ABSTRACT

Title of Document:	**MECHANISMS OF INNATE IMMUNE EVASION BY *MYCOBACTERIUM TUBERCULOSIS*.**
	Swati Shah, Doctor of Philosophy, 2014
Directed By:	Associate Professor, Volker Briken, PhD Cell Biology and Molecular Genetics

Tuberculosis (Tb) is second only to HIV/AIDS as the most fatal disease caused by a single infectious agent. *Mycobacterium tuberculosis* (*Mtb*) is the causative agent of the disease and is an intracellular pathogen that first infects phagocytes in the lungs. To successfully establish an infection in the host and setup a replicative niche, the bacteria has to be able to evade detection and manipulate innate immune responses in an effort to delay induction of inflammation and the subsequent onset of adaptive immunity. The investigations in this dissertation are focused on three different innate immune evasion mechanisms employed by mycobacteria: (1) inhibition of inflammasome activation (2) secretion of virulence factors and (3) inhibition of apoptosis.

First we demonstrated that unlike the avirulent mycobacterial species, *Mtb* limits the secretion of IL-1β by inhibiting AIM2 inflammasome activation. Experiments revealed that *Mtb* was able to inhibit AIM2 activation and IFN-β production induced by *M. smegmatis*/dsDNA (AIM2 ligand) in an ESX-1 dependent manner. *Mtb* did not affect the expression of AIM2 or IL-1β but rather, inhibited the activation of the inflammasome itself as evidenced by a decrease in caspase-1 cleavage. In another study, we characterized one of the three gene regions (*Rv1037c-Rv1040c*)

duplicated from the ESX-5 secretion system for its role in protein secretion, its effects on host cell immune responses and its virulence in a zebrafish model. We named this four gene region as ESX-5a. Even though the ESX-5a region is required for the secretion of Alanine-L dehydrogenase (ALD), it does not contain genes encoding for the components of a secretion system. Instead, a western blot analysis of the cell supernatants of an ESX-5 system mutant showed that ALD was exported via the parent ESX-5 secretion system. The *Mtb* ESX-5a mutant was found to be defective in inducing inflammasome activation and the *M. marinum* mutant was attenuated in the fish model. The final study focuses on screening for anti-apoptotic genes. *In vitro* infections with *Mtb* transposon mutants helped identify *Rv1048c* as a novel anti-apoptotic gene. As a result, we have identified several novel mechanisms by which *Mtb* achieves a survival advantage during initial infection.

MECHANISMS OF INNATE IMMUNE EVASION BY *MYCOBACTERIUM TUBERCULOSIS*.

By

Swati Shah

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2014

Advisory Committee:
Professor Volker Briken, PhD, Chair
Professor Kevin McIver, PhD
Professor Vincent Lee, PhD
Professor Daniel C. Stein, PhD
Professor Catherine Fenselau, PhD

Dedication

I dedicate this dissertation to:

To my parents Jayesh and Pushpa Shah for making everything in my life possible.

Acknowledgements

First and foremost I would like to profusely thank my advisor, Dr. Volker Briken for mentoring me through my graduate studies. You have been an extremely patient teacher and a guide as you've taught me to research, think and communicate like a scientist, for which I am eternally grateful. I would also like to thank my committee members- Dr. Kevin McIver, Dr. Dan Stein and Dr. Vincent Lee for the support, time and valuable suggestions they have given to me.

I would like to thank my lab members- Jessica, Ben, Serdar and Hana for helping my find my way through the lab early and Amro, from whom I have learnt a great deal. Amro- thanks you for the all phenomenal times and food that you brought to the lab! Gaya - our long downtown trips to blow off steam were essential to my survival. Lalitha- its funny how everyone always mistook us for sisters! We have worked together a lot and your insights during all our work discussions has been invaluable. Thank you for that! Jeff- our lab's "autoclave fairy" and Schwarzenegger impersonator, thank you for the numerous times you've helped me with stuff. Sarah- the lab has been a lot of fun with you around! Thanks a lot for your encouraging words and helpful comments on my dissertation. I wish both you and Jeff the very best of luck in your research!

Finally, I have reserved a special thanks for the most important people in my life. Saurabh- you have been my biggest support ever since I came to the US. You have always been the biggest source of comfort when my frustrations tipped over. Thank you so very much for that. To my parents and brother who are so far away in India- I miss you all and thanks for never letting me forget that you are my safety net. Mama

and Papa- you both have always let me pursue everything I have wanted to do and making you proud of me is still the greatest joy in my life.

Table of Contents

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-LO-	5-Lipooxygenase
AA-	Arachidonic Acid
AIDS-	Acquired Immunodeficiency Syndrome
AIM2-	Absent in Melanoma 2
AK-	Adenylate Kinase
ALD-	Alanine-L Dehydrogenase
Apaf-1-	Apoptosis Protease Activating Factor-1
APC-	Antigen Presenting Cell
ASC-	Apoptosis associated Speck-like containing a CARD
Ask-1-	Apoptosis signal-regulating Kinase-1
ATP-	Adenosine tri-phosphate
BCG-	Bacillus Calmette-Guerin
BMDC-	Bone Marrow derived Dendritic Cell
BMDM-	Bone Marrow Derived Macrophage
BSA-	Bovine Serum Albumin
CARD-	Caspase Activation and Recruitment Domain
CF-	Culture Filtrate
ChIP-	Chromatin Immunoprecipitation
CL-	Cell Lysate
COX2-	Cyclooxygenase 2
DAMP-	Danger Associated Molecular Patterns
DC-	Dendritic Cell
DC-SIGN-	DC Specific ICAM3 Grabbing Non-integrin
DISC-	Death Inducing Signaling Complex
DMEM-	Dulbecco's Modified Eagle Medium
DNA-	Deoxyribonucleic Acid
dsDNA-	Double stranded DNA
eDNA-	Extracellular DNA
ELISA-	Enzyme linked Immunosorbent Assay
ERK 1/ 2-	Extracellular-signal regulated Kinases
ESAT6-	6 kDa Early Secreted Antigenic Target
ESCRT-	Endosomal Sorting Complex Required for Transport
ESX-	ESAT6 Secretion System
FADD-	Fas Associated Death Domain
FAP-	Fibronectin Attachment Protein
FCS-	Fetal Calf Serum
FM-	Foamy Macrophages
GM-CSF-	Granulocyte Macrophage Colony Forming Unit

HIN-	Hemopoeitic expression, Inteferon-inducibility, Nuclear Localization
HIV-	Human Immunodeficiency Virus
HRP-	Horseradish Peroxidase
IFN-	Interferon
Ifnar1-	Interferon alpha receptor subunit 1
IL-	Interleukin
IL-1R-	Interleukin 1 receptor
iNOS-	Inducible Nitric Oxide Synthase
IR-	Immune Response
IRF3-	Interferon Regulatory Factor 3
JNK-	C Jun N-terminal Kinase
LCCM-	L- cell Conditioned Media
LoGoF-	Loss of Gain of Function
LPS-	Lipopolysaccharide
LRR-	Leucine rich repeat
LVS-	Live Vaccine Strain
LXA4-	Lipoxin A4
Man-Lam-	Mannose capped Lipoarabinomannan
MAP3K-	Mitogen Activated Protein Kinase Kinase Kinase
MDR-	Multi-drug Resistant
Mfort-	*M. fortuitum*
MGC-	Giant multinucleated Cell
MHC-	Major Histocompatibility Complex
Mkan-	*M. kansasii*
Mm-	*M. marinum*
MOI-	Multiplicity of Infection
MOMP-	Mitochondrial Outer Membrane Permeabilzation
MR-	Mannose Receptor
Msme-	*M. smegmatis*
Mtb-	*M. tuberculosis*
NADH-	Nicotinamide adenine dinucleotide
NF-κB-	Nuclear Factor kappa light chain enhancer of activated B cells
NLR-	Nucleotide-binding oligomerization domain like Receptor
NO-	Nitric Oxide
NOX-	NADPH Oxidase
NTM-	Non-tuberculous Mycobacteria
NuoG-	NADG-ubiquinone oxidoreductase chain G (belonging to Type I NADH dehydrogenase)
PAK-	*Pseudomonas aeruginosa* strain
PAMP-	Pathogen Associated Molecular Pattern

PBS-	Phosphate Buffered Saline
PGE2-	Prostaglandin E2
PknE-	Protein kinase E
PknG-	Protein kinase G
PMA-	Phorbol Myristate Acetate
PRR-	Pathogen Recognition Receptor
PYD-	Pyrin Domain
qRT-PCR-	Quantitative Real Time Polymerase Chain Reaction
RD1-	Region of Difference 1
RNA-	Ribonucleic Acid
RNI-	Reactive Nitrogen Intermediate
ROS-	Reactive Oxygen Species
RPMI-	Roswell Park Memorial Institute Medium
SDS-PAGE-	Sodium dodecyl sulphate- Polyacrylamide Gel Electrophoresis
SodA-	Superoxide Dismutase A
Syt-7-	Synanptotagmin-7
T7SS-	Type VII Secretion System
TACO-	Tryptophan-Aspartate containing Coat Protein
Tb-	Tuberculosis
TBK-1-	TANK binding kinase-1
TBS-	Tris buffered Saline
TH-	T-helper
THP-1-	Human Monocytic cell line
TLR-	Toll like Receptor
Tn-	Transposon
TNF-	Tumor Necrosis Factor
TNFR-	Tumor Necrosis Factor Receptor
TRADD-	TNF Receptor Associated Death Domain
TUNEL-	Terminal deoxynucleotidyl transferase dUTP nick end labeling assay
UI-	Uninfected
WB-	Western Blot
WHO-	World Health Organization
WT-	Wildtype
XDR-	Extremely Drug Resistant
ZMP1-	Zinc Metalloprotease 1

CHAPTER 1: INTRODUCTION

1.1 Tuberculosis

Tuberculosis (Tb) still remains one of the biggest global health concerns. The 2013 global Tb report issued by the World Health Organization (WHO) states that in 2012 alone, about 8.6 million people were diagnosed with Tb and 1.3 million fatalities have occurred, including ~0.3 million deaths among HIV positive people. Tb is a debilitating disease that primarily infects the lungs but can spread to other parts of the body.

1.1.1 History

Tb is one of the oldest known diseases afflicting humans, since the first infected specimen excavated from the Mediterranean Sea was dated to be around 9,000 years old [105]. However, there is evidence indicating the origin of *Mycobacterium tuberculosis* (*Mtb*) as a human pathogen occurred over 70,000 years ago. Since then it was believed that the organism migrated out of Africa along with the humans and was transmitted due to increase in population density [49]. Over the years, Tb has been known by other names such as consumption, white plague, phthisis etc. In the absence of any knowledge about cause or treatment regimens, Tb was the leading cause of death in 17th century Europe owing to poor sanitation and over-crowded cities which created ideal environment for rapid spread of *Mtb* [15]. It was only since the early 19th century that the details about the cause and transmissible nature of the disease arose, but the only treatment options available were rest and retreat to cold climates. The first cure for Tb was found only in the mid-20th century with the discovery of streptomycin by

Selman Waksman [57]. Many notable people, namely – Frederic Chopin, John Keats, Eleanor Roosevelt, Thoreau and Voltaire succumbed to the disease.

1.1.2 Causative Agent

The causative agent of Tb is *Mycobacterium tuberculosis* (*Mtb*). This organism belongs to a group of closely related members called the *M. tuberculosis* complex. They are known to cause Tb in humans (*Mtb*, *M. africanum* etc) and animals (*M. bovis*, *M. microti* etc) [36]. Other mycobacterial species found in the environment such as *M. avium* and *M. kansasii*, cause lung infections similar to Tb in immunocompromised people and are classified as non-tuberculous mycobacteria (NTM) [206]. *Mtb* is a facultative, intracellular pathogen that exclusively resides and persists within a human host. Mycobacterial species have a unique membrane composition made up of lipids called mycolic acid forming the "mycomembrane" that contributes to its unusual properties [169]. They are weakly Gram positive but identified by acid fast staining techniques such as Ziehl-Neelsen stain. The virulent mycobacterial species are also extremely slow growing with a doubling time of ~24 hours.

1.1.3 Disease Progression

Mtb is spread directly from person to person through aerosol droplets released from active Tb patients through coughing or sneezing. They are highly infectious with 1-2 bacilli being enough to mount an infection [168]. Upon entry into the lungs, *Mtb* first encounters the alveolar macrophages and is phagocytized by them. They can also be taken up by the dendritic cells circulating in the lungs.

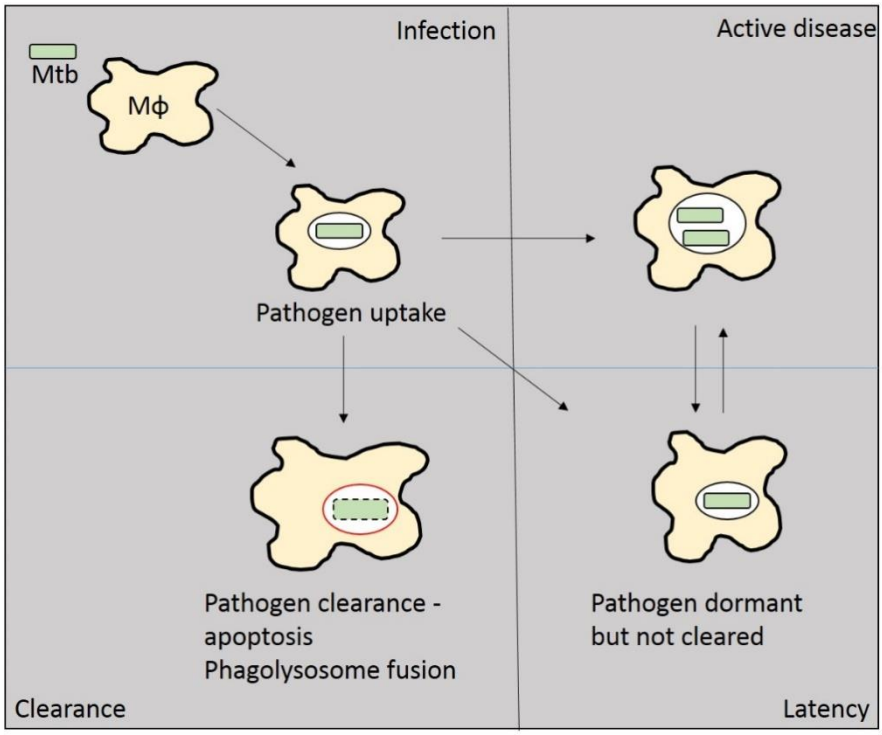

Figure 1: Bacterial fate after infection [adapted from 64].
Bacteria are engulfed by phagocytes upon inhalation and can meet with three fates: clearance, active infection or latency

Upon entry and phagocytosis, *Mtb* can: 1) be **cleared by the immune system** 2) **disseminate causing active disease** or 3) **establish a latent infection** (Fig 1).

Mtb can cause an active disease in immunocompromised individuals, patients taking immunosuppressive drugs and people suffering from malnutrition or old age. Here the bacteria induce necrosis, a form of cell death that induces membrane lysis that does not affect its viability [61]. Upon release, the bacteria can re-infect neighboring cells causing disease dissemination. In the case of latent infection, the bacteria remain dormant. Additionally, the infected macrophages secrete cytokines and chemokines to recruit other immune cells to the site of infection. The bacteria are walled off within a structure called the granuloma, which is an organized collection of immune cells. The granuloma consists of infected macrophages in the center surrounded by macrophages that fuse to form giant multinucleated cells (MGC) or differentiate in lipid rich foamy macrophages (FM). The outer rim of the granuloma is made up og T and B lymphocytes. The bacteria contained within this structure remain in a dormant non-replicative state [72]. However, they can re-activate at any time during the life of the infected individual owing to a variety of host factors such as malnutrition, old age and HIV infection. During such time, the center of the granuloma undergoes necrosis (forming a caseum), ultimately causing the disintegration of the entire structure and dissemination of the bacteria (Fig 2) [39].

1.1.4 Epidemiology

One-third of the world is latently infected with Tb, with the highest incidences seen in developing countries from regions of sub-Saharan Africa and parts of Southeast Asia.

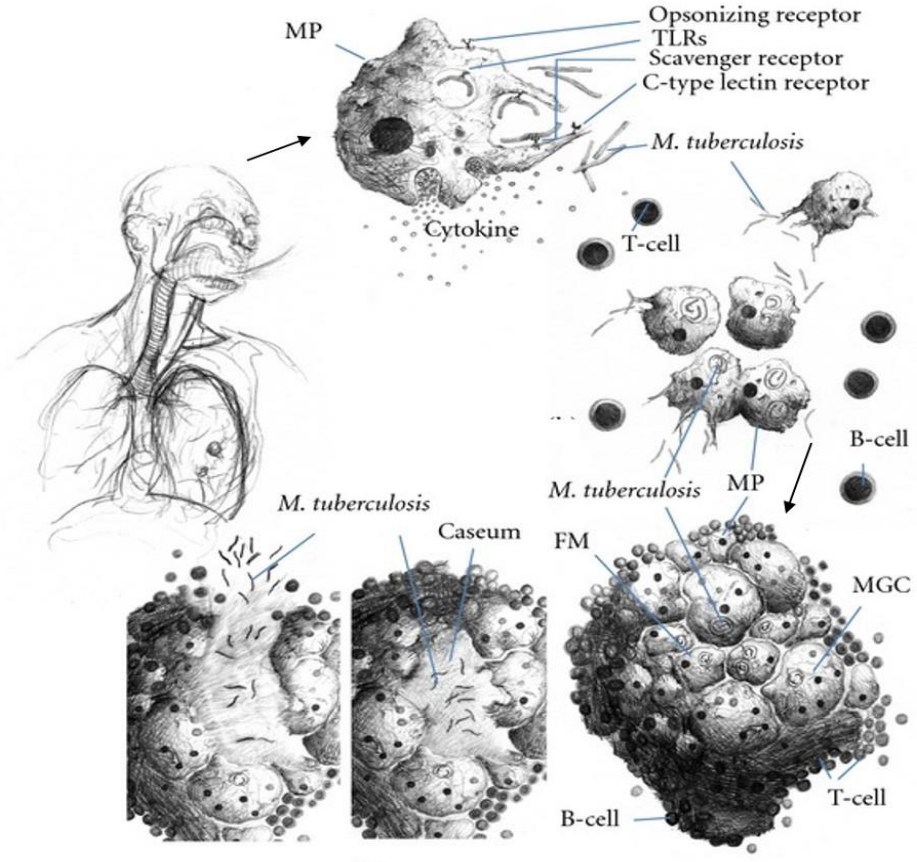

Figure 2: Formation of the granuloma [204].
The figure describes the steps leading to formation of granuloma leading to latency and escape of bacteria during reactivation.

A properly functioning immune system is imperative for controlling the disease, which is why the resurgence of Tb is closely tied to the high HIV infection rates [16]. However, the rates of new Tb cases have been decreasing over the past few years due to the efforts of various organizations to diagnose and treat both diseases (WHO 2013 Factsheet). About 90% of the affected individuals do not develop the active form of infection, a condition referred to as latent Tb. During this time the infected people are asymptomatic and non-contagious. However, the dormant bacteria can reactivate at any time and cause an active infection. The infected individuals then display the following symptoms: fever, night sweats, weight loss, chest pain and a chronic cough resulting in bloody discharge of sputum [68].

1.1.5 Treatment and Prevention

The development of Tb therapy began in 1882 when Robert Koch identified *Mtb* as the causative agent of the disease. The discovery of X-rays by Roentgen made tracking the disease progression easier. However, the only treatment options available during this period were fresh air, rest and a good diet. The afflicted people were sent to places called "sanatoriums" where they were quarantined until they succumbed to the disease. The first concrete step in treating Tb was the discovery of streptomycin by Selman Waksman and Albert Schatz (1943). Shortly after, there were a barrage of drugs discovered, namely, isoniazid, rifampin, pyrazinamide and ethambutol which formed the first line antibiotics [198]. Even today, all four of these drugs are prescribed as a combination chemotherapy that typically lasts 6-9 months. Isoniazid is highly toxic and can cause neuropathy, which is why it is given along with pyridoxal phosphate or

vitamin B_6 to prevent nerve damage. These drugs are to be taken for 4 months after which only isoniazid and ethambutol are taken for 2 additional months. All of these drugs have different modes of action against *Mtb*; for example, inhibiting cell wall synthesis (isoniazid) or transcription (rifampin) (Fig 3) [194].

The above regimen is good only for drug susceptible *Mtb*. In many cases patients do not complete the full antibiotic course because the regimen is too long or that they start feeling better after a few weeks. This led to the development of drug resistant strains and disease relapse, in which case the second and third line antibiotics are used [190]. The second line drugs include flouroquinolones, aminoglycosides and cyclosporine among others. These drugs are known to cause more severe side effects or in some cases are expensive and not readily available. The multi-drug resistant (MDR) strain of *Mtb* is found to be resistant to both isoniazid and rifampin [174]. However, ineffective chemotherapy with the second line drugs led to development of extremely drug resistant (XDR) strain in the early 2000s. The XDR strain is resistant to isoniazid, rifampin, second line drugs such as flouroquinolones and at least three injectable drugs (amikacin, kanamycin and capreomycin) leaving patients with very few treatment options [42].

Albert Calmette and Camille Guerin generated the vaccine *M. bovis* BCG after passaging *M. bovis* for 11 years and the BCG vaccine was first administered in 1921 [79]. To date, BCG remains as the only approved vaccine and is administered worldwide.

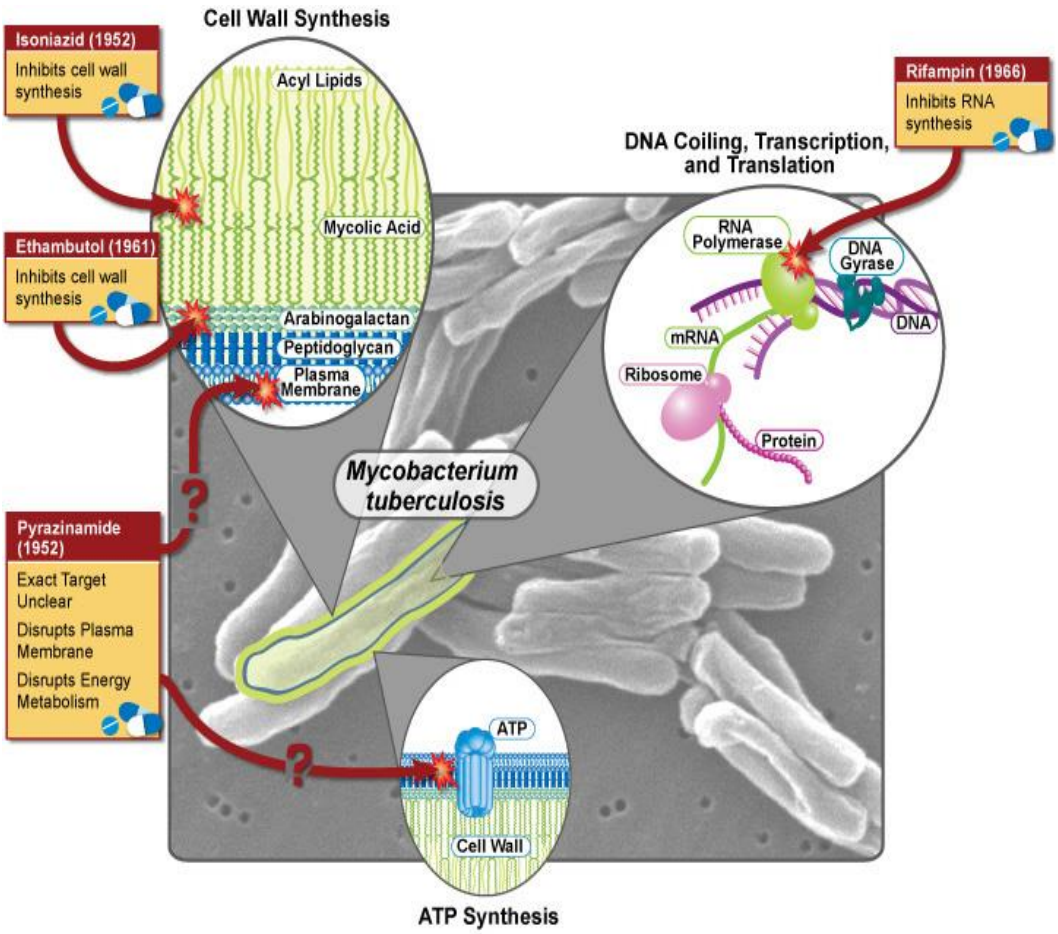

Figure 3: Mechanisms of action of first line antibiotics against *Mtb* (NIAID).
Both isoniazid and ethambutol inhibit cell wall synthesis. Pyrazinamide disrupts energy metabolism and rifampin affects RNA synthesis.

However, use of the BCG vaccine has been decreasing over the years because of its variable efficacy in protecting adults from pulmonary Tb (0-80%), which is in part based on the specific vaccine strain used and the geographical location [47, 80]. Another drawback of BCG is that it poorly stimulates CD8 T-cells to mount a robust adaptive immune response [31, 187].

1.2 Mycobacteria and Immune Response

Immune response (IR) against invasion by a pathogen can be classified into two distinct types: 1) Innate Immunity and 2) Adaptive Immunity.

Innate immunity is a primary response mediated by detection of pathogen associated molecular patterns (PAMPs) leading to a localized inflammatory response to recruit other immune cells to the site of infection. Its functions include signaling events leading to the destruction of the pathogen as well as processing antigens to stimulate the second IR, which is adaptive immunity. When compared to innate IR, the adaptive response is more specific leading to cellular and humoral immunity and also results in long term memory. Some of the components and signaling processes required for mediating a successful immune response against *Mtb* are listed below.

1.2.1 Detection of *Mtb*

Innate IR involves the detection of *Mtb* by a host of cell types such as macrophages, dendritic cells (DC) etc., which are then capable of ingesting the bacteria. Multiple *Mtb* ligands are recognized by various receptors such as Toll Like Receptor 2

(TLR2) in cooperation with dectin-1 [239], DC specific intracellular adhesion molecule 3 grabbing non-integrin (DC-SIGN), Mannose Receptor (MR) and Complement Receptor CR3 displayed by the alveolar macrophages before being phagocytized [74, 220]. The uptake of *Mtb* by DCs is primarily mediated by DC-SIGN [94].

The TLRs are a family of membrane bound pattern recognition receptors (PRRs) that detect microbial ligands and activate inflammatory pathways as a result [9]. Downstream signaling of TLR-ligand binding leads to activation of various transcription factors like NF-κB and AP-1, which in turn induce the transcription of various inflammatory cytokines. *In vitro* infections with *Mtb* show that TLR2 plays a very important role as it detects lipopeptides when associated with TLR1 and TLR6. A mycobacterial protein called the 19 kDa lipoprotein is recognized by TLR2/1 [222], while an extract of *Mtb* called soluble Tb factor is detected by TLR2/6 [38]. Deletion of TLR9 in DCs caused a partial reduction in IL12p40 production and while a TLR2/TLR9 double deletion completely abrogated IL12p40 secretion [155]. The above data demonstrates that TLR9 has a role to play in *Mtb* infection control. The individual role of TLRs *in vivo* has been difficult to show because of redundancy and many conflicting reports [71, 188]. However, the deletion of multiple TLRs increases susceptibility to infection in mice.

Nucleotide-binding oligomerization domain-like receptors (NLR) are a family of cytoplasmic receptors that interact with PAMPs or danger associated molecular patterns (DAMPs) within the cell and are shown to be very important for innate immunity against *Mtb*. These proteins contain 3 major domains: (1) a central nucleotide binding domain, (2) a C-terminal ligand binding domain and a (3) a variable N-terminal

domain involved in protein-protein interactions. The N-terminal domain is usually a caspase recruitment domain (CARD) or pyrin domain (PYD) [86]. One of the major consequences of NLR ligation is the formation of a multi-protein complex called the inflammasome, which leads to various cellular effects such as cell death by pyroptosis and maturation of the inflammatory cytokines: Interleukin 1β (IL-1β) and Interleukin 18 (IL-18). There are many NLRs activated upon bacterial infection as discussed below. Of these, multiple studies have shown that *Mtb* infection solely activates the NLRP3 inflammasome [1, 128, 153]. Absent in melanoma 2 (AIM2) is a member of a family of receptors called the AIM2 like receptors (ALRs) that can also activate the inflammasome after binding to double stranded DNA (dsDNA) [76, 191]. Thus, the initiation of immunity against the pathogen starts with detecting different pathogenic components via various cellular receptors.

1.2.2 Cytokines

Cytokines are signaling proteins secreted by cells to shape the immune response. They have multiple roles in immune response, such as maturation of immune cells, initiating either cell mediated or humoral response and recruitment of other immune cells to the site of infection. Cytokines involved in cell recruitment to the site of infection are called chemokines. The cytokines shown to be most important for controlling *Mtb* infection are Tumor Necrosis Factor (TNF), Interferon gamma (IFNγ), Interleukin 12 (IL-12) and IL-1β, all of which are pro-inflammatory in nature.

TNF produced by macrophages and T-cells is important for macrophage activation. It also has an important role of inducing apoptotic cell death in infected

macrophages [160]. It helps in maintaining the structure of granuloma as both TNF and TNF receptor (TNFR) knockout mice are highly susceptible to *Mtb* infection and show impaired granuloma formation [17,84]. Studies involving *M. marinum* infected zebrafish showed that in the absence of TNF, the granulomas had highly necrotic centers and disintegrated more rapidly [182]. This phenotype of high susceptibility is also reflected in humans who are being treated with TNF inhibitors [124]. In a macaque model, the addition of TNF-neutralizing antibodies caused the animals with latent infection to rapidly developed active Tb [136].

IL-12 is mainly secreted by macrophages, DCs and neutrophils in response to ligation of TLR-9 and TLR-2 [11]. It plays an important role in T-cell differentiation into TH1 (T Helper1) type IR which is important for controlling intracellular pathogens [52, 171, 226]. Mice deficient in IL-12p40 and IL-12p35 are highly susceptible to *Mtb* infections [127]. As a result of TH1 response, IFNγ is produced by CD4 and CD8 T-cells. This cytokine is essential to *Mtb* infection control as it induces the production of inducible nitric acid synthase (iNOS) in macrophages that is required for mycobactericidal effects and in its absence there is increased dissemination of the pathogen within the mouse [51].

IL-1β

IL-1β is another important cytokine required for the control of *Mtb* infections. The absence of IL-1β or its receptor causes mice to succumb within 40 days of an *Mtb* challenge while the infected WT mice survive for more than a year [89, 153]. IL-1β is highly pro-inflammatory, thus the production of mature IL-1β is a tightly regulated two-step process. It is produced as an inactive precursor that must undergo cleavage to

form a mature 17 kDa fragment that is then secreted outside the cell. Another layer of regulation comes in the form of decoy receptors and IL-1R antagonists, which disrupt the IL-1β/IL-1R interaction [65]. Even though most *in vitro* studies show that IL-1β secretion is inflammasome mediated, *in vivo* IL-1β can be cleaved by caspase-1 independent mechanisms [153] and this process is shown to occur in neutrophils with the help of cathepsins and elastases [99]. IL-1β is directly involved in killing of mycobacteria as it is required for up-regulation of TNF secretion and increased caspase-3 activation [116]. Autophagy is considered to be an anti-inflammatory cellular process that inhibits inflammasome components and thus limits IL-1β production. However, a recent study has described a new role for IL-1β signaling mediated autophagic response that is required for killing mycobacteria in a TANK binding kinase-1 (TBK-1) dependent manner [178]. The process of generating the mature cytokine is by the activation of an inflammasome and is described below.

Inflammasome Activation

Firstly, inactive pro-IL-1β and pro-IL-18 are produced due to TLR ligation with bacterial products (signal 1) leading to NF-κB dependent transcription of *il1β* and *il18.* This pro form of IL-1β needs to be cleaved before it can be secreted and initiate its signaling events via the IL1 receptor (IL1R). Therefore, the second step involves the formation of a multi-protein complex called the inflammasome, which is initiated by the NLR binding to a PAMP/DAMP (signal 2) [148].

Some of the PAMPs recognized by various NLRs are: flagellin (NLRC4) [85] and anthrax lethal toxin (NLRP1) [30]. Recent studies have shown that NLRP3 does

not directly bind to a ligand, but various cellular insults result in potassium depletion of the cytosol that in turn leads to its activation. Some of the other known effectors are ATP, alum, cholera toxins etc. [165]. The inflammasome can also be activated by AIM2. The inflammasome consists of activated NLR bound to the Apoptosis associated Speck-like protein containing a CARD (ASC) protein via its pyrin domain. The ASC protein binds to pro-caspase-1via the CARD region. However, not all NLRs require the adaptor protein ASC (e.g. NLRC4) as they can directly recruit caspase-1. The oligomerization of caspase-1 results in auto-proteolytic cleavage to release activated caspase-1. This cleaved caspase-1 in turn cleaves pro- IL-1β and pro-IL-18 into mature IL-1β and IL-18, respectively (Fig 4). Thus, the inflammasome activation is critical for the maturation of IL-1β and IL-18.

1.2.3 Innate Immune Cell Types

Phagocytes are important for mounting an innate IR against *Mtb*. Once the pathogen is internalized, macrophages unleash a host pathogen killing mechanisms such as production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), anti-bacterial peptides, maturation of phagosome resulting in phagolysosome fusion and degradation of bacterial cell wall by lysosomal hydrolases [reviewed in 161]. Additionally, the cells undergo apoptotic cell death (discussed below) to prevent bacterial escape. The bacteria are killed by a process called efferocytosis which involves the uptake of bacteria-containing apoptotic bodies by neighboring phagocytes. The apoptotic bodies are also phagocytized by DCs which in turn mediate cross-priming resulting in activation of CD8 T-cells (section 4.2.2).

