

ABSTRACT

Title of Document: CHARACTERIZATION OF THE IMMUNE RESPONSE INDUCED BY NON-PATHOGENIC MYCOBACTERIA IN MACROPHAGES AND DENDRITIC CELLS

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The capacity of the host to mount an effective immune response (IR) is crucial for the protection against invading pathogens. The apoptotic and proinflammatory responses of infected cells are important innate immune mechanisms. Consequently, the ability of persistent intracellular pathogens, such as the human pathogen *Mycobacterium tuberculosis* (*Mtb*), to inhibit infection-induced apoptosis of macrophages is important for its virulence in the host. Facultative-pathogenic mycobacterial species, like *M. kansasii* (*Mkan*), can cause disseminating disease in individuals with immune deficiencies. In contrast, non-pathogenic mycobacteria, like *M. smegmatis* (*Msme*), are not known to cause disseminating disease even in immunocompromised individuals. We hypothesized that this difference in phenotype could be explained by the strong induction of an innate IR by non-pathogenic mycobacteria.

Here we analyze the mechanisms by which non-pathogenic mycobacteria induce a strong IR in their macrophage and dendritic cell (DC) host, specifically the induction of host cell apoptosis and the host inflammasome response via the secretion of IL-1 β . The

comparison of two non-pathogenic mycobacterial species (*Msmc* and *Mkan*) with two facultative-pathogenic mycobacterial species (*M.kan* and *M. bovis* BCG) demonstrated that only the non-pathogenic mycobacteria induce strong apoptosis in murine bone marrow derived macrophages (BMDM) and dendritic cells (BMDC), which was dependent upon caspase-3 activation and TNF secretion. Consistently, BMDMs responded by secreting relatively large amounts of TNF and by upregulating the expression of *Il-12*. We also demonstrated that *Msmc* infection of BMDCs strongly induces the secretion of IL-1 β . This induction was dependent upon the presence of functional ASC and was partially independent of NLRP3. Interestingly this induction was also partially dependent on AIM2 and IFN- β . This AIM2-dependent induction was observed in infection with non-pathogenic and opportunistic mycobacteria, and attenuated but not virulent *Mtb*. Surprisingly, caspase-1/11 deficient BMDCs still secreted substantial but reduced amounts of IL-1 β upon *Msmc* infection.

In conclusion, we demonstrate a strong induction of the innate IR by non-pathogenic mycobacteria as measured by host cell apoptosis, and IL-12 / TNF / IL-1 β cytokine induction. We also demonstrate the partially caspase-1/11 and NLRP3 - independent, partially AIM2-dependent, but ASC-dependent IL-1 β secretion in *Msmc* infected BMDCs. Our findings support the hypothesis that the strong induction of the innate IR is a major reason for the lack of pathogenicity in non-pathogenic mycobacteria.

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Dedication

I dedicate this dissertation to:

My mother Najla whose unconditional love and support keep me going.

To:

My late father Youssef whose spirit is always with me.

And to:

My brother Mounir who never let me doubt myself, always supported me, and kept me
balanced.

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Table of Contents

Dedication	ii
Acknowledgments	iii
Table of Contents	v
List of Figures	viii
List of Abbreviations	x
Chapter 1 Introduction	1
1.1 Tuberculosis (TB)	1
1.1.1 Tuberculosis, the disease	1
1.1.2 Treatment and prevention	4
1.1.2.1 Drug therapies	4
1.1.2.2 <i>Bacillus Calmette-Guérin</i> (BCG) vaccine	5
1.1.2.3 Current state of TB Infections	6
1.2 Mycobacteria	7
1.2.1 Virulent pathogenic mycobacteria	9
1.2.2 Opportunistic facultative-pathogenic mycobacteria	9
1.2.3 Non-pathogenic mycobacteria	10
1.2.4 <i>M. smegmatis</i> , a potential vaccine platform	11
1.3 Immune response and mycobacteria	12
1.3.1 The immune system	13
1.3.1.1. Cytokines	13
1.3.1.2 Pattern recognition receptors (PRR)	14
1.3.1.3 Cell types involved in innate immunity	15
1.3.1.4 Cell types involved in adaptive immunity	20
1.3.2 Manipulation of the immune response by <i>Mtb</i>	22
1.3.2.1 Inhibition of phagolysosome fusion	22
1.3.2.2 Resistance to ROS and RNI	23
1.3.2.3 Inhibition of host cell apoptosis	24
1.3.3 Non-pathogenic and facultative pathogenic mycobacteria and the immune response	25
1.4 Programmed cell death	27
1.4.1 Apoptosis	27
1.4.1.1 Caspases	28
1.4.1.2 Intrinsic apoptosis pathway	28
1.4.1.3 Extrinsic apoptosis pathway	29
1.4.1.4 TNF signaling: dual role in apoptosis and survival	30
1.4.2 Caspase-independent apoptosis	30
1.4.3 Autophagy	33
1.4.4 Necrosis	33
1.4.5 Pyroptosis	34
1.4.6 Pyronecrosis	35
1.5 Inflammasome and mycobacteria	35

1.5.1 The inflammasome	36
1.5.1.1 Inflammasome components	36
1.5.1.2 Inflammasome activation and IL-1 β / IL-18 processing	36
1.5.1.3. Types of inflammasomes	37
1.5.2 Mycobacteria-induced inflammasome activation and the role of IL-1 β in mycobacterial infections	41
1.6 Specific aims and significance	42
Chapter 2. Materials and methods	44
2.1 Materials	44
2.2 Bacteria and culture conditions	45
2.3 Cell culture conditions and infection	45
2.4 Bone-marrow derived macrophages and dendritic cells.	45
2.5 Apoptosis assays	46
2.6 Enzyme-linked immunosorbent assays (ELISA)	47
2.7 ROS detection assays.	47
2.8 Enzymes inhibition and proteins neutralization assays	48
2.9 Determination of infection rate and bacterial load	48
2.10 Statistical analysis	49
Chapter 3. Results and discussion	49
3.1 Mechanisms of non-pathogenic mycobacterial species <i>M. smegmatis</i> and <i>M.</i> <i>fortuitum</i> induced apoptosis in macrophages	49
3.1.1 Non-pathogenic mycobacteria induce increased host cell apoptosis in macrophages compared to facultative-pathogenic mycobacteria	50
3.1.2 Non-pathogenic mycobacteria strongly induce the secretion of TNF and IL-12 in macrophages.	53
3.1.3 Non-pathogenic mycobacteria induce apoptosis via TNF and caspase-3 signaling pathways	55
3.1.4 Non-pathogenic mycobacteria do not induce apoptosis in C57Bl/6 BMDMs	58
3.1.5 Discussion	60
3.2 Differences in apoptosis induced by facultative-pathogenic and non-pathogenic mycobacteria in BALB/c and C57Bl/6 dendritic cells	65
3.2.1 <i>Msm</i> e and BCG infect BALB/c and C57Bl/6 dendritic cells at the same rate	66
3.2.2 Non-pathogenic mycobacteria induce increased host cell apoptosis in BALB/c and C57Bl/6 dendritic cells compared to facultative- pathogenic mycobacteria	67
3.2.3 Facultative-pathogenic mycobacteria induce more apoptosis in BMDC than BMDMs	68
3.2.4 Differences in ROS response to mycobacterial infection between C57Bl/6 macrophages and dendritic cells	68
3.2.5 Discussion	70
3.3 Molecular mechanisms of <i>Msm</i> e-induced IL-1 β secretion in dendritic cell and macrophages	73

3.3.1 <i>Msmc</i> -induced IL-1 β secretion in dendritic cells is dependent on ASC but partially independent of NLRP3 and caspases-1/11.....	74
3.3.2 <i>Msmc</i> induces apoptosis but not pyroptosis or pyronecrosis in dendritic cells.	77
3.3.3 <i>Msmc</i> and <i>Mtb</i> -induced secretion of IL-1 β in macrophages is partially independent of casapase-1/11	78
3.3.4 L cell-conditioned medium has an inhibitory effect on the <i>Msmc</i> -induced IL-1 β secretion in BMDMs	81
3.3.5 The IL-1 receptor is not required for <i>Msmc</i> -mediated IL-1 β secretion and has no role in apoptosis induction	84
3.3.6 <i>Msmc</i> -induced IL-1 β production is independent of caspases-3, -8, and -9	86
3.3.7 <i>Msmc</i> -induced IL-1 β production is independent of Cathepsins B, L, S and Calpain	88
3.3.8 Characterization of the caspase-1/11-independent IL-1 β secretion in dendritic cells	90
3.3.9 <i>Msmc</i> -induced IL-1 β production in dendritic cells is partially dependent on AIM2 but independent of NLRP6, NLRP10, NLRP12, and IPAF	94
3.3.10 AIM2 plays a role in the IL-1 β secretion by dendritic cells infected with non-pathogenic and attenuated but not virulent mycobacteria	96
3.3.11 IFN- β is important for the AIM2-dependent <i>Msmc</i> -induced IL-1 β secretion in BMDCs	99
3.3.12 Discussion	102
Chapter 4. Summary and future directions	109
Bibliography.....	114

List of Figures

Figure 1. Global instances in TB in 2010 according to the World Health Organization (WHO)	2
Figure 2. Extrinsic and intrinsic pathways of apoptosis	31
Figure 3. TNF signaling via TNFR1 leading to both cells death (apoptosis) and survival.....	32
Figure 4. Mechanism of NLRP3 inflammasome complex formation	40
Figure 5: Differences in apoptosis induced by facultative-pathogenic versus non-pathogenic mycobacteria in primary murine macrophages	51
Figure 6: Difference in apoptosis induction between facultative and non-pathogenic mycobacteria in a human macrophage cell line.....	52
Figure 7: Differences in TNF secretion and IL-12 induction between facultative-pathogenic and non-pathogenic mycobacteria-infected macrophages	54
Figure 8: Macrophage apoptosis induction by a nonpathogenic mycobacteria is caspase-3- and TNF-dependent	57
Figure 9: Mycobacteria do not induce rapid apoptosis in BMDM originating from C57Bl/6 mice	59
Figure 10. Rate of infection and intracellular growth of <i>Msm</i> and <i>BCG</i> in BMDCs.....	66
Figure 11: Differences in apoptosis induced by facultative-pathogenic and non-pathogenic mycobacteria in BALB/c and C57Bl/6 dendritic cells	69
Figure 12: Differences in ROS response to mycobacterial infection between C57Bl/6 macrophages and dendritic cells	71
Figure 13. <i>Msm</i> -induced IL-1 β secretion is dependent on ASC but partially independent of NLRP3 and caspases-1/11	75
Figure 14. <i>Msm</i> and <i>Mtb</i> -induced Secretion of IL-1 β in macrophages is partially independent of caspase-1/11	79
Figure 15. L cell-conditioned medium has a inhibitory effect on the <i>Msm</i> -induced IL-1 β secretion in BMDMs	83

Figure 16. The IL-1 receptor is not required for <i>Msmc</i> mediated IL-1 β secretion and has no role in apoptosis induction	85
Figure 17. <i>Msmc</i> -induced IL-1 β production is independent of caspases-3, -8, and -9.....	87
Figure 18. <i>Msmc</i> -induced IL-1 β secretion is independent of Cathepsins B, L, S and Calpain.	89
Figure 19. The caspase-1-independent IL-1 β secretion is independent of dectin-1	91
Figure 20. Characterization of the caspase-1/11-independent IL-1 β secretion in dendritic cells	93
Figure 21. <i>Msmc</i> -induced IL-1 β secretion is partially dependent on AIM2 but independent of NLRP6, NLRP10, and NLRP12	95
Figure 22. AIM2 plays a role in the IL-1 β secretion by dendritic cells infected with non-pathogenic and attenuated but not virulent mycobacteria	98
Figure 23. IFN- β is important for the AIM2-dependent <i>Msmc</i> -induced IL-1 β secretion in BMDCs	101

List of Abbreviations

In alphabetical order

AIM2 – Absent in melanoma-2
AIF – Apoptosis inducing factor
AK – Adenylate kinase
Apaf - apoptotic protease activating factor
AP1 – Activator protein 1
ASC – Apoptosis-associated speck-like protein containing a casapse-recruitment domain
BCG - Bacillus Calmette-Guérin
BMDM – Bone marrow derived macrophage
BMDC – Bone marrow derived dendritic cell
cAMP – Cyclic adenosine monophosphate
CARD – Caspase recruitment domains
Cat - Cathepsin
CD4+ – Helper T cells
CD8+ – Cytotoxic T cells
c-FLIP – Cellular FLICE like inhibitory protein
CFU – Colony forming units
CIA – Caspase independent apoptosis
CREB – Cyclic AMP response element binding protein
DAMP – Danger-associated molecular pattern
DC – Dendritic cell
DED – Death effector domain
DHE – Dihydroethidium
DISC – Death inducing signaling complex
DMEM – Dulbecco's modified eagle medium
ELISA - Enzyme linked immunosorbent assay
ER – Endoplasmic reticulum
FADD – Fas-associated death domain
FCS – Fetal calf serum
GFP – Green fluorescence protein
HIV - Human immunodeficiency virus
IAP – Inhibitor of apoptosis
IL – Interleukin
IFN γ – Interferon gamma
IFN- β – Interferon beta
iNOS – Inducible nitric oxide synthase
IR – Immune response
JNK – June N-terminal kinase
K – Kinase
LAM – Lipoarabinomannan
LCCM – L cell-conditioned medium

LRR – Leucine-rich repeat
LVS – Live vaccine strain
Man-LAM – Mannose capped lipoarabinomannan
MAP – Mitogen activated protein
MAPK – MAP kinase
MDR – Multi-drug resistant
Mfort – *Mycobacterium fortuitum*
MHC – Major histocompatibility complex
Mkan – *Mycobacterium kansasii*
MOI – Multiplicity of infection
MOMP – Mitochondrial outer membrane permeabilization
Msme – *Mycobacterium smegmatis*
MSU – Monosodium urate
Mtb – *Mycobacterium tuberculosis*
MTBC – *Mycobacterium tuberculosis* complex
NADH – Nicotinamide adenine dinucleotide
NADPH – Nicotinamide adenine dinucleotide phosphate
NF- κ B – Nuclear factor- κ B
NLR – NOD-like receptor
NOD – Nucleotide and oligomerization domain
NOX – NADPH oxidase
PAMP – Pathogen associated molecular patterns
PBS – Phosphate buffered saline
PBST – PBS-tween
PCD – Programmed cell death
PFA – Paraformaldehyde
PI – Propidium iodine
PI3 – Phosphoinositide 3
PI-LAM – Phosphoinositide-modified lipoarabinomannan
PknE – Protein kinase E
PMA – Phorbol myristate acetate
PRR – Pattern recognition receptor
PTX – Pentoxifylline
PYD – Pyrin domain
RNI – Reactive nitrogen intermediates
RD – Region of difference
PRR – Pattern recognition receptor
ROS – Reactive oxygen species
SOD – Superoxide dismutase
ST – staurosporine
TB – Tuberculosis
TCR – T cell receptor
Th1 – CD4⁺ T helper cell type 1
Th2 – CD4⁺ T helper cell type 2
TLR – Toll like receptors
TNF – Tumor necrosis factor

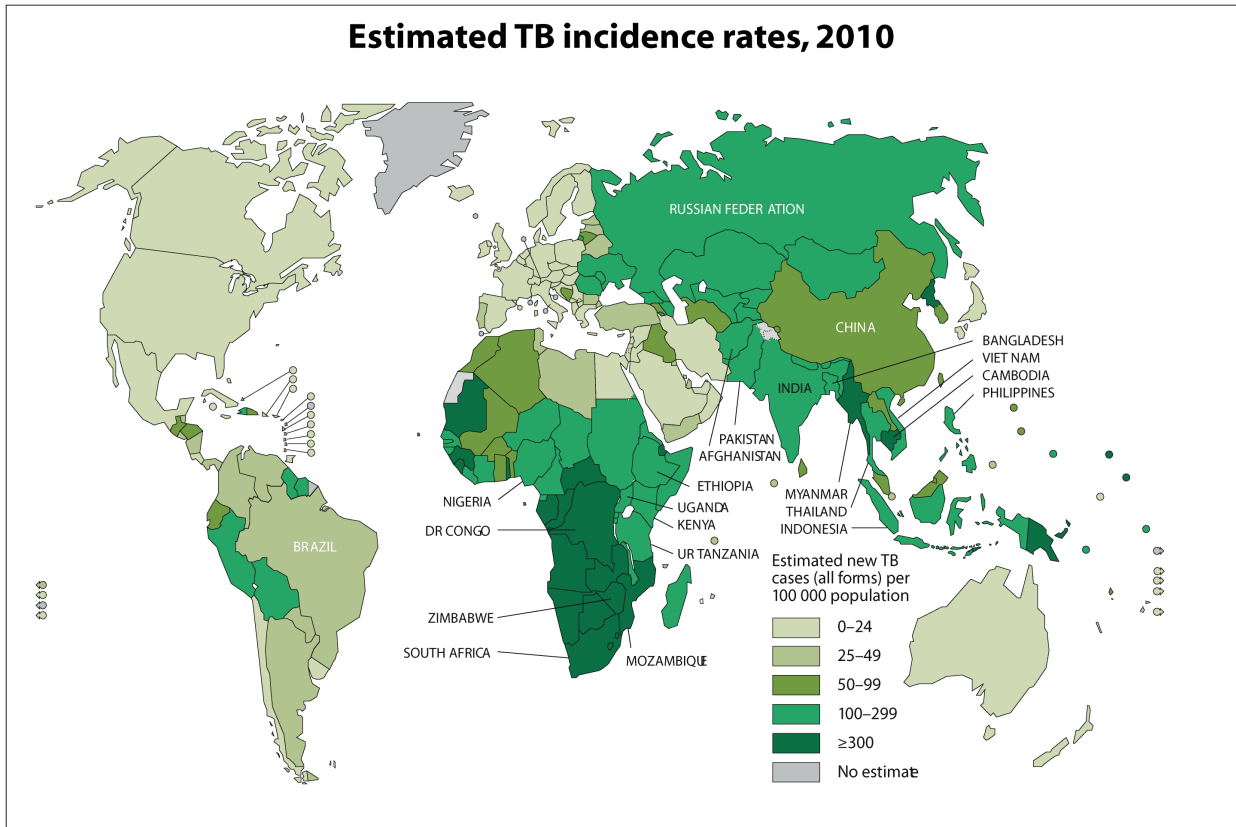
TNF^{-/-} – *TNF* knockout
TNFR – TNF receptor
TRADD – TNFR-associated death domain
TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling
TXNIP – Thioredoxin-interacting protein
UI – Uninfected
UT – Untreated
WHO – World Health Organization
WT – Wild type
XDR – Extreme drug resistant

CHAPTER 1. INTRODUCTION

1.1 Tuberculosis (TB).

1.1.1 Tuberculosis, the disease.

Tuberculosis is a disease that has been in existence since antiquity causing human mortality for thousands of years from pre-historic era to our current day [1] [2]. In the past TB used to be called “consumption” and regarded as “vampirism” as one infected person in the family died from TB, other infected family members would become diseased and lose their health slowly, which was regarded as the first diseased person draining the life out of the rest of the family [3]. It was also called ‘consumption’ because it was perceived to consume people from within with a severe bloody cough, fever, and heavy wasting. TB remains a global health problem with ten million new cases and causing the death of more than two million people every year [4]. *Mycobacterium tuberculosis (Mtb)*, the etiologic agent of TB, infects approximately two billion people worldwide in most parts of Africa, India, and parts of Eastern Europe (Figure 1) [4]. Tuberculosis manifests itself as a pulmonary disease with a high infection rate as it transmits via the aerosols [5], which are formed when a person simply coughs or sneezes. *Mtb* primarily infects alveolar macrophages in the lungs, and other phagocytic white blood cells, which provide a niche for the bacteria to survive, multiply, and propagate. Once infected, macrophages may die via necrosis and release the bacteria into the extracellular environment leading to infection of other macrophages and phagocytic white blood cells such as dendritic cells and neutrophils. During infection with *Mtb*, macrophages and other white blood cells are recruited to the site of infection where they induce an inflammatory response. The newly recruited and infected macrophages and white blood cells will then start forming a



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

Source: *Global Tuberculosis Control 2011*. WHO, 2011.



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Adapted from [6].

Figure 1. Global instances in TB in 2010 according to the World Health Organization (WHO). The WHO Global TB control report 2011 showing a geographical distribution of estimated new TB cases per 100,000 population.

granuloma at the infection site [7-10]. The granuloma consists of a core of infected macrophages that sometimes undergo necrosis and get surrounded by lymphocytes [7]. The infected macrophages release IL-12 and IL-23 to promote a Th1 lymphocyte primary response, which then activate the immune system by the release of interferon- γ (IFN- γ) and TNF [11]. These immunomodulatory signals are sensed by the immune system, which gets activated but fails to clear the infection. Within granulomas, *Mtb* can survive for decades without getting cleared but is unable to cause any new pathology [7] [12]. During this dormant phase of TB called latency, individuals do not develop any symptoms of the disease and are generally not contagious. However, 10% of individuals infected with *Mtb* may develop active TB when they become immunocompromised or immunosuppressed either from old age, malnutrition, treatment with immunosuppressant drugs, or commonly from infection with other pathogens such as the human immunodeficiency virus (HIV) [13-15]. In the event where latent TB develops into active disease, the infecting bacteria induce necrosis and caseation within the granuloma, which results in the release of *Mtb* into the lungs tissue and airways. This invasion of lung tissues and airways often leads to the propagation and dissemination of the bacteria into other tissues in the body leading to pathology in the liver, bone, spleen, and brain [16]. The disease is contagious in individuals with active TB, which manifests in symptoms of consumption that include excessive cough, bloody mucus, fever, fatigue, night sweats, unexplained weight loss, chills, and loss of appetite [17] [18].

1.1.2 Treatment and prevention.

1.1.2.1 *Drug therapies.*

TB existed among humans for thousands of years at times when antibiotics and vaccines did not exist. The best treatment for TB was thought to be bed rest, good nutrition and proper ventilation of the breathing environment by providing fresh air for patients [19]. Robert Koch, the discoverer of *Mtb*, proposed the first attempted medical cure for the disease in 1882. Koch used tuberculin, an extract from the bacterium *Mtb* and tried to cure infected individuals. His attempt failed but tuberculin is still used today for the diagnosis of TB infections in humans via the PPD skin test and other adjuvant drug therapies [20]. In 1946 Albert Schatz and Selman Waksman discovered streptomycin, which was then adopted as the first effective drug against TB infections [21]. Other new antibiotic drugs were discovered in the following years including isoniazid, rifampicin, pyrazinamide, and ethambutol [22]. These drugs have shown to be effective since they caused a substantial decrease in the mortality rate due to TB associated illnesses [22]. However, there are many problems associated with the use of these first line antibiotics against TB. First, some of them can cause severe hepatotoxicity [23] [24]. Second, the course of treatment with these drugs is fairly long (approx. 6-9 months) which causes toxicity and socio-medical problems [25-27]. The prolonged treatment with the drugs puts more stress on tissues and organs and may lead to tissue injury, as is the case with isoniazid [24]. This fairly long treatment also is a socio-medical problem since infected individuals mostly reside in poor areas of the world and they fail to adhere to the treatment regimen for this long period of time. Failure to follow the precise treatment regimen leads to the emergence of drug resistant strains of *Mtb* [26]. Third, these antibiotics are unable to effectively clear bacteria that are not

genetically drug resistant from infected individuals as drug-susceptible bacteria are often found to be persistent in patients that already underwent drug therapy [25] [28]. This might be due the hiding of bacteria in granulomas, sequestered in tissues away from the drugs. Alternatively, *Mtb* might also adopt a physiological state inside the granuloma that renders them insensitive to the drugs [28].

1.1.2.2 *Bacillus Calmette-Guérin (BCG) vaccine.*

Microbiologists Albert Calmette and Camille Guérin created the first and only known TB vaccine called 'BCG' in 1908 and it has been used worldwide as a vaccine against TB [29] [30]. However, the vaccine's efficacy currently ranges from zero to eighty percent with highest efficacy against TB infections in infants as reported in clinical trials [31] [32]. Initially, the BCG vaccine was created by passaging pathogenic *Mycobacterium bovis* (*M. bovis*) in culture for eleven years until it lost its virulence in humans. BCG was then shipped to different parts of the world where each country continued passaging the bacteria in culture to maintain their own stock [33]. The variability in the efficacy of the vaccine over a wide range is suggested to be due to genomic differences among the BCG substrains from different regions of the world [34]. This is due to the independent passaging of the original parent strain at various sites [31]. This difference in efficacy might also be due to differences in BCG-induced immunity in different populations due to different environmental exposures [35]. Indeed, I co-authored a study in which we demonstrated that different *M. bovis* BCG substrains from various regions of the globe confer different levels of protection against *Mtb* infections in mice. The different BCG substrains induced different levels of adaptive T cell immune response (IR), which we

demonstrated to be linked to the differences in the number of genomic regions of deletions (RD) among the different BCG substrains [31].

1.1.2.3 Current state of TB Infections.

The 20th century was marked by a decrease in the mortality rate associated with TB infections due to improvements in public health and general public awareness, as well as the discovery and use of the BCG vaccine and chemotherapeutic TB drugs [36]. Despite this decrease in mortalities, TB was never eradicated and still is endemic in many parts of the world including Africa, Southeast Asia, and Western Europe causing a global health threat [37] (Figure 1). The 1980s have seen a rise of drug-resistant strains of TB such as the increase in TB infections in Britain, which diminished all hopes that the disease could have been eradicated [36] [38]. The incidences of multi-drug-resistant (MDR) TB and extreme-drug-resistant (XDR) TB have also risen resulting in the declaration of a global health emergency by the World Health Organization (WHO) in 1993 [38] [39]. MDR *Mtb* is resistant to at least one of the two most powerful first line drugs isoniazid and rifampicin [36]. XDR *Mtb* emerged as a consequence of chemotherapies against MDR-TB involving second line drugs [38]. XDR *Mtb* is resistant to first line drugs, fluoroquinolones, and at least one of the injectable drugs (amikancin, kanamycin, or capreomycin) [36-38].

