* hyphae have also previously been shown to cleave pro-IL-1 β into mature IL-1 β (Beausejour et al. 1998). A similar secreted protease that has the direct capability to mimic caspase-1, making the host caspase-1 redundant, and interfere with IL-1 β processing has yet to be identified in *M. tuberculosis*.

However, there are candidates that might have an indirect role. Interestingly, a study a few years ago proposed that *M. tuberculosis* actually *inhibits* AIM2 inflammasome induction, rather than activates it, whilst avirulent mycobacteria were capable of inducing AIM2 (Shah et al. 2013). Perhaps a mycobacterial effector that is secreted via the ESX-1 secretion system can inhibit activation of AIM2. *M. tuberculosis* Rv3364c is secreted from the phagosome and binds cathepsin G, a trypsin-like serine protease; this reduces cathepsin G activity and in turn decreases caspase-1 activity and cell death, but IL-1 β was not measured in this study (Danelishvili et al. 2012). Rv3668c is another serine protease with a signal sequence that is highly conserved amongst pathogenic mycobacteria (Zhao et al. 2014). When Rv3668c was expressed in *M. smegmatis* it led to the transcription and translation of IL-1 β and caspase-1 in a pathway that was mediated by ERK1/2 and NF- κ B (Zhao et al. 2014). Strains may also modulate the host through a differential destruction of extracellular matrix. Prolyl oligopeptidases (POPs) are a group of enzymes that can hydrolyse peptides — mammalian POPs can act with MMPs to break down collagen following LPS stimulation with the generated peptides serving as a neutrophil chemoattractant (O'Reilly et al. 2009). Recently, *M. tuberculosis* POPs have been shown to immunomodulate murine macrophages by inducing the secretion of IL-1 β , TNF- α and IL-6 (Portugal et al. 2017).

Further experiments would need to ask whether Dectin-1 inhibition might alter the transcription of pro-IL-1 β in response to the clinical isolates, or whether pharmacological inhibition of caspase-8 has any effect on processing. The next immediate step would be to determine whether antibodies against Dectin-1 affect the transcription and post-transcriptional control of IL-1 β . It would also be pertinent to look at whether IL-1 β processing still occurs in a *caspase-1/11*^{-/-} background. To look at whether inflammasome priming is associated with *M. tuberculosis* internalisation, future experiments would look at whether blocking phagocytosis with cytochalasin D prior to infection affects the expression of pro-IL-1 β . Putative bacterial proteases would initially be identified by incubating bacterial lysates with purified pro-IL-1 β under optimised buffer conditions. The addition of a series of steps, including adding protease inhibitors, would provide us with some information whether such a protease is present within *M. tuberculosis*. Examining the culture filtrate would be the next step, as a secreted protease would be more likely to effect a direct cleavage of a host protein. Future work would also look at the role of potential host proteases that are involved in pro-IL-1 β cleavage, using specific protease inhibitors.

3.4.4 H37RV, 212, 411 and their differential effect on the inflammasome

In order to compare the differences in inflammasome activation between the strains, I looked at these strains since they all induced similar levels of pro-IL-1 β expression in wild-type cells as well as a similar

levels of bacterial uptake. Thereafter, there are stark differences in the release of IL-1 β . It is well known that the lineages, in particular the Beijing lineage (lineage 2), have disseminated globally (Merker et al. 2015) and are characterised by different variants of the regions of difference (RD) (Gagneux et al. 2006). Sub-lineages have also been shown to have distinct clinical phenotypes in guinea pig models with variation in the degree of cytokine production and lung damage (Kato-Maeda et al. 2012). In addition, a different study has shown that the proportion of cases with extra-pulmonary TB may depend on the sub-lineage (Kato-Maeda et al. 2010). 212 and 411 both belong to Beijing lineage 2, but spoligotyping (Krishnan et al. 2011) has subdivided 411 into a 'Beijing-like' sub-lineage. This might explain the clear differences in immunological phenotype that we see throughout this thesis, with 212 consistently inducing lower levels of IL-1 β in comparison to the 411 strain. It is interesting to observe that very little activated caspase-1 is detected following infection with 212 in wild-type cells, which corresponds to an equivalently low level of IL-1 β .

Whilst in the previous section I discussed the possibility of caspase-1-independent conversion of IL-1 β (which would need to be verified in a *Caspase-1/11*^{-/-} background), H37Rv, 212 and 411 all appeared to be completely dependent on caspase-1/11 for processing. As with the panel of clinical isolates, I ask the same question as to what is causing this differential activation of caspase-1 and subsequent IL-1 β processing. The result that no caspase-1 or IL-1 β processing occurs in response to 212 (see figure 3.20) suggests that something intrinsic to the strain is able to negatively regulate inflammasome activation. A previous study has demonstrated strain-specific differences in the activation of the NLRP3 inflammasome following infection of mouse macrophages with various enterohaemorrhagic *E. coli* isolates, with inflammasome activation attributed to the pore-forming toxin α -haemolysin (Schaale et al. 2016). It would be worthwhile testing K⁺ efflux, lysosomal rupture and ROS production as potential candidates for contributing to the difference in inflammasome activation as well as infecting *Nlrp3*^{-/-} cells. Equally, it is possible that 212 is acting in a similar manner to observations by Shah et al. 2013, who have demonstrated that H37Rv is actively inhibiting the activation of the AIM2 inflammasome. Thus, in a modified recapitulation of their experiment, infecting cells initially with H37Rv, followed by 212, in an *Nlrp3*^{-/-} background might provide some insight as to whether the difference in inflammasome activity is mediated by inhibition imposed by 212.

3.4.5 Inflammasome activation is partially dependent on NLRP3 and AIM2 suggesting the involvement of an additional sensor

Several studies have shown that IL-1 β is almost fully dependent on NLRP3 following mycobacterial infection (McElvania Tekippe et al. 2010; Walter et al. 2010; Abdalla et al. 2012) and a handful of studies have demonstrated that AIM2 also plays a role in activation (e.g. Shah et al. 2013). Here, for

the first time, using knock-outs of caspase-1/11, NLRP3/AIM2 and ASC, I have shown that, although reduced, inflammasome activation can clearly occur in the absence of both NLRP3 and AIM2, but is dependent on both caspase-1/11 and ASC. This difference might be due to the fact that I have been using immortalised murine BMDMs, rather than primary murine or human cells. Additionally, the timings of infection have a role — early inflammasome activation is likely to be associated with shorter time frames, whereas longer infections will allow the bacteria to escape into the cytosol and therefore additionally activate AIM2 (Briken et al. 2013). Given that in my experimental set-up, all the cells were from the same background and underwent similar experimental conditions, this strongly suggests that an as yet unidentified inflammasome is capable of activating caspase-1/11 and IL-1 β . Previous studies using knock-outs have shown that NLRC4 is unlikely to play a role in caspase-1 processing during *M. tuberculosis* infection (McElvania Tekippe et al. 2010; Abdalla et al. 2012). Certainly this makes sense since no molecules in *M. tuberculosis* capable of activating NAIP have to date been identified.

A recent study identified the NLRP7 inflammasome as a mediator for caspase-1 activation in the *M. bovis* Beijing strain (Zhou et al. 2016a). Zhou et al. 2016a showed that *Nlrp4* gene expression was upregulated and NLRP7 co-localised with caspase-1 and ASC following infection. Moreover, caspase-1 and IL-1 β processing was reduced by NLRP7-targeting siRNA treatment, as well as after gene expression of pro-inflammatory cytokines and chemokines such as TNF- α and CCL3 was reduced by NLRC4-targeted siRNA (Zhou et al. 2016a). Microbial lipopeptides signalling via TLR2 have also previously been shown to activate the NLRP7 inflammasome (Khare et al. 2012), therefore the idea that this might play a role in *M. tuberculosis* inflammasome activation is highly attractive, especially given that NLRP7 uses both caspase-1 and ASC as the inflammasome scaffold. However, NLRP7 is a human protein, and it does not have an orthologue in mice — NLRP2, from which NLRP7 is believed to have emerged via gene duplication is believed to be a paralogue (Duñez-Guzmán et al. 2014). Future work could examine the contribution of NLRP2 during *M. tuberculosis* infection in *Nlrp2*^{-/-} cells. A complete abrogation of IL-1 β release using *Nlrp2*^{-/-}/*Nlrp3*^{-/-}/*Aim2*^{-/-} mice would demonstrate the role of NLRP7 in *M. tuberculosis*. It would also be interesting to observe whether, in the context of NLRP2 activation, TLR2 signalling is always a prerequisite, especially given the multitude of surface receptors that are involved in *M. tuberculosis* recognition. An alternative thinking would be that another DNA sensor is activated following *M. tuberculosis* infection. 13 DNA sensors have been identified in the mouse (Brunette et al. 2012). Co-transfection of 3 of the sensors (either AIM2, MNDAL or MNDAL) with pro-IL-1 β in HEK293T cells can lead to IL-1 β cleavage and ASC co-localisation with the DNA sensor (Brunette et al. 2012). Thus, looking into this area of inflammasome activation may also provide some novel insights.

In this study, I have also shown whilst there is variation in cell death between clinical isolates, there

is no association between IL-1 β release and cell death. Unlike the comparative reduction in IL-1 β release, there was no difference in cell death between the different knock-outs, suggesting that during *M. tuberculosis* infection *in vitro*, cell death (which in our experimental system was measured by LDH leakage from damaged cells; Chan et al. 2013) is inflammasome-independent. Abdalla et al. 2012 have also observed that *M. tuberculosis* induces cell death, as measured by DNA fragmentation, but did not observe differences in caspase-1/11, ASC or NLRP3 knock-out dendritic cells. However, adenylate kinase (AK) is also a measure of cytotoxicity and cell lysis (alongside LDH), and AK activity was similar between wild-type, uninfected and *Caspase1/11*^{-/-} whereas there was increased lysis in infected *Nlrp3*^{-/-} and *Asc*^{-/-} dendritic cells, suggesting that in this study ASC and NLRP3 protected against necrosis but caspase-1/11 had no effect. The differences between Abdalla et al. 2012 and my findings might be due to the different cell lines used, though the signal stability of AK assays is lower than LDH release assays (Niles et al. 2008). I did not measure nuclear degradation or chromatin cleavage to ascertain the mechanism of cell death in this study but future work might evaluate whether the degree of nuclear degradation is similar to Abdalla et al. 2012 using a TUNEL assay or annexin staining. Caspase-independent cell death following virulent *M. tuberculosis* infection has been reported previously (Lee et al. 2006; O'Sullivan et al. 2007). More recently, the C-terminal domain of the mycobacterial outer membrane protein CpnT was found to be cleaved and secreted, and found to induce necrotic cell death in a manner that is not attenuated by pan-caspase inhibitors (Danilchanka et al. 2014). These studies and my findings suggest that cell death is due to cell lysis but not pyroptosis.

3.4.6 Genetic and pharmacological inhibition of the inflammasome is associated with reduced bacterial replication

I have shown that inflammasome inhibition through either genetic means or pharmacological blockade reduces bacterial intracellular survival. As tabulated in 3.1, there are very few studies that have looked at the effect of knocking out inflammasome components on mycobacterial replication *in vitro*.

Fremond et al. 2007, Mayer-Barber et al. 2010 and Mayer-Barber et al. 2014 have previously shown that *IL-1R*^{-/-} mice have markedly increased susceptibility to *M. tuberculosis* infection compared to wild-type *in vivo*. Similarly, (Verway et al. 2013; Gleeson et al. 2016) showed that *IL-1R*^{-/-} cells display reduced resistance to bacterial replication compared to wild-type. Furthermore, addition of exogenous IL-1 α (Jayaraman et al. 2013) led to a reduction in CFU formation. In our experimental set-up, it appears that blocking inflammasome activation is associated with reducing bacterial replication. Thus, two hypotheses are at play, which might at first appear contentious - that IL-1 β signalling (through IL-1R) may aid bacterial clearance and blockade of inflammasome activation also supports bacterial

eradication. This implies that inflammasome blockade leads to activation of additional pathways that are independent of IL-1 β signalling. Therefore, it would be prudent in future experiments to examine how *IL-1R*^{-/-} cells respond to *M. tuberculosis* infection in my experimental system. Additionally, the use of more potent and targeted antagonists of IL-1 β , such as canakinumab may provide more information than the IL-1R antagonist, anakinra, which as we have seen demonstrates little pharmacological inhibition during *M. tuberculosis* infection and marries with findings by Wilkinson et al. 1999.

Information exists about how type 1 IFN regulates IL-1 β and NLRP3 but there are few studies on how the inflammasome impacts type 1 IFN. Increased type 1 IFN signalling is implicated in virulent and aggressive *M. tuberculosis* infection (Manca et al. 2005; Berry et al. 2010). However, bacterial escape into the cytosol is detected by cGAS which stimulates the production of type 1 IFNs through the adapter protein STING, but cGAS also targets *M. tuberculosis* to the autophagy pathway (Watson et al. 2015). Liu et al. 2016a showed that siRNA knockdown of AIM2 in J774A.1 cells increased LC3-II and reduced p62 expression following *M. bovis* infection and inhibited bacterial replication *in vitro*, therefore implying that AIM2 inhibits autophagy and potentially enhances bacterial survival. Furthermore, the authors showed increased levels of type 1 IFN due to activation of the STING pathway in AIM2 knock-down cells and showed that exogenous addition of type 1 IFN to wild-type BMDMs increased autophagy activation (Liu et al. 2016a). To this end, I have shown that *Nlrp3*^{-/-}/*AIM2*^{-/-} BMDMs exhibit reduced bacterial replication compared to wild-type cells (see figure 3.32) and enhanced autophagy due to genetic knockout of AIM2 could explain this finding. Additionally, recent studies have demonstrated that NLRP3 inhibition during hypoxia and oxidative stress was protective *in vitro* and *in vivo* and induced autophagy (Pavillard et al. 2017; Cosin-Roger et al. 2017), perhaps again suggesting that inflammasome inhibition activates the autophagy pathway to lead to bacterial degradation.

In my experiments, I chose a short time-frame (using a 24 hour protocol) for infection and data analysis - this adopted to minimise cell death. Inflammasome inhibition during this time-frame correlates with a model of mycobacterial early clearance. Recent studies have used proteomics to identify groups of potential biomarkers that signal bacterial clearance at 5 days following infection compared to infection at day 1 (Kaewseekhao et al. 2015). Thus, I would need to extend the time-frame of this model *in vitro* to establish whether bacterial replication remains suppressed following continuous inflammasome inhibition. *In vivo* studies would help to identify whether mycobacterial clearance does indeed occur prior to the onset of adaptive immunity as suggested by my experimental model. However, in order to understand the mechanism behind bacterial clearance, future experiments would need to extract samples for autophagy markers at earlier time points as well as performing confocal microscopy.

An additional way by which inflammasome inhibition and adjunctive therapy may augment host defense against mycobacteria is by modulating bacterial gene expression through the change in the environment. At 24 hours, genes related to hypoxia-related stress, general stress, cell wall biosynthesis and iron metabolism are known to be modulated following intracellular infection (Koo et al. 2012). Moreover, Subbian et al. 2011 have shown that inhibition of PDE-4 leads to reduced expression of mycobacterial stress response genes compared to infected and untreated rabbits. Thus, in our model, the reduction in the inflammatory signalling pathways following inflammasome inhibition may change the extent to which *M. tuberculosis* can survive intracellularly. Importantly, MCC950 had no direct effect upon the bacteria, implying that the changes I saw were intrinsic to host cell responses. Furthermore, given that treatment of *Nlrp3*^{-/-} cells with MCC950 did not alter IL-1 β translation, this suggests that the pathways altered by this pharmacological agent are specific to inflammasome signalling.

The use of MCC950 as a post-translational inhibitor of NLRP3 during this project mostly mimicked the effects of *Nlrp3* knock-out, and there was no evidence that the inhibitor targeted the proteasome given that pro-IL-1 β expression levels were similar between the treatment groups (see figure 3.43), suggesting that the mechanism of bacterial clearance is similar to the genetic knock-out. Glibenclamide and JNJ-47965567 could have been examined also but glibenclamide does carry the risk of hypoglycaemia (Ting et al. 2010) and there were very few studies in the literature that harness JNJ-47965567 as a potential HDT agent. However, given that thioredoxin is believed to be essential in mycobacterial host defense (Lin et al. 2016), I believe it would be interesting to examine the levels of the NLRP3 binding partner, TXNIP, in patients with diabetes and TB to determine whether they correlate with disease severity in such individuals and whether glibenclamide treatment reduces levels of TXNIP and increases levels of thioredoxin. MCC950 is gaining increasing momentum as an attractive small molecule inhibitor of inflammasome activation. It is being deployed in many settings that require inflammasome attenuation and appears to have a reasonable safety profile *in vivo* (Daniels et al. 2016; Hout et al. 2017). Multiple studies have demonstrated that MCC950 is relatively specific (Coll et al. 2015; Primiano et al. 2016), and in my work, I have shown that it has no effect on TNF- α (see figure 3.41), however, how MCC950 affects other cytokines and chemokines during *M. tuberculosis* infection needs to be considered. In addition, there may well be off-target effects of MCC950, other than NLRP3, that have yet to be explored, given that my work suggests a trend to a further reduction in IL-1 β release by the *Nlrp3*^{-/-} BMDMs by comparison with wild-type BMDMs.

Finally, I looked to see how clinical isolates fared over 24 hours in wild-type and *Nlrp3*^{-/-} cells. Given that H37Rv and 411 induced the inflammasome and high levels of IL-1 β at 24 hours (see figure 3.18), I hypothesised that not only would their cytokine induction be reduced but that their replicative ability would be diminished, given what we have seen in the previous section. Indeed, whilst at

early time frames, the absence of NLRP3 did not confer any protection, at 24 hours, the bacterial replication of both strains was reduced compared to their wild-type counterpart (see figure 3.48). This may suggest a temporal effect induced by the inflammasome as has been suggested by previous studies (Tate et al. 2016). Following infection with influenza A virus, NLRP3 appears to contribute to an early inflammatory environment which is protective, but this subsequently progresses to an excessive inflammatory response that is associated with increased mortality which can be mitigated by the use of MCC950 (Tate et al. 2016; Coates et al. 2017). Thus it would certainly be interesting to explore the *M. tuberculosis* infection period between 8 and 24 hours and even beyond that to ascertain whether we see an early protective effect. The low IL-1 β inducer, 212, exhibited a trend to reduced bacterial replication in *Nlrp3*^{-/-} cells, but this was not statistically significant (see figure 3.48). Differences in cytokine induction and bacterial uptake between mycobacterial isolates is not new (Koo et al. 2012; Sarkar et al. 2012; Reiling et al. 2013). However, I believe these final data show that a homogenous administration of inflammasome inhibitors is unlikely to be of benefit to all individuals. It is clearly important to use an array of clinical isolates rather than solely H37Rv in order to infer whether a drug treatment is relevant or useful. In this case, bacterial replication was not increased following infection by 212 in *Nlrp3*^{-/-} cells, but as seen using *IL-1R*^{-/-} mice, completely curtailing IL-1 signalling may also be detrimental. Indeed, there may be other reasons for the reduction in bacterial replication, but bacterial uptake between wild-type and the knock-out cells was similar at 4 hours and a specific reduction in intracellular replication was observed at 24 hours rather than earlier time frames in *Nlrp3*^{-/-} cells (see figure 3.48), implying that the inflammasome and IL-1 β may drive increased bacterial growth. A study by Koo et al. 2012 examined the host and bacterial transcriptional profile following infection with two clinical isolates, CDC1551 and HN878. In comparison with HN878, CDC1551 elicited a robust host cell response that was counteracted by an increased bacterial stress response (Koo et al. 2012). Krishnan et al. 2011 have previously examined in detail the immune responses of a wider panel of clinical isolates other than the ones used here. It is exciting to think that these isolates would elicit a differential activation of the bacterial stress response and so offer insightful information.

3.5 Summary

I started my thesis looking how the melanocortin system could be modified on the basis of previous genetic studies showing SNPs in *MC3R* showing increased susceptibility to TB disease. *In vitro* and *in vivo* data demonstrated that administration of melanocortin receptor agonists did not affect bacterial replication or cytokine release. However, a change in bacterial numbers is not necessary to be considered as an important immunotherapy. In the context of MCR agonism, I measured TNF- α

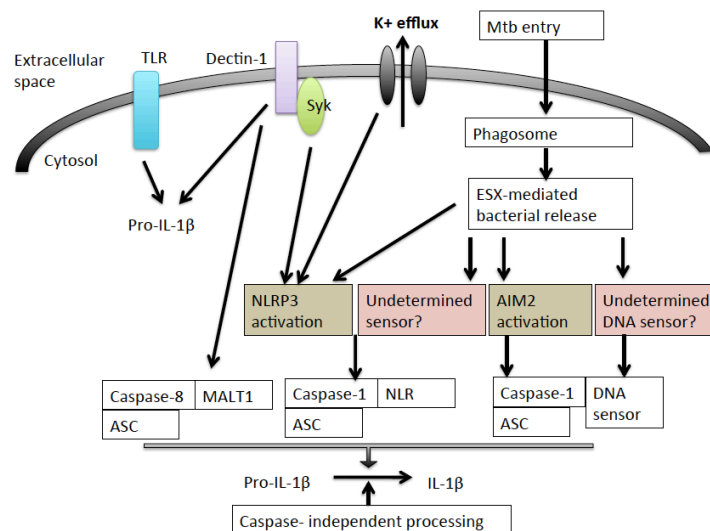


Figure 3.49: Proposed additional inflammasome activation during *M. tuberculosis* infection. In addition to previously discussed mechanisms of IL-1 β processing, it is likely that an additional sensor is able to process IL-1 β , which may be an as yet unidentified NLR or DNA sensor. In addition, there may be strain variances that confer differential priming of pro-IL-1 β and activation of the caspase-8 non-canonical inflammasome.

and IL-6 as an output, however, these are quite specific markers and it is entirely plausible that a functional outcome may occur that does not include this. Furthermore, standard anti-TB treatment was not used as an additional adjunct to MSH in either our *in vitro* nor *in vivo* models, which would have been a useful comparison.

In this chapter I have examined how clinical isolates affect inflammasome activation and then utilised this information to investigate different methods of inflammasome blockade as a potential means for HDT. I have shown that clinical isolates lead to a differential activation of the inflammasome and IL-1 β processing. Given the ability of IL-1 β processing to occur in the absence of caspase-1 activation in some of the strains, it is tantalising to think that there is a mycobacterial analogue that is able to do the same job as this cysteine protease. Additionally different strains might vary in their activation of Dectin-1 and caspase-8 signalling. This might explain the differences observed *in vitro* in caspase-1 redundancy (see figure 3.49). I then concentrated on 3 strains and showed that, following infection, IL-1 β is still processed in the absence of NLRP3 and AIM2 (although these strains require the ASC and caspase-1/11 platform) suggesting that another inflammasome sensor is responsible for IL-1 β activation (see figure 3.50).

Using genetic knock-outs, I then showed that inflammasome signalling in my experimental set-up augments bacterial replication and that pharmacological inhibition of NLRP3 was able to reduce bacterial replication. Finally, re-visiting the clinical isolates and using *Nlrp3*^{-/-} cells as a surrogate for pharmacological inhibition, I have shown that in high IL-1 β inducers, growth rate is dependent on inflammasome activity, highlighting that host-directed therapy truly has potential, but to work optimally some knowledge of the host and the bacteria may be required.