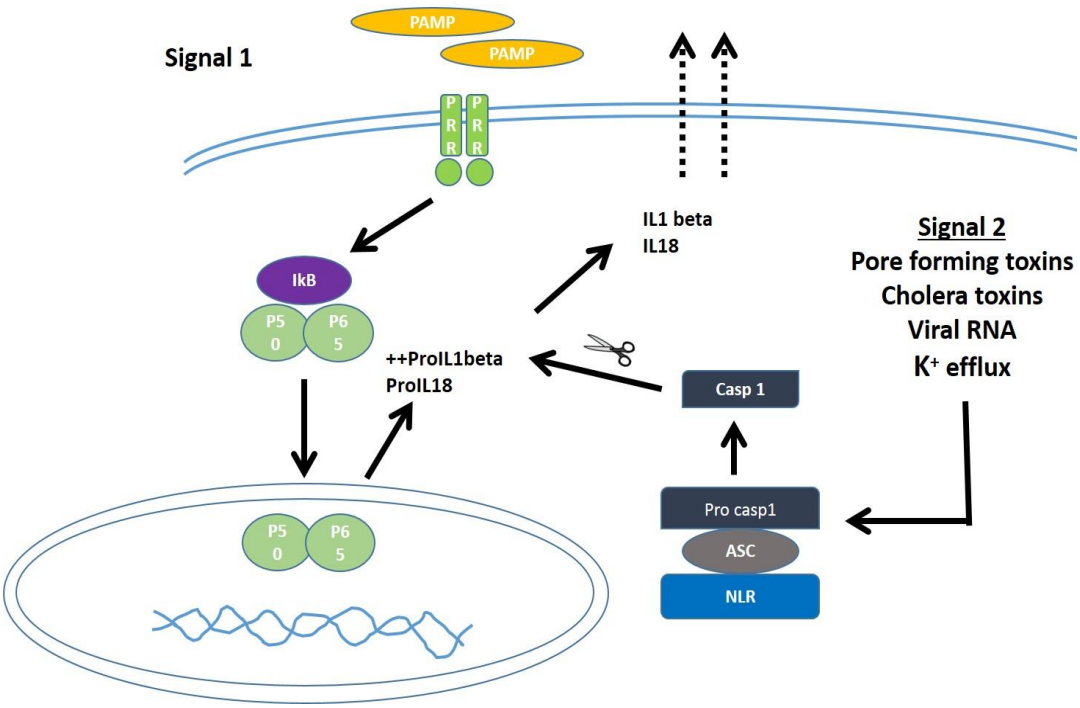

Figure 4: IL-1β secretion by NLRP3 inflammasome activation.
Maturation of IL-1β and IL-18 is a two-step process involving the transcription of cytokine precursors through signal 1 and cleavage to mature form by active caspase-1, mediated by signal 2.

Neutrophils are phagocytic cells that quickly migrate to the site of infection in response to chemokines secreted by activated macrophages. These cells contribute to early defense by inducing a strong respiratory burst (influx of ROS into the phagosome) and by producing "defensins", enzymes known to kill mycobacteria [150]. The neutrophils are short lived as they rapidly undergo apoptosis and the apoptotic blebs are engulfed by DCs. DCs acquiring *Mtb* infected neutrophilic apoptotic bodies are better at migrating to lymph nodes and activating CD4 T-cells to mount an adaptive IR when compared to DCs that directly acquired *Mtb* [27].

While macrophages and neutrophils are major mediators of innate immunity, DCs are the most potent antigen presenting cells (APCs) that act as a bridge between the innate and adaptive IR. Immature DCs present in the lungs either directly phagocytize invading bacteria or the remnants of infected macrophage/neutrophils apoptotic bodies. As a result, the DCs mature and migrate to the lymph nodes where they process the bacterial antigens via MHC class I and II molecules and present the processed peptides to prime naïve T-cells [12,158].

Apoptosis – Mechanism and Characteristics

Cell death by apoptosis is one of the mechanisms of innate IR initiated by macrophages upon infection. Apoptosis is a process of programmed cell death, wherein a cell on receiving specific stimuli undergoes a series of orderly steps that ultimately leads to its demise. Apoptosis is very important for embryonic development, selection of leukocytes, general homeostasis and host defense [73]. Cells undergoing this form of cell death show signs of shrinkage due to their cytoskeleton breakdown. Other

hallmark features are membrane blebbing, DNA condensation and fragmentation. The flipping of inner cell membrane to the outside causes the exposure of phosphatidylserine and this is an early stage marker of apoptosis. However, unlike in necrosis, the caustic cell contents are not spilt into the extracellular milieu but contained within an impermeable "apoptotic envelope" [179]. With time, the cell breaks into smaller vesicles called "apoptotic bodies" which are then phagocytized by the surrounding cells before they can cause any damage. Hence, this form of cell death is immunologically silent. Apoptosis is mediated by a group of cysteine-aspartic proteases called caspases via two different pathways: intrinsic or extrinsic pathways [73] (Fig 5).

Intrinsic Pathway of Apoptosis

The intrinsic pathway is generally activated in response to cell damage by toxins, free radicals, radiation or DNA damage and involves the mitochondria [73]. Under normal conditions, the mitochondrial membrane is coated by anti-apoptotic proteins of the Bcl-2 family. These proteins bind to and sequester the pro-apoptotic proteins of the Bcl-2 family. All members of this family consist of homology domains called BH1-BH4. Anti-apoptotic members have all four domains while pro-apoptotic members may either have all domains or only the BH3 domain. Stress creates an imbalance between the two factions of this family and this leads to mitochondrial outer membrane permeabilization (MOMP) [8]. As a consequence, cytochrome c is released into the cytoplasm. This leads to the association of cytochrome c with apoptosis protease activating factor (Apaf-1).

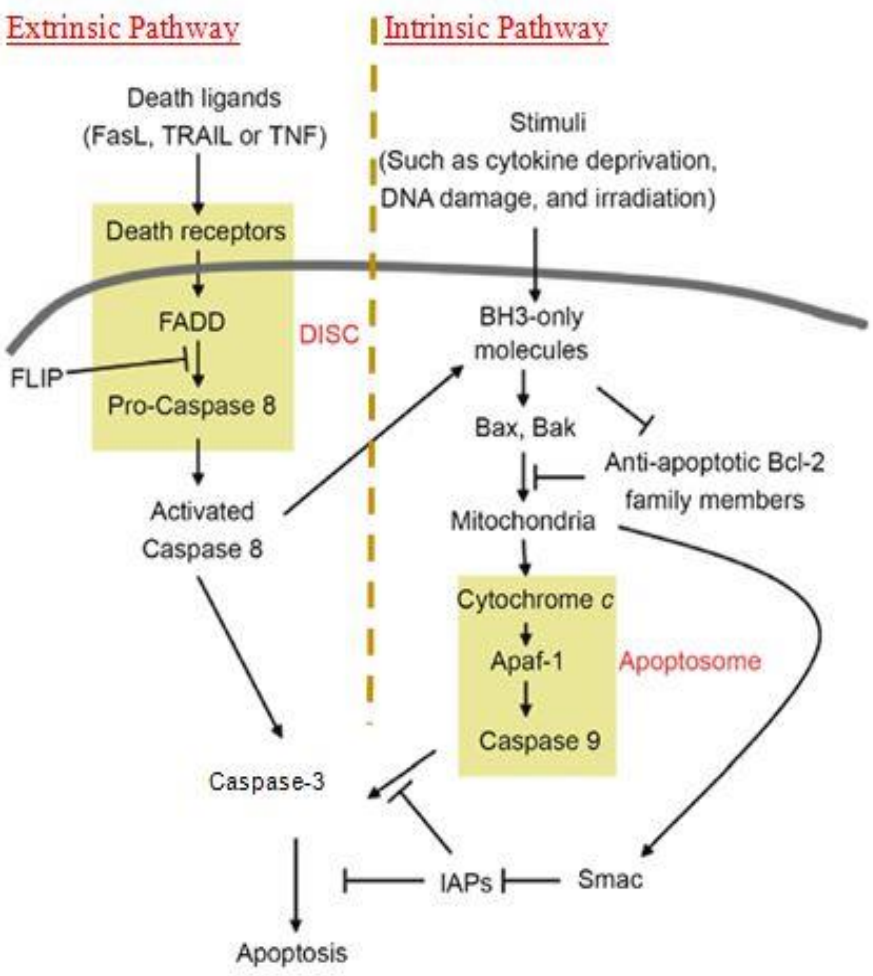

Figure 5: Schematics of apoptotic pathways [adapted from 237].
The figure describes the steps involved in both extrinsic and intrinsic pathways of apoptosis and the point of intersection between both pathways at the level of caspase-3 activation.

The Apaf-1 binds to the (initiator) pro-caspase-9 through its CARD and activates it, thus forming a complex of proteins called the apoptosome. The activated caspase-9 is released and activates the effector caspase-3 [189]. The activated caspase-3 in turn cleaves the inhibitor ICAD/DFF-45 to allow the endonuclease activity of CAD leading to DNA fragmentation [137] (Fig 5).

Extrinsic Pathway of Apoptosis:

The extrinsic pathway is induced by external signals such as binding of specific ligands to the Tumour Necrosis Factor Receptor 1 (TNFR1) and Fas [232]. The next step in this signaling cascade is the recruitment of death domains. During a Fas ligand-Fas interaction, the Fas associated death domain (FADD) binds to the cytoplasmic tail of the receptor. In case of TNF-TNFR association, TNF receptor associated death domain (TRADD) is recruited, which in turn recruits the FADD protein. Caspase-8 and 10 (also called initiator caspases) can now bind to FADD protein. This complex is referred to as the death inducing signaling complex (DISC). The pro-caspases are now cleaved into their active form. From this point onwards the pathway can proceed two ways. The activated caspase-8 cleaves the pro-caspase-3 (effector caspase) into its active form or it cleaves the protein BID into tBID (truncated), which then intertwines with the intrinsic pathway by associating with other Bcl-2 family members on the mitochondrial surface and causing MOMP [134, 139] (Fig 5).

Apoptotic death of infected macrophages not only affects bacterial viability but it is also important for mediating cross presentation by DCs to naïve T-cells to initiate an adaptive IR.

1.2.4 Adaptive Immune Response

The adaptive immune response is the secondary response generated by the host immune system against foreign invasion. The T and B lymphocytes initiate a highly specific immune response that is tailor-made against the invading pathogen. B cells mount a humoral response by generating antibodies. T-cells are activated by processed antigens presented to it by APCs and mount a cell mediated IR. T-cells form two distinct populations: T Helper cells (CD4+) and cytotoxic T-cells (CD8+). Depending on the cytokine secreted, the CD4 cells can induce either an inflammatory TH1 response or an anti-inflammatory TH2 response. Macrophages and dendritic cells secrete IL-12 to push the CD4 cells toward a TH1 type response. Once stimulated, the CD4 T-cells can now produce IFNγ, IL-2 and TNF, which are known to activate macrophages and make them more efficient at killing pathogens [82]. Cytotoxic or CD8 T-cells are mostly activated by cross-presentation pathways involving DCs. The activated CD8 T-cells secrete IFNγ, perforins and granulysins that can lyse host cells and kill bacteria directly [214].The importance of the CD4 T-cell population during *Mtb* infections is evidenced by the finding that people with very low CD4 T-cell counts (HIV patients) show increased susceptibility to active Tb [200]. The macrophages and neutrophil apoptotic bodies containing *Mtb* and its antigens are phagocytized by DCs, which then process the mycobacterial antigens via MHC class I molecules and present them to naïve CD8 T-cells to activate them [67, 235]. Both the T-cell populations are also very important for granuloma formation and the absence of either one or both lineages make mice highly susceptible to Tb [82]. Thus, adaptive IR is very important for the control of mycobacterial infections.

1.3 *Mtb* Evasion of Host Immune Responses

Mtb successfully persists within a macrophage phagosome, which by nature is a harsh environment. To survive, it uses various strategies to avoid being detected and killed by the immune cells. Some of the immune evasion mechanisms employed by *Mtb* are listed below.

1.3.1 Secretion of Virulence Factors

Mtb has a specialized protein secretion system referred to as the Type VII secretion system (T7SS), which encompasses five individual systems of similar gene groups labeled as ESX 1-5. All of these systems contain the usual elements of a secretory system, such as: a transmembrane protein acting as a channel and an ATPase. Additionally, the T7SS encode for two small, secreted proteins belonging to the ESAT6 family and are flanked by *pe* and *ppe* genes (except in ESX-4). The group of proteins belonging to a family called PE or PPE, are exclusive to the mycobacterial genus and found encoded by the genome of slow growing mycobacteria only. The PE/PPE protein families derive their names from the Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs found in the N-terminal of these proteins. Of the 5 T7SSs, both ESX 1 and ESX 5 are important for virulence in *Mtb* [4, 29, 180].

EsxA (a member of the ESAT6 family) is secreted by the ESX-1 system and is required for multiple processes such as, co-dependent secretion of other virulence factors, perforating the phagosomal membrane [209] and abrogating NF-κB activity by directly binding to and inhibiting TLR2 function [176]. EsxH and EsxG are the ESAT6 family proteins contained in and secreted by the ESX-3 system. The ESX-3 system is

involved in the secretion of proteins involved in iron and zinc uptake [201]. These proteins directly bind to components of the endosomal sorting complex required for transport (ESCRT) pathway and impair phagosome maturation [156]. The ESX-5 system is required for the secretion of various members of the PE/PPE family. Single gene deletion mutants of ESX-5 components or their secreted proteins are attenuated during mouse infections [29, 197]. Due to the sheer number of PE/PPE proteins, it is difficult to tease out individual functions, but the current hypothesis is that these proteins interact with a variety of host components and aid in immune evasion. In fact, members of the PE/PPE family are involved in modulating host responses by interacting with TLR2 [13,167], preventing vacuole acidification [117, 215] and limiting antigen presentation [32].

1.3.2 Inhibition of Phagolysosome Fusion

When a macrophage engulfs a foreign particle, the phagosome undergoes a series of maturation events by fusing with the early and late endosomes and finally fuses with the lysosomes. The lysosome is a storehouse of various hydrolytic enzymes that can digest the foreign bodies. Through various stages of maturation the phagosome acquires various markers that direct its fusion with other vesicles. The interior of the phagolysosome becomes increasingly acidic due to the recruitment of the vacuolar proton ATPase and this activates the lysosomal hydrolases [161].

Virulent *Mtb* strains have developed the means to prevent the acidification of the vacuole and hence can arrest phagosome maturation [217]. *Mtb* containing phagosomes are also found to be coated with tryptophan-aspartate containing coat

(TACO) protein, which prevents it from fusing with the lysosome [78]. Release of Ca^{2+} from intracellular stores to increase its cytosolic concentration is required for the membrane fusion between two vesicles. However, the lipoprotein Man-Lam (Mannose capped Lipoarabinomannan), found only in pathogenic *Mtb*, is able to inhibit the increase in Ca^{2+} concentrations and its downstream signaling [192]. Other mycobacterial lipids such as trehalose dimycolate [113] and proteins like SapM [231] and PknG [233] have also been implicated in prevention of phagolysosome fusion.

1.3.3 Inhibition of ROS and RNI

Activated macrophages and neutrophils can efficiently kill microbes through the generation of ROS and RNI. ROS is generated by the assembly of the NOX2 complex on the phagosome membrane. The process of NOX2 activation is very tightly regulated and requires the assembly of several proteins on the cytoplasmic side. Once active, it can transfer electrons across the membrane to generate superoxides [19]. The generated ROS, is not only highly toxic but also plays additional roles in cell signaling. ROS production affects the activation of Ask-1 (a member of the mitogen activated protein kinase- MAP3K family) which in turn activates Jun N-terminal kinase (JNK) and p38 pathways. Both JNK and p38 are involved in induction of apoptosis [112, 221]. ROS is also required for TNF synthesis by macrophages, which is absolutely necessary for apoptosis induction and controlling *Mtb* growth [160]. Thus, *Mtb* have evolved ways to neutralize the effect of ROS by secreting superoxide dismutase A (SodA) via the SecA2 secretion system. Thus, deleting the *secA2* gene also resulted in increased ROS, apoptosis and mycobacterial killing [106]. A mycobacterial gene,

nuoG encodes for a subunit of type- I NADH dehydrogenase and is involved in mitigating ROS production in *Mtb* infected macrophages [160].

Nitric Oxide (NO) and other reactive intermediates are generated by the enzyme called inducible nitric oxide synthase (iNOS). Since the RNI produced are highly reactive, proximity to the target is very important and so the iNOS is recruited to the bacteria containing phagosome by the actin cytoskeleton [60,159]. The importance of RNI activity is evidenced when the mice with the deletion of iNOS succumb to an *Mtb* challenge faster than WT mice. However, the importance of iNOS in controlling mycobacterial infections is still a point of contention in humans [140]. Two *Mtb* proteins associated with proteasome functions, Mpa and PafA, are important for NO detoxification [58, 59], but their exact mechanism of action is still unknown. *Mtb* also interferes with the process of iNOS recruitment to the phagosome by preventing the interaction between iNOS, a scaffolding protein called EBP50 and the actin filament, thus reducing the toxic effect of RNI. The *Mtb* effectors that prevent iNOS recruitment are unknown [60, 159]. Thus, *Mtb* has developed multiple mechanisms by which it can neutralize the deleterious effects of both ROS and RNI.

1.3.4 Inhibition of Inflammasome Activation

NLR family proteins scavenge the cytosol for PAMPs and DAMPs. Upon detection of pathogens, they induce inflammatory cascades which eventually lead to the onset of innate IR. The main components of the inflammasome are: NLRs, ASC and pro-caspase-1. The inflammasome activation leads to production of active caspase-1. This protease functions to induce a form of cell death called pyroptosis which helps

in restricting the growth of intracellular pathogens. Another study shows that, caspase-1 activates lipid metabolic pathways for repairing the membrane damaged by toxins [103]. However, one of the major functions of active caspase-1 is to convert the inactive pro forms of IL-1β, IL-18 and IL-33 to their mature forms. There are many means by which several pathogens subvert inflammasome activation. The *Yersinia* effector, YopK is secreted into the host cytosol and interacts with the Type III secreted proteins, thereby preventing their recognition by NLRs [35]. Another *Yersinia* protein, YopM directly binds to pro-caspase-1 active site and prevents its recruitment to the inflammasome [130]. *Legionella* inhibits the transcription of the adaptor protein ASC, thereby mitigating the secretion of IL-1β [7]. The secreted effectors of *P.aeruginosa*, EsxoU and ExoS suppress NLRC4 inflammasome activation by an unknown mechanism [90, 218]. Other bacteria such as *Salmonella, Chlamydia* and *Francisella* also evade inflammasome activation [53].

The importance of IL-1β in controlling *Mtb* infections is quite evident from *in vivo* survival studies using IL-1β$^{-/-}$ mice that die within 4 weeks of being infected with *Mtb* [153]. Most *in vitro* infections of macrophages and DCs show NLRP3 to be the sole NLR to be activated by *Mtb* as infected NLRP3 knockout macrophages do not secrete any IL-1β [1, 41, 153, 162]. During *Mtb* infections, IFNγ produced by CD4 cells results in the increased NO levels. NLRP3 undergoes S-nitrosylation directly by NO, resulting in the inhibition of inflammasome activation [163]. It has also been shown that *Mtb* inhibits NLRP3 inflammasome via IFNβ mediated induction of anti-inflammatory IL-10. The IL-10 signaling then leads to suppression of proIL-1β expression [152, 170]. One report has shown that AIM2 deletion in mice caused

increased susceptibility to *Mtb* infection [195]. Considering that no activation of AIM2 is seen in cells infected with *Mtb*, it gives rise to the possibility that *Mtb* could also inhibit the AIM2 inflammasome.

1.3.5 Inhibition of Apoptosis Induction

Induction of apoptosis in infected cells is an important innate defense mechanism employed by phagocytes to restrict bacterial growth via a process called efferocytosis. This process is described as the uptake of apoptotic bodies by neighboring phagocytes (described in section 4.1.2) and as a result can stimulate adaptive immunity. However, induction of host cell apoptosis would be detrimental to *Mtb* survival and it employs multiple strategies to manipulate cell death pathways to its advantage.

Virulent *Mtb* strains can inhibit apoptosis and subsequently push the cell towards a more necrotic form of cell death while avirulent strains are unable to evade killing by apoptosis (Fig 6). Necrosis allows the bacteria to be released into the extracellular milieu unharmed and gives them the opportunity to re-infect neighboring cells, thus causing spread of infection [123, 179]. Virulent *Mtb* inhibit apoptosis by enhancing the release of soluble TNFR2 factors by the cell and the subsequent neutralization of TNF [87]. Upon infection, it also reduces the surface expression of Fas receptors abrogating the Fas mediated extrinsic apoptotic pathway [172]. *Mtb* can also inhibit intrinsic apoptotic pathways by modulating the expression of various pro- and anti-apoptotic proteins of the BCL-2 family. It up-regulates an anti-apoptotic gene *mcl-1* and promotes survival via the mitochondria [208].

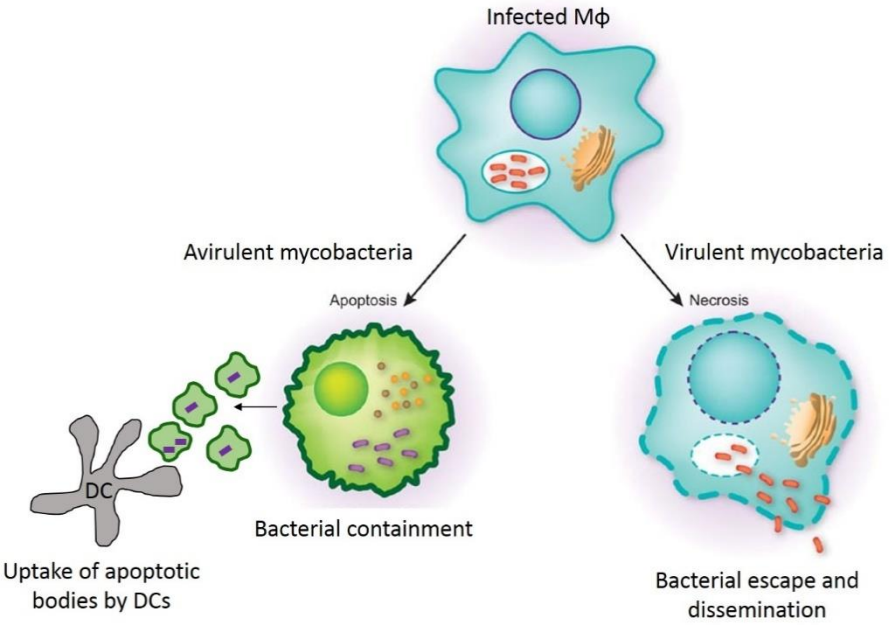

Figure 6: Cell death modality induced by virulent and avirulent *Mtb* strains [adapted from 179].
Avirulent strain of *Mtb* (H37*Ra*) induces apoptosis in infected cells while virulent *Mtb* (H37*Rv*) inhibits apoptosis and instead pushes the cell towards necrosis.

Another study shows that only virulent *Mtb* can up-regulate the anti-apoptotic protein, Bcl-w [210] while the pro-apoptotic Bad is inactivated upon infection [141]. More recently, it was shown that macrophages infected with an avirulent *Mtb* strain (H37*Ra*) underwent apoptosis and this process involved the formation of an "apoptotic envelope". The process begins with the exposure of phosphatidylserine on the cell surface and the deposition of phospholipid binding protein, annexin-1. The next step is the cross-linking at the amino terminal of annexin-1 to complete the apoptotic envelope. In case of virulent *Mtb* (H37*Rv*) infected cells, the amino terminal of annexin-1 was cleaved by proteolysis preventing the cross-linking and hence the formation of the envelope. As a result, the cells could not undergo apoptosis [91]. Another report shows that eicosanoid biosynthetic pathways are involved in selecting cell death modality. Membrane phospholipids when acted upon by cytosolic phospholipase A_2 produce arachidonic acid (AA). This is the precursor for multiple prostaglandins (PG) and lipoxins (LX). Avirulent *Mtb* induces the conversion of AA to prostaglandin E2 (PGE2) by cyclooxygenase 2 (COX2). When PGE2 interacts with receptors EP2 and EP4, they prevent damage specifically to the mitochondrial inner membrane and promote lysosome-mediated plasma membrane repair, ultimately leading to apoptosis (Fig 3). PGE2 is involved in synthesis of plasminogen activator inhibitor type 2 (serpin B2 protease inhibitor), which blocks the cleavage of annexin-1 amino terminal. It is also induces the transcription of synanptotagmin-7 (syt-7), a Ca^{++} sensor that allows for lysosomal repair of the plasma membrane. H37*Rv* instead, induces the conversion of AA into LXA4 through 5-lipooxygenase (5-LO), which inhibits the activity of COX2 leading to mitochondrial inner membrane damage,

prevention of plasma membrane repair and finally, death by necrosis to allow for bacterial dissemination [21,44, 66] (Fig 7).

1.4 Summary and Significance

The scope of *Mtb* infection worldwide is staggering. With the emergence of HIV and drug resistant strains, the existing treatment and preventative measures are outdated and incapable of combating Tb. Current research is again focused on finding new antibiotics and vaccines. Therefore, to be able to engineer new therapeutics, it is imperative that we improve our understanding of the host-pathogen interactions occurring during active and latent infections. With the knowledge of host immune responses and counter measures employed by the bacteria we can identify new targets for drug design and vaccine strategies. For example, knowing that inhibition of apoptosis delays the onset of adaptive immunity, deletion of various anti-apoptotic genes alone or in combination, can lead to development of better vaccine strains. The following chapters describing three studies of different mechanisms by which *Mtb* inhibits innate immune responses has been the focus of my research. In chapter 2 we describe a novel mechanism of inflammasome inhibition by *Mtb*. We show that *Mtb* actively inhibits the activation of the AIM2 inflammasome by secreting an unknown factor via the ESX-1 secretion system. In chapter 3, we demonstrate that a duplicated ESAT6 region (ESX-5a) of *Mtb* aids in the export of virulence factors via the parent ESX-5 secretion system. We have also shown that the ESX-5a substrates activate the inflammasome and are involved in virulence. Finally in chapter 4, we identified a novel anti-apoptotic gene using loss of function genetic approaches.

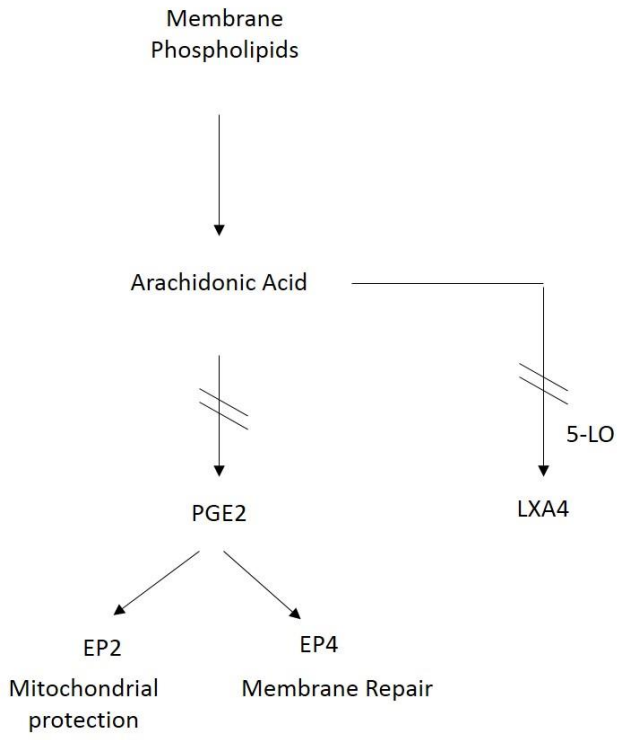

Figure 7: Eicosanoid pathways in cell death [adapted from 21].
This pathway describes the role of eicosanoid biosynthetic pathways in determining cell death modality.

CHAPTER 2: IMMUNE EVASION BY INHIBITION OF AIM2 INFLAMMASOME

2.1 Abstract

After phagocytosis, *Mtb* extracellular DNA (eDNA) gains access to the host cell cytosol via the ESX-1 secretion system. The mycobacterial eDNA induces host cell IFN-β production that then reduces the NLRP3-inflammasome activation to minimize IL-1β secretion. However, the cytosolic eDNA of *Mtb* does not induce activation of the AIM2-inflammasome, even though AIM2 recognizes and binds to cytosolic DNA. Here we show that in contrast to *Mtb*, non-virulent *M. smegmatis* (*Msme*) induces AIM2-inflammasome activation. Importantly, *Mtb* inhibits the AIM2-inflammasome activation induced by either infection with *Msme* or transfection of dsDNA in an ESX-1 dependent mechanism. The inhibition did not involve changes in AIM2 mRNA or protein levels but led to decreased activation of caspase-1. In conclusion, we report a novel immune evasion mechanism of *Mtb* that involves the ESX-1-dependent, direct or indirect, suppression of the host cell AIM2-inflammasome activation during infection.

2.2 Introduction

2.2.1 Inflammasome Activation by *Mtb*

IL-1β is important for host immune defense against *Mtb*, since several studies demonstrated that IL-1β and IL-1-receptor deficient mice are more susceptible to *Mtb* infections [153, 154]. In macrophages and dendritic cells the production of mature IL-1β is dependent on activation of the inflammasome [199]. The NLR proteins such as NLRP3 and NLRC4 are one family of cytosolic receptors, which upon ligand binding mediate inflammasome activation. In the case of *Mtb* the sole NLR capable of inducing inflammasome activation is NLRP3 [1, 69, 153, 154, 162, 236].

The significance of type I IFN signaling for activation of inflammasome responses was first reported for *Francisella* infected macrophages (Fig 8) [104]. Nevertheless, during the course of *Mtb* infections IFN-β has the opposite effect and suppresses activation of the NLRP3 inflammasome, suggesting that induction of IFN-β could correlate with increased virulence [152, 170]. Mice deficient in Interferon Regulatory Factor 3 (IRF-3), a major signaling component of the type I IFN host cell signaling pathway, are much more resistant to *Mtb* infections [147].

2.2.2 *Mtb* and the AIM2 Inflammasome

The induction of host cell type I IFN signaling after *Mtb* infection is dependent upon the type VII secretion system (ESX-1) mediated translocation of *Mtb* extracellular DNA (eDNA) into the host cell cytosol (Fig 9) [147, 212]. It is confounding that this cytosolic *Mtb* DNA is not recognized by the host cell inflammasome component AIM2 which should lead to subsequent inflammasome activation. AIM2 binds to dsDNA of

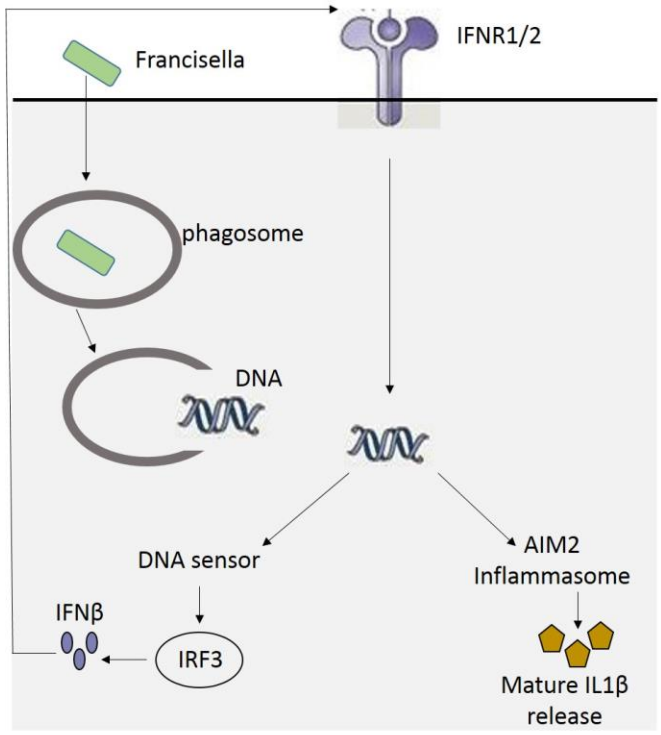

Figure 8: IFN-β regulation of AIM2 inflammasome in *F. tularensis* [adapted from 98].
F. tularensis DNA induces the production of IFN-β which upon autocrine signaling through the IFN receptor activates the AIM2 inflammasome via an unknown mechanism.

intracellular pathogens such as *Francisella* and *Listeria* [107, 196]. There is evidence that transfected *Mtb* dsDNA can interact with AIM2 and activate the inflammasome. It is also shown that AIM2 is important for host resistance to *Mtb* infection [195].

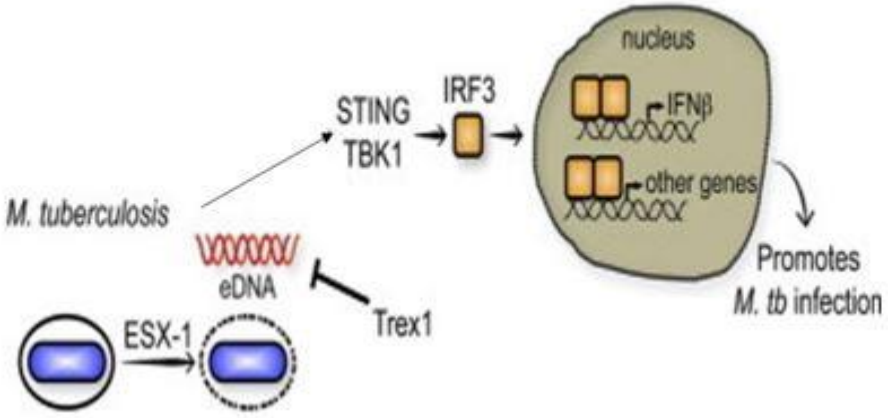

Figure 9: Secretion of *Mtb* eDNA and IFN-β activation [adapted from 147].
Secreted EsxA perforates the phagosomal membrane allowing *Mtb* eDNA to access the host cytosol. The cytosolic eDNA interacts with a DNA sensor and then activates a type I interferon signature.

2.3 Materials and Methods

Cell culture and animals

C57Bl/6 WT mice were obtained from The Jackson Laboratories. *Nlrc4*$^{-/-}$, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Nlrp6*$^{-/-}$, *Nlrp10*$^{-/-}$ mice were provided by Dr. R. Flavell and Millennium Pharmaceuticals. *Aim2*$^{-/-}$ [183] and *Aim2/Nlrp3*$^{-/-}$ double knockout mice were obtained from Dr. K. A. Fitzgerald. The *Ifnar1*$^{-/-}$ mice from Dr. A. Sher (NIH). *IFN-β*$^{-/-}$ mice were provided by Dr. S. N. Vogel. All studies were approved by the IACUC at the University of Maryland and were conducted in accordance with the National Institutes of Health Guide.