In addition, the emergence of HIV in the 1990s and the incidences of HIV-TB co-infections especially in Africa and Asia increased the rate of mortality in TB patients [40]. HIV-positive patients infected with *Mtb* are more likely to become symptomatic and suffer from active TB than HIV-negative patients [41]. HIV compromises the immune system and causes a progressive loss of Th1 cell mediated IR, which is required to defend against *Mtb*

infections, thus making it harder for HIV-TB co-infected individuals to control *Mtb* infections [14] [15] [42]. This is a major health risk since it was shown that most HIV patients with XDR-TB die with a median survival time of 18 days post infection [42].

1.2 Mycobacteria.

Mycobacterium is a genus of bacteria that belongs to the family of Mycobacteriaceae and contains more than fifty well-characterized species, which vary in their environmental niches, their physical growth, and their ability to cause disease in different hosts [43] [44]. They are intracellular, rod-shaped, aerobic, Gram-positive bacilli known for their thick cell walls rich with lipids and unique mycolic acids. They retain the carbol fuchsin dye in the presence of acidic alcohol in an acid fast staining [45]. Species in the mycobacterium genus are classified into three main groups for the purpose of diagnosis and treatment: *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium leprae*, and nontuberculous mycobacteria (NTM).

The *Mycobacterium tuberculosis* complex (MTBC) is a group of phylogenetically closely related sub-species sharing more than 99% identity at the nucleotide level for some loci and are known to cause TB in human and diverse animal hosts. These include the human pathogens *Mtb* and *Mycobacterium africanum* and the animal pathogens *M. bovis*, *Mycobacterium microti*, and *Mycobacterium canettii* [4] [43]. Bacteria in the MTBC are essentially clonal with little evidence of horizontal gene transfer with the exception of *Mycobacterium canettii* [4] [46]. These species are best adapted to their particular host species with a direct link between host-specific virulence and transmission despite the few incidences of animal-animal or human-animal transmission [4] [47]. For example *M. bovis*

used to be a significant cause of human TB primarily in children who consumed raw milk but its infection rate in humans has decreased since the introduction of pasteurization [48].

Mycobacterium leprae (*M. leprae*) is the etiological agent of leprosy (also known as Hansen's disease), a chronic granulomatous disease of the peripheral nerves and mucosa of the upper respiratory tract that has plagued human population for thousands of years [49]. *M. leprae*'s genome has been exposed to extreme reductive evolution reducing its genome size to almost half of the last common mycobacterial ancestor shared with *Mtb*. As a result, *M. leprae* have lost more than two thousand genes and only half of its genes remained functional [50] [51].

Nontuberculous mycobacteria (NTM), also called environmental mycobacteria or atypical mycobacteria, include all other mycobacterial species that do not belong to the MTBC or *M. leprae* group [52]. These species do not cause TB or leprosy. Members of this group include *Mycobacterium smegmatis* (*Msme*), *Mycobacterium fortuitum* (*Mfort*), *Mycobacterium kansasii* (*Mkan*), *Mycobacterium marinum*, and *Mycobacterium avium* (*M. avium*) in addition to many other species [53]. NTM are environmental species widely distributed in a variety environmental reservoirs such as water, protozoans, soil, marshland, rivers, and estuaries [53]. Disease in humans caused by NTM is believed to be acquired from environmental exposures. Unlike TB and leprosy, there has been no evidence of animal-human or human-human transmission in immunocompetent hosts [52]. Some species in the NTM group such as *Mkan* and *M. avium* may cause a variety of infections including pulmonary disease, the prevalence of which is increasing [54]. Nontuberculous mycobacterial species show significant variations in their growth rate, colony morphology, and virulence [55].

Based on the ability to cause disease in their host, mycobacteria can be organized into three groups: virulent pathogenic, opportunistic facultative-pathogenic, and non-pathogenic [45].

1.2.1 Virulent pathogenic mycobacteria.

Pathogenic virulent mycobacteria such as *Mtb* and *M. leprae* are known to cause serious disease in healthy humans [45] [49]. *Mtb* is the primary etiological agent of pulmonary TB and is the most virulent member of the MTBC. *Mtb* is a rod-shaped, aerobic, Gram-positive bacillus. Like other mycobacteria species, it has a very thick cell wall that contains a large number of mycolic acids, peptidoglycans, arabinogalactans, lipoarabinomannans, and phosphatidylinositol mannosides [56]. These components of the *Mtb* cell wall are shown to assist the bacillus in evading the host IR as well as resisting therapeutic antibacterial compounds [57] [58].

1.2.2 Opportunistic facultative-pathogenic mycobacteria.

Other mycobacterial species are considered opportunistic facultative-pathogenic. These species such as *M. avium*, *M. kansasii* (*Mkan*), and even the vaccine strain BCG can cause disseminating infections in humans that are defective in the adaptive IR, but unlike *Mtb*, do not cause disease in healthy individuals [59]. *M. avium* and *Mkan* are often to be found as opportunistic pathogens in immunosuppressed individuals due to AIDS [59]. The cell wall of these bacteria contains some modifications to its lipids and mycolic acids that are different from *Mtb* [60].

Mkan is a biosafety level (BSL) -2 organism and was first isolated from human pulmonary secretions [61]. It is considered the most virulent among environmental NTM and the second most common nontuberculous mycobacteria isolated from clinical specimens after *M. avium* [62] [63]. It can cause pulmonary disease in immunocompetent patients with preexisting pulmonary conditions like asthma and chronic obstructive pulmonary disease as well as disseminating extrapulmonary infections in immunocompromised humans with various immunodeficiencies and chronic illnesses such as AIDS patients [59] [64]. *Mkan* causes symptoms that are similar to those of *Mtb* infections, however, like other NTM, it does not transmit to other hosts and is sensitive to antibiotics [63].

1.2.3 Non-pathogenic mycobacteria.

Mycobacterial species such as *Msmc* and *Mfort* are considered non-pathogenic since they do not cause disseminating infections even in immunocompromised and immunosuppressed individuals [59]. However, *Msmc* and *Mfort* have been implicated to be causative agents of community-acquired and health care-associated diseases [59]. *Msmc* was first isolated from genital secretions (smegma) of a patient with syphilis in 1885, hence the name 'smegmatis' [65] [66]. It was considered to be an environmental saprophyte of no clinical significance until a case of lung disease in a patient with underlying lipoid pneumonia caused by *Msmc* was reported in 1986 [59] [67]. It is known to be a cause of localized non-disseminating infections of community-acquired disease involving cellulitis, localized abscesses, and osteomyelitis of wound sites following a traumatic event [68]. *Msmc* has also been implicated in health care-associated diseases

including sporadic cases of infected pacemaker site, catheter sepsis, sternal wound infection following cardiac surgery, and infections following plastic surgery [59] [67] [69]. *Msmc* and *Mfort* contain modifications to their cell lipids and mycolic acids that are implied in their severe attenuation [70] [71]. These bacilli are fast growers when compared to pathogenic and facultative-pathogenic mycobacteria. They double at a fast rate of approximately 3 hours compared to 24 hours for *Mtb* and BCG [66]. Despite the fact that mycobacterial species share the same mycobacterium ancestor, non-pathogenic environmental species have a larger genome size (approx. 6Mb) than pathogenic *Mtb* (approx. 4Mb) [59]. This could be attributed to *Mtb* undergoing genome downsizing and extensive lateral gene transfer to become a specialized pathogen of humans without retaining an environmental niche. Conversely, non-pathogenic environmental species like *Msmc* have maintained a relatively large genome in order to retain their capacity to resist the various environmental stresses and survive in an environmental niche [72].

Msmc can be a useful model to study certain aspects of mycobacterial molecular mechanisms of *Mtb* proteins since it grows fairly rapidly and is a BSL-2 organism even after transfection with genes from *Mtb* [73]. *Msmc* also has a number of properties that potentially make it an effective vaccine platform against a variety of bacterial and viral diseases [74] (section 1.2.4).

1.2.4 *M. smegmatis*, a potential vaccine platform.

Msmc has several properties that may make it an effective vaccine vector. Many *Msmc* strains do not cause any disseminating disease and are commensal in humans [73] [75]. The bacillus is also rapidly killed by lysosomal proteases in the phagolysosome of

infected cells unlike other mycobacterial species such as the vaccine strain BCG, which survives in infected cells for months by inhibiting the fusion of the phagosome with lysosomal vesicles [76-80]. Furthermore, *Msmc* induces cytokine production by host macrophages better than pathogenic mycobacteria [71] [81-83] [84] and can activate and induce the maturation of dendritic cells (DC) more efficiently than BCG by upregulating major histocompatibility complex (MHC) class I and costimulatory molecules [85]. *Msmc* is shown to be more efficient than BCG in accessing the MHC-I pathway for presentation of mycobacterial antigens [86]. These immunogenic properties of *Msmc* may make it a potentially good vaccine platform that is needed for the elicitation of cell-mediated immunity. Several groups highlighted the prominent potential of recombinant *Msmc* expressing protective antigens of different pathogens and molecules that modulate the IR as a vaccine vector and/or immunotherapy against viral infections such as HIV [74] [87] and Hepatitis B [88], cancer treatment [85] [89], and bacterial infections such as *Mtb* [90-92] and *Helicobacter pylori* infections [93].

1.3 Immune response and mycobacteria.

The exposure to foreign bodies and microbial infections induces an immune response (IR) in the host. The IR is divided into two categories, the innate IR and the adaptive IR (also called acquired IR). The innate IR is the first defensive response that manifests in the host upon encountering the microbe. It is fast, non-specific, localized to the site of infection, and does not confer any chronic protection [94] [95]. In contrast, the adaptive IR is delayed and requires the mounting of an initial innate IR [96] [97]. It is specific and targeted toward the type of infectious microbe. The adaptive IR manifests in

two types, cellular or humoral [98]. Virulent pathogens like *Mtb* have the capacity to manipulate the host IR in order to survive and cause disease while less pathogenic bacteria like *Msmc* are less potent in such manipulation [99]. In the following section we review the fundamentals of the IR in mammals as well as the interaction of mycobacterial species with the immune system.

1.3.1 The immune system.

1.3.1.1. *Cytokines.*

Cytokines play a major role in the regulation of the IR. They are cell-signaling protein molecules secreted by a variety of immune cells in order to regulate the IR, recruit other immune cells (property of chemokines), and shift the IR in either a humoral or a cell-mediated direction [97] [100] [101]. Proinflammatory cytokines such as the tumor necrosis factor (TNF) [102], interferon- γ (IFN- γ) [103], IL-1 β [104], IL-18 [104], and IL-12, the latter being proinflammatory during the primary IR [105], are all important in the IR against mycobacterial infections. IL-12 is secreted by macrophages upon infection and leads to the stimulation of T cells and the release of IFN- γ during the primary IR [105]. IFN- γ in turn activates macrophages making them more efficient in killing pathogens [106]. TNF is secreted by infected macrophages and stimulated T cells and can further activate macrophages, which may secrete IL-12 to further stimulate the T cells [105] [107]. IL-1 β and IL-18 are secreted by macrophages, DCs, and neutrophils via a pathway that involves activation of the inflammasome (section 1.5) and lead to the induction of IFN- γ secretion, angiogenesis, and neutrophil influx to remove cellular debris [108]. All of these

cytokines are required for an efficient IR against *Mtb* infections since mice deficient in any of them are highly susceptible to *Mtb* infections [102] [106] [109].

1.3.1.2 Pattern recognition receptors (PRR).

Pattern recognition receptors (PRR) are cell surface or cytosolic receptors that specifically recognize pathogen associated molecular patterns (PAMP), which are secreted by or expressed on the surface of microbial pathogens and not by mammalian cells [110]. PRRs are classified into two categories: cell surface PRRs and cytosolic PRRs.

Cell surface PRRs.

The mammalian family of toll like receptors (TLR) is comprised of PRRs expressed on the cell surface. TLRs are vital to the initiation of the innate IR leading to activation of the adaptive immunity [111]. TLR signaling upon sensing of microbial PAMPs on the cell surface leads to the activation of transcription factors such as nuclear factor κ B (NF- κ B), which are required for the expression of genes important for the IR [112]. *Mtb* cell wall components such as the mannose-capped lipoarabinomannans (Man-LAM) are shown to bind TLR-2 of DCs, which is shown to play important role in the IR against *Mtb* [113-115].

Cytosolic PRRs.

Other types of PRRs are localized to the cytoplasm. Unlike TLR, these receptors consist of soluble proteins that survey the cytoplasm for signs that indicate the presence of intracellular invaders. These include the nucleotide-binding domain leucine rich repeat (NLR) family of proteins, the PYHIN family proteins such as absent in melanoma-2

protein (AIM2), and the Rig-like helicases (RLH) [110]. Cytosolic PRRs can sense microbial components in the cytoplasm and lead to the assembly and activation of the inflammasome. Inflammasome activation then leads to the activation of proteases such as caspase-1, which can cleave pro-IL-1 β and pro-IL-18 into their mature secreted form [108] (section 1.5). IL-1 β and IL-18 induced secretion upon inflammasome activation are shown to play an important role in the IR against mycobacterial infections [116-123]

1.3.1.3 Cell types involved in innate immunity.

The innate IR is not specific to a particular pathogen. It depends on a group of proteins and immune cells that recognize conserved features of pathogens and become quickly activated to help destroy invaders [124]. The main cell types involved in the innate IR are the natural killer cells, eosinophils, basophils, and the phagocytic cells including monocytes, mast cells, macrophages, DCs, and neutrophils [124]. Here I will briefly discuss the two most important ones, which are most relevant for the purpose of our study: macrophages and dendritic cells.

Macrophages.

Macrophages are “professional” phagocytes. They are the result of the differentiation of monocytes in tissues. Monocytes originate in the bone marrow from a common myeloid progenitor, circulate in the blood, then migrate from the circulation into tissues throughout the body to differentiate into macrophages or DCs [125] [126]. Through their phagocytic capacity, macrophages remove cellular debris, dead tissue, dying cells (necrotic or apoptotic), as well as foreign bodies such as microbial pathogens [127]. They

express a multitude of cell-surface receptors such as the scavenger receptors and TLRs. They also express intracellular receptors such as the NLRs. These receptors can detect signals from foreign bodies and microbes as well as signals that are not normally found in healthy mammalian cells [126] [127]. Macrophages are divided into subpopulations based on their anatomical location and functional phenotype, which include tissue homeostasis and wound healing, host defense against pathogens, and immunoregulatory functions. These subpopulations include bone-resident macrophages (osteoclasts), alveolar macrophages in the lungs, histiocytes in connective tissue, Kupffer cells in the liver, and microglial cells in the central nervous system in addition to many others in different tissues [125]. Based on their functions as proinflammatory and microbicidal or immune suppressors and tissue repairers, macrophages are divided into two subsets: classically activated macrophages (M1) and alternatively activated macrophages (M2). M2 macrophages include all other macrophages that are not classically activated including ‘regulatory’ macrophages, tumor associated macrophages (TAM), and myeloid-derived suppressor cells (MCSC) [126] [128]. M2 macrophages exhibit immune suppressive activity while M1 macrophages exhibit proinflammatory and microbicidal activity [128] [129] [129]. Here we will briefly review the M1 and M2 macrophages. M1 macrophages are of great importance to our discussion since they are one of the main types of phagocytic cells that are involved in the IR against mycobacterial infections [128].

Classically activated macrophages (M1).

Tissue-resident resting macrophages can engulf pathogens and foreign bodies via phagocytosis leading to their sequestration inside vacuoles called phagosomes. The

phagosome then matures, acidifies, and fuses with lysosomal vesicles to form the phagolysosome. The degradative enzymes contained in lysosomal vesicles, now in the phagolysosome, result in the breakdown of the phagosomal contents and the killing of sequestered microbes [130]. At this point macrophages are called 'naïve' since they have not been activated yet. Naïve macrophages become classically activated by exposure to two signals. Signal 1 consists of the ligation of IFN- γ (secreted by other activated macrophages and T cells) to surface receptors, which primes naïve macrophages for activations. The second signal (signal 2) is exogenous TNF binding to TNF receptor (TNFR) or an inducer of TNF such as TLR signaling via the ligation of PAMPS to TLR, which induces endogenous TNF production by the macrophage itself [131]. The macrophage then upregulates its antimicrobial capacity and produces nitric oxide (NO) and reactive oxygen species (ROS) [130]. NO and ROS are antimicrobial agents important for the killing of pathogens [132].

M1 Macrophages are also potent antigen presenting cells (APC). Once activated, they can upregulate the expression of major histocompatibility complex (MHC) class II molecules, which can present bacterial antigens on the cell surface for recognition by and activation of T cells [133]. Macrophages are the primary host cell for mycobacteria, especially alveolar macrophages, which are the main bacterial host in pulmonary TB [134].

Alternatively activated macrophages (M2).

Naïve resident tissue macrophages have the ability to respond to external stimuli very rapidly leading to drastic alterations in their gene expression. This rapid response leads to the activation of different types of macrophages [135]. The activation of naïve

macrophages can take a different path and result in alternatively activated macrophages (M2) that have regulatory and anti-inflammatory functions, which are different from the functions of classically activated macrophages. These M2 macrophages fail to produce NO and are compromised in their ability to limit the growth of intracellular pathogens [136]. They are also not efficient APCs and can inhibit T Cell proliferation. They secrete anti-inflammatory interleukin (IL)-10 and IL-1 receptor antagonist [131]. M2 macrophages are formed in the presence of IL-4 and/or IL-13, which are secreted primarily by T helper type-2 (Th2) cells [137], distinct from IFN- γ -mediated classical activation. M2 macrophages have been implicated in a range of physiological and pathological processes including tissue homeostasis, wound repair, regulation of inflammation, angiogenesis, metabolic functions, and tumorigenesis. They also play an important role in immunity against parasitic infections [136], allergic responses, and hypersensitivity [138].

Dendritic cells (DC) and antigen presentation.

Dendritic cells represent a heterogeneous cell population and reside in peripheral tissues especially at sites of interaction with the environment like the skin, mucosae, and lymphoid organs. They are phagocytic cells and the main APCs of the immune system. Hematopoietic stem cells in the bone marrow give rise to DC precursors that enter the circulation then reside in tissues as immature phagocytic cells [139].

DCs capture exogenous antigens by phagocytosis, macropinocytosis, or via interaction with a variety of cell surface receptors (mostly C-type lectin family members) and endocytosis. DCs also process endogenous antigens from intracellular bacteria, viruses, or host's cell own proteins. Antigens get processed and presented on MHC class -I or -II molecules on

the surface of DCs. The cells then migrate to lymphoid tissue where they present antigens loaded on MHC molecules to CD4⁺, CD8⁺ or memory T cells; thereby initiating immune responses [140]. Two types of antigen presentation pathways exist, notably MHC-I and MHC-II antigen presentation.

MHC-I antigen presentation involves the presentation of endogenous cytoplasmic antigens derived from intracellular bacteria, viruses, or the host cell's own proteins. Antigens in the cytosol are digested by an enzyme complex called the proteasome, then are transported to the endoplasmic reticulum (ER) where they get mounted on MHC-I molecules and exported to the cell surface through the Golgi apparatus for presentation to naïve CD8⁺ T cells in order to activate them and generate effector cytotoxic T lymphocytes (CTL) [141] [142]. The MHC-I antigen presentation enables the immune system to detect infected or transformed cells displaying proteins from pathogens or modified-self, respectively, on their surface MHC-I molecules [143].

MHC-II antigen presentation involves the presentation of exogenous antigens contained in phagosomes or endosomes following phagocytosis or endocytosis of bacteria, parasites, and toxins. These antigens are processed in the endosome via the activity of lysosome-associated enzymes, then get loaded on MHC-II molecules and trafficked to the cell surface for presentation to CD4⁺ T-helper cells [142].

Exogenous antigens contained in endocytic compartments can also be loaded on MHC-I molecules for presentation to CD8⁺ T cells via a process called cross-presentation. Exogenous antigens, which are classically processed in the phagosome or endosome and loaded on MHC-II molecules, are instead loaded on MHC-I molecules during cross-presentation. This cross-presentation is reported to occur via two different pathways:

‘cytosolic’ and ‘vacuolar’ pathways. The ‘cytosolic’ pathway involves the processing of exogenous antigens by the proteasome and transport of peptides to the ER where they get loaded on MHC-I molecules, which then get transported to the cell surface. By contrast, the ‘vacuolar’ pathway is suggested to involve the processing and loading of antigens on MHC-I molecules in endocytic compartments [143].

Mycobacterial antigens in apoptotic bodies, which result from the apoptotic death of infected cells can be taken up by APCs such as DCs and get cross-presented by MHC-I molecules to CD8⁺ T cells. This cross-presentation by DCs to CD8⁺ T cells, which leads to the activation and differentiation of CD8⁺ T cells into cytotoxic lymphocytes, is important during *Mtb* infections since DCs were shown to be necessary for the activation of tuberculosis-specific T cells [144], and are also shown to be bacterial hosts [145].

1.3.1.4 Cell types involved in adaptive immunity.

Mammalian cells involved in the adaptive IR include two types of lymphocytes, notably B cells and T cells. Unlike phagocytic cells of the innate IR, B cells and T cells specifically recognize and mount a targeted IR against antigens [146].

B cells

B cells are lymphocytes that can recognize antigens via interaction with their B cell receptors and differentiate into specialized plasma cells. Plasma cells produce antibodies that can recognize specific antigens and lead to their neutralization. This type of adaptive IR is called humoral IR [146]. We will not discuss B cells in detail since they are not

relevant for the purpose of this discussion as the host IR against mycobacteria do not involve B cells and humoral immunity.

T cells

T cells are important for another type of adaptive IR called cell-mediated response. They express specific receptors called T cell receptors (TCR) on their cell surface, which specifically bind to antigens presented on MHC molecules of APCs leading to a localized IR [147]. T cells exist in two distinct classes: helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$).

$CD4^+$ helper T cells do not possess any cytotoxic or phagocytic capabilities. However, they secrete cytokines (IL-2, IL4, and IFN- γ), which activate leukocytes to kill pathogens. Once stimulated, $CD4^+$ T cells differentiate into either Th1 or Th2 helper T cells depending on the cytokine environment [148]. Th1 helper T cells secrete IFN- γ , which activates macrophages and stimulate B cells to secrete specific antibodies required for the opsonization and neutralization of antigens via an inflammatory cell-mediated immunity (Th1 response). Th2 helper T cells secrete IL-4, which stimulates B cells to secrete antibodies used in the neutralization of antigens via anti-inflammatory humoral immunity (Th2 response) [148]. $CD4^+$ T cells are important during *Mtb* infections. TNF and IL-12 secreted by *Mtb*-infected macrophages lead to the stimulation of $CD4^+$ T cells to secrete IFN- γ , which activates macrophages rendering them more efficient in killing the bacilli [149].

$CD8^+$ T cells, also called cytotoxic T cells, possess TCRs that recognize antigens presented on MHC-I molecules on the surface of APCs. This specific recognition leads to

their activation and the release of cytotoxins (perforin, granzymes, and granulysin), which form pores in the plasma membrane of infected cells. Cytotoxins can then penetrate infected cells and lead to their killing via apoptosis. CD8⁺ T cells also secrete IFN- γ , which activates macrophages [150] [151].

Both types of T cells (CD4⁺ and CD8⁺) are important for the IR against *Mtb* since mice deficient in either cell type are more susceptible to *Mtb* infections [152] [153].

1.3.2 Manipulation of the immune response by *Mtb*.

Mtb, through evolution, has developed many mechanisms to evade the host IR and persist inside its macrophage host. These mechanisms include inhibition of the fusion of the phagosome with the lysosome, resistance to ROS and reactive nitrogen intermediates (RNI), inhibition of host cell apoptosis, and formation of granulomas.

1.3.2.1 *Inhibition of phagolysosome fusion.*

Mtb is known to actively inhibit the fusion of the phagosome-containing bacilli with lysosomal vacuoles [154]. Such fusion, if allowed, delivers lysosomal degradative enzymes to the phagosome leading to the killing of the bacillus. This inhibition is suggested to involve *Mtb* cell wall component Man-LAM [152] [153] and trehalose dimycolate [155], which along with other proteins [156] [157] inhibit the phosphoinositide 3 (PI3) kinases preventing the accumulation of PI3P necessary for the recruitment of early endosome antigen 1 (EEA1). This inhibition prevents SNARE from interacting with EEA1 leading to a defect in endosomal trafficking and inability of the lysosome to fuse with the phagosome [157]. This inhibition leads to hindered antigen processing and less presentation of *Mtb*-

antigens on MHC-II, and/or MHC-I molecules via cross presentation [158] since mycobacterial antigens remain enclosed in phagosomes and are not released outside the phagosome. The formation of *Mtb*-containing apoptotic bodies released in the extracellular environment following apoptosis of infected cells is shown to compensate for this impediment by presenting mycobacterial antigens to neighboring bystander APCs [144].

1.3.2.2 Resistance to ROS and RNI.

Virulent *Mtb* has the capacity to neutralize the ROS produced by the host cell NADPH oxidase (NOX) -2 complex in the phagosome. This neutralization of ROS is accomplished via the secretion of bacterial superoxide dismutase (SOD) A and C [159] [160], and catalase G (KatG) in the phagosome. These bacterial enzymes are shown to be important for the virulence of *Mtb* as deletion or silencing of any of them results in attenuated mycobacteria [29] [161]. *Mtb* also possesses anti-apoptotic genes such as *nuoG*, which inhibits the accumulation of ROS in the phagosome, prevents the increase of TNF secretion, and inhibits host cell apoptosis [162] [163].