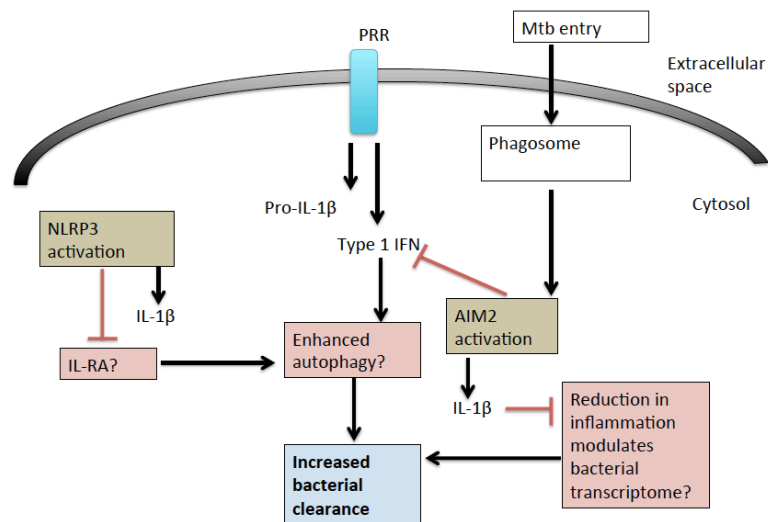


Figure 3.50: Proposed mechanism of how reducing inflammasome activity may aid a reduction in bacterial replication. Inflammasome inhibition may alter bacterial replication through two distinct mechanisms. Firstly, in a 'normal' state of inflammation, inflammasome activation through NLRP3 and AIM2 may negatively regulate the levels of IL-1RA and type 1 IFN respectively. In the absence of this activation, the increased levels of IL-1RA and type-1 IFN may enhance autophagic processes. Secondly, the reduction in inflammatory signalling may alter the bacterial transcriptome conferring a reduction in genes that define persistence augmenting the hosts susceptibility to anti-microbial defenses.

3.6 Future work

Future work would need to ask the following questions:

1. What additional markers of inflammasome regulation can be measured?

In this chapter, I have solely focused on measuring IL-1 β and LDH as a direct output of inflammasome activation. However, inflammasome activation is well described to additionally correlate with IL-18 processing and gasdermin D activation (Evavold et al. 2018). Although not discussed in this chapter, IL-18 helps to induce IFN- γ production (Broz et al. 2016). Future work should examine IL-18 release and utilise *Gsdmd*^{-/-} cells to evaluate whether gasdermin D controls the role of IL-1 β .

2. Does the *M. tuberculosis* genome harbour a protease that is capable of processing IL-1 β ?

Given that caspase-1-independent processing of IL-1 β was not ubiquitous amongst the strains tested, it seems unlikely from an evolutionary perspective that a mycobacterial bacterial protease, akin to the highly virulent protease SpeB secreted by *S. pyogenes*, would have so much variation between clinical isolates. However, this would be worth pursuing as would the possibility of an inhibitor that is secreted by the ESX-1 secretion system.

3. What is the additional sensor that is driving caspase-1 activation and IL-1 β processing in the absence of AIM2 and NLRP3?

Given that recent studies have demonstrated a role for NLRP7 in IL-1 β in human studies, could NLRP2 the mouse paralogue be implicated here? Future work would need to consider the role of both NLRP7 and NLRP2 in human and murine cells respectively following *M. tuberculosis* infection. In addition, the role of other DNA sensors would need to be reviewed.

4. To determine the mechanism by which inflammasome inhibition is able to modulate bacterial replication.

I hypothesise that the predominant method is through enhancement of autophagy although other potential defence mechanisms such as nitric oxide release would need to be excluded. Further studies would need to evaluate whether activation of BMDMs with IFN- γ would alter inflammasome activity. Mishra et al. [2013](#) has shown previously that IFN- γ -activated cells have increased nitric oxide production and subsequent suppression of the NLRP3 inflammsome — would this change in a system where there is pre-existing inflammasome inhibition?

We would additionally need to verify this work at earlier and later infection time points. Proof of concept would explore whether *Nlrp3*^{-/-} or pharmacological treatment would enhance mycobacterial clearance *in vivo* on its own, or with an antimycobacterial agent such as isoniazid, which has predominantly bacteriostatic activity (Koo et al. [2011](#)).

Secondary effects on bacterial gene expression are likely to occur so this would also need to be explored *in vitro*. Bacterial and host transcriptional profiling, in addition to immunological analysis and the possibility of laser-scanning mass spectrometry to provide detail on drug delivery pharmacokinetics (Dartois [2014](#)), would provide us with a wealth of information that would support the utility of HDT during treatment of *M. tuberculosis*. But there is work to be done, not least because TB disease is so difficult to model effectively. There are major obstacles moving between the *in vitro* culture system model and human disease, including the most obvious — that cell cultures are not able to mimic the complex tissue matrix that comprises the granuloma and thus cannot reliably estimate tissue drug concentrations. Even plasma drug concentrations do not correlate with concentrations at the infected site (Dartois [2014](#)). Thus, in the last few years, there have been some major developments aimed at advancing our knowledge in this field. Firstly, using animal (rabbit) models, HPLC coupled to tandem mass spectrometry is able to model intra-lesional drug distributions, and MALDI mass spectrometric imaging (MALDI-MSI) is further able to delineate the distribution of small molecules across pulmonary compartments (Dartois [2014](#)). These images can also provide valuable information about inflammatory signalling, Marakalala et al. [2016](#) have shown that the central granuloma is mostly pro-inflammatory with abundant eicosanoids, whereas the surrounding caseum is characterised by an anti-inflammatory phenotype. MALDI-MSI has also been used to

identify cholesterol abundance in mice following adjunctive simvastatin treatment (Dutta et al. 2016). More recently, generation of a complex 3-dimensional cell culture within a microfluidic plate was used to establish the pharmacokinetic dynamics of anti-mycobacterial therapy (Bielecka et al. 2017). These latest platforms alongside CT-PET imaging will almost certainly revolutionise how we model standard chemotherapy and measure the efficacy of new treatments, such as HDT.

3.7 Potential clinical applications

As I have discussed in the first chapter and hoped to have iterated in this chapter, host-directed therapy cannot adopt a one size fits all approach. However, in terms of my work, I believe that there will be groups which might benefit from HDT. Firstly, extensive cavitary disease may lead to subsequent fibrosis (Kim et al. 2001). Secondly, a recent study has demonstrated that cavitary disease is highest in patients with diabetes and drug-resistant infection (Zhang et al. 2016). Thus, given that diabetes is associated with dysregulated inflammasome activity (Schulze et al. 2004), it is conceivable that patients with both these pathologies, as well as certain patients with drug-resistant infection would benefit from inflammasome inhibition.

Another cohort of patients likely to benefit are those infected with HIV who develop IRIS. It is well established that patients with IRIS have transcriptional and proteomic evidence of increased inflammasome activity (Tadokera et al. 2011; Lai et al. 2015). Moreover, TB meningitis-associated IRIS, which has a mortality of up to 30 %, is associated with an inflammasome-activated response in serum and CSF (Marais et al. 2017). Such patients are currently treated with steroids, but with its broad inhibition of pro-inflammatory transcriptional pathways and adverse effects, there is a pressing need for alternatives.

3.7.1 Inflammasome modulation and the potential role in precision medicine

One of the principle aims of HDT is to shorten the length of standard mycobacterial chemotherapy, but such a treatment currently remains elusive. Current standards employ a relatively uniform treatment regimen and weight based dosing with limited therapeutic drug monitoring (TDM). Additionally outcomes are mainly defined by sputum clearance and clinical and radiological recovery.

However, given that inflammasome blockade enhances bacterial clearance and reduces inflammation *in vitro*, I envisage that this may enhance anti-microbial penetration. However, as I have demonstrated the efficacy of this might depend on the strain of the infected bacteria, whereby inhibition of strains which induce inflammasome activation might lead to a more favourable response than strains which

have little inflammsome activation. In the same manner as Tobin et al. [2010](#), the pharmacodynamic demonstration would need to be trialed in a range of different host-genotypes to ensure that inflammasome inhibition does not enhance bacterial replicative activity.

Tailored therapy might utilise regular TDM in order to modulate drug concentrations and use host and bacterial transcriptional profiles to define treatment response. Specifically, these markers would also be used to highlight those patients that might benefit from shortened therapy.

Whilst there is clearly much to be done, I hope that this work demonstrates that the inflammasome, which quite rightly has long been heralded as the saviour of the immune response, may also serve to propagate bacterial control and is thus potentially a hugely exciting and novel pathway that can be exploited for TB therapy.

Bibliography

- Aachoui, Youssef et al. (2013). Caspase-11 protects against bacteria that escape the vacuole. *Science* 339.6122, pp. 975–8. DOI: [10.1126/science.1230751](https://doi.org/10.1126/science.1230751).
- Abdalla, Hana et al. (2012). *Mycobacterium tuberculosis* infection of dendritic cells leads to partially caspase-1/11-independent IL-1 β and IL-18 secretion but not to pyroptosis. *PLoS One* 7.7, e40722. DOI: [10.1371/journal.pone.0040722](https://doi.org/10.1371/journal.pone.0040722).
- Abel, Brian et al. (2002). Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. *J Immunol* 169.6, pp. 3155–62.
- Abubakar, I et al. (2013). Systematic review and meta-analysis of the current evidence on the duration of protection by bacillus Calmette–Guérin vaccination against tuberculosis. *Health Technol Assess* 17.37, pp. 1–372, v–vi. DOI: [10.3310/hta17370](https://doi.org/10.3310/hta17370).
- Acosta-Rodriguez, Eva V et al. (2007). Interleukins 1 β and 6 but not transforming growth factor- β are essential for the differentiation of interleukin 17–producing human T helper cells. *Nat Immunol* 8.9, pp. 942–949. DOI: [10.1038/ni1496](https://doi.org/10.1038/ni1496).
- Adams, L A et al. (2011). Polymorphisms in MC3R promoter and CTSZ 3'UTR are associated with tuberculosis susceptibility. *European Journal of Human Genetics* 19.6, pp. 676–681. DOI: [10.1038/ejhg.2011.1](https://doi.org/10.1038/ejhg.2011.1).
- Agarwal, Nisheeth et al. (2009). Cyclic AMP intoxication of macrophages by a *Mycobacterium tuberculosis* adenylate cyclase. *Nature* 460.7251, pp. 98–102. DOI: [10.1038/nature08123](https://doi.org/10.1038/nature08123).
- Aglietti, Robin A et al. (2016). GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. *Proc Natl Acad Sci U S A* 113.28, pp. 7858–63. DOI: [10.1073/pnas.1607769113](https://doi.org/10.1073/pnas.1607769113).
- Agnholt, Jorgen et al. (2001). Infliximab downregulates interferon- γ production in activated gut T-lymphocytes from patients with Crohn's disease. *Cytokine* 15.4, pp. 212–222. DOI: [10.1006/cyto.2001.0919](https://doi.org/10.1006/cyto.2001.0919).
- Ahmed, T J et al. (2013). Curbing inflammation through endogenous pathways: focus on melanocortin peptides. *Int J Inflam* 2013, p. 985815. DOI: [10.1155/2013/985815](https://doi.org/10.1155/2013/985815).
- Al-Jamal, Wafa T. et al. (2011). Liposomes: From a clinically established drug delivery system to a nanoparticle platform for theranostic nanomedicine. *Acc Chem Res* 44.10, pp. 1094–1104. DOI: [10.1021/ar200105p](https://doi.org/10.1021/ar200105p).

- Alexander, W S et al. (1999). SOCS1 is a critical inhibitor of interferon γ signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* 98.5, pp. 597–608.
- Amaral, Eduardo P et al. (2014). Pulmonary infection with hypervirulent mycobacteria reveals a crucial role for the P2X7 receptor in aggressive forms of tuberculosis. *PLoS Pathog* 10.7, e1004188. DOI: [10.1371/journal.ppat.1004188](https://doi.org/10.1371/journal.ppat.1004188).
- Amaral, Eduardo P. et al. (2016). Innate immunity in tuberculosis: how the sensing of mycobacteria and tissue damage modulates macrophage death. *Microbes and Infection* 18.1, pp. 11–20. DOI: [10.1016/j.micinf.2015.09.005](https://doi.org/10.1016/j.micinf.2015.09.005).
- Andreu, Nuria et al. (2017). Primary macrophages and J774 cells respond differently to infection with *Mycobacterium tuberculosis*. *Sci Rep* 7, p. 42225. DOI: [10.1038/srep42225](https://doi.org/10.1038/srep42225).
- Arthur, J. Simon C. et al. (2013). Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol* 13.9, pp. 679–692. DOI: [10.1038/nri3495](https://doi.org/10.1038/nri3495).
- Arulkumaran, Nishkantha et al. (2011). A potential therapeutic role for P2X7 receptor (P2X7R) antagonists in the treatment of inflammatory diseases. *Expert Opin Investig Drugs* 20.7, pp. 897–915. DOI: [10.1517/13543784.2011.578068](https://doi.org/10.1517/13543784.2011.578068).
- Ashenafi, Senait et al. (2014). Progression of clinical tuberculosis is associated with a Th2 immune response signature in combination with elevated levels of SOCS3. *Clinical Immunology* 151.2, pp. 84–99. DOI: [10.1016/j.clim.2014.01.010](https://doi.org/10.1016/j.clim.2014.01.010).
- Assis, Patricia A et al. (2014). *Mycobacterium tuberculosis* expressing phospholipase C subverts PGE2 synthesis and induces necrosis in alveolar macrophages. *BMC Microbiol* 14, p. 128. DOI: [10.1186/1471-2180-14-128](https://doi.org/10.1186/1471-2180-14-128).
- Ataide, Marco A. et al. (2014). Malaria-induced NLRP12/NLRP3-dependent caspase-1 activation mediates inflammation and hypersensitivity to bacterial superinfection. *PLoS Pathog* 10.1, e1003885. DOI: [10.1371/journal.ppat.1003885](https://doi.org/10.1371/journal.ppat.1003885).
- Aubert, Daniel F. et al. (2016). A *Burkholderia* type VI effector deamidates Rho GTPases to activate the Pyrin inflammasome and trigger inflammation. *Cell Host Microbe* 19.5, pp. 664–674. DOI: [10.1016/j.chom.2016.04.004](https://doi.org/10.1016/j.chom.2016.04.004).
- Awomoyi, Agnes A et al. (2005). Polymorphism in IL1B: IL1B-511 association with tuberculosis and decreased lipopolysaccharide-induced IL-1 β in IFN- γ primed ex-vivo whole blood assay. *J Endotoxin Res* 11.5, pp. 281–6. DOI: [10.1179/096805105X58706](https://doi.org/10.1179/096805105X58706).
- Bafica, Andre et al. (2005). TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*. *J Exp Med* 202.12, pp. 1715–24. DOI: [10.1084/jem.20051782](https://doi.org/10.1084/jem.20051782).
- Bahn, Yong-Sun et al. (2007). Genome-wide transcriptional profiling of the cyclic AMP-dependent signaling pathway during morphogenic transitions of *Candida albicans*. *Eukaryot Cell* 6.12, pp. 2376–90. DOI: [10.1128/EC.00318-07](https://doi.org/10.1128/EC.00318-07).

- Bai, Guangchun et al. (2009). cAMP levels within *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG increase upon infection of macrophages. *FEMS Immunol Med Microbiol* 55.1, pp. 68–73. DOI: [10.1111/j.1574-695X.2008.00500.x](https://doi.org/10.1111/j.1574-695X.2008.00500.x).
- Balgi, Aruna D. et al. (2009). Screen for chemical modulators of autophagy reveals novel therapeutic inhibitors of mTORC1 signaling. *PLoS One* 4.9, e7124. DOI: [10.1371/journal.pone.0007124](https://doi.org/10.1371/journal.pone.0007124).
- Ballinger, Megan N. et al. (2015). IRAK-M promotes alternative macrophage activation and fibroproliferation in bleomycin-induced lung injury. *The Journal of Immunology* 194.4.
- Bandyopadhyay, Sayantani et al. (2013). MicroRNA-133a-1 regulates inflammasome activation through uncoupling protein-2. *Biochem Biophys Res Commun* 439.3, pp. 407–412. DOI: [10.1016/j.bbrc.2013.08.056](https://doi.org/10.1016/j.bbrc.2013.08.056).
- Barrow, E L et al. (1998). Use of microsphere technology for targeted delivery of rifampin to *Mycobacterium tuberculosis*-infected macrophages. *Antimicrob Agents Chemother* 42.10, pp. 2682–9.
- Basu, Joyoti et al. (2012). Mycobacterial signaling through Toll-like receptors. *Front Cell Infect Microbiol* 2, p. 145. DOI: [10.3389/fcimb.2012.00145](https://doi.org/10.3389/fcimb.2012.00145).
- Bauernfeind, F. et al. (2012). NLRP3 inflammasome activity is negatively controlled by miR-223. *J Immunol* 189.8, pp. 4175–4181. DOI: [10.4049/jimmunol.1201516](https://doi.org/10.4049/jimmunol.1201516).
- Bauernfeind, Franz G et al. (2009). Cutting edge: NF- κ B activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 183.2, pp. 787–91. DOI: [10.4049/jimmunol.0901363](https://doi.org/10.4049/jimmunol.0901363).
- Bean, A G et al. (1999). Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. *J Immunol* 162.6, pp. 3504–11.
- Beausejour, A et al. (1998). Proteolytic activation of the interleukin-1 β precursor by *Candida albicans*. *Infect Immun* 66.2, pp. 676–681.
- Bekker, L G et al. (1998). Selective increase in plasma tumor necrosis factor- α and concomitant clinical deterioration after initiating therapy in patients with severe tuberculosis. *J Infect Dis* 178.2, pp. 580–4.
- Bekker, L G et al. (2000). Immunopathologic effects of tumor necrosis factor α in murine mycobacterial infection are dose dependent. *Infect Immun* 68.12, pp. 6954–61.
- Belcher, John D et al. (2014). Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood* 123.3, pp. 377–90. DOI: [10.1182/blood-2013-04-495887](https://doi.org/10.1182/blood-2013-04-495887).
- Belhocine, Kamila et al. (2012). *Francisella* infection triggers activation of the AIM2 inflammasome in murine dendritic cells. *Cell Microbiol* 14.1, pp. 71–80. DOI: [10.1111/j.1462-5822.2011.01700.x](https://doi.org/10.1111/j.1462-5822.2011.01700.x).

- Berda-Haddad, Yaël et al. (2011). Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1 α . *Proc Natl Acad Sci U S A* 108.51, pp. 20684–9. DOI: [10.1073/pnas.1116848108](https://doi.org/10.1073/pnas.1116848108).
- Berghaus, Londa J et al. (2010). Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. *Comp Immunol Microbiol Infect Dis* 33.5, pp. 443–54. DOI: [10.1016/j.cimid.2009.07.001](https://doi.org/10.1016/j.cimid.2009.07.001).
- Berry, Matthew P R et al. (2010). An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* 466.7309, pp. 973–7. DOI: [10.1038/nature09247](https://doi.org/10.1038/nature09247).
- Bhardwaj, R et al. (1997). Evidence for the differential expression of the functional α -melanocyte-stimulating hormone receptor MC-1 on human monocytes. *J Immunol* 158.7, pp. 3378–84.
- Bhatt, Kamlesh et al. (2007). Host innate immune response to *Mycobacterium tuberculosis*. *J Clin Immunol* 27.4, pp. 347–362. DOI: [10.1007/s10875-007-9084-0](https://doi.org/10.1007/s10875-007-9084-0).
- Bhattacharya, Anindya et al. (2013). Pharmacological characterization of a novel centrally permeable P2X7 receptor antagonist: JNJ-47965567. *Br J Pharmacol* 170.3, pp. 624–640. DOI: [10.1111/bph.12314](https://doi.org/10.1111/bph.12314).
- Bielecka, Magdalena K et al. (2017). A bioengineered three-dimensional cell culture platform integrated with microfluidics to address antimicrobial resistance in tuberculosis. *MBio* 8.1, e02073–16. DOI: [10.1128/mBio.02073-16](https://doi.org/10.1128/mBio.02073-16).
- Bitto, A et al. (2011). Melanocortins protect against multiple organ dysfunction syndrome in mice. *Br J Pharmacol* 162.4, pp. 917–928. DOI: [10.1111/j.1476-5381.2010.01098.x](https://doi.org/10.1111/j.1476-5381.2010.01098.x).
- Blackmore, Timothy K. et al. (2008). Therapeutic use of infliximab in tuberculosis to control severe paradoxical reaction of the brain and lymph nodes. *Clin Infect Dis* 47.10, e83–e85. DOI: [10.1086/592695](https://doi.org/10.1086/592695).
- Blankley, Simon et al. (2016). A 380-gene meta-signature of active tuberculosis compared with healthy controls. *Eur Respir J*, ERJ-02121–2015. DOI: [10.1183/13993003.02121-2015](https://doi.org/10.1183/13993003.02121-2015).
- Bloom, Chloe I et al. (2012). Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PLoS One* 7.10, e46191. DOI: [10.1371/journal.pone.0046191](https://doi.org/10.1371/journal.pone.0046191).
- Blumenthal, Antje et al. (2009). RP105 facilitates macrophage activation by *Mycobacterium tuberculosis* lipoproteins. *Cell Host Microbe* 5.1, pp. 35–46. DOI: [10.1016/j.chom.2008.12.002](https://doi.org/10.1016/j.chom.2008.12.002).
- Böhm, Markus et al. (2016). α -MSH modulates cell adhesion and inflammatory responses of synovial fibroblasts from osteoarthritis patients. *Biochem Pharmacol* 116, pp. 89–99. DOI: [10.1016/j.bcp.2016.07.003](https://doi.org/10.1016/j.bcp.2016.07.003).
- Borghi, Monica et al. (2015). Pathogenic NLRP3 inflammasome activity during *Candida* infection is negatively regulated by IL-22 via activation of NLRC4 and IL-1Ra. *Cell Host Microbe* 18.2, pp. 198–209. DOI: [10.1016/j.chom.2015.07.004](https://doi.org/10.1016/j.chom.2015.07.004).

- Bradfute, Steven B et al. (2013). Autophagy as an immune effector against tuberculosis. *Curr Opin Microbiol* 16.3, pp. 355–65. DOI: [10.1016/j.mib.2013.05.003](https://doi.org/10.1016/j.mib.2013.05.003).
- Briken, Volker et al. (2013). *Mycobacterium tuberculosis* and the host cell inflammasome: a complex relationship. *Front Cell Infect Microbiol* 3.October, p. 62. DOI: [10.3389/fcimb.2013.00062](https://doi.org/10.3389/fcimb.2013.00062).
- Brough, David et al. (2007). Caspase-1-dependent processing of pro-interleukin-1 β is cytosolic and precedes cell death. *J Cell Sci* 120.Pt 5, pp. 772–781. DOI: [10.1242/jcs.03377](https://doi.org/10.1242/jcs.03377).
- Brown, Gordon D. et al. (2001). Immune recognition: A new receptor for β -glucans. *Nature* 413.6851, pp. 36–37. DOI: [10.1038/35092620](https://doi.org/10.1038/35092620).
- Broz, Petr et al. (2010). Differential requirement for caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe* 8.6, pp. 471–483. DOI: [10.1016/j.chom.2010.11.007](https://doi.org/10.1016/j.chom.2010.11.007).
- Broz, Petr et al. (2016). Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol* 16.7, pp. 407–420. DOI: [10.1038/nri.2016.58](https://doi.org/10.1038/nri.2016.58).
- Brunette, Rebecca L. et al. (2012). Extensive evolutionary and functional diversity among mammalian AIM2-like receptors. *J Exp Med* 209.11.
- Bruns, H. et al. (2012). Abelson tyrosine kinase controls phagosomal acidification required for killing of *Mycobacterium tuberculosis* in human macrophages. *J Immunol* 189.8, pp. 4069–4078. DOI: [10.4049/jimmunol.1201538](https://doi.org/10.4049/jimmunol.1201538).
- Bunschoten, A. et al. (2013). Development and prospects of dedicated tracers for the molecular imaging of bacterial infections. *Bioconjug Chem* 24.12, pp. 1971–1989. DOI: [10.1021/bc4003037](https://doi.org/10.1021/bc4003037).
- Butler, Rachel E et al. (2012). The balance of apoptotic and necrotic cell death in *Mycobacterium tuberculosis* infected macrophages is not dependent on bacterial virulence. *PLoS One* 7.10, e47573. DOI: [10.1371/journal.pone.0047573](https://doi.org/10.1371/journal.pone.0047573).
- Canna, Scott W et al. (2014). An activating NLRP4 inflammasome mutation causes autoinflammation with recurrent macrophage activation syndrome. *Nat Genet* 46.10, pp. 1140–1146. DOI: [10.1038/ng.3089](https://doi.org/10.1038/ng.3089).
- Capsoni, F et al. (2009). Melanocortin peptides inhibit urate crystal-induced activation of phagocytic cells. *Arthritis Res Ther* 11.5, R151. DOI: [10.1186/ar2827](https://doi.org/10.1186/ar2827).
- Capsoni, F et al. (2015). α -Melanocyte-stimulating-hormone (α -MSH) modulates human chondrocyte activation induced by proinflammatory cytokines. *BMC Musculoskelet Disord* 16, p. 154. DOI: [10.1186/s12891-015-0615-1](https://doi.org/10.1186/s12891-015-0615-1).
- Carlos, Daniela et al. (2017). Mitochondrial DNA activates the NLRP3 inflammasome and predisposes to type 1 diabetes in murine model. *Front Immunol* 8, p. 164. DOI: [10.3389/fimmu.2017.00164](https://doi.org/10.3389/fimmu.2017.00164).
- Carlsson, Fredric et al. (2010). Host-detrimental role of Esx-1-mediated inflammasome activation in mycobacterial infection. *PLoS Pathog* 6.5, e1000895. DOI: [10.1371/journal.ppat.1000895](https://doi.org/10.1371/journal.ppat.1000895).