Bacteria

M. smegmatis (mc^{2}155), *M. tuberculosis* H37*Rv* (ATCC 25618), H37*Ra* (ATC25177) were obtained from Dr. W.R. Jacobs Jr. (AECOM). *M. fortuitum* (ATCC 6841) and *M. kansasii* strain Hauduroy (ATCC 12478) were obtained from ATCC. *Mtb ΔesxA* and *ΔexoU* PAK (*P. aeruginosa*) were kindly provided by Dr. L. Gao and Dr. V. Lee, respectively. *M. smegmatis Δesx1, ΔeccCb* mutant, *ΔeccCb* complemented strains were kind gifts of Dr. K. Derbyshire [81]. *F. tularenesis* Live Vaccine Strain (LVS) was obtained from Dr. Kevin McIver.

Ex vivo infection

Bacterial infections of BMDCs and BMDMs were performed as described [1, 28]. For induction of AIM2 inflammasome, BMDCs were pre-treated with 20ng/ml LPS (Invivogen) for 1 hour and then infected with H37*Rv* for 4 hours. Infected BMDCs

were then washed twice with PBS and transfected with 0.5ug/ml poly (dA:dT) (Sigma) using Lipofectamine LTX Plus reagent (Invitrogen) for 2 hours. The transfection was performed according to the manufacturer's instructions and the supernatants were harvested 2 hours post transfection.

IFN-β Neutralization

BMDCs from C57Bl/6 mice were treated with anti-IFN-β neutralizing antibody 7F-D3 (5 μg/ml) (Abcam) for 1 hour and infected with *M. smegmatis* at MOI 10:1 for two hours as previously described. Cells were then washed with PBS and incubated for an additional 20 h in DMEM chase media. Supernatants were collected for ELISA.

Cell death assays

The adenylate kinase (AK) release assay, Toxilight®BioAssay (Lonza) was used to quantify necrotic cell death. The assay was performed according to the manufacturer's instructions.

Cytokine Measurement and Immunoblotting

ELISA was used to measure secreted IL-1β (BD Biosciences) and IFN-β LEGEND MAX™ (BioLegend) respectively. For immunoblotting the cell lysate preparation and western blotting was performed as described earlier [1]. The primary antibodies used were: anti-IL-1β (R&D systems) at 0.15μg/ml in 0.1% BSA, anti-caspase-1 (Santa Cruz) at 1:300, anti-AIM2 (Santa Cruz) at 1:500, anti-Tubulin (Cell Signaling) at 1:1000. The above 3 antibodies were diluted in 5% milk with TBST. The secondary

antibodies used were: Donkey anti-goat (Jackson) at 1:25,000, goat anti-rabbit (Jackson) at 1:50,000 and goat anti-mouse (Jackson) at 1:50,000 dilutions respectively.

Real time PCR

BMDCs were harvested 8 hours post-infection using TRIzol (Invitrogen). Real time PCR was done using SYBR green PCR master mix (Roche) with *GAPDH* as the house keeping gene. The primers used were: *Aim2*: 5'-GTCACCAGTTCCTCAGTTGT-3' and 5'-CACCTCCATTGTCCCTGTTTTAT-3' *Gapdh*: 5'-ATGGGATTTCCATTGATGACA- 3' and 5'- CCACCCATGGCAAATTCC-3'; *Mx1*: 5'-TGTGCAGGCACTATGAGGAG-3' and 5'-ACTCTGGTCCCCAATGACAG-3'; *PKR*: 5'-GCACCGGGTTTTGTATCGA-3' and 5'-GGAGCACGAAGTACAAGCGC-3'.

Statistical analysis

Statistical analysis was performed on at least three independent experiments using GraphPad Prism 5.0 software and One-way ANOVA with Tukey's post-test unless otherwise noted in the figure legends. Shown are representative results of triplicate values with standard deviation. The range of p-values is indicated as follows: * $0.01<p<0.05$; ** $0.001<p<0.01$ and *** $0.0001<p<0.001$.

2.4 Results and Discussion

2.4.1 Non-virulent Mycobacteria Induce AIM2 Inflammasome Activation

The non-virulent mycobacterial species such as *Msme* induce a very potent pro-inflammatory immune response and host cell apoptosis when compared to more virulent mycobacterial species [28] but the activation of host cell inflammasome mediated by *Msme* infection has not been analyzed. BMDCs isolated from various mouse strains were infected with *Msme* and the amount of IL-1β in the supernatant was detected and normalized to IL-1β levels secreted by WT BMDCs (Fig. 1A). There were no differences in cell lysis between UI and infected conditions as determined by AK release assay, which showed that the IL-1β present in the supernatant was mainly the mature form. Surprisingly, in NLRP3$^{-/-}$ cells the amount of secreted IL-1β dropped by less than 50%. This is unexpected because the inflammasome activation in *Mtb* is completely dependent upon the presence of NLRP3 [69, 153, 154, 162]. NLRP6, NLRP12, NLRC4, and NLRP10 did not significantly contribute to *Msme*-induced inflammasome activation (Fig 10a). Interestingly however, the deficiency of AIM2, resulted in approximately 40% reduction in IL-1β response in BMDCs (Fig 10a) and almost 75% reduction in BMDMs (Fig 10b). The partial reductions in IL-1β secretion suggested redundancy between AIM2 and NLRP3 pathways. Consistently, the IL-1β production in *Aim2/Nlrp3*$^{-/-}$ cells was further reduced when compared to any of the single deletions. However, *Msme* infected *Aim2/Nlrp3*$^{-/-}$ BMDCs were still able to secrete up to 40% of the IL-1β (Fig 10a). This result suggests that *Msme* cytosolic components are also recognized by one or more unidentified NLRs. The *Mtb*-induced activation of NLRP3 is dependent upon the *Mtb* ESX-1 secretion system [1,129, 213].

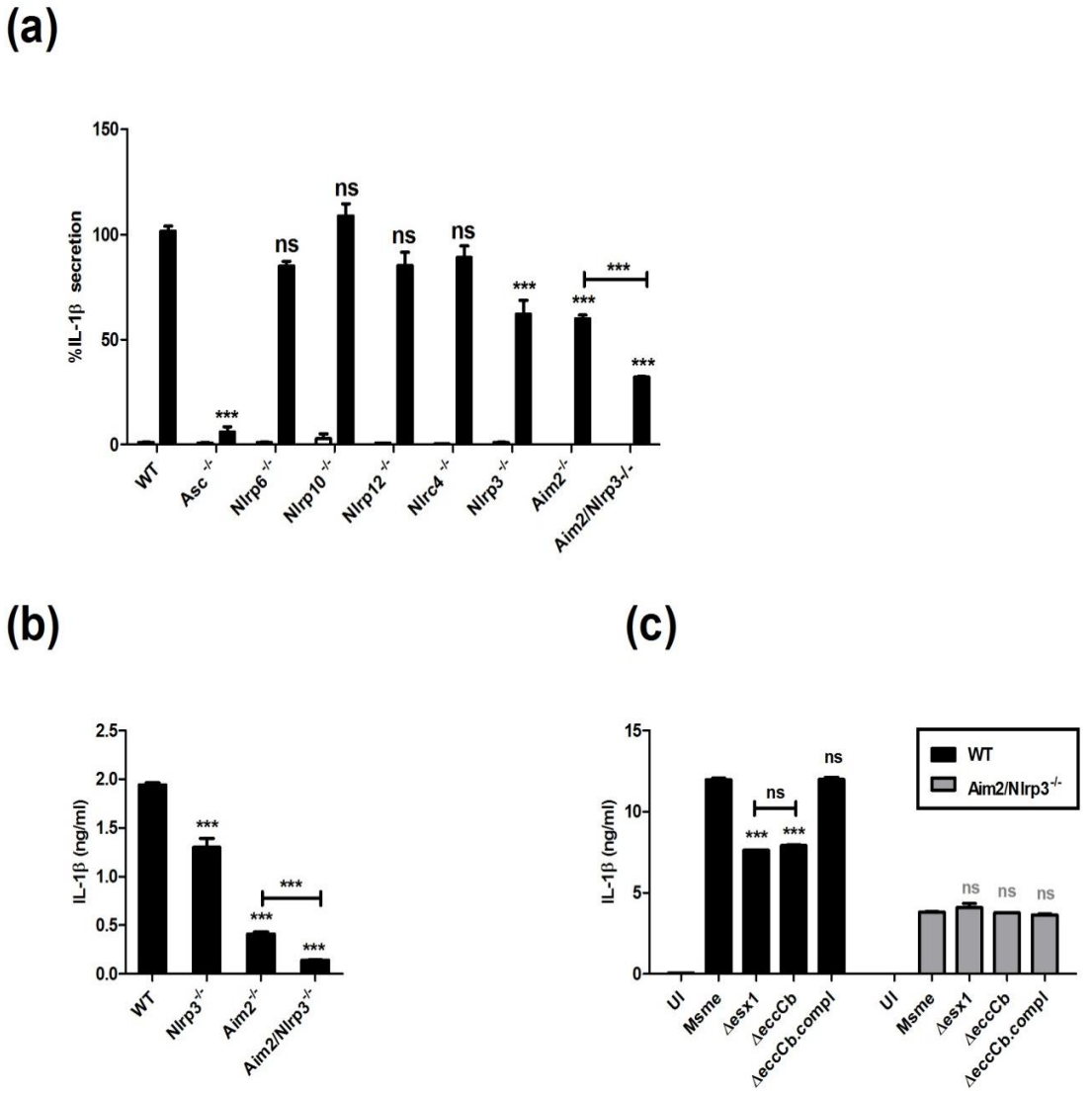

Figure 10: ***M. smegmatis* (*Msme*) ESX-1 dependent AIM2 inflammasome activation. (a)** BMDCs and **(b)** BMDMs from various indicated knockout mice were either left uninfected (UI, grey bars) or infected with *Msme* (black bars). The cell supernatants were harvested 16 hours post infection (hpi) and analyzed for IL-1β secretion by ELISA. ***0.0001< p < 0.001, WT **(c)** BMDCs from WT and *Aim2/Nlrp3*$^{-/-}$ mice were infected with different *Msme* strains (*Msme, Δesx1, ΔeccCb*, compl) and the supernatants were analyzed for IL-1β secretion. Data are shown as the mean and standard deviation of triplicate measurements of one representative experiment out of three except for 1b and 1c where the data is one representative experiment out of two, ***0.0001< p < 0.001 *Msme, M. smegmatis* [202].

The core ESX-1 secretion complex is conserved in *Msme* making it a compelling model to study the mechanisms of ESX-1-mediated protein secretion [50, 173]. Similar to *Mtb*, a functional *Msme* ESX-1 secretion system is required for AIM2/NLRP3 dependent secretion of IL-1β because two different *Msme* mutants with defective ESX-1 show an almost 50% reduction of IL-1β secretion when compared to the WT *Msme* in WT BMDCs. However, this difference is abolished in *Aim2*/*Nlrp3*$^{-/-}$ BMDCs (Fig 10c).

Next, we wanted to investigate if other mycobacterial species activate the AIM2 inflammasome. We thus infected WT and AIM2-deficient BMDCs with *Msme*, *M. fortuitum* (*Mfort*), *M. kansasii* (*Mkan*), the attenuated *M. tuberculosis* H37Ra and the virulent *M. tuberculosis* H37Rv. We monitored IL-1β secretion for all the infections (Fig 11a). In general, there was an inverse correlation between virulence of the species and the amount of IL-1β induction, with the least virulent species inducing the most IL-1β in WT BMDCs (Fig 11a). The infection of AIM2-deficient BMDCs by these mycobacterial species allowed us to determine the fraction of total IL-1β secretion that was dependent upon presence of AIM2. As expected the *Francisella*-induced inflammasome activation was completely dependent on AIM2, whereas about 40-50% of the *Msme*, *Mfort* and *Mkan* induction was dependent on AIM2 (Fig 11a). The attenuated *Mtb* strain H37Ra induced the lowest amount of AIM2 inflammasome activation and the virulent *Mtb* strains H37Rv did not induce any AIM2 activation (Fig 11a).

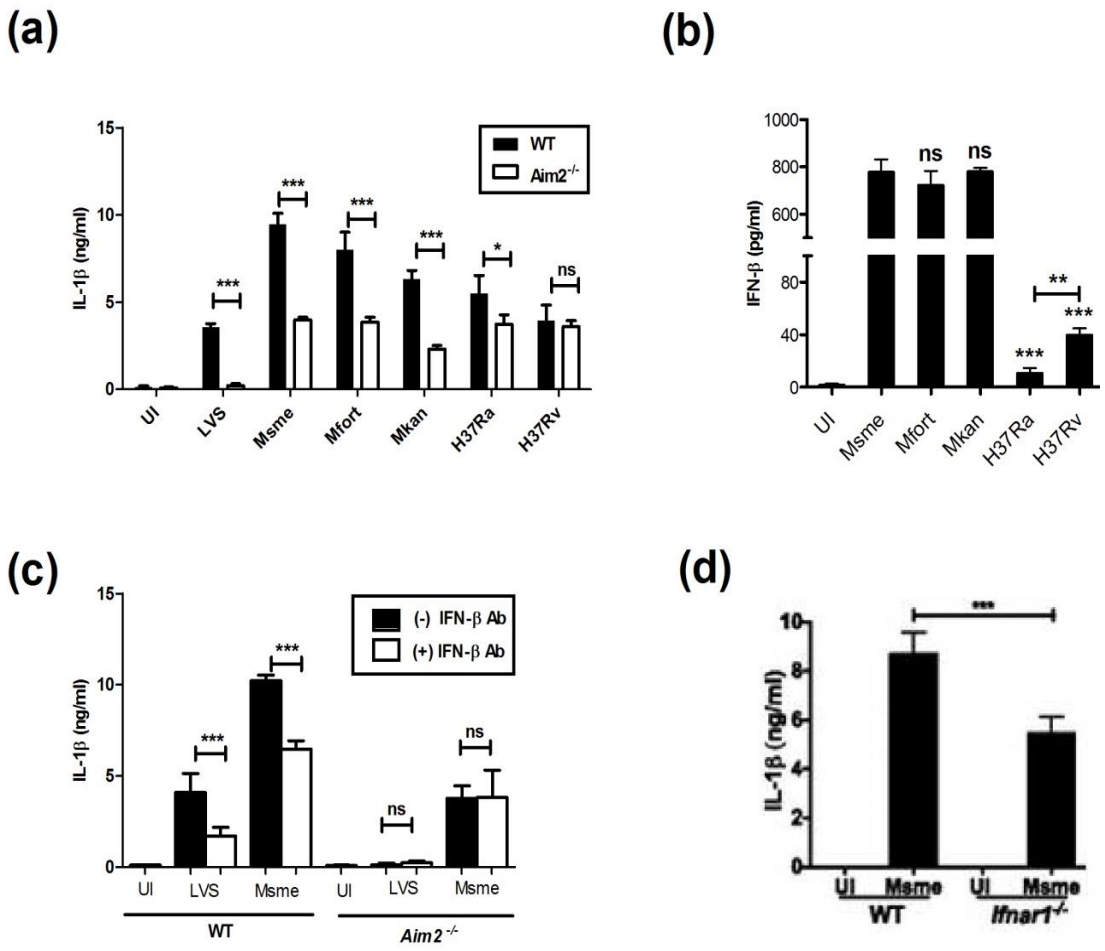

Figure 11: Non-virulent mycobacteria induce IFN-β dependent AIM2 inflammasome activation. (a) WT and *Aim2*$^{-/-}$ BMDCs were infected with *Msme*, *M. fortuitum* (*Mfort*), *M. kansasii* (*Mkan*), attenuated *M. tuberculosis* H37Ra and virulent *M. tuberculosis* H37Rv. *Francisella* Live Vaccine Strain (LVS) infection is used as a positive control. The secreted IL-1β was measured at 16hpi by ELISA. **(b)** IFN-β ELISA of supernatants from WT BMDCs infected with indicated mycobacterial species. ***0.0001< p < 0.001 *Msme* **(c)** IL-1β ELISA of supernatants from WT and *Aim2*$^{-/-}$ *Msme* infected BMDCs in the presence or absence of IFN-β neutralizing antibodies. LVS is used as a positive control. **(d)** IL-1β ELISA of supernatants from *Ifnar1*$^{-/-}$ or WT BMDCs infected with *Msme* or left uninfected. Data is shown as the mean and standard deviation of triplicate measurements of one representative experiment out of three [202].

2.4.2 Non-virulent Mycobacteria Induce IFN-β Dependent AIM2 Inflammasome Activation

The AIM2 inflammasome activation by *Francisella* is stimulated by IFN-β [104]. In order to investigate the potential importance of IFN-β in AIM2-inflammasome activation by non-virulent mycobacteria we analyzed the supernatants of BMDCs infected with various mycobacterial species for IFN-β production. Interestingly, the three species (*Msme*, *Mfort* and *Mkan*) that mediated the strongest AIM2-inflammasome activation also induced a very pronounced IFN-β production of approximately 800 pg/ml which was a 20 fold increase over the amount of IFN-β produced by BMDCs after infection with *Mtb* H37Rv (Fig 11b). In order to demonstrate that this IFN-β secretion is important for AIM2-inflammasome activation, we infected BMDCs from WT and *Aim2*$^{-/-}$ mice with *F. tularensis* LVS and *Msme* in the absence or presence of neutralizing IFN-β antibodies and measured the IL-1β secretion after 24h (Fig 11c). The addition of neutralizing IFN-β antibodies significantly reduced the IL-1β secretion after infection with either *F. tularensis* LVS or *Msme* (Fig 11c). Consistently, the production of IL-1β was reduced by similar levels when BMDCs of WT or *Ifnar1*$^{-/-}$ mice were infected (Fig 11d). The neutralization of IFN-β had no effect on the IL-1β production after *F. tularensis* LVS or *Msme* infection of *Aim2*$^{-/-}$ BMDCs. These results suggest that IFN-β plays a similar role in the induction of AIM2 inflammasome activation by non-virulent mycobacterial species as it does after *Francisella* infections.

2.4.3 *Mtb* Inhibits AIM2 Dependent IL-1β Production

We addressed the hypothesis that *Mtb* can actively inhibit AIM2 inflammasome activation by performing mixed infection experiments. BMDCs from *Nlrp3*$^{-/-}$ mice were infected with *Mtb* or the *esxA* deletion *Mtb* mutant (*ΔesxA*) and either 4 or 8 hours post infection (hpi) the supernatants were harvested for analysis of IL-1β secretion (Fig 12a and b). In *Msme*-infected BMDCs we detected about 100 pg/ml after 4hpi and 3000 pg/ml after 8hpi. Interestingly, when *Mtb* infected BMDCs were challenged with *Msme*, we observed a 2-3 fold reduction in the amount of secreted IL-1β when compared to BMDCs infected only with *Msme*. The *Mtb* mediated inhibition was dependent on functional ESX-1 secretion system since infection with the *ΔesxA* mutant did not inhibit IL-1β secretion after challenge with *Msme* (Fig 12a and b).

We also found that the rate of infection of *Msme* was not affected by the prior infection with *Mtb* as analyzed via flow cytometry using GFP-labeled *Msme* (Fig 13a). A similar inhibition by *Mtb* was also observed for IL-18 induced by *Msme* infections (Fig 13b). There were no differences in necrosis induction at 8hpi (Fig. 12b). To confirm the specific inhibition of the AIM2 inflammasome, we transfected the *Mtb* or *ΔesxA* infected *Nlrp3*$^{-/-}$ BMDCs with 0.5 μg/ml of poly (dA:dT) in the absence (Fig. 12c) or presence (Fig. 12d) of LPS pre-treatment. Consistent with the previous finding, analysis of IL-1β showed that *Mtb* does inhibit the activation of the AIM2 inflammasome when compared to *ΔesxA* infected cells in both cases.

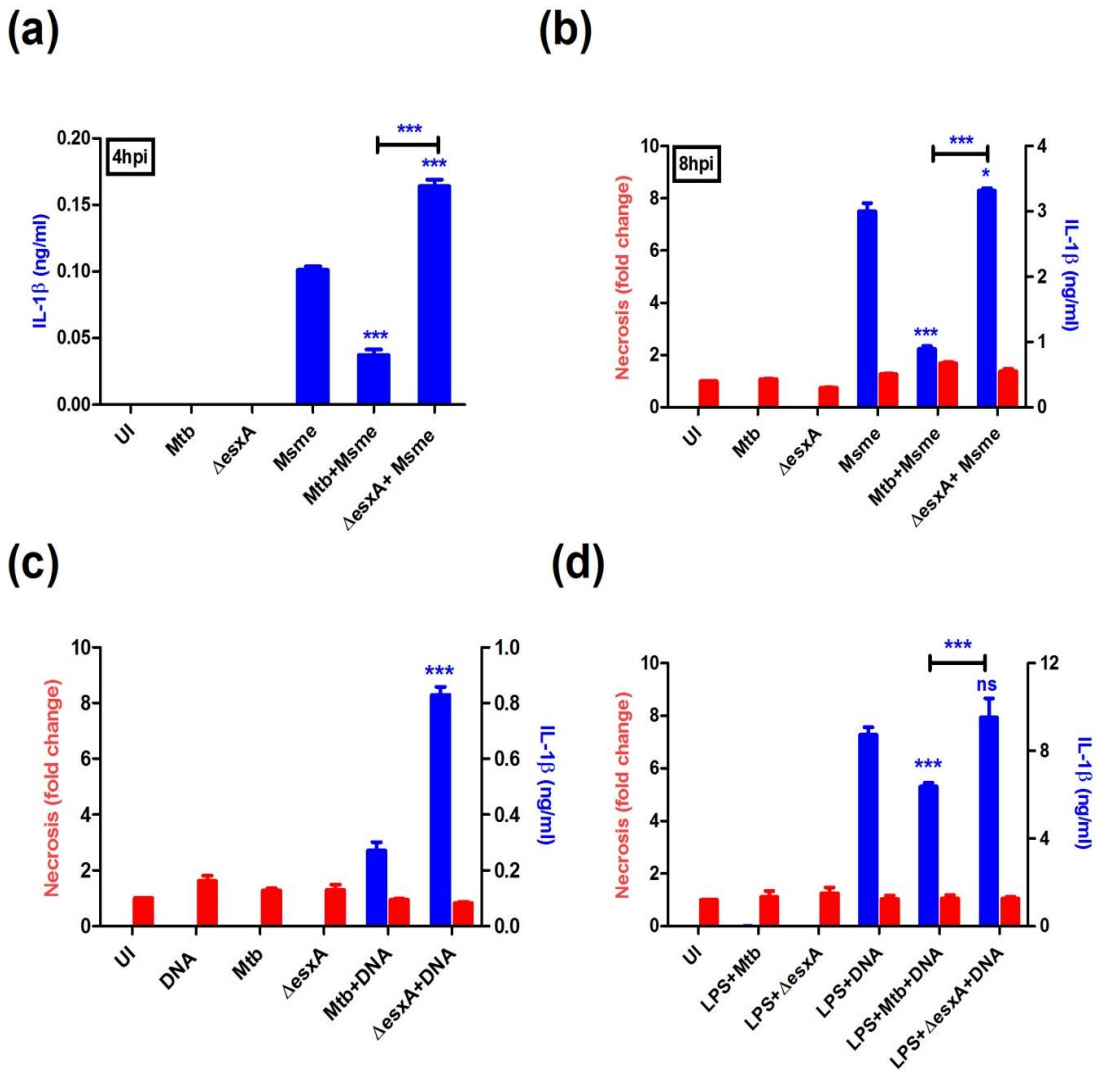

Figure 12: *Mtb* inhibits AIM2 dependent IL-1β production. *Nlrp3*$^{-/-}$ BMDCs were first infected with *Mtb* or the *Mtb ΔesxA* mutant and then with *Msme*. Secreted IL-1β (blue bars) was measured by ELISA at **(a)** 4hpi **(b)** 8hpi, ***0.0001< p < 0.001 *Msme*. Necrotic cell death (red bars) was assayed is represented as fold change over uninfected (UI). *Nlrp3*$^{-/-}$ BMDCs in the **(c)** absence or **(d)** presence of LPS pre-treatment were first infected with *Mtb* or the *Mtb ΔesxA* mutant and then transfected with poly (dA:dT) for 2 hours. Both secretion of IL-1β and necrosis were measured as before, ***0.0001< p < 0.001 LPS+DNA. Data is shown as the mean and standard deviation of triplicate measurements of one representative experiment out of three [202].

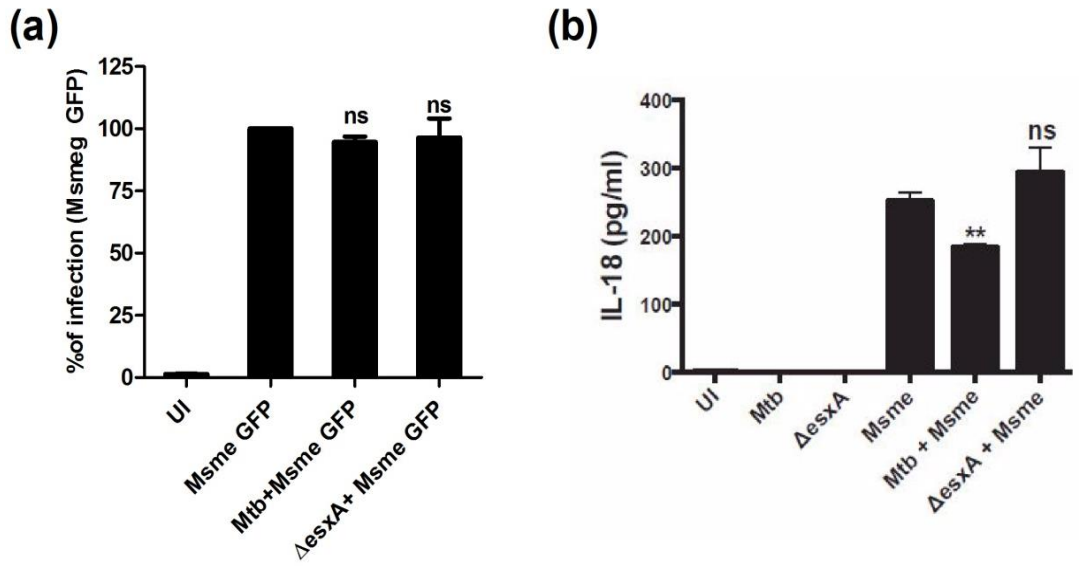

Figure 13: Rate of infection of *Msme*-GFP and inhibition of IL-18 secretion by *Mtb*. (a) Nlrp3-/- BMDCs left uninfected (UI), infected with either *Mtb* or *Mtb ΔesxA* first and then infected with *Msme*-GFP or with just *Msme*-GFP for 2 hours. Cells were harvested and analyzed by flow cytometry for GFP positive cells, ***0.0001< p < 0.001 *Msme* GFP. **(b)** Supernatants from experiments described in Fig.3B were analyzed for IL-18 using a mouse IL-18 ELISA kit (MBL International), ***0.0001< p < 0.001 *Msme*. Data is shown as the mean and standard deviation of triplicate measurements of one representative experiment out of three [202].

Since *Mtb* is able to actively inhibit the AIM2 inflammasome, we wanted to see if it exerted similar inhibitory effects on some of the other inflammasomes. Co-infection experiments with *Pseudomonas aerigunosa* (activates the NLRC4 inflammasome) demonstrated that *Mtb* was not able to inhibit NLRC4 inflammasome activation (Fig 14a and b). The mRNA and protein expression of pro-IL-1β was unaffected as were the proteins levels of both pro-caspase-1 and cleaved caspase-1. Tubulin is the loading control in cell lysates (Fig 14b).

2.4.4 Mechanism of AIM2 Inflammasome Inhibition

Finally, to further support our hypothesis that *Mtb* mediates inhibition of AIM2 inflammasome activation, we investigated the mechanism of this inhibition. The immunoblots of cell lysates from infected BMDCs at 6 hpi showed that there is no difference in AIM2 protein expression (Fig 15a). Also, the protein expression of pro-IL-1β and pro-caspase-1 was not affected by *Mtb*. However, immunoblots of the corresponding supernatants showed that there is decreased secretion of the p10 fragment of caspase-1 and the mature IL-1β (p17) fragment in *Mtb* infected cells challenged with *Msme* when compared to *Msme* or *MtbΔesxA*/*Msme* infected cells even though similar amounts of protein was loaded for each condition (Fig 15a). Interestingly, *Mtb* infection reduced the amount of *Msme*-induced IFN-β secretion in an ESX-1-dependent manner (Fig 15b). Even the addition of high amounts of exogenous IFN-β (400 ng/ml) could only partially overcome the *Mtb*-mediated inhibition of *Msme*-induced IL-1β secretion (Fig 15c). These results demonstrate that *Mtb* is able to limit IFN-β production in infected host cells, which may explain some of its capacity to inhibit the IFN-β-dependent AIM2 inflammasome activation.

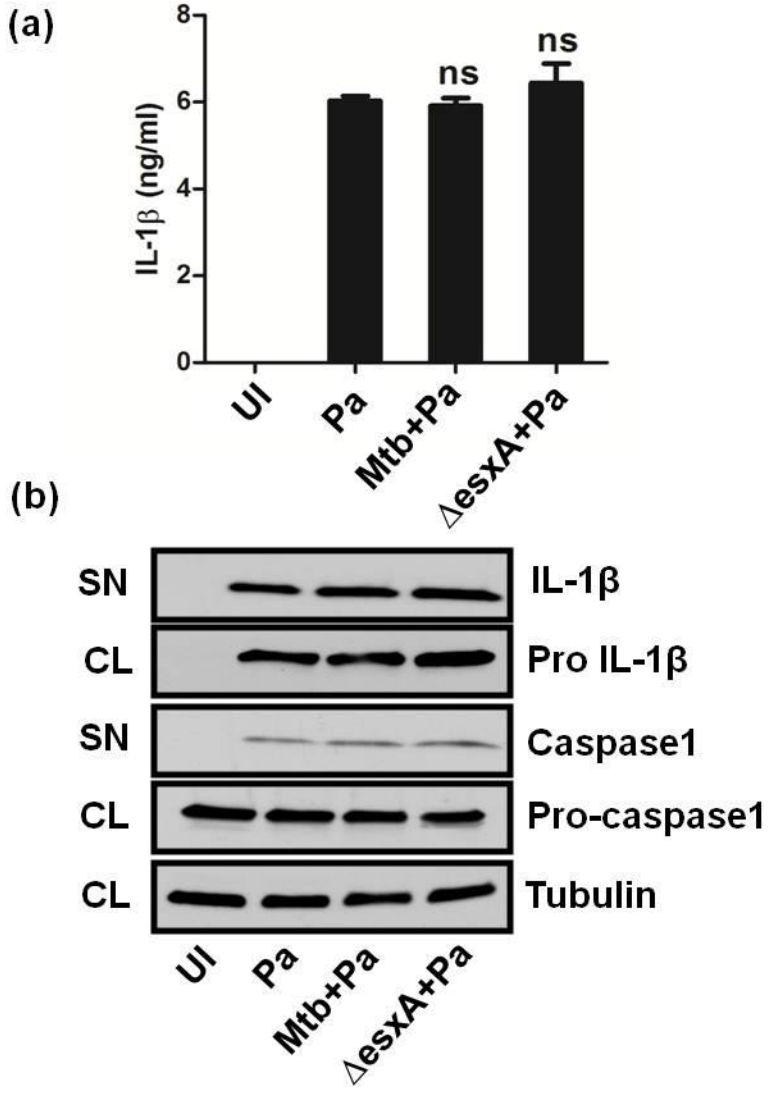

Figure 14: *Mtb* does not inhibit NLRC4 inflammasome activation. Nlrp3-/-BMDCs left uninfected (UI), infected with *P. aeruginosa ΔexoU* only (Pa) or first infected with *Mtb* (*Mtb*) or *MtbΔesxA* (Δ*Mtb*) and then *P.aeruginosa* **(a)** IL-1β secretion measured by ELISA in supernatants at 3hpi, ***0.0001< p < 0.001 Pa **(b)** Western blot of supernatants (SN) for cleavage fragments of caspase-1 and IL-1β and cell lysates (CL) detecting pro-caspase-1, pro-IL-1β and tubulin. Experiments shown are representative out of three total [202].

To investigate if *Mtb* may also inhibit IFN-β signaling, we used IFN-$\beta^{-/-}$ BMDCs infected with *Mtb* and *MtbΔesxA* followed by treatment with IFN-β. The transcription of IFN-β–inducible genes *Mx1* and *PKR* was analyzed by RT-PCR (Fig 15d + e). In both cases *Mtb* infection reduced the IFN-β mediated increase in transcription but this inhibition was not dependent upon ESX-1, since the *MtbΔesxA* mutant showed a similar reduction.