Furthermore, *Mtb* is also shown to be able to resist the effects of iNOS-dependent RNIs, which are toxic to the bacteria. The activation of macrophages with IFN- γ results in the induction of iNOS and the production of bactericidal NO [164]. Nevertheless, *Mtb* expresses a four-proteins NADH-dependent peroxynitrate reductase complex that enables it to defend itself against RNIs. The deletion of one these proteins, *dlaT*, resulted in an attenuated *Mtb* with diminished growth *in vitro* and high susceptibility to killing by RNIs [165]. In addition, the proteasome of *Mtb* was shown to be required for the resistance to RNI since deficiency in proteasomal adenosine triphosphate resulted in the attenuation of

Mtb in vivo and exposure of *Mtb* to proteasomal protease inhibitor rendered the bacillus more susceptible to RNI [166] [167]. Other mycobacterial proteins have also been implicated in the resistance of *Mtb* to RNIs including MsrA and MsrB. *Mtb* deficient in both proteins is shown to be more susceptible to RNIs [168].

1.3.2.3 Inhibition of host cell apoptosis.

Apoptosis plays an important role in the innate and adaptive IR against intracellular pathogens [169]. The apoptotic but not necrotic response of macrophages infected with intracellular mycobacteria could lead to the killing of the bacteria via different mechanisms. Intracellular mycobacteria could be directly killed when their host macrophage undergoes apoptosis [170]. Furthermore, apoptotic vesicles containing mycobacteria can be phagocytosed by stimulated uninfected bystander macrophages, which can then kill the bacteria more efficiently. These bystander macrophages get stimulated via cell surface receptor signaling induced by the binding of TNF and IFN- γ [171]. In addition, mycobacterial antigens in apoptotic bodies can be taken up by APCs such as macrophages and DCs and get cross-presented by MHC-I and CD1b molecules to CD8⁺ T cells which leads to the activation and differentiation of CD8⁺ T cells into tuberculosis-specific cytotoxic lymphocytes [171]. *Mtb* has the capacity to inhibit host cell apoptosis as one of the mechanism employed by the bacillus to resist the host IR and be able to hide and persist within macrophages and DCs [169]. This inhibition of apoptosis by *Mtb* was shown to involve several mechanisms. First, *Mtb* can down-regulate the expression of Fas ligand (FasL) expression leading to diminished Fas-mediated apoptosis of host cells [172] (section 1.4.1.3) . *Mtb* is also reported to induce the production of soluble TNF receptor (TNFR)-2,

which then competes with TNFR-1 for TNF binding and signaling [173]. TNFR-1 signaling is important for the induction of apoptosis in the extrinsic apoptosis pathway [174] (section 1.4.1.3). Furthermore, virulent *Mtb* can also up-regulate the expression of host anti-apoptotic genes of the *Bcl-2* family such as *mcl-1* and *bfl-1* in infected cells [175]. *Mtb* also possesses anti-apoptotic genes such as *nuoG*, which inhibits the accumulation of ROS in the phagosome, prevents the increase of TNF secretion, and inhibits host cell apoptosis [163]. Other *Mtb* anti-apoptotic genes include *secA2*, which encodes a protein required for the production of SODA, catalase G (*katG*) and the protein kinase E gene *pknE* [176].

1.3.3 Non-pathogenic and facultative pathogenic mycobacteria and the immune response.

Non-pathogenic mycobacteria such as *Msmc* and *Mfort* are strongly attenuated and do not cause disseminating infections even in immunosuppressed individuals [59].

Facultative-pathogenic mycobacteria such as *Mkan* and BCG were shown to be less attenuated than non-pathogenic mycobacteria but less virulent than *Mtb*. They cause disseminating infections in humans that are defective in the adaptive IR, but unlike *Mtb*, do not cause disease in healthy individuals [59].

The capacity of infected macrophages to undergo apoptosis following infection is an efficient mechanism of the innate IR [169]. Host cell susceptibility or resistance to mycobacterial infections was suggested to be linked to the capacity of the infected macrophages to undergo necrosis or apoptosis, respectively [177].

Mycobacteria from the facultative-pathogenic and non-pathogenic groups were shown to induce higher levels of apoptosis in infected macrophages than virulent *Mtb*. Facultative-pathogenic mycobacterial species *Mkan* and BCG induce more apoptosis than virulent *Mtb* in infected primary human alveolar macrophages [178]. Non-pathogenic mycobacterial species *Msme* induces significantly higher levels of apoptosis in infected human macrophage-like cell line THP-1 cells [162] and J774 macrophages [163] than virulent *Mtb*.

The host-cell cytokine response during mycobacterial infections is regulated by mitogen-activated protein kinase (MAPK) pathways [179]. The facultative-pathogenic *M. avium* induced a profoundly different host cell signaling response when compared to the non-pathogenic *Msme* [82]. In particular, the infection with *Msme* led to an increased p38 and ERK1/2 MAPKs activity in mouse bone marrow derived macrophages (BMDM), which was necessary for increased TNF secretion [82]. In addition, sphingosine kinase, phosphoinositide-specific phospholipase C, and conventional protein kinase C were all implicated in *Msme*-induced activation of Erk1/2 [180]. One downstream target of the MAPK p38 was determined to be the transcription factor cyclic AMP response element binding protein (CREB), which was more activated in *Msme*-infected cells than *M. avium*-infected cells [181].

The characterization of the strong IR mounted by the host against facultative-pathogenic and non-pathogenic mycobacteria compared to the diminished IR against virulent mycobacteria (*Mtb*) is of great importance for the understanding and unveiling of mechanisms employed by virulent *Mtb* to manipulate the host IR in order to persist and cause disease. This comparative analysis is one of the main subjects of our study.

1.4 Programmed cell death.

Programmed cell death (PCD) is the death of cells in any form, provided that it is mediated by intracellular pathways [182]. PCD pathways include apoptosis, programmed necrosis, pyroptosis, pyronecrosis, and autophagy.

1.4.1 Apoptosis.

Apoptosis is a form of PCD employed by multicellular organisms for tissue development, selection of lymphocytes during development of the immune system, general homeostasis, and host defense against invading pathogens. During apoptosis, biochemical events lead to characteristic morphological cell changes and subsequent death. These changes include cell shrinkage and blebbing, nuclear fragmentation, chromatin condensation, fragmentation of chromosomal DNA, and the formation of an impermeable cellular envelope [183]. Following these events, the cell eventually disintegrates into vesicles called apoptotic bodies. Apoptotic bodies are then taken up by neighboring phagocytes such as macrophages, DCs, and neutrophils. In this fashion mycobacteria-infected cells that undergo apoptosis are able to sequester mycobacterial antigens in apoptotic bodies, which get ingested by neighboring APCs and get presented on MHC-I molecules leading to the activation of cell-mediated IR [171] [184].

Apoptosis is mediated by proteases called caspases, which can be activated via intrinsic or extrinsic pathways (Figure 2) [185].

1.4.1.1 Caspases.

Caspases are highly conserved cysteine-aspartic proteases responsible for the cleavage of a multitude of cellular substrates as part of the apoptosis pathway. Genes encoding caspases are expressed to yield an immature uncleaved form of the protein called pro-caspase. Inactive pro-caspases are cleaved by other proteases in order to become active [182]. Two types of caspases are involved in the apoptosis process: initiator caspases (caspases 2,8,9,10,) and effector caspases (caspases 3,6,7) [186] [187]. A third type includes inflammatory caspases (caspases 1,4,5 in humans and caspases 1,11,12 in mice) [188]. Initiator caspases are the first proteases to be activated in response to apoptotic stimuli. Many pro-caspases contains a caspase recruitment domain (CARD) and death effector domain (DED). CARD is required for the interaction between caspases while DED is required for the interaction of pro-caspases with the formed activation complex (DISC or apoptosome) in response to an apoptosis-inducing signal. Activated initiator caspases cleave effector pro-caspases to yield active effector caspases. The active effector caspases then cleave a cascade of intracellular proteins to carry out the cell death program [187] [189].

1.4.1.2 Intrinsic apoptosis pathway.

The intrinsic apoptosis pathway is activated in response to internal stress experienced by the cell such as extensive DNA damage, radiation exposure, or loss of survival factors [174] [187]. This pathway involves the mitochondria, which affected by internal stress, gets depolarized and its outer membrane gets permeabilized. This process is called mitochondrial outer membrane permeabilization (MOMP) [190]. In this pathway the

pro-apoptotic Bcl-2 family member proteins such as Bax, Bak, and Bok translocate to the mitochondria upon stimulation causing the release of cytochrome C from the mitochondria. Cytochrome C then binds to the apoptotic protease activating factor-1 (Apaf-1). Apaf-1 contains a CARD domain and can form the oligomeric apoptosome complex, which includes cytochrome c, Apaf-1, and procaspase-9. The apoptosome complex then activates the initiator caspase pro-caspase-9. This leads to cleavage and activation of caspase-9 and the initiation of a cascade leading to activation of caspase-3 and induction of apoptosis [174] [187] [190] (Figure2).

1.4.1.3 Extrinsic apoptosis pathway.

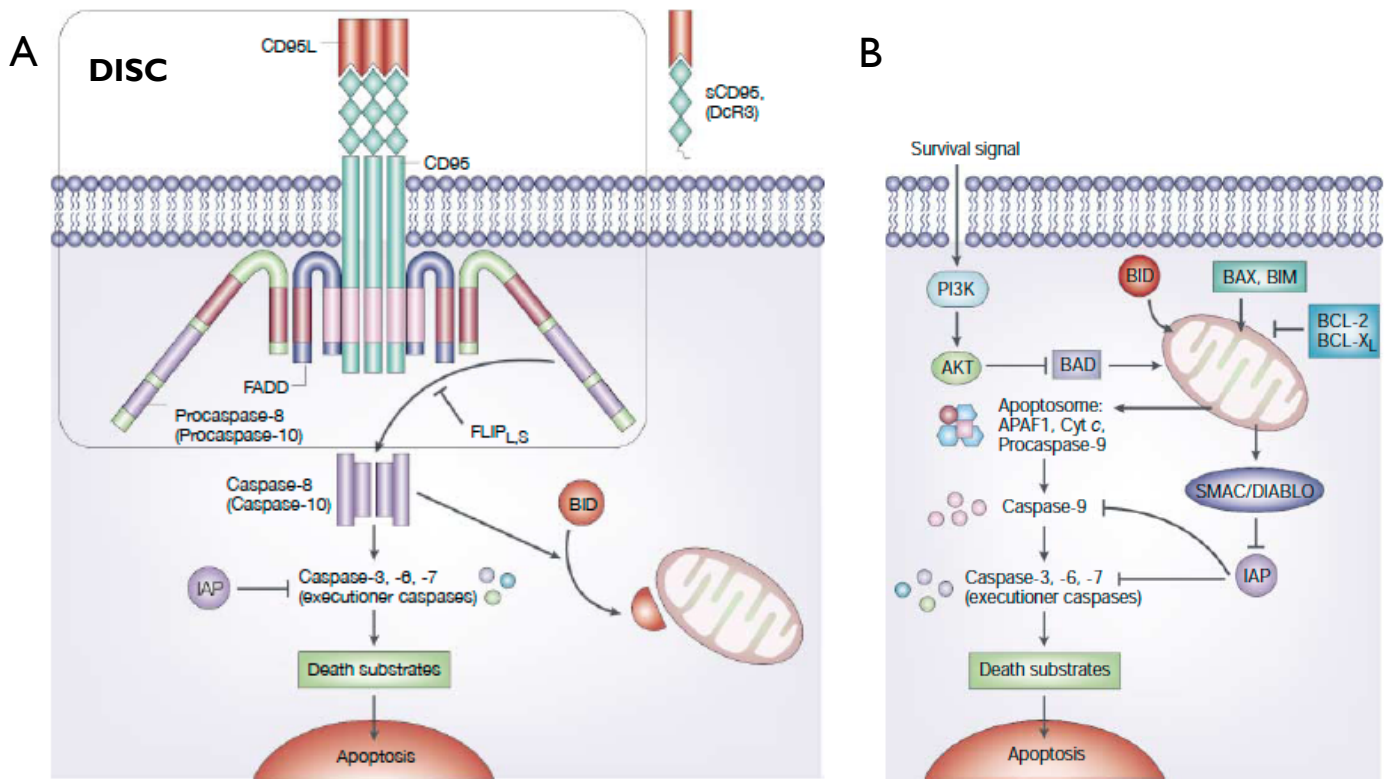
The extrinsic apoptosis pathway involves external signals and the binding of ligands such as Fas ligand (FasL) and TNF to cell surface receptors. FasL binds to Fas on the cell surface and leads to the activation and direct binding of Fas cytoplasmic death domain to Fas-associated death domain (FADD) [174]. In the case of TNF signaling, TNF binds to TNFR-1 on the cell surface via interaction of their death domains. Subsequently a TNFR-1 associated death domain (TRADD) binds to TNFR-1 and recruits FADD protein. In both pathways FADD recruits and binds to the initiator pro-caspase-8 to form a complex called DISC (death-inducing signaling complex). Pro-caspase-8 gets activated within the complex DISC then gets released to further activate effector caspase-3. The activation of caspase-3 is where both intrinsic and extrinsic apoptosis pathways converge and continue a cascade of events leading to cell death [174] [187] (Figure 2).

1.4.1.4 TNF signaling: dual role in apoptosis and survival.

In addition to being an inducer of apoptotic cell death, TNF is also important for the induction of proinflammatory response and cell survival. TNF binding to TNFR-1 may lead to recruitment and binding to the proteins RIP1, TRADD, TRAF2, and cIAP1 and activation of either Jun/Fos or NF- κ B, followed by gene transcription, and production of inflammatory mediators and survival proteins. On the other hand, TNF binding to the death receptor TNFR-1 may also initiate Jun N-terminal kinase (JNK) activation, which can inhibit TNF-induced apoptosis and lead to activation of caspase-8 and downstream effector caspases such as caspase-3 resulting in apoptosis [191] (Figure 3). It is important to note that intracellular levels of ROS can activate MAP kinases leading to JNK activation. Sustained JNK activation can shift the TNF signaling pathway toward host cell apoptosis instead of NF- κ B driven cell survival pathway [191]. This is significant since mycobacteria can inhibit the influx of ROS into the phagosome via the action of NuoG, which diminishes JNK activation and apoptosis [163].

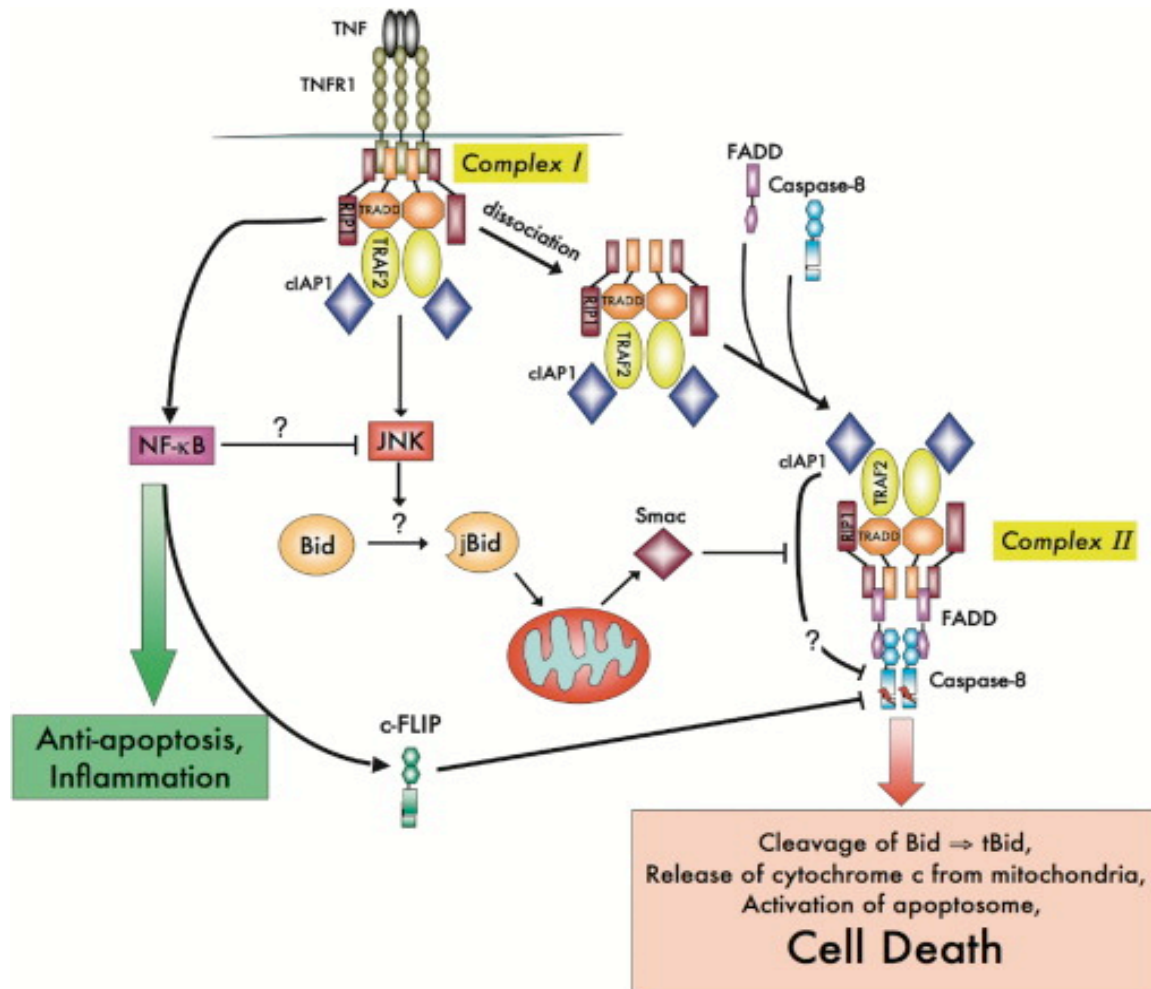
1.4.2 Caspase-independent apoptosis.

Caspase-independent apoptosis is defined as a cell death pathway that leads to DNA fragmentation and does not depend on caspases for initiation and activation. It is often mediated by proteolytic enzymes released from the mitochondria that are not dependent on caspases for activation. For example, Apoptosis inducing factor (AIF) [192-194] and endonuclease G [195], are enzymes released from the mitochondria and are involved in initiating a caspase-independent pathway of apoptosis leading to chromatin condensation and DNA fragmentation. Lysosomal enzymes like cathepsins are sometimes released from



Adapted from [174].

Figure 2. Extrinsic and intrinsic pathways of apoptosis. (A) The extrinsic apoptosis pathway. Upon Fas ligation (CD95 in the figure) FADD and procaspases-8 and -9 are recruited to the cytoplasmic side of the receptor forming the DISC complex. Caspases-8 and -10 become activated and go on to cleave and activate the effector caspases. (B) The intrinsic apoptosis pathway. In the presence of a death signal or the absence of pro-life signals, levels of the pro-apoptotic Bcl-2 family proteins (Bax, Bad, Bim) can overcome the number of anti-apoptotic proteins (Bcl-2, Bcl-xL) and induce MOMP. This mitochondrial permeabilization allows for the release of the pro-apoptotic proteins cytochrome C and SMAC/Diablo. Cytochrome C binds to APAF1 to create the apoptosome, which can bind to and activate the initiator caspase-9, which in turn activates the effector caspases-3, -6, and -7 to induce apoptosis. SMAC/Diablo can bind to inhibitor of apoptosis proteins that can inhibit caspase activation. Although these two pathways utilize different initiator caspases, they both converge at the level of caspase-3.



Adapted from [191].

Figure 3. TNF signaling via TNFR-1 leading to both cells death (apoptosis) and survival. Binding of TNF to TNFR-1 leads to recruitment of RIP1, TRADD, TRAF2, and cIAP1 to form complex I. Complex I transduces signals leading to NF-κB translocation. At later time points, RIP1, TRADD, and TRAF2 dissociate from TNFR-1 and recruit FADD and caspase-8 to form complex II. In the absence of NF-κB activity from complex I, complex II can initiate caspase-8 activation and cell death. NF-κB inhibits cell death through upregulation of antiapoptotic genes such as c-FLIP, which directly inhibits caspase activation in complex II or through suppression of JNK activity, possibly through upregulation of A20. Sustained TNFR-1-mediated JNK activation results in the cleavage of Bid to jBid, which then induces the preferential release of SMAC from the mitochondria. Released SMAC interferes with cIAP1-mediated inhibition of caspase-8.

'leaky' lysosomes and can induce apoptosis via a caspase-independent pathway [196-198]. *Mtb* has been shown to induce the lysosomal CIA pathway when infecting host cells at high multiplicity of infection (MOI) [199].

1.4.3 Autophagy.

Autophagy is a catabolic process that involves the degradation of the cell's own components through lysosomal degradation often in response to stress and/or starvation. It involves the sequestration of large cytoplasmic components and organelles in autophagic vacuoles and the subsequent digestion of the vacuoles contents by lysozymes upon fusion of the autophagic vacuoles with lysosomic vesicles [200] [201]. Macro autophagy, which involves the sequestration and digestion of most of the cytoplasm leads to cell death in a caspase-independent manner [202]. Autophagy also plays a role in the host defense against mycobacterial infections since *Mtb*-infected cells that undergo autophagy are shown to be able to reduce the viability of the bacilli [203] [204]. This is probably possible since autophagic vacuoles can sequester and digest *Mtb*-containing phagosomes leading to the killing of the bacteria. This process would overcome the need of the phagosome to fuse with the lysosome.

1.4.4 Necrosis.

In contrast to apoptosis, necrosis is sometimes considered to be passive and therefore un-programmed form of cell death. It is the premature death of cells induced by external factors such as infection, inflammation, toxins, trauma from injury, and oxygen deprivation [205]. Necrosis is independent of caspases and usually begins with cell

swelling, chromatin digestion, and disruption of the plasma and organelle membranes. At the nuclear level, necrosis is distinguished from apoptosis by the persistence of the DNA content, which remains uncondensed [206]. The integrity of the plasma membrane is another key difference between apoptosis and necrosis since necrosis results in the disruption of the plasma membrane and the expulsion of cytosolic content when the cell bursts while apoptosis maintains the integrity of the membrane [205]. This feature is important in the context of immune and inflammatory response since the necrotic cell death results in the expulsion of cellular components into the local environment. Some of these components can become proinflammatory. In addition, necrotic macrophages can release proinflammatory cytokines such as TNF and IL-12 [206].

1.4.5 Pyroptosis.

Pyroptosis is a newly identified PCD pathway that somehow resembles both apoptosis and necrosis. It is activated by microbial pathogens during inflammation including *Listeria* and *Salmonella* species. It is similar to apoptosis in that it is dependent on caspases and results in DNA damage. However, unlike apoptosis, it does not depend on classical apoptotic caspases (caspase-3, -8, and -9) but is rather dependent on caspase-1 [206]. Pyroptosis is similar to necrosis in that the plasma membrane breaks down. Recent studies implicated the involvement of the apoptosome, which is described as a large complex comprised of ASC (apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD)) dimers. This is important from an inflammation standpoint since ASC is an adaptor molecule that interacts with NOD-like receptors (NLR) and is required for the activation of most inflammasomes (section 1.5). This is in addition to the

activation of caspase-1, which is also a major caspase involved in inflammasome activation [108].

1.4.6 Pyronecrosis.

Pyronecrosis is another newly identified proinflammatory PCD pathway that depends on NRL, ASC, and Cathepsin B for activation. It resembles necrosis in that it does not depend on caspases, results in membrane degradation, does induce chromatin condensation, and does not involve DNA fragmentation [206].

1.5 Inflammasomes and mycobacteria.

Pathogens, upon invasion of the host, are first faced by the innate IR carried on by phagocytic cells such as macrophages, monocytes, and neutrophils [207]. The activation of macrophages and neutrophils, which leads to the release of bactericidal ROS and RNI, is dependent on proinflammatory cytokines such as IL-1 β and IL-18. These proinflammatory cytokines are secreted primarily from infected macrophages and monocytes. The inactive precursors of IL-1 β and IL-18 (pro-IL-1 β and pro-IL-18, respectively) require processing and cleavage by caspase-1 to become active. Caspase-1 is an inflammatory caspase that gets activated within a multimeric protein platform called the inflammasome [207]. In the following we review the components of inflammasomes, the mechanisms of inflammasome activation, and the interaction of mycobacteria with inflammasome components leading to IL-1 β secretion.

1.5.1 The inflammasome.

1.5.1.1. *Inflammasome components.*

The Inflammasome is a multimeric protein complex that classically (with some exceptions) contain a member of the nucleotide and oligomerization domain (NOD)-like receptor (NLR) family, the adapter molecule apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1 [208]. NLRs are cytosolic PRRs that, similar to cell surface TLRs, detect a variety of conserved microbial components and danger signals induced by microbes within intracellular compartments. These microbial components and danger signals are called pathogen associated molecular patterns (PAMP) and danger associated molecular patterns (DAMP), respectively. Examples of PAMPs and DAMPs sensed by NLRs are microbial cell wall components, microbial DNA, bacterial RNA, flagella, muramyl dipeptide (MDP), uric acid crystals, and β -amyloid [208] [209].

Another identified inflammasome called AIM2 contains a PYHIN protein family member called absent in melanoma-2 (AIM2) instead of an NLR [208]. NLRs share a domain organization that is usually comprised of an amino-terminal protein-protein interaction domain such as the caspase recruitment domain (CARD) or pyrin domain, a NACHT domain that is required for nucleotide binding and self oligomerization, and a number of carboxy-terminal leucine-rich repeat (LRR) motifs involved in sensing PAMPs and DAMPs [108] [210].

1.5.1.2 *Inflammasome activation and IL-1 β / IL-18 processing.*

ASC adapter protein plays an important role in the interaction between NLRs or AIM2 with caspase-1 [208]. Upon detection of cellular stress via the sensing of PAMPs

and/or DAMPs, NLR molecules oligomerize through homotypic interactions between NACHT domains leading to the exposure of their pyrin domain (PYD), which can then interact with PYD of ASC. As a result, CARD of ASC interacts with CARD of inactive pro-caspase-1 leading to auto-activation of caspase-1. Active caspase-1 can then cleave proinflammatory cytokines precursors pro-IL-1 β and pro-IL-18 into their mature active form (IL-1 β and IL-18) [108].