- Carmona, Jenny et al. (2013). *Mycobacterium tuberculosis* strains are differentially recognized by TLRs with an impact on the immune response. *PLoS One* 8.6, e67277. DOI: [10.1371/journal.pone.0067277](https://doi.org/10.1371/journal.pone.0067277).
- Carniglia, Lila et al. (2013). Effect of NDP- α -MSH on PPAR- γ and β expression and anti-inflammatory cytokine release in rat astrocytes and microglia. *PLoS One* 8.2, e57313. DOI: [10.1371/journal.pone.0057313](https://doi.org/10.1371/journal.pone.0057313).
- Carniglia, Lila et al. (2016). [Nle4, D-Phe7]- α -MSH inhibits Toll-like receptor (TLR)2- and TLR4-induced microglial activation and promotes a M2-like phenotype. *PLoS One* 11.6, e0158564. DOI: [10.1371/journal.pone.0158564](https://doi.org/10.1371/journal.pone.0158564).
- Carow, Berit et al. (2011). Silencing suppressor of cytokine signaling-1 (SOCS1) in macrophages improves *Mycobacterium tuberculosis* control in an interferon- γ (IFN- γ)-dependent manner. *J Biol Chem* 286.30, pp. 26873–87. DOI: [10.1074/jbc.M111.238287](https://doi.org/10.1074/jbc.M111.238287).
- Castiglia, Virginia et al. (2016). Type I interferon signaling prevents IL-1 β -driven lethal systemic hyperinflammation during invasive bacterial infection of soft tissue. *Cell Host Microbe* 19.3, pp. 375–387. DOI: [10.1016/j.chom.2016.02.003](https://doi.org/10.1016/j.chom.2016.02.003).
- Castillo, Eliseo F et al. (2012). Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proc Natl Acad Sci U S A* 109.46, E3168–76. DOI: [10.1073/pnas.1210500109](https://doi.org/10.1073/pnas.1210500109).
- Catania, A et al. (2000). Plasma concentrations and anti-L-cytokine effects of α -melanocyte stimulating hormone in septic patients. *Crit Care Med* 28.5, pp. 1403–7.
- Catania, A et al. (2004). Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharmacol Rev* 56.1, pp. 1–29. DOI: [10.1124/pr.56.1.1](https://doi.org/10.1124/pr.56.1.1).
- Catania, A et al. (2006). Antimicrobial properties of α -MSH and related synthetic melanocortins. *ScientificWorldJournal* 6, pp. 1241–1246. DOI: [10.1100/tsw.2006.227](https://doi.org/10.1100/tsw.2006.227).
- Catania, A et al. (2010). The melanocortin system in control of inflammation. *ScientificWorldJournal* 10, pp. 1840–1853. DOI: [10.1100/tsw.2010.173](https://doi.org/10.1100/tsw.2010.173).
- Caws, Maxine et al. (2008). The influence of host and bacterial genotype on the development of disseminated disease with *Mycobacterium tuberculosis*. *PLoS Pathog* 4.3. DOI: [10.1371/journal.ppat.1000034](https://doi.org/10.1371/journal.ppat.1000034).
- Chacón-Salinas, R et al. (2005). Differential pattern of cytokine expression by macrophages infected *in vitro* with different *Mycobacterium tuberculosis* genotypes. *Clin Exp Immunol* 140.3, pp. 443–9. DOI: [10.1111/j.1365-2249.2005.02797.x](https://doi.org/10.1111/j.1365-2249.2005.02797.x).
- Chakraborty, Pampi et al. (2013). Drug resistant clinical isolates of *Mycobacterium tuberculosis* from different genotypes exhibit differential host responses in THP-1 cells. *PLoS One* 8.5, e62966. DOI: [10.1371/journal.pone.0062966](https://doi.org/10.1371/journal.pone.0062966).

- Chan, Francis Ka-Ming et al. (2013). Detection of necrosis by release of lactate dehydrogenase activity. *Methods Mol Biol* 979, pp. 65–70. DOI: [10.1007/978-1-62703-290-2_7](https://doi.org/10.1007/978-1-62703-290-2_7).
- Chandra, Pallavi et al. (2016). Targeting drug-sensitive and -resistant strains of *Mycobacterium tuberculosis* by inhibition of Src family kinases lowers disease burden and pathology. *mSphere* 1.2, e00043–15. DOI: [10.1128/mSphere.00043-15](https://doi.org/10.1128/mSphere.00043-15).
- Chao, Michael C et al. (2010). Letting sleeping *dos* lie: does dormancy play a role in tuberculosis? *Annu Rev Microbiol* 64.1, pp. 293–311. DOI: [10.1146/annurev.micro.112408.134043](https://doi.org/10.1146/annurev.micro.112408.134043).
- Charnley, Mirren et al. (2008). Anti-microbial action of melanocortin peptides and identification of a novel X-Pro-d/I-Val sequence in Gram-positive and Gram-negative bacteria. *Peptides* 29.6, pp. 1004–1009. DOI: [10.1016/j.peptides.2008.02.004](https://doi.org/10.1016/j.peptides.2008.02.004).
- Chavarría-Smith, Joseph et al. (2013). Direct proteolytic cleavage of NLRP1B is necessary and sufficient for inflammasome activation by anthrax lethal factor. *PLoS Pathog* 9.6, e1003452. DOI: [10.1371/journal.ppat.1003452](https://doi.org/10.1371/journal.ppat.1003452).
- Chavarría-Smith, Joseph et al. (2016). Functional and evolutionary analyses identify proteolysis as a general mechanism for NLRP1 inflammasome activation. *PLoS Pathog* 12.12, e1006052. DOI: [10.1371/journal.ppat.1006052](https://doi.org/10.1371/journal.ppat.1006052).
- Chen, Chun-Jen et al. (2007). Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* 13.7, pp. 851–856. DOI: [10.1038/nm1603](https://doi.org/10.1038/nm1603).
- Chen, Mingkuan et al. (2015). Internalized *Cryptococcus neoformans* Activates the Canonical Caspase-1 and the Noncanonical Caspase-8 Inflammasomes. *J Immunol* 195.10, pp. 4962–4972. DOI: [10.4049/jimmunol.1500865](https://doi.org/10.4049/jimmunol.1500865).
- Chen, Wei et al. (2012). Endogenous IRAK-M attenuates postinfarction remodeling through effects on macrophages and fibroblasts. *Arterioscler Thromb Vasc Biol* 32.11, pp. 2598–608. DOI: [10.1161/ATVBAHA.112.300310](https://doi.org/10.1161/ATVBAHA.112.300310).
- Chen, Wei et al. (2013). The melanocortin 1 receptor (MC1R) inhibits the inflammatory response in RAW 264.7 cells and atopic dermatitis (AD) mouse model. *Mol Biol Rep* 40.2, pp. 1987–1996. DOI: [10.1007/s11033-012-2256-x](https://doi.org/10.1007/s11033-012-2256-x).
- Chen, Yih-Yuan et al. (2014). The pattern of cytokine production *in vitro* induced by ancient and modern Beijing *Mycobacterium tuberculosis* strains. *PLoS One* 9.4, e94296. DOI: [10.1371/journal.pone.0094296](https://doi.org/10.1371/journal.pone.0094296).
- Chhajlani, Vijay et al. (1996). Identification of ligand binding residues in extracellular loops of the melanocortin 1 receptor. *Biochem Biophys Res Commun* 219.2, pp. 521–525. DOI: [10.1006/bbrc.1996.0266](https://doi.org/10.1006/bbrc.1996.0266).
- Chivero, Ernest T. et al. (2017). HIV-1 Tat primes and activates microglial NLRP3 inflammasome-mediated neuroinflammation. *J Neurosci* 37.13, pp. 3599–3609. DOI: [10.1523/JNEUROSCI.3045-16.2017](https://doi.org/10.1523/JNEUROSCI.3045-16.2017).

- Choi, Augustine M K et al. (2013). Autophagy in human health and disease. *N Engl J Med* 368.7, pp. 651–62. DOI: [10.1056/NEJMra1205406](https://doi.org/10.1056/NEJMra1205406).
- Choulaki, C. et al. (2015). Enhanced activity of NLRP3 inflammasome in peripheral blood cells of patients with active rheumatoid arthritis. *Arthritis Research and Therapy* 17.1. DOI: [10.1186/s13075-015-0775-2](https://doi.org/10.1186/s13075-015-0775-2).
- Chu, Charleen T et al. (2013). Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells. *Nat Cell Biol* 15.10, pp. 1197–205. DOI: [10.1038/ncb2837](https://doi.org/10.1038/ncb2837).
- Chu, J. et al. (2009). Cholesterol-dependent cytolysins induce rapid release of mature IL-1 from murine macrophages in a NLRP3 inflammasome and cathepsin B-dependent manner. *J Leukoc Biol* 86.5, pp. 1227–1238. DOI: [10.1189/jlb.0309164](https://doi.org/10.1189/jlb.0309164).
- Coates, Bria M. et al. (2017). Inhibition of the NOD-like receptor protein 3 inflammasome is protective in juvenile influenza A virus infection. *Front Immunol* 8.JUL. DOI: [10.3389/fimmu.2017.00782](https://doi.org/10.3389/fimmu.2017.00782).
- Coll, Rebecca C et al. (2015). A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med* 21.3, pp. 248–55. DOI: [10.1038/nm.3806](https://doi.org/10.1038/nm.3806).
- Collins, Angela C. et al. (2015). Cyclic GMP-AMP synthase is an innate immune DNA sensor for *Mycobacterium tuberculosis*. *Cell Host Microbe* 17.6, pp. 820–828. DOI: [10.1016/j.chom.2015.05.005](https://doi.org/10.1016/j.chom.2015.05.005).
- Comas, Iñaki et al. (2013). Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. *Nat Genet* 45.10, pp. 1176–82. DOI: [10.1038/ng.2744](https://doi.org/10.1038/ng.2744).
- Connolly, L E et al. (2007). Why is long-term therapy required to cure tuberculosis? *PLoS Med* 4.3, e120. DOI: [10.1371/journal.pmed.0040120](https://doi.org/10.1371/journal.pmed.0040120).
- Conos, S A et al. (2016). Cell death is not essential for caspase-1-mediated interleukin-1 β activation and secretion. *Cell Death Differ*, pp. 1–12. DOI: [10.1038/cdd.2016.69](https://doi.org/10.1038/cdd.2016.69).
- Cooke, G S et al. (2008). Mapping of a novel susceptibility locus suggests a role for MC3R and CTSZ in human tuberculosis. *Am J Respir Crit Care Med* 178.2, pp. 203–207. DOI: [10.1164/rccm.200710-15540C](https://doi.org/10.1164/rccm.200710-15540C).
- Cosin-Roger, Jesus et al. (2017). Hypoxia ameliorates intestinal inflammation through NLRP3/mTOR downregulation and autophagy activation. *Nat Commun* 8.1, p. 98. DOI: [10.1038/s41467-017-00213-3](https://doi.org/10.1038/s41467-017-00213-3).
- Coussens, Anna et al. (2009). 1 α ,25-dihydroxyvitamin D₃ inhibits matrix metalloproteinases induced by *Mycobacterium tuberculosis* infection. *Immunology* 127.4, pp. 539–548. DOI: [10.1111/j.1365-2567.2008.03024.x](https://doi.org/10.1111/j.1365-2567.2008.03024.x).
- Critchley, Julia A et al. (2013). Corticosteroids for prevention of mortality in people with tuberculosis: a systematic review and meta-analysis. *Lancet Infect Dis* 13.3, pp. 223–237. DOI: [10.1016/S1473-3099\(12\)70321-3](https://doi.org/10.1016/S1473-3099(12)70321-3).

- Cullen, Sean P. et al. (2015). Diverse activators of the NLRP3 inflammasome promote IL-1 β secretion by triggering necrosis. *Cell Rep* 11.10, pp. 1535–1548. DOI: [10.1016/j.celrep.2015.05.003](https://doi.org/10.1016/j.celrep.2015.05.003).
- Cutuli, M et al. (2000). Antimicrobial effects of α -MSH peptides. *J Leukoc Biol* 67.2, pp. 233–239.
- Dai, Xiuju et al. (2017). Epidermal keratinocytes sense dsRNA via the NLRP3 inflammasome, mediating interleukin (IL)-1 β and IL-18 release. *Exp Dermatol*. DOI: [10.1111/exd.13334](https://doi.org/10.1111/exd.13334).
- Danelishvili, Lia et al. (2012). Inhibition of the plasma-membrane-associated serine protease cathepsin G by *Mycobacterium tuberculosis* Rv3364c suppresses caspase-1 and pyroptosis in macrophages. *Front Microbiol* 2, p. 281. DOI: [10.3389/fmicb.2011.00281](https://doi.org/10.3389/fmicb.2011.00281).
- Daniels, Michael J D et al. (2016). Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer's disease in rodent models. *Nat Commun* 7, p. 12504. DOI: [10.1038/ncomms12504](https://doi.org/10.1038/ncomms12504).
- Danilchanka, Olga et al. (2014). An outer membrane channel protein of *Mycobacterium tuberculosis* with exotoxin activity. *Proc Natl Acad Sci U S A* 111.18, pp. 6750–5. DOI: [10.1073/pnas.1400136111](https://doi.org/10.1073/pnas.1400136111).
- Dartois, V (2014). The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nat Rev Microbiol* 12.3, pp. 159–167. DOI: [10.1038/nrmicro3200](https://doi.org/10.1038/nrmicro3200).
- Davila, Sonia et al. (2008). Genetic association and expression studies indicate a role of Toll-like receptor 8 in pulmonary tuberculosis. *PLoS Genet* 4.10, e1000218. DOI: [10.1371/journal.pgen.1000218](https://doi.org/10.1371/journal.pgen.1000218).
- Davis, J. Muse et al. (2009). The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 136.1, pp. 37–49. DOI: [10.1016/j.cell.2008.11.014](https://doi.org/10.1016/j.cell.2008.11.014).
- Dawson, Rod et al. (2009). Immunomodulation with recombinant interferon- γ 1b in pulmonary tuberculosis. *PLoS One* 4.9, e6984. DOI: [10.1371/journal.pone.0006984](https://doi.org/10.1371/journal.pone.0006984).
- Deguine, Jacques et al. (2014). MyD88: a central player in innate immune signaling. *F1000Prime Rep* 6, p. 97. DOI: [10.12703/P6-97](https://doi.org/10.12703/P6-97).
- Deng, J. C. et al. (2006). Sepsis-induced suppression of lung innate immunity is mediated by IRAK-M. *J Clin Invest* 116.9, pp. 2532–42. DOI: [10.1172/JCI28054](https://doi.org/10.1172/JCI28054).
- Deng, Jiangping et al. (2004). α -melanocyte-stimulating hormone inhibits lung injury after renal ischemia/reperfusion. *Am J Respir Crit Care Med* 169.6, pp. 749–56. DOI: [10.1164/rccm.200303-3720C](https://doi.org/10.1164/rccm.200303-3720C).
- Deretic, Vojo et al. (2013). Autophagy in infection, inflammation and immunity. *Nat Rev Immunol* 13.10, pp. 722–37. DOI: [10.1038/nri3532](https://doi.org/10.1038/nri3532).
- Deschodt-Lanckman, Monique et al. (1990). Degradation of α -melanocyte stimulating hormone (α -MSH) by CALLA/endopeptidase 24.11 expressed by human melanoma cells in culture. *Int J Cancer* 46.6, pp. 1124–1130. DOI: [10.1002/ijc.2910460629](https://doi.org/10.1002/ijc.2910460629).

- Dey, Bappaditya et al. (2015). A bacterial cyclic dinucleotide activates the cytosolic surveillance pathway and mediates innate resistance to tuberculosis. *Nat Med* 21.4, pp. 401–6. DOI: [10.1038/nm.3813](https://doi.org/10.1038/nm.3813).
- Dick, Mathias S et al. (2016). ASC filament formation serves as a signal amplification mechanism for inflammasomes. *Nat Commun* 7, p. 11929. DOI: [10.1038/ncomms11929](https://doi.org/10.1038/ncomms11929).
- Dinarello, Charles A (2009). Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 27, pp. 519–50. DOI: [10.1146/annurev.immunol.021908.132612](https://doi.org/10.1146/annurev.immunol.021908.132612).
- Divangahi, Maziar et al. (2008). NOD2-deficient mice have impaired resistance to *Mycobacterium tuberculosis* infection through defective innate and adaptive immunity. *J Immunol* 181.10, pp. 7157–65.
- Divanovic, Senad et al. (2005). Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. *Nat Immunol* 6.6, pp. 571–578. DOI: [10.1038/ni1198](https://doi.org/10.1038/ni1198).
- Doi, K et al. (2008). AP214, an analogue of α -melanocyte-stimulating hormone, ameliorates sepsis-induced acute kidney injury and mortality. *Kidney Int* 73.11, pp. 1266–74. DOI: [10.1038/ki.2008.97](https://doi.org/10.1038/ki.2008.97).
- Donnarumma, G et al. (2004). α -MSH reduces the internalization of *Staphylococcus aureus* and down-regulates HSP 70, integrins and cytokine expression in human keratinocyte cell lines. *Exp Dermatol* 13.12, pp. 748–754. DOI: [10.1111/j.0906-6705.2004.00218.x](https://doi.org/10.1111/j.0906-6705.2004.00218.x).
- Dooley, D P et al. (1997). Adjunctive corticosteroid therapy for tuberculosis: a critical reappraisal of the literature. *Clin Infect Dis* 25.4, pp. 872–87.
- Dorhoi, A et al. (2012). Activation of the NLRP3 inflammasome by *Mycobacterium tuberculosis* is uncoupled from susceptibility to active tuberculosis. *Eur J Immunol* 42.2, pp. 374–384. DOI: [10.1002/eji.201141548](https://doi.org/10.1002/eji.201141548).
- Dorhoi, Anca et al. (2016). Pathology and immune reactivity: understanding multidimensionality in pulmonary tuberculosis. *Semin Immunopathol* 38.2, pp. 153–166. DOI: [10.1007/s00281-015-0531-3](https://doi.org/10.1007/s00281-015-0531-3).
- Dostert, Catherine et al. (2008). Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320.5876, pp. 674–7. DOI: [10.1126/science.1156995](https://doi.org/10.1126/science.1156995).
- Dostert, Catherine et al. (2009). Malarial hemozoin is a Nalp3 inflammasome activating danger signal. *PLoS One* 4.8, e6510. DOI: [10.1371/journal.pone.0006510](https://doi.org/10.1371/journal.pone.0006510).
- D’Oswaldo, Andrea et al. (2015). Transcription factor ATF4 induces NLRP1 inflammasome expression during endoplasmic reticulum stress. *PLoS One* 10.6, e0130635. DOI: [10.1371/journal.pone.0130635](https://doi.org/10.1371/journal.pone.0130635).
- Drennan, Michael B et al. (2004). Toll-like receptor 2-deficient mice succumb to *Mycobacterium tuberculosis* infection. *Am J Pathol* 164.1, pp. 49–57. DOI: [10.1016/S0002-9440\(10\)63095-7](https://doi.org/10.1016/S0002-9440(10)63095-7).

- Duñéñez-Guzmán, Edgar A. et al. (2014). The evolution of reproduction-related NLRP genes. *J Mol Evol* 78.3-4, pp. 194–201. DOI: [10.1007/s00239-014-9614-3](https://doi.org/10.1007/s00239-014-9614-3).
- Dutra, Fabianno F et al. (2014). Hemolysis-induced lethality involves inflammasome activation by heme. *Proc Natl Acad Sci U S A* 111.39, E4110–8. DOI: [10.1073/pnas.1405023111](https://doi.org/10.1073/pnas.1405023111).
- Dutta, Noton K. et al. (2016). Statin adjunctive therapy shortens the duration of TB treatment in mice. *J Antimicrob Chemother* 71.6, pp. 1570–1577. DOI: [10.1093/jac/dkw014](https://doi.org/10.1093/jac/dkw014).
- Eklund, Daniel et al. (2014). Human gene variants linked to enhanced NLRP3 activity limit intramacrophage growth of *Mycobacterium tuberculosis*. *J Infect Dis* 209.5, pp. 749–53. DOI: [10.1093/infdis/jit572](https://doi.org/10.1093/infdis/jit572).
- Eldridge, Matthew J G et al. (2015). Antimicrobial inflammasomes: unified signalling against diverse bacterial pathogens. *Curr Opin Microbiol* 23, pp. 32–41. DOI: [10.1016/j.mib.2014.10.008](https://doi.org/10.1016/j.mib.2014.10.008).
- Elliott, J Michael et al. (2009). Crystal structure of procaspase-1 zymogen domain reveals insight into inflammatory caspase autoactivation. *J Biol Chem* 284.10, pp. 6546–53. DOI: [10.1074/jbc.M806121200](https://doi.org/10.1074/jbc.M806121200).
- Engle, Matthias et al. (2002). Induction of TNF in human alveolar macrophages as a potential evasion mechanism of virulent *Mycobacterium tuberculosis*. *The Journal of Immunology* 168.3.
- Eom, Young Woo et al. (2014). The role of growth factors in maintenance of stemness in bone marrow-derived mesenchymal stem cells. *Biochem Biophys Res Commun* 445.1, pp. 16–22. DOI: [10.1016/j.bbrc.2014.01.084](https://doi.org/10.1016/j.bbrc.2014.01.084).
- Esmail, H et al. (2014). The ongoing challenge of latent tuberculosis. *Philos Trans R Soc Lond B Biol Sci* 369.1645, p. 20130437. DOI: [10.1098/rstb.2013.0437](https://doi.org/10.1098/rstb.2013.0437).
- Espert, Lucile et al. (2015). Autophagy in *Mycobacterium tuberculosis* and HIV infections. *Front Cell Infect Microbiol* 5, p. 49. DOI: [10.3389/fcimb.2015.00049](https://doi.org/10.3389/fcimb.2015.00049).
- Evavold, Charles L. et al. (2018). The pore-forming protein gasdermin D regulates interleukin-1 secretion from living macrophages. *Immunity* 48.1, 35–44.e6. DOI: [10.1016/j.immuni.2017.11.013](https://doi.org/10.1016/j.immuni.2017.11.013).
- Ewald, S. E. et al. (2014). NLRP1 is an inflammasome sensor for *Toxoplasma gondii*. *Infect Immun* 82.1, pp. 460–468. DOI: [10.1128/IAI.01170-13](https://doi.org/10.1128/IAI.01170-13).
- Eyles, Joanne L et al. (2002). Negative regulation of interleukin-12 signaling by suppressor of cytokine signaling-1. *J Biol Chem* 277.46, pp. 43735–40. DOI: [10.1074/jbc.M208586200](https://doi.org/10.1074/jbc.M208586200).
- Fabri, Mario et al. (2011). Vitamin D is required for IFN- γ -mediated antimicrobial activity of human macrophages. *Sci Transl Med* 3.104, 104ra102. DOI: [10.1126/scitranslmed.3003045](https://doi.org/10.1126/scitranslmed.3003045).
- Fang, Rendong et al. (2014). Type I interferon signaling regulates activation of the absent in melanoma 2 inflammasome during *Streptococcus pneumoniae* infection. *Infect Immun* 82.6, pp. 2310–7. DOI: [10.1128/IAI.01572-14](https://doi.org/10.1128/IAI.01572-14).