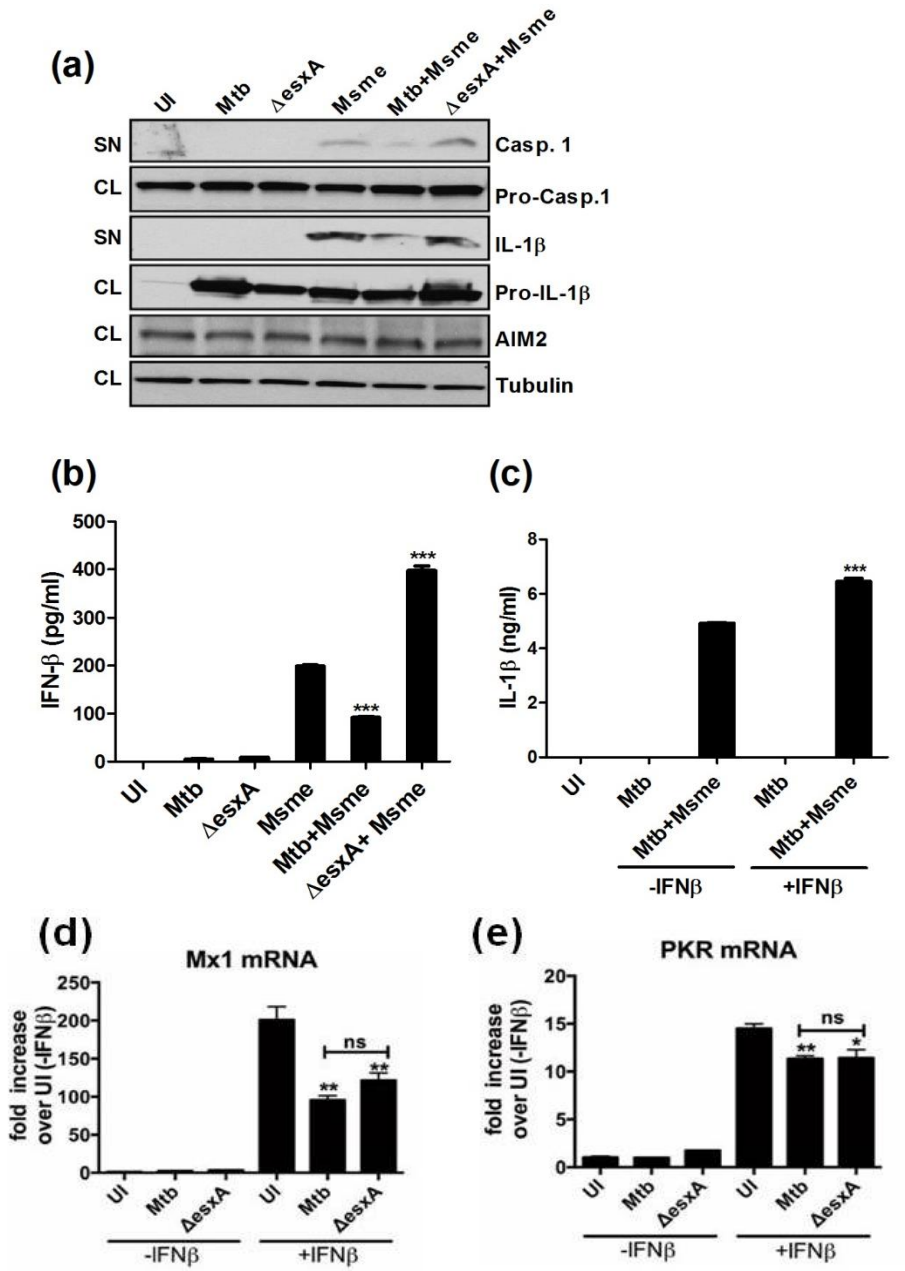

Figure 15: Mechanism of AIM2 inflammasome inhibition. *Nlrp3*$^{-/-}$ BMDCs were infected first with *Mtb* or the *Mtb ΔesxA* mutant and then with *Msme.* In **(a)** Western blots of supernatants (SN) showing active cleavage fragments of caspase-1 (p10) and IL-1β (p17) and cell lysates (CL) detecting pro-caspase-1 (p45), pro-IL-1β (p35), tubulin (p55) and AIM2 (p38) protein levels. **(b)** Secreted IFN-β was measured from supernatants collected 8hpi by ELISA, ***0.0001< p < 0.001 *Msme*. **(c)** Secreted IL-1β from supernatants of untreated *Nlrp3*$^{-/-}$ BMDCs or those treated with IFN-β (400ng/ml) and then infected as indicated was measured 8hpi by ELISA, ***0.0001< p < 0.001 *Mtb+Msme*. **(d+e)** IFN-β$^{-/-}$ mice were infected with indicated bacteria and treated or not with IFN-β (700pg/ml) and the mRNA levels of *Mx1* and *PKR* were analyzed after 4h via qRT-PCR. Data is of one representative experiment out of three [202].

2.4.5 Discussion

The inhibition of IL-1β production by *Mtb* has been reported before [138] but the inhibition of IFN-β signaling by *Mtb* has not been shown previously to our knowledge. The precise molecular mechanism of the *Mtb*-mediated AIM2 inflammasome inhibition remains to be elucidated. It seems unlikely that limiting IFN-β production is the only pathway for *Mtb* to suppress AIM2 inflammasome activation since external addition of IFN-β did not induce IL-1β secretion in *Mtb* infected *Nlrp3*$^{-/-}$ BMDCs (Fig 15c) and only partially restored the *Mtb*-mediated inhibition of *Msme* induced IL-1β. Hence, *Mtb* may secrete another effector that could inhibit signaling of the IFN-α/β receptor and/or directly modify AIM2 inflammasome activation. Indeed we provide evidence that *Mtb* inhibits IFN-β signaling. A detailed analysis of the large number of IFN-β regulated genes may reveal a subset whose expression can only be inhibited by *Mtb* with a functional ESX-1 system. This subset of genes would be the most likely to contain candidates for mediating the AIM2-inflammasome inhibition. Overall, the co-secretion into the host cell cytosol of *Mtb* eDNA and a putative AIM2-inhibitor and/or IFN-β signaling inhibitor via the ESX-1 system may allow *Mtb* to take advantage of the type I IFN-mediated inhibition of the NLRP3-inflammasome without the *Mtb* eDNA inducing activation of the AIM2-inflammasome. The recent finding that *Aim2*$^{-/-}$ mice are very susceptible to *Mtb* infections supports the potential role of AIM2 inflammasome inhibition for optimal virulence of *Mtb* [195]. Our discovery of a novel immune evasion mechanism engaged by *Mtb* opens the door for investigations into the identification of the *Mtb* genes involved in this inhibition and subsequent analysis of their importance for virulence of *Mtb*.

CHAPTER 3: AN ESAT-6 REGION DUPLICATED FROM ESX-5 SECRETION SYSTEM INVOLVED IN SECRETION OF VIRULENCE FACTORS

3.1 Abstract

The ESX-5 secretion system of *Mycobacterium tuberculosis* (*Mtb*) is important for bacterial virulence and the secretion of the large PE/PPE protein family that constitutes 10% of the *Mtb* genome. A four-gene region of ESX-5 is duplicated three times in the *Mtb* genome but the function of these duplicates is unknown. Here we investigate one of these duplicates, the *esxI*, *esxJ*, *ppe15* and *pe8* (ESX-5a) region. ESX-5a deletion mutants in the *Mtb* and *M. marinum* (*Mm*) background are both deficient in secretion of the PE/PPE family of proteins. Surprisingly, using unbiased proteomics approaches, we also identified another protein that is not a member this family, thus expanding the range of ESX-5 secretion substrates. In addition, we demonstrate that the ESX-5a is important for virulence of *M. marinum* in the zebrafish model. In conclusion, the ESX-5a region is non-redundant with its ESX-5 paralog and is necessary for secretion of a specific subset of proteins that are important for bacterial virulence. Our findings point to an important role of the three ESX-5 duplicate regions in the selection of substrates for secretion via ESX-5 and hence they provide the basis for a novel model of the molecular mechanism of this type VII secretion system.

3.2 Introduction

3.2.1 Mycobacterial Type VII Secretion System

Mtb is an extremely successful pathogen that employs various strategies to evade immune responses and persist within the host [20, 211]. Integral to this manipulation of the host by *Mtb* is the secretion of virulence factors by various secretion systems [135]. There are five different T7SS in *Mtb* (ESX-1 to ESX-5) [3, 109]. The different ESX systems were most likely generated via duplications of the ancestral system ESX-4 in the chronological order of ESX-1, ESX-3, ESX-2 and lastly ESX-5 [95]. All the ESX secretion systems have a set of common genes, which form the core components of their secretion machinery among which are genes encoding for two members of the ESAT-6 like family (Esx proteins) [109, 223].

The region of difference 1 (RD1) is a 9.5 kbp stretch comprising of 9 genes of ESX-1 that is deleted in all strains of the live tuberculosis vaccine *M. bovis BCG* [22]. The importance of ESX-1 for virulence was first demonstrated by expressing the entire RD1 locus in the BCG vaccine strain and restoring virulence in the mouse model of tuberculosis [180, 181]. ESX-1 is essential for full virulence of *Mtb* in mice [110, 133, 213]. In *M. marinum* (*Mm*), it is required for the growth of bacteria within macrophages, cell-to-cell spread and virulence in the zebrafish [92]. ESX-1 is required for the co-secretion of two ESAT-6 family proteins EsxA and EsxB [213]. The other known substrates are Rv3881c [238] and Rv3864 [40].

3.2.2 ESX-5 Secretion System

The ESX-5 system is the most recently evolved of all the T7SSs and is only found in slow growing mycobacterial species [96]. It is important for the secretion of

the majority of the PE and PPE family of proteins [5, 6]. The PE/PPE families are unique to mycobacteria and are found mostly in pathogenic and slow growing mycobacteria where they expanded over time to now occupy approximately 10% of the *Mtb* genome. The PE proteins have a subfamily of proteins called PE_PGRS which stands for polymorphic GC-rich sequences. A number of these proteins are either surface exposed on the bacterium or secreted into the host milieu [48, 96]. ESX-5 mediates the suppression of secretion of host cell pro-inflammatory cytokines TNF, IL-6 and IL-12 after infection of macrophages with *Mm* [4]. After the bacteria escape from the phagosome into the host cell cytosol ESX-5 is required for the induction of caspase-independent cell death in macrophages, which allows the bacteria to exit the host cell and infect new cells [2]. Hence it is not surprising that *Mtb* ESX-5 deficient mutants are attenuated in SCID mice [29] and *Mtb* lacking the ESX-5 PE-PPE proteins are strongly attenuated in immunocompetent mice [197].

3.2.3 ESX-5 Accessory Systems

Besides their isolated location in the *Mtb* genome one *pe* and a *ppe* genes are also found flanking genes encoding for the ESAT-6-like proteins in each ESX loci except for the ESX-4 system [96, 109]. This shows that they most likely were first associated with the ESX-1 system and then co-duplicated thereof until they further expanded independently of ESX secretion systems [96].

The two ESAT-6-like proteins and their flanking PE/PPE proteins from the ESX-5 system underwent three more duplication events (Fig 16) [96]. One of those loci is found in the *Mtb* genome starting at Rv1037c to Rv1040 (ESX-5a) containing the *esxI, esxJ, ppe15* and *pe8* genes. EsxI and EsxJ are themselves secreted into the

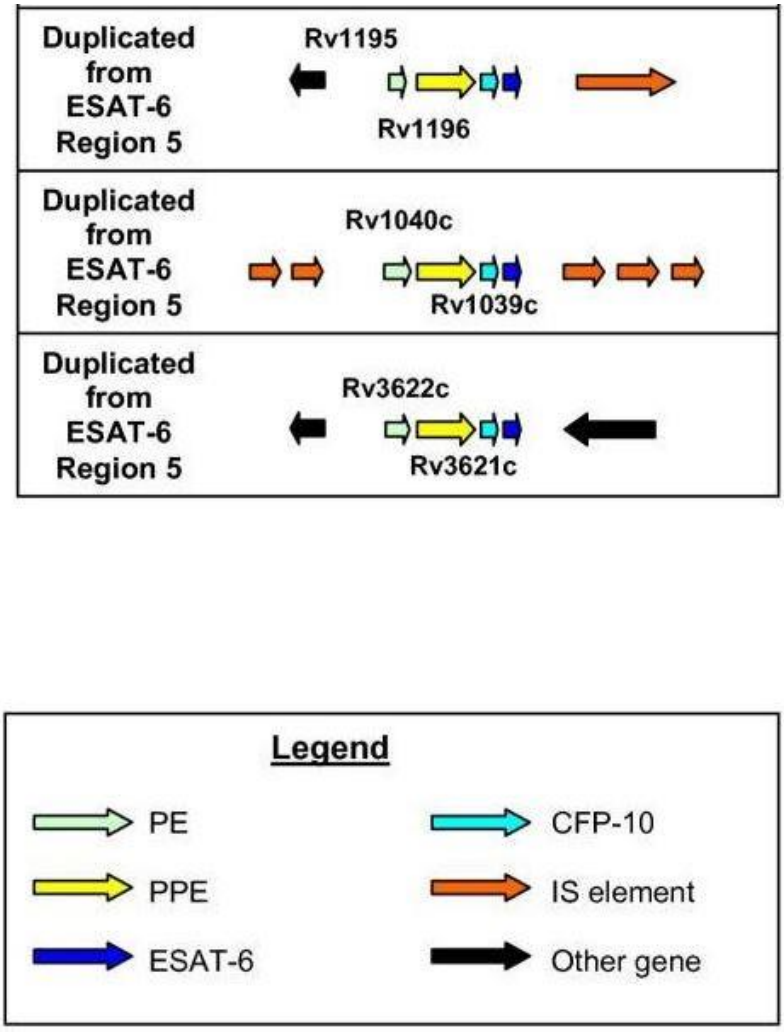

Figure 16: Duplicated *esat6/pe/ppe* regions from the ESX-5 secretion system [adapted from 96]
Shown here is the genomic location of the three regions duplicated from the ESX-5 locus.

extracellular medium [142]. Interestingly, an EsxJ derived peptide constitutes a CD8 T-cell epitope found in human individuals with latent or active tuberculosis [102]. The biological function of any of the three ESX-5 duplication regions has not been investigated to date.

In the present study, we have characterized the role of ESX-5a in protein secretion, inflammasome activation, host cell cytokine secretion, host cell death induction and virulence during *in vivo* infections. *Mm* is a fish pathogen that is genetically very similar to *Mtb*. This mycobacterial species has been used as a model organism to study protein secretion via the T7SS and host-pathogen interactions using the zebrafish larvae [3, 4, 5]. *Mtb* ESX-5a deletion mutants did not secrete any alanine-L-dehydrogenase (ALD) and showed partial reduction in secretion of PE/PPE family of proteins, which are ESX-5 substrates. The ΔESX-5a mutant does not activate the host cell inflammasome as much as wild-type *Mtb* and also shows reduced induction of the secretion of some pro-inflammatory cytokines. Nevertheless, the mutant shows no difference in apoptotic and necrotic cell death induction. Finally, the *Mm* ΔESX-5a mutant showed reduced virulence in the adult zebrafish model.

3.3 Materials and Methods

Mice

C57Bl/6 wild-type mice were obtained from The Jackson Laboratories. Mice were maintained under pathogen-free conditions and used between 6 to 12 weeks of age. All studies were approved by the Institutional Care and Use Committee of The University of Maryland at College Park and were conducted in accordance with the IACUC guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture

Bone marrow cells were obtained from the femurs and tibia of mice. Cells were then cultured in 100 *Mm* dishes for 7- 9 days in DMEM supplemented with Penicillin (100 U/ml), Streptomycin (100 μg/ml), 2-mercaptoethanol (50 μM) (all from Invitrogen), 10% heat-inactivated FCS (Hyclone), and either 200U/ml GM-CSF (Peprotech) to generate bone marrow-derived dendritic cells (BMDCs) or 20% L929 cell supernatant (LCCM) to generate bone marrow derived macrophages (BMDMs). The generation of BMDCs was performed as described [1]. Human myelomonocytic cell line THP-1 (ATCC TIB-202) was cultured and differentiated using phorbol myristate acetate (PMA) (Sigma #P8139) as described in [160].

Bacteria

M. marinum (*Mm*) strain M, obtained from Dr.Gao (University of Maryland, College Park) was cultured and maintained as mentioned previously [93]. *M. smegmatis* (*Msme*) mc^2155 and *M. tuberculosis* (*Mtb*) H37*Rv* (ATCC 25618) were obtained from Dr. W.R. Jacobs Jr. (AECOM).

Generation of deletion mutants in *Mm* and *Mtb*

Regions flanking the *Mm* genes *esxP* and *esxN_2* (homologs of *Mtb esxI* and *esxJ*) was amplified by PCR and inserted on either sides of kanamycin resistant (Kan^r) cassette contained in a pBluescript plasmid. The left fragment was generated using primers LLFP_esx (GATGGTACCTCTAGACCCATCGCCGCTGGCATTGG) and LLRP_esx (GCAGTCGACGATTCCTGTCTCCTTATGTTGAAGTCCGC) and the right fragment was generated using RLFP_esx (GCCGACTAGTTACAAGAGCAGGACAACAGTGGC) and RLRP_esx (GAATGAGCTCTCTAGAGGTAATACCGACCACCCACAC). This entire sequence was cut out from the plasmid and ligated into pLYG304.zeo. This knockout plasmid was electroporated into WT *Mm* and the resulting double crossover mutants *Δesx- I/J* were selected as described earlier [93]. The *Mtb* mutant *ΔESX-5a* was generated recombineering approach described in [14]. About 400-600bp flanking sequences on either side of the gene region Rv1037c-Rv1040c were amplified using PCR. The left fragment primers were Rvesx_LLFP (GCAACTCGAGCCGCCGTCAGGTGATCGAATCAG) and Rvesx_LLRP (GGAGAAGCTTGCTGGCTTAAGGCCCGCGCC) and the right fragment primers

were Rvesx_RLFP (GGCATCTAGAGCTGCTGTCTCCTTGTCTCGAAGTCG) and Rvesx_RL RP (GTGAGGTACCATCCCCACCGCGATATTCCTAGC). These were then ligated on either sides of a hygromycin resistance gene on the pMSG360 plasmid. This recombinant plasmid was then linearized and electroporated into a specialized strain of *E.coli* (EL350/phAE87). A recombination event between the phasmid phAE87 and the linearized plasmid created a new phasmid containing the hygromycin marker and the flanking sequences. This phasmid DNA was extracted and transformed in *Msm* to generate recombinant phages that was then used for *Mtb* transduction. The hygromycin resistant *Mtb* colonies were picked and screened by southern blotting to confirm the knockout. EL350/phAE87 and pMSG360 were kind gifts from Dr. Michael S. Glickman, Memorial Sloan-Kettering Cancer Centre.

Southern Blot

Knockout confirmation in the mutants was done using Southern blot. The DNA probes for both *Mm* (FP- GGCAAACGCTCTCGATACC and RP- GCCGAATCGCGGAT AGATTAC) and *Mtb* (FP- CCGGACCGAACGGGATGAAC and RP-GTGCGTCT GGCGGCAGAAAC) mutants were biotinylated using BrightStar® Psoralen-Biotin Nonisotopic Labeling Kit (Ambion #AM1480) as per the manufacturer's instructions. The genomic DNA digested using the restriction enzymes (Fermentas) PstI (Δesx- I/J) and BamHI (ΔESX-5a) and run on an agarose gel. The DNA from the gel was transferred to a nylon membrane and exposed to the biotinylated hybrization probe. The bands were detected using BrightStar® BioDetect™ Kit (Ambion #1930).

Complementation of mutants

The complementation of the *Mm* mutant *Δesx-I/J* was done by amplifying the gene fragment containing both the genes and ligating it in the plasmid, pLYG206.zeo [93]. This recombinant plasmid was electroporated into *Δesx-I/J to* generate the complement. Similarly, the fragment containing *esxI* and *esxJ* genes was amplified by PCR using the primers FP (GCACGAATTCAGCG GCCGGCAGGTTTCAC) and RP (GACCATCGATACGGCGCGGGCCTTAAGC) and ligated into pMV261. This recombinant plasmid was electroporated into *ΔESX-5a* to obtain a complement.

Cell Culture and Infection

Cultured medium containing differentiated BMDCs in suspension was collected and centrifuged. Cell pellets were then re-suspended in infection media containing only GM-CSF and seeded in 24 well plates. Bacteria were grown to an OD_{600} ranging from 0.5 to 0.8 and centrifuged. The pellet was re-suspended in PBS (Cellgro) supplemented with 0.05% Tween-80 and centrifuged at 80g to remove clumps. Infections were carried out at a multiplicity of infection (MOI) of 10:1 for 4h in infection media containing 10% non-heat inactivated FCS. The extracellular bacteria were then removed by washing twice with PBS and the cells were incubated in chase media containing GM-CSF and 100μg/ml of gentamicin (Invitrogen) for 24h. In case of BMDMs the cells were seeded at least 16h before infection in complete media containing 20% LCCM. The cells were then washed and infection media was added to it (without LCCM) and infected 3h post LCCM starvation in conditions similar to BMDCs with the exception of GM-CSF addition. THP-1 cells were seeded in 24 well

plates with PMA for 18-22h and then washed twice with PBS to remove the PMA. They were then re-suspended with infection media containing 5% of pooled human serum (Sigma) and infected with bacteria at an MOI of 3:1 for 4hrs. The extracellular bacteria were washed off and cells were incubated with chase media for 3 days.

Cell death assays

The adenylate kinase (AK) release assay, Toxilight®BioAssay (Lonza, Cat # LT07-217) was used to quantify necrotic cell death. The assay was performed according to the manufacturer's instructions using cell free supernatants harvested 1or 3 days post infection. The TUNEL assay was performed to reveal apoptosis-induced DNA fragmentation in THP-1 cells using the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science #11-684-795-910). The assay was carried out as described by the manufacturer and the percentage of stained cells was analyzed using flow cytometry. For the detection of apoptosis in BMDM and BMDCs, hypodiploid stain was used. Cells were collected after infection, pelleted and re-suspended in propidium iodine (PI)/RNase buffer (BD Pharmingen #550825) for 20min and the percentage of hypodiploid positive cells was determined by flow cytometry.

Cytokine Measurement

ELISA was used to measure secreted IL-1β and pro-IL-1β in cells supernatant collected from infected cells at the indicated time points using BD OptEIA™ Set Mouse IL-1β kit (BD Biosciences cat# 559603), BD OptEIA™ Set Human IL-1β kit (#557953) and Mouse IL-1 pro-form ELISA (ebioscience # 88-8014) respectively. The analysis of

multiple cytokines in the BDMC supernatants was performed using the magnetic 10-plex mouse cytokine panel (Life Technologies Cat# LMC0001M) and analyzed with the MAGPIX® system (Life Technologies).

Preparation of culture filtrates (CF) and cell lysates (CL)

Mm CF and CL preparation was done as previously described [238]. *Mtb* were cultures grown in 7H9 media to log phase, washed and then transferred to Sautons' media (with 0.05% Tween 80) at a starting OD of 0.05. These cultures were grown at 37°C in shaker (~100rpm) for 4 days (~OD = 0.8) and passaged again in 50ml of fresh Sautons' media (with 0.005% Tween 80) to an initial OD of 0.05. The bacteria were allowed to grow for another 4 days and spun down to separate the pellet and supernatant. The CF was filtered and then concentrated using Amicon Ultra centrifugal filter units (Millipore#UFC900308). The CL was generated by re-suspending the bacterial pellet in 20*Mm* Tris-HCL (pH 7.6) and breaking up the cells using bead beating as described [238]. BMDC lysates preparation was performed as described earlier [1]. To detect cleavage fragments of caspase-1 and IL-1β, supernatants of infected cells were collected at indicated time points and subjected to methanol/chloroform extraction. The precipitated proteins were pelleted and re-suspended in reducing sample buffer and boiled.

Western blot

10µg of BMDC lysates were loaded onto an Any KD TGX SDS-PAGE gel (Bio-Rad #567-1123) and transferred to PVDF membranes. The blots were blocked using 5%

dry milk for 1 hour and then incubated overnight with the primary antibody. This was followed by incubation with HRP-conjugated secondary antibody for 1 hour. The membrane was then developed using the chemiluminescent pico substrate (Pierce #34078) and exposed on films (GeneMate #F-9023). The primary antibodies used were: anti-IL-1β (R&D systems #AF401NA) at 0.15µg/ml in 0.1% BSA, anti-caspase-1 (Santa Cruz #sc-514) at 1:300 and anti-Tubulin (Cell Signaling #9774) at 1:1000. The above 2 antibodies were diluted in 5% dry milk with TBST. The secondary antibodies used were: Donkey anti-goat (Jackson #705-035-003) at 1:25,000, goat anti-rabbit (Jackson #111-035-003) at 1:50,000 and goat anti-mouse (Jackson#115-035-174) at 1:50,000 dilutions respectively.

The bacterial CF and CL western blotting was done as mentioned above. The primary antibodies used were: ALD (NR-13651), SodA (NR-13810) and GroEL2 (NR-13813) - from BEI resources used at dilutions recommended by them. Rabbit polyclonal serum FAP [184] was used at 1:8000. The anti-PGRS and anti-PPE41 antibodies were kindly provided by Dr. Wilbert Bitter, VU University Medical Centre, Amsterdam and used at a 1:1000 and 1:20,000 respectively [6].

2D gel electrophoresis

100µg of CF proteins were precipitated using the Readyprep 2-D clean-up Kit (Bio-Rad #163-2130) and dissolved in 200µl of rehydration buffer. This solution was loaded onto an IEF tray and the first dimension was run on an 11cm IPG strip, pH 4-7 when placed in an IEF cell (Bio-Rad). The running conditions were: rehydration for 12 h, 250 V for 20 min with linear ramp, 8000 V for 2.5h with linear ramp, and 8000 V with

rapid ramp at 20°C. Following this, the IPG strip was placed onto a 4-20% gel and the second dimension was run at 150V for 1hr. The gel was removed, washed and stained with Bio- safe coomassie for 90 min and de-stained overnight in DI water. A GS-800 densitometer (Bio-Rad) was used to image the 2D gel.

Mass spectrometry analysis

Coomassie stained gel spots were analyzed according to the method of Shevchenko *et. al.* with minor modifications [203]. Briefly, gel spots were excised with a transfer pipette and destained via three consecutive 30 min incubations at room temperature in 50/50 (v/v) 100*Mm* NH_4HCO_3/acetonitrile. After the stain was removed, the excised gel pieces were dehydrated in 100% acetonitrile and subsequently rehydrated on ice in 100*Mm* NH_4HCO_3 containing trypsin (Promega) at 20 ng/µL. The gel bands were allowed to fully rehydrate for 2 hours on ice, and then buffer was added to keep the gel pieces hydrated over the course of the digestion. Proteins were digested at 37º C overnight. The resulting peptides were extracted by adding an equal volume of 5% formic acid in acetonitrile (v/v) and incubating for 30 minutes on a shaker. The peptides were removed and lyophilized to near dryness.

Peptides were analyzed using an LTQ-Orbitrap XL (ThermoFisher) mass spectrometer coupled to a Prominence nano liquid chromatograph (nLC) (Shimadzu). As peptides eluted from the nLC precursor mass analysis was performed at high resolution while simultaneously fragmenting the 5 most abundant multiply charged precursors using collision-induced dissociation. The resulting spectra were searched against a mycobacterial database using the MASCOT search engine (Matrix

ScienceLondon, U.K.). Precursor masses were searched with a mass tolerance of 10 ppm while product ion masses were searched within 0.8 Da. Peptides were considered significant if identified with E-values less than 0.5.

Real time PCR

BMDCs were harvested 0 and 8h post-infection using TRIzol (invitrogen). Total RNA was extracted from these fractions using chloroform and precipitated with iso-propyl alcohol. The residual DNA was digested using *Ambion® TURBO™ DNase* as per the manufacturer's instructions. Real time PCR was done using SYBR green PCR master mix (Roche) with *GAPDH* as the house-keeping gene. The samples were run on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System and the data was analyzed using the CFX software (version 3.0). The primers used were: IL-1β: 5'-AGCCTCGTGCTGTCGGACCC-3'/ 5'- TGAGGCCCAAGGCCACAGGT- 3' AND GapDH: 5'- ATGGGATTTCCATTGATGACA- 3'/ 5'-CCACCCATGGCAAATTCC-3'

Survival studies using zebrafish

The zebrafish were obtained from Dr. Du, University of Maryland, Baltimore County. All studies were approved by the Institutional Care and Use Co*Mm*ittee of The University of Maryland at College Park and were conducted in accordance with the IACUC guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Prior to infection, the fish were exposed to 0.02% tricaine (ethyl 3-aminobenzoate methanesulphonate) (Sigma-Aldrich #E10521) for 2-3 min and then

injected intraperitonially with 5–10 µl of *Mm* suspension in PBS containing $1x10^4$ bacilli. The control fish were injected with PBS only. For survival studies, eight fish were infected with each *Mm* strain.

Statistical analysis

Statistical analysis was performed on at least three independent experiments using GraphPad Prism 5.0 software and One-way ANOVA with Tukey's post-test unless otherwise noted in the figure legends. Shown are representative results of triplicate values with standard deviation. The range of p-values is indicated as follows: * $p=0.01< p < 0.5$; ** $p = 0.001< p< 0.01$ and *** $p = 0.0001< p < 0.001$.

3.4 Results and discussion

3.4.1 Generation of *Mm* and *Mtb* Deletion Mutants

The *Mm* mutant was generated by deleting both *esxP* and *esxN_1* (homologs of *Mtb esxI* and *esxJ* respectively) genes from the ESX-5a loci and is referred to as Δesx-I/J. The *Mtb* mutant (ΔESX-5a) lacks the entire ESX5a region which includes the four genes: *esxI, esxJ, ppe15* and *pe8* (Fig 17). The gene deletions in both the mutants were confirmed using southern blotting (Fig 17).

3.4.2 Identification of a Novel Secreted Substrate of ESX-5a

The secretome of *Mm* (Fig 18a top panel) and the ESX-5a mutant - Δesx-I/J (Fig 18a bottom panel) was analyzed by subjecting their short-term culture filtrates (CF) to 2D gel electrophoresis (pH 4-7 and 6-70KDa). Based on a visual inspection, one major spot (labeled as 1) was found to be to be missing from the 2D gel of the mutant CF. In all our 2D gel electrophoresis repeats with multiple *Mm* WT and Δesx-I/J CF samples, we found only spot 1 to be consistently absent in the mutant secretome. All other differences in secretion patterns between the mutant and WT could not be reproduced. For example, in the representative 2D gel image shown in Fig 18a, some of the spots seen in the Δesx-I/J CF are missing from the WT sample. These differences can be attributed to minor differences in protein amounts loaded onto the gel or due to excessive de-staining of coomassie from one of the blots. Spot 1 was identified as alanine-L-dehydrogenase (ALD) by mass spectrometry analysis and this was further confirmed by western blotting (Fig 18b).

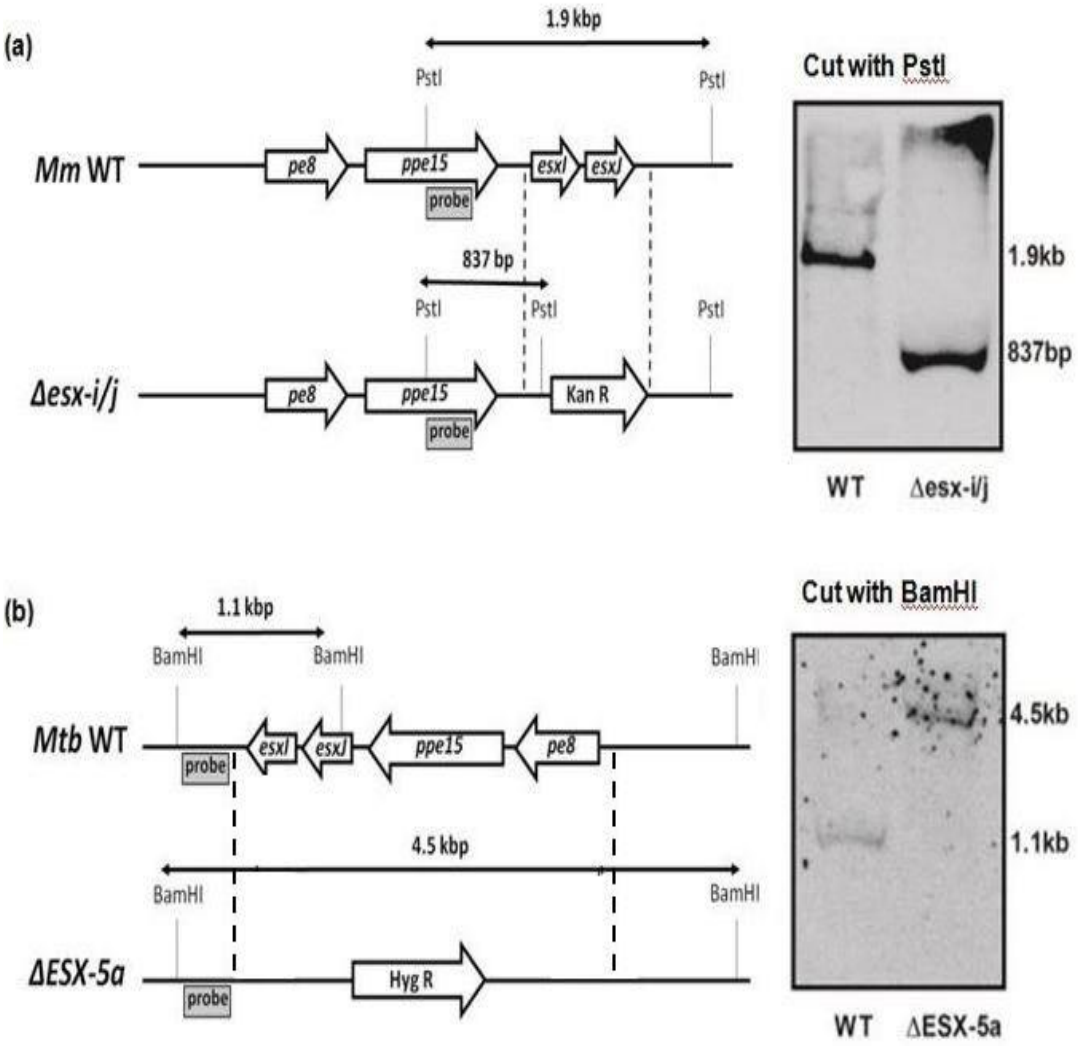

Figure 17: Southern blot confirmation of *Mm* and *Mtb* deletion mutants. Schematic diagrams (left) of the genome regions in *Mm* and *Mtb* that contain the ESX-5a region and the Southern blots (right) are shown to confirm the deletion of **(a)** the *esxI* and *esxJ* genes from *Mm* and of **(b)** the *esxI, esxJ, ppe15* and *pe8* genes from *Mtb*. (Work done by Shah S & Briken V.)

Here, we have used Fibronectin attachment protein (FAP) as a loading control. FAP is known to be exported via the SecA1 system, making it a good control as its secretion is not affected by any mutations made to the components of the T7SS. FAP is detected by a polyclonal anti-serum and shows up on the western blot as two closely spaced bands at 45 kDa with the top band usually more prominent than the lower band. GroEL, a 65 kDa cytoplasmic protein, is used as a lysis indicator. The ALD protein amounts in CF and CL from one representative blot was quantified using the imageJ software. The data is represented as a densitometric ratio of ALD to FAP (loading control) and is also relative to WT *Mm*. The quantification clearly shows a defect in both secretion and also in the expression of ALD by the mutant. Thus, in addition to a secretion defect, ESX-5a deletion could result in two possibilities: it affects either expression or stability of ALD. To see if this phenotype was conserved in *Mtb*, short term CF and cell lysates (CL) were collected from both the *Mtb* and the ΔESX-5a mutant and run on a SDS-PAGE gel. When the blots were probed with anti-ALD, no protein could be detected in the CL itself (lanes 1 and 2 of Fig 19a).