The activation of IL-1 β and IL-18 is a two-step process that requires two signals. Signal 1 involves the pattern recognition of microbes by host cells, which induces the expression and transcription of *pro-IL-1 β* and *pro-IL-18*. The second signal (signal 2) involves the activation of the inflammasome by DAMPs and/or PAMPs, which results in the activation of caspase-1 and the cleavage of pro-IL-1 β and pro-IL-18 to yield their mature active form [207].

1.5.1.3. Types of inflammasomes.

To date, several types of inflammasomes have been identified. However, only few are well characterized. They vary in the type of activating PAMPs or DAMPs from different microorganisms as well in some of their components. The most studied inflammasomes include NLRP1, NLRP3, IPAF (also known as NLRC4), NLRC5, NOD-1, NOD-2, and AIM2 inflammasomes [108] [208] [211].

NLRP3 inflammasome.

The NLRP3 inflammasome, also called cryopyrin or NALP3, is the most well-characterized inflammasome. It is expressed in myeloid cells and is up-regulated in

response to the stimulation of macrophages and DCs to PAMPs [212]. It consists of the cytosolic PRR NOD-like receptor-3 (NLRP3) proteins, which upon sensing of microbial components or danger signals gets oligomerized and exposes its PYD domain. The exposed PYD domain then interacts with PYD of ASC leading the activation of caspase-1 via CARD-CARD domains interaction. NLRP3 does not possess a CARD domain so it requires ASC to activate caspase-1, which only contain a CARD domain but no PYD domain [108]. The activation of caspase-1 via NLRP3 inflammasome activation leads to the activation and secretion of IL-1 β and IL-18 [108] (Figure 4).

The NLRP3 inflammasome has been shown to play an important role in the inflammatory response induced by several activators such as low intracellular potassium concentrations, *influenza A*, *Neisseria gonorrhoeae*, bacterial toxins, cholesterol crystals, monosodium urate (MSU), and mycobacterial infections [108], the latter being of great importance for the purpose of our analysis. NLRP3 inflammasome has also been implicated in metabolic disorders (obesity and type-2 diabetes) [213], cancer [214], intestinal inflammation [215], and liver injury [216]. In addition to its proinflammatory role in innate immunity, the NLRP3 inflammasome has also been suggested to play an important role in shaping the adaptive IR via the secretion of IL-1 β and IL-18 [108].

Several models have been described for the activation of the NLRP3 inflammasome: the channel mode, the lysosome rupture model, and the ROS model [108].

- The channel model involves the release of ATP at the site of cellular injury or necrosis leading to the activation of the P2X7 ATP-gated ion channel, which results in a rapid efflux of potassium ions from the cell. This efflux of K⁺ ions leads to the recruitment of pore-forming pannexin-1 hemichannels, which then allow extracellular NLRP3

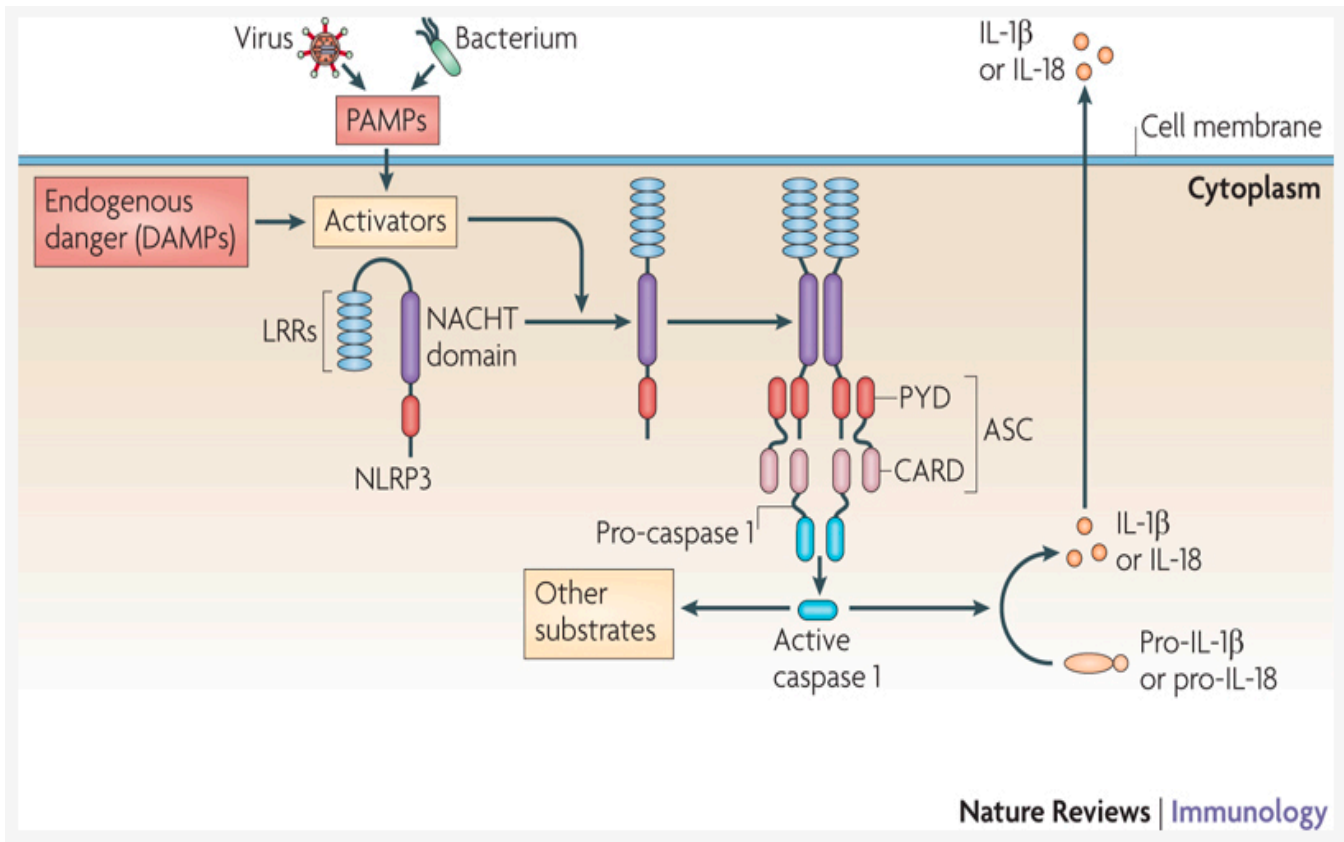
activators such as bacterial products to access the cytoplasm and activate NLRP3. This activation model only allows small activators to cross the plasma membrane into the cytoplasm but not large molecules [108].

- The lysosome rupture model involves inefficient clearance of activating particles via lysosomal digestion following phagocytosis, leading to the release of cathepsin B into the cytoplasm and activation of NLRP3 receptor molecules. This model of activation is efficient for large activator molecules as they are internalized via phagocytosis [108].

The ROS model is dependent on the generation of ROS. ROS generation is frequently accompanied by K^+ efflux and it is possible that low intracellular K^+ concentrations trigger ROS production and vice versa. Treatment with NLRP3 agonists leads to the association of NLRP3 with thioredoxin-interacting protein (TXNIP, also known as VDUP1) in a ROS-dependent manner. TXNIP is constitutively bound and inhibited by oxidoreductase thioredoxin. An increase in ROS concentration leads to the dissociation of the TXNIP-thioredoxin complex, which results in release of unbound TXNIP and subsequent binding to NLRP3 molecules and activation of the inflammasome [108].

AIM2 inflammasome.

The AIM2 inflammasome is activated upon sensing of double stranded DNA (dsDNA) in the cytoplasm, as observed with *Francisella* infections [217]. Unlike other NLR inflammasomes, which use an NLR family protein as a PRR, the AIM2 inflammasome uses a PYHIN family protein member called absent in melanoma-2 (AIM2) as the PRR for the sensing of microbial dsDNA. AIM2 is localized to the cytoplasm and it



Adapted from [108].

Figure 4. Mechanism of NLRP3 inflammasome complex formation. Under healthy cellular conditions, NLR family, pyrin domain-containing 3 (NLRP3) is auto-repressed owing to an internal interaction between the NACHT domain and LRRs. This auto-repression is removed in the presence of pathogen-associated molecular patterns (PAMPs) from microorganisms or damage-associated molecular patterns (DAMPs) from endogenous danger signals. This results in exposure of the NACHT domain. In turn, NLRP3 oligomerizes and recruits apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD) and pro-caspase-1, triggering the activation of caspase-1 and the maturation and secretion of proinflammatory cytokines such as interleukin-1 β (IL-1 β) and IL-18. Other cytoplasmic proteins, such as enzymes of the glycolytic pathway, are also substrates of active caspase-1. CARD, caspase-recruitment domain; LRRs, leucine-rich repeats; NACHT, NAIP, CIITA, HET-E and TP1; PYD, pyrin domain.

contains a PYD domain that engages ASC via PYD-PYD interaction leading to the activation of caspase-1 and subsequent maturation of pro-IL-1 β and pro-IL-18. A recent report suggested that AIM2 plays an important role in *Mtb* infections since mice deficient in Aim2 were more susceptible to *Mtb* infection than wild type (WT) mice [108] [212].

Other inflammasome types.

Other inflammasomes such as NLRP1, IPAF (also called NLRC4), have been described while others such as NLRP6, NLRP10, and NLRP12 are identified but not well characterized. IPAF requires ASC for the activation of caspase-1 since it contains a PYD domain but no CARD. NLRP1 unlike other NLRs contains both PYD and CARD domains and has the ability to either activate caspase-1 directly and/or via ASC [208] [212].

1.5.2 Mycobacteria-induced inflammasome activation and the role of IL-1 β in mycobacterial infections.

IL-1 β and IL-18 are shown to play an important role in the host defense against *Mtb* as was reported *in vivo* in studies where mice deficient in IL-1 receptor (IL-1R), IL-1 β or IL-18 were more susceptible to *Mtb* infections [116] [117] [122] [218] [219].

Inflammasome activation leads to secretion of IL-1 β and IL-18, which are important for the host defense against *Mtb* infections. *Mtb*-infected BMDMs and THP-1 cells induced IL-1 β secretion, which was dependent on ASC, NLRP3, and caspase-1 [220] indicating an important role for NLRP3 inflammasome in the immune response against *Mtb*. However, *in vivo* infections of mice deficient in *Asc* and *caspase-1* did not display any decrease in IL-1 β secretion compared to WT mice, and these knockout mice were less susceptible to *Mtb*

infections than *Il-1 β* knockout mice. Furthermore, a recent study showing that mice deficient in *Aim2* were more susceptible to *Mtb* infections than WT mice suggests an important role of AIM2 in the immune response against *Mtb* infections [221].

The discrepancy between *in vivo* and *in vitro* *Mtb*-induced secretion of IL-1 β suggests that cell types other than macrophages are involved in the processing and secretion of IL-1 β . These cell types might also involve mechanisms other than inflammasome activation leading to the secretion of IL-1 β . Our analysis of mycobacteria-infected DCs, which are also an important host for mycobacteria besides macrophages [145] [230-232], addresses these questions.

1.6 Specific aims and significance.

Mtb infects approximately two billion people worldwide, making it a major global health problem. The only available vaccine BCG was shown to be unreliable since it does not confer consistent protection in humans. Furthermore, the drug chemotherapies used against TB are becoming ineffective due the emergence of drug-resistant *Mtb* in addition to them being inconvenient due to the rather long 6-9 months treatment. It is therefore evident that the development of a new vaccine as well as new antibiotics capable of clearing TB infections are needed to keep fighting this disease and prevent it from spreading beyond control. *Mtb* has evolved to acquire mechanisms that allow it to manipulate the host IR, and hide and persist within the host. The pathogen inhibits host cell apoptosis, prevents the fusion of phagosome with lysosome, and resists ROS and RNI. In comparison, less pathogenic mycobacteria of the facultative-pathogenic group such as *Mkan* and BCG as well as non-pathogenic mycobacteria such as *Msmc* and *Mfort*, do not possess such

capacities to resist the host IR. Non-pathogenic *Msmc* and *Mfort* have been shown to be strongly attenuated and do not cause disseminating infections even in immunocompromised individuals. These mycobacterial species induce a very strong host innate IR, potent cytokine production by host macrophages, and can activate and induce the maturation of DCs for presentation of antigens on MHC molecules better than pathogenic and opportunistic mycobacteria such as *Mtb* and BCG, respectively. *Msmc* has also been shown to be a potentially good vaccine platform for several diseases like tuberculosis, cancer, AIDS, Hepatitis B, gastritis, and gastric ulcers. It is therefore important to exploit and further analyze the immunogenic properties of non-pathogenic mycobacteria as a strategy to study and better understand the mechanisms of the host innate IR. The comparison of non-pathogenic to more pathogenic mycobacterial species can also be used as a strategy to understand how pathogenic mycobacteria developed mechanisms to evade the host IR and cause disease. These analyses will also provide us with better strategies for the use of non-pathogenic mycobacteria as a recombinant vaccine vector for various microbial infections, viral infections, and diseases.

The focus of our research was the analysis of the mechanisms of the IR induced by non-pathogenic mycobacteria in macrophages and DCs, two main cell types involved in innate immunity and the induction of the adaptive IR. Therefore, we first aimed to analyze the mechanisms by which non-pathogenic mycobacteria strongly induce apoptosis, an important innate immune mechanism, in macrophages and dendritic cells. Then we aimed to analyze the molecular mechanisms of *Msmc*-induced IL-1 β secretion in DCs and macrophages.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials.

WT BALB/c and C57BL/6 mice were obtained from the National Cancer Institute. *Casp1^{-/-}*, *Nlrp3^{-/-}*, *Asc^{-/-}*, and *Il1r1^{-/-}* mice have been described elsewhere {Adachi, 1998 #118; Mariathasan, 2004 #119; Sutterwala, 2006 #120}. *Aim2^{-/-}* mice were obtained from Dr. Fitzgerald (University of Massachusetts Medical School). Caspase-specific inhibitors, cathepsin and calpain inhibitors, and their analogs were purchased from Calbiochem (www.emdbiosciences.com). Neutralizing anti-mouse TNF antibody was purchased from Peprotech Inc (www.peprotech.com). Anti-mouse IFN- β antibody was purchased from abcam (www.abcam.com). Anti-mouse dectin-1 antibody was purchased from InvivoGen (www.invivogen.com). All other reagents were purchases from Sigma (www.sigma.com) unless otherwise noted.

2.2 Bacteria and culture conditions.

M. smegmatis strain (mc² 155) was obtained from Dr. William Jacobs Jr. *M. fortuitum* strain (ATCC 6841) and *M. kansasii* strain Hauduroy (ATCC 12478) were obtained from the American Type Culture Collection (<http://www.atcc.org>). *M. bovis* BCG Pasteur strain was obtained from the Trudeau Culture Collection (Saranac Lake, New York, United States). *M. tuberculosis H37Rv* (ATCC 25618) and *M. tuberculosis H37Ra* (ATCC 25177) were obtained from Dr. W.R. Jacobs Jr. (Albert Einstein College of Medicine). *F. tularensis* Live Vaccine Strain (LVS) (ATCC 29684) was obtained from Dr. Kevin McIver (University of Maryland, College Park). GFP-expressing BCG and *M. smegmatis* were generated by subcloning the enhanced GFP gene (Clontech, <http://www.clontech.com>)

into the mycobacterial episomal expression vector pMV261. The resulting plasmid (pYU921) was transfected into competent cells by electroporation as previously described. *M. smegmatis*, *M. fortuitum*, *M. kansasii*, and *M. bovis* BCG were cultured in 7H9 broth with 0.5% glycerol, 0.5% dextrose, and 0.05% TWEEN-80. For selective media, 40 µg/ml kanamycin was added. *F. tularensis* LVS was cultured in MH broth supplemented with 0.015% anhydrous CaCl₂, 0.02% hydrous MgCl₂, 0.1% dextrose, 0.025% ferric pyrophosphate, and 2% Isovitalax supplement (Invitrogen, USA).

2.3 Cell culture conditions and infection.

For the apoptosis and IL-1β secretion assays, 5 x 10⁵ BMDMs and BMDCs in DMEM supplemented with 10% fetal calf serum, and 2% HEPES (infection media) were seeded on each well of a 24 well plates. Bacteria were grown to an OD₆₀₀ ranging from 0.2 to 0.8, passed through a 26 Gauge needle three times and allowed to settle for 10 minutes. The infection was carried out at a multiplicity of infection (MOI) of 1:1, 3:1, and 10:1 for the apoptosis assays and MOI of 10:1 for the IL-1β secretion assay. Infections were carried out for two hours in duplicate wells, after which extracellular bacteria were removed by three washes using 1x phosphate buffered saline (PBS). The cells were then incubated in DMEM infection medium supplemented with 100 µg/ml gentamicin (Invitrogen) for 20 hours and the apoptosis assay was performed.

2.4 Bone-marrow derived macrophages and dendritic cells.

BALB/c and C57Bl/6 mice were maintained under pathogen-free conditions and used between 6 to 12 weeks of age and sacrificed by CO₂ asphyxiation or isoflurane

inhalation followed by cervical dislocation in accordance with IACUC approved protocols. The anterior limbs were flushed with DMEM supplemented with 2% fetal calf serum. Flushed bone marrow cells were then pelleted and treated with 1x red blood cells lysis buffer (eBiosciences) for 10 minutes then washed with 1x PBS. For macrophage differentiation, cells were then plated on Petri dishes in DMEM supplemented with 10% heat inactivated fetal calf serum, 15% L cell-conditioned medium (LCCM), 1% Penicillin/Streptomycin, then incubated at 37°C / 5% CO₂. Cells were supplemented with additional medium on days 3 and 6. On day 7, all non-adherent cells were washed off and the remaining adherent bone marrow derived macrophages were seeded on appropriate plates for infection. To derive dendritic cells, cells were incubated in medium as described for macrophages but containing 20 ng/ml murine GM-CSF (Peprotech) instead of LCCM. 5 x 10⁶ cells/plate were added to Petri dishes containing 10 ml medium and an additional 9 ml medium/well were added on days three and six. On day 8, all non-adherent dendritic cells were collected and seeded on appropriate plates for infection.

2.5 Apoptosis assays.

In most of the experiments the flow cytometry-based hypodiploid assay was used for the detection of apoptosis after infection of BMDMs and BMDCs. Cells were collected after infection, pelleted and resuspended in propidium iodine (PI)/RNase buffer (BD Pharmingen) for 20 minutes and the percentage of hypodiploid-positive cells was determined by flow cytometry in duplicates in the FL-2 channel at 580 nm (FACS-Calibur, BD Biosciences). The TUNEL assay was performed to reveal apoptosis-induced DNA fragmentation in tissue culture cells using the In Situ Cell Death Kit, Fluorescein (Roche

Applied Science). The assay was carried out as described by the manufacturer and as described previously [162] and the percentage of stained cells was analyzed using flow cytometry.

2.6 Enzyme-linked immunosorbent assays (ELISA).

For the TNF, IL-12, IL-1 β , and pro- IL-1 β secretion assays, 5×10^5 BMDMs or BMDCs in DMEM infection media were seeded onto each well of 24 well plates (500 μ l/well) and infected with bacteria as previously indicated. The culture supernatants were then collected 20 hours after incubation in infection media supplemented with 100 μ g/ml gentamicin. The amount of TNF, IL-12, IL-1 β , and pro- IL-1 β in supernatants were then measured via ELISA (BD Bioscience for TNF and IL-12 detection, and eBioscience for IL-1 β and pro- IL-1 β detection).

2.7 ROS detection assays.

Reactive oxygen species (ROS) in BMDMs and BMDCs were detected at 2 hours post-infection using the ROS sensitive dye dihydroethidium (DHE) (Invitrogen). BMDMs or BMDCs were deprived of LCCM or rGM-CSF respectively 16 hours prior to infection and maintained in cytokine free media without phenol red for the length of the experiment. Post-infection, cells were washed once in HBSS and then incubated in 2 μ M DHE for 15 minutes. Cells were washed three times with HBSS, fixed with 4% paraformaldehyde and analyzed by flow cytometry.

2.8 Enzymes inhibition and protein neutralization assays.

BMDMs and BMDCs from BALB/c and/or C57Bl/6 mice were treated with a pan caspase -inhibitor (100 μ M), caspase-3 inhibitor negative control (100 μ M), caspase-1 inhibitor (100 μ M), caspase-8 inhibitor (100 μ M), caspase-9 inhibitor (100 μ M), cathepsin B/S/L inhibitor (100 μ M), and calpain inhibitor (100 μ M) (all from Calbiochem), anti-murine TNF neutralizing antibody (5 μ g/ml), isotype control antibody (5 μ g/ml) (both from BD Bioscience), anti-IFN- β neutralizing antibody (5 μ g/ml) (Abcam), anti-dectin-1 neutralizing antibody (5 μ g/ml) (InvivoGen), or pentoxifylline (Sigma, 100 μ g/ml) for one hour at 37°C / 5% CO₂ and infected with *M. smegmatis* at MOI 10:1 for two hours as previously described. Cells were then washed three times in 1x PBS and incubated for an additional 20 h in DMEM infection media supplemented with the appropriate inhibitors and controls and the apoptosis assay was performed.

2.9 Determination of infection rate and bacterial load.

For the rate of infection assay, BALB/c and C57Bl/6 BMDMs and BMDCs were infected with GFP-expressing *Msmc* and BCG for two hours at MOIs 3:1 and 10:1. At this point cells were washed and the number of infected cells was determined by flow cytometry (GFP fluorescence intensity shifts) (0 hours post-infection time point). A second set of cells were washed with 1x PBS and incubated for an additional 20 hours in infection medium supplemented with 100 μ g/ml gentamicin as previously described and the number of infected cells was determined by flow cytometry (GFP fluorescence intensity shifts) (20 hours post-infection time point).

For the bacterial load assay, BALB/c and C57Bl/6 BMDMs and BMDCs were infected with GFP-expressing *Msmc* and BCG for 2 hours at MOIs 3:1 and 10:1. At this point cells were washed and the numbers of colony forming units (CFU) in infected cells were quantified on agar plates (0 hours post-infection time point). A second set of cells were washed with 1x PBS and incubated for an additional 20 hours in infection medium supplemented with 100 µg/ml gentamicin as previously described and the numbers of CFUs in infected cells were quantified on agar plates (20 hours post-infection time point)

2.10 Statistical analysis.

Statistical analysis was performed on at least three independent experiments using GraphPad Prism 5.0 software and One-way ANOVA with Tukey 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: ns = not significant, * = $0.01 < p < 0.05$, ** = $0.001 < p < 0.01$, and *** = $0.0001 < p < 0.001$.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Mechanisms of non-pathogenic mycobacterial species *M. smegmatis* and *M. fortuitum* induced apoptosis in macrophages.

Non-pathogenic mycobacteria such as *Msmc* and *Mfort* were shown to be strongly attenuated [59]. Facultative pathogenic mycobacteria such as *Mkan* and BCG were shown to be less attenuated than non-pathogenic mycobacteria but also less virulent than *Mtb* [59].

The following studies address the mechanisms by which non-pathogenic mycobacterial species (*Msmc* and *Mfort*) induce a rapid host cell innate IR.

3.1.1 Non-pathogenic mycobacteria induce increased host cell apoptosis in macrophages compared to facultative-pathogenic mycobacteria.

In order to test the apoptotic response of macrophages when infected with non-pathogenic compared to facultative-pathogenic mycobacteria, we used bone marrow derived macrophages (BMDM) from BALB/c mice and infected them with *Msmc*, *Mfort*, *Mkan*, or BCG for duration of 2 hours. The cells were then washed and incubated in infection medium supplemented with gentamicin for an additional 20 hours. The percentage of apoptotic cells was determined by quantifying the fraction of cells in hypodiploid state via flow cytometry (Figure 5). 75-80% of BMDMs infected with *Msmc* and *Mfort* were hypodiploid positive, which was significantly different from BMDMs infected with facultative-pathogenic *Mkan* and BCG ($p < 0.001$) (Figure 5). Concurrently, BMDMs infected with BCG and *Mkan* did not show any significant increase in the levels of apoptosis compared to the untreated control (UT) cells during the entire course of this short-term 22 hours infection ($p > 0.05$) (Figure 5).