- Ferioti, Claudia et al. (2015). Expression of dectin-1 and enhanced activation of NALP3 inflammasome are associated with resistance to paracoccidioidomycosis. *Front Microbiol* 6, p. 913. DOI: [10.3389/fmicb.2015.00913](https://doi.org/10.3389/fmicb.2015.00913).
- Fernandes-Alnemri, Teresa et al. (2013). Cutting edge: TLR signaling licenses IRAK1 for rapid activation of the NLRP3 inflammasome. *J Immunol* 191.8, pp. 3995–9. DOI: [10.4049/jimmunol.1301681](https://doi.org/10.4049/jimmunol.1301681).
- Ferwerda, Gerben et al. (2005). NOD2 and Toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*. *PLoS Pathog* 1.3, pp. 279–85. DOI: [10.1371/journal.ppat.0010034](https://doi.org/10.1371/journal.ppat.0010034).
- Flynn, J L et al. (1995). Tumor necrosis factor- α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* 2.6, pp. 561–72.
- Fossati-Jimack, Liliane et al. (2013). Phagocytosis is the main CR3-mediated function affected by the lupus-associated variant of CD11b in human myeloid cells. *PLoS One* 8.2, e57082. DOI: [10.1371/journal.pone.0057082](https://doi.org/10.1371/journal.pone.0057082).
- Franchi, L. et al. (2009). Cutting edge: TNF- α mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J Immunol* 183.2, pp. 792–796. DOI: [10.4049/jimmunol.0900173](https://doi.org/10.4049/jimmunol.0900173).
- Fremond, Cecile M et al. (2007). IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to *Mycobacterium tuberculosis* infection. *J Immunol* 179.2, pp. 1178–89.
- Fukao, Taro et al. (2007). An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell* 129.3, pp. 617–31. DOI: [10.1016/j.cell.2007.02.048](https://doi.org/10.1016/j.cell.2007.02.048).
- Gagneux, S. et al. (2006). Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* 103.8, pp. 2869–2873. DOI: [10.1073/pnas.0511240103](https://doi.org/10.1073/pnas.0511240103).
- Gaidt, Moritz M. et al. (2016). Human monocytes engage an alternative inflammasome pathway. *Immunity* 44.4, pp. 713–715. DOI: [10.1016/j.immuni.2016.01.012](https://doi.org/10.1016/j.immuni.2016.01.012).
- Gandotra, S. et al. (2007). Nucleotide-binding oligomerization domain protein 2-deficient mice control infection with *Mycobacterium tuberculosis*. *Infect Immun* 75.11, pp. 5127–5134. DOI: [10.1128/IAI.00458-07](https://doi.org/10.1128/IAI.00458-07).
- Gao, X-F et al. (2011). Adjunctive therapy with interferon- γ for the treatment of pulmonary tuberculosis: a systematic review. *Int J Infect Dis* 15.9, e594–600. DOI: [10.1016/j.ijid.2011.05.002](https://doi.org/10.1016/j.ijid.2011.05.002).
- García-Borrón, Jose C et al. (2014). MC1R, the cAMP pathway, and the response to solar UV: extending the horizon beyond pigmentation. *Pigment Cell Melanoma Res* 27.5, pp. 699–720. DOI: [10.1111/pcmr.12257](https://doi.org/10.1111/pcmr.12257).

- Garlanda, C et al. (2007). Damping excessive inflammation and tissue damage in *Mycobacterium tuberculosis* infection by Toll IL-1 receptor 8/single Ig IL-1-related receptor, a negative regulator of IL-1/TLR signaling. *J Immunol* 179.5, pp. 3119–3125.
- Garlanda, Cecilia et al. (2013). The interleukin-1 family: back to the future. *Immunity* 39.6, pp. 1003–1018. DOI: [10.1016/j.immuni.2013.11.010](https://doi.org/10.1016/j.immuni.2013.11.010).
- Gelman, Julia S et al. (2012). Quantitative peptidomics to measure neuropeptide levels in animal models relevant to psychiatric disorders. *Methods Mol Biol* 829, pp. 487–503. DOI: [10.1007/978-1-61779-458-2_31](https://doi.org/10.1007/978-1-61779-458-2_31).
- Gengenbacher, Martin et al. (2012). *Mycobacterium tuberculosis*: success through dormancy. *FEMS Microbiol Rev* 36.3, pp. 514–32. DOI: [10.1111/j.1574-6976.2012.00331.x](https://doi.org/10.1111/j.1574-6976.2012.00331.x).
- Gerritsen, M E et al. (1997). CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc Natl Acad Sci U S A* 94.7, pp. 2927–32.
- Getting, S J (2006). Targeting melanocortin receptors as potential novel therapeutics. *Pharmacol Ther* 111.1, pp. 1–15. DOI: [10.1016/j.pharmthera.2005.06.022](https://doi.org/10.1016/j.pharmthera.2005.06.022).
- Getting, S J et al. (1999). POMC gene-derived peptides activate melanocortin type 3 receptor on murine macrophages, suppress cytokine release, and inhibit neutrophil migration in acute experimental inflammation. *J Immunol* 162.12, pp. 7446–53.
- Getting, S J et al. (2003). Dissection of the anti-inflammatory effect of the core and C-terminal (KPV) α -melanocyte-stimulating hormone peptides. *J Pharmacol Exp Ther* 306.2, pp. 631–637. DOI: [10.1124/jpet.103.051623](https://doi.org/10.1124/jpet.103.051623).
- Getting, S J et al. (2006). The melanocortin peptide HP228 displays protective effects in acute models of inflammation and organ damage. *Eur J Pharmacol* 532.1-2, pp. 138–144. DOI: [10.1016/j.ejphar.2005.12.023](https://doi.org/10.1016/j.ejphar.2005.12.023).
- Gibney, Katherine B et al. (2008). Vitamin D deficiency is associated with tuberculosis and latent tuberculosis infection in immigrants from sub-Saharan Africa. *Clin Infect Dis* 46.3, pp. 443–6. DOI: [10.1086/525268](https://doi.org/10.1086/525268).
- Gleeson, Laura E et al. (2016). Cutting edge: *Mycobacterium tuberculosis* induces aerobic glycolysis in human alveolar macrophages that is required for control of intracellular bacillary replication. *J Immunol* 196.6, pp. 2444–9. DOI: [10.4049/jimmunol.1501612](https://doi.org/10.4049/jimmunol.1501612).
- Glick, Danielle et al. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol* 221.1, pp. 3–12. DOI: [10.1002/path.2697](https://doi.org/10.1002/path.2697).
- Gombart, Adrian F (2009). The vitamin D-antimicrobial peptide pathway and its role in protection against infection. *Future Microbiol* 4.9, pp. 1151–65. DOI: [10.2217/fmb.09.87](https://doi.org/10.2217/fmb.09.87).
- Gomez, James E et al. (2004). *M. tuberculosis* persistence, latency, and drug tolerance. *Tuberculosis* 84.1-2, pp. 29–44. DOI: [10.1016/j.tube.2003.08.003](https://doi.org/10.1016/j.tube.2003.08.003).

- Grandemange, Sylvie et al. (2016). A new autoinflammatory and autoimmune syndrome associated with NLRP1 mutations: NAIAD (*NLRP1*-associated autoinflammation with arthritis and dyskeratosis). *Ann Rheum Dis*, pp. 1191–1198. DOI: [10.1136/annrheumdis-2016-210021](https://doi.org/10.1136/annrheumdis-2016-210021).
- Green, Justin A. et al. (2009). Dexamethasone, cerebrospinal fluid matrix metalloproteinase concentrations and clinical outcomes in tuberculous meningitis. *PLoS One* 4.9, e7277. DOI: [10.1371/journal.pone.0007277](https://doi.org/10.1371/journal.pone.0007277).
- Grieco, Paolo et al. (2003). Novel α -melanocyte stimulating hormone peptide analogues with high candidacidal activity. *J Med Chem* 46.5, pp. 850–855. DOI: [10.1021/jm0204338](https://doi.org/10.1021/jm0204338).
- Grieco, Paolo et al. (2013). Novel α -MSH peptide analogues with broad spectrum antimicrobial activity. *PLoS One* 8.4. DOI: [10.1371/journal.pone.0061614](https://doi.org/10.1371/journal.pone.0061614).
- Gringhuis, Sonja I et al. (2012). Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1 β via a noncanonical caspase-8 inflammasome. *Nat Immunol* 13.3, pp. 246–54. DOI: [10.1038/ni.2222](https://doi.org/10.1038/ni.2222).
- Grode, Leander et al. (2005). Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guérin mutants that secrete listeriolysin. *J Clin Invest* 115.9, pp. 2472–9. DOI: [10.1172/JCI24617](https://doi.org/10.1172/JCI24617).
- Grode, Leander et al. (2013). Safety and immunogenicity of the recombinant BCG vaccine VPM1002 in a phase 1 open-label randomized clinical trial. *Vaccine* 31.9, pp. 1340–8. DOI: [10.1016/j.vaccine.2012.12.053](https://doi.org/10.1016/j.vaccine.2012.12.053).
- Groß, Christina J. et al. (2016). K⁺ efflux-independent NLRP3 inflammasome activation by small molecules targeting mitochondria. *Immunity* 45.4, pp. 761–773. DOI: [10.1016/j.immuni.2016.08.010](https://doi.org/10.1016/j.immuni.2016.08.010).
- Guarda, Greta et al. (2011). Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* 34.2, pp. 213–223. DOI: [10.1016/j.immuni.2011.02.006](https://doi.org/10.1016/j.immuni.2011.02.006).
- Guo, Haitao et al. (2014). HIV-1 infection induces interleukin-1 β production via TLR8 protein-dependent and NLRP3 inflammasome mechanisms in human monocytes. *J Biol Chem* 289.31, pp. 21716–26. DOI: [10.1074/jbc.M114.566620](https://doi.org/10.1074/jbc.M114.566620).
- Guo, Sheng et al. (2015). The NLRP3 inflammasome and IL-1 β accelerate immunologically mediated pathology in experimental viral fulminant hepatitis. *PLoS Pathog* 11.9, e1005155. DOI: [10.1371/journal.ppat.1005155](https://doi.org/10.1371/journal.ppat.1005155).
- Gupta, Ashish K. et al. (2000). α -MSH inhibits induction of C/EBP β -DNA binding activity and NOS2 gene transcription in macrophages. *Kidney Int* 57.6, pp. 2239–2248. DOI: [10.1046/j.1523-1755.2000.00084.x](https://doi.org/10.1046/j.1523-1755.2000.00084.x).
- Gupta, R. et al. (2014). RNA and β -hemolysin of Group B *Streptococcus* induce interleukin-1 β (IL-1 β) by activating NLRP3 inflammasomes in mouse macrophages. *J Biol Chem* 289.20, pp. 13701–13705. DOI: [10.1074/jbc.C114.548982](https://doi.org/10.1074/jbc.C114.548982).

- Gurung, P. et al. (2014). FADD and Caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes. *J Immunol* 192.4, pp. 1835–1846. DOI: [10.4049/jimmunol.1302839](https://doi.org/10.4049/jimmunol.1302839).
- Gurung, Prajwal et al. (2015). Mitochondria: diversity in the regulation of the NLRP3 inflammasome. *Trends Mol Med* 21.3, pp. 193–201. DOI: [10.1016/j.molmed.2014.11.008](https://doi.org/10.1016/j.molmed.2014.11.008).
- Gutierrez, Maximiliano G. et al. (2004). Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* 119.6, pp. 753–766. DOI: [10.1016/j.cell.2004.11.038](https://doi.org/10.1016/j.cell.2004.11.038).
- Hagar, Jon A et al. (2013). Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* 341.6151, pp. 1250–3. DOI: [10.1126/science.1240988](https://doi.org/10.1126/science.1240988).
- Hahn, Katherine J et al. (2015). Treatment with anakinra, a recombinant IL-1 receptor antagonist, unlikely to induce lasting remission in patients with CGD colitis. *Am J Gastroenterol* 110.6, pp. 938–939. DOI: [10.1038/ajg.2015.135](https://doi.org/10.1038/ajg.2015.135).
- Haque, Shabirul et al. (2016). HIV promotes NLRP3 inflammasome complex activation in murine HIV-associated nephropathy. *Am J Pathol* 186.2, pp. 347–358. DOI: [10.1016/j.ajpath.2015.10.002](https://doi.org/10.1016/j.ajpath.2015.10.002).
- Hara, Hideki et al. (2013). Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat Immunol* 14.12, pp. 1247–55. DOI: [10.1038/ni.2749](https://doi.org/10.1038/ni.2749).
- Harijith, Anantha et al. (2014). Reactive oxygen species at the crossroads of inflammasome and inflammation. *Front Physiol* 5, p. 352. DOI: [10.3389/fphys.2014.00352](https://doi.org/10.3389/fphys.2014.00352).
- Harris, James E et al. (2007a). IFN γ synergizes with IL-1 β to up-regulate MMP-9 secretion in a cellular model of central nervous system tuberculosis. *FASEB Journal* 21.2, pp. 356–365.
- Harris, James E et al. (2007b). T helper 2 cytokines inhibit autophagic control of intracellular *Mycobacterium tuberculosis*. *Immunity* 27.3, pp. 505–517. DOI: [10.1016/j.immuni.2007.07.022](https://doi.org/10.1016/j.immuni.2007.07.022).
- Harris, James E et al. (2008). Tumor necrosis factor blockers influence macrophage responses to *Mycobacterium tuberculosis*. *J Infect Dis* 198.12, pp. 1842–50. DOI: [10.1086/593174](https://doi.org/10.1086/593174).
- Harris, James E et al. (2010). How tumour necrosis factor blockers interfere with tuberculosis immunity. *Clin Exp Immunol* 161.1, no–no. DOI: [10.1111/j.1365-2249.2010.04146.x](https://doi.org/10.1111/j.1365-2249.2010.04146.x).
- Harris, James E et al. (2011). Autophagy controls IL-1 β secretion by targeting pro-IL-1 β for degradation. *J Biol Chem* 286.11, pp. 9587–9597. DOI: [10.1074/jbc.M110.202911](https://doi.org/10.1074/jbc.M110.202911).
- Hawn, Thomas R et al. (2013). Host-directed therapeutics for tuberculosis: can we harness the host? *Microbiol Mol Biol Rev* 77.4, pp. 608–27. DOI: [10.1128/MMBR.00032-13](https://doi.org/10.1128/MMBR.00032-13).

- Haycock, J W et al. (2000). α -melanocyte-stimulating hormone reduces impact of proinflammatory cytokine and peroxide-generated oxidative stress on keratinocyte and melanoma cell lines. *J Biol Chem* 275.21, pp. 15629–36. DOI: [10.1074/JBC.275.21.15629](https://doi.org/10.1074/JBC.275.21.15629).
- He, Yuan et al. (2013). The protein kinase PKR is critical for LPS-induced iNOS production but dispensable for inflammasome activation in macrophages. *Eur J Immunol* 43.5, pp. 1147–52. DOI: [10.1002/eji.201243187](https://doi.org/10.1002/eji.201243187).
- He, Yuan et al. (2016). Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci* 41.12, pp. 1012–1021. DOI: [10.1016/j.tibs.2016.09.002](https://doi.org/10.1016/j.tibs.2016.09.002).
- Heid, Michelle E et al. (2013). Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *J Immunol* 191.10, pp. 5230–8. DOI: [10.4049/jimmunol.1301490](https://doi.org/10.4049/jimmunol.1301490).
- Heinrich, P C et al. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J Pt 2*, pp. 297–314.
- Hellmich, Kristina A et al. (2012). Anthrax lethal factor cleaves mouse Nlrp1b in both toxin-sensitive and toxin-resistant macrophages. *PLoS One* 7.11, e49741. DOI: [10.1371/journal.pone.0049741](https://doi.org/10.1371/journal.pone.0049741).
- Hesker, Pamela R et al. (2012). Genetic loss of murine pyrin, the Familial Mediterranean Fever protein, increases interleukin-1 β levels. *PLoS One* 7.11, e51105. DOI: [10.1371/journal.pone.0051105](https://doi.org/10.1371/journal.pone.0051105).
- Hnizdo, E et al. (2000). Chronic pulmonary function impairment caused by initial and recurrent pulmonary tuberculosis following treatment. *Thorax* 55.1, pp. 32–38.
- Hoffman, Hal M et al. (2009). Recurrent febrile syndromes: what a rheumatologist needs to know. *Nat Rev Rheumatol* 5.5, pp. 249–56. DOI: [10.1038/nrrheum.2009.40](https://doi.org/10.1038/nrrheum.2009.40).
- Hölscher, Christoph et al. (2008). Containment of aerogenic *Mycobacterium tuberculosis* infection in mice does not require MyD88 adaptor function for TLR2, -4 and -9. *Eur J Immunol* 38.3, pp. 680–94. DOI: [10.1002/eji.200736458](https://doi.org/10.1002/eji.200736458).
- Homolka, Susanne et al. (2010). Functional genetic diversity among *Mycobacterium tuberculosis* complex clinical isolates: delineation of conserved core and lineage-specific transcriptomes during intracellular survival. *PLoS Pathog* 6.7, e1000988. DOI: [10.1371/journal.ppat.1000988](https://doi.org/10.1371/journal.ppat.1000988).
- Hornung, Veit et al. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9.8, pp. 847–856. DOI: [10.1038/ni.1631](https://doi.org/10.1038/ni.1631).
- Hout, Gerardus P J van et al. (2017). The selective NLRP3-inflammasome inhibitor MCC950 reduces infarct size and preserves cardiac function in a pig model of myocardial infarction. *Eur Heart J* 38.11, pp. 828–836. DOI: [10.1093/eurheartj/ehw247](https://doi.org/10.1093/eurheartj/ehw247).
- Hruby, Victor J. et al. (1993). Design, synthesis, and conformation of superpotent and prolonged acting melanotropins. *Ann N Y Acad Sci* 680.1 The Melanotro, pp. 51–63. DOI: [10.1111/j.1749-6632.1993.tb19674.x](https://doi.org/10.1111/j.1749-6632.1993.tb19674.x).

- Hu, Yu et al. (2010). Tripartite-motif protein 30 negatively regulates NLRP3 inflammasome activation by modulating reactive oxygen species production. *The Journal of Immunology* 185.12.
- Hu, Zehan et al. (2013). Crystal structure of NLRC4 reveals its autoinhibition mechanism. *Science* 341.6142.
- Huang, Q H et al. (1998). Systemic α -MSH suppresses LPS fever via central melanocortin receptors independently of its suppression of corticosterone and IL-6 release. *Am J Physiol* 275.2 Pt 2, R524–30.
- Hussell, Tracy et al. (2014). Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol* 14.2, pp. 81–93. DOI: [10.1038/nri3600](https://doi.org/10.1038/nri3600).
- Ichinohe, Takeshi et al. (2009). Inflammasome recognition of influenza virus is essential for adaptive immune responses. *J Exp Med* 206.1, pp. 79–87. DOI: [10.1084/jem.20081667](https://doi.org/10.1084/jem.20081667).
- Inglis, Diane O et al. (2013). Ras signaling gets fine-tuned: regulation of multiple pathogenic traits of *Candida albicans*. *Eukaryot Cell* 12.10, pp. 1316–25. DOI: [10.1128/EC.00094-13](https://doi.org/10.1128/EC.00094-13).
- Ioerger, Thomas R et al. (2010). Variation among genome sequences of H37Rv strains of *Mycobacterium tuberculosis* from multiple laboratories. *J Bacteriol* 192.14, pp. 3645–53. DOI: [10.1128/JB.00166-10](https://doi.org/10.1128/JB.00166-10).
- Ishikawa, Eri et al. (2009). Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J Exp Med* 206.13, pp. 2879–88. DOI: [10.1084/jem.20091750](https://doi.org/10.1084/jem.20091750).
- Ivanyi, J. et al. (2013). Nonsteroidal antiinflammatory drugs for adjunctive tuberculosis treatment. *J Infect Dis* 208.2, pp. 185–188. DOI: [10.1093/infdis/jit153](https://doi.org/10.1093/infdis/jit153).
- Iyer, Shankar S et al. (2013). Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 39.2, pp. 311–23. DOI: [10.1016/j.immuni.2013.08.001](https://doi.org/10.1016/j.immuni.2013.08.001).
- Izzo, A.A. et al. (2004). A matrix metalloproteinase inhibitor promotes granuloma formation during the early phase of *Mycobacterium tuberculosis* pulmonary infection. *Tuberculosis* 84.6, pp. 387–396. DOI: [10.1016/j.tube.2004.07.001](https://doi.org/10.1016/j.tube.2004.07.001).
- Jacobs, M et al. (2000). Fatal *Mycobacterium bovis* BCG infection in TNF-LT- α -deficient mice. *Clin Immunol* 94.3, pp. 192–9. DOI: [10.1006/clin.2000.4835](https://doi.org/10.1006/clin.2000.4835).
- Jayaraman, Pushpa et al. (2013). IL-1 β promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *J Immunol* 190.8, pp. 4196–204. DOI: [10.4049/jimmunol.1202688](https://doi.org/10.4049/jimmunol.1202688).
- Jeyanathan, M et al. (2014). Pulmonary *M. tuberculosis* infection delays Th1 immunity via immunoadaptor DAP12-regulated IRAK-M and IL-10 expression in antigen-presenting cells. *Mucosal Immunol* 7.3, pp. 670–683. DOI: [10.1038/mi.2013.86](https://doi.org/10.1038/mi.2013.86).
- Ji, Hai-xia et al. (2013). The synthetic melanocortin (CKPV)₂ exerts anti-fungal and anti-inflammatory effects against *Candida albicans* vaginitis via inducing macrophage M2 polarization. *PLoS One* 8.2, e56004. DOI: [10.1371/journal.pone.0056004](https://doi.org/10.1371/journal.pone.0056004).

- Jin, Tengchuan et al. (2012). Structures of the HIN domain:DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. *Immunity* 36.4, pp. 561–71. DOI: [10.1016/j.immuni.2012.02.014](https://doi.org/10.1016/j.immuni.2012.02.014).
- Jin, Tengchuan et al. (2013). Structure of the absent in melanoma 2 (AIM2) pyrin domain provides insights into the mechanisms of AIM2 autoinhibition and inflammasome assembly. *J Biol Chem* 288.19, pp. 13225–35. DOI: [10.1074/jbc.M113.468033](https://doi.org/10.1074/jbc.M113.468033).
- Jo, Eun-Kyeong et al. (2015). Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol* 13.2, pp. 148–159. DOI: [10.1038/cmi.2015.95](https://doi.org/10.1038/cmi.2015.95).
- Jones, J. W. et al. (2010). Absent in melanoma 2 is required for innate immune recognition of *Francisella tularensis*. *Proc Natl Acad Sci U S A* 107.21, pp. 9771–9776. DOI: [10.1073/pnas.1003738107](https://doi.org/10.1073/pnas.1003738107).
- Jordens, I et al. (2001). The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr Biol* 11.21, pp. 1680–5.
- Juliana, Christine et al. (2012). Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *J Biol Chem* 287.43, pp. 36617–22. DOI: [10.1074/jbc.M112.407130](https://doi.org/10.1074/jbc.M112.407130).
- Kaewseekhao, Benjawan et al. (2015). Comparative proteomics of activated THP-1 cells infected with *Mycobacterium tuberculosis* identifies putative clearance biomarkers for tuberculosis treatment. *PLoS One* 10.7, e0134168. DOI: [10.1371/journal.pone.0134168](https://doi.org/10.1371/journal.pone.0134168).
- Kailasan Vanaja, Sivapriya et al. (2014). Bacterial RNA:DNA hybrids are activators of the NLRP3 inflammasome. *Proc Natl Acad Sci U S A* 111.21, pp. 7765–70. DOI: [10.1073/pnas.1400075111](https://doi.org/10.1073/pnas.1400075111).
- Kalamidas, Stefanos A. et al. (2006). cAMP synthesis and degradation by phagosomes regulate actin assembly and fusion events: consequences for mycobacteria. *J Cell Sci* 119.17.
- Kalantari, Parisa et al. (2014). Dual engagement of the NLRP3 and AIM2 inflammasomes by *Plasmodium*-derived hemozoin and DNA during malaria. *Cell Rep* 6.1, pp. 196–210. DOI: [10.1016/j.celrep.2013.12.014](https://doi.org/10.1016/j.celrep.2013.12.014).
- Kalden, D H et al. (1999). Mechanisms of the antiinflammatory effects of α -MSH. Role of transcription factor NF- κ B and adhesion molecule expression. *Ann N Y Acad Sci* 885, pp. 254–61.
- Kang, Peter B et al. (2005). The human macrophage mannose receptor directs *Mycobacterium tuberculosis* lipoarabinomannan-mediated phagosome biogenesis. *J Exp Med* 202.7, pp. 987–99. DOI: [10.1084/jem.20051239](https://doi.org/10.1084/jem.20051239).
- Kannengiesser, Klaus et al. (2008). Melanocortin-derived tripeptide KPV has anti-inflammatory potential in murine models of inflammatory bowel disease. *Inflamm Bowel Dis* 14.3, pp. 324–331. DOI: [10.1002/ibd.20334](https://doi.org/10.1002/ibd.20334).
- Kariagina, Anastasia et al. (2004). Hypothalamic-pituitary cytokine network. *Endocrinology* 145.1, pp. 104–12. DOI: [10.1210/en.2003-0669](https://doi.org/10.1210/en.2003-0669).
- Kato-Maeda, M et al. (2010). Differences among sublineages of the East-Asian lineage of *Mycobacterium tuberculosis* in genotypic clustering. *Int J Tuberc Lung Dis* 14.5, pp. 538–44.