Earlier reports have shown that ALD is up regulated during various stress conditions [25, 193]. Thus *Mtb* cultures were exposed to reactive oxygen species (ROS) for 0, 1.5 or 3 h and the cell lysates were prepared. Upon probing the CL blots with anti-ALD we could see some expression at 3 h in *Mtb* lysate and to a lesser extent in the mutant (Fig 19a).

To conclusively show that there is an ALD secretion defect in the mutant we constitutively expressed ALD in both WT and ΔESX-5a. Analysis of the western blot

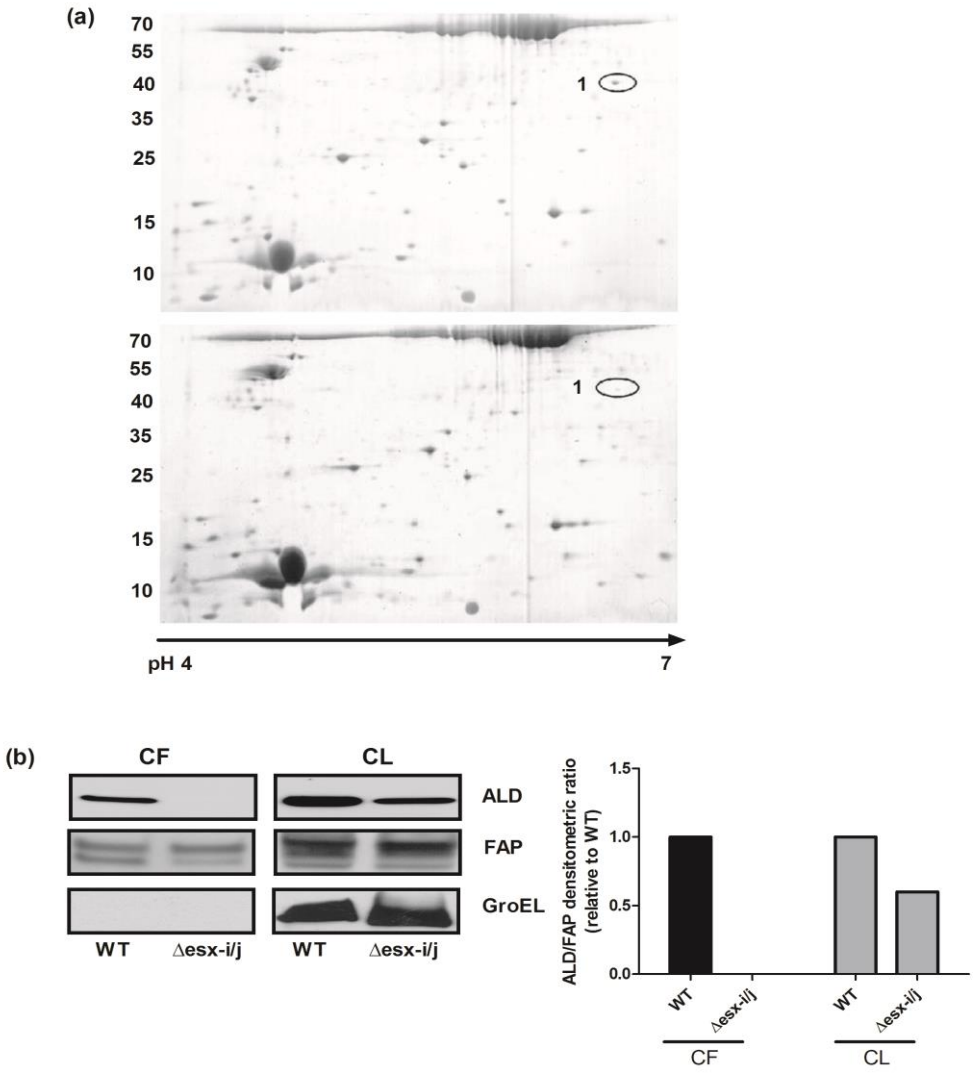

Figure 18: Secretion defects in the *Mm* ESX-5a mutant. (a) 2D gel electrophoresis was performed on secreted proteins from *M. marinum* (top) and the ESX-5a deficient *Mm*Δesx i/j mutant (bottom). The encircled protein spot was consistently absent in the mutant proteome. It was collected from the *Mm* gel and identified as alanine-L-dehydrogenase via mass spectroscopy analysis. The 2D gel image is one representative of 2 independent experiments. **(b)** *M. marinum* (WT) and the ESX-5a mutant (Δesx i/j) secreted proteins (CF) and bacterial cell lysates (CL) were probed for ALD. The bar graph shows the quantification of ALD in CF and CL normalized to FAP and relative to WT *Mm*. Detection of GroEL is used as a quality control for the CFs, while the FAP signal serves as a loading control. All quantification was done imageJ. Data shown is one representative experiment out of three. (Work done by Shah S, Canon J & Briken V.)

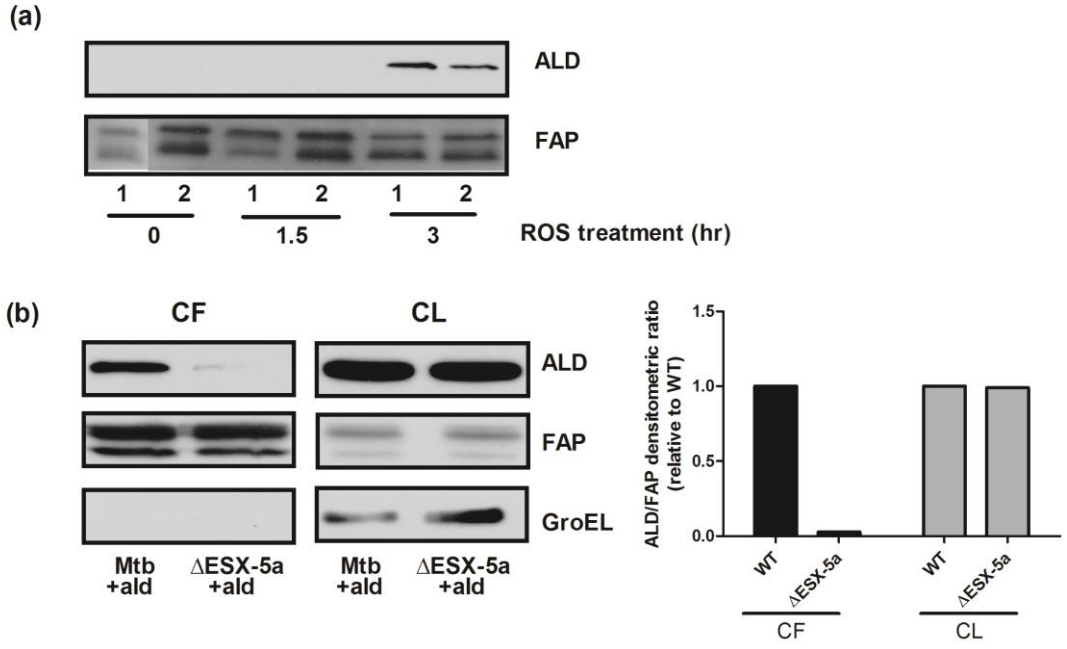

Figure 19: Secretion defects in the *Mtb* ΔESX-5a mutant. (a) *Mtb* (1) and *Mtb*ΔESX-5a mutant (2) cultures were exposed to superoxide for different times as indicated and the corresponding cell lysates (CL) were probed for ALD (top) and FAP (bottom) by western blotting. In this particular image an empty lane between the 1st and 2nd lane has been cropped. **(b)** The secreted proteins (CF) and CL from *Mtb* and *Mtb*ΔESX-5a strains constitutively expressing ALD were blotted for ALD, FAP and GroEL. The bar graph shows the quantification of ALD in CF and CL normalized to FAP and relative to WT *Mtb*. All data shown is one representative experiment out of three. (Work done by Shah S & Briken V.)

and the quantification shows that only WT *Mtb* secretes ALD even though both *Mtb* and ΔESX-5a now express comparable amounts of the protein (Fig 19b).This result also indicates that the ESX-5a deletion might not affect ALD expression. Instead, it points to the possibility that inability of the mutant to export the ALD somehow affects the stability of the protein itself. This confirms that ESX5a is necessary for secretion of ALD in *Mtb* and *Mm*.

3.4.3 ESX-5a Contributes to the Secretion of ESX-5 Substrates

Since the ESX-5a region has been duplicated from the ESX-5 locus we wanted to see if the secretion of any ESX-5 substrates were affected in ΔESX-5a. When probed for the PGRS proteins, the *Mm* mutant was partially defective in the secretion of some of these proteins (Fig 20a). The antibody used here recognizes the PGRS motif and thus identifies multiple proteins from the family. The three PGRS protein bands labeled as 1, 2 and 3 were quantified and represented as a densitometric ratio of PGRS to the FAP relative to WT *Mm*. The ESX-5a deletion also affected the expression of two PGRS proteins (bands 1 and 3), which could possibly be a stability issue like with ALD. From the *Mtb* CF blots, we found that the mutant was partially defective in the secretion of PPE41, another known ESX-5 substrate and one PGRS protein (Fig 20b). In both these cases, quantification of the blots (done as before) shows that defect is solely in secretion of the proteins (Fig 20b). The above data also indicates that the accessory region could play a role in the secretion of ESX-5 substrates which points to some redundancy between the two loci.

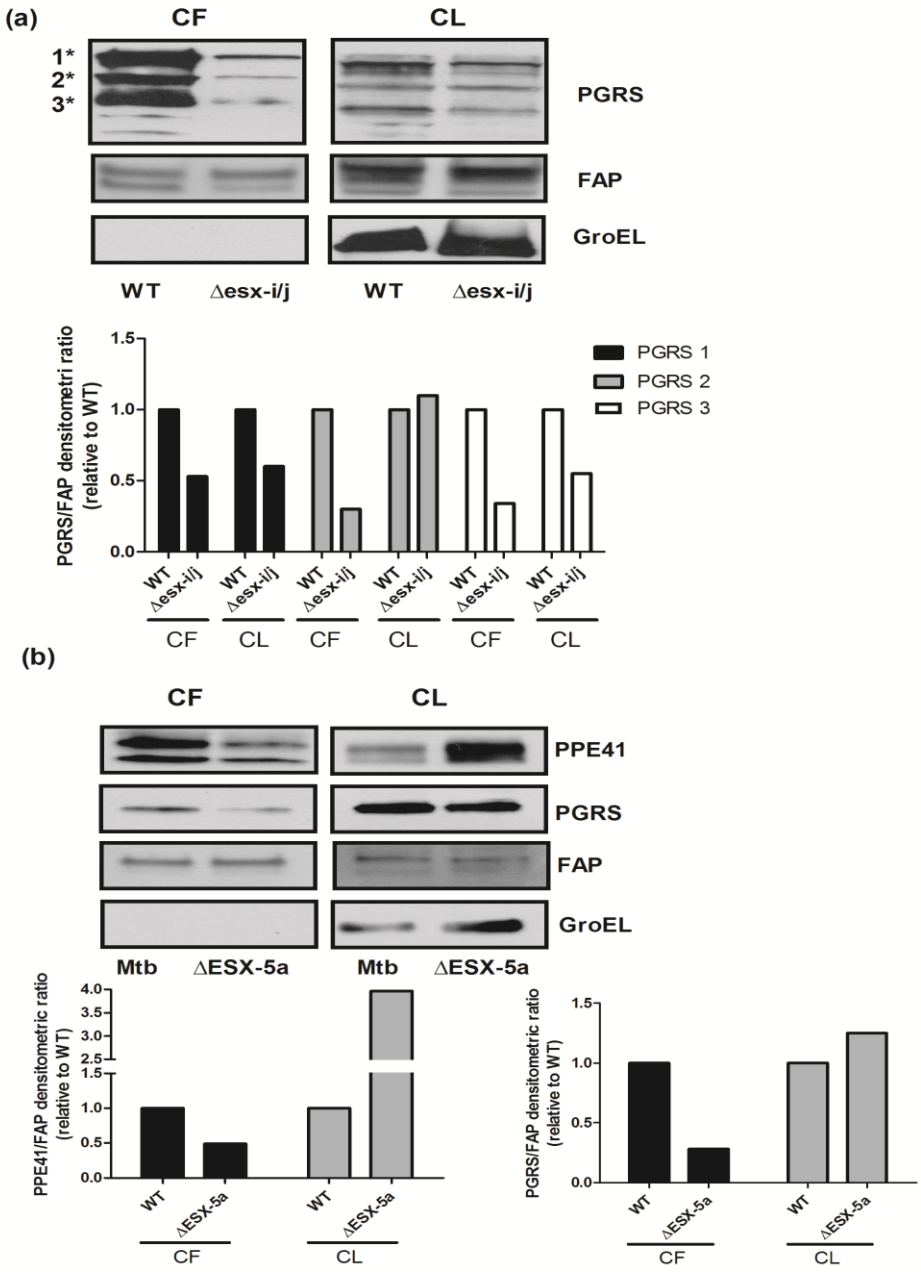

Figure 20: ESX-5a contributes to the secretion of ESX-5 substrates (a) *Mm* (WT) and the ESX-5a mutant (*Δesx i/j*) CF and CL were probed for PGRS family of proteins (PE sub-family) Three PGRS bands labeled as 1, 2, 3 were quantified and are shown below the blot **(b)** The *Mtb* and *Mtb*ΔESX-5a CF and CL were probed with antibodies against the ESX-5 substrates: PPE41 (PPE family member) and the PGRS-containing proteins with the quantification of CF and CL bands shown below. All quantification was done using the imageJ software and is normalized to the respective FAP protein bands (loading control) and is relative to WT. All data shown is one representative experiment out of three. (Work done by Shah S & Briken V.)

3.4.4 Secretion of ALD Requires the ESX-5 System

The ESX-5a region lacks its own secretion machinery and since we found evidence of crosstalk with its parent system, we hypothesized that the ESX-5a substrates were also exported via the ESX-5 secretion system. *Mm* cultures: WT and the mutant with transposon insertions in the ATPase ($eccA_5$) of the ESX-5 system were grown in sautons media. Their short-term CF and CL were collected, run on an SDS-PAGE gel and subjected to western blotting. The blot was then probed with anti-ALD. The CL of $eccA_5$::Tn showed ALD protein concentrations comparable to that of WT *Mm* and yet did not secrete any of it unlike the WT *Mm* (Fig 21) indicating the requirement of an intact ESX-5 system for its secretion. The western blot quantification was done ALD for both CF and CL and is represented as a ratio of ALD to FAP band intensity relative to WT *Mtb*. The absence of GroEL in the CF indicated that the supernatants were devoid of any cytosolic contamination. The blots were probed with anti-PGRS to show that both the transposon mutants were also defective in secretion of ESX-5 substrates. The eccA5::Tn was defective in the secretion of only some of the PGRS proteins. The increased secretion of some PGRS proteins seen in the CF of eccA5::Tn could be due to the difference in protein amounts loaded (FAP bands in eccA5::Tn CF are stronger than WT). These results thus confirm our hypothesis that the ESX-5a substrates are secreted through the parent ESX-5 system.

3.4.5 Deletion of ESX-5a Does Not Affect Host Cell Death Induction

The interaction of *Mtb* with its host cell in regard to cell death induction is complex [34].

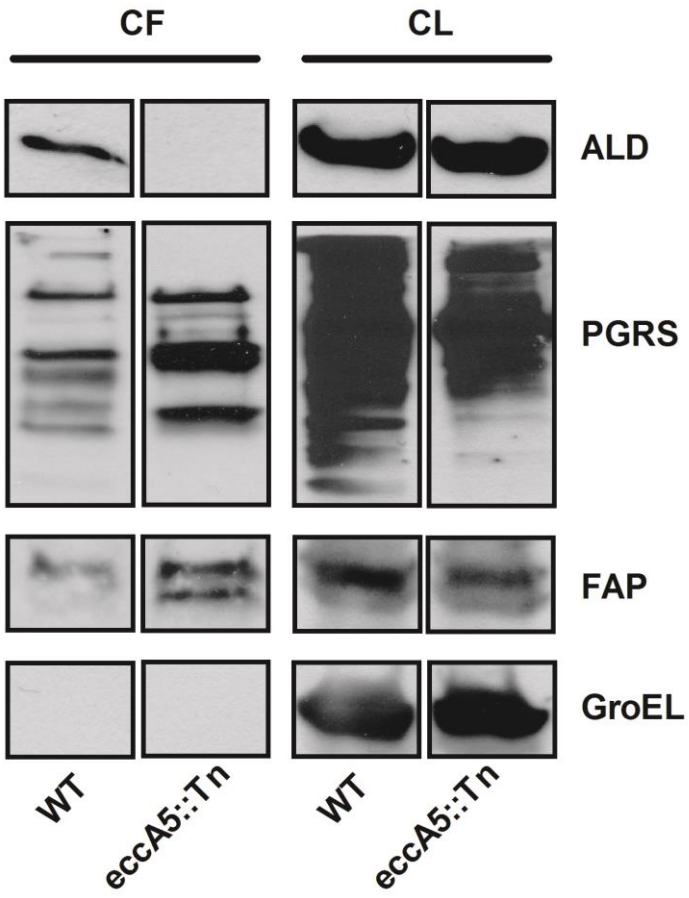

Figure 21: ALD is secreted via the ESX-5 system. Cell lysates (CL) and cell supernatant filtrates (CF) were collected from *Mm* wild-type (WT) and transposon insertions mutant in the ESX-5 ATPase (eccA5::Tn). The western blots were probed for ALD, GroEL, FAP and PGRS. Data shown is one representative experiment out of three. Work done by Shah S & Briken V.)

It is most likely that *Mtb* inhibits cell death induction during initial phase of replication but induces cell death later on in order to exit the host cell [20]. Esx-5 deficient *Mtb* and *Mm* induce less host cell death [2]. To assess apoptotic cell death in macrophages, THP-1 cells were infected with *Mtb*, ΔESX-5a and its complement (ΔESX-5aC) for 3 days. The apoptosis rates were calculated as the percent of TUNEL positive cells using flow cytometry. Both *Mtb* and the mutant induced very similar rates of apoptotic death of about 20% while the complement induced rates slightly higher (Fig 22a). This small increase could be attributed to the fact that in the complement the ESX-5a region is constitutively expressed which may lead to higher protein levels when compared to *Mtb*. Death by necrosis was measured by the release of cytosolic adenylate kinase (AK) into the supernatant. The cells were collected for measuring apoptosis while the cell supernatants were collected to measure necrosis. Both the mutant and the complement showed no difference in necrotic cell death when compared to *Mtb*. All three bacterial strains induced about 3 fold higher levels of AK in the supernatant than uninfected cells (Fig 22a). Cells treated with 1% Triton X-100 were used as a positive control. In primary mouse bone marrow derived dendritic cells (BMDC) infected with the bacterial strains for 24h the hypodiploid positive population in *Mtb* and mutant was around 47% and this was slightly higher in the complement at about 60%. Similarly, there was no difference in the necrosis induced as well (Fig 22b).

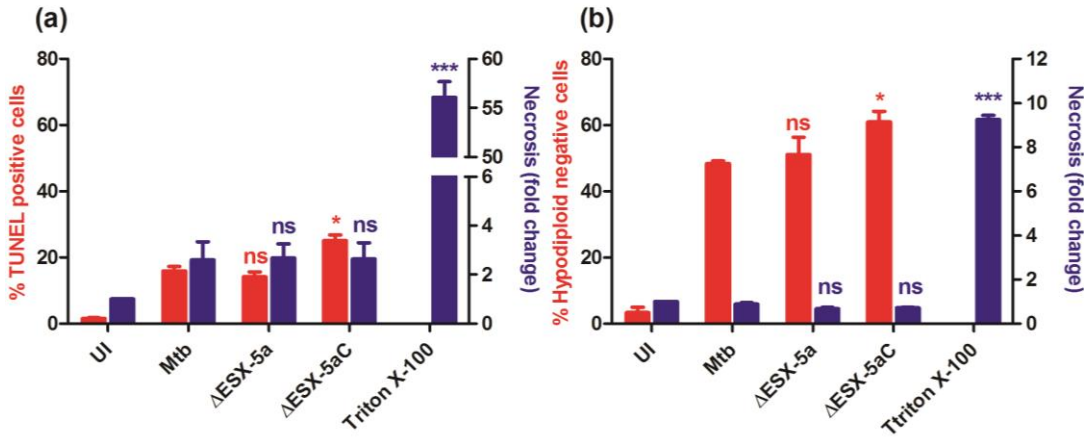

Figure 22: ESX-5a deletion has no effect on host cell death induction. Apoptotic cell death (red bars along the left Y axis) induction was measured in THP-1 macrophages by TUNEL assay at day 3 **(a)** and in BMDCs by hypodiploid staining at day 1 **(b)** while the release of adenylate kinase into the supernatant as a measure of necrosis (blue bars along the right Y axis) is shown as fold change over uninfected cells (UI) in **(a)** and **(b)**. ***0.0001< p < 0.001, *Mtb*. Data are shown as the means ± standard deviation (SD) of triplicate measurements of one representative experiment out of three. (Work done by Shah S & Briken V.)

3.4.6 Inflammasome Activation is Reduced in Bacteria Lacking a Functional ESX-5a

The activation of the inflammasome and the secretion of mature IL-1β by BMDMs and BMDCs after *Mtb* infection are dependent on a functional ESX-1 secretion system [1,129, 212]. In *Mm*, both ESX-1 and ESX-5 secretion systems have been shown to be required for IL-1β secretion [2]. So we investigated the effect of ESX-5a deletion on IL-1β secretion in THP-1 cells, BMDMs and BMDCs. THP-1 cells were infected with *Mtb*, ΔESX-5a and ΔESX-5aC at an MOI 3 and the supernatants were assayed for IL-1β secretion by ELISA 3 days post infection. The mutant showed around 50% reduction in cytokine secretion when compared to both *Mtb* and complement (Fig 23a).

BMDMs and BMDCs were infected at an MOI of 10 and the cell-free supernatants were analyzed for IL-1β secretion 24 h post infection. BMDCs infected with the mutant also showed a 50% reduction in secretion of IL-1β (Fig 23b) while in BMDMs it is completely abrogated (Fig 23c). All supernatants were tested for release of AK as a control for contamination with cytosolic contents due to necrosis and there was found to be minimal induction after infection with all three strains and no differences between the strains (blue bars in Fig 22b). Additionally, the BMDC supernatants showed slight reduction in the secretion of some other pro-inflammatory cytokines (Fig 24).

Inflammasome activation is a two-signal process ultimately leading to the production of mature inflammatory cytokines like IL-18 and IL-1β. The first signal leads to the induction of pro-IL1β synthesis.

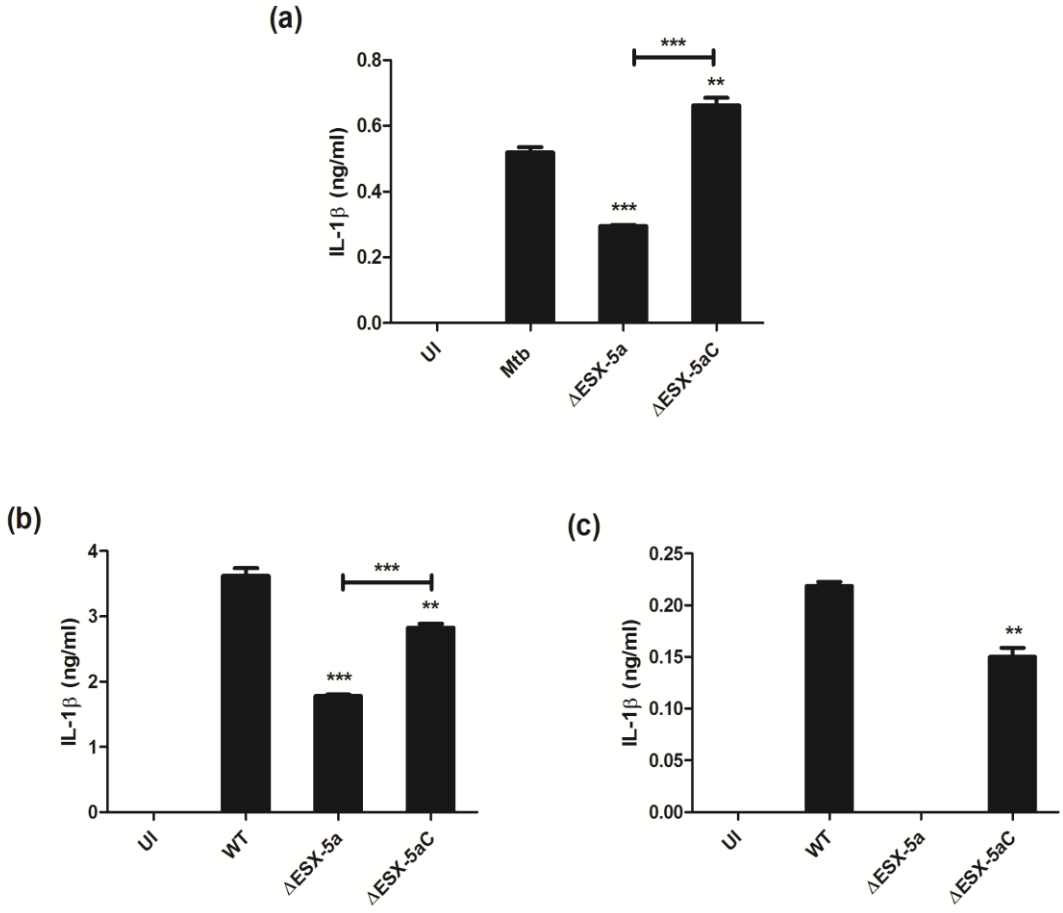

Figure 23: ESX-5a deletion in *Mtb* reduces the secretion of IL-1β by infected host cells. IL-1β ELISAs were performed on supernatants from cells infected with *Mtb*, ΔESX-5a or its complement (ΔESX-5aC) in **(a)** THP-1 cells at day 3 post infection or **(b)** mouse BMDC and **(c)** mouse BMDMs at 24h. ***0.0001< p < 0.001, *Mtb*. Data are shown as the means ± SD of triplicate measurements of one representative experiment out of three. (Work done by Shah S & Briken V.)

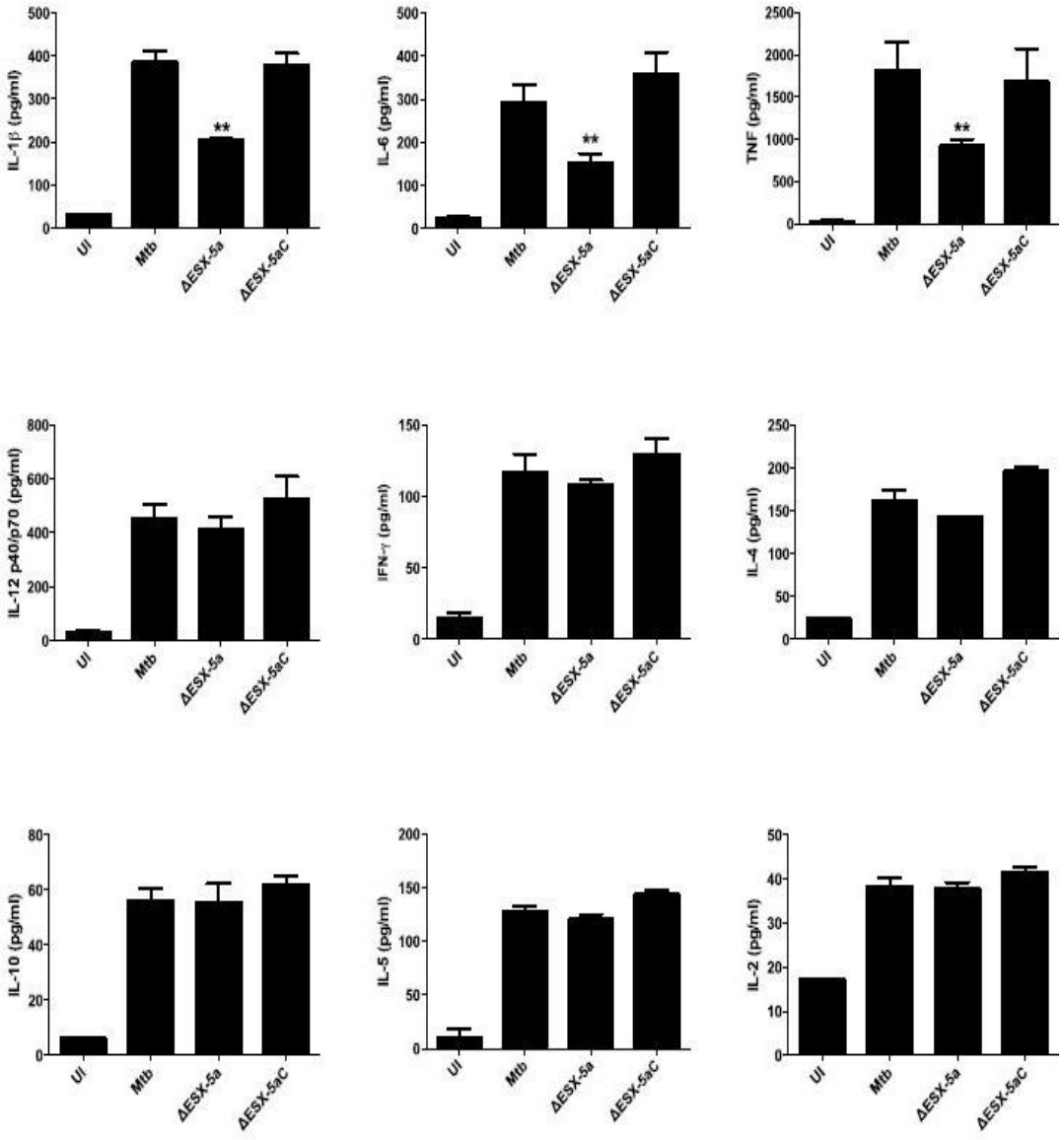

Figure 24: Multiplexed cytokine analysis of infected BMDCs. BMDCs were infected with *Mtb*, the ESX-5a mutant (ΔESX-5a) and complemented mutant strain (ΔESX-5aC) and after 24h the supernatants were analyzed for expression of the indicated cytokines using a multiplexed magnetic mouse cytokine panel. ***0.0001< p < 0.001, *Mtb*. Data are shown as the means ± SD of triplicate measurements of one representative experiment out of three. (Work done by Shah S & Briken V.)

The second is required for caspase-1 activation via the inflammasome. In order to identify at what point the ΔESX-5a mutant resulted in reduced activation the mRNA and cell lysates of infected BMDCs were collected to check for expression of pro-IL-1β at both transcriptional and translational levels. We observed that there was no difference in expression of pro-IL-1β mRNA between mutant and *Mtb* after four hours of infection (Fig 25a). The protein levels of pro-IL-1 β were also found to be similar (Fig 25b). So next we analyzed caspase-1 activation by blotting for activated, cleaved caspase-1 (p10) in the supernatants of infected BMDCs. All infection conditions used the same number of cells and from these, the entire supernatant was collected. This was done to ensure that any variation in the amount of protein detected was not due to differences in concentration of loaded samples. The proteins were extracted from these supernatant samples were then probed for caspase-1. Signal for activated caspase-1 was clearly visible in supernatants of both *Mtb* and complement infected cells but reduced in the mutant infected cell supernatants (Fig 25b). The complement infected sample showed more of the cleaved caspase-1 fragment, possibly because the ESX-5a region in the complement is constitutively expressed. In conclusion, the absence of ESX-5a proteins reduces the generation of mature IL-1β via reduced activation of the inflammasome leading to the generation of less active caspase-1.

3.4.7 ESX-5a Contributes to Virulence of *Mm* in Zebrafish

Zebrafish is an *in vivo* model for *Mm* infections that has been extensively used to understand mycobacterial pathogenicity mechanisms that can be translated into the human pathogen *Mtb* [182, 225].

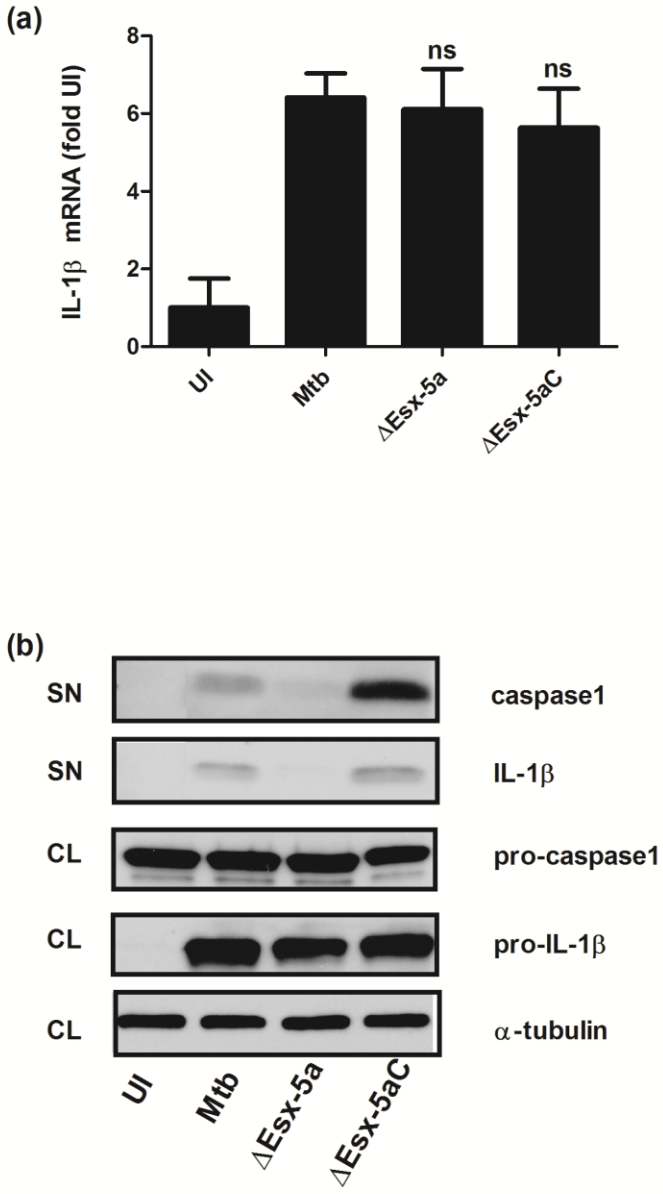

Figure 25: ESX-5a deletion in *Mtb* reduces the host cell inflammasome activation. **(a)** The mRNA levels of pro IL-1β were measured in BMDCs infected with *Mtb*, the ESX-5a mutant (ΔESX-5a) and complemented mutant strain (ΔESX-5aC) right after the 4h infection period using quantitative RT-PCR. ***0.0001< p < 0.001, *Mtb* **(b)** Western blots of supernatants (SN) collected at 6h post infection showing the active fragments of caspase-1 (10kDa) and IL-1β (17kDa), respectively. The corresponding cell lysates (CL) were probed for procaspase-1 (45kDa), pro-IL-1β (35kDa). Tubulin (55kDa) signal serves as the loading control. Data are shown as the means ± SD of triplicate measurements of one representative experiment out of three.