This observed difference in apoptotic induction is also conserved in human macrophage-like cells (THP-1 cell line). THP-1 cells are a good model for the study of the behavior of primary human alveolar macrophages in response to mycobacterial infections [222]. PMA (Phorbol myristate acetate)-differentiated THP-1 cells were infected with *Msmc*, *Mfort*, *Mkan*, or BCG for 2 hours then washed and incubated in infection medium supplemented

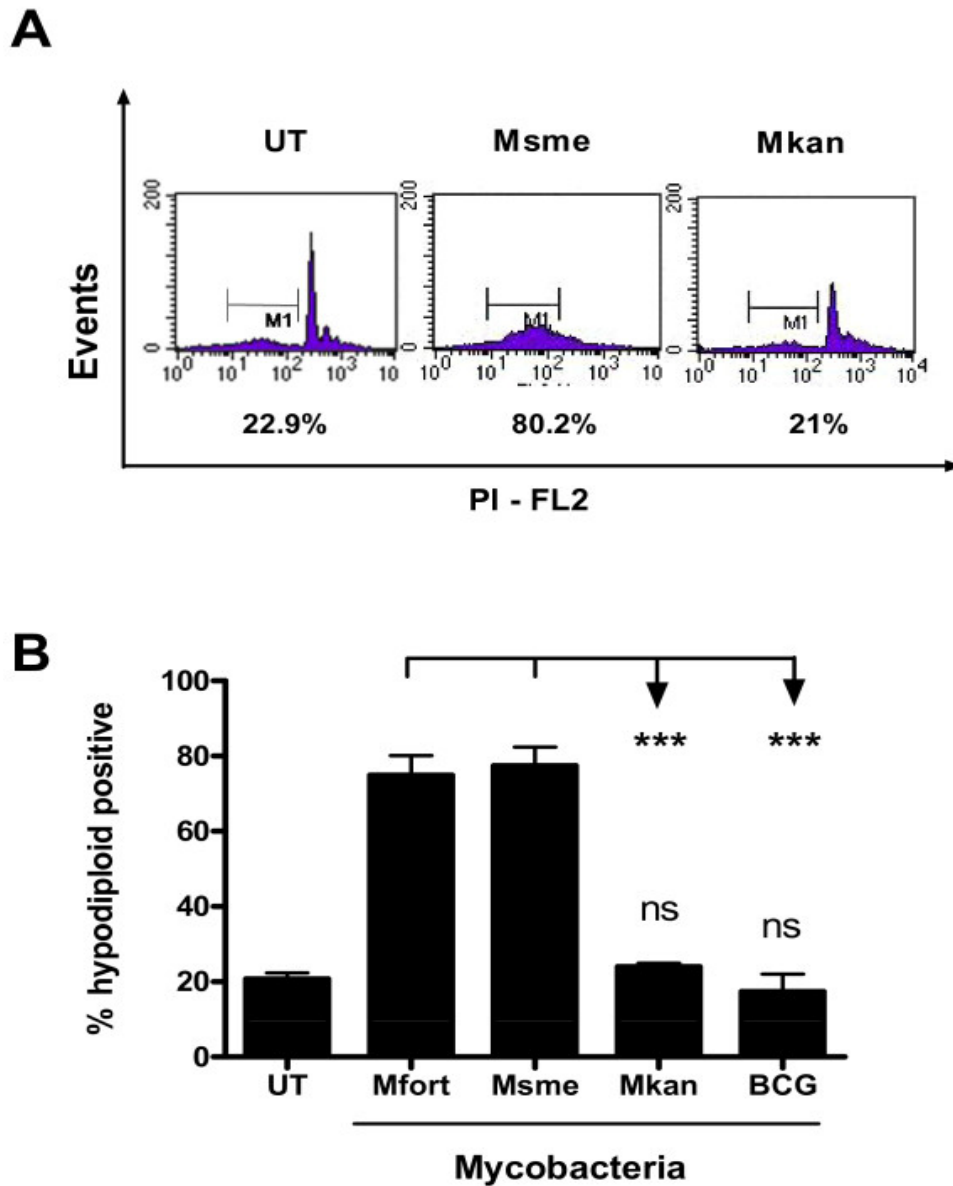


Figure 5. Differences in apoptosis induced by facultative-pathogenic versus non-pathogenic mycobacteria in primary murine macrophages. Differentiated BALB/c BMDMs were infected at an MOI of 10:1 with *M. smegmatis* (*Msme*), *M. fortuitum* (*Mfort*), *M. kansasii* (*Mkan*), *M. bovis* BCG or left untreated (UT). The percentage of apoptotic cells was determined using a propidium iodide (PI) based staining protocol to detect the population of hypodiploid cells via flow cytometry at 20 hours after infection. Representative histograms are shown in **A**. **B**. The average and standard deviation of three independent experiments is shown. For this and all subsequent figures asterisks indicate statistical significance with ns = $p > 0.05$, * = $0.05 > p > 0.01$, ** = $0.01 > p > 0.001$ and *** = $p < 0.001$ which was determined using one way ANOVA using GraphPad Prism 5.0 software.

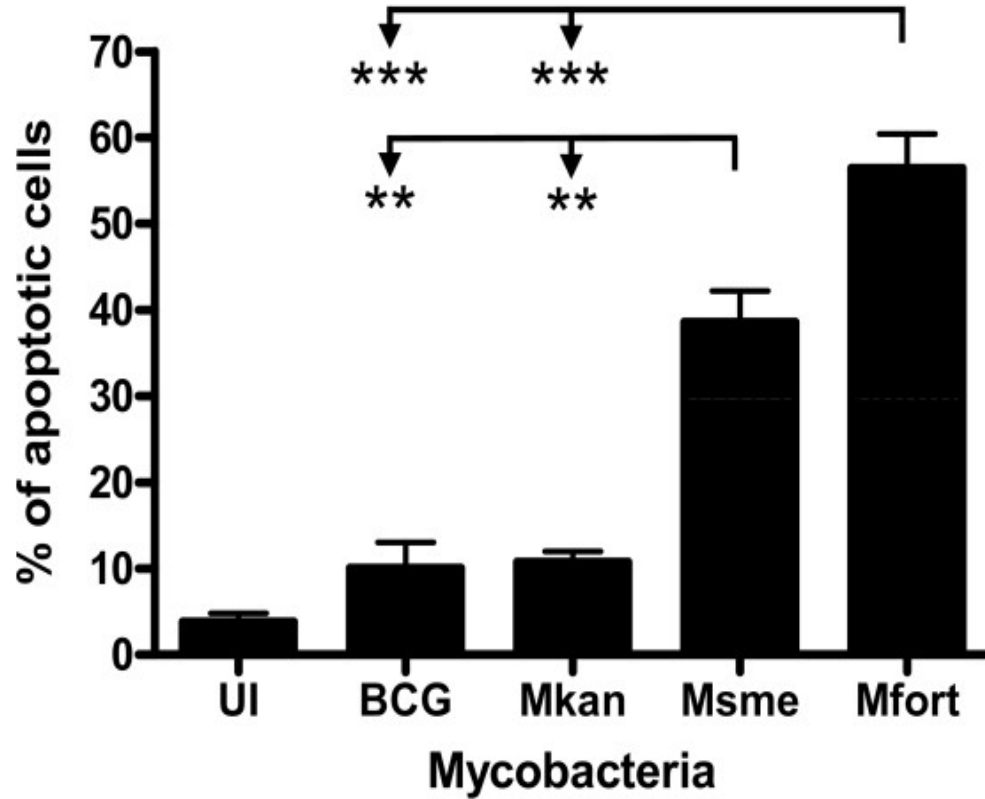


Figure 6. Difference in apoptosis induction between facultative-pathogenic and non-pathogenic mycobacteria in a human macrophage cell line. PMA-differentiated THP-1 cells were at an MOI of 10:1 with *M. smegmatis* (*Msme*), *M. fortuitum* (*Mfort*), *M. kansasii* (*Mkan*), *M. bovis* BCG or left uninfected (UI) and the amount of apoptosis was determined 20 hours after infection using TUNEL assay and flow cytometry on duplicate samples. The results are the mean and standard deviation of three independent experiments.

with gentamicin for an additional 20 hours. The percentage of apoptotic cells was determined by TUNEL assay. *Msmc*-infected cells showed a 4-fold increase in apoptosis (approx. 40% of total cells) and *Mfort*-infected cells showed a 6-fold increase in apoptosis (approx. 60% of total cells) (Figure 6) when compared to cells infected with facultative-pathogenic *Mkan* and BCG where approximately 10% of infected cells underwent apoptosis (Figure 6).

These results indicate that non-pathogenic mycobacteria strongly induce higher levels of apoptosis when compared to facultative-pathogenic mycobacteria. This difference in the apoptotic response supports the hypothesis that non-pathogenic mycobacteria induce a very potent innate IR when compared to facultative-pathogenic mycobacteria.

3.1.2 Non-pathogenic mycobacteria strongly induce the secretion of TNF and IL-12 in macrophages.

TNF is a central proinflammatory cytokine that mediates and regulates innate immunity. TNF binding to TNFR-1 may lead to activation of either Jun/Fos or NF-KB, followed by gene transcription, and production of inflammatory mediators and survival proteins. On the other hand, TNF binding to the death receptor TNFR1 may also initiate JNK protein kinase activation followed by activation of caspase-8 and downstream effector caspases such as caspase-3 resulting in apoptosis of the cell [223].

In order to examine the effect of TNF on the induction of apoptosis by non-pathogenic mycobacteria, BALB/c BMDMs were infected with *Msmc*, *Mfort*, *Mkan*, or BCG at three different MOIs (1:1, 3:1, and 10:1) for 2 hours. The cells were then washed and incubated in infection medium supplemented with gentamicin for an additional 20

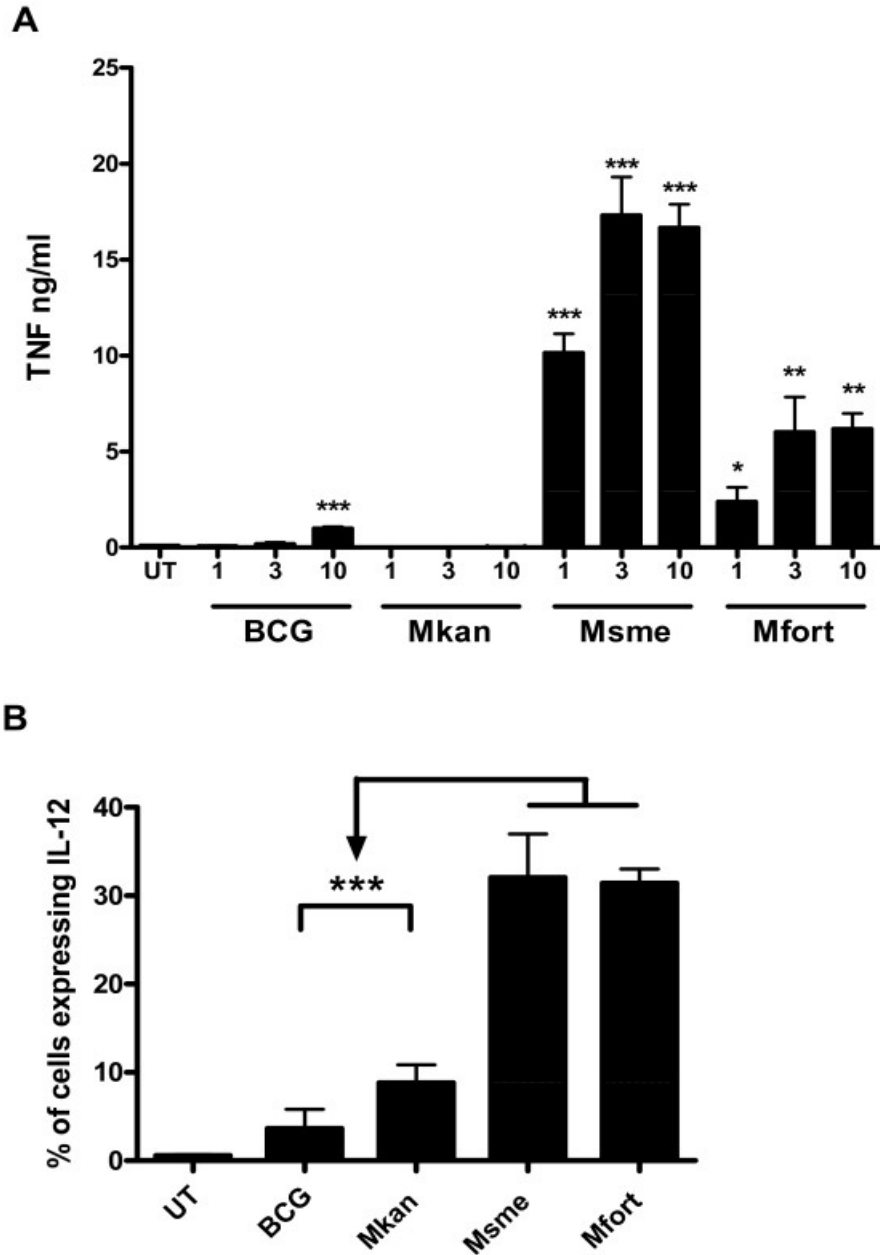


Figure 7. Differences in TNF secretion and *Il-12 p40* induction between facultative-pathogenic and non-pathogenic mycobacteria-infected macrophages. **A.** BALB/c BMDMs were infected at MOIs of 1:1, 3:1, and 10:1 with *M. smegmatis* (*Msme*), *M. fortuitum* (*Mfort*), *M. kansasii* (*Mkan*), *M. bovis* BCG, or left untreated (UT). Cells were infected in triplicates for two hours then washed and incubated in infection media with 100 μ g/ml gentamycin for an additional 20 hours. Culture supernatants were then collected and the amount of secreted TNF was determined using ELISA. The values are the mean and standard deviation of triplicate readings and they are representative of three independent experiments. **B.** The induction of *Il-12 p40* gene expression was analyzed by infecting RAW/pIL-12-GFP macrophages with the indicated bacteria for two hours at an MOI of 10:1. The GFP-expression was analyzed on 5,000 cells 16 hours later and the mean and standard deviation of three independent experiments are shown.

hours. The amounts of secreted TNF in the supernatant were measured via ELISA. BMDMs infected with *Msmc* secreted 10 to 18 times more TNF than BMDMs infected with facultative-pathogenic *Mkan* or BCG, which did not secrete any significant amounts of TNF (Figure 7A). Concurrently, BMDMs infected with *Mfort* also secreted approximately 10-fold more TNF than macrophages infected with BCG or *Mkan* (Figure 7A). In a similar fashion, non-pathogenic mycobacteria had a much stronger impact on the expression of *Il-12* gene in RAW/pIL-12 GFP reporter cell line infected with *Msmc* and *Mfort* at MOI 10:1 compared to cells infected with BCG or *Mkan* at similar MOI (Figure 7B). *Msmc* and *Mfort* infections resulted in p40 promoter-driven GFP expression in approximately 30% of infected cells, whereas infection with BCG or *Mkan* resulted in GFP expression of merely 5-10% of infected cells (Figure 7B).

These results demonstrate a stronger induction of two proinflammatory cytokines (TNF and IL-12) in macrophages when infected with non-pathogenic mycobacterial species (*Msmc* and *Mfort*) compared to facultative-pathogenic mycobacteria (BCG and *Mkan*).

3.1.3 Non-pathogenic mycobacteria induce apoptosis via TNF and caspase-3 signaling pathways.

We showed that non-pathogenic mycobacteria induce a strong apoptotic response and TNF secretion in BALB/c macrophages (Figures 5B and 7A) when compared to facultative-pathogenic mycobacteria. Apoptosis of eukaryotic cells could follow either a caspase-dependent or caspase-independent pathway. All caspase-dependent pathways lead

to the activations of effector caspases-3, -6, and 7 [224]. Caspase-independent pathways involve lysozymes and do not lead to the activation of caspases.

In order to determine which pathway is involved in the macrophage apoptotic response to non-pathogenic mycobacteria infection, we pretreated BALB/c BMDMs with caspase-3 inhibitor, TNF neutralizing antibody, Pentoxifylline (PTX, chemical inhibitor of TNF synthesis), the appropriate controls, or left the cells untreated then infected them with *Msmc* at MOI 10:1 for two hours. The cells were then washed and incubated in infection medium supplemented with gentamicin as well as the same concentrations of inhibitors and antibodies for an additional 20 hours. Host cell apoptosis was determined on 10,000 cells using the hypodiploid flow cytometry assay. Cells treated with the caspase-3 inhibitor showed a significant 20-fold decrease in apoptosis (1.2% apoptotic cells) when compared to the untreated (UT) *Msmc*-infected control (20% apoptotic cells) and to cells treated with the inactive chemical analog of the caspase-3 inhibitor (16.8%) (Figure 8). In a similar fashion, TNF neutralizing antibody (1.1% apoptotic cells) and Pentoxifylline (PTX) treated cells (5.5% apoptotic cells) both showed a significant decrease in apoptosis compared to UT *Msmc*-infected cells (20% apoptotic cells) or the non-specific antibody-treated cells (21% apoptotic cells) (Figure 8). These results demonstrate that apoptosis of BMDMs induced by non-pathogenic mycobacteria is dependent upon TNF secretion and caspase-3 activation.

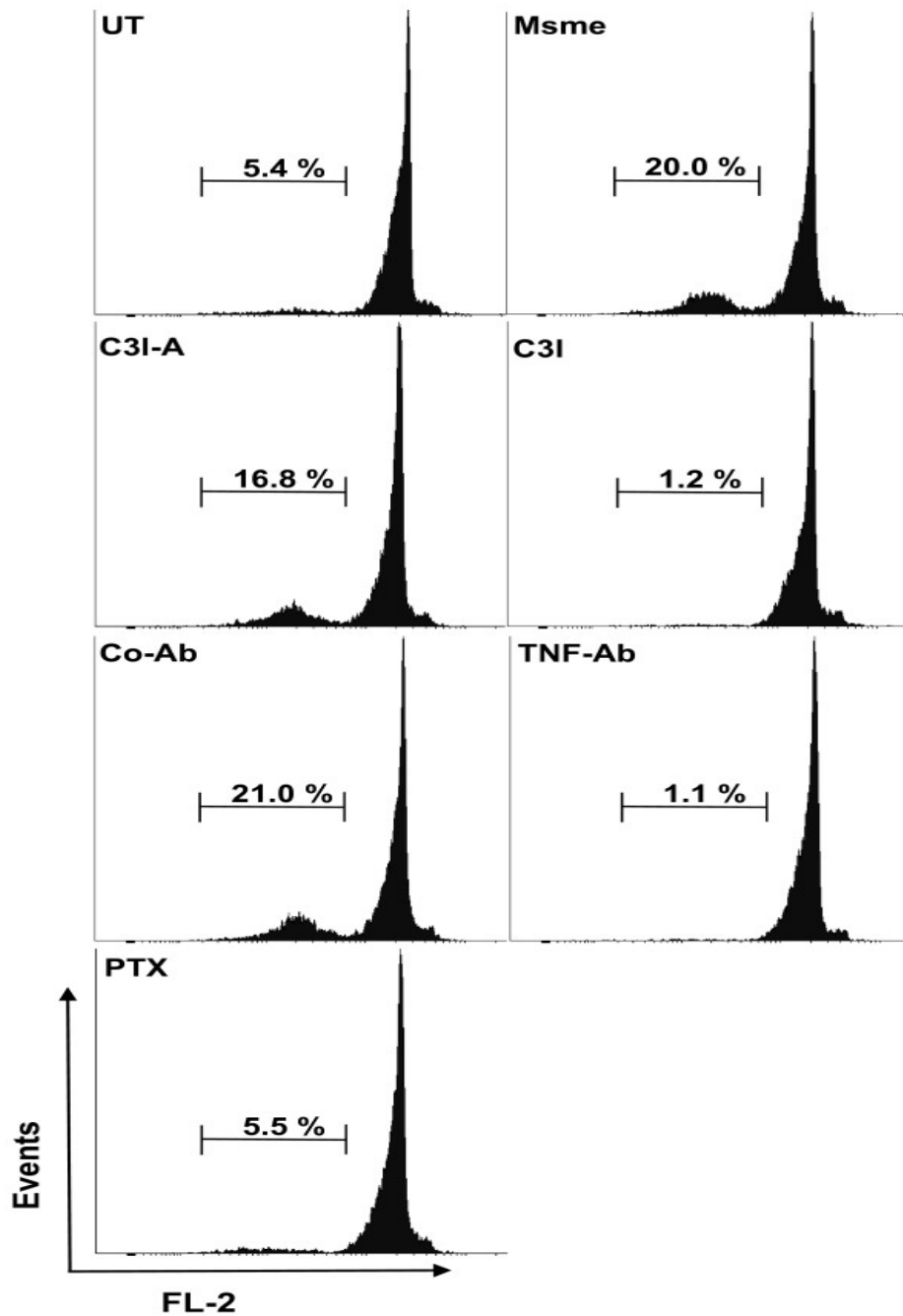


Figure 8. Macrophage apoptosis induction by a nonpathogenic mycobacteria is caspase-3 and TNF-dependent. BMDMs from BALB/c mice were left untreated and uninfected (UT) or infected with *M. smegmatis* at MOI 10:1 and then either left in medium (*Msme*) or treated with caspase-3 inhibitor (C3I), nonspecific chemical analog (C3I-A) neutralizing TNF antibody (TNF-Ab), nonspecific control Ab (Co-Ab) and TNF synthesis inhibitor pentoxifylline (PTX). The percentage of apoptotic cells out of 10,000 total cells was determined after 20 h using the hypodiploid PI flow cytometry assay and a representative histogram of two independent experiments performed in duplicates is shown.

3.1.4 Non-pathogenic mycobacteria do not induce apoptosis in C57Bl/6 BMDMs.

We showed that non-pathogenic mycobacteria induce a strong apoptotic response and TNF secretion in BALB/c macrophages compared to facultative-pathogenic mycobacteria. We also demonstrated that this apoptotic induction in macrophages is dependent upon TNF and caspase-3 activation. These results were all observed in BMDMs originating from BALB/c mice. In order to account for the different genotypes of the host's macrophages, we also derived macrophages from C57Bl/6 mouse bone marrow and infected them with non-pathogenic and facultative-pathogenic mycobacteria as was done for BALB/c BMDMs. C57Bl/6 BMDMs infected with *Msmc* and *Mfort* did not show any increased levels of apoptosis when compared to BCG or *Mkan* infected cells or untreated cells (UT) as the percentages of hypodiploid positive cells (Figure 9A) and TUNEL positive cells (Figure 9B) were similar across all conditions ($p > 0.05$). There was no significant increase in apoptosis in any infected BMDMs. These results demonstrate that the apoptotic response upon infection with non-pathogenic mycobacteria is dependent on the genotype of the host.

Since non-pathogenic mycobacteria did not induce apoptosis in infected C57BL/6 BMDMs in contrary to BALB/c BMDMs (compare Figures 5B to Figures 9A and 9B) it was important to check the levels of secreted TNF by C57BL/6 macrophages upon infection with non-pathogenic mycobacteria. C57BL/6 BMDMs secreted nearly 5-fold more TNF when infected with *Msmc* or *Mfort* compared to infections with BCG or *Mkan*, the latter of which did not induce the secretion of any TNF (Figure 9C). The total amount of TNF secreted after *Msmc* infection is reduced in C57BL/6 versus BALB/c BMDMs (compare Figures 7A and 9C). For example at an MOI of 10:1, *Msmc* induced the secretion

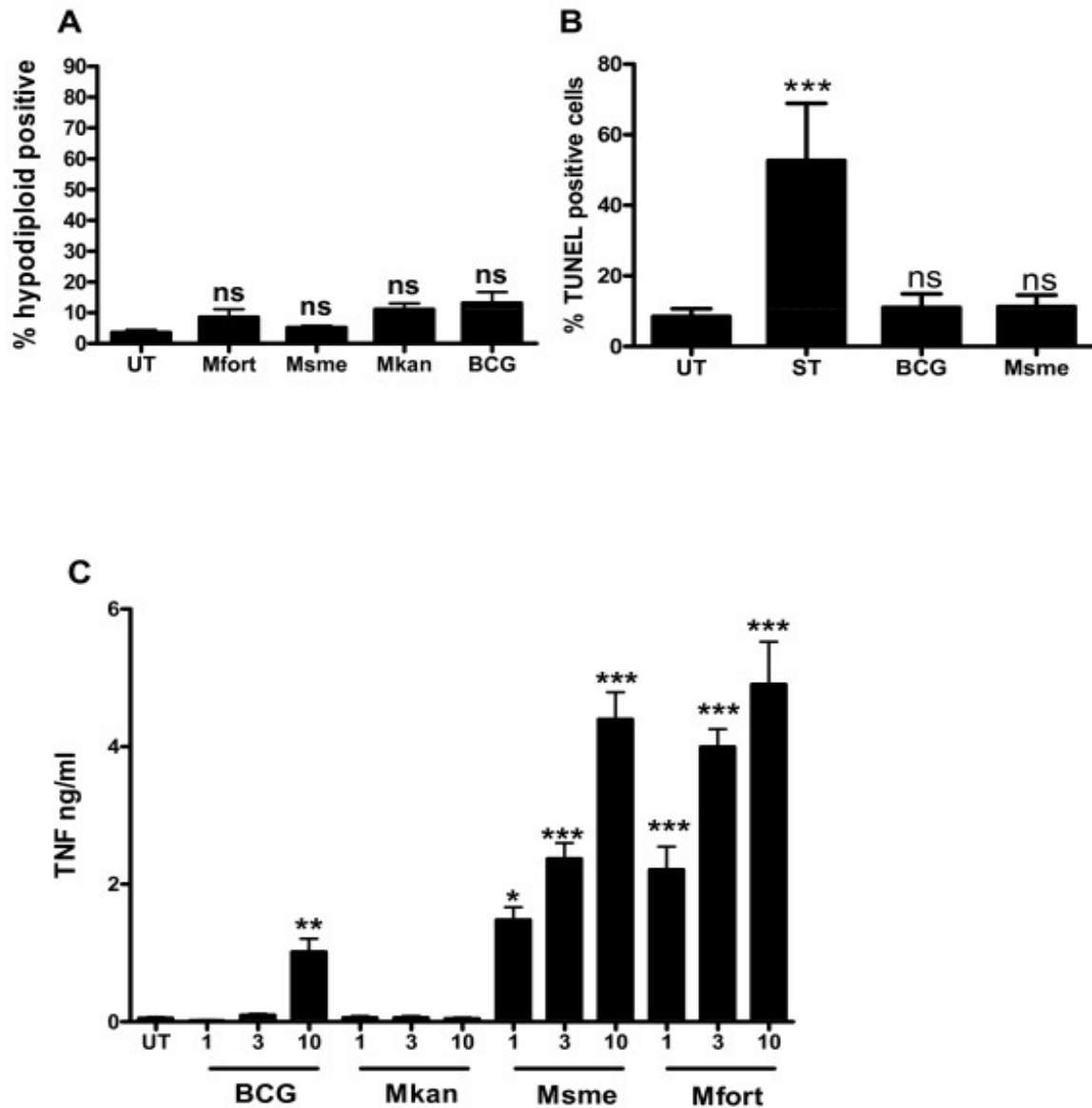


Figure 9. Mycobacteria do not induce rapid apoptosis in BMDMs originating from C57Bl/6 mice. **A.** Differentiated C57Bl/6 BMDMs were infected at an MOI of 10:1 with *M. smegmatis* (*Msme*), *M. fortuitum* (*Mfort*), *M. kansasii* (*Mkan*), *M. bovis* BCG or left untreated (UT). The percentage of apoptotic cells was determined using a propidium iodide based staining protocol to detect the population of hypodiploid cells via flow cytometry at 20 hours after infection. **B.** C57Bl/6 BMDMs were infected as in A. or incubated with the apoptosis inducer staurosporine (ST) and the amount of apoptosis was detected using TUNEL staining and flow cytometry analysis. **C.** Macrophages were infected at MOIs of 1:1, 3:1, and 10:1 with *M. smegmatis* (*Msme*), *M. fortuitum* (*Mfort*), *M. kansasii* (*Mkan*), *M. bovis* BCG, or left untreated (UT). Culture supernatants of triplicate wells were collected after 20 hours and the amounts of secreted TNF was determined using ELISA. In A. and B. the data shown is the mean and standard deviation of three independent experiments. In C. the values are the mean and standard deviation of triplicate readings of one experiment and they are representative of three independent experiments.

of 16.7 ± 2.7 ng/ml TNF in BALB/c BMDMs (Figure 7A) but only the secretion of 4.4 ± 0.7 ng/ml TNF in C57BL/6 BMDMs ($p < 0.01$) (Figure 9C). This difference in TNF secretion between BALB/c and C57BL/6 BMDMs could be interpreted as evidence for the role of decreased TNF secretion in the absence of *Msmc*-induced apoptosis. On the other hand, the amounts of TNF secretion induced by infection of either mouse strains with *Mfort* are similar (compare Figures 7A and 9C). For example at an MOI of 10:1, *Mfort* induces the secretion of 6.2 ± 2.0 ng/ml TNF in BALB/c BMDMs (Figure 7A) and the secretion of 4.9 ± 1.1 ng/ml TNF in C57BL/6 BMDMs ($p > 0.01$) (Figure 9C). In contrast to this unchanged amount of secreted TNF in C57BL/6 BMDMs, *Mfort* like *Msmc* only induces apoptosis in BALB/c but not C57BL/6 BMDMs (compare Figures 5B and 9A).