- Kato-Maeda, Midori et al. (2012). Beijing sublineages of *Mycobacterium tuberculosis* differ in pathogenicity in the guinea pig. *Clin Vaccine Immunol* 19.8, pp. 1227–37. DOI: [10.1128/CVI.00250-12](https://doi.org/10.1128/CVI.00250-12).
- Kaufman, Adam C. et al. (2015). Fyn inhibition rescues established memory and synapse loss in Alzheimer mice. *Ann Neurol* 77.6, pp. 953–971. DOI: [10.1002/ana.24394](https://doi.org/10.1002/ana.24394).
- Kaufmann, Stefan HE et al. (2013). Inflammation in tuberculosis: interactions, imbalances and interventions. *Curr Opin Immunol* 25.4, pp. 441–449. DOI: [10.1016/j.coi.2013.05.005](https://doi.org/10.1016/j.coi.2013.05.005).
- Kaufmann, Stefan He et al. (2014). The BCG replacement vaccine VPM1002: from drawing board to clinical trial. *Expert Rev Vaccines* 13.5, pp. 619–30. DOI: [10.1586/14760584.2014.905746](https://doi.org/10.1586/14760584.2014.905746).
- Kawai, Taro et al. (2007). Signaling to NF- κ B by Toll-like receptors. *Trends Mol Med* 13.11, pp. 460–469. DOI: [10.1016/j.molmed.2007.09.002](https://doi.org/10.1016/j.molmed.2007.09.002).
- Kawasaki, Takumi et al. (2014). Toll-like receptor signaling pathways. *Front Immunol* 5, p. 461. DOI: [10.3389/fimmu.2014.00461](https://doi.org/10.3389/fimmu.2014.00461).
- Kayagaki, N. et al. (2013). Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* 341.6151, pp. 1246–1249. DOI: [10.1126/science.1240248](https://doi.org/10.1126/science.1240248).
- Kayagaki, N et al. (2015). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* 526.7575, pp. 666–671. DOI: [10.1038/nature15541](https://doi.org/10.1038/nature15541).
- Kayagaki, Nobuhiko et al. (2011). Non-canonical inflammasome activation targets caspase-11. *Nature* 479.7371, pp. 117–121. DOI: [10.1038/nature10558](https://doi.org/10.1038/nature10558).
- Keane, J et al. (2000). Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *J Immunol* 164.4, pp. 2016–20.
- Keane, J et al. (2001). Tuberculosis associated with infliximab, a tumor necrosis factor α -neutralizing agent. *N Engl J Med* 345.15, pp. 1098–1104. DOI: [10.1056/NEJMoa011110](https://doi.org/10.1056/NEJMoa011110).
- Khalifa, Reham A. et al. (2013). Resazurin microtiter assay plate method for detection of susceptibility of multidrug resistant *Mycobacterium tuberculosis* to second-line anti-tuberculous drugs. *Egypt J Chest Dis Tuberc* 62.2, pp. 241–247. DOI: [10.1016/j.ejcdt.2013.05.008](https://doi.org/10.1016/j.ejcdt.2013.05.008).
- Khan, Faiz A et al. (2010). Treatment of active tuberculosis in HIV-coinfected patients: a systematic review and meta-analysis. *Clin Infect Dis* 50.9, pp. 1288–99. DOI: [10.1086/651686](https://doi.org/10.1086/651686).
- Khare, Sonal et al. (2012). An NLRP7-containing inflammasome mediates recognition of microbial lipopeptides in human macrophages. *Immunity* 36.3, pp. 464–476. DOI: [10.1016/j.immuni.2012.02.001](https://doi.org/10.1016/j.immuni.2012.02.001).
- Kim, B.-H. et al. (2011). A Family of IFN- γ -Inducible 65-kD GTPases Protects Against Bacterial Infection. *Science* 332.6030, pp. 717–721. DOI: [10.1126/science.1201711](https://doi.org/10.1126/science.1201711).
- Kim, Hyae Young et al. (2001). Thoracic sequelae and complications of tuberculosis. *Radiographics* 21.4, pp. 839–858. DOI: [10.1148/radiographics.21.4.g01j106839](https://doi.org/10.1148/radiographics.21.4.g01j106839).

- Kim, Jwa-Jin et al. (2012). Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action. *Cell Host Microbe* 11.5, pp. 457–468. DOI: [10.1016/j.chom.2012.03.008](https://doi.org/10.1016/j.chom.2012.03.008).
- Kim, Sarah et al. (2010). *Listeria monocytogenes* is sensed by the NLRP3 and AIM2 inflammasome. *Eur J Immunol* 40, pp. 1545–1551. DOI: [10.1002/eji.201040425](https://doi.org/10.1002/eji.201040425).
- Kim, Young Keun et al. (2016). NOD-like receptors in infection, immunity, and diseases. *Yonsei Med J* 57.1, p. 5. DOI: [10.3349/ymj.2016.57.1.5](https://doi.org/10.3349/ymj.2016.57.1.5).
- Kiran, Dilara et al. (2016). Host-directed therapy targeting the *Mycobacterium tuberculosis* granuloma: a review. *Semin Immunopathol* 38.2, pp. 167–83. DOI: [10.1007/s00281-015-0537-x](https://doi.org/10.1007/s00281-015-0537-x).
- Kleinnijenhuis, Johanneke et al. (2011). Innate immune recognition of *Mycobacterium tuberculosis*. *Clin Dev Immunol* 2011, pp. 1–12. DOI: [10.1155/2011/405310](https://doi.org/10.1155/2011/405310).
- Kofoed, Eric M et al. (2011). Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477.7366, pp. 592–5. DOI: [10.1038/nature10394](https://doi.org/10.1038/nature10394).
- Kolonko, Margot et al. (2014). WASH-driven actin polymerization is required for efficient mycobacterial phagosome maturation arrest. *Cell Microbiol* 16.2, pp. 232–46. DOI: [10.1111/cmi.12217](https://doi.org/10.1111/cmi.12217).
- Koo, Ingrid C. et al. (2008). ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection. *Cell Microbiol* 10.9, pp. 1866–1878. DOI: [10.1111/j.1462-5822.2008.01177.x](https://doi.org/10.1111/j.1462-5822.2008.01177.x).
- Koo, M S et al. (2011). Phosphodiesterase 4 inhibition reduces innate immunity and improves isoniazid clearance of *Mycobacterium tuberculosis* in the lungs of infected mice. *PLoS One* 6.2, e17091. DOI: [10.1371/journal.pone.0017091](https://doi.org/10.1371/journal.pone.0017091).
- Koo, Mi-Sun et al. (2012). Strain specific transcriptional response in *Mycobacterium tuberculosis* infected macrophages. *Cell Commun Signal* 10.1, p. 2. DOI: [10.1186/1478-811X-10-2](https://doi.org/10.1186/1478-811X-10-2).
- Kortmann, Jens et al. (2015). Cutting edge: inflammasome activation in primary human macrophages is dependent on flagellin. *J Immunol* 195.3, pp. 815–819. DOI: [10.4049/jimmunol.1403100](https://doi.org/10.4049/jimmunol.1403100).
- Krishnan, N et al. (2013). Pathways of IL-1 β secretion by macrophages infected with clinical *Mycobacterium tuberculosis* strains. *Tuberculosis (Edinb)* 93.5, pp. 538–547. DOI: [10.1016/j.tube.2013.05.002](https://doi.org/10.1016/j.tube.2013.05.002).
- Krishnan, Nitya et al. (2011). *Mycobacterium tuberculosis* lineage influences innate immune response and virulence and is associated with distinct cell envelope lipid profiles. *PLoS One* 6.9, e23870. DOI: [10.1371/journal.pone.0023870](https://doi.org/10.1371/journal.pone.0023870).
- Kubica, G. P. et al. (1972). Designation of strain H37Rv as the neotype of *Mycobacterium tuberculosis*. *Int J Syst Bacteriol* 22.2, pp. 99–106. DOI: [10.1099/00207713-22-2-99](https://doi.org/10.1099/00207713-22-2-99).
- Kyrmizi, I. et al. (2013). Corticosteroids block autophagy protein recruitment in *Aspergillus fumigatus* phagosomes via targeting Dectin-1/Syk kinase signaling. *J Immunol* 191.3, pp. 1287–1299. DOI: [10.4049/jimmunol.1300132](https://doi.org/10.4049/jimmunol.1300132).

- Laarhoven, Arjan van et al. (2013). Low induction of proinflammatory cytokines parallels evolutionary success of modern strains within the *Mycobacterium tuberculosis* Beijing genotype. *Infect Immun* 81.10, pp. 3750–6. DOI: [10.1128/IAI.00282-13](https://doi.org/10.1128/IAI.00282-13).
- Lai, Rachel P. J. et al. (2013). The immunopathogenesis of the HIV tuberculosis immune reconstitution inflammatory syndrome. *Eur J Immunol* 43.8, pp. 1995–2002. DOI: [10.1002/eji.201343632](https://doi.org/10.1002/eji.201343632).
- Lai, Rachel P J et al. (2015). HIV-tuberculosis-associated immune reconstitution inflammatory syndrome is characterized by Toll-like receptor and inflammasome signalling. *Nat Commun* 6, p. 8451. DOI: [10.1038/ncomms9451](https://doi.org/10.1038/ncomms9451).
- Lam, C W et al. (2005). *In vitro* and *in vivo* induction of heme oxygenase 1 in mouse macrophages following melanocortin receptor activation. *J Immunol* 174.4, pp. 2297–2304.
- Lam, C W et al. (2006). Melanocortin receptor signaling in RAW264.7 macrophage cell line. *Peptides* 27.2, pp. 404–412. DOI: [10.1016/j.peptides.2005.01.031](https://doi.org/10.1016/j.peptides.2005.01.031).
- Lamkanfi, M et al. (2002). Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ* 9.4, pp. 358–361. DOI: [10.1038/sj/cdd/4400989](https://doi.org/10.1038/sj/cdd/4400989).
- LaRock, Christopher N et al. (2016). IL-1 β is an innate immune sensor of microbial proteolysis. *Sci Immunol* 1.2, eaah3539. DOI: [10.1126/sciimmunol.aah3539](https://doi.org/10.1126/sciimmunol.aah3539).
- Larsen, Rasmus et al. (2010). A central role for free heme in the pathogenesis of severe sepsis. *Sci Transl Med* 2.51.
- Layoun, Antonio et al. (2015). Isolation of murine peritoneal macrophages to carry out gene expression analysis upon Toll-like receptors stimulation. *J Vis Exp* 98, e52749. DOI: [10.3791/52749](https://doi.org/10.3791/52749).
- Lazear, Helen M. et al. (2013). IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling. *PLoS Pathog* 9.1, e1003118. DOI: [10.1371/journal.ppat.1003118](https://doi.org/10.1371/journal.ppat.1003118).
- Lee, Hye-Mi et al. (2012). *Mycobacterium abscessus* activates the NLRP3 inflammasome via Dectin-1–Syk and p62/SQSTM1. *Immunol Cell Biol* 90.6, pp. 601–610. DOI: [10.1038/icb.2011.72](https://doi.org/10.1038/icb.2011.72).
- Lee, Jinhee et al. (2006). Macrophage apoptosis in response to high intracellular burden of *Mycobacterium tuberculosis* is mediated by a novel caspase-independent pathway. *J Immunol* 176.7, pp. 4267–74.
- Lee, Jinhee et al. (2011). *Mycobacterium tuberculosis* induces an atypical cell death mode to escape from infected macrophages. *PLoS One* 6.3, e18367. DOI: [10.1371/journal.pone.0018367](https://doi.org/10.1371/journal.pone.0018367).
- Legrand-Poels, Sylvie et al. (2014). Free fatty acids as modulators of the NLRP3 inflammasome in obesity/type 2 diabetes. *Biochem Pharmacol* 92.1, pp. 131–41. DOI: [10.1016/j.bcp.2014.08.013](https://doi.org/10.1016/j.bcp.2014.08.013).
- Leisching, Gina et al. (2016). The host response to a clinical MDR mycobacterial strain cultured in a detergent-free environment: a global transcriptomics approach. *PLoS One* 11.4, e0153079. DOI: [10.1371/journal.pone.0153079](https://doi.org/10.1371/journal.pone.0153079).

- Levinsohn, Jonathan L. et al. (2012). Anthrax lethal factor cleavage of Nlrp1 is required for activation of the inflammasome. *PLoS Pathog* 8.3, e1002638. DOI: [10.1371/journal.ppat.1002638](https://doi.org/10.1371/journal.ppat.1002638).
- Li, Dan et al. (2015). SIGIRR participates in negative regulation of LPS response and tolerance in human bladder epithelial cells. *BMC Immunol* 16.1, p. 73. DOI: [10.1186/s12865-015-0137-5](https://doi.org/10.1186/s12865-015-0137-5).
- Li, Dayu et al. (2008). Diminishment of α -MSH anti-inflammatory activity in MC1r siRNA-transfected RAW264.7 macrophages. *J Leukoc Biol* 84.1, pp. 191–8. DOI: [10.1189/jlb.0707463](https://doi.org/10.1189/jlb.0707463).
- Liang, Jiancong et al. (2017). Novel Nlrp4 mutation causes a syndrome of perinatal autoinflammation with hemophagocytic lymphohistiocytosis, hepatosplenomegaly, fetal thrombotic vasculopathy, and congenital anemia and ascites. *Pediatr Dev Pathol*, p. 1093526616686890. DOI: [10.1177/1093526616686890](https://doi.org/10.1177/1093526616686890).
- Liao, Huanhuan et al. (2014). Effects of long-term serial cell passaging on cell spreading, migration, and cell-surface ultrastructures of cultured vascular endothelial cells. *Cytotechnology* 66.2, pp. 229–38. DOI: [10.1007/s10616-013-9560-8](https://doi.org/10.1007/s10616-013-9560-8).
- Liao, K.-C. et al. (2013). Activation of the Nlrp1b inflammasome by reduction of cytosolic ATP. *Infect Immun* 81.2, pp. 570–579. DOI: [10.1128/IAI.01003-12](https://doi.org/10.1128/IAI.01003-12).
- Lin, Kan et al. (2016). *Mycobacterium tuberculosis* thioredoxin reductase is essential for thiol redox homeostasis but plays a minor role in antioxidant defense. *PLoS Pathog* 12.6, e1005675. DOI: [10.1371/journal.ppat.1005675](https://doi.org/10.1371/journal.ppat.1005675).
- Lin, Keng-Mean et al. (2014). IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. *Proc Natl Acad Sci U S A* 111.2, pp. 775–80. DOI: [10.1073/pnas.1320294111](https://doi.org/10.1073/pnas.1320294111).
- Ling, Guang Sheng et al. (2014). Integrin CD11b positively regulates TLR4-induced signalling pathways in dendritic cells but not in macrophages. *Nat Commun* 5, pp. 709–712. DOI: [10.1038/ncomms4039](https://doi.org/10.1038/ncomms4039).
- Lipton, J M (1990). Modulation of host defense by the neuropeptide α -MSH. *Yale J Biol Med* 63.2, pp. 173–82.
- Lipton, J M et al. (1991). Central administration of the peptide α -MSH inhibits inflammation in the skin. *Peptides* 12.4, pp. 795–8.
- Liu, Chunfa et al. (2016a). AIM2 inhibits autophagy and IFN- β production during *M. bovis* infection. *Oncotarget* 7.30, pp. 46972–46987. DOI: [10.18632/oncotarget.10503](https://doi.org/10.18632/oncotarget.10503).
- Liu, Ke-Jun et al. (2013). Saracatinib (AZD0530) is a potent modulator of ABCB1-mediated multidrug resistance *in vitro* and *in vivo*. *Int J Cancer* 132.1, pp. 224–35. DOI: [10.1002/ijc.27649](https://doi.org/10.1002/ijc.27649).
- Liu, Philip T et al. (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 311.5768, pp. 1770–3. DOI: [10.1126/science.1123933](https://doi.org/10.1126/science.1123933).
- Liu, Xing et al. (2016b). Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* 535.7610, pp. 153–158. DOI: [10.1038/nature18629](https://doi.org/10.1038/nature18629).

- Lopez-Castejon, Gloria et al. (2011). Understanding the mechanism of IL-1 β secretion. *Cytokine Growth Factor Rev* 22.4, pp. 189–195. DOI: [10.1016/j.cytogfr.2011.10.001](https://doi.org/10.1016/j.cytogfr.2011.10.001).
- López-Castejón, Gloria et al. (2012). Current status of inflammasome blockers as anti-inflammatory drugs. *Expert Opin Investig Drugs* 21.7, pp. 995–1007. DOI: [10.1517/13543784.2012.690032](https://doi.org/10.1517/13543784.2012.690032).
- Lowe, David M. et al. (2012). Neutrophils in tuberculosis: friend or foe? *Trends Immunol* 33.1, pp. 14–25. DOI: [10.1016/j.it.2011.10.003](https://doi.org/10.1016/j.it.2011.10.003).
- Lu, Ben et al. (2012). Novel role of PKR in inflammasome activation and HMGB1 release. *Nature* 488.7413, pp. 670–674. DOI: [10.1038/nature11290](https://doi.org/10.1038/nature11290).
- Lu, G. et al. (2014). The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science* 343.6168, pp. 305–309. DOI: [10.1126/science.1244917](https://doi.org/10.1126/science.1244917).
- Luca, Antonella de et al. (2014). IL-1 receptor blockade restores autophagy and reduces inflammation in chronic granulomatous disease in mice and in humans. *Proc Natl Acad Sci U S A* 111.9, pp. 3526–3531. DOI: [10.1073/pnas.1322831111](https://doi.org/10.1073/pnas.1322831111).
- Lyadova, Irina V et al. (2010). In mice, tuberculosis progression is associated with intensive inflammatory response and the accumulation of Gr-1 cells in the lungs. *PLoS One* 5.5, e10469. DOI: [10.1371/journal.pone.0010469](https://doi.org/10.1371/journal.pone.0010469).
- Ma, J. et al. (2014). Tuberculosis antigen-induced expression of IFN- α in tuberculosis patients inhibits production of IL-1 β . *FASEB J* 28.7, pp. 3238–3248. DOI: [10.1096/fj.13-247056](https://doi.org/10.1096/fj.13-247056).
- Maartens, Gary et al. (2015). Linezolid for treating tuberculosis: a delicate balancing act. *EBioMedicine* 2.11, pp. 1568–9. DOI: [10.1016/j.ebiom.2015.10.014](https://doi.org/10.1016/j.ebiom.2015.10.014).
- Maaser, C et al. (2006). Crucial role of the melanocortin receptor MC1R in experimental colitis. *Gut* 55.10, pp. 1415–22. DOI: [10.1136/gut.2005.083634](https://doi.org/10.1136/gut.2005.083634).
- Madhuri et al. (2009). *In vitro* antimicrobial activity of α -melanocyte stimulating hormone against major human pathogen *Staphylococcus aureus*. *Peptides* 30.9, pp. 1627–1635. DOI: [10.1016/j.peptides.2009.06.020](https://doi.org/10.1016/j.peptides.2009.06.020).
- Mahamed, Deeqa et al. (2017). Intracellular growth of *Mycobacterium tuberculosis* after macrophage cell death leads to serial killing of host cells. *Elife* 6, e22028. DOI: [10.7554/eLife.22028](https://doi.org/10.7554/eLife.22028).
- Maiga, Mamoudou et al. (2012). Successful shortening of tuberculosis treatment using adjuvant host-directed therapy with FDA-approved phosphodiesterase inhibitors in the mouse model. *PLoS One* 7.2, e30749. DOI: [10.1371/journal.pone.0030749](https://doi.org/10.1371/journal.pone.0030749).
- Maiga, Mamoudou et al. (2015). Efficacy of adjunctive tofacitinib therapy in mouse models of tuberculosis. *EBioMedicine* 2.8, pp. 866–71. DOI: [10.1016/j.ebiom.2015.07.014](https://doi.org/10.1016/j.ebiom.2015.07.014).
- Malherbe, Stephanus T et al. (2016). Persisting positron emission tomography lesion activity and *Mycobacterium tuberculosis* mRNA after tuberculosis cure. *Nat Med* 22.10, pp. 1094–1100. DOI: [10.1038/nm.4177](https://doi.org/10.1038/nm.4177).

- Man, Si Ming et al. (2015). Regulation of inflammasome activation. *Immunol Rev* 265.1, pp. 6–21. DOI: [10.1111/imr.12296](https://doi.org/10.1111/imr.12296).
- Manca, Claudia et al. (2005). Hypervirulent *M. tuberculosis* W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. *J Interferon Cytokine Res* 25.11, pp. 694–701. DOI: [10.1089/jir.2005.25.694](https://doi.org/10.1089/jir.2005.25.694).
- Manca, Claudia et al. (2013). Host targeted activity of pyrazinamide in *Mycobacterium tuberculosis* infection. *PLoS One* 8.8, e74082. DOI: [10.1371/journal.pone.0074082](https://doi.org/10.1371/journal.pone.0074082).
- Mandrika, I et al. (2001). Effects of melanocortin peptides on lipopolysaccharide/interferon- γ -induced NF- κ B DNA binding and nitric oxide production in macrophage-like RAW 264.7 cells: evidence for dual mechanisms of action. *Biochem Pharmacol* 61.5, pp. 613–21.
- Manna, S K et al. (1998). α -melanocyte-stimulating hormone inhibits the nuclear transcription factor NF- κ B activation induced by various inflammatory agents. *J Immunol* 161.6, pp. 2873–2880.
- Mantovani, Alberto et al. (2008). Cancer-related inflammation. *Nature* 454.7203, pp. 436–444. DOI: [10.1038/nature07205](https://doi.org/10.1038/nature07205).
- Marais, Suzaan et al. (2017). Inflammasome activation underlying central nervous system deterioration in HIV-associated tuberculosis. *J Infect Dis* 215.5, pp. 677–686. DOI: [10.1093/infdis/jiw561](https://doi.org/10.1093/infdis/jiw561).
- Marakalala, Mohlopheni J et al. (2016). Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nat Med* 22.5, pp. 531–538. DOI: [10.1038/nm.4073](https://doi.org/10.1038/nm.4073).
- Marakalala, Mohlopheni Jackson et al. (2010). The role of Syk/CARD9-coupled C-type lectin receptors in immunity to *Mycobacterium tuberculosis* infections. *Clin Dev Immunol* 2010, p. 567571. DOI: [10.1155/2010/567571](https://doi.org/10.1155/2010/567571).
- Mariathasan, Sanjeev et al. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430.6996, pp. 213–218. DOI: [10.1038/nature02664](https://doi.org/10.1038/nature02664).
- Marquina-Castillo, Brenda et al. (2009). Virulence, immunopathology and transmissibility of selected strains of *Mycobacterium tuberculosis* in a murine model. *Immunology* 128.1, pp. 123–33. DOI: [10.1111/j.1365-2567.2008.03004.x](https://doi.org/10.1111/j.1365-2567.2008.03004.x).
- Martel-Gallegos, Guadalupe et al. (2013). Oxidative stress induced by P2X7 receptor stimulation in murine macrophages is mediated by c-Src/Pyk2 and ERK1/2. *Biochim Biophys Acta* 1830.10, pp. 4650–4659. DOI: [10.1016/j.bbagen.2013.05.023](https://doi.org/10.1016/j.bbagen.2013.05.023).
- Martineau, A R et al. (2007). A single dose of vitamin D enhances immunity to mycobacteria. *Am J Respir Crit Care Med* 176.2, pp. 208–213. DOI: [10.1164/rccm.200701-0070C](https://doi.org/10.1164/rccm.200701-0070C).
- Martineau, Adrian R et al. (2011). High-dose vitamin D₃ during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *Lancet* 377.9761, pp. 242–50. DOI: [10.1016/S0140-6736\(10\)61889-2](https://doi.org/10.1016/S0140-6736(10)61889-2).
- Martinon, Fabio et al. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL- β . *Mol Cell* 10.2, pp. 417–26.