To determine the effect of the ESX-5a region on virulence of mycobacteria as indicated by survival, the zebrafish were injected intraperitoneally (i.p) with PBS, *Mm*, ΔesxI/J and ΔesxI/J-C (complement) at a CFU of 10^4/fish and monitored for up to 80 days. There were 10 fish per experimental group and the PBS injected fish were kept as a control group to negate the possibility of death due to needle injuries. Most of the *Mm* and complement bacteria infected fish died within 3-5 weeks whereas a bulk of the ΔesxI/J mutant infected fish started dying only after 5 weeks (Fig 26). This shows that the absence of these ESX-5a proteins significantly reduced the virulence of the bacteria.

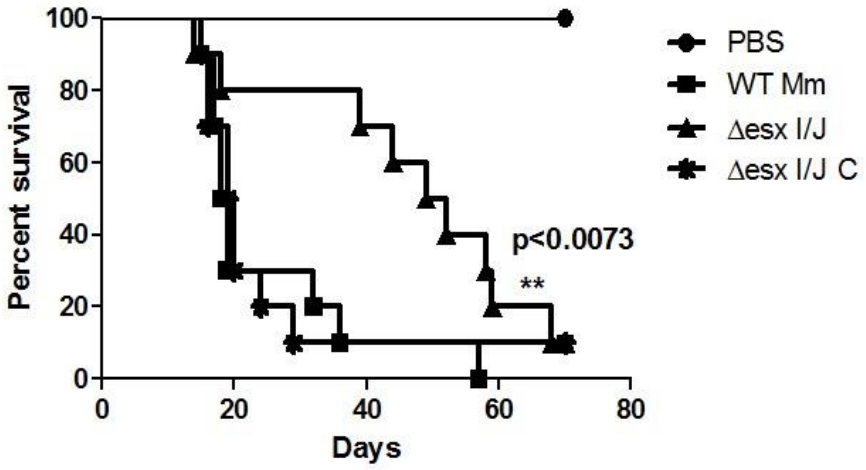

Figure 26: Virulence defect of the *Mm* ESX-5a mutant. Adult zebrafish were injected intraperitoneally (ip) with 10^4 bacteria per fish of *M. marinum* (WT *Mm*), the *Mm* ESX-5a deficient Δesx i/j mutant (Δesx i/j) and the complemented mutant strain (Δesx-i/jC) and monitored for survival. There were 10 fish per group (n=10) with saline (PBS) injected fish as a control group. **0.001< p < 0.01, WT *Mm*. Data are shown as the means ± SD of triplicate measurements of one representative experiment out of three. Statistical analysis was performed using log-rank test. (Work done by Shah S and Briken V.)

3.4.8 Discussion

We demonstrate that the deletion of ESX-5a locus partially affects the secretion of some ESX-5 system substrates like PPE41 and PGRS proteins (Fig 20). Since the ESX-5a region does not include genes that code for the core secretory machinery, such as a channel protein, it must utilize another secretion system for protein export. Our finding that there is an overlap in secretion substrates between the ESX-5 and ESX-5a system points to the possibility of crosstalk between the ESX-5 secretion system and ESX-5a. This is supported by our finding that *Mm* transposon mutant of AAA ATPase ($eccA_5$) is also deficient in the secretion of ALD (Fig 21) as is the *Mm* ESX-5a deletion mutant (Δesxi/j) (Fig 18a+18b). Hence, we propose a model in which the ESX-5 system encodes for the core secretion machinery whereas ESX-5a is important for chaperoning a subset of ESX-5 secreted proteins (Fig 27). This model suggest that all three ESX-5 duplicated regions: Rv1195-Rv1198, Rv1037c-Rv1040c and Rv3619c-Rv3622c could potentially play a role in protein secretion in conjunction with the parent ESX-5 and that all these different loci contribute to the overall secretory functioning of the ESX-5 system.

Mtb has 168 members of the PE/PPE family of proteins in it genome, which accounts for about 10% of the total genome. The function of these proteins is not well understood. Their expression was hypothesized to provide antigenic variability in order to favor evasion of the host immune response. One problem with the study of individual members of a large protein family is the high chance of redundancy and thus loss-of-function genetic approaches are often futile.

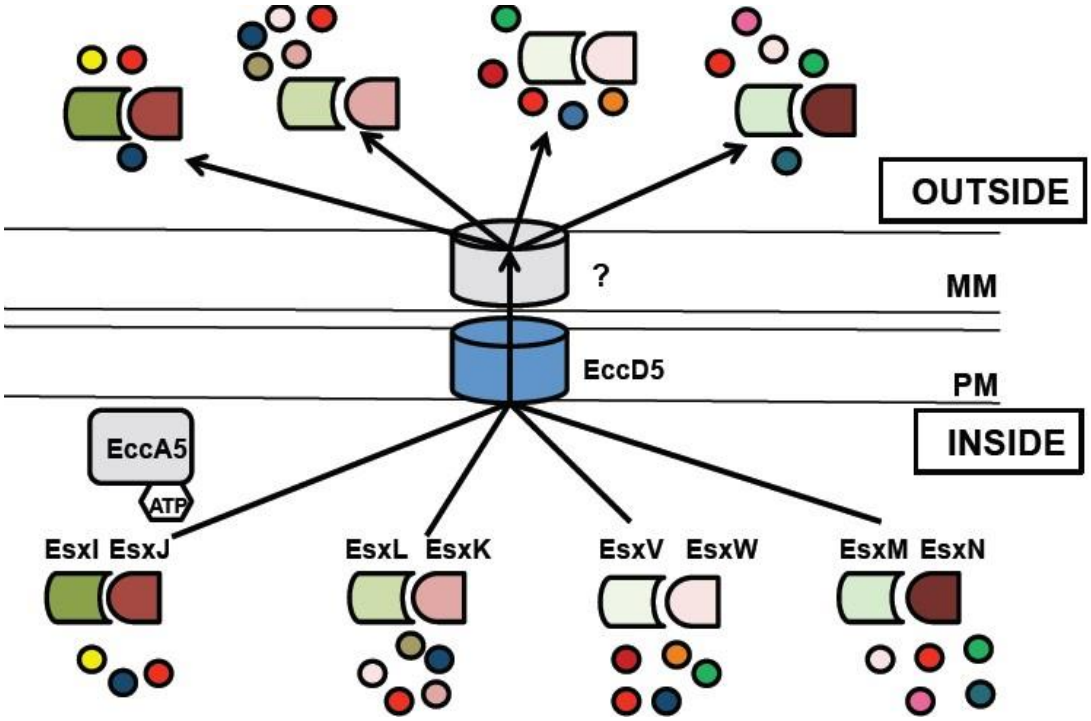

Figure 27: Model of ESX-5 and replicate ESAT-6 regions function in protein secretion. The ESX-5 ESAT-6 region Esx protein pair (EsxM and EsxN) and its duplicate paralogs are indicated. The circles represent putative secretion substrates associated with each of these systems. We propose that they all feed into the core components of the ESX-5 system represented here by the putative channel protein $EccD_5$ and the ATPase $EccA_5$. The plasma membrane (PM) and the mycomembrane formed by mycobacterial lipids (MM) are indicated.

Our results led to the hypothesis that the ESAT-6 duplicate regions of ESX-5 are important for selection of substrates to be secreted by ESX-5. Thus by deleting each of the three duplicated regions and determining the phenotype of the mutant and the secretion defect it should be possible to attribute a function a smaller subset of the PE/PPE protein family. For example, the ESX-5a mutant has no cell death phenotype, which is different from an ESX-5 mutant, and therefore we can conclude that the ESX-5a substrates are not involved in cell death induction. This approach may help in characterizing the function of various PE/PPE proteins.

The molecular mechanism of substrate selection for type VII secretion systems is a matter of great interest and intense research efforts. Recently, a short peptide motif (YxxxD/E) in the C-terminus of proteins was identified as a general signal for type VII secretion [55]. However, this signal does not determine ESX specificity since exchanging it on ESX-1 and ESX-5 substrates did not change their respective secretion pathway [55]. Another characteristic of ESX and PE/PPE secretion substrates is the formation of heterodimers, which are essential for successful secretion and which result in the generation of a characteristic four-helix bundle [109]. These characteristics have been used to develop an algorithm to predict novel T7SS substrates [55]. Interestingly, ALD does not have the structural features or an YxxxD/E motif, which would identify it as a T7SS substrate. Consequently, it is possible that another group of T7SS substrates exist which remains to be identified. This task maybe complicated by the fact that other T7SS may only be highly expressed in *Mtb* after infection of host cells, similar to ALD, which makes these T7SS impossible to identify using the standard *ex vivo* proteomics approaches.

Mtb infection activates the host cell inflammasome which results in the generation of mature secreted IL-1β. The active inflammasome consists of a variable sensing component (most often an NLR), the adaptor protein ASC and pro-caspase-1 [199, 224]. In the case of *Mtb* infections of macrophages or dendritic cells only the NLRP3-inflammasome is activated [1, 33, 41, 70, 153, 154, 162, 236]. There are several agonists that induce activation of the NLRP3 inflammasome such as an increase in ROS, nigericin, ATP, bacterial toxins and particulate matter [114, 165, 219]. All of these agonist share that they induce K^+ efflux, which is the common trigger for the NLRP3-inflammasome [165]. A central role was recently attributed to the mitochondria, since during mitochondrial dysfunction cardiolipin is relocated to the outer mitochondrial membrane where it can bind to and activate NLRP3 [114]. Here we demonstrate that the *Mtb*ΔESX-5a mutant does not induce NLRP3-inflammasome activation as efficiently as the wild-type bacteria. We show that this defect is not due to a lack of signaling to induce pro-IL-1β or pro-caspase-1 transcription or translation (Fig 25a+25b) but because of the disruption of activation of the inflammasome (Fig 25b). In the absence of either the ESX-1 or ESX-5 systems there is a reduction of mature IL-1β secretion by the host cells into the supernatants [1, 2, 129, 213] . It is thus somewhat surprising that the deletion of the ESX-5a region also has such a strong effect on inflammasome activation. One interpretation of the results is that the reduced inflammasome activation observed with the ESX-5 mutant is actually due to a lack of secreted substrates dependent on the ESX-5a region and hence the deletion of ESX-5a has a similar effect to ESX-5 deletion. It seems unlikely that the inflammasome phenotype of the *Mtb* ESX-5a mutant is linked to cell death induction as it has been

reported for the *Mm* ESX-5 mutant [2] since cell death levels are not affected by the ESX-5a deletion in *Mtb* (Fig 22). This implies that the secreted ESX-5a substrates have no role to play in host cell death induction.

The appearance of the ESX-5 system in the genomes of slow growing mycobacteria demarcates them from the fast growing mycobacterial species [96]. Most of its secreted substrates are from the PE/PPE protein family that is considerably expanded in the slow growing species [6, 29, 96]. Since all of the pathogenic mycobacterial species adapted towards infection of a host are also slow growing, this correlation suggests that the ESX-5 system is involved in virulence. Indeed, the *Mtb* ESX-5 deletion mutants were attenuated upon *in vitro* infection of macrophages and *in vivo* infections using the mouse model [29, 197]. Our study shows that *Mm* ESX-5a mutant is attenuated in the adult zebrafish when compared to both the wild-type *Mm* and the complement mutant strain (Fig 26). This result indicates that the substrates of this accessory ESX-5 secretion system are necessary for full virulence of mycobacteria. Another possibility for the attenuation seen in mutant infected fish could be due to the inability of the bacteria to replicate normally even though the mutant did not show any growth defects when cultured *in vitro*. In any case, the decreased *in vivo* fitness of the mutant could attribute to virulence attenuation. Consistently, the transcription of two genes of the ESX-5a region (*esxI and esxJ*) is highly up-regulated in bacteria isolated from human tuberculosis patients or from the lungs of infected mice [37]. Thus, it would be interesting to see if the *Mtb* ΔESX-5a is attenuated in mice as seen with the *Mm* mutant in zebrafish. Altogether, our findings point towards an important function

of the three replicate ESAT-6 regions of ESX-5 in protein secretion and virulence of *Mtb*.

CHAPTER 4: IMMUNE EVASION BY INHIBITION OF HOST CELL APOPTOSIS

4.1 Abstract

Mtb is a highly infectious organism and is one of the leading causes of death in third world countries. *Mtb* primarily resides within macrophages and inhibits host cell apoptosis to protect its replicative niche. Apoptosis is an important innate defense mechanism employed by the host immune cells to clear intracellular pathogens. A gain of function screen using avirulent *M. smegmatis* (*Msme*) and *M.kansasii (Mkan)* identified three unique genomic regions of *Mtb* with anti-apoptotic activity. Using different experimental approaches, gene products from one of the regions (M24) were systematically assessed for their ability to inhibit host cell apoptosis. One of the genes from the M24 region - *Rv1048c* was found to have an anti-apoptotic function. *Rv1048c* encodes a conserved hypothetical protein and is contained on a genomic island that is only found among the members of the *Mtb* complex.

4.2 Introduction

4.2.1 Cell Death Mechanisms for Host Defense

Multiple modes of cell death are important host immune defense mechanisms in the event of a microbial infection, so much so that many pathogens are known to manipulate cell death pathways for gaining a survival advantage. There are various cell death modalities such as apoptosis, pyroptosis, necroptosis etc. that are important for controlling infections. Pyroptosis is an inflammatory mode of programmed cell death that is dependent on caspase-1 activation. This form of cell death was required for the clearance of intracellular bacteria such as *S.typhimurium, L.pneumophilia and B.thailandensis* [157]. Necroptosis, or programmed necrosis, is dependent on the activity of RIPK1 and RIPK3 for cell lysis and release of DAMPs (Danger Associated Molecular Patterns) such as HMGB1, IL-1α etc. along with its cytoplasmic contents [121]. Necroptosis is important for the control of Vaccinia Virus infection [45]. Apoptosis is an immunologically silent form of programmed cell death that is a primary innate defense mechanism against many viral and bacterial pathogens. It serves to play two important roles in controlling infections. One, it eliminates the pathogen along with its replicative niche without inducing an inflammatory response and two, the resulting apoptotic bodies with the pathogenic antigens are taken up DCs, processed and presented via MHC class I molecules to cytotoxic CD8 T-cells to ultimately mount an adaptive immune response [235].

4.2.2 Effects of Apoptosis on Mycobacterial Survival

Macrophage cell death by apoptosis is known to affect the viability of various mycobacterial species [66, 88, 164]. However, the death of invading pathogens cannot be completely attributed to the apoptosis of the primary infected cells. When the cells undergo apoptosis they are broken down into smaller vesicles that are then take up by other neighbouring phagocytes in a process known as "efferocytosis" (Fig 28) [149]. It has been shown that uninfected macrophages contribute to controlling bacterial growth *in vitro* [88]. Another group has shown that same phenomenon *in vivo* by using a novel LIVE/DEAD *Mtb* reporter strain. They demonstrated that most of the bacteria contained in efferocytic cells were dead and that apoptotic vesicle + *Mtb*- containing phagosomes in these cells can now seem to mature as they become acidic and also accumulate the lysosomal marker Lamp1 on their surface [149].

Using zebrafish infected with its natural pathogen, *M. marinum* (*Mm*), it was found that the initiation of apoptosis is important for the recruitment of neutrophils to the site of the granulomas. Here the neutrophils did not seem to phagocytize extracellular *Mtb* but acquired them through efferocytosis. After which, the ingested mycobacteria were killed by the generation of ROS via the NOX2 complex [240].

Induction of apoptosis is an innate immune response mounted by the host against foreign invasion. Subsequently, the apoptotic blebs containing bacterial antigens are taken up by dendritic cells which process them through the MHC class I molecules and present the peptides to naïve CD8 T-cells leading to the onset of adaptive immunity. This process is known as "cross-presentation" [26].

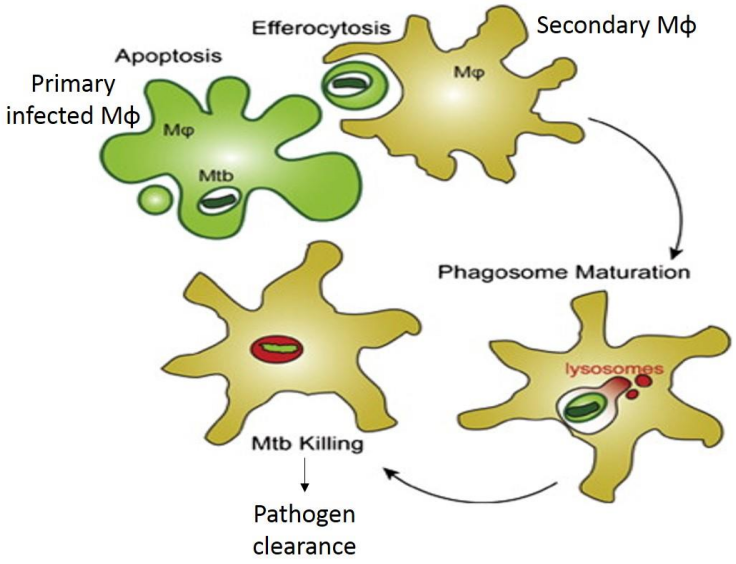

Figure 28: *Mtb* killing by efferocytosis [adapted from 149].
The neighboring phagocytes engulf apoptotic bodies released from primary infected macrophages. These apoptotic bodies contain live bacteria. The phagosome of the secondary macrophages containing *Mtb*+apoptotic bodies can now undergo fusion with the lysosome resulting in pathogen clearance.

The vaccination of mice with apoptotic vesicles containing *Mtb*, was enough to prime naïve T-cells against a virulent *Mtb* challenge [235]. Divangahi et al, show that inhibition of apoptosis by *Mtb* delays the onset of adaptive immunity by preventing cross-presentation via CD11c DCs through a series of elegant experiments. In the presence of LXA4, infected macrophages tend to die from necrosis. Therefore by knocking out this gene, the macrophages are found to have a higher propensity for undergoing apoptotic cell death upon mycobacterial infection. The alveolar macrophages from the LXA4 knockout mice were intratracheally transferred into WT mice which were then infected with virulent *Mtb*. These mice were found to have a higher percentage of CD8 T-cells specific for Tb10.4 antigens in the lungs and peripheral lymph nodes when compared to mice that received WT alveolar macrophages. The apoptosis dependent expansion of T-cell population specific to *Mtb* antigens requires the presence of CD11c+ DCs and an intact MHC class I system. [67].

4.2.3 Mechanisms of Mycobacterial Inhibition of Apoptosis

Virulent *Mtb* has developed several ways to inhibit host cell apoptosis early on and later push the cell to undergo necrosis. This way the bacteria are released from the cells and can re-infect other cells leading to disease dissemination whereas avirulent mycobacteria induce host cell apoptosis upon infection [123].

Virulent *Mtb* does still induce very low levels of programmed cell death due to proteins like EsxA [62] and 19kDa lipoproteins [46]. These bacterial effectors however are important for virulence and therefore to compensate, it is quite possible that *Mtb* employs other anti-apoptotic mechanisms to actively suppress apoptosis levels to the

minimum early on in the infection and later cause necrosis to promote bacterial escape to the extracellular milieu [179]. It has also been reported that virulent *Mtb* is able to inhibit apoptosis when infected *in vitro* at a low MOI (Multiplicity of Infection) but at higher MOIs, it induces cell death independent of caspases. This form of host cell death resembles necrosis at later stages and is most likely a mechanism to promote its extracellular dissemination [131].

TNF is indispensable for apoptosis induction in host cells and virulent *Mtb* is known to increase secretion of soluble TNFR2 from host cells to neutralize the cytokine [87]. TNF can signal for either cell survival or apoptosis. ROS is a major determinant which pushes the signaling pathway towards JNK and p38 MAPK, thereby ensuring that the cell undergoes TNF dependent apoptosis [160]. Recently a slew of studies showed that virulent *Mtb* manipulates the eicosanoid biosynthetic pathways to push the cell away from apoptosis and induce necrosis instead. This involved inhibiting PGE2 production and thereby increasing amounts of LXA4. As a result, the cells could no longer repair the damage done to the plasma membrane or complete the formation of the "apoptotic envelope" [Reviewed in 21].

Bacterial Effectors in Apoptosis Inhibition

Proof of mycobacterial inhibition of host cell apoptosis is validated by the identification of numerous pro-apoptotic deletion mutants. Some of the proteins identified as anti-apoptotic were SecA2 and PknE (protein kinase E). SecA2 encodes an accessory secretion system required for the export of virulence factors like SodA (Superoxide Dismutase A) which is involved in neutralizing superoxides generated by

the host cells. The *secA2* deletion mutant induced high levels of host cell apoptosis. This phenotype could be complemented when SodA with an antigen85 signal peptide was added to allow its secretion through SecA1 system implying that the actual anti-apoptotic effector is SodA [106]. The deletion mutant of *PknE* was more susceptible to nitric oxide exposure and also induced higher levels of host cell apoptosis as compared to WT *Mtb* [115]. The promoter of PknE is up-regulated during nitrosative stress and the protein is able to cause increased phosphorylation of a MAPK (ERK 1/2), which ultimately leads to host cell survival [175]. Another study identified Rv3654c and Rv3655c proteins as being anti-apoptotic. These two proteins belong to a "type IV pili" operon and are secreted into the macrophage cytosol where they interact with host proteins PSF and ALO17 respectively and affect the availability of caspase-8. As a result, *Mtb* is able to interfere with the extrinsic apoptotic pathway [56].

Previously, a gain of function screen was done to systematically identify anti-apoptotic genes in virulent *Mtb*. Around 40kbp fragments of the genome of the virulent *Mtb* Erdman strain were ligated into the episomal cosmid pYUB415. These clones were then transformed into avirulent mycobacterial species that are known to strongly induce apoptosis, such as *Mkansasii (Mkan)* and *M. smegmatis (Msme)* [125]. THP-1 cells were infected with the *Mkan* transformants and the cells were harvested 5 days post infection (dpi) for a TUNEL assay to gauge the levels of apoptosis. Both WT *Mkan* and *Mkan* with empty cosmid (CO) infected conditions were almost completely apoptotic while only about 10% of *Mtb* infected cells were dying. *Mkan+* J21 and M24 infected cells resembled the *Mtb* infected condition. Therefore, it was concluded that the J21 and M24 cosmids carry anti-apoptotic genes and that these *Mkan* transformants

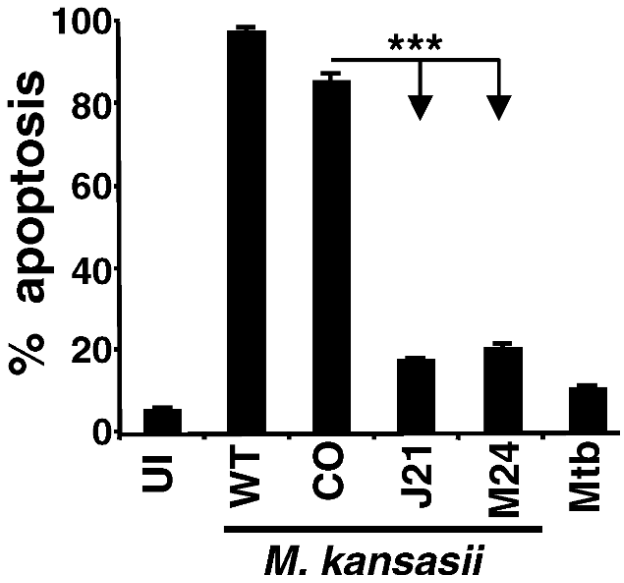

Figure 29: Apoptosis induction by *Mkan*+J21 and *Mkan*+M24.
THP-1 cells were infected with WT *Mkan*, *Mkan* transformed with empty cosmid (CO), J21 and M24 and WT *Mtb*. The cells were harvested 5dpi and the levels of apoptosis shown as percent TUNEL positive cells. Results are represented as an average of three independent experiments. Statistical significance is relative to WT *Mkan* and is indicated as * $0.01<p<0.05$, ** $0.001<p<0.01$, *** $p<0.001$ (ANOVA with Tukey post-test). [230]

have gained the function of apoptosis inhibition (Fig 29). A third anti-apoptotic, *Mtb* genomic region to be identified was called K20. The first anti-apoptotic gene to be identified using this screen was from the J21 cosmid, namely, *nuoG*. An *Mtb* mutant of NuoG, a protein of the Type I NADH dehydrogenase complex was found to induce higher levels of apoptosis in infected BMDMs as well as THP-1 macrophages [230]. The pro-apoptotic phenotype of *ΔnuoG* could be attributed to the induction of phagosomal ROS via NOX2 system that ultimately led to TNF production and apoptosis [160].

In the current study, the M24 cosmid consisting of 38 *Mtb* genes (Fig 30), was subjected to multiple loss of function genetic approaches to identify the gene(s) responsible for inhibiting apoptosis. The M24 region consists of many operons, such as the *kdp* operon (regulates potassium uptake), the Esx-5a region (involved in protein secretion and virulence), Rv1041c-Rv1055 (a genomic island mostly encoding for proteins of unknown function). From the M24 region, a novel anti-apoptotic gene called *Rv1048c* was identified. This gene encodes for a conserved hypothetical protein of an unknown function and is found on a genomic island that is exclusively present in the members of the *Mtb* complex.

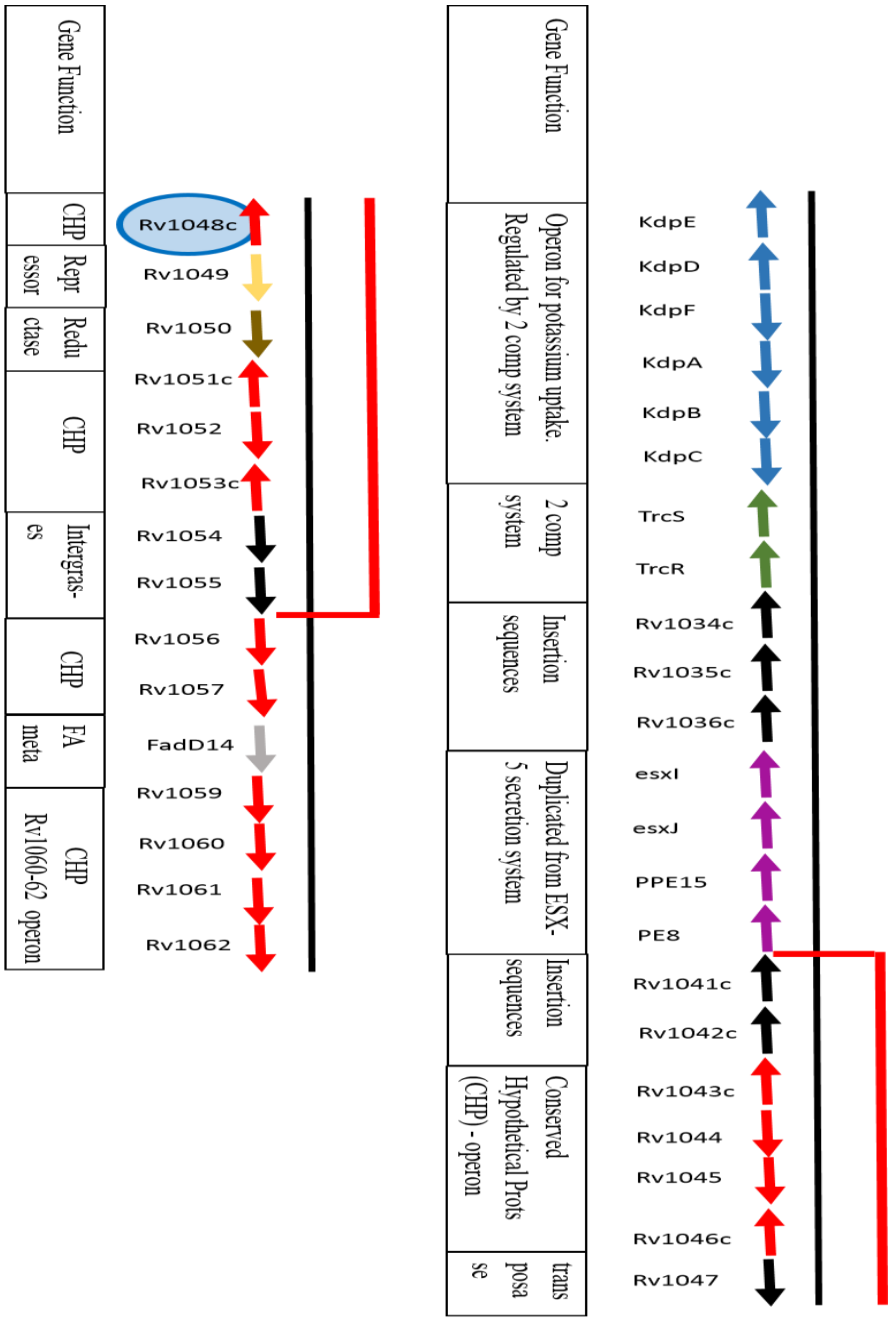

Figure 30: *Mtb* genomic fragment in M24 cosmid
Genes within known operons or in same functional groups are in similar colours. The red line indicates the genomic island (*Rv1041c-Rv1055*) that is unique to the *Mtb* complex and the gene found in a blue circle *(Rv1048c)* is the identified anti-apoptotic gene.

4.3 Materials and Methods

Bacterial strains

Mtb CDC1551 WT strain was a kind gift from Dr. Gilla Kaplan. The CDC1551 transposon insertions were obtained from the TARGET repository at BEI resources. *M. smegmatis* (*Msme*) mc^2155 and *M. tuberculosis* (*Mtb*) H37*Rv* (ATCC 25618) were obtained from Dr. W.R. Jacobs Jr. (AECOM). *M. kansasii* strain Hauduroy (ATCC 12478) was purchased from American Type Culture Collection (ATCC). The Bacterial strains were cultured as mentioned before [230].

Cell Culture and infection

The cell system used was THP-1(ATCC TIB-202), a human myelomonocytic cell line. THP-1 cells were grown in RPMI (ATCC) medium with 10% heat inactivated fetal calf serum (Hyclone). The cells were seeded in 24 well plates at a density of 0.75 million cells/ml/well and differentiated with PMA from Sigma (phorbol 12-myristate 13-acetate) at a 2000x dilution for 18-22 hours. They were then washed twice with PBS and left in 500ul of RPMI media with 5% human serum (Sigma) and infected at an MOI 3 for 4 hours. After which, the chase medium containing RPMI+10% calf serum and 100µg/ml gentamicin (Invitrogen) and harvested 3-4 days post infection.

TUNEL Assay

The TUNEL assay was performed to reveal apoptosis-induced DNA fragmentation in THP-1 cells using the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science #11-684-795-910). The assay was carried out as described by the manufacturer and the percentage of stained cells was analyzed using flow cytometry.

Mtb gene deletion mutant generation by Recombineering

The *Mtb* mutant *ΔESX-5a* was generated using the recombineering approach described in [14]. About 400-600bp flanking sequences on either side of the gene region Rv1037c-Rv1040c were amplified using PCR. The left fragment primers were Rvesx_LLFP (GCAACTCGAGCCGCCGTCAGGTGATCGAATCAG) and Rvesx_LLRP (GGAGAAGCTTGCTGGCTTAAGGCCCGCGCC) and the right fragment primers were Rvesx_RLFP(GGCATCTAGAGCTGCTGTCTCCTTGTCTCGAAGTCG) and Rvesx_RL RP (GTGAGGTACCATCCCCACCGCGATATTCCTAGC). These were then ligated on either sides of a hygromycin resistance gene on the pMSG360 plasmid. This recombinant plasmid was then linearized and electroporated into a specialized strain of *E.coli* (EL350/phAE87). A recombination event between the phasmid phAE87 and the linearized plasmid created a new phasmid containing the hygromycin marker and the flanking sequences. This phasmid DNA was extracted and transformed in *Msme* to generate recombinant phages that was then used for *Mtb* transduction. The hygromycin resistant *Mtb* colonies were picked and screened by southern blotting to confirm the knockout. EL350/phAE87 and pMSG360 were kind gifts from Dr. Michael S. Glickman, Memorial Sloan-Kettering Cancer Centre.

"LoGoF" (Loss of Gain of Function) Screen

The original gain of function cosmid labelled as M24 was subjected to transposon mutagenesis using HyperMu transposon (Epicentre Technology) according to the manufacturer's instructions. Thus a library of M24 cosmids, deficient in the expression

of a single gene, was generated. The clones were screened and sequenced to identify the transposon insertion sites. These were then transformed into WT *Mkan*. Hence, these transformants carry a mutant gain-of function M24 cosmid where one of the genes in the cosmid is inactivated. Therefore they are called Loss of Gain of Function ("LoGoF") mutants.

Complementation of *Rv1048c*

Rv1048c gene was amplified by PCR and the gene product was then cloned into an integrating plasmid, pDB60 [14]. This plasmid was transformed into *Rv1048c::Tn* strain and plated onto 7H10 with 20µg/ml of streptomycin. The complementation was confirmed by PCR.

Statistical analysis

Statistical analysis was performed on at least three independent experiments using GraphPad Prism 5.0 software and One-way ANOVA with Tukey's post-test unless otherwise noted in the figure legends. Shown are representative results of triplicate values with standard deviation. The range of p-values is indicated as follows: * $p=0.01< p < 0.5$; ** $p = 0.001< p< 0.01$ and *** $p = 0.0001< p < 0.001$.