3.1.5 Discussion.

Apoptosis plays an important role in the innate IR against intracellular pathogens. The apoptotic but not necrotic response of macrophages infected with intracellular mycobacteria could lead to killing of the bacteria via different mechanisms. Intracellular mycobacteria could be directly killed when their host macrophage undergoes apoptosis [170]. Furthermore, apoptotic vesicles containing mycobacteria can be phagocytosed by stimulated uninfected bystander macrophages, which can then kill the bacteria more efficiently. These bystander macrophages get stimulated via cell surface receptor signaling induced by the binding of TNF and IFN- γ [171]. In addition, mycobacterial antigens in apoptotic bodies can be taken up by APCs such as macrophages and DCs and get cross-presented by MHC-I molecules to CD8⁺ T cells which leads to the activation and differentiation of CD8⁺ T cells into tuberculosis-specific cytotoxic lymphocytes [171]. This

cross presentation by MHC-I is very important since pathogenic *Mtb* inhibits the fusion of the mycobacteria-containing phagosome with the lysosome [158] [171]. Furthermore, recent studies have demonstrated that virulent *Mtb* expresses proteins that are shown to be involved in the active inhibition of host cell apoptosis. These proteins include the superoxide dismutase A (SodA), catalase G (KatG), and NuoG that is part of the NDH-1 protein complex [160] [163] [225]. The deletion of any of these genes in *Mtb* strongly attenuates the bacteria suggesting that host cell apoptosis inhibition is a virulence pathway.

Facultative-pathogenic mycobacteria cause disseminating disease in individuals deficient in the adaptive IR. To the contrary, humans deficient in the adaptive IR do not get disseminating disease when infected with non-pathogenic mycobacteria such as *Msmc* and *Mfort*. The inability of non-pathogenic mycobacteria to cause disease in individuals lacking adaptive immunity suggests that the innate IR in these individuals is sufficient to defend against these species of mycobacteria. Non-pathogenic mycobacteria are killed early in infection, which suggests that their attenuation might be due to their strong induction of apoptosis in their macrophage host. Our results showing that non-pathogenic mycobacteria induce increased host cell apoptosis in macrophages compared to facultative-pathogenic mycobacteria (Figures 5 and 6) strongly support the hypothesis that the inability of non-pathogenic species of mycobacteria to cause disease could be due to their capacity to induce a strong innate IR.

This strong induction of apoptosis in macrophages is fast as it is observed within 22 hours post infection as shown in Figure 5. It is important to pay attention to this relatively fast induction of apoptosis by non-pathogenic *Msmc* and *Mfort* when compared to facultative-pathogenic mycobacteria since the latter have been shown to induce

significantly more apoptosis in primary human alveolar macrophages than four different strains of *Mtb* after 5 days of infection [178]. Therefore, it is important to note that facultative-pathogenic mycobacteria do induce significant amounts of apoptosis in infected host cells but this host response only takes place late in infection (5 days post infection). The fast 22 hours post-infection apoptotic response is only seen in non-pathogenic mycobacterial infections suggesting the presence of potent mycobacterial ligands in non-pathogenic species capable of inducing host cell signaling [162].

The cell wall of mycobacteria contains several components with immunomodulatory activities. The differential modification of the mycobacterial cell wall lipoglycan ‘lipoarabinomannan’ (LAM) by different mycobacterial species is correlated with the ability of these species to induce apoptosis and IL-12 production in macrophages, two very important innate immunity components [70] [226]. The mannose cap modification on LAM of *Mtb* (Man-LAM) does not induce apoptosis and IL-12 production in macrophages and is found to be involved in the inhibition of phagosome maturation and IFN- γ signaling in macrophages whereas; the addition of phosphoinositide residues on LAM of *Msmc* (PI-LAM) have an opposite effect since it induces a strong proinflammatory response in macrophages and DCs by inducing apoptosis and IL-12 production [70] [226]. These lipid modifications of the cell wall (PI-LAM) are shown to mediate their biological activity via interaction with TLR-2 [70]. We have shown in published data [71] that PI-LAM purified from non-pathogenic *Msmc* and *Mfort* induced a significant increase in apoptosis and IL-12 secretion when compared to Man-LAM purified from facultative-pathogenic *Mkan* and BCG in THP-1 macrophages . We have also shown in this published study that the purified PI-LAM and Man-LAM only interact with TLR-2 surface receptors

but not TLR-4 [71]. These results suggest that the PI-component in non-pathogenic mycobacteria is the ligand of TLR-2.

Previous studies have demonstrated increased cytokine secretion such as TNF in primary mouse macrophages, human peripheral blood derived macrophages, and neutrophils in response to non-pathogenic *Msmc* infection compared to opportunistic facultative-pathogenic *M. avium* [81] [83] [227]. It was also shown that virulent *Mtb* induces very little secretion, if not negligible, of TNF in mouse BMDMs [162] and neutrophils [227]. Our results confirm these findings and add to them by extending the analysis to include additional non-pathogenic mycobacteria (*Mfort*) and additional facultative-pathogenic mycobacteria (BCG and *Mkan*). This broad analysis of proinflammatory cytokines secretion by macrophages infected with several non-pathogenic and facultative-pathogenic mycobacterial species indicate that the strong proinflammatory response elicited by infected macrophages might be a more general characteristic of non-pathogenic mycobacteria. The increased TNF secretion in murine BMDMs infected with *Msmc* has been shown to be dependent on increased stimulation of cAMP/protein Kinase A and Calcium/Calmodulin/Calmodulin Kinase pathways, which result in prolonged activation of MAPKs p38 and ERK1/2 [83]. Furthermore, *Msmc* but not *M. avium* have been shown to induce an increase in *Tnf* and *Nos2* promoter activity in RAW macrophage cell line, which was also dependent on Ets and NF- κ B transcription factors [83] [228]. Our results showing that apoptosis induced by *Msmc* and *Mfort* is dependent on TNF adds to these previous findings by linking the increase in TNF secretion to the pro-apoptotic capacity of non-pathogenic mycobacteria (Figure 8) and by characterizing this apoptosis pathway as being caspase-dependent (Figure 8).

The comparison of *Msmc*-induced TNF secretion in BALB/c versus C57Bl/6 BMDMs revealed that secreted TNF was reduced to almost half the amount in C57Bl/6 BMDMs compared to BALB/c BMDMs (compare Figures 7A and 9C). In parallel, apoptosis induced by *Msmc* in C57Bl/6 BMDMs was absent when compared to the increased induction of apoptosis in BALB/c BMDMs (compare Figures 5A and 9C). These findings provide evidence for the role of decreased TNF secretion in the absence of apoptosis induced by *Msmc* in C57Bl/6 BMDMs. However, this correlation was not entirely observed in *Mfort* infected BALB/c and C57Bl/6 BMDMs (Figures 5A and 9C). Our results showed similar amounts of TNF secreted by BALB/c and C57Bl/6 BMDMs when infected with *Mfort* but the induction of apoptosis was still absent in C57Bl/6 BMDMs (Figure 9A) compared to increased induction in BALB/c BMDMs (Figure 5B). As a result we hypothesized that a certain amount of TNF secretion is necessary but not sufficient to mediate apoptosis induction in infected BMDMs. A recent study by Miller *et al.* [163] has shown a similar pattern between the induction of TNF secretion and apoptosis induction in host macrophages. A pro-apoptotic *Mtb* mutant ($\Delta nuoG$) still induced increased TNF secretion but not host cell apoptosis in C57Bl/6 BMDMs lacking functional phagocyte NADPH oxidase (NOX2), thus incapable of generating reactive oxygen species (ROS) in the phagosome. It is therefore logical to speculate that BALB/c and C57Bl/6 NOX2 enzymes react differently upon phagocytosis of non-pathogenic mycobacteria. In this hypothesis, the BALB/c NOX2 complex would induce a stronger and more prolonged activity resulting in greater release of phagosomal ROS. This increase in ROS could to a sustained JNK activation and shift the TNF signaling pathway toward host cell apoptosis instead of NF- κ B driven cell survival pathway [229].

3.2 Differences in apoptosis induced by facultative-pathogenic and non-pathogenic mycobacteria in BALB/c and C57Bl/6 dendritic cells.

Thus far we examined the innate IR in BMDMs from BALB/c and C57Bl/6 mice induced by non-pathogenic and facultative-pathogenic mycobacteria. *Mtb* primarily infects and resides in alveolar macrophages of infected humans. However, alveolar macrophages are not the only host for *Mtb*. It has been shown that in the lungs of infected mice, *Mtb* resides in large percentage in DCs [145] [230-232]. DCs are also shown to be necessary for the activation of *Mtb*-specific T cells and the transport of the bacilli from the lungs to the local lymph nodes [144] [145]. Therefore, we sought to examine the difference in the apoptotic response induced by non-pathogenic and facultative-pathogenic mycobacteria in BALB/c and C57Bl/6 bone marrow derived dendritic cells (BMDC) and compare it to the response we already characterized in BMDMs.

3.2.1 *Msmc* and BCG infect BALB/c and C57Bl/6 dendritic cells at the same rate.

In order to account for any difference in the rate of infection of non-pathogenic *Msmc* and facultative-pathogenic BCG we examined the infection rate of both species in BMDCs. BALB/c and C57Bl/6 BMDCs were infected with GFP-expressing *Msmc* and BCG for two hours at MOI 3:1 and 10:1 then washed and incubated in media with gentamicin for an additional 20 hours. The number of infected cells was determined by flow cytometry (GFP fluorescence intensity shifts) at 0 hours and 22 hours post infection. The rate of infection was similar across all conditions and cells (Figure 10A).

Msmc is a relatively fast growing bacterium (approx. three hours duplication time) when compared to the slow growing BCG (approx. 24 hours duplication time). In order to

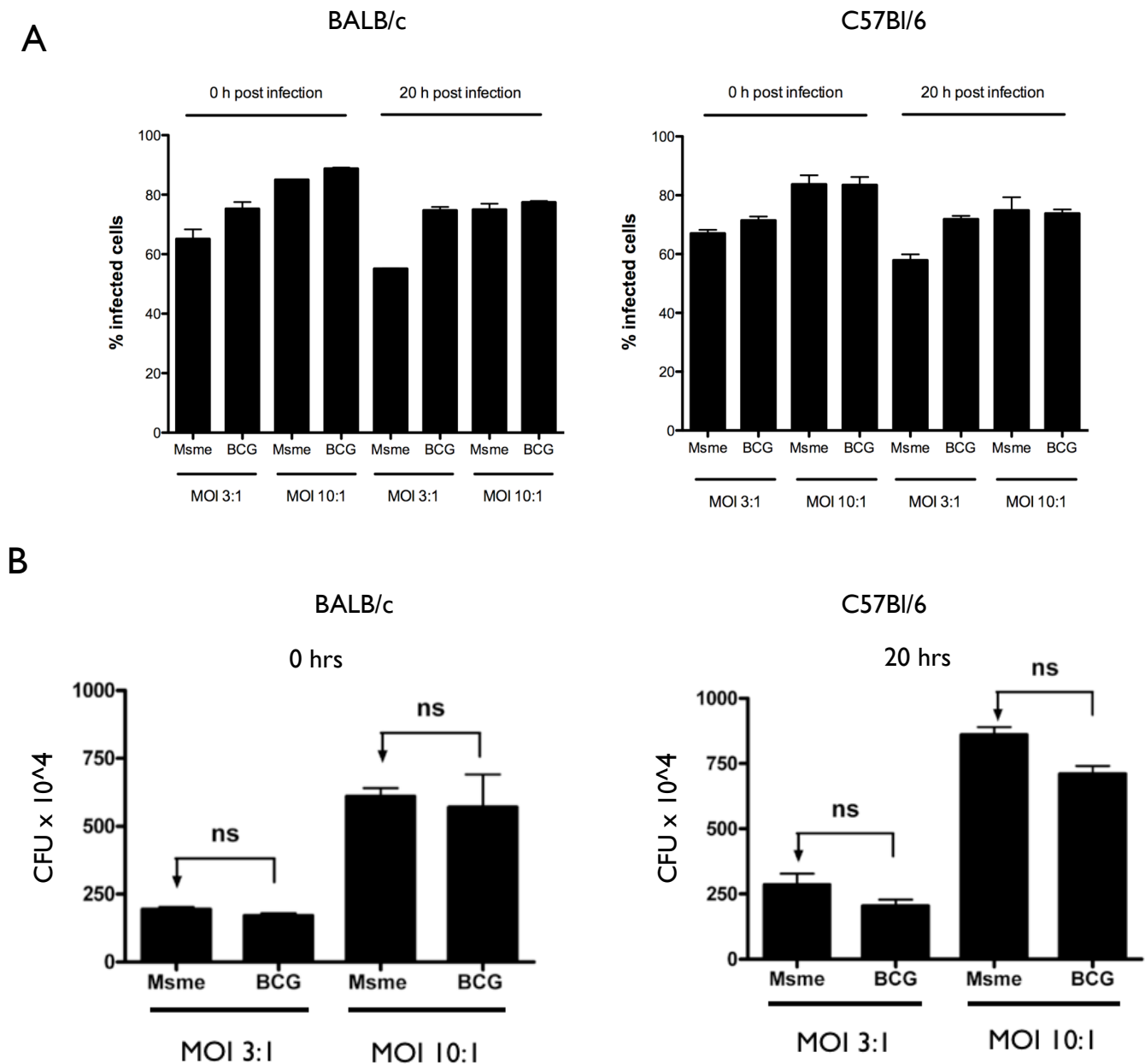


Figure 10. Rate of infection and intracellular growth of *Msme* and BCG in BMDCs. **A.** BALB/c and C57Bl/6 BMDCs were infected with GFP-expressing *Msme* and BCG for two hours at MOI 3:1 and 10:1 then washed and incubated in media with gentamycin for an additional 20 hours. The number of infected cells was determined by flow cytometry (GFP fluorescence intensity shifts) at 0 h and 20 h post infection. **B.** BALB/c and C57Bl/6 BMDCs were infected with *Msme* and BCG at MOI 3:1 and 10:1. The numbers of colony forming units (CFU) in infected cells were quantified on agar plates at 0 h and 20 hours post infection.

account for this significant difference in growth rate, we used an infection time of 2 hours, which is not enough for fast growing *Msmc* to duplicate. We also examined the bacterial load in BALB/c and C57Bl/6 BMDCs infected with *Msmc* and BCG at MOIs 3:1 and 10:1. The numbers of colony forming units (CFU) in infected cells were quantified on agar plates at 0 hours and 20 hours post infection. The numbers of CFUs were similar across all conditions and cells ($p > 0.05$) (Figure 10B).

3.2.2 Non-pathogenic mycobacteria induce increased host cell apoptosis in BALB/c and C57Bl/6 dendritic cells compared to facultative-pathogenic mycobacteria.

In order to test the apoptotic response of DCs when infected with non-pathogenic compared to facultative-pathogenic mycobacteria, we infected BMDCs from BALB/c and C57Bl/6 mice with *Msmc* or BCG for duration of 2 hours. The cells were then washed and incubated in infection medium supplemented with gentamicin for an additional 20 hours. The percentage of apoptotic cells was determined by quantifying the fraction of cells in hypodiploid state via flow cytometry (Figure 11). Both BALB/c and C57Bl/6 BMDCs showed significantly higher levels of apoptosis induced by non-pathogenic *Msmc* compared to facultative-pathogenic BCG (compare Figures 11A and 11B). Approximately 60% of both BALB/c and C57Bl/6 BMDMs infected with *Msmc* were hypodiploid positive ($p < 0.0001$) (Figures 5B and 9A), which was significantly higher than BALB/c BMDCs infected with BCG (25% hypodiploid positive cells) (Figure 11B) and BCG-infected C57Bl/6 BMDCs (15% hypodiploid positive cells) compared to approximately 5% hypodiploid positive untreated cells (Figure 11A).

3.2.3 Facultative-pathogenic mycobacteria induce more apoptosis in BMDC than BMDMs.

Despite the fact that the levels of apoptosis in BCG-infected BALB/c and C57Bl/6 BMDCs are significantly lower than those of *Msmc*-infected BMDCs, these levels of apoptosis (25% for BALB/c and 15% for C57Bl/6) are significantly higher than the levels of apoptosis in BMDMs (compare Figures 5B and 9A to Figure 11). These results suggest that BMDCs are inherently more susceptible for undergoing apoptosis upon infection with facultative-pathogenic mycobacteria than BMDMs in the BALB/c background. These results also indicate that there is a profound difference between BMDMs and BMDCs in C57Bl/6 mice in regard to apoptosis induction upon infection with non-pathogenic mycobacteria since *Msmc*-infected C57Bl/6 BMDMs did not show any levels of apoptosis, whereas about 60% of *Msmc*-infected C57Bl/6 BMDCs were apoptotic (compare Figures 9A to 11A).

3.2.4 Differences in ROS response to mycobacterial infections between C57Bl/6 macrophages and dendritic cells.

Previous studies have shown an increased activity of NOX2 enzyme complex in DCs compared to a lower level of activity in macrophages [233]. This increased NOX2 activity induces an increase in phagosomal ROS, which is required to maintain a neutral PH in the phagosome to prevent the degradation of antigens required for cross presentation by DCs and subsequent recognition by T cells [233]. This increase in ROS level in the phagosome of DCs have been shown to shift the balance of TNFR-1 signaling toward cell death via apoptosis by activating JNK [223] [229]. In order to address the potential role of

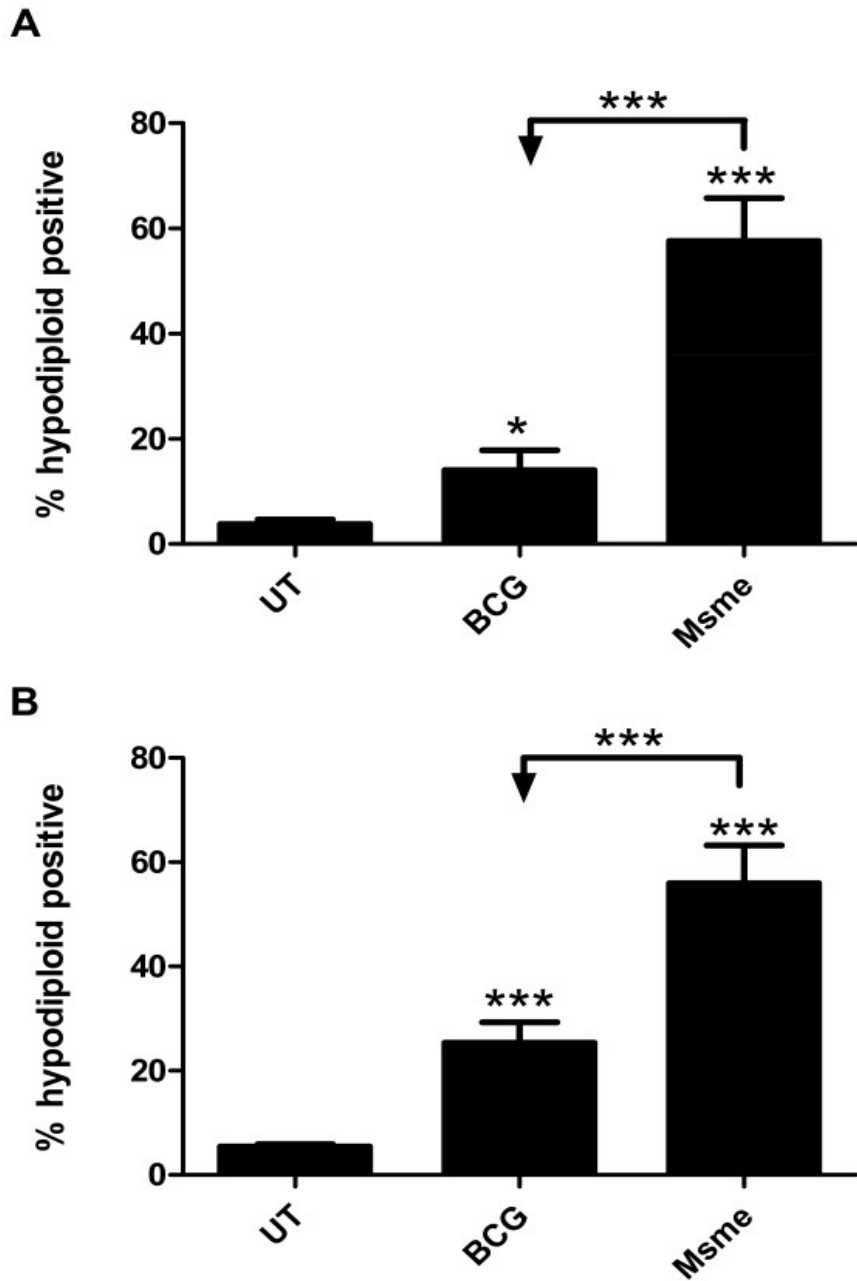


Figure 11. Differences in apoptosis induced by facultative-pathogenic and non-pathogenic mycobacteria in BALB/c and C57Bl/6 dendritic cells. C57Bl/6 (A) and BALB/c (B) bone marrow derived dendritic cells (BMDC) were infected at an MOI of 10:1 with *M. smegmatis* (*Msme*), *M. bovis* BCG or left untreated (UT). After two hours, the cells were washed and incubated in infection media with 100 $\mu\text{g/ml}$ gentamycin for an additional 20 hours. The percentage of hypodiploid cells of a total of 10,000 cells was determined using flow cytometry. The values of the means and standard deviations of three independent experiments are shown.

ROS in the apoptosis induction in C57Bl/6 macrophages and DCs, we infected C57Bl/6 BMDMs and BMDCs with *Msmc* or BCG for 2 hours followed by 20 hours incubation in infection medium supplemented with gentamicin, or incubated the cells with opsonized zymosan positive control for 4 or 24 hours, or left them untreated (UT). We then measured the amount of ROS accumulation in the cells using dihydroethidium (DHE) and quantified it by flow cytometry. In BMDMs, only the opsonized zymosan positive control induced an increase in ROS accumulation after 24 hours incubation (Figure 12A). In contrast, in BMDCs, *Msmc* induced high levels of ROS accumulation when compared to untreated cells (UT) (Figure 12B). Furthermore, BCG also induced a significant amount of ROS accumulation in DCs (Figure 12B). However, this increase in ROS accumulation in BCG-infected BMDCs was lower than the one induced in *Msmc*-infected BMDCs (Figure 12B).