- Masman, Marcelo F. et al. (2006). Synthesis and conformational analysis of His-Phe-Arg-Trp-NH₂ and analogues with antifungal properties. *Bioorg Med Chem* 14.22, pp. 7604–7614. DOI: [10.1016/j.bmc.2006.07.007](https://doi.org/10.1016/j.bmc.2006.07.007).
- Master, Sharon S. et al. (2008). *Mycobacterium tuberculosis* Prevents Inflammasome Activation. *Cell Host and Microbe* 3.4, pp. 224–232. DOI: [10.1016/j.chom.2008.03.003](https://doi.org/10.1016/j.chom.2008.03.003).
- Masters, Seth L et al. (2012). NLRP1 inflammasome activation induces pyroptosis of hematopoietic progenitor cells. *Immunity* 37.6, pp. 1009–23. DOI: [10.1016/j.immuni.2012.08.027](https://doi.org/10.1016/j.immuni.2012.08.027).
- Mastrocola, Raffaella et al. (2016). Pharmacological Inhibition of NLRP3 Inflammasome Attenuates Myocardial Ischemia/Reperfusion Injury by Activation of RISK and Mitochondrial Pathways. *Oxid Med Cell Longev* 2016, pp. 1–11. DOI: [10.1155/2016/5271251](https://doi.org/10.1155/2016/5271251).
- Matusiak, Magdalena et al. (2015). Flagellin-induced NLRC4 phosphorylation primes the inflammasome for activation by NAIP5. *Proc Natl Acad Sci U S A* 112.5, pp. 1541–6. DOI: [10.1073/pnas.1417945112](https://doi.org/10.1073/pnas.1417945112).
- Maya-Mendoza, A et al. (2014). Immortalised breast epithelia survive prolonged DNA replication stress and return to cycle from a senescent-like state. *Cell Death Dis* 5.7, e1351–e1351. DOI: [10.1038/cddis.2014.315](https://doi.org/10.1038/cddis.2014.315).
- Mayer-Barber, K D et al. (2010). Caspase-1 independent IL-1 β production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling *in vivo*. *J Immunol* 184.7, pp. 3326–3330. DOI: [10.4049/jimmunol.0904189](https://doi.org/10.4049/jimmunol.0904189).
- Mayer-Barber, K D et al. (2014). Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature* 511.7507, pp. 99–103. DOI: [10.1038/nature13489](https://doi.org/10.1038/nature13489).
- Mayer-Barber, Katrin D et al. (2016). Clash of the cytokine titans: counter-regulation of interleukin-1 and type I interferon-mediated inflammatory responses. *Cell Mol Immunol*. DOI: [10.1038/cmi.2016.25](https://doi.org/10.1038/cmi.2016.25).
- McElvania Tekippe, Erin et al. (2010). Granuloma formation and host defense in chronic *Mycobacterium tuberculosis* infection requires PYCARD/ASC but not NLRP3 or caspase-1. *PLoS One* 5.8, e12320. DOI: [10.1371/journal.pone.0012320](https://doi.org/10.1371/journal.pone.0012320).
- McNab, Finlay et al. (2015). Type I interferons in infectious disease. *Nat Rev Immunol* 15.2, pp. 87–103. DOI: [10.1038/nri3787](https://doi.org/10.1038/nri3787).
- Meissner, Felix et al. (2010). Inflammasome activation in NADPH oxidase defective mononuclear phagocytes from patients with chronic granulomatous disease. *Blood* 116.9, pp. 1570–3. DOI: [10.1182/blood-2010-01-264218](https://doi.org/10.1182/blood-2010-01-264218).
- Merker, Matthias et al. (2015). Evolutionary history and global spread of the *Mycobacterium tuberculosis* Beijing lineage. *Nat Genet* 47.3, pp. 242–249. DOI: [10.1038/ng.3195](https://doi.org/10.1038/ng.3195).
- Mertens, Marty et al. (2009). Anakinra for rheumatoid arthritis. *Cochrane Database Syst Rev* 1, p. CD005121. DOI: [10.1002/14651858.CD005121.pub3](https://doi.org/10.1002/14651858.CD005121.pub3).

- Meunier, Etienne et al. (2014). Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature* 509.7500, pp. 366–70. DOI: [10.1038/nature13157](https://doi.org/10.1038/nature13157).
- Meunier, Etienne et al. (2015). Guanylate-binding proteins promote activation of the AIM2 inflammasome during infection with *Francisella tularensis*. *Nat Immunol* 16.5, pp. 476–484. DOI: [10.1038/ni.3119](https://doi.org/10.1038/ni.3119).
- Miao, Edward A et al. (2011). Caspase-1-induced pyroptotic cell death. *Immunol Rev* 243.1, pp. 206–14. DOI: [10.1111/j.1600-065X.2011.01044.x](https://doi.org/10.1111/j.1600-065X.2011.01044.x).
- Minguet, Susana et al. (2005). Adenosine and cAMP are potent inhibitors of the NF- κ B pathway downstream of immunoreceptors. *Eur J Immunol* 35.1, pp. 31–41. DOI: [10.1002/eji.200425524](https://doi.org/10.1002/eji.200425524).
- Mishra, Bibhuti B. et al. (2010). *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. *Cell Microbiol* 12.8, pp. 1046–1063. DOI: [10.1111/j.1462-5822.2010.01450.x](https://doi.org/10.1111/j.1462-5822.2010.01450.x).
- Mishra, Bibhuti B et al. (2013). Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1 β . *Nat Immunol* 14.1, pp. 52–60. DOI: [10.1038/ni.2474](https://doi.org/10.1038/ni.2474).
- Mitchison, D A (2000). Role of individual drugs in the chemotherapy of tuberculosis. *Int J Tuberc Lung Dis* 4.9, pp. 796–806.
- Mitoma, Hiroki et al. (2013). The DHX33 RNA helicase senses cytosolic RNA and activates the NLRP3 inflammasome. *Immunity* 39.1, pp. 123–35. DOI: [10.1016/j.immuni.2013.07.001](https://doi.org/10.1016/j.immuni.2013.07.001).
- Moayeri, Mahtab et al. (2010). Inflammasome sensor Nlrp1b-dependent resistance to anthrax is mediated by caspase-1, IL-1 signaling and neutrophil recruitment. *PLoS Pathog* 6.12, e1001222. DOI: [10.1371/journal.ppat.1001222](https://doi.org/10.1371/journal.ppat.1001222).
- Mogensen, Trine H (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* 22.2, 240–73, Table of Contents. DOI: [10.1128/CMR.00046-08](https://doi.org/10.1128/CMR.00046-08).
- Molina-Holgado, E et al. (2000). Induction of COX-2 and PGE₂ biosynthesis by IL-1 β is mediated by PKC and mitogen-activated protein kinases in murine astrocytes. *Br J Pharmacol* 131.1, pp. 152–9. DOI: [10.1038/sj.bjp.0703557](https://doi.org/10.1038/sj.bjp.0703557).
- Moltke, Jakob von et al. (2012). Rapid induction of inflammatory lipid mediators by the inflammasome *in vivo*. *Nature* 490.7418, pp. 107–111. DOI: [10.1038/nature11351](https://doi.org/10.1038/nature11351).
- Moltke, Jakob von et al. (2013). Recognition of bacteria by inflammasomes. *Annu Rev Immunol* 31.1, pp. 73–106. DOI: [10.1146/annurev-immunol-032712-095944](https://doi.org/10.1146/annurev-immunol-032712-095944).
- Montero-Melendez, T et al. (2011). The melanocortin agonist AP214 exerts anti-inflammatory and proresolving properties. *Am J Pathol* 179.1, pp. 259–269. DOI: [10.1016/j.ajpath.2011.03.042](https://doi.org/10.1016/j.ajpath.2011.03.042).
- Montero-Melendez, Trinidad et al. (2015). Biased agonism as a novel strategy to harness the proresolving properties of melanocortin receptors without eliciting melanogenic effects. *J Immunol* 194.7, pp. 3381–3388. DOI: [10.4049/jimmunol.1402645](https://doi.org/10.4049/jimmunol.1402645).

- Moreira, A L et al. (1997). Effect of cytokine modulation by thalidomide on the granulomatous response in murine tuberculosis. *Tuber Lung Dis* 78.1, pp. 47–55.
- Moriwaki, Kenta et al. (2015). A RIPK3-caspase 8 complex mediates atypical pro-IL-1 β processing. *J Immunol* 194.4, pp. 1938–44. DOI: [10.4049/jimmunol.1402167](https://doi.org/10.4049/jimmunol.1402167).
- Morrone, Seamus R et al. (2014). Cooperative assembly of IFI16 filaments on dsDNA provides insights into host defense strategy. *Proc Natl Acad Sci U S A* 111.1, E62–71. DOI: [10.1073/pnas.1313577111](https://doi.org/10.1073/pnas.1313577111).
- Morrone, Seamus R. et al. (2015). Assembly-driven activation of the AIM2 foreign-dsDNA sensor provides a polymerization template for downstream ASC. *Nat Commun* 6, p. 7827. DOI: [10.1038/ncomms8827](https://doi.org/10.1038/ncomms8827).
- Mortaz, Esmail et al. (2015). Interaction of pattern recognition receptors with *Mycobacterium tuberculosis*. *J Clin Immunol* 35.1, pp. 1–10. DOI: [10.1007/s10875-014-0103-7](https://doi.org/10.1007/s10875-014-0103-7).
- Mortellaro, Alessandro et al. (2015). Novel perspectives on non-canonical inflammasome activation. *Immunotargets Ther* 4, p. 131. DOI: [10.2147/ITT.S57976](https://doi.org/10.2147/ITT.S57976).
- Motta, Vinicius et al. (2014). NOD-like receptors: versatile cytosolic sentinels. *Physiol Rev* 95.1, pp. 149–78. DOI: [10.1152/physrev.00009.2014](https://doi.org/10.1152/physrev.00009.2014).
- Mouriaux, Frédéric et al. (2016). Effects of long-term serial passaging on the characteristics and properties of cell lines derived from uveal melanoma primary tumors. *Invest Ophthalmol Vis Sci* 57.13, pp. 5288–5301. DOI: [10.1167/iovs.16-19317](https://doi.org/10.1167/iovs.16-19317).
- Muñoz-Planillo, Raúl et al. (2009). A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome. *J Immunol* 183.6, pp. 3942–8. DOI: [10.4049/jimmunol.0900729](https://doi.org/10.4049/jimmunol.0900729).
- Muñoz-Planillo, Raúl et al. (2013). K⁺ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38.6, pp. 1142–53. DOI: [10.1016/j.immuni.2013.05.016](https://doi.org/10.1016/j.immuni.2013.05.016).
- Nadiri, Amal et al. (2006). The inflammatory caspases: key players in the host response to pathogenic invasion and sepsis. *J Immunol* 177.7, pp. 4239–45.
- Nagai, Yoshinori et al. (2005). The radioprotective 105/MD-1 complex links TLR2 and TLR4/MD-2 in antibody response to microbial membranes. *J Immunol* 174.11, pp. 7043–9.
- Nakahira, Kiichi et al. (2011). Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* 12, pp. 222–30. DOI: [10.1038/ni.1980](https://doi.org/10.1038/ni.1980).
- Napier, Ruth J. et al. (2015). Low doses of imatinib induce myelopoiesis and enhance host anti-microbial immunity. *PLoS Pathog* 11.3, pp. 1–27. DOI: [10.1371/journal.ppat.1004770](https://doi.org/10.1371/journal.ppat.1004770).

- Neiman-Zenevich, Jana et al. (2014). Distinct regions of NLRP1B are required to respond to anthrax lethal toxin and metabolic inhibition. *Infect Immun* 82.9, pp. 3697–703. DOI: [10.1128/IAI.02167-14](https://doi.org/10.1128/IAI.02167-14).
- Nelson, Michael E. et al. (2012). Melanotan II injection resulting in systemic toxicity and rhabdomyolysis. *Clin Toxicol* 50.10, pp. 1169–1173. DOI: [10.3109/15563650.2012.740637](https://doi.org/10.3109/15563650.2012.740637).
- Netea, Mihai G et al. (2010). IL-1 β processing in host defense: beyond the inflammasomes. *PLoS Pathog* 6.2, e1000661. DOI: [10.1371/journal.ppat.1000661](https://doi.org/10.1371/journal.ppat.1000661).
- Ng, G Z et al. (2016). The MUC1 mucin specifically inhibits activation of the NLRP3 inflammasome. *Genes Immun* 17.3, pp. 203–206. DOI: [10.1038/gene.2016.10](https://doi.org/10.1038/gene.2016.10).
- Niles, Andrew L et al. (2008). Update on *in vitro* cytotoxicity assays for drug development. *Expert Opin Drug Discov* 3.6, pp. 655–669. DOI: [10.1517/17460441.3.6.655](https://doi.org/10.1517/17460441.3.6.655).
- Novikov, Aleksey et al. (2011). *Mycobacterium tuberculosis* triggers host type I IFN signaling to regulate IL-1 β production in human macrophages. *J Immunol* 187.5, pp. 2540–7. DOI: [10.4049/jimmunol.1100926](https://doi.org/10.4049/jimmunol.1100926).
- Nygaard, Haakon B et al. (2015). A phase Ib multiple ascending dose study of the safety, tolerability, and central nervous system availability of AZD0530 (saracatinib) in Alzheimer's disease. *Alzheimers Res Ther* 7.1, p. 35. DOI: [10.1186/s13195-015-0119-0](https://doi.org/10.1186/s13195-015-0119-0).
- O'Garra, Anne et al. (2013). The immune response in tuberculosis. *Annu Rev Immunol* 31, pp. 475–527. DOI: [10.1146/annurev-immunol-032712-095939](https://doi.org/10.1146/annurev-immunol-032712-095939).
- Okada, Masahiro et al. (2014). The lysosome rupture-activated TAK1-JNK pathway regulates NLRP3 inflammasome activation. *J Biol Chem* 289.47, pp. 32926–36. DOI: [10.1074/jbc.M114.579961](https://doi.org/10.1074/jbc.M114.579961).
- Ong, Catherine W M et al. (2014). Tuberculosis, pulmonary cavitation, and matrix metalloproteinases. *Am J Respir Crit Care Med* 190.1, pp. 9–18. DOI: [10.1164/rccm.201311-2106PP](https://doi.org/10.1164/rccm.201311-2106PP).
- O'Reilly, Philip J. et al. (2009). Neutrophils contain prolyl endopeptidase and generate the chemotactic peptide, PGP, from collagen. *J Neuroimmunol* 217.1-2, pp. 51–54. DOI: [10.1016/j.jneuroim.2009.09.020](https://doi.org/10.1016/j.jneuroim.2009.09.020).
- Orlowski, G. M. et al. (2015). Multiple cathepsins promote pro-IL-1 β synthesis and NLRP3-mediated IL-1 β activation. *J Immunol* 195.4, pp. 1685–1697. DOI: [10.4049/jimmunol.1500509](https://doi.org/10.4049/jimmunol.1500509).
- O'Sullivan, Mary P. et al. (2007). A caspase-independent pathway mediates macrophage cell death in response to *Mycobacterium tuberculosis* infection. *Infect Immun* 75.4, pp. 1984–1993. DOI: [10.1128/IAI.01107-06](https://doi.org/10.1128/IAI.01107-06).
- Ottani, Alessandra et al. (2013). Modulation of the JAK/ERK/STAT signaling in melanocortin-induced inhibition of local and systemic responses to myocardial ischemia/reperfusion. *Pharmacol Res* 72, pp. 1–8. DOI: [10.1016/j.phrs.2013.03.005](https://doi.org/10.1016/j.phrs.2013.03.005).

- Owolabi, Olumuyiwa et al. (2016). Elevated serum 25-hydroxy (OH) vitamin D levels are associated with risk of TB progression in Gambian adults. *Tuberculosis (Edinb)* 98, pp. 86–91. DOI: [10.1016/j.tube.2016.02.007](https://doi.org/10.1016/j.tube.2016.02.007).
- Ozaki, Ema et al. (2015). Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. *J Inflamm Res* 8, pp. 15–27. DOI: [10.2147/JIR.S51250](https://doi.org/10.2147/JIR.S51250).
- Ozinsky, A. et al. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc Natl Acad Sci U S A* 97.25, pp. 13766–13771. DOI: [10.1073/pnas.250476497](https://doi.org/10.1073/pnas.250476497).
- Palomino, J. C. et al. (2002). Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 46.8, pp. 2720–2722. DOI: [10.1128/AAC.46.8.2720](https://doi.org/10.1128/AAC.46.8.2720).
- Paramel Varghese, Geena et al. (2016). NLRP3 inflammasome expression and activation in human atherosclerosis. *J Am Heart Assoc* 5.5.
- Parry, G C et al. (1997). Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF- κ B-mediated transcription. *J Immunol* 159.11, pp. 5450–6.
- Pasquinelli, Virginia et al. (2004). Expression of signaling lymphocytic activation molecule-associated protein interrupts IFN- γ production in human tuberculosis. *J Immunol* 172.2, pp. 1177–85.
- Pathak, S. K. et al. (2005). *Mycobacterium tuberculosis* lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin-12 p40 production in macrophages. *J Biol Chem* 280.52, pp. 42794–42800. DOI: [10.1074/jbc.M506471200](https://doi.org/10.1074/jbc.M506471200).
- Pathak, Sushil Kumar et al. (2007). Direct extracellular interaction between the early secreted antigen ESAT-6 of *Mycobacterium tuberculosis* and TLR2 inhibits TLR signaling in macrophages. *Nat Immunol* 8.6, pp. 610–618. DOI: [10.1038/ni1468](https://doi.org/10.1038/ni1468).
- Pavillard, Luís E. et al. (2017). NLRP3-inflammasome inhibition prevents high fat and high sugar diets-induced heart damage through autophagy induction. *Oncotarget* 8.59, pp. 99740–99756. DOI: [10.18632/oncotarget.20763](https://doi.org/10.18632/oncotarget.20763).
- Pazyar, Nader et al. (2012). An overview of interleukin-1 receptor antagonist, anakinra, in the treatment of cutaneous diseases. *Curr Clin Pharmacol* 7.4, pp. 271–5.
- Pellegrini, Carolina et al. (2017). Canonical and non-canonical activation of NLRP3 inflammasome at the crossroad between immune tolerance and intestinal inflammation. *Front Immunol* 8, p. 36. DOI: [10.3389/fimmu.2017.00036](https://doi.org/10.3389/fimmu.2017.00036).
- Pereira, Milton et al. (2016). CARD9 negatively regulates NLRP3-induced IL-1 β production on *Salmonella* infection of macrophages. *Nat Commun* 7, p. 12874. DOI: [10.1038/ncomms12874](https://doi.org/10.1038/ncomms12874).
- Pérez, Esther et al. (2004). Molecular dissection of the role of two methyltransferases in the biosynthesis of phenolglycolipids and phthiocerol dimycoserolate in the *Mycobacterium tuberculosis* complex. *J Biol Chem* 279.41, pp. 42584–92. DOI: [10.1074/jbc.M406134200](https://doi.org/10.1074/jbc.M406134200).

- Perregaux, D G et al. (2001). Identification and characterization of a novel class of interleukin-1 post-translational processing inhibitors. *J Pharmacol Exp Ther* 299.1, pp. 187–97.
- Perroud, Bertrand et al. (2009). *In vivo* multiplex quantitative analysis of 3 forms of α melanocyte stimulating hormone in pituitary of prolyl endopeptidase deficient mice. *Mol Brain* 2, p. 14. DOI: [10.1186/1756-6606-2-14](https://doi.org/10.1186/1756-6606-2-14).
- Peters-Golden, Marc (2009). Putting on the brakes: cyclic AMP as a multipronged controller of macrophage function. *Sci Signal* 2.75, pe37. DOI: [10.1126/scisignal.275pe37](https://doi.org/10.1126/scisignal.275pe37).
- Petrilli, V et al. (2007). Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14.9, pp. 1583–1589. DOI: [10.1038/sj.cdd.4402195](https://doi.org/10.1038/sj.cdd.4402195).
- Pierce, Kristen L. et al. (2001). Classical and new roles of β -arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci* 2.10, pp. 727–733. DOI: [10.1038/35094577](https://doi.org/10.1038/35094577).
- Pilla, Danielle M et al. (2014). Guanylate binding proteins promote caspase-11-dependent pyroptosis in response to cytoplasmic LPS. *Proc Natl Acad Sci U S A* 111.16, pp. 6046–51. DOI: [10.1073/pnas.1321700111](https://doi.org/10.1073/pnas.1321700111).
- Poeck, Hendrik et al. (2010). SYK kinase signaling and the NLRP3 inflammasome in antifungal immunity. *J Mol Med* 88.8, pp. 745–752. DOI: [10.1007/s00109-010-0631-4](https://doi.org/10.1007/s00109-010-0631-4).
- Poli, V (1998). The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J Biol Chem* 273.45, pp. 29279–82.
- Portevin, Damien et al. (2011). Human macrophage responses to clinical isolates from the *Mycobacterium tuberculosis* complex discriminate between ancient and modern lineages. *PLoS Pathog* 7.3, e1001307. DOI: [10.1371/journal.ppat.1001307](https://doi.org/10.1371/journal.ppat.1001307).
- Portugal, Brina et al. (2017). *Mycobacterium tuberculosis* prolyl oligopeptidase induces *in vitro* secretion of proinflammatory cytokines by peritoneal macrophages. *Front Microbiol* 8, p. 155. DOI: [10.3389/fmicb.2017.00155](https://doi.org/10.3389/fmicb.2017.00155).
- Price, Nicholas M et al. (2003). Unopposed matrix metalloproteinase-9 expression in human tuberculous granuloma and the role of TNF- α -dependent monocyte networks. *J Immunol* 171.10, pp. 5579–86.
- Primiano, Michael J. et al. (2016). Efficacy and pharmacology of the NLRP3 inflammasome inhibitor CP-456,773 (CRID3) in murine models of dermal and pulmonary inflammation. *The Journal of Immunology* 197.6.
- Prosser, Gareth et al. (2017). The bacillary and macrophage response to hypoxia in tuberculosis and the consequences for T cell antigen recognition. *Microbes and Infection* 19.3, pp. 177–192. DOI: [10.1016/j.micinf.2016.10.001](https://doi.org/10.1016/j.micinf.2016.10.001).
- Public Health England (2013). *Tuberculosis (TB): annual notification and mortality data (1913 onwards)*. Tech. rep.
- Purkayastha, Sudarshana et al. (2011). Uncoupling the mechanisms of obesity and hypertension by targeting hypothalamic IKK- β and NF- κ B. *Nat Med* 17.7, pp. 883–7. DOI: [10.1038/nm.2372](https://doi.org/10.1038/nm.2372).