4.4 Results and Discussion

4.4.1 Identification of Anti-apoptotic Gene(s) from the M24 Cosmid Using Loss of Function Genetic Approaches

Two large gene deletions from the M24 region were made in the *Mtb* strain H37*Rv* namely, ΔESX-5a (4 genes from *esxI* to *pe8*) and Δ25/26 (5 genes from *fadD14* to *Rv1062*) (refer to Fig 30). The gene deletions were confirmed by PCR for the deletion. The WT and two mutants were then used to infect THP-1 cells at an MOI 3:1. The cells were harvested 3dpi and levels of apoptosis was measured using the TUNEL assay. If the gene(s) of interest was deleted then that bacterial strain is expected to induce more cell death when compared to the WT infected cells. However, all the infected strains induced similar levels of apoptosis (10-15%) (Fig 31a). Thus, the above 9 genes do not seem to have an anti-apoptotic function.

The next approach was the LoGoF (Loss of- Gain of Function) screen using a panel of *Mkan*+ Tn::M24 clones. The genes with transposon (Tn) insertions in the M24 cosmids were *Rv1030* (*kdpB*), *Rv1038c* (*esxJ*) and *Rv1028A* (*kdpF*). The 3 strains in addition to WT and *Mkan*+M24 were infected into THP-1 cells at an MOI 3:1. The cells were harvested 3dpi and cell death was measured using TUNEL assay. The WT *Mkan* induced as high levels of apoptosis (~40%) as expected while the *Mkan*+M24 induced approximately 15-20% apoptosis in THP-1 cells. If any one of those genes had an anti-apoptotic function then the transposon interruption of that gene in the cosmid would confer a loss of inhibition of apoptosis function to that transformant which would then have a TUNEL positive cell percent close to WT *Mkan*.

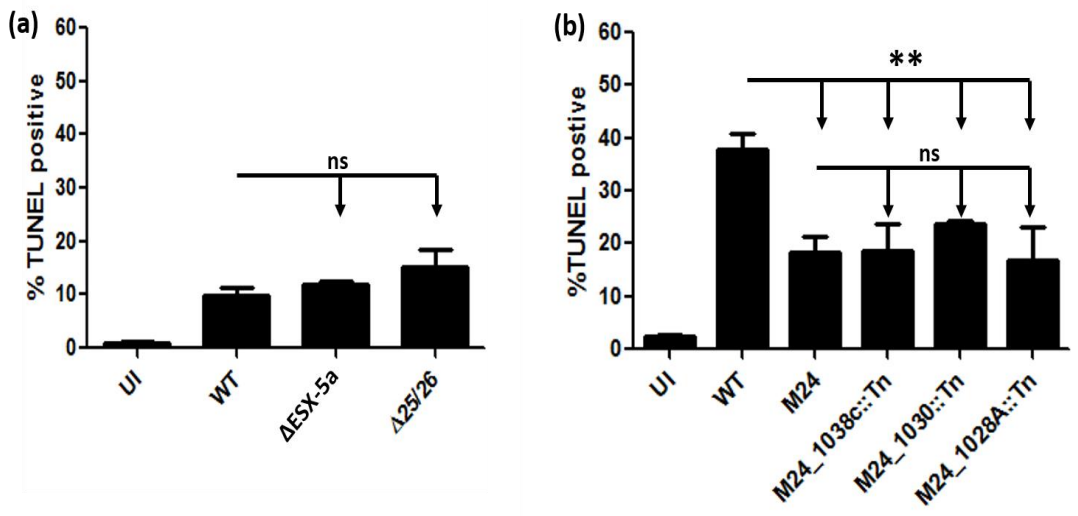

Figure 31: Identification of anti-apoptotic genes in the M24 cosmid. TUNEL assay done on THP-1 cells infected with **(a)** multiple gene knockouts of H37*Rv* or (b) *Mkan* WT and LoGoF strains. In each case the MOI is 3:1 and cells were harvested 3dpi. The figures is one representative experiment out of three. Statistical significance is indicated as * $0.01<p<0.05$, ** $0.001<p<0.01$, *** $p<0.001$, ns non-significant (ANOVA with Tukey post-test).

However, all the clones with mutant M24 cosmids behaved like the WT M24 transformant, showing that genes were not involved in apoptosis inhibition (Fig 31b). Since two genes from the *kdp* operon do not exhibit the function of interest, we ruled out the entire *kdp* operon from consideration bringing the total to 15 genes that did not have an anti-apoptotic function.

The final loss of function approach was to use a host of *Mtb* single gene mutants. A ready library of transposon mutants made in a different clinical *Mtb* strain called CDC1551, was available from a repository called TARGET. THP-1 cells were infected with these CDC1551 transposon mutants at an MOI of 3:1 and the cells were harvested for a TUNEL assay 3dpi. The transposon insertions were in the genes *Rv1045, Rv1048c* and *Rv1058*. Out of the three Tn mutants, Tn::1048-542 showed significantly higher rate of apoptosis (~45-50%) when compared to WT CDC1551 infected cells (Fig 32a). To confirm that the pro-apoptotic phenotype of the mutant is due to abrogation of *Rv1048c* and not because of polar effects, a complement was generated. THP-1 cells were again infected with the Tn mutant, complement and WT *Mtb* as before. The anti-apoptotic phenotype was thus confirmed to be due to *Rv1048c* (Fig 32b).

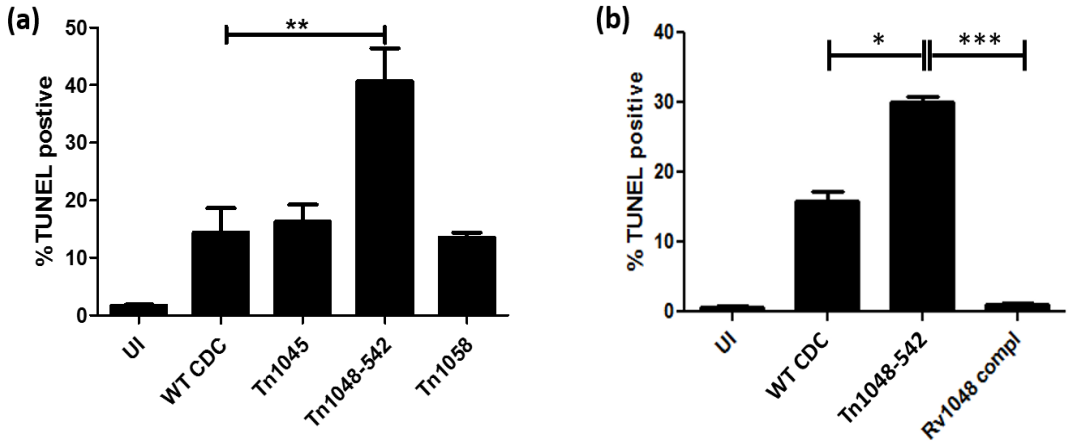

Figure 32: *Rv1048c* is the anti-apoptotic gene from M24 cosmid. TUNEL assay was performed on THP1 cells infected with WT and **(a)** Tn mutants in CDC1551 background **(b)** Tn1048-542 mutant and complement. In each case the MOI is 3:1 and cells were harvested 3dpi. The figures is one representative experiment out of three. Statistical significance is indicated as * $0.01<p<0.05$, ** $0.001<p<0.01$, *** $p<0.001$, ns non-significant (ANOVA with Tukey post-test).

4.3.2 Discussion

Induction of apoptosis benefits some pathogens such as *Coxiella, Chlamydia* and *Yersinia* as it helps to mitigate inflammatory responses while avoiding detection [23,118]. On the flip side, bacteria such as virulent *Mtb* prevent apoptosis induction, instead prefer pushing the cell death modality to necrosis as it helps with dissemination and infection spread [131]. *Mtb* is an intracellular pathogen that takes residence within the macrophages setting up its own replicative niche. To do so, it is known to inhibit multiple signaling events occurring during apoptosis such as secretion of soluble TNFR2 to neutralize TNF or manipulating the eicosanoid biosynthetic pathways. This inhibitory effect was seen only with virulent *Mtb* and not with avirulent strains such as *Msme, Mkan* and *Mtb* H37*Ra* [125].

A gain of function genetic screen was conducted to identify the gene(s) responsible for apoptosis inhibition by transforming cosmids containing *Mtb* DNA fragments into *Msme* and *Mkan* [230]. As a result, the search was narrowed down to three non-overlapping DNA regions. The first anti-apoptotic gene to be identified from these three gene regions was *nuoG*. The M24 cosmid consists of 38 genes, many of which form operons and have a wide range of functions. Out of these, the genomic island region (*Rv1041c to Rv1055*) was of great interest because it is found exclusively in the *Mtb* complex.

Different loss of function genetic approaches were used to identify the gene responsible for apoptosis inhibition. Firstly, a host of single gene transposon mutants of *Mtb* in the CDC1551 background were obtained. Secondly, the M24 cosmid was subjected to transposon mutagenesis and a library of mutant cosmids were generated.

These cosmids were transformed in to *Mkan* to perform a unique screen named as LoGoF. In this screen, the transformant has a mutant M24 cosmid with single gene interruption. Therefore, if the gene of interest was mutated, the the *Mkan* transformant would lose the ability to inhibit apoptosis and this clone would induce macrophage apoptosis at levels similar to WT *Mkan*. Both these screens target single genes which would not yield results in case functional redundancy between genes or the requirement of multiple gene to orchestrate the inhibitory effect. Therefore the third approach was used to generate multiple gene deletions mutants of *Mtb* H37*Rv*.

While infecting THP-1 cells with the CDC1551 mutants at an MOI 3:1, it was seen that the loss of *Rv1048c* function caused significantly more apoptotic cell death than the WT infected cells as measured by TUNEL assay. To negate the possibility of polar effects in the mutant, a complement was generated. Upon THP-1 infection, the complement induced much lesser levels of apoptosis than the WT while the mutant went the other way showing that *Rv1048c* is responsible for the anti-apoptotic function (Fig 32a+ 32b).

Following the identification of *Rv1048c*, it is important to characterize the function of this protein and understand how it affects host cell apoptosis inhibition. *Rv1048c* lies within the genomic island mentioned previously. It is designated as hypothetical protein and is highly conserved among members of the *Mtb* complex with homology greater than 85%. It is not found in any of the other mycobacterial species and does not share homology with any protein of known function. The protein has no known conserved domains offering very little clues as to how it could affect host cell pathways. Therefore, elucidating the subcellular location of this protein would help indicate if it

directly interacts with host cell effectors (upon export to bacterial cell surface or into the phagosome) or has indirect influence on the host signaling by regulating other bacterial effectors (cytoplasmic or membrane bound). Based on the analysis using programs such as signalP [177], the protein did not have any known signal sequences. The general secretion signal motif of T7SSs is YxxxD/E [55], which is absent in the Rv1048c protein. These factors indicate that Rv1048c is most likely to be retained intracellularly and indirectly affects host cell apoptosis. *Rv1048c* is found to be up-regulated under certain stress conditions like high temperature [216] and in the presence of detergents [145,146]. Additionally, an analysis using the PHYRE2 program shows that Rv1048c contains a region that shares homology with a "winged helix" DNA binding domain and multiple antibiotic resistance regulators (MarR) family of transcriptional regulators [126]. The MarR family reportedly regulate the expression of proteins conferring resistance to antibiotics, oxidative stress agents and some pathogenic factors [10]. Indeed, the bio-informatic data needs experimental validation and this can be done by assessing if Rv1048c can actually bind to DNA using assays such as ChIP (Chromatin Immunoprecipitation) assays followed by RNA sequencing. This would help in identifying the group of proteins whose expression *Rv1048c* regulates, a subset of which may directly influence the inhibition of host cell apoptosis.

An interesting aspect that warrants further investigation is whether the function of *Rv1048c* as an anti-apoptotic effector is independent of previously identified genes such as *nuoG, sodA* etc. This question can be addressed by generating double mutants of *Rv1048c* with other genes. If this mutant induces more apoptosis in macrophages

when compared to the single mutants then the two gene products feed into independent pathways. This premise does make sense considering how the bacteria seem to modulate multiple host effectors (Fig 33).

With the reemergence of Tb and the rise of drug resistant strains in the recent times, it is imperative that we update our arsenal of therapeutics against the disease. Understanding the mechanism of host cell apoptosis inhibition by *Mtb* will help identify new drug targets. Pro-apoptotic mutants are promising vaccine candidates as they tend to stimulate a faster and a more robust T-cell response. Identifying anti-apoptotic genes that function independently could prove to be very useful for vaccine design, as multiple gene deletion mutants inducing higher rates of apoptosis could potentially stimulate a more robust adaptive immune response.

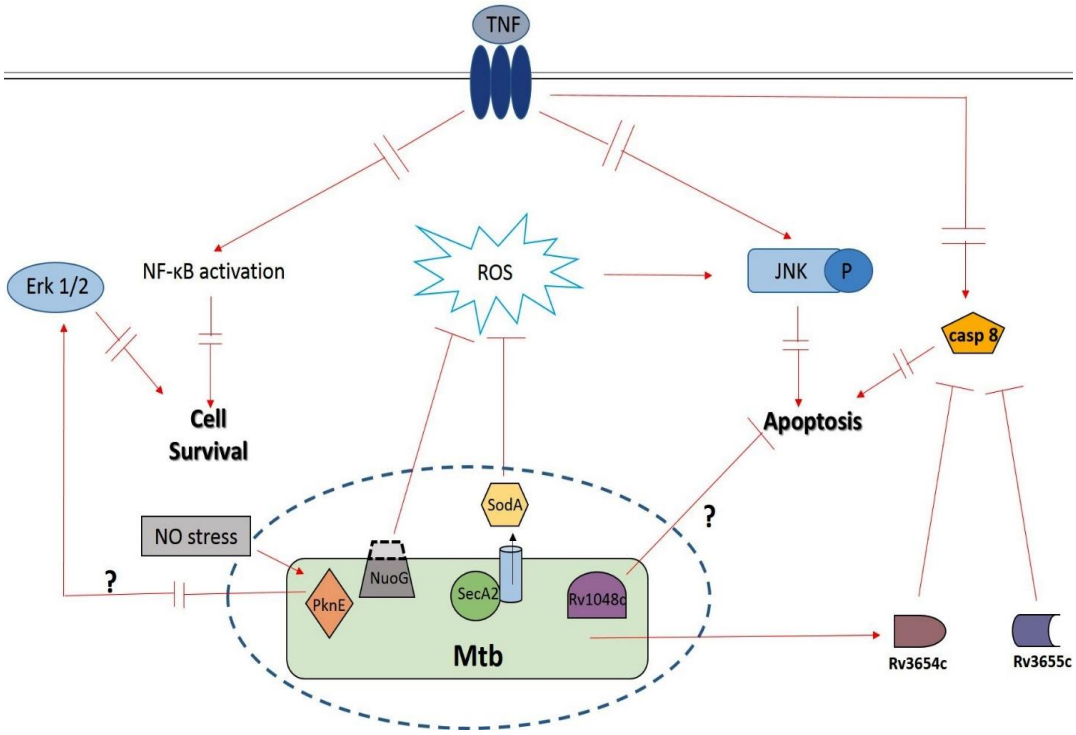

Figure 33: Mechanisms of apoptosis inhibition by known *Mtb* anti-apoptotic proteins

The figure describes the effects of various *Mtb* anti-apoptotic genes on host cell signaling resulting in the inhibition of apoptosis.

CHAPTER 5: CONCLUSIONS AND FUTURE IMPLICATIONS

In this dissertation we have described three major early evasion tactics employed by virulent *Mtb* to survive an onslaught by the host immune system. We have shown that *Mtb* inhibits *Msme*/dsDNA induced activation of the AIM2 inflammasome in an ESX-1 dependent manner (chapter 2). We also characterized the role of the ESX-5a region in virulence, protein secretion and its effect on host cell processes (chapter 3). In chapter 4, we describe the use of various loss of function genetic approaches to identify a novel anti-apoptotic gene (*Rv1048c*) from the M24 cosmid region.

Inflammasome activation is a major innate immune response mounted by the host and can have several consequences on the survival of invading pathogens. It can lead to cell death by pyroptosis and/or onset of inflammation by the secretion of cytokines such as IL-1β and IL-18 [185]. Therefore, many pathogens have found ways to subvert the activation of inflammasomes (reviewed in section 1.3.4). In the case of infection with virulent *Mtb*, the only NLR responsible for secretion of IL-1β in infected macrophages and DCs is NLRP3 [1,128, 153]. However, our results show that avirulent mycobacterial species are also able to activate AIM2 (Fig 11a). This cytosolic receptor is known to indiscriminately bind to double-stranded DNA of microbial, viral, mammalian or synthetic origin [166, 183]. Pathogens such as *F. tularensis* and *L. monocytogenes* are known to activate the AIM2 inflammasome by accessing the host cytosol after infection. In the process, a few organisms die, resulting in the release of bacterial dsDNA into the cytosol [77, 119, 196]. *Mtb* on the other hand, are known to stay within the phagosome for several days after infection [108, 205] but still exports eDNA, quite early on, into the cytosol via its ESX-1 secretion system. The DNA sensor

IFI16/IFI204 detects the cytosolic eDNA which in turns leads to IFN-β production [147, 212]. However, we demonstrated that in spite of the presence of eDNA, the AIM2 inflammasome was not activated. Instead, *Mtb* actively inhibited the induction of AIM2 inflammasome by *Msme* infection or by transfection of artificial poly dA:dT in an ESX-1 dependent manner, implying that the secretion system was also involved in exporting the inhibitory factor (Fig 12).

As in earlier reports with *Francisella* we found that IFN-β is required for the activation of AIM2 during *Msme* infection because there was a reduction in AIM2 dependent IL-1β secretion in the presence of IFN-β neutralizing antibodies (Fig 11c). Based on these results, we hypothesized that the inhibition of IFN-β production is beneficial to the bacteria and this contradicts many earlier studies showing that the induction of a type I IFN signature actually leads to increased *Mtb* virulence [24,143,144, 147, 152, 212]. This was believed to be the case because IFN-β suppresses the activation of NLRP3 inflammasome and limits IL-1β secretion. The mechanism involves the induction of the anti-inflammatory cytokine, IL-10 by IFN-β signaling which in turn inhibits pro IL-1β production [152, 170]. However, none of these studies compared production of IFN-β in *Mtb* with other avirulent mycobacterial species, and indeed *Mtb* infected cells produced 20 fold less IFN-β when compared to the avirulent strains (Fig 11b). However, the inhibition is not complete as *Mtb* infection still induces some IFN-β production which in turn may be sufficient to limit NLRP3 dependent maturation of pro IL-1β and IL-18. *Mtb* was also able to reduce *Msme*- induced IFN-β secretion in an ESX-1 dependent manner. These results give rise to the possibility that *Mtb* inhibits AIM2 inflammasome activation indirectly by suppressing IFN-β

production and/or its downstream signaling. It has been reported that IFN-β increases *aim2* transcription [120, 227]. However, we found no increase in levels of AIM2 expression mirroring the results of another study with *Francisella* [77]. Instead, the point of inhibition lay at the level of caspase-1 activation by the inflammasome (Fig 15). We also showed that exogenous addition of IFN-β did not completely reverse the inhibition of AIM2 inflammasome by *Mtb* (Fig 15c). Therefore, it seems more likely that there are multiple inhibitory mechanisms which interfere with the signaling events of both IFN-β and AIM2.

The exact mechanism of inhibition still remains to be elucidated. A recent study has demonstrated that the mouse DNA sensor p202 has two (Hemopoeitic expression, Interferon-inducibility, Nuclear localization) HIN domains, of which only the HIN1 domain binds to dsDNA while the HIN2 can dimerize with the AIM2's HIN domain thereby preventing it from binding with dsDNA and activating the inflammasome [241]. Similarly, increased expression of another DNA sensor IFI16 (murine homolog-p204) was able to inhibit AIM2 inflammasome mediated caspase-1 activation [229]. Another group showed that an oligonucleotide containing the TTAGGG motif competed with cytosolic dsDNA to bind AIM2 and prevented further activation of the inflammasome [122] giving rise to possibility that *Mtb* eDNA may also contain certain inhibitory motifs. It would be interesting to see if *Mtb* exploits these mechanisms to its own advantage. It is also possible that one or several of the IFN-β regulated genes affect the activity of the AIM2 inflammasome. Since the inhibitory effect is ESX-1 dependent, it will be conducive to identify IFN-β regulated genes that are differentially expressed upon infection with WT or *ΔesxA Mtb* by RNA sequencing technology or

qRT-PCR and see if any these targets are involved in AIM2 inflammasome dependent IL-1β secretion. Another important avenue of investigation would be to identify which ESX-1 dependent *Mtb* effector mediates AIM2 inflammasome inhibition. The gain of function screen utilized previously to identify anti-apoptotic genes could also be used to pinpoint gene products that are involved in AIM2 inflammasome inhibition. Once narrowed down, loss of function genetic approaches can be used to identify the exact gene effector/s that inhibit the activation of AIM2.

In chapter 3, we described the functions of a duplicated ESX region in protein secretion and its effect on virulence. This four gene region termed as ESX-5a was duplicated from the *esx/pe/ppe* gene region of ESX-5 secretion system [96]. This region is involved in the secretion of ALD and a few other PE/PPE proteins via the parent ESX-5 system (Fig 18 to 21). Additionally, the EsxI and EsxJ proteins of the ESX-5a region are also secreted. On looking closely at its impact on the host immune system we found that deleting the Esx-5a region decreased the amount of secreted IL-1β by about 50% (Fig 23). A similar phenotype was seen in ESX-1 and ESX-5 mutants where the amounts of secreted IL-1β was reduced to background levels [2, 128]. Survival studies with the zebrafish model of infection show that the *M.marinum* ESX-5a mutant is attenuated (Fig 26) as it has been shown earlier with the *Mtb* ESX-5 mutant in mice [29, 197]. This result currently being confirmed in the mouse model with the *Mtb* ΔESX-5a. The above two results taken together may seem counterintuitive because it would be expected that both ESX-5 and ESX-5a mutants which limit IL-1β secretion ought to be more virulent, but in reality are highly attenuated. However, the secreted mycobacterial proteins execute various functions once exported into the host milieu to

help with bacterial survival and thus cannot avoid being detected by the host immune system, which leads to the subsequent induction of inflammation. Therefore, the bacteria secretes additional effectors to inhibit the inflammatory pathways (Fig 34) much like the YopK protein of *Yersinia spp* which is possibly secreted with the purpose of preventing the recognition of other Type III secreted factors by the NLRs and impairing inflammasome activation [35].

Identifying the role of ESX-5a in secretion of proteins via the ESX-5 system has led us to develop a model in which the other duplicated ESX regions are responsible for the export of various ESX-5 protein subsets and thus contribute to overall functioning of this secretion system (Fig 27). For example, ESX-5a deletion does not increase host cell death induction while the parent ESX-5 mutant has been shown to induce a late-stage caspase-independent death pathway [2]. It is possible that one of the other duplicated regions secrete effectors that mediate this phenotype. This hypothesis still requires experimental validation and if true, then the strain with deletions of all the duplicated regions would be more attenuated when compared to strains missing the individual regions.

Studies in the future should include assays to decipher the function of ALD upon release into the host milieu and its contribution to virulence. *Mtb* ALD has a dual function as both an alanine dehydrogenase and a glycine dehydrogenase [97]. As a result there have been wide ranging suggestions about its actual function. ALD is shown to be important for peptidoglycan synthesis [43, 111], utilizing alanine during nutrient starvation conditions [25] and for anerobic growth [75].

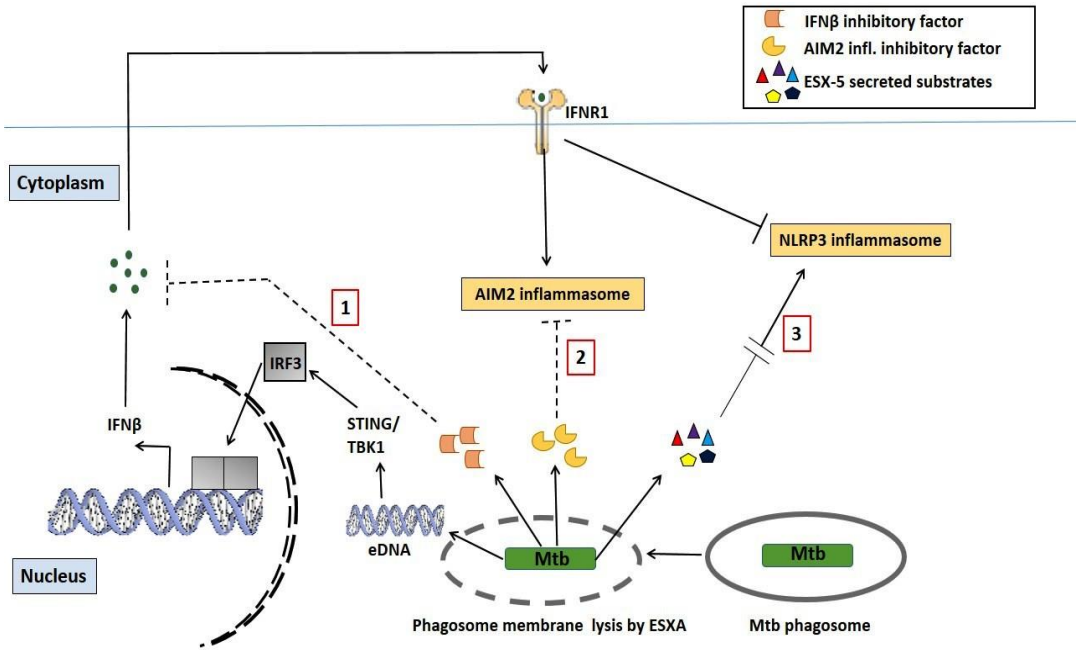

Figure 34: Overall effect of *Mtb* on inflammasome pathways

(1) Inhibition of IFN-β (2) Inhibition of AIM2 inflammasome activation (3) Activation of NLRP3 inflammasome by ESX-5 secreted proteins

ald mutants of *B.subtilis* and *M.xanthus* were defective for sporulation [207, 234]. All of these point to a role for ALD during latent infection thereby making it an attractive drug target. Raynaud et al, hypothesized that secreted ALD could convert alanine (abundantly present in the host cell) to pyruvate and generate ammonium as a by-product which can then block phagosomal acidification [186]. This hypothesis can be tested by generating an ALD *Mtb* mutant and analyzing infected cells to see if the mutant is now unable to block acidification. Additionally, the ESX-5a mutant should also be tested to see if it prevents acidification of the phagosome, implying that secretion of ALD is also important for this phenotype. The ESX-5a genes can be utilized in the field of vaccine design. Members of the ESAT6 family are found to be highly immunogenic [119] and EsxJ was recently identified as an important CD8 T-cell antigen [132]. Kaufmann and his colleagues have proposed an improved BCG vaccine candidate: BCG ΔureC::*hly*, which is urease deficient (urease prevents phagosomal acidification) and expresses listeriolysin O (LLO), a porin from *L. monocytogenes* that can perforate the phagosomal membrane at an acidic pH, allowing access to the host cytosol [100, 101]. This recombinant vaccine strain showed increased stimulation of CD4 T-cell response when compared to conventional BCG but the CD8 T-cell population counts however, did not improve very much [63]. Potentially a BCG ΔureC::*hly* strain overexpressing *esxI* and *esxJ* can increase stimulation of CD8 T-cell populations and we could test its efficacy as a vaccine.

Induction of host cell apoptosis is an important innate immune defense mechanism that provides the basis for other mechanisms such as- efferocytosis and cross-priming to stimulate T-cell mediated killing of pathogens [67, 149, 235]. Using

a gain of genetic screen, three *Mtb* genomic regions were identified as containing anti-apoptotic genes [230]. Secondary loss of function genetic approaches were applied to the M24 region. We identified a novel anti-apoptotic gene: *Rv1048c*, adding to a growing list of anti-apoptotic genes (Fig 33). *Rv1048c* is an interesting gene with an unknown function and is contained on a genomic island, which is present only among the members of the virulent *Mtb* complex [18]. An investigation into the mechanism by which Rv1048c inhibits host cell apoptosis is required. It will be important to elucidate if Rv1048c directly interacts with host cell effectors to modulate the apoptotic response. If the effect happens to be indirect, then there could be other genes (potentially from the same genomic island) whose functions it regulates to orchestrate an anti-apoptotic phenotype. Therefore, it would be worthwhile to continue testing the remaining M24 genes for their ability to suppress apoptosis in macrophages.

The results provided in this thesis have described several novel mechanisms employed by *Mtb* in an effort to modulate host innate immune responses and ensure its own survival. Despite the need for further investigation, these studies show promise of having practical implications in the development of preventative and curative remedies against Tb.

BIBLIOGRAPHY

1. Abdalla H, Srinivasan L, Shah S, Mayer-Barber KD, Sher A, et al. (2012) *Mycobacterium tuberculosis* infection of dendritic cells leads to partially caspase-1/11-independent IL-1ß and IL-18 secretion but not to pyroptosis. PLoS ONE.

2. Abdallah AM, Bestebroer J, Savage ND, de Punder K, van Zon M, et al. (2011) Mycobacterial secretion systems ESX-1 and ESX-5 play distinct roles in host cell death and inflammasome activation. J Immunol 187: 4744-4753.

3. Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, et al. (2007) Type VII secretion--mycobacteria show the way. Nat Rev Microbiol 5: 883-891.

4. Abdallah AM, Savage ND, van Zon M, Wilson L, Vandenbroucke-Grauls CM, et al. (2008) The ESX-5 secretion system of *Mycobacterium marinum* modulates the macrophage response. J Immunol 181: 7166-7175.

5. Abdallah AM, Verboom T, Hannes F, Safi M, Strong M, et al. (2006) A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. Mol Microbiol 62: 667-679.

6. Abdallah AM, Verboom T, Weerdenburg EM, Gey van Pittius NC, Mahasha PW, et al. (2009) PPE and PE_PGRS proteins of *Mycobacterium marinum* are transported via the type VII secretion system ESX-5. Mol Microbiol 73: 329-340.

7. Abdelaziz DH, Gavrilin MA, Akhter A, Caution K, Kotrange S, et al. (2011) Asc-dependent and independent mechanisms contribute to restriction of *Legionella pneumophila* infection in murine macrophages. Front Microbiol 2: 18.

8. Adams JM, Cory S (2007) Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr Opin Immunol 19: 488-496.

9. Aderem A, Ulevitch RJ (2000) Toll-like receptors in the induction of the innate immune response. Nature 406: 782-787.

10. Aravind L, Anantharaman V, Balaji S, Babu MM, Iyer LM (2005) The many faces of the helix-turn-helix domain: transcription regulation and beyond. FEMS Microbiol Rev 29: 231-262.

11. Bafica A, Scanga CA, Feng CG, Leifer C, Cheever A, et al. (2005) TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*. J Exp Med 202: 1715-1724.

12. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392: 245-252.

13. Bansal K, Sinha AY, Ghorpade DS, Togarsimalemath SK, Patil SA, et al. (2010) Src homology 3-interacting domain of Rv1917c of *Mycobacterium tuberculosis* induces selective maturation of human dendritic cells by regulating PI3K-MAPK-NF-kappaB signaling and drives Th2 immune responses. J Biol Chem 285: 36511-36522.

14. Barkan D, Hedhli D, Yan HG, Huygen K, Glickman MS (2012) *Mycobacterium tuberculosis* lacking all mycolic acid cyclopropanation is viable but highly attenuated and hyperinflammatory in mice. Infect Immun 80: 1958-1968.

15. R. B (1994) *Tuberculosis : pathogenesis, protection, and control*.: ASM Press.

16. Bartlett JG (2007) Tuberculosis and HIV infection: partners in human tragedy. J Infect Dis 196 Suppl 1: S124-125.

17. Bean AG, Roach DR, Briscoe H, France MP, Korner H, 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: 3504-3511.

18. Becq J, Gutierrez MC, Rosas-Magallanes V, Rauzier J, Gicquel B, et al. (2007) Contribution of horizontally acquired genomic islands to the evolution of the tubercle bacilli. Mol Biol Evol 24: 1861-1871.

19. Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87: 245-313.

20. Behar SM, Divangahi M, Remold HG (2010) Evasion of innate immunity by *Mycobacterium tuberculosis*: is death an exit strategy? Nat Rev Microbiol 8: 668-674.

21. Behar SM, Martin CJ, Booty MG, Nishimura T, Zhao X, et al. (2011) Apoptosis is an innate defense function of macrophages against *Mycobacterium tuberculosis*. Mucosal Immunol 4: 279-287.

22. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, et al. (1999)

Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284: 1520-1523.

23. Bergsbaken T, Cookson BT (2007) Macrophage activation redirects *Yersinia*-infected host cell death from apoptosis to caspase-1-dependent pyroptosis. PLoS Pathog 3: e161.

24. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, et al. (2010) An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 466: 973-977.

25. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K (2002) Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. Mol Microbiol 43: 717-731.

26. Bevan MJ (2006) Cross-priming. Nat Immunol 7: 363-365.

27. Blomgran R, Ernst JD (2011) Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during *Mycobacterium tuberculosis* infection. J Immunol 186: 7110-7119.

28. Bohsali A, Abdalla H, Velmurugan K, Briken V (2010) The non-pathogenic mycobacteria *M. smegmatis* and *M. fortuitum* induce rapid host cell apoptosis via a caspase-3 and TNF dependent pathway. BMC Microbiol 10: 237.

29. Bottai D, Di Luca M, Majlessi L, Frigui W, Simeone R, et al. (2012) Disruption of the ESX-5 system of *Mycobacterium tuberculosis* causes loss of PPE protein secretion, reduction of cell wall integrity and strong attenuation. Mol Microbiol 83: 1195-1209.

30. Boyden ED, Dietrich WF (2006) Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. Nat Genet 38: 240-244.

31. Brandt L, Orme I (2002) Prospects for new vaccines against tuberculosis. Biotechniques 33: 1098, 1100, 1102.

32. Brennan MJ, Delogu G (2002) The PE multigene family: a 'molecular mantra' for mycobacteria. Trends Microbiol 10: 246-249.

33. Briken V, Ahlbrand SE, Shah S (2013) *Mycobacterium tuberculosis* and the host cell inflammasome: a complex relationship. Front Cell Infect Microbiol 3: 62.

34. Briken V, Miller JL (2008) Living on the edge: inhibition of host cell apoptosis by

Mycobacterium tuberculosis. Future Microbiol 3: 415-422.

35. Brodsky IE, Palm NW, Sadanand S, Ryndak MB, Sutterwala FS, et al. (2010) A *Yersinia* effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. Cell Host Microbe 7: 376-387.

36. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, et al. (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci U S A 99: 3684-3689.

37. Bukka A, Price CT, Kernodle DS, Graham JE (2011) *Mycobacterium tuberculosis* RNA Expression Patterns in Sputum Bacteria Indicate Secreted Esx Factors Contributing to Growth are Highly Expressed in Active Disease. Front Microbiol 2: 266.

38. Bulut Y, Faure E, Thomas L, Equils O, Arditi M (2001) Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and *Borrelia burgdorferi* outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. J Immunol 167: 987-994.

39. Cardona PJ (2011) A spotlight on liquefaction: evidence from clinical settings and experimental models in tuberculosis. Clin Dev Immunol 2011: 868246.

40. Carlsson F, Joshi SA, Rangell L, Brown EJ (2009) Polar localization of virulence-related Esx-1 secretion in mycobacteria. PLoS Pathog 5: e1000285.

41. Carlsson F, Kim J, Dumitru C, Barck KH, Carano RA, et al. (2010) Host-detrimental role of Esx-1-mediated inflammasome activation in mycobacterial infection. PLoS Pathog 6: e1000895.

42. Control CfD (2006) Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drug—Worldwide, 2000–2004. *MMWR Weekly*.

43. Chen JM, Alexander DC, Behr MA, Liu J (2003) *Mycobacterium bovis* BCG vaccines exhibit defects in alanine and serine catabolism. Infect Immun 71: 708-716.

44. Chen M, Divangahi M, Gan H, Shin DS, Hong S, et al. (2008) Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. J Exp Med 205: 2791-2801.

45. Cho YS, Challa S, Moquin D, Genga R, Ray TD, et al. (2009) Phosphorylation-

driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137: 1112-1123.

46. Ciaramella A, Cavone A, Santucci MB, Garg SK, Sanarico N, et al. (2004) Induction of apoptosis and release of interleukin-1 beta by cell wall-associated 19-kDa lipoprotein during the course of mycobacterial infection. J Infect Dis 190: 1167-1176.

47. Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, et al. (1994) Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 271: 698-702.

48. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, et al. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393: 537-544.

49. Comas I, Coscolla M, Luo T, Borrell S, Holt KE, et al. (2013) Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. Nat Genet 45: 1176-1182.

50. Converse SE, Cox JS (2005) A protein secretion pathway critical for *Mycobacterium tuberculosis* virulence is conserved and functional in *Mycobacterium smegmatis*. J Bacteriol 187: 1238-1245.

51. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, et al. (1993) Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 178: 2243-2247.

52. Cooper AM, Mayer-Barber KD, Sher A (2011) Role of innate cytokines in mycobacterial infection. Mucosal Immunol 4: 252-260.

53. Cunha LD, Zamboni DS (2013) Subversion of inflammasome activation and pyroptosis by pathogenic bacteria. Front Cell Infect Microbiol 3: 76.

54. Daleke MH, Cascioferro A, de Punder K, Ummels R, Abdallah AM, et al. (2011) Conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) protein domains target LipY lipases of pathogenic mycobacteria to the cell surface via the ESX-5 pathway. J Biol Chem 286: 19024-19034.

55. Daleke MH, Ummels R, Bawono P, Heringa J, Vandenbroucke-Grauls CM, et al. (2012) General secretion signal for the mycobacterial type VII secretion pathway. Proc Natl Acad Sci U S A 109: 11342-11347.

56. Danelishvili L, Yamazaki Y, Selker J, Bermudez LE (2010) Secreted *Mycobacterium tuberculosis* Rv3654c and Rv3655c proteins participate in the suppression of macrophage apoptosis. PLoS One 5: e10474.

57. Daniel TM (2006) The history of tuberculosis. Respir Med 100: 1862-1870.

58. Darwin KH, Ehrt S, Gutierrez-Ramos JC, Weich N, Nathan CF (2003) The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. Science 302: 1963-1966.

59. Darwin KH, Lin G, Chen Z, Li H, Nathan CF (2005) Characterization of a *Mycobacterium tuberculosis* proteasomal ATPase homologue. Mol Microbiol 55: 561-571.

60. Davis AS, Vergne I, Master SS, Kyei GB, Chua J, et al. (2007) Mechanism of inducible nitric oxide synthase exclusion from mycobacterial phagosomes. PLoS Pathog 3: e186.

61. Degterev A, Yuan J (2008) Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol 9: 378-390.

62. Derrick SC, Morris SL (2007) The ESAT6 protein of *Mycobacterium tuberculosis* induces apoptosis of macrophages by activating caspase expression. Cell Microbiol 9: 1547-1555.

63. Desel C, Dorhoi A, Bandermann S, Grode L, Eisele B, et al. (2011) Recombinant BCG ΔureC hly+ induces superior protection over parental BCG by stimulating a balanced combination of type 1 and type 17 cytokine responses. J Infect Dis 204: 1573-1584.

64. Dietrich J, Doherty TM (2009) Interaction *of Mycobacterium tuberculosis* with the host: consequences for vaccine development. APMIS 117: 440-457.

65. Dinarello CA (2009) Interleukin-1beta and the autoinflammatory diseases. N Engl J Med 360: 2467-2470.

66. Divangahi M, Chen M, Gan H, Desjardins D, Hickman TT, et al. (2009) *Mycobacterium tuberculosis* evades macrophage defenses by inhibiting plasma membrane repair. Nat Immunol 10: 899-906.

67. Divangahi M, Desjardins D, Nunes-Alves C, Remold HG, Behar SM (2010) Eicosanoid pathways regulate adaptive immunity to *Mycobacterium tuberculosis.* Nat Immunol 11: 751-758.

68. Dolin ebGLM, John E. Bennett, Raphael (2010) Mandell, Douglas, and Bennett's principles and practice of infectious diseases (7th ed.). Philadelphia, PA. Churchill Livingstone/Elsevier p.

69. Dorhoi A, Nouailles G, Jorg S, Hagens K, Heinemann E, et al. (2011) Activation of the NLRP3 inflammasome by *Mycobacterium tuberculosis* is uncoupled from susceptibility to active tuberculosis. Eur J Immunol.

70. Dorhoi A, Nouailles G, Jorg S, Hagens K, Heinemann E, et al. (2012) Activation of the NLRP3 inflammasome by *Mycobacterium tuberculosis* is uncoupled from susceptibility to active tuberculosis. Eur J Immunol 42: 374-384.

71. Drennan MB, Nicolle D, Quesniaux VJ, Jacobs M, Allie N, et al. (2004) Toll-like receptor 2-deficient mice succumb to *Mycobacterium tuberculosis* infection. Am J Pathol 164: 49-57.

72. Ehlers S, Schaible UE (2012) The granuloma in tuberculosis: dynamics of a host-pathogen collusion. Front Immunol 3: 411.

73. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35: 495-516.

74. Ernst JD (1998) Macrophage receptors for *Mycobacterium tuberculosis*. Infect Immun 66: 1277-1281.

75. Feng Z, Cáceres NE, Sarath G, Barletta RG (2002) *Mycobacterium smegmatis* L-alanine dehydrogenase (Ald) is required for proficient utilization of alanine as a sole nitrogen source and sustained anaerobic growth. J Bacteriol 184: 5001-5010.

76. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 458: 509-513.

77. Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, et al. (2010) The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*. Nat Immunol 11: 385-393.

78. Ferrari G, Langen H, Naito M, Pieters J (1999) A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 97: 435-447.

79. Fine PE CI, Milstein JB, Clements CJ (1999) Issues relating to the use of BCG in immunization programs. Geneva WHO.

80. Fine PE (1995) Variation in protection by BCG: implications of and for heterologous immunity. Lancet 346: 1339-1345.

81. Flint JL, Kowalski JC, Karnati PK, Derbyshire KM (2004) The RD1 virulence locus of *Mycobacterium tuberculosis* regulates DNA transfer in *Mycobacterium smegmatis*. Proc Natl Acad Sci U S A 101: 12598-12603.

82. Flynn JL, Chan J (2001) Immunology of tuberculosis. Annu Rev Immunol 19: 93-129.

83. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, et al. (1993) An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J Exp Med 178: 2249-2254.

84. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, et al. (1995) Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity 2: 561-572.

85. Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozören N, et al. (2006) Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. Nat Immunol 7: 576-582.

86. Franchi L, Warner N, Viani K, Nuñez G (2009) Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev 227: 106-128.

87. Fratazzi C, Arbeit RD, Carini C, Balcewicz-Sablinska MK, Keane J, et al. (1999) Macrophage apoptosis in mycobacterial infections. J Leukoc Biol 66: 763-764.

88. Fratazzi C, Arbeit RD, Carini C, Remold HG (1997) Programmed cell death of *Mycobacterium avium* serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. J Immunol 158: 4320-4327.

89. Fremond CM, Togbe D, Doz E, Rose S, Vasseur V, et al. (2007) IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to *Mycobacterium tuberculosis* infection. J Immunol 179: 1178-1189.

90. Galle M, Schotte P, Haegman M, Wullaert A, Yang HJ, et al. (2008) The *Pseudomonas aeruginosa* Type III secretion system plays a dual role in the regulation of caspase-1 mediated IL-1beta maturation. J Cell Mol Med 12: 1767-1776.

91. Gan H, Lee J, Ren F, Chen M, Kornfeld H, et al. (2008) *Mycobacterium*

tuberculosis blocks crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages to maintain virulence. Nat Immunol 9: 1189-1197.

92. Gao LY, Guo S, McLaughlin B, Morisaki H, Engel JN, et al. (2004) A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. Mol Microbiol 53: 1677-1693.

93. Gao LY, Laval F, Lawson EH, Groger RK, Woodruff A, et al. (2003) Requirement for *kasB* in *Mycobacterium* mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. Mol Microbiol 49: 1547-1563.

94. Geijtenbeek TB, Van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, et al. (2003) Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med 197: 7-17.

95. Gey Van Pittius NC, Gamieldien J, Hide W, Brown GD, Siezen RJ, et al. (2001) The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. Genome Biol 2: RESEARCH0044.

96. Gey van Pittius NC, Sampson SL, Lee H, Kim Y, van Helden PD, et al. (2006) Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (esx) gene cluster regions. BMC Evol Biol 6: 95.

97. Giffin MM, Modesti L, Raab RW, Wayne LG, Sohaskey CD (2012) *ald* of *Mycobacterium tuberculosis* encodes both the alanine dehydrogenase and the putative glycine dehydrogenase. J Bacteriol 194: 1045-1054.

98. González-Navajas JM, Lee J, David M, Raz E (2012) Immunomodulatory functions of type I interferons. Nat Rev Immunol 12: 125-135.

99. Greten FR, Arkan MC, Bollrath J, Hsu LC, Goode J, et al. (2007) NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. Cell 130: 918-931.

100. Grode L, Ganoza CA, Brohm C, Weiner J, Eisele B, et al. (2013) Safety and immunogenicity of the recombinant BCG vaccine VPM1002 in a phase 1 open-label randomized clinical trial. Vaccine 31: 1340-1348.

101. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, et al. (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guérin mutants that secrete listeriolysin. J Clin Invest 115:

2472-2479.

102. Grotzke JE, Siler AC, Lewinsohn DA, Lewinsohn DM (2010) Secreted immunodominant *Mycobacterium tuberculosis* antigens are processed by the cytosolic pathway. J Immunol 185: 4336-4343.

103. Gurcel L, Abrami L, Girardin S, Tschopp J, van der Goot FG (2006) Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival. Cell 126: 1135-1145.

104. Henry T, Brotcke A, Weiss DS, Thompson LJ, Monack DM (2007) Type I interferon signaling is required for activation of the inflammasome during *Francisella* infection. J Exp Med 204: 987-994.

105. Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OY, et al. (2008) Detection and molecular characterization of 9,000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. PLoS One 3: e3426.

106. Hinchey J, Lee S, Jeon BY, Basaraba RJ, Venkataswamy MM, et al. (2007) Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. J Clin Invest 117: 2279-2288.

107. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, et al. (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458: 514-518.

108. Houben D, Demangel C, van Ingen J, Perez J, Baldeón L, et al. (2012) ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. Cell Microbiol 14: 1287-1298.

109. Houben EN, Korotkov KV, Bitter W (2013) Take five - Type VII secretion systems of Mycobacteria. Biochim Biophys Acta.

110. Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, et al. (2003) The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc Natl Acad Sci U S A 100: 12420-12425.

111. Hutter B, Singh M (1999) Properties of the 40 kDa antigen of *Mycobacterium tuberculosis*, a functional L-alanine dehydrogenase. Biochem J 343 Pt 3: 669-672.

112. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, et al. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275: 90-94.

113. Indrigo J, Hunter RL, Actor JK (2003) Cord factor trehalose 6,6'-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. Microbiology 149: 2049-2059.

114. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, et al. (2013) Mitochondrial Cardiolipin Is Required for *Nlrp3* Inflammasome Activation. Immunity.

115. Jayakumar D, Jacobs WR, Narayanan S (2008) Protein kinase E of *Mycobacterium tuberculosis* has a role in the nitric oxide stress response and apoptosis in a human macrophage model of infection. Cell Microbiol 10: 365-374.

116. Jayaraman P, Sada-Ovalle I, Nishimura T, Anderson AC, Kuchroo VK, et al. (2013) IL-1β promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. J Immunol 190: 4196-4204.

117. Jha SS, Danelishvili L, Wagner D, Maser J, Li YJ, et al. (2010) Virulence-related *Mycobacterium avium subsp hominissuis MAV_2928* gene is associated with vacuole remodeling in macrophages. BMC Microbiol 10: 100.

118. John B, Hunter CA (2008) Immunology. Neutrophil soldiers or Trojan Horses? Science 321: 917-918.

119. Jones GJ, Gordon SV, Hewinson RG, Vordermeier HM (2010) Screening of predicted secreted antigens from *Mycobacterium bovis* reveals the immunodominance of the ESAT-6 protein family. Infect Immun 78: 1326-1332.

120. Jones JW, Kayagaki N, Broz P, Henry T, Newton K, et al. (2010) Absent in melanoma 2 is required for innate immune recognition of *Francisella tularensis*. Proc Natl Acad Sci U S A 107: 9771-9776.

121. Kaczmarek A, Vandenabeele P, Krysko DV (2013) Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity 38: 209-223.

122. Kaminski JJ, Schattgen SA, Tzeng TC, Bode C, Klinman DM, et al. (2013) Synthetic oligodeoxynucleotides containing suppressive TTAGGG motifs inhibit AIM2 inflammasome activation. J Immunol 191: 3876-3883.

123. Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, et al. (1997) Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. Infect Immun 65: 298-304.

124. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, et al. (2001) Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med 345: 1098-1104.

125. Keane J, Remold HG, Kornfeld H (2000) Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. J Immunol 164: 2016-2020.

126. Kelley LA, Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4: 363-371.

127. Khader SA, Pearl JE, Sakamoto K, Gilmartin L, Bell GK, et al. (2005) IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-gamma responses if IL-12p70 is available. J Immunol 175: 788-795.

128. Koo IC, Wang C, Raghavan S, Morisaki JH, Cox JS, et al. (2008) ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection. Cell Microbiol 10: 1866-1878.

129. Kurenuma T, Kawamura I, Hara H, Uchiyama R, Daim S, et al. (2009) The RD1 locus in the *Mycobacterium tuberculosis* genome contributes to activation of caspase-1 via induction of potassium ion efflux in infected macrophages. Infect Immun 77: 3992-4001.

130. LaRock CN, Cookson BT (2012) The *Yersinia* virulence effector YopM binds caspase-1 to arrest inflammasome assembly and processing. Cell Host Microbe 12: 799-805.

131. Lee J, Remold HG, Ieong MH, Kornfeld H (2006) Macrophage apoptosis in response to high intracellular burden of *Mycobacterium tuberculosis* is mediated by a novel caspase-independent pathway. J Immunol 176: 4267-4274.

132. Lewinsohn DM, Swarbrick GM, Cansler ME, Null MD, Rajaraman V, et al. (2013) Human *Mycobacterium tuberculosis* CD8 T Cell Antigens/Epitopes Identified by a Proteomic Peptide Library. PLoS One 8: e67016.

133. Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, et al. (2003) Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation.

J Infect Dis 187: 117-123.

134. Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94: 491-501.

135. Ligon LS, Hayden JD, Braunstein M (2012) The ins and outs of *Mycobacterium tuberculosis* protein export. Tuberculosis (Edinb) 92: 121-132.

136. Lin PL, Rutledge T, Green AM, Bigbee M, Fuhrman C, et al. (2012) CD4 T cell depletion exacerbates acute *Mycobacterium tuberculosis* while reactivation of latent infection is dependent on severity of tissue depletion in cynomolgus macaques. AIDS Res Hum Retroviruses 28: 1693-1702.

137. Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89: 175-184.

138. Liu YC, Simmons DP, Li X, Abbott DW, Boom WH, et al. (2012) TLR2 signaling depletes IRAK1 and inhibits induction of type I IFN by TLR7/9. J Immunol 188: 1019-1026.

139. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94: 481-490.

140. MacMicking J, Xie QW, Nathan C (1997) Nitric oxide and macrophage function. Annu Rev Immunol 15: 323-350.

141. Maiti D, Bhattacharyya A, Basu J (2001) Lipoarabinomannan from *Mycobacterium tuberculosis* promotes macrophage survival by phosphorylating Bad through a phosphatidylinositol 3-kinase/Akt pathway. J Biol Chem 276: 329-333.

142. Malen H, Berven FS, Fladmark KE, Wiker HG (2007) Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. Proteomics 7: 1702-1718.

143. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, et al. (2001) Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta. Proc Natl Acad Sci U S A 98: 5752-5757.

144. Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, 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: 694-701.

145. Manganelli R (2007) Polyphosphate and stress response in mycobacteria. Mol Microbiol 65: 258-260.

146. Manganelli R, Voskuil MI, Schoolnik GK, Smith I (2001) The *Mycobacterium tuberculosis* ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. Mol Microbiol 41: 423-437.

147. Manzanillo PS, Shiloh MU, Portnoy DA, Cox JS (2012) *Mycobacterium tuberculosis* activates the DNA-dependent cytosolic surveillance pathway within macrophages. Cell Host Microbe 11: 469-480.

148. Mariathasan S, Monack DM (2007) Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nat Rev Immunol 7: 31-40.

149. Martin CJ, Booty MG, Rosebrock TR, Nunes-Alves C, Desjardins DM, et al. (2012) Efferocytosis is an innate antibacterial mechanism. Cell Host Microbe 12: 289-300.

150. Martineau AR, Newton SM, Wilkinson KA, Kampmann B, Hall BM, et al. (2007) Neutrophil-mediated innate immune resistance to mycobacteria. J Clin Invest 117: 1988-1994.

151. Master SS, Rampini SK, Davis AS, Keller C, Ehlers S, et al. (2008) *Mycobacterium tuberculosis* prevents inflammasome activation. Cell Host Microbe 3: 224-232.

152. Mayer-Barber KD, Andrade BB, Barber DL, Hieny S, Feng CG, et al. (2011) Innate and adaptive interferons suppress IL-1alpha and IL-1beta production by distinct pulmonary myeloid subsets during *Mycobacterium tuberculosis* infection. Immunity 35: 1023-1034.

153. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, et al. (2010) Caspase-1 independent IL-1beta production is critical for host resistance to *Mycobacterium tuberculosis* and does not require TLR signaling in vivo. J Immunol 184: 3326-3330.

154. McElvania Tekippe E, Allen IC, Hulseberg PD, Sullivan JT, McCann JR, 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: e12320.

155. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, et al. (1999) Human toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. J Immunol 163: 3920-3927.

156. Mehra A, Zahra A, Thompson V, Sirisaengtaksin N, Wells A, et al. (2013) *Mycobacterium tuberculosis* type VII secreted effector EsxH targets host ESCRT to impair trafficking. PLoS Pathog 9: e1003734.

157. Miao EA, Leaf IA, Treuting PM, Mao DP, Dors M, et al. (2010) Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. Nat Immunol 11: 1136-1142.

158. Mihret A (2012) The role of dendritic cells in *Mycobacterium tuberculosis* infection. Virulence 3: 654-659.

159. Miller BH, Fratti RA, Poschet JF, Timmins GS, Master SS, et al. (2004) Mycobacteria inhibit nitric oxide synthase recruitment to phagosomes during macrophage infection. Infect Immun 72: 2872-2878.

160. Miller JL, Velmurugan K, Cowan MJ, Briken V (2010) The type I NADH dehydrogenase of *Mycobacterium tuberculosis* counters phagosomal NOX2 activity to inhibit TNF-alpha-mediated host cell apoptosis. PLoS Pathog 6: e1000864.

161. Mindell JA (2012) Lysosomal acidification mechanisms. Annu Rev Physiol 74: 69-86.

162. Mishra BB, Moura-Alves P, Sonawane A, Hacohen N, Griffiths G, et al. (2010) *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell Microbiol 12: 1046-1063.

163. Mishra BB, Rathinam VA, Martens GW, Martinot AJ, Kornfeld H, et al. (2013) Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1β. Nat Immunol 14: 52-60.

164. Molloy A, Laochumroonvorapong P, Kaplan G (1994) Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guérin. J Exp Med 180: 1499-1509.

165. Muñoz-Planillo R, Kuffa P, Martínez-Colón G, Smith BL, Rajendiran TM, et al. (2013) K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity 38: 1142-1153.

166. Muruve DA, Pétrilli V, Zaiss AK, White LR, Clark SA, et al. (2008) The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature 452: 103-107.

167. Nair S, Ramaswamy PA, Ghosh S, Joshi DC, Pathak N, et al. (2009) The PPE18 of *Mycobacterium tuberculosis* interacts with TLR2 and activates IL-10 induction in macrophage. J Immunol 183: 6269-6281.

168. Nicas M, Nazaroff WW, Hubbard A (2005) Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. J Occup Environ Hyg 2: 143-154.

169. Niederweis M, Danilchanka O, Huff J, Hoffmann C, Engelhardt H (2010) Mycobacterial outer membranes: in search of proteins. Trends Microbiol 18: 109-116.

170. Novikov A, Cardone M, Thompson R, Shenderov K, Kirschman KD, et al. (2011) *Mycobacterium tuberculosis* triggers host type I IFN signaling to regulate IL-1beta production in human macrophages. J Immunol 187: 2540-2547.

171. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, et al. (2013) The immune response in tuberculosis. Annu Rev Immunol 31: 475-527.

172. Oddo M, Renno T, Attinger A, Bakker T, MacDonald HR, et al. (1998) Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. J Immunol 160: 5448-5454.

173. Ohol YM, Goetz DH, Chan K, Shiloh MU, Craik CS, et al. (2010) *Mycobacterium tuberculosis* MycP1 protease plays a dual role in regulation of ESX-1 secretion and virulence. Cell Host Microbe 7: 210-220.

174. Ormerod LP (2005) Multidrug-resistant tuberculosis (MDR-TB): epidemiology, prevention and treatment. Br Med Bull 73-74: 17-24.

175. Parandhaman DK, Hanna LE, Narayanan S (2014) PknE, a serine/threonine protein kinase of *Mycobacterium tuberculosis* initiates survival crosstalk that also impacts HIV coinfection. PLoS One 9: e83541.

176. Pathak SK, Basu S, Basu KK, Banerjee A, Pathak S, 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: 610-618.

177. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8: 785-786.

178. Pilli M, Arko-Mensah J, Ponpuak M, Roberts E, Master S, et al. (2012) TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. Immunity 37: 223-234.

179. Porcelli SA, Jacobs WR (2008) Tuberculosis: unsealing the apoptotic envelope. Nat Immunol 9: 1101-1102.

180. Pym AS, Brodin P, Brosch R, Huerre M, Cole ST (2002) Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis BCG* and *Mycobacterium microti*. Mol Microbiol 46: 709-717.

181. Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, et al. (2003) Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat Med 9: 533-539.

182. Ramakrishnan L (2013) Looking within the zebrafish to understand the tuberculous granuloma. Adv Exp Med Biol 783: 251-266.

183. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, et al. (2010) The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol 11: 395-402.

184. Ratliff TL, McCarthy R, Telle WB, Brown EJ (1993) Purification of a mycobacterial adhesin for fibronectin. Infect Immun 61: 1889-1894.

185. Ratsimandresey RA, Dorfleutner A, Stehlik C (2013) An Update on PYRIN Domain-Containing Pattern Recognition Receptors: From Immunity to Pathology. Front Immunol 4: 440.

186. Raynaud C, Etienne G, Peyron P, Lanéelle MA, Daffé M (1998) Extracellular enzyme activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis*. Microbiology 144 (Pt 2): 577-587.

187. Reece ST, Kaufmann SH (2008) Rational design of vaccines against tuberculosis directed by basic immunology. Int J Med Microbiol 298: 143-150.

188. Reiling N, Hölscher C, Fehrenbach A, Kröger S, Kirschning CJ, 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: 3480-3484.

189. Riedl SJ, Shi Y (2004) Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 5: 897-907.

190. Rittershaus ES, Baek SH, Sassetti CM (2013) The normalcy of dormancy: common themes in microbial quiescence. Cell Host Microbe 13: 643-651.

191. Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, et al. (2009) HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science 323: 1057-1060.

192. Rojas M, García LF, Nigou J, Puzo G, Olivier M (2000) Mannosylated lipoarabinomannan antagonizes *Mycobacterium tuberculosis*-induced macrophage apoptosis by altering Ca+2-dependent cell signaling. J Infect Dis 182: 240-251.

193. Rosenkrands I, Slayden RA, Crawford J, Aagaard C, Barry CE, 3rd, et al. (2002) Hypoxic response of *Mycobacterium tuberculosis* studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. J Bacteriol 184: 3485-3491.

194. Sacchettini JC, Rubin EJ, Freundlich JS (2008) Drugs versus bugs: in pursuit of the persistent predator *Mycobacterium tuberculosis*. Nat Rev Microbiol 6: 41-52.

195. Saiga H, Kitada S, Shimada Y, Kamiyama N, Okuyama M, et al. (2012) Critical role of AIM2 in *Mycobacterium tuberculosis* infection. Int Immunol.

196. Sauer JD, Witte CE, Zemansky J, Hanson B, Lauer P, et al. (2010) *Listeria monocytogenes* triggers AIM2-mediated pyroptosis upon infrequent bacteriolysis in the macrophage cytosol. Cell Host Microbe 7: 412-419.

197. Sayes F, Sun L, Di Luca M, Simeone R, Degaiffier N, et al. (2012) Strong immunogenicity and cross-reactivity of *Mycobacterium tuberculosis* ESX-5 type VII secretion: encoded PE-PPE proteins predicts vaccine potential. Cell Host Microbe 11: 352-363.

198. Scheindlin S (2006) The fight against tuberculosis. Mol Interv 6: 124-130.

199. Schroder K, Tschopp J (2010) The inflammasomes. Cell 140: 821-832.

200. Selwyn PA, Hartel D, Lewis VA, Schoenbaum EE, Vermund SH, et al. (1989) A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. N Engl J Med 320: 545-550.

201. Serafini A, Boldrin F, Palù G, Manganelli R (2009) Characterization of a *Mycobacterium tuberculosis* ESX-3 conditional mutant: essentiality and rescue by iron and zinc. J Bacteriol 191: 6340-6344.

202. Shah S, Bohsali A, Ahlbrand SE, Srinivasan L, Rathinam VA, et al. (2013) Cutting edge: *Mycobacterium tuberculosis* but not nonvirulent mycobacteria inhibits IFN-β and AIM2 inflammasome-dependent IL-1β production via its ESX-1 secretion system. J Immunol 191: 3514-3518.

203. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1: 2856-2860.

204. Silva Miranda M, Breiman A, Allain S, Deknuydt F, Altare F (2012) The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for the bacteria? Clin Dev Immunol 2012: 139127.

205. Simeone R, Bobard A, Lippmann J, Bitter W, Majlessi L, et al. (2012) Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. PLoS Pathog 8: e1002507.

206. Simpson GL, Raffin TA, Remington JS (1982) Association of prior nocardiosis and subsequent occurrence of nontuberculous mycobacteriosis in a defined, immunosuppressed population. J Infect Dis 146: 211-219.

207. Siranosian KJ, Ireton K, Grossman AD (1993) Alanine dehydrogenase (*ald*) is required for normal sporulation in Bacillus subtilis. J Bacteriol 175: 6789-6796.

208. Sly LM, Hingley-Wilson SM, Reiner NE, McMaster WR (2003) Survival of *Mycobacterium tuberculosis* in host macrophages involves resistance to apoptosis dependent upon induction of antiapoptotic Bcl-2 family member Mcl-1. J Immunol 170: 430-437.

209. Smith J, Manoranjan J, Pan M, Bohsali A, Xu J, et al. (2008) Evidence for pore formation in host cell membranes by ESX-1-secreted ESAT-6 and its role in *Mycobacterium marinum* escape from the vacuole. Infect Immun 76: 5478-5487.

210. Spira A, Carroll JD, Liu G, Aziz Z, Shah V, et al. (2003) Apoptosis genes in human alveolar macrophages infected with virulent or attenuated *Mycobacterium tuberculosis*: a pivotal role for tumor necrosis factor. Am J Respir Cell Mol Biol 29: 545-551.

211. Stanley SA, Cox JS (2013) Host-Pathogen Interactions During *Mycobacterium tuberculosis* infections. Curr Top Microbiol Immunol 374: 211-241.

212. Stanley SA, Johndrow JE, Manzanillo P, Cox JS (2007) The Type I IFN response to infection with *Mycobacterium tuberculosis* requires ESX-1-mediated secretion and contributes to pathogenesis. J Immunol 178: 3143-3152.

213. Stanley SA, Raghavan S, Hwang WW, Cox JS (2003) Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. Proc Natl Acad Sci U S A 100: 13001-13006.

214. Stenger S, Mazzaccaro RJ, Uyemura K, Cho S, Barnes PF, et al. (1997) Differential effects of cytolytic T cell subsets on intracellular infection. Science 276: 1684-1687.

215. Stewart GR, Patel J, Robertson BD, Rae A, Young DB (2005) Mycobacterial mutants with defective control of phagosomal acidification. PLoS Pathog 1: 269-278.

216. Stewart GR, Wernisch L, Stabler R, Mangan JA, Hinds J, et al. (2002) Dissection of the wongheat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays. Microbiology 148: 3129-3138.

217. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, et al. (1994) Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263: 678-681.

218. Sutterwala FS, Mijares LA, Li L, Ogura Y, Kazmierczak BI, et al. (2007) Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. J Exp Med 204: 3235-3245.

219. Sutterwala FS, Ogura Y, Szczepanik M, Lara-Tejero M, Lichtenberger GS, et al. (2006) Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity 24: 317-327.

220. Tailleux L, Pham-Thi N, Bergeron-Lafaurie A, Herrmann JL, Charles P, et al. (2005) DC-SIGN induction in alveolar macrophages defines privileged target host cells for mycobacteria in patients with tuberculosis. PLoS Med 2: e381.

221. Takeda K, Noguchi T, Naguro I, Ichijo H (2008) Apoptosis signal-regulating kinase 1 in stress and immune response. Annu Rev Pharmacol Toxicol 48: 199-225.

222. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, et al. (2002) Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 169: 10-14.

223. Tekaia F, Gordon SV, Garnier T, Brosch R, Barrell BG, et al. (1999) Analysis of the proteome of *Mycobacterium tuberculosis* in silico. Tuber Lung Dis 79: 329-342.

224. Ting JP, Willingham SB, Bergstralh DT (2008) NLRs at the intersection of cell death and immunity. Nat Rev Immunol 8: 372-379.

225. Tobin DM, Ramakrishnan L (2008) Comparative pathogenesis of *Mycobacterium marinum* and *Mycobacterium tuberculosis*. Cell Microbiol 10: 1027-1039.

226. Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3: 133-146.

227. Tsuchiya K, Hara H, Kawamura I, Nomura T, Yamamoto T, et al. (2010) Involvement of absent in melanoma 2 in inflammasome activation in macrophages infected with *Listeria monocytogenes*. J Immunol 185: 1186-1195.

228. Tunbridge AJ, Stevanin TM, Lee M, Marriott HM, Moir JW, et al. (2006) Inhibition of macrophage apoptosis by *Neisseria meningitidis* requires nitric oxide detoxification mechanisms. Infect Immun 74: 729-733.

229. Veeranki S, Duan X, Panchanathan R, Liu H, Choubey D (2011) IFI16 protein mediates the anti-inflammatory actions of the type-I interferons through suppression of activation of caspase-1 by inflammasomes. PLoS One 6: e27040.

230. Velmurugan K, Chen B, Miller JL, Azogue S, Gurses S, et al. (2007) *Mycobacterium tuberculosis nuoG* is a virulence gene that inhibits apoptosis of infected host cells. PLoS Pathog 3: e110.

231. Vergne I, Chua J, Lee HH, Lucas M, Belisle J, et al. (2005) Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 102: 4033-4038.

232. Wajant H (2002) The Fas signaling pathway: more than a paradigm. Science 296: 1635-1636.

233. Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, et al. (2004) Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. Science 304: 1800-1804.

234. Ward MJ, Lew H, Zusman DR (2000) Social motility in *Myxococcus xanthus* requires FrzS, a protein with an extensive coiled-coil domain. Mol Microbiol 37: 1357-1371.

235. Winau F, Weber S, Sad S, de Diego J, Hoops SL, et al. (2006) Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. Immunity 24: 105-117.

236. Wong KW, Jacobs WR, Jr. (2011) Critical role for NLRP3 in necrotic death triggered by *Mycobacterium tuberculosis*. Cell Microbiol 13: 1371-1384.

237. Xu G, Shi Y (2007) Apoptosis signaling pathways and lymphocyte homeostasis. Cell Res 17: 759-771.

238. Xu J, Laine O, Masciocchi M, Manoranjan J, Smith J, et al. (2007) A unique *Mycobacterium* ESX-1 protein co-secretes with CFP-10/ESAT-6 and is necessary for inhibiting phagosome maturation. Mol Microbiol.

239. Yadav M, Schorey JS (2006) The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. Blood 108: 3168-3175.

240. Yang CT, Cambier CJ, Davis JM, Hall CJ, Crosier PS, et al. (2012) Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. Cell Host Microbe 12: 301-312.

241. Yin Q, Sester DP, Tian Y, Hsiao YS, Lu A, et al. (2013) Molecular mechanism for p202-mediated specific inhibition of AIM2 inflammasome activation. Cell Rep 4: 327-339.