3.2.5 Discussion.

It was important to characterize the apoptotic response in DCs upon infection with non-pathogenic and facultative-pathogenic mycobacteria since DCs are also a host for *Mtb* [145] [230-232]. We have shown that BMDMs and BMDCs display the same pattern of apoptosis induction upon infection with non-pathogenic compared to facultative-pathogenic mycobacteria, with the former inducing much higher levels of apoptosis. However, in contrast to BMDMs, BCG-infected BMDCs showed a significant increase in apoptosis induction after one day of infection, which was not observed in BMDMs (Figures 5B, 9A, and 11). Concurrently, The significant levels of apoptosis induction in *Msmc*-infected C57Bl/6 BMDCs compared to the absence of apoptosis in *Msmc*-infected C57Bl/6

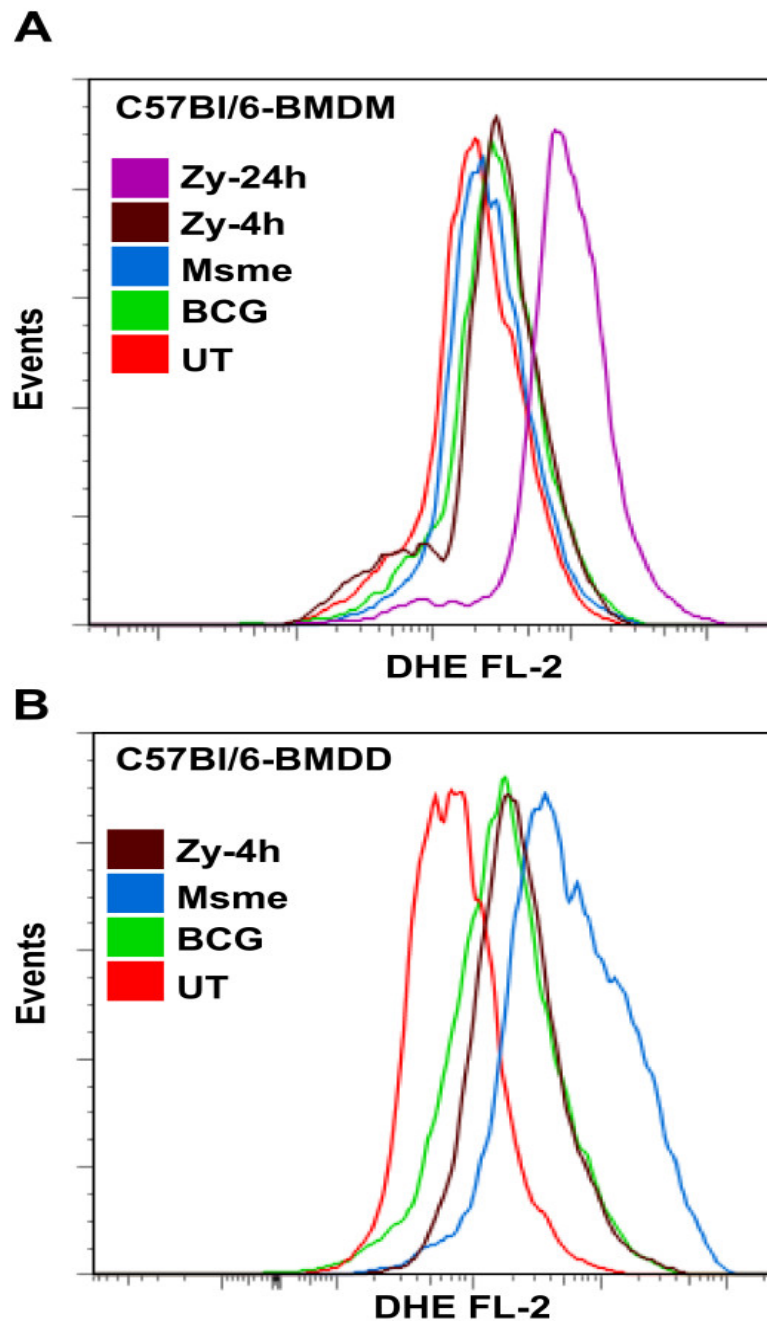


Figure 12. Differences in ROS response to mycobacterial infections between C57BI/6 macrophages and dendritic cells. Cells were infected as described in figure 8 and ROS were detected two hours after infection using dihydroethidium (DHE). **A.** BMDM or **B.** BMDC left untreated (UT), infected with BCG, *M. smegmatis* (*Msme*) or incubated with opsonized zymosan particles for 4 hours or 24 hours. The increase in DHE mediated fluorescence (FL-2) was analyzed by flow cytometry of 10,000 total cells. A representative of three independent experiments is shown. [Experiment performed in collaboration with Dr. Hana Abdalla].

BMDMs (compare Figures 9C and 11A) indicate that there is an underlying difference between BMDCs and BMDMs in C57Bl/6 mice in regard to the induction of apoptosis upon infection with non-pathogenic mycobacteria. This difference could be attributed to an increase in ROS accumulation in BMDC phagosomes upon infection with *Msmc*. Our results showed that the amount of ROS in C57Bl/6 BMDCs was significantly increased upon infection with *Msmc* when compared to *Msmc*-infected C57Bl/6 BMDMs (Figure 12).

Dendritic cells play an important role in the host defense against *Mtb* acting as professional APCs. Antigen-specific T cells recognize antigens presented on MHC molecules on the surface of DCs. Antigens from intracellular bacteria are presented on MHC-I molecules on the surface of DCs via cross-presentations [233]. This presentation of antigens is important for the initiation of the adaptive cytotoxic IR [233] [234]. In order to prevent degradation of antigens in the phagosome, the phagosomal acidity should be tightly regulated to a neutral PH. This regulation of the PH in the phagosome is due to NOX2 recruitment into the phagosome, which induces the production of low levels of ROS causing an active and maintained alkalinization of the phagosome [163]. Consequently, this increase in phagosomal ROS can lead to a sustained JNK activation and shift the TNF signaling pathway toward host cell apoptosis instead of NF- κ B driven cell survival pathway [163]. Our results support the argument that DCs are more susceptible to infection-induced apoptosis due to their capacity to generate high levels of ROS due to sustained NOX2 activity when compared to the rapid induction and inactivation of NOX2 in macrophages [163].

3.3 Molecular mechanisms of *M. smegmatis*-induced IL-1 β secretion in dendritic cells and macrophages.

The inflammasome was shown to play an important role in the host defense against *Mtb* as was reported *in vivo* in studies where mice deficient in the IL-1 receptor (*Il-1r*), *Il-1 β* , or *Il-18* genes were more susceptible to *Mtb* infections [116] [117] [122] [218] [219]. *Mtb* infections in BMDMs and THP-1 cell line induced IL-1 β secretion, which was dependent on ASC, NLRP3, and caspase-1 [104] [122] [218] [235-237]. However, *in vivo* infections of mice deficient in *Asc* and *caspase-1* did not display any decrease in IL-1 β secretion compared to wild type (WT) mice. These knockout mice were also less susceptible to *Mtb* infections than *Il-1 β* deficient mice [116] [117] [122] [218] [219]. This discrepancy between *in vivo* and *in vitro* macrophages suggests that cell types other than macrophages are involved in the processing and secretion of IL-1 β . These cell types might also involve mechanisms other than inflammasome activation leading to secretion of IL-1 β .

Alveolar DCs are host cells for *Mtb* besides macrophages *in vivo* highlighting their importance for host defense against tuberculosis [145] [230-232]. DCs were shown to be necessary for the activation of *Mtb*-specific T cells and the transport of *Mtb* from the lungs to the local lymph nodes [163]. Unlike in macrophage [121] [220] [236-239], the interaction between mycobacteria and host cell inflammasome has not been well analyzed in dendritic cells. As stated, previous studies have established the importance of IL-1 β in the host defense against *Mtb* infections [240]. Therefore, it is important to characterize the regulation of IL-1 β in host DCs upon infection with mycobacteria. The following studies address the regulation of IL-1 β secretion in DCs upon infection with the proinflammatory non-pathogenic mycobacterial species *Msmc*. We also compare the capacity of a multitude

of virulent, opportunistic, and non-pathogenic mycobacterial species to induce secretion of IL-1 β in addition to the difference in the involvement of several inflammasome components in IL-1 β secretion in DCs.

3.3.1 *Msmc*-induced IL-1 β secretion in dendritic cells is dependent on ASC but partially independent of NLRP3 and caspases-1, and -11.

In order to characterize the components that are involved in inflammasome activation and IL-1 β secretion induced by *Msmc* in DCs, we used BMDCs from C57Bl/6 WT mice and mice deficient in *Nlrp3* (*Nlrp3*^{-/-}), *Asc* (*Asc*^{-/-}), or *Caspase-1/11* (*Casp1/11*^{-/-}) and infected them with *Msmc* for duration of two hours at MOI 10:1 or left them uninfected. The cells were then washed and incubated in infection medium supplemented with gentamicin for an additional 20 hours. The amounts of secreted IL-1 β in the supernatants were measured via ELISA. *Caspase-1* and *Caspase-11* genes are tightly adjacent to each other in the mouse genome and separated by only 1,500 base pairs, therefore, backcrossing over multiple generations yielded a caspase-1 knockout that is also lacking caspase-11. As a result the *caspase-1* knockout strains is in fact a *Caspase-1/11*^{-/-} double knockout [241]. WT BMDCs infected with *Msmc* secreted large amounts of IL-1 β (approx. 13 \pm 0.6 ng/ml) while *Asc*^{-/-} BMDCs did not secrete any significant amounts of IL-1 β upon infection with *Msmc* compared to the uninfected control (Figure 13A). This indicated that *Msmc*-induced IL-1 β secretion in BMDCs requires ASC. Furthermore, *Casp1/11*^{-/-} and *Nlrp3*^{-/-} BMDCs showed a significant decrease in IL-1 β secretion upon infection with *Msmc* (Figure 13A), which indicates that caspase-1/11, and NLRP3 are important for the secretion of IL-1 β . However, this decrease in IL-1 β was only partial and the amount of secreted cytokine was

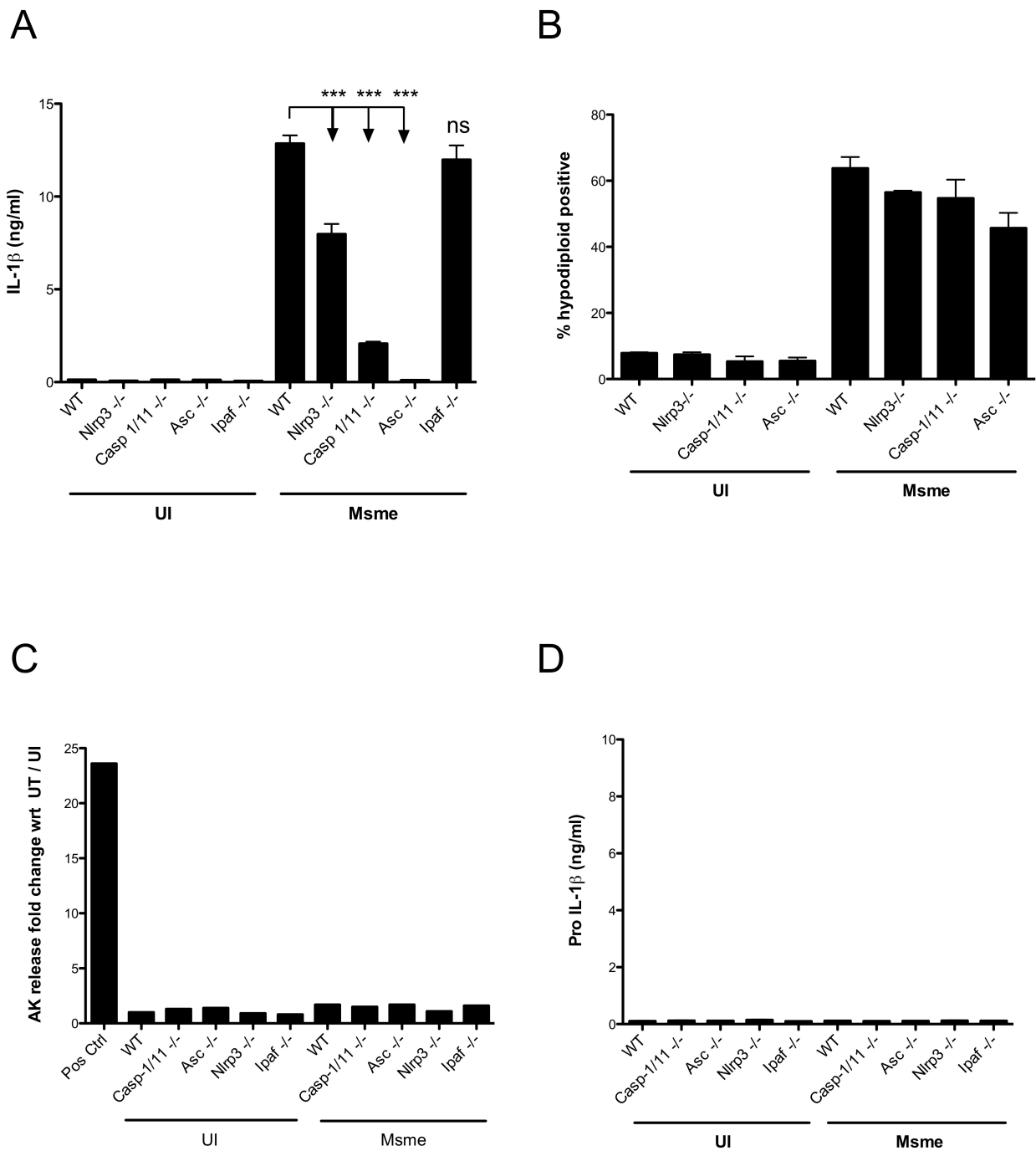


Figure 13. *Msme*-induced IL-1 β secretion is dependent on ASC but partially independent of NLRP3 and caspases-1/11. BMDCs were derived from WT, caspase-1/11^{-/-}, ASC^{-/-}, NLRP3^{-/-}, and IPAF^{-/-} mice and infected with *Msme* or left uninfected (UI). The supernatants were harvested after 22 hours and the amount of secreted IL-1 β (A) and pro-IL-1 β (D) were detected by ELISA. The amounts of AK in the supernatant were measured via Toxilight AK release colorimetric assay (C). The positive control (Pos Ctrl) indicates complete cell lysis via treatment with Triton-X (B). Shown are the percentages of hypodiploid positive cells. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

still significant (approx. 7.5 ng/ml for *Nlrp3*^{-/-} and 3 ng/ml for *Asc*^{-/-} compared to 13 ng/ml for the WT, Figure 13A).

It is well established that LPS stimulation (signal 1) leads to the expression of the *Il-1 β* gene and the production of the pro-form of the cytokine (pro- IL-1 β). Pro- IL-1 β is then cleaved via caspase-1 to yield the mature active form of IL-1 β [218]. The IL-1 ELISA detects the amount of both pro- IL-1 β and mature IL-1 β . Additional control experiments were performed in order to confirm that IL-1 β detected in the supernatant is the mature secreted form of the cytokine. This was done to account for the possibility that *Msm*-infected BMDCs might have been lysed via necrosis like processes such as pyroptosis or pyronecrosis, which were shown to be induced by inflammasome activation via caspase-1 and ASC respectively [242]. Such events would result in the release of pro- IL-1 β in the supernatant in which case the detected IL-1 β would not entirely be of the mature form. To account for this possibility we measured the amount of pro-IL-1 β in the supernatant via an ELISA kit (ebioscience) that only detects the pro-form of the cytokine. Figure 13D shows that no pro- IL-1 β was detected in any supernatant for all conditions. This indicates that all the IL-1 β in the supernatant is the mature secreted form of the cytokine.

Our results demonstrated that the induction of IL-1 β secretion in *Msm*-infected BMDCs requires ASC but is partially independent of NLRP3, and caspase-1/11. These results suggest that NLRP3 and caspases-1/11 are important for the secretion of some of the IL-1 β but also other components are involved in this cytokine secretion. The NLRP3 inflammasome has been extensively analyzed and is shown to be the main inflammasome responsible for the processing of pro- IL-1 β into the mature form (IL-1 β) by caspase-1 upon infection with *Mtb* in macrophages (BMDMs and THP-1 cells). This pathway

involves ASC as an indispensable adapter molecule [108] [207] [211] [220] [243]. Our results suggest that *Msmc* infection of BMDCs do activate the NLRP3 inflammasome leading to secretion of IL-1 β but also other pathways that require ASC are involved in IL-1 β secretion.

3.3.2 *Msmc* induces apoptosis but not pyroptosis or pyronecrosis in dendritic cells.

Previous studies have shown that activation of NLR inflammasomes can lead to cell death via processes that resemble apoptosis or necrosis, called pyroptosis and pyronecrosis respectively. Pyroptosis is caspase-1-dependent and leads to the dimerization of ASC into pyroptosomes while pyronecrosis is independent of caspases but requires ASC [242]. Both pathways involve the loss of plasma membrane integrity resulting in the release of cytosolic proteins into the extracellular environment, which unlike apoptosis, maintains the integrity of the plasma membrane [248-250]. We previously showed that *Msmc* induces high levels of apoptosis of infected host macrophages and DCs (Figures 5, 6, and 11). Here we extend our analysis to test whether DCs infected with *Msmc* undergo these forms of cells death. We infected WT, *Nlrp3*^{-/-}, *Asc*^{-/-}, and *Casp1/11*^{-/-} BMDCs with *Msmc* at MOI 10:1 and analyzed the permeabilization of the cell plasma membrane via the detection of the cytosolic enzyme adenylate kinase (AK) in the supernatant, which would be released extracellularly (supernatant) if the cell membrane was compromised. In addition, we determined the percentage of apoptotic cells by quantifying the fraction of cells in the hypodiploid state via flow cytometry. First, the percentage of hypodiploid positive cells in *Nlrp3*^{-/-}, *Asc*^{-/-}, and *Casp1/11*^{-/-} were all similar to the WT (approx. 75%) as shown in Figure 13C. These results agree with the previously reported data demonstrating that *Msmc*

induces apoptosis in BMDCs (Figure 11) and also add to them by indicating that none of the examined inflammasome components are important for the induction of cell death via apoptosis. Furthermore, *Msmc* did not lead to any increase in plasma membrane permeability of infected BMDCs as shown in Figure 13C where only a minor 1 to 2-fold increase in AK release was detected for all conditions compared to the cell lysis positive control, which showed a 24-fold increase in AK release with respect to the WT uninfected cells (WT/UI) (Figure 13C). These results highlight the absence of pyroptosis and pyronecrosis cell death in *Msmc*-infected BMDCs.

3.3.3 *Msmc* and *Mtb* -induced secretion of IL-1 β in macrophages is partially independent of caspase-1/11.

Several groups have studied inflammasome activation and IL-1 β secretion in response to *Mtb* infections in monocytes and macrophages [121] [220] [236-239] [244]. The secretion of IL-1 β in *Mtb*-infected macrophages was shown to be dependent on ASC, NLRP3, and caspase-1 *in vivo* [104]. In addition, *Mtb*-infected BMDMs were shown to secrete small amounts of IL-1 β *in vitro* ranging from 0.4 to 0.7 ng/ml as reported by several groups [238] [245] [237] [245] compared to the significantly larger amounts of IL-1 β (approx. 3.8 ng/ml) in *Mtb*-infected BMDCs as we have shown in Figures 22 and 23. No analysis of IL-1 β secretion in response to non-pathogenic mycobacteria has been reported in macrophages. Therefore, we sought to extend our analysis of the *Msmc* and *Mtb* -induced secretion of IL-1 β to include macrophages in addition to our analysis in DCs. In order to check the level of IL-1 β secretion induced by *Msmc* and *Mtb* in macrophages, we deprived WT and *caspase-1/11*^{-/-} C57Bl/6 BMDMs from L cell-conditioned medium

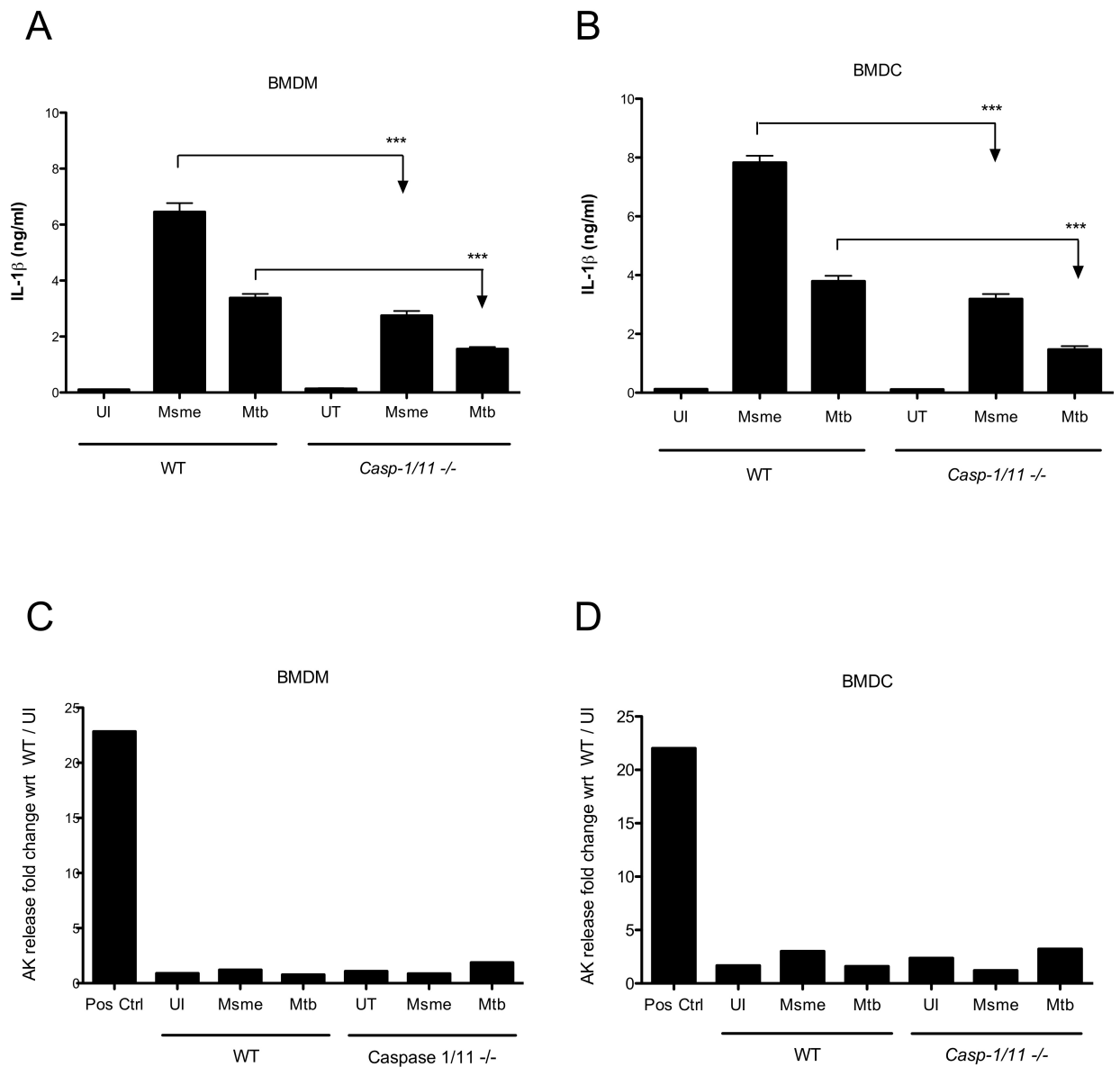


Figure 14. *Msme* and *Mtb* -induced Secretion of IL-1 β in macrophages is partially independent of caspase-1/11. BMDMs (A and C) and BMDCs (B and D) were derived from WT and caspase-1/11 mice and infected with *Msme*, *Mtb H37Rv*, or left uninfected (UI). The supernatants were harvested after 22 hours and the amount of secreted IL-1 β (A and B) were detected by ELISA. The amounts of AK in the supernatant were measured via AK release colorimetric assay (C and D). The positive control (Pos Ctrl) indicates complete cell lysis via treatment with Triton-X. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three. [Experiment performed in collaboration with Dr. Hana Abdalla].

(LCCM) for 6 hours and infected them with virulent *Mtb H37Rv* and *Msmc* at MOI 10:1 for duration of 2 hours or left them uninfected. The cells were then washed and incubated in infection medium without LCCM and supplemented with gentamicin for an additional 20 hours. The amounts of secreted IL-1 β in the supernatant were measured via ELISA. WT and *caspase-1/11*^{-/-} C57Bl/6 BMDCs were also infected with *Msmc* and *Mtb* as previously described.

WT BMDMs infected with *Msmc* secreted large amounts of IL-1 β (6.5 ± 0.3 ng/ml) while *Casp1/11*^{-/-} BMDCs showed a significant decrease in IL-1 β secretion upon infection with *Msmc* (Figure 14A), which indicates that caspase-1/11 is important for the secretion of IL-1 β in macrophages. However, this decrease in IL-1 β was only partial and the amount of secreted cytokine was still significant (approx. 2.7 ± 0.16 ng/ml) when compared to the uninfected control (Figure 14A). These results demonstrate that, like in BMDCs, the *Msmc*-induced IL-1 β secretion in BMDMs is also partially independent of Caspase-1/11. In comparison, *Mtb* also showed the same pattern of Caspase-1/11-partially independent IL-1 β secretion in infected macrophages. The infection of WT BMDMs with *Mtb* induced the secretion of 3.4 ± 0.1 ng/ml IL-1 β compared to a reduced but still significant secretion of 1.6 ± 0.1 ng/ml IL-1 β by *Caspase1/11*^{-/-} BMDMs (Figure 14A). All detected IL-1 β in the supernatant was of the secreted mature form since no cytosolic AK enzyme was detected in the supernatants of infected cells, which indicates that no cell lysis occurred as shown in Figure 14C.

WT and *Casp1/11*^{-/-} BMDCs infected in parallel with *Msmc* and *Mtb* also showed the same pattern of IL-1 β secretion. *Msmc* induced the secretion of 7.8 ± 0.2 ng/ml and 3.2 ± 0.2 ng/ml IL-1 β in WT and *Casp1/11*^{-/-} BMDCs, respectively. Infections with *Mtb* also

induced the secretion of 3.8 ± 0.2 ng/ml and 1.5 ± 0.1 ng/ml IL-1 β in WT and *Casp1/11^{-/-}* BMDCs, respectively (Figure 14B). All detected IL-1 β in the supernatant was of the secreted mature form since no cytosolic AK enzyme was detected in the supernatants of infected cells, which indicates that no cell lysis occurred as shown in Figure 14D.

3.3.4. L cell-conditioned medium has an inhibitory effect on the *Msm*-induced IL-1 β secretion in BMDMs.

It was surprising that the amounts of IL-1 β secreted by our *Mtb*-infected BMDMs (Figure 14A) are significantly higher than the amounts reported by several research groups in *Mtb*-infected BMDMs (0.4 to 0.7 ng/ml) [238] [245] [237] [245]. In addition, the Caspase-1/11-partially independent IL-1 β secretion by *Mtb*-infected macrophages was not seen in those published studies. One explanation of the discrepancy between our results and published literature could be that we deprived our BMDMs from LCCM 18 hours prior to infection and kept them without LCCM during the course of infection while all other research groups did not include these deprivation conditions in their experiments [238] [237] [245]. To test this hypothesis, we pretreated fully differentiated BMDMs with different concentrations of LCCM (6% and 20%) or completely deprived them from this supplement 18 hours prior to infection with *Msm*, and kept them under these same conditions during infection. Cells were infected at MOI 10:1 for duration of 2 hours, washed, then incubated in media with gentamicin for an additional 20 hours. The amounts of secreted IL-1 β in the supernatants were measured via ELISA. *Msm*-infected BMDMs that were completely deprived from LCCM secreted approximately 7.6 ± 0.8 ng/ml IL-1 β compared to 5.4 ± 0.4 ng/ml and 2.6 ± 0.2 ng/ml for the 6% and 20% LCCM-supplemented

BMDMs, respectively (Figure 15A). All detected IL-1 β in the supernatant was of the secreted mature form since no cytosolic AK enzyme was detected in the supernatants of infected cells, which indicates that no cell lysis occurred as shown in Figure 15B. These results support our hypothesis by demonstrating that LCCM has an inhibitory effect on the capacity of BMDMs to secrete IL-1 β upon infection with mycobacteria.