- Py, Bénédicte F et al. (2013). Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Mol Cell* 49.2, pp. 331–8. DOI: [10.1016/j.molcel.2012.11.009](https://doi.org/10.1016/j.molcel.2012.11.009).
- Qazi, Omar et al. (2013). Can microRNAs keep inflammasomes in check? *Front Genet* 4, p. 30. DOI: [10.3389/fgene.2013.00030](https://doi.org/10.3389/fgene.2013.00030).
- Qu, Yan et al. (2016). NLRP3 recruitment by NLRC4 during *Salmonella* infection. *J Exp Med*.
- Rajan, Jayant V et al. (2011). The NLRP3 inflammasome detects encephalomyocarditis virus and vesicular stomatitis virus infection. *J Virol* 85.9, pp. 4167–72. DOI: [10.1128/JVI.01687-10](https://doi.org/10.1128/JVI.01687-10).
- Rajaram, M. V. S. et al. (2010). *Mycobacterium tuberculosis* activates human macrophage peroxisome proliferator-activated receptor linking mannose receptor recognition to regulation of immune responses. *J Immunol* 185.2, pp. 929–942. DOI: [10.4049/jimmunol.1000866](https://doi.org/10.4049/jimmunol.1000866).
- Rajaram, Murugesan V.S. et al. (2014). Macrophage immunoregulatory pathways in tuberculosis. *Semin Immunol* 26.6, pp. 471–485. DOI: [10.1016/j.smim.2014.09.010](https://doi.org/10.1016/j.smim.2014.09.010).
- Rajora, N et al. (1996). α -MSH production, receptors, and influence on neopterin in a human monocyte/macrophage cell line. *J Leukoc Biol* 59.2, pp. 248–253.
- Ralph, Anna P. et al. (2013). L-arginine and vitamin d adjunctive therapies in pulmonary tuberculosis: a randomised, double-blind, placebo-controlled trial. *PLoS One* 8.8, e70032. DOI: [10.1371/journal.pone.0070032](https://doi.org/10.1371/journal.pone.0070032).
- Rao, Martin et al. (2013). The tuberculosis vaccine candidate Bacillus Calmette-Guérin $\Delta ureC::hly$ coexpressing Human interleukin-7 or -18 enhances antigen-specific T cell responses in mice. *PLoS One* 8.11, e78966. DOI: [10.1371/journal.pone.0078966](https://doi.org/10.1371/journal.pone.0078966).
- Rauch, I et al. (2009). Anti-*Candida* activity of α -melanocyte-stimulating hormone (α -MSH) peptides. *J Leukoc Biol* 85.3, pp. 371–2. DOI: [10.1189/jlb.1008614](https://doi.org/10.1189/jlb.1008614).
- Rauch, Isabella et al. (2016). NAIP proteins are required for cytosolic detection of specific bacterial ligands *in vivo*. *J Exp Med* 213.5, pp. 657–65. DOI: [10.1084/jem.20151809](https://doi.org/10.1084/jem.20151809).
- Reiling, Norbert et al. (2002). Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. *J Immunol* 169.7, pp. 3480–3484. DOI: [10.4049/jimmunol.169.7.3480](https://doi.org/10.4049/jimmunol.169.7.3480).
- Reiling, Norbert et al. (2013). Clade-specific virulence patterns of *Mycobacterium tuberculosis* complex strains in human primary macrophages and aerogenically infected mice. *MBio* 4.4, e00250–13. DOI: [10.1128/mBio.00250-13](https://doi.org/10.1128/mBio.00250-13).
- Restrepo, Blanca I. (2016). Metformin: candidate host-directed therapy for tuberculosis in diabetes and non-diabetes patients. *Tuberculosis* 101, S69–S72. DOI: [10.1016/j.tube.2016.09.008](https://doi.org/10.1016/j.tube.2016.09.008).
- Restrepo, Blanca I. et al. (2014). Phagocytosis via complement or Fc- γ receptors is compromised in monocytes from type 2 diabetes patients with chronic hyperglycemia. *PLoS One* 9.3, e92977. DOI: [10.1371/journal.pone.0092977](https://doi.org/10.1371/journal.pone.0092977).

- Richmond, Jillian M. et al. (2012). Mannose-capped lipoarabinomannan from *Mycobacterium tuberculosis* preferentially inhibits sphingosine-1-phosphate-induced migration of Th1 cells. *The Journal of Immunology* 189.12.
- Risco, Ana et al. (2012). p38 γ and p38 δ kinases regulate the Toll-like receptor 4 (TLR4)-induced cytokine production by controlling ERK1/2 protein kinase pathway activation. *Proc Natl Acad Sci U S A* 109.28, pp. 11200–5. DOI: [10.1073/pnas.1207290109](https://doi.org/10.1073/pnas.1207290109).
- Riza, Anca Lelia et al. (2014). Clinical management of concurrent diabetes and tuberculosis and the implications for patient services. *Lancet Diabetes Endocrinol* 2.9, pp. 740–53. DOI: [10.1016/S2213-8587\(14\)70110-X](https://doi.org/10.1016/S2213-8587(14)70110-X).
- Rode, Anna K O et al. (2017). Vitamin D counteracts *Mycobacterium tuberculosis*-induced cathelicidin downregulation in dendritic cells and allows Th1 differentiation and IFN γ secretion. *Front Immunol* 8, p. 656. DOI: [10.3389/fimmu.2017.00656](https://doi.org/10.3389/fimmu.2017.00656).
- Romberg, Neil et al. (2014). Mutation of NLRC4 causes a syndrome of enterocolitis and autoinflammation. *Nat Genet* 46.10, pp. 1135–1139. DOI: [10.1038/ng.3066](https://doi.org/10.1038/ng.3066).
- Romero, María M. et al. (2012). Clinical isolates of *Mycobacterium tuberculosis* differ in their ability to induce respiratory burst and apoptosis in neutrophils as a possible mechanism of immune escape. *Clin Dev Immunol* 2012, pp. 1–11. DOI: [10.1155/2012/152546](https://doi.org/10.1155/2012/152546).
- Rothfuchs, Antonio Gigliotti et al. (2007). Dectin-1 interaction with *Mycobacterium tuberculosis* leads to enhanced IL-12p40 production by splenic dendritic cells. *J Immunol* 179.6, pp. 3463–71.
- Roux, Anne-Laure et al. (2011). Overexpression of proinflammatory TLR-2-signalling lipoproteins in hypervirulent mycobacterial variants. *Cell Microbiol* 13.5, pp. 692–704. DOI: [10.1111/j.1462-5822.2010.01565.x](https://doi.org/10.1111/j.1462-5822.2010.01565.x).
- Rovetta, Ana I et al. (2014). IFN γ -mediated immune responses enhance autophagy against *Mycobacterium tuberculosis* antigens in patients with active tuberculosis. *Autophagy* 10.12, pp. 2109–21. DOI: [10.4161/15548627.2014.981791](https://doi.org/10.4161/15548627.2014.981791).
- Roy, A et al. (2014). Effect of BCG vaccination against *Mycobacterium tuberculosis* infection in children: systematic review and meta-analysis. *BMJ* 349.
- Ru, Yusha et al. (2016). α -Melanocyte-stimulating hormone ameliorates ocular surface dysfunctions and lesions in a scopolamine-induced dry eye model via PKA-CREB and MEK-Erk pathways. *Sci Rep* 5.1, p. 18619. DOI: [10.1038/srep18619](https://doi.org/10.1038/srep18619).
- Russell, Ryan C. et al. (2013). ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat Cell Biol* 15.7, pp. 741–750. DOI: [10.1038/ncb2757](https://doi.org/10.1038/ncb2757).
- Sadatom, Daichi et al. (2017). Mitochondrial function is required for extracellular ATP-induced NLRP3 inflammasome activation. *J Biochem* 161.6, pp. 503–12. DOI: [10.1093/jb/mvw098](https://doi.org/10.1093/jb/mvw098).
- Sagunenko, V et al. (2013). AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ* 20.9, pp. 1149–1160. DOI: [10.1038/cdd.2013.37](https://doi.org/10.1038/cdd.2013.37).

- Saiga, H et al. (2012). Critical role of AIM2 in *Mycobacterium tuberculosis* infection. *Int Immunol* 24.10, pp. 637–644. DOI: [10.1093/intimm/dxs062](https://doi.org/10.1093/intimm/dxs062).
- Saiga, Hiroyuki et al. (2015). The recombinant BCG $\Delta ureC::hly$ vaccine targets the AIM2 inflammasome to induce autophagy and inflammation. *J Infect Dis* 211.11, pp. 1831–41. DOI: [10.1093/infdis/jiu675](https://doi.org/10.1093/infdis/jiu675).
- Saitoh, Tatsuya et al. (2008). Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1 β production. *Nature* 456.7219, pp. 264–268. DOI: [10.1038/nature07383](https://doi.org/10.1038/nature07383).
- Salahuddin, Nawal et al. (2013). Vitamin D accelerates clinical recovery from tuberculosis: results of the SUCCINCT Study [Supplementary Cholecalciferol in recovery from tuberculosis]. A randomized, placebo-controlled, clinical trial of vitamin D supplementation in patients with pulmonary tuberculosis. *BMC Infect Dis* 13, p. 22. DOI: [10.1186/1471-2334-13-22](https://doi.org/10.1186/1471-2334-13-22).
- Salgame, Padmini (2011). MMPs in tuberculosis: granuloma creators and tissue destroyers. *J Clin Invest* 121.5, pp. 1686–8. DOI: [10.1172/JCI57423](https://doi.org/10.1172/JCI57423).
- Sampson, Samantha L et al. (2004). Protection elicited by a double leucine and pantothenate auxotroph of *Mycobacterium tuberculosis* in guinea pigs. *Infect Immun* 72.5, pp. 3031–7. DOI: [10.1128/IAI.72.5.3031-3037.2004](https://doi.org/10.1128/IAI.72.5.3031-3037.2004).
- Saresella, Marina et al. (2016). The NLRP3 and NLRP1 inflammasomes are activated in Alzheimer's disease. *Mol Neurodegener* 11.1, p. 23. DOI: [10.1186/s13024-016-0088-1](https://doi.org/10.1186/s13024-016-0088-1).
- Sarkar, Abira et al. (2003). α -Melanocyte-stimulating hormone induces cell death in mast cells: involvement of NF- κ B. *FEBS Lett* 549.1-3, pp. 87–93. DOI: [10.1016/S0014-5793\(03\)00797-X](https://doi.org/10.1016/S0014-5793(03)00797-X).
- Sarkar, Rajesh et al. (2012). Modern lineages of *Mycobacterium tuberculosis* exhibit lineage-specific patterns of growth and cytokine induction in human monocyte-derived macrophages. *PLoS One* 7.8. DOI: [10.1371/journal.pone.0043170](https://doi.org/10.1371/journal.pone.0043170).
- Sastalla, Inka et al. (2013). Transcriptional analysis of the three Nlrp1 paralogs in mice. *BMC Genomics* 14.1, p. 188. DOI: [10.1186/1471-2164-14-188](https://doi.org/10.1186/1471-2164-14-188).
- Sawyer, T K et al. (1980). 4-Norleucine, 7-D-phenylalanine- α -melanocyte-stimulating hormone: a highly potent α -melanotropin with ultralong biological activity. *Proc Natl Acad Sci U S A* 77.10, pp. 5754–8.
- Scarlett, Jarrad M. et al. (2007). Regulation of central melanocortin signaling by interleukin-1 β . *Endocrinology* 148.9, pp. 4217–4225. DOI: [10.1210/en.2007-0017](https://doi.org/10.1210/en.2007-0017).
- Schaale, K et al. (2016). Strain- and host species-specific inflammasome activation, IL-1 β release, and cell death in macrophages infected with uropathogenic *Escherichia coli*. *Mucosal Immunol* 9.1, pp. 124–136. DOI: [10.1038/mi.2015.44](https://doi.org/10.1038/mi.2015.44).
- Schaible, U E et al. (1998). Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. *J Immunol* 160.3, pp. 1290–6.

- Schenk, Mirjam et al. (2014). Interleukin-1 β triggers the differentiation of macrophages with enhanced capacity to present mycobacterial antigen to T cells. *Immunology* 141.2, pp. 174–80. DOI: [10.1111/imm.12167](https://doi.org/10.1111/imm.12167).
- Schiebler, M. et al. (2015). Functional drug screening reveals anticonvulsants as enhancers of mTOR-independent autophagic killing of *Mycobacterium tuberculosis* through inositol depletion. *EMBO Mol Med* 7.2, pp. 127–139. DOI: [10.15252/emmm.201404137](https://doi.org/10.15252/emmm.201404137).
- Schoeman, Johan F. et al. (2004). Adjunctive thalidomide therapy for childhood tuberculous meningitis: results of a randomized study. *J Child Neurol* 19.4, pp. 250–257. DOI: [10.1177/088307380401900402](https://doi.org/10.1177/088307380401900402).
- Schoeman, Johan F. et al. (2006). Intractable intracranial tuberculous infection responsive to thalidomide: report of four cases. *J Child Neurol* 21.4, pp. 301–308. DOI: [10.1177/08830738060210040801](https://doi.org/10.1177/08830738060210040801).
- Scholzen, T E et al. (2000). Expression of proopiomelanocortin peptides in human dermal microvascular endothelial cells: evidence for a regulation by ultraviolet light and interleukin-1. *J Invest Dermatol* 115.6, pp. 1021–8. DOI: [10.1046/j.1523-1747.2000.00174.x](https://doi.org/10.1046/j.1523-1747.2000.00174.x).
- Scholzen, T. E. et al. (2003). α -melanocyte stimulating hormone prevents lipopolysaccharide-induced vasculitis by down-regulating endothelial cell adhesion molecule expression. *Endocrinology* 144.1, pp. 360–370. DOI: [10.1210/en.2002-220651](https://doi.org/10.1210/en.2002-220651).
- Schulze, P Christian et al. (2004). Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem* 279.29, pp. 30369–74. DOI: [10.1074/jbc.M400549200](https://doi.org/10.1074/jbc.M400549200).
- Schumann, R R et al. (1998). Lipopolysaccharide activates caspase-1 (interleukin-1-converting enzyme) in cultured monocytic and endothelial cells. *Blood* 91.2, pp. 577–84.
- Schwartz, J. T. et al. (2012). *Francisella tularensis* inhibits the intrinsic and extrinsic pathways to delay constitutive apoptosis and prolong human neutrophil lifespan. *J Immunol* 188.7, pp. 3351–3363. DOI: [10.4049/jimmunol.1102863](https://doi.org/10.4049/jimmunol.1102863).
- Schwebach, J Reid et al. (2002). Glucan is a component of the *Mycobacterium tuberculosis* surface that is expressed *in vitro* and *in vivo*. *Infect Immun* 70.5, pp. 2566–75. DOI: [10.1128/IAI.70.5.2566-2575.2002](https://doi.org/10.1128/IAI.70.5.2566-2575.2002).
- Seddon, Jo et al. (2013). Procollagen III N-terminal propeptide and desmosine are released by matrix destruction in pulmonary tuberculosis. *J Infect Dis* 208.10, pp. 1571–1579. DOI: [10.1093/infdis/jit343](https://doi.org/10.1093/infdis/jit343).
- Serezani, Carlos H et al. (2008). Cyclic AMP: master regulator of innate immune cell function. *Am J Respir Cell Mol Biol* 39.2, pp. 127–32. DOI: [10.1165/rcmb.2008-0091TR](https://doi.org/10.1165/rcmb.2008-0091TR).
- Sexton, D J et al. (1998). Ceftriaxone once daily for four weeks compared with ceftriaxone plus gentamicin once daily for two weeks for treatment of endocarditis due to penicillin-susceptible streptococci. *Clin Infect Dis* 27.6, pp. 1470–4. DOI: [10.1086/515038](https://doi.org/10.1086/515038).

- Shah, Swati et al. (2013). Cutting edge: *Mycobacterium tuberculosis* but not nonvirulent mycobacteria inhibits IFN- β and AIM2 inflammasome-dependent IL-1 β production via its ESX-1 secretion system. *J Immunol* 191.7, pp. 3514–8. DOI: [10.4049/jimmunol.1301331](https://doi.org/10.4049/jimmunol.1301331).
- Sharma, Deepika et al. (2016). The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. *J Cell Biol* 213.6.
- Shenderov, Kevin et al. (2013). Cord factor and peptidoglycan recapitulate the Th17-promoting adjuvant activity of mycobacteria through mincle/CARD9 signaling and the inflammasome. *J Immunol* 190.11, pp. 5722–30. DOI: [10.4049/jimmunol.1203343](https://doi.org/10.4049/jimmunol.1203343).
- Shenoy, Avinash R et al. (2012). GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. *Science* 336.6080, pp. 481–5. DOI: [10.1126/science.1217141](https://doi.org/10.1126/science.1217141).
- Shi, Jianjin et al. (2014). Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 514.7521, pp. 187–192. DOI: [10.1038/nature13683](https://doi.org/10.1038/nature13683).
- Shi, Mude et al. (2008). TRIM30 α negatively regulates TLR-mediated NF- κ B activation by targeting TAB2 and TAB3 for degradation. *Nat Immunol* 9.4, pp. 369–377. DOI: [10.1038/ni1577](https://doi.org/10.1038/ni1577).
- Shimada, Kenichi et al. (2012). Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36.3, pp. 401–414. DOI: [10.1016/j.immuni.2012.01.009](https://doi.org/10.1016/j.immuni.2012.01.009).
- Shinyama, Hiroshi et al. (2003). Regulation of melanocortin-4 receptor signaling: agonist-mediated desensitization and internalization. *Endocrinology* 144.4, pp. 1301–1314. DOI: [10.1210/en.2002-220931](https://doi.org/10.1210/en.2002-220931).
- Shiratori, Kenji et al. (2004). Inhibition of endotoxin-induced uveitis and potentiation of cyclooxygenase-2 protein expression by α -melanocyte-stimulating hormone. *Investigative Ophthalmology and Visual Science* 45.1, pp. 159–164. DOI: [10.1167/iovs.03-0492](https://doi.org/10.1167/iovs.03-0492).
- Simeone, Roxane et al. (2015). Cytosolic access of *Mycobacterium tuberculosis*: critical impact of phagosomal acidification control and demonstration of occurrence *in vivo*. *PLoS Pathog* 11.2, e1004650. DOI: [10.1371/journal.ppat.1004650](https://doi.org/10.1371/journal.ppat.1004650).
- Sims, John E. et al. (2010). The IL-1 family: regulators of immunity. *Nat Rev Immunol* 10.2, p. 117. DOI: [10.1038/nri2691](https://doi.org/10.1038/nri2691).
- Singh, Madhuri et al. (2013). Combination of α -melanocyte stimulating hormone with conventional antibiotics against methicillin resistant *Staphylococcus aureus*. *PLoS One* 8.9, e73815. DOI: [10.1371/journal.pone.0073815](https://doi.org/10.1371/journal.pone.0073815).
- Singh, Madhuri et al. (2014). α -melanocyte stimulating hormone: an emerging anti-inflammatory antimicrobial peptide. *Biomed Res Int* 2014, p. 874610. DOI: [10.1155/2014/874610](https://doi.org/10.1155/2014/874610).
- Singhal, Amit et al. (2014). Metformin as adjunct antituberculosis therapy. *Sci Transl Med* 6.263.
- Song, Hui et al. (2016). The E3 ubiquitin ligase TRIM31 attenuates NLRP3 inflammasome activation by promoting proteasomal degradation of NLRP3. *Nat Commun* 7, p. 13727. DOI: [10.1038/ncomms13727](https://doi.org/10.1038/ncomms13727).

- Stamm, Chelsea E et al. (2015). Sensing of *Mycobacterium tuberculosis* and consequences to both host and bacillus. *Immunol Rev* 264.1, pp. 204–19. DOI: [10.1111/imr.12263](https://doi.org/10.1111/imr.12263).
- Star, R A et al. (1995). Evidence of autocrine modulation of macrophage nitric oxide synthase by α -melanocyte-stimulating hormone. *Proc Natl Acad Sci U S A* 92.17, pp. 8016–8020.
- Stein, Catherine M et al. (2008). Genome scan of *M. tuberculosis* infection and disease in Ugandans. *PLoS One* 3.12, e4094. DOI: [10.1371/journal.pone.0004094](https://doi.org/10.1371/journal.pone.0004094).
- Subbian, S et al. (2011). Phosphodiesterase-4 inhibition alters gene expression and improves isoniazid-mediated clearance of *Mycobacterium tuberculosis* in rabbit lungs. *PLoS Pathog* 7.9, e1002262. DOI: [10.1371/journal.ppat.1002262](https://doi.org/10.1371/journal.ppat.1002262).
- Subbian, Selvakumar et al. (2016). Adjunctive phosphodiesterase-4 inhibitor therapy improves antibiotic response to pulmonary tuberculosis in a rabbit model. *EBioMedicine* 4, pp. 104–114. DOI: [10.1016/j.ebiom.2016.01.015](https://doi.org/10.1016/j.ebiom.2016.01.015).
- Sugawara, I et al. (2001). Role of interleukin (IL)-1 type 1 receptor in mycobacterial infection. *Microbiol Immunol* 45.11, pp. 743–50.
- Sugawara, Isamu et al. (2003). Mycobacterial infection in TLR2 and TLR6 knockout mice. *Microbiol Immunol* 47.5, pp. 327–36.
- Sui, Jiangdong et al. (2012). NLRP1 gene polymorphism influences gene transcription and is a risk factor for rheumatoid arthritis in Han Chinese. *Arthritis Rheum* 64.3, pp. 647–654. DOI: [10.1002/art.33370](https://doi.org/10.1002/art.33370).
- Sun, Haiji et al. (2007). A method of oligochip for single nucleotide polymorphism genotyping in the promoter region of the interleukin-1 β gene and its clinical application. *Oligonucleotides* 17.3, pp. 336–344. DOI: [10.1089/oli.2007.0071](https://doi.org/10.1089/oli.2007.0071).
- Suzuki, Itaru et al. (1996). Binding of melanotropic hormones to the melanocortin receptor MC1R on human melanocytes stimulates proliferation and melanogenesis. *Endocrinology* 137.5, pp. 1627–1633. DOI: [10.1210/en.137.5.1627](https://doi.org/10.1210/en.137.5.1627).
- Swaminathan, Soumya et al. (2010). Efficacy of a 6-month versus 9-month Intermittent Treatment Regimen in HIV-infected Patients with Tuberculosis. *Am J Respir Crit Care Med* 181.7, pp. 743–751. DOI: [10.1164/rccm.200903-04390C](https://doi.org/10.1164/rccm.200903-04390C).
- Sweeney, Timothy E et al. (2016). Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis. *Lancet Respir Med* 4.3, pp. 213–24. DOI: [10.1016/S2213-2600\(16\)00048-5](https://doi.org/10.1016/S2213-2600(16)00048-5).
- Tadokera, R et al. (2011). Hypercytokinaemia accompanies HIV-tuberculosis immune reconstitution inflammatory syndrome. *Eur Respir J* 37.5, pp. 1248–59. DOI: [10.1183/09031936.00091010](https://doi.org/10.1183/09031936.00091010).
- Taherzadeh, S et al. (1999). α -MSH and its receptors in regulation of tumor necrosis factor- α production by human monocyte/macrophages. *Am J Physiol* 276.5 Pt 2, R1289–94.

- Tan, Hong Yien et al. (2016). Aberrant inflammasome activation characterizes tuberculosis-associated immune reconstitution inflammatory syndrome. *J Immunol* 196.10, pp. 4052–63. DOI: [10.4049/jimmunol.1502203](https://doi.org/10.4049/jimmunol.1502203).
- Tannous, Bakhos A et al. (2009). *Gaussia* luciferase reporter assay for monitoring biological processes in culture and *in vivo*. *Nat Protoc* 4.4, pp. 582–591. DOI: [10.1038/nprot.2009.28](https://doi.org/10.1038/nprot.2009.28).
- Tate, Michelle D. et al. (2016). Reassessing the role of the NLRP3 inflammasome during pathogenic influenza A virus infection via temporal inhibition. *Sci Rep* 6.1, p. 27912. DOI: [10.1038/srep27912](https://doi.org/10.1038/srep27912).
- Tavares, J L et al. (1997). Thalidomide reduces tumour necrosis factor- α production by human alveolar macrophages. *Respir Med* 91.1, pp. 31–9.
- Taylor, A W (2013). α -melanocyte stimulating hormone (α -MSH) is a post-caspase suppressor of apoptosis in RAW 264.7 macrophages. *PLoS One* 8.8, e74488. DOI: [10.1371/journal.pone.0074488](https://doi.org/10.1371/journal.pone.0074488).
- Tenthorey, Jeannette L. et al. (2014). Molecular basis for specific recognition of bacterial ligands by NAIP/NLRC4 inflammasomes. *Mol Cell* 54.1, pp. 17–29. DOI: [10.1016/j.molcel.2014.02.018](https://doi.org/10.1016/j.molcel.2014.02.018).
- Thuong, Nguyen T. T. et al. (2017). Leukotriene A4 hydrolase genotype and HIV infection influence intracerebral inflammation and survival from tuberculous meningitis. *J Infect Dis* 215.7, pp. 1020–1028. DOI: [10.1093/infdis/jix050](https://doi.org/10.1093/infdis/jix050).
- Thwaites, Guy E (2013). Adjunctive corticosteroids for all forms of tuberculosis? *Lancet Infect Dis* 13.3, pp. 186–188. DOI: [10.1016/S1473-3099\(13\)70009-4](https://doi.org/10.1016/S1473-3099(13)70009-4).
- Ting, Harold J et al. (2010). Repurposing an old drug for a new use: glybenclamide exerts antiplatelet activity by interacting with the thromboxane A₂ receptor. *Acta Pharmacol Sin* 31.2, pp. 150–159. DOI: [10.1038/aps.2009.195](https://doi.org/10.1038/aps.2009.195).
- Tobin, David M et al. (2010). The *Ita4h* locus modulates susceptibility to mycobacterial infection in zebrafish and humans. *Cell* 140.5, pp. 717–30. DOI: [10.1016/j.cell.2010.02.013](https://doi.org/10.1016/j.cell.2010.02.013).
- Tobin, David M et al. (2012). Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. *Cell* 148.3, pp. 434–46. DOI: [10.1016/j.cell.2011.12.023](https://doi.org/10.1016/j.cell.2011.12.023).
- Todorovic, Aleksandar et al. (2005). A review of melanocortin receptor small molecule ligands. *Peptides* 26.10, pp. 2026–36. DOI: [10.1016/j.peptides.2004.11.024](https://doi.org/10.1016/j.peptides.2004.11.024).
- Torre-Minguela, Carlos de et al. (2017). The NLRP3 and Pyrin Inflammasomes: Implications in the Pathophysiology of Autoinflammatory Diseases. *Front Immunol* 8, p. 43. DOI: [10.3389/fimmu.2017.00043](https://doi.org/10.3389/fimmu.2017.00043).
- Torrelles, Jordi B. et al. (2006). Fine discrimination in the recognition of individual species of phosphatidyl-*myo*-inositol mannosides from *Mycobacterium tuberculosis* by C-type lectin pattern recognition receptors. *The Journal of Immunology* 177.3.

- Torrelles, Jordi B et al. (2008). Identification of *Mycobacterium tuberculosis* clinical isolates with altered phagocytosis by human macrophages due to a truncated lipoarabinomannan. *J Biol Chem* 283.46, pp. 31417–28. DOI: [10.1074/jbc.M806350200](https://doi.org/10.1074/jbc.M806350200).
- Torrelles, Jordi B et al. (2010). Diversity in *Mycobacterium tuberculosis* mannosylated cell wall determinants impacts adaptation to the host. *Tuberculosis (Edinb)* 90.2, pp. 84–93. DOI: [10.1016/j.tube.2010.02.003](https://doi.org/10.1016/j.tube.2010.02.003).
- Torres-Juarez, Flor et al. (2015). LL-37 immunomodulatory activity during *Mycobacterium tuberculosis* infection in macrophages. *Infect Immun* 83.12, pp. 4495–503. DOI: [10.1128/IAI.00936-15](https://doi.org/10.1128/IAI.00936-15).
- Tramontana, J M et al. (1995). Thalidomide treatment reduces tumor necrosis factor α production and enhances weight gain in patients with pulmonary tuberculosis. *Mol Med* 1.4, pp. 384–397.
- Tsao, T C et al. (1999). Increased TNF- α , IL-1 β and IL-6 levels in the bronchoalveolar lavage fluid with the upregulation of their mRNA in macrophages lavaged from patients with active pulmonary tuberculosis. *Tuber Lung Dis* 79.5, pp. 279–85.
- Tsao, T C et al. (2000). Imbalances between tumor necrosis factor- α and its soluble receptor forms, and interleukin-1 β and interleukin-1 receptor antagonist in BAL fluid of cavitary pulmonary tuberculosis. *Chest* 117.1, pp. 103–9.
- Tseng, Y H et al. (1998). Transcriptional regulation of interleukin-1 β gene by interleukin-1 β itself is mediated in part by Oct-1 in thymic stromal cells. *J Biol Chem* 273.20, pp. 12633–41. DOI: [10.1074/JBC.273.20.12633](https://doi.org/10.1074/JBC.273.20.12633).
- Tsuchiya, K. et al. (2010). Involvement of Absent in Melanoma 2 in inflammasome activation in macrophages infected with *Listeria monocytogenes*. *J Immunol* 185.2, pp. 1186–1195. DOI: [10.4049/jimmunol.1001058](https://doi.org/10.4049/jimmunol.1001058).
- Van Opdenbosch, Nina et al. (2014). Activation of the NLRP1b inflammasome independently of ASC-mediated caspase-1 autoproteolysis and speck formation. *Nat Commun* 5, p. 3209. DOI: [10.1038/ncomms4209](https://doi.org/10.1038/ncomms4209).
- Vanaja, Sivapriya Kailasan et al. (2015). Mechanisms of inflammasome activation: Recent advances and novel insights. *Trends Cell Biol* 25.5, pp. 308–315. DOI: [10.1016/j.tcb.2014.12.009](https://doi.org/10.1016/j.tcb.2014.12.009).
- Vance, Russell E (2015). The NAIP/NLRC4 inflammasomes. *Curr Opin Immunol* 32, pp. 84–89. DOI: [10.1016/j.coi.2015.01.010](https://doi.org/10.1016/j.coi.2015.01.010).
- Vashisht, Rohit et al. (2014). Systems level mapping of metabolic complexity in *Mycobacterium tuberculosis* to identify high-value drug targets. *J Transl Med* 12.1, p. 263. DOI: [10.1186/s12967-014-0263-5](https://doi.org/10.1186/s12967-014-0263-5).
- Verver, S et al. (2005). Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. *Am J Respir Crit Care Med* 171.12, pp. 1430–1435. DOI: [10.1164/rccm.200409-12000C](https://doi.org/10.1164/rccm.200409-12000C).

- Verway, Mark et al. (2013). Vitamin D induces interleukin-1 β expression: paracrine macrophage epithelial signaling controls *M. tuberculosis* infection. *PLoS Pathog* 9.6, e1003407. DOI: [10.1371/journal.ppat.1003407](https://doi.org/10.1371/journal.ppat.1003407).
- Vogelzang, Alexis et al. (2014). Central memory CD4⁺ T cells are responsible for the recombinant Bacillus Calmette-Guérin $\Delta ureC::hly$ vaccine's superior protection against tuberculosis. *J Infect Dis* 210.12, pp. 1928–37. DOI: [10.1093/infdis/jiu347](https://doi.org/10.1093/infdis/jiu347).
- Wadleigh, David J et al. (2000). Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *J Biol Chem* 275.9, pp. 6259–66. DOI: [10.1074/jbc.275.9.6259](https://doi.org/10.1074/jbc.275.9.6259).
- Wagener, Frank A D T G et al. (2003). Different faces of the heme-heme oxygenase system in inflammation. *Pharmacol Rev* 55.3, pp. 551–71. DOI: [10.1124/pr.55.3.5](https://doi.org/10.1124/pr.55.3.5).
- Waite, Andrea L et al. (2009). Pyrin and ASC co-localize to cellular sites that are rich in polymerizing actin. *Exp Biol Med (Maywood)* 234.1, pp. 40–52. DOI: [10.3181/0806-RM-184](https://doi.org/10.3181/0806-RM-184).
- Wald, David et al. (2003). SIGIRR, a negative regulator of Toll-like receptor–interleukin 1 receptor signaling. *Nat Immunol* 4.9, pp. 920–927. DOI: [10.1038/ni968](https://doi.org/10.1038/ni968).
- Wallis, Robert S (2014). Corticosteroid effects on sputum culture in pulmonary tuberculosis: a meta-regression analysis. *Open Forum Infect Dis* 1.1, ofu020. DOI: [10.1093/ofid/ofu020](https://doi.org/10.1093/ofid/ofu020).
- Wallis, Robert S et al. (2004). A study of the safety, immunology, virology, and microbiology of adjunctive etanercept in HIV-1-associated tuberculosis. *AIDS* 18.2, pp. 257–64.
- Wallis, Robert S. et al. (2015). Advancing host-directed therapy for tuberculosis. *Nat Rev Immunol* 15.4, pp. 255–263. DOI: [10.1038/nri3813](https://doi.org/10.1038/nri3813).
- Walter, Kerstin et al. (2010). NALP3 is not necessary for early protection against experimental tuberculosis. *Immunobiology* 215.9-10, pp. 804–811. DOI: [10.1016/j.imbio.2010.05.015](https://doi.org/10.1016/j.imbio.2010.05.015).
- Wan, Jerry et al. (2017). Novel approaches to vitiligo treatment via modulation of mTOR and NF- κ B pathways in human skin melanocytes. *Int J Biol Sci* 13.3, pp. 391–400. DOI: [10.7150/ijbs.17318](https://doi.org/10.7150/ijbs.17318).
- Wang, Changqi et al. (2013). Characterization of murine macrophages from bone marrow, spleen and peritoneum. *BMC Immunol* 14.1, p. 6. DOI: [10.1186/1471-2172-14-6](https://doi.org/10.1186/1471-2172-14-6).
- Wang, Xiaqiong et al. (2014). RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway. *Nat Immunol* 15.12, pp. 1126–1133. DOI: [10.1038/ni.3015](https://doi.org/10.1038/ni.3015).
- Wang, Yanli et al. (2015). Serum IL-1 β and IL-18 correlate with ESR and CRP in multidrug-resistant tuberculosis patients. *J Biomed Res* 29.5, pp. 426–8. DOI: [10.7555/JBR.29.20150077](https://doi.org/10.7555/JBR.29.20150077).
- Wang, Yue et al. (2017). MicroRNA-9 inhibits NLRP3 inflammasome activation in Human atherosclerosis inflammation cell models through the JAK1/STAT signaling pathway. *Cell Physiol Biochem* 41.4, pp. 1555–1571. DOI: [10.1159/000470822](https://doi.org/10.1159/000470822).

- Wassermann, Ruth et al. (2015). *Mycobacterium tuberculosis* differentially activates cGAS- and inflammasome-dependent intracellular immune responses through ESX-1. *Cell Host Microbe* 17.6, pp. 799–810. DOI: [10.1016/j.chom.2015.05.003](https://doi.org/10.1016/j.chom.2015.05.003).
- Watson, Robert O et al. (2015). The cytosolic sensor cGAS detects *Mycobacterium tuberculosis* DNA to induce type I interferons and activate autophagy. *Cell Host Microbe* 17.6, pp. 811–9. DOI: [10.1016/j.chom.2015.05.004](https://doi.org/10.1016/j.chom.2015.05.004).
- Wei, Meili et al. (2016). NLRP3 activation was regulated by DNA methylation modification during *Mycobacterium tuberculosis* infection. *Biomed Res Int* 2016, p. 4323281. DOI: [10.1155/2016/4323281](https://doi.org/10.1155/2016/4323281).
- Weiss, Günter et al. (2015). Macrophage defense mechanisms against intracellular bacteria. *Immunol Rev* 264.1, pp. 182–203. DOI: [10.1111/imr.12266](https://doi.org/10.1111/imr.12266).
- Wejse, Christian et al. (2007). Serum 25-hydroxyvitamin D in a West African population of tuberculosis patients and unmatched healthy controls. *Am J Clin Nutr* 86.5, pp. 1376–83.
- Wejse, Christian et al. (2009). Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial. *Am J Respir Crit Care Med* 179.9, pp. 843–50. DOI: [10.1164/rccm.200804-5670C](https://doi.org/10.1164/rccm.200804-5670C).
- Welin, Amanda et al. (2011). Human macrophages infected with a high burden of ESAT-6-expressing *M. tuberculosis* undergo caspase-1- and cathepsin B-independent necrosis. *PLoS One* 6.5, e20302. DOI: [10.1371/journal.pone.0020302](https://doi.org/10.1371/journal.pone.0020302).
- Wen, Andy Y. et al. (2010). The role of the transcription factor CREB in immune function. *The Journal of Immunology* 185.11.
- WHO (2016). *Global tuberculosis report 2015*. Tech. rep.
- Wikberg, J E (1999). Melanocortin receptors: perspectives for novel drugs. *Eur J Pharmacol* 375.1-3, pp. 295–310.
- Wikberg, Jarl E.S. et al. (2000). New aspects on the melanocortins and their receptors. *Pharmacol Res* 42.5, pp. 393–420. DOI: [10.1006/phrs.2000.0725](https://doi.org/10.1006/phrs.2000.0725).
- Wilkinson, R J et al. (1999). Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1 β on tuberculosis. *J Exp Med* 189.12, pp. 1863–74. DOI: [10.1084/JEM.189.12.1863](https://doi.org/10.1084/JEM.189.12.1863).
- Wilkinson, Robert J et al. (2000). Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. *The Lancet* 355.9204, pp. 618–621. DOI: [10.1016/S0140-6736\(99\)02301-6](https://doi.org/10.1016/S0140-6736(99)02301-6).
- Witola, William H et al. (2011). NALP1 influences susceptibility to human congenital toxoplasmosis, proinflammatory cytokine response, and fate of *Toxoplasma gondii*-infected monocytic cells. *Infect Immun* 79.2, pp. 756–66. DOI: [10.1128/IAI.00898-10](https://doi.org/10.1128/IAI.00898-10).

- Wolf Horrell, Erin M et al. (2016). Melanocortin 1 receptor: structure, function, and regulation. *Front Genet* 7, p. 95. DOI: [10.3389/fgene.2016.00095](https://doi.org/10.3389/fgene.2016.00095).
- Wong, Ka-Wing et al. (2011). Critical role for NLRP3 in necrotic death triggered by *Mycobacterium tuberculosis*. *Cell Microbiol* 13.9, pp. 1371–84. DOI: [10.1111/j.1462-5822.2011.01625.x](https://doi.org/10.1111/j.1462-5822.2011.01625.x).
- Wu, Shaoping et al. (2011). Vitamin D, vitamin D receptor, and macroautophagy in inflammation and infection. *Discov Med* 11.59, pp. 325–35.
- Xiao, Ye Da et al. (2016). Thioredoxin-interacting protein mediates NLRP3 inflammasome activation involved in the susceptibility to ischemic acute kidney injury in diabetes. *Oxid Med Cell Longev* 2016, pp. 1–17. DOI: [10.1155/2016/2386068](https://doi.org/10.1155/2016/2386068).
- Yadav, Mahesh et al. (2006). The β -glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood* 108.9, pp. 3168–75. DOI: [10.1182/blood-2006-05-024406](https://doi.org/10.1182/blood-2006-05-024406).
- Yamada, H et al. (2000). Protective role of interleukin-1 in mycobacterial infection in IL-1 α/β double-knockout mice. *Lab Invest* 80.5, pp. 759–67.
- Yang, Dahai et al. (2015). Caspase-11 requires the pannexin-1 channel and the purinergic P2X7 pore to mediate pyroptosis and endotoxic shock. *Immunity* 43.5, pp. 923–932. DOI: [10.1016/j.immuni.2015.10.009](https://doi.org/10.1016/j.immuni.2015.10.009).
- Yang, Jieliang et al. (2013a). Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proc Natl Acad Sci U S A* 110.35, pp. 14408–13. DOI: [10.1073/pnas.1306376110](https://doi.org/10.1073/pnas.1306376110).
- Yang, X et al. (1998). Autoproteolytic activation of pro-caspases by oligomerization. *Mol Cell* 1.2, pp. 319–25.
- Yang, Y. et al. (2013b). The AIM2 inflammasome is involved in macrophage activation during infection with virulent *Mycobacterium bovis* strain. *J Infect Dis* 208.11, pp. 1849–1858. DOI: [10.1093/infdis/jit347](https://doi.org/10.1093/infdis/jit347).
- Yates, Tom A et al. (2016). The transmission of *Mycobacterium tuberculosis* in high burden settings. *Lancet Infect Dis* 16.2, pp. 227–238. DOI: [10.1016/S1473-3099\(15\)00499-5](https://doi.org/10.1016/S1473-3099(15)00499-5).
- Yim, Howard CH et al. (2016). The kinase activity of PKR represses inflammasome activity. *Cell Res* 26.3, pp. 367–379. DOI: [10.1038/cr.2016.11](https://doi.org/10.1038/cr.2016.11).
- Yoon, Sun-Woo et al. (2003). α -Melanocyte-stimulating hormone inhibits lipopolysaccharide-induced tumor necrosis factor- α production in leukocytes by modulating protein kinase A, p38 kinase, and nuclear factor κ B signaling pathways. *J Biol Chem* 278.35, pp. 32914–20. DOI: [10.1074/jbc.M302444200](https://doi.org/10.1074/jbc.M302444200).
- Yoshimura, Akihiko et al. (2007). SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol* 7.6, pp. 454–465. DOI: [10.1038/nri2093](https://doi.org/10.1038/nri2093).

- Yu, Chang et al. (2017). Sulforaphane improves outcomes and slows cerebral ischemic/reperfusion injury via inhibition of NLRP3 inflammasome activation in rats. *Int Immunopharmacol* 45, pp. 74–78. DOI: [10.1016/j.intimp.2017.01.034](https://doi.org/10.1016/j.intimp.2017.01.034).
- Yu, Chien-Hsiung et al. (2015). RP105 engages phosphatidylinositol 3-kinase p110 δ to facilitate the trafficking and secretion of cytokines in macrophages during mycobacterial infection. *J Immunol* 195.8, pp. 3890–3900. DOI: [10.4049/jimmunol.1500017](https://doi.org/10.4049/jimmunol.1500017).
- Yu, Jiujiu et al. (2014). Inflammasome activation leads to Caspase-1-dependent mitochondrial damage and block of mitophagy. *Proc Natl Acad Sci U S A* 111.43, pp. 15514–9. DOI: [10.1073/pnas.1414859111](https://doi.org/10.1073/pnas.1414859111).
- Yuk, Jae-Min et al. (2009). Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe* 6.3, pp. 231–243. DOI: [10.1016/j.chom.2009.08.004](https://doi.org/10.1016/j.chom.2009.08.004).
- Zhai, Z et al. (2017). NLRP1 promotes tumor growth by enhancing inflammasome activation and suppressing apoptosis in metastatic melanoma. *Oncogene*. DOI: [10.1038/onc.2017.26](https://doi.org/10.1038/onc.2017.26).
- Zhang, Guoliang et al. (2014). Allele-specific induction of IL-1 β expression by C/EBP β and PU.1 contributes to increased tuberculosis susceptibility. *PLoS Pathog* 10.10, e1004426. DOI: [10.1371/journal.ppat.1004426](https://doi.org/10.1371/journal.ppat.1004426).
- Zhang, Liqun et al. (2016). Risk factors for pulmonary cavitation in tuberculosis patients from China. *Emerg Microbes Infect* 5.10, e110. DOI: [10.1038/emi.2016.111](https://doi.org/10.1038/emi.2016.111).
- Zhang, Xia et al. (2008). The isolation and characterization of murine macrophages. *Curr Protoc Immunol* Chapter 14, Unit 14.1. DOI: [10.1002/0471142735.im1401s83](https://doi.org/10.1002/0471142735.im1401s83).
- Zhang, Yan et al. (2009). Melanocortin potentiates leptin-induced STAT3 signaling via MAPK pathway. *J Neurochem* 110.1, pp. 390–399. DOI: [10.1111/j.1471-4159.2009.06144.x](https://doi.org/10.1111/j.1471-4159.2009.06144.x).
- Zhang, Zhiqiang et al. (2011). DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. *Immunity* 34.6, pp. 866–878. DOI: [10.1016/j.immuni.2011.03.027](https://doi.org/10.1016/j.immuni.2011.03.027).
- Zhao, Quanju et al. (2014). *Mycobacterium tuberculosis* serine protease Rv3668c can manipulate the host-pathogen interaction via Erk-NF- κ B axis-mediated cytokine differential expression. *J Interferon Cytokine Res* 34.9, pp. 686–98. DOI: [10.1089/jir.2013.0071](https://doi.org/10.1089/jir.2013.0071).
- Zhao, Yue et al. (2011). The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 477.7366, pp. 596–600. DOI: [10.1038/nature10510](https://doi.org/10.1038/nature10510).
- Zhao, Yue et al. (2015). The NAIP-NLRC4 inflammasome in innate immune detection of bacterial flagellin and type III secretion apparatus. *Immunol Rev* 265.1, pp. 85–102. DOI: [10.1111/imr.12293](https://doi.org/10.1111/imr.12293).
- Zhong, Zhenyu et al. (2013). TRPM2 links oxidative stress to NLRP3 inflammasome activation. *Nat Commun* 4, p. 1611. DOI: [10.1038/ncomms2608](https://doi.org/10.1038/ncomms2608).

- Zhong, Zhenyu et al. (2016). Autophagy, NLRP3 inflammasome and auto-inflammatory/immune diseases. *Clin Exp Rheumatol* 34.4 Suppl 98, pp. 12–6.
- Zhou, G et al. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108.8, pp. 1167–74. DOI: [10.1172/JCI13505](https://doi.org/10.1172/JCI13505).
- Zhou, Rongbin et al. (2010). Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 11.2, pp. 136–40. DOI: [10.1038/ni.1831](https://doi.org/10.1038/ni.1831).
- Zhou, Rongbin et al. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469.7329, pp. 221–5. DOI: [10.1038/nature09663](https://doi.org/10.1038/nature09663).
- Zhou, Yang et al. (2015). Inflammasomes-dependent regulation of IL-1 β secretion induced by the virulent *Mycobacterium bovis* Beijing strain in THP-1 macrophages. *Antonie Van Leeuwenhoek* 108.1, pp. 163–71. DOI: [10.1007/s10482-015-0475-6](https://doi.org/10.1007/s10482-015-0475-6).
- Zhou, Yang et al. (2016a). Virulent *Mycobacterium bovis* Beijing strain activates the NLRP7 inflammasome in THP-1 macrophages. *PLoS One* 11.4, e0152853. DOI: [10.1371/journal.pone.0152853](https://doi.org/10.1371/journal.pone.0152853).
- Zhou, Zhong'e et al. (2016b). Metformin inhibits advanced glycation end products-induced inflammatory response in murine macrophages partly through AMPK activation and RAGE/NF κ B pathway suppression. *J Diabetes Res* 2016, p. 4847812. DOI: [10.1155/2016/4847812](https://doi.org/10.1155/2016/4847812).
- Ziglam, H.M. et al. (2004). Immunomodulating activity of rifampicin. *J Chemother* 16.4, pp. 357–361. DOI: [10.1179/joc.2004.16.4.357](https://doi.org/10.1179/joc.2004.16.4.357).
- Zumla, A. et al. (2015). Inflammation and tuberculosis: host-directed therapies. *J Intern Med* 277.4, pp. 373–387. DOI: [10.1111/joim.12256](https://doi.org/10.1111/joim.12256).
- Zumla, Alimuddin et al. (2016). Host-directed therapies for infectious diseases: current status, recent progress, and future prospects. *Lancet Infect Dis* 16.4, e47–e63. DOI: [10.1016/S1473-3099\(16\)00078-5](https://doi.org/10.1016/S1473-3099(16)00078-5).
- Zurek, Birte et al. (2012). TRIM27 negatively regulates NOD2 by ubiquitination and proteasomal degradation. *PLoS One* 7.7, e41255. DOI: [10.1371/journal.pone.0041255](https://doi.org/10.1371/journal.pone.0041255).