LCCM is a source of macrophage colony-stimulating factor (M-CSF), which is essential for the differentiation of monocytes into macrophages [246] [247]. It was therefore logical to ask whether it is the M-CSF in LCCM that is responsible for this inhibitory effect on IL-1 β secretion in BMDMs. To test this hypothesis, we measured the exact amount of M-CSF in the LCCM that was used in the BMDM infection experiments (presented in Figure 15A) by ELISA and repeated the infection of BMDMs with *Msmc* using recombinant mouse M-CSF at concentrations matching the percentages of LCCM used in the previous experiment (presented in Figure 15A). The LCCM we used contained approximately 158 ng/ml M-CSF. Therefore, we pretreated fully differentiated BMDMs with 9.48 ng/ml and 31.6 ng/ml recombinant M-CSF or completely deprived them from M-CSF (as equivalents for the 6%, 20%, and no LCCM, respectively) for 18 hours then infected with *Msmc* as previously described. The same M-CSF conditions were maintained throughout the 22 hours infection. We then measured the amounts of secreted IL-1 β by ELISA. There was no significant difference in *Msmc*-induced IL-1 β secretion between BMDMs deprived from LCCM and the ones supplemented with 9.48 ng/ml M-CSF. However, BMDMs supplemented with 31.6 ng/ml M-CSF showed a slightly significant decrease in IL-1 β secretion (approx. 5 ng/ml) compared to BMDMs deprived of M-CSF (approx. 7.4 ng/ml) (Figure 15C). These results suggest that M-CSF plays a small role in

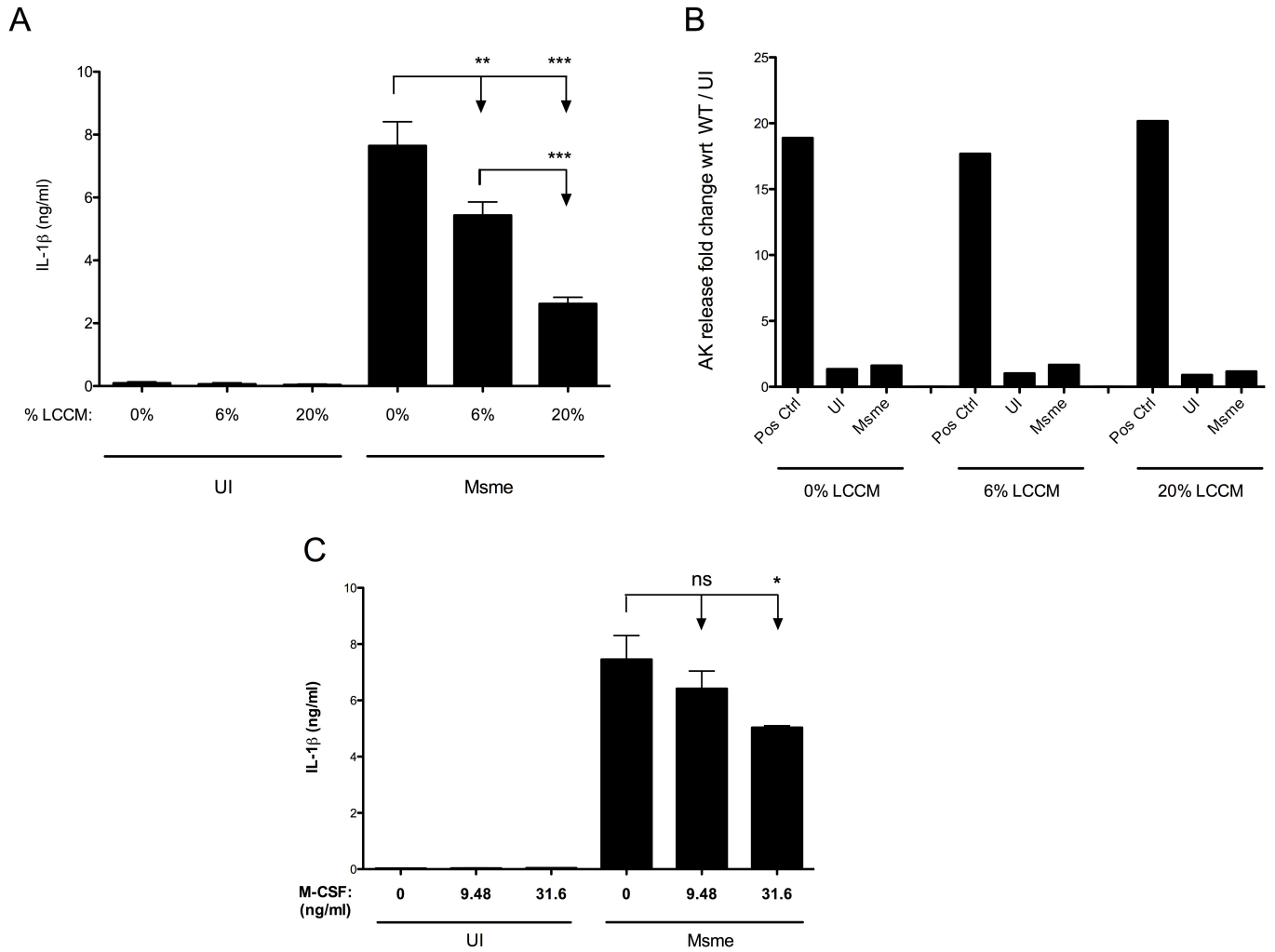


Figure 15. L cell-conditioned medium has an inhibitory effect on the *Msme*-induced IL-1 β secretion in BMDMs. **A.** BMDMs were derived from WT mice then supplemented with 6%, 20%, or left without L cell-conditioned medium (LCCM) for 18 hours then infected with *Msme* at MOI 10:1 left uninfected (UI). The supernatants were harvested after 22 hours and the amount of secreted IL-1 β were detected by ELISA. The amounts of AK in the supernatant were measured via AK release colorimetric assay (**B**) The positive control (Pos Ctrl) indicates complete cell lysis via treatment with Triton-X. **C.** BMDMs derived from WT mice were supplemented with 9.48 ng/ml, 31.6 ng/ml, or left without recombinant macrophage colony-stimulating factor (M-CSF) for 18 hours then infected with *Msme* at MOI 10:1 left uninfected (UI). The supernatants were harvested after 22 hours and the amount of secreted IL-1 β were detected by ELISA. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

the inhibition of the *Msmc*-induced IL-1 β secretion in BMDMs. However, The comparison of these results (Figure 15C) with the effect of complete LCCM on IL-1 β secretion (Figure 15A) suggests that M-CSF it is not the only components of LCCM that is responsible for this inhibition

3.3.5 The IL-1 receptor is not required for *Msmc*-mediated IL-1 β secretion and has no role in apoptosis induction in dendritic cells.

We further sought to investigate whether signaling via the IL-1 receptor is required for the *Msmc*-induced secretion of IL-1 β and to check whether IL-1R signaling is involved in the *Msmc*-induced apoptosis in DCs. We infected WT and *Il-1r* deficient (*Il-1r*^{-/-}) BMDCs with *Msmc* as previously described. We also pre-treated WT BMDCs with the drug Anakinra, an antagonist of IL-1R, and infected those cells with *Msmc* as well. We then measured the amounts of secreted IL-1 β in the supernatants and quantified the number of apoptotic cells via the hypodiploid cytometric assay. The lack of IL-1R did not have any effect on the *Msmc*-induced secretion of IL-1 β . This indicates that IL-1 β secretion does not have a positive autocrine feedback via IL-1R signaling. Furthermore, the lack of IL-1R did not have any effect on the apoptotic response induced by *Msmc* in dendritic cells. *Msmc* infection in both WT and *Il-1r*^{-/-} BMDCs induced similar levels of apoptosis (approx. 75% hypodiploid positive cells) (Figure 16B). Concurrently, treatment of WT BMDCs with Anakinra did not have any effect on the induction of apoptosis by *Msmc* in DCs since both Anakinra-treated and untreated BMDCs showed similar levels of apoptosis upon infection with *Msmc* (approx. 68% hypodiploid positive cell). These results suggest that signaling of IL-1 β via IL-1R is not involved in the induction of apoptosis in *Msmc*-infected DCs.

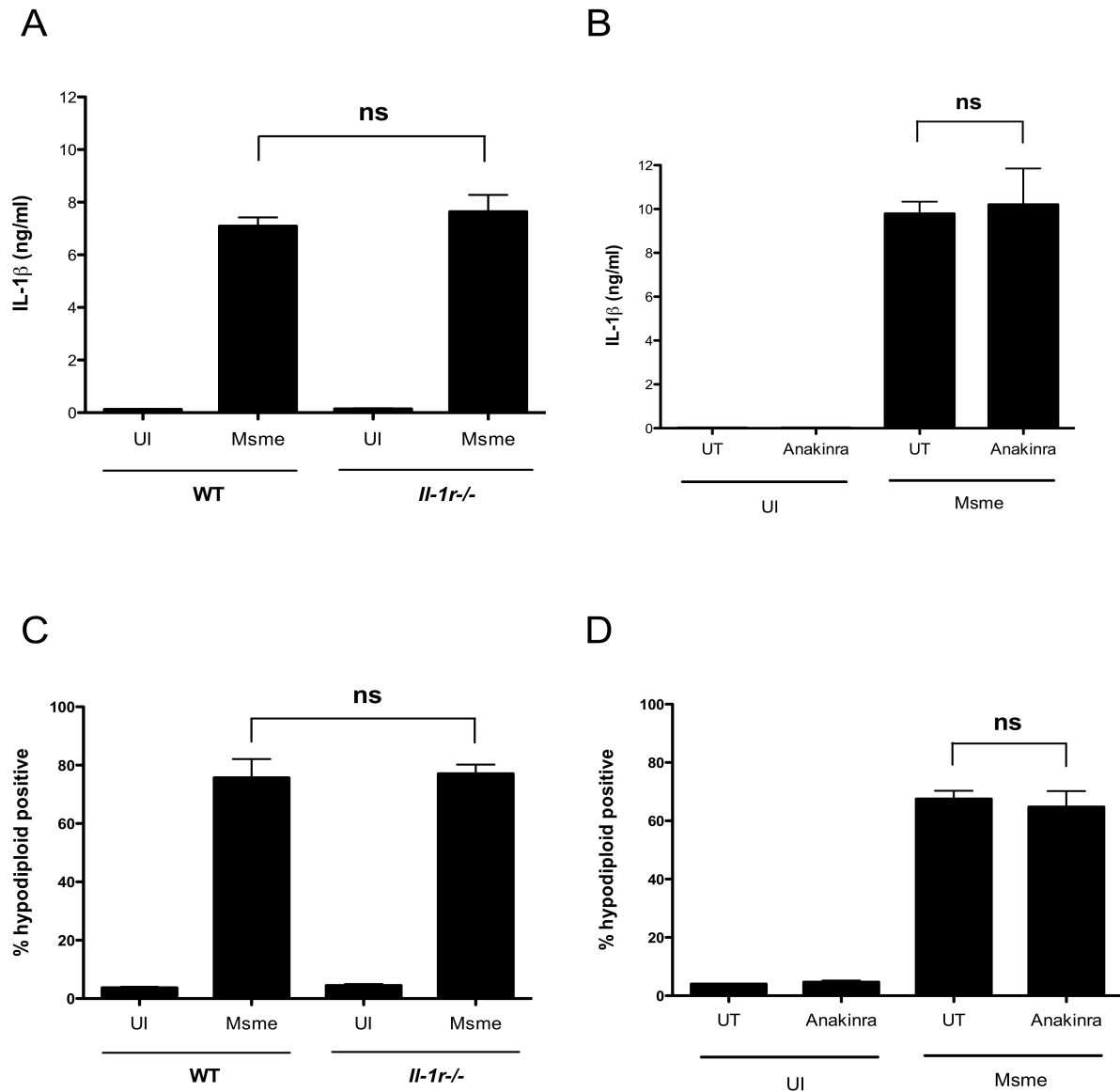


Figure 16. The IL-1 receptor is not required for *Msme*-induced IL-1 β secretion and has no role in apoptosis induction. A and C. BMDCs from WT and *Il-1r* deficient (*Il-1r*^{-/-}) mice were infected with *Msme*. **B and D.** BMDCs from WT mice were treated with Anakinra (IL-1R antagonist) or left untreated (UT) then infected with *Msme* or left uninfected (UI). The amount of secreted IL-1 β in the supernatants were measured 22 hours after infection by ELISA (**A and B**). The percentage of apoptotic cells was also measured 22 hours post infection via the hypodiploid cytometric assay (**C and D**). Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

3.3.6 *Msmc*-induced IL-1 β secretion in dendritic cells is independent of caspases-3, -8, and -9.

As previously stated, *in vivo Mtb* infection studies have implicated the presence of caspase-1-independent production of IL-1 β in contrast to caspase-1-dependent IL-1 β production in BMDMs infections *in vitro*, which suggests the involvement of cell types other than macrophages such as DCs in the production of IL-1 β [104]. We have shown that decreased (compared to WT) but significant amount of secreted IL-1 β was independent of caspase-1/11 in BMDCs infected with *Msmc* (Figure 13A). Therefore, it is possible that other protease(s) might be involved in the processing of pro- IL-1 β and the secretion of mature IL-1 β . To test this hypothesis we checked for the involvement of caspases other than caspase-1 and-11 in the production of IL-1 β . We pre-treated *Casp-1/11*^{-/-} BMDCs with a general pan caspase inhibitor (z-vad-fmk), caspase-3 inhibitor, caspase-8 inhibitor, caspase-9 inhibitor, caspase inhibitor chemical analog (negative control), or left the cells untreated for duration of 4 hours then infected them with *Msmc* for 2 hours. We then washed the cells and incubated them in infection medium supplemented with gentamicin while maintaining the same concentrations of inhibitors in the medium. The amounts of secreted IL-1 β were measured in the supernatant via ELISA. Interestingly, the general pan caspase inhibitor further reduced the secretion of IL-1 β in *Casp1/11*^{-/-} BMDCs to 1.9 ± 0.1 ng/ml compared to the untreated cells (3.4 ± 0.3 ng/ml) upon infection with *Msmc* (Figure 17A). This significant decrease in IL-1 β secretion indicates the involvement of a caspase(s) other than caspase-1 in the production of IL-1 β . The inhibition of caspases-3, -8, or -9 did not result in any significant decrease in the amount of secreted IL-1 β (Figure 17A). All the

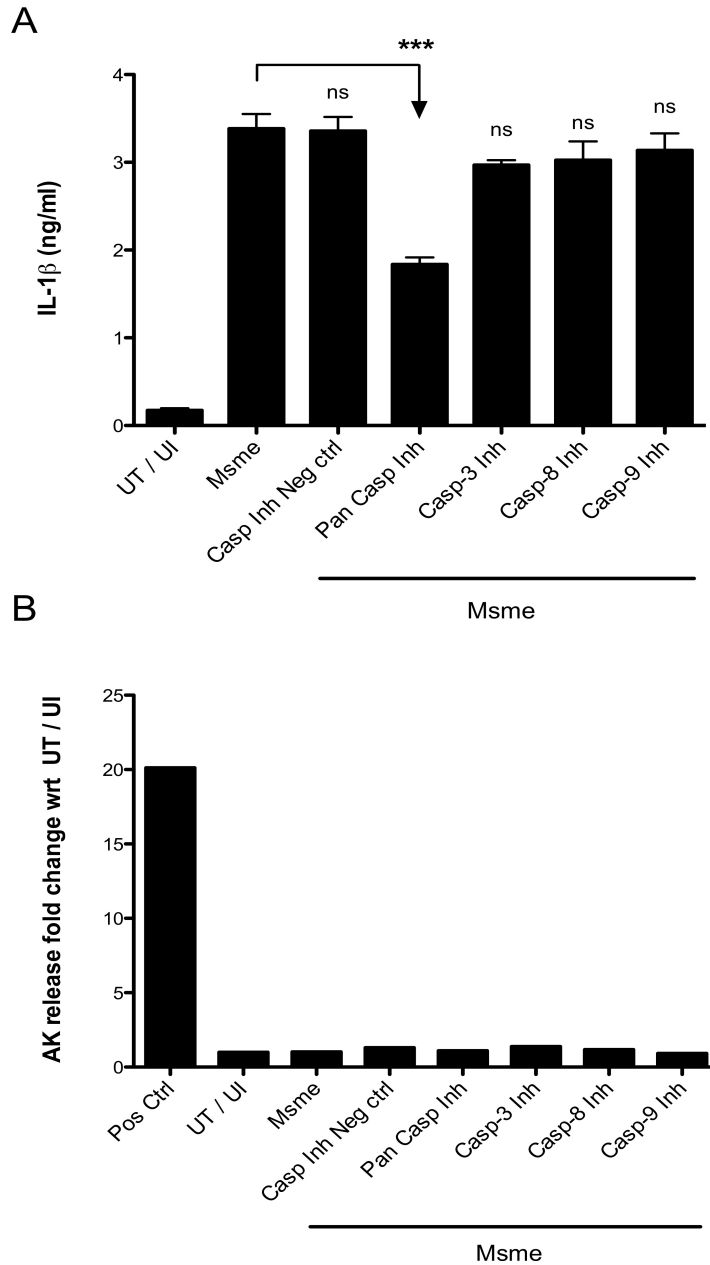


Figure 17. *Msme*-induced IL-1 β secretion is independent of caspases-3, -8, and -9. BMDCs were pre-treated with capase-3 inhibitor (casp 3 inh), capase-8 inhibitor (casp 8 inh), capase-9 inhibitor (casp 9 inh), a general caspase inhibitor (pan casp inh), inactive chemical analog (casp inh – ctrl) or left the cells untreated (UT) then infected them with *Msme* for duration of 2 hours. The untreated cells were also left uninfected (UT /UI). The cells were then washed and incubated in media supplemented with gentamicin and the same concentrations of inhibitors. Culture supernatants where collected 20 hours later and assayed for the amounts of secreted IL-1 β by ELISA (**A**) or the amounts of AK released by the AK release colorimetric assay (**B**). The positive control (Pos Ctrl) indicates complete cell lysis via treatment with Triton-X. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

IL-1 β detected in the supernatant was of the secreted mature form since no cytosolic AK enzyme was detected in the supernatants of infected cells, which indicates that no cell lysis occurred as shown in Figure 17B. These results demonstrate the involvement of a caspase other than caspase-1 in the production of IL-1 β in *Msmc*-infected BMDCs. They further extend the analysis and eliminate three main caspases, -3, -8, and -9 from the pool of possible caspases that might be involved in the production of IL-1 β in *Msmc*-infected BMDCs.

3.3.7 *Msmc*-induced IL-1 β secretion in dendritic cells is independent of Cathepsins B, L, S and Calpain.

The cysteine proteases cathepsins are reported to be involved in inflammasome activation and IL-1 β secretion. Previous studies have shown that inflammasome activation and subsequent secretion of IL-1 β were dependent on Cathepsin B (CatB) and NLRP3 inflammasome activation via P2X7 receptor stimulated by the acute-phase protein serum amyloid A (SAA) [251-253]. The release of cathepsin B and the production of ROS were also reported to be involved in *Mkan*-induced inflammasome activation [220]. Furthermore, CatB, cathepsin L (CatL), and cathepsin S (CatS) along with NLRP3 were also shown to be required for IL-1 β secretion during inflammatory Colitis in mice [252]. CatB inhibition also attenuated the secretion of IL-1 β from *Nlrp3* and *Asc* deficient microglia [252]. Another protease, calpain, was also reported to be involved in the processing of IL-1 α in mice and BMDCs via a caspase-1-independent pathway [254]. We therefore extended our analysis of the caspase-1/11-independent IL-1 β secretion in *Msmc*-infected BMDCs to test whether cathepsins (CatB, CatS, and CatL) and calpain are involved in the production of

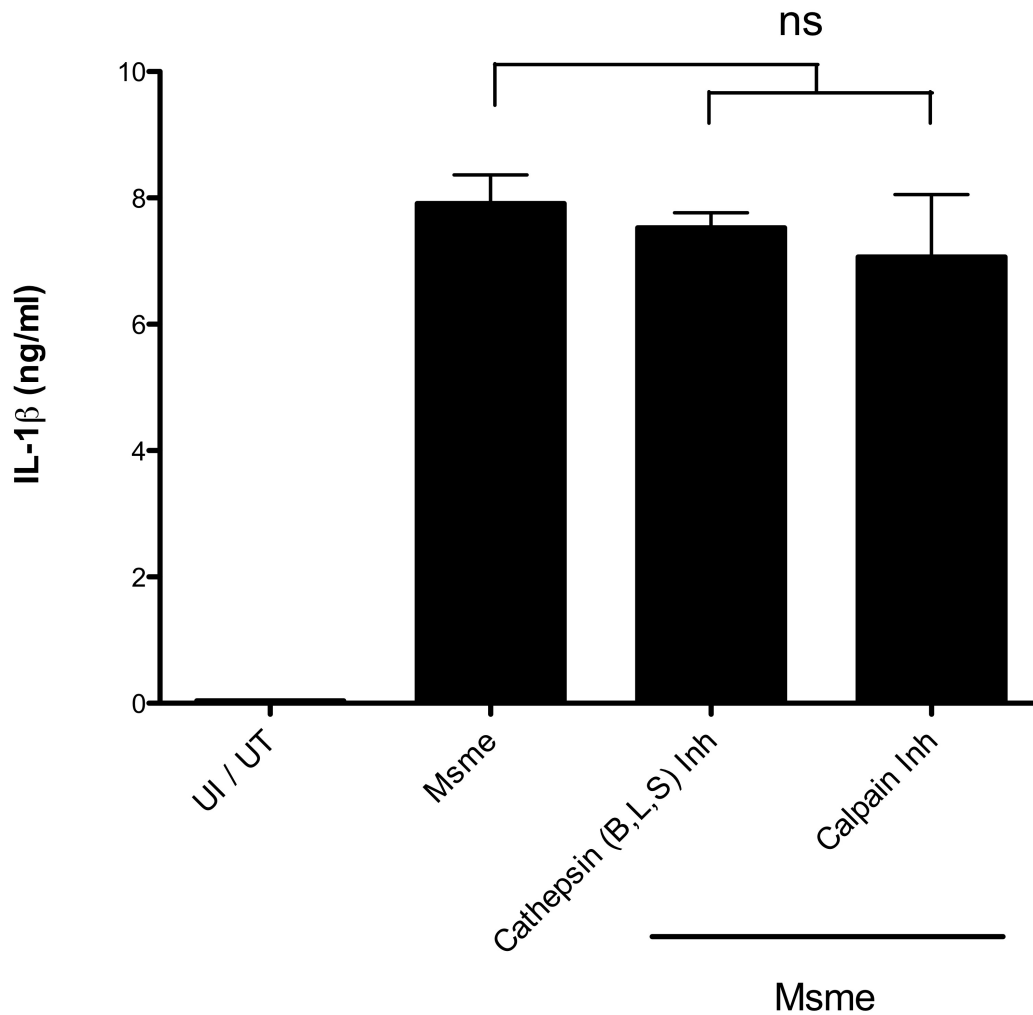


Figure 18. *Msme*-induced IL-1 β secretion is independent of Cathepsins B, L, S and Calpain. BMDCs were pre-treated with inhibitor of Cathepsins B, S, and L (cathepsin (B,L,S) inh) or left untreated for duration of 4 hours then infected them with *Msme* at MOI 10:1 duration of two hours. The untreated cells were also left uninfected (UT /UI). Cells were then washed and incubated in infection medium with gentamicin for an additional 20 hours while maintaining the same concentration of inhibitor in the media. The amounts of secreted IL-1 β were measured in the supernatant after 20 hours by ELISA. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

the cytokine. We pre-treated BMDCs with inhibitor of cathepsins B, S, and L, inhibitor of calpain, or left them untreated for duration of 4 hours then infected them with *Msmc* at MOI 10:1 as previously described for duration of 2 hours. The cells were then washed and incubated in infection medium with gentamicin for an additional 20 hours while maintaining the same concentration of inhibitors in the media. As shown in Figure 18, the amounts of secreted IL-1 β in the supernatant were similar for all conditions compared to untreated *Msmc*-infected cells (7-7.8 ng/ml, $p > 0.05$). Our results indicate that none of the examined proteases (CatB, CatS, CatS, and calpain) were involved in the production of IL-1 β in *Msmc*-infected BMDCs (Figure 18).

3.3.8 Characterization of the caspase-1/11-independent IL-1 β secretion in dendritic cells.

A recent study has shown that in addition to the NLRP3-caspase-1-dependent IL-1 β secretion in BMDMs and BMDCs, there is a caspase-8 pathway that promotes IL-1 β precursor maturation and secretion independently of inflammasome activation [255]. This NLRP3-caspase-1-independent caspase-8-dependent production of IL-1 β was demonstrated to be independent of ASC. Both the NLRP3-caspase-1-dependent and the caspase-8-dependent-ASC-independent processing of IL-1 β relied on RIP3 kinase activation in response to the inhibition of inhibitors of apoptosis (IAP) in macrophages and DCs [255] [256]. Another study revealed dectin-1 as an extracellular pathogen sensor involved in the induction and processing of IL-1 β via a non-canonical caspase-8 inflammasome [257]. These recently published reports prompted us to re-examine the involvement of caspase-8 in IL-1 β secretion and extend our analysis by examining the involvement of dectin-1 in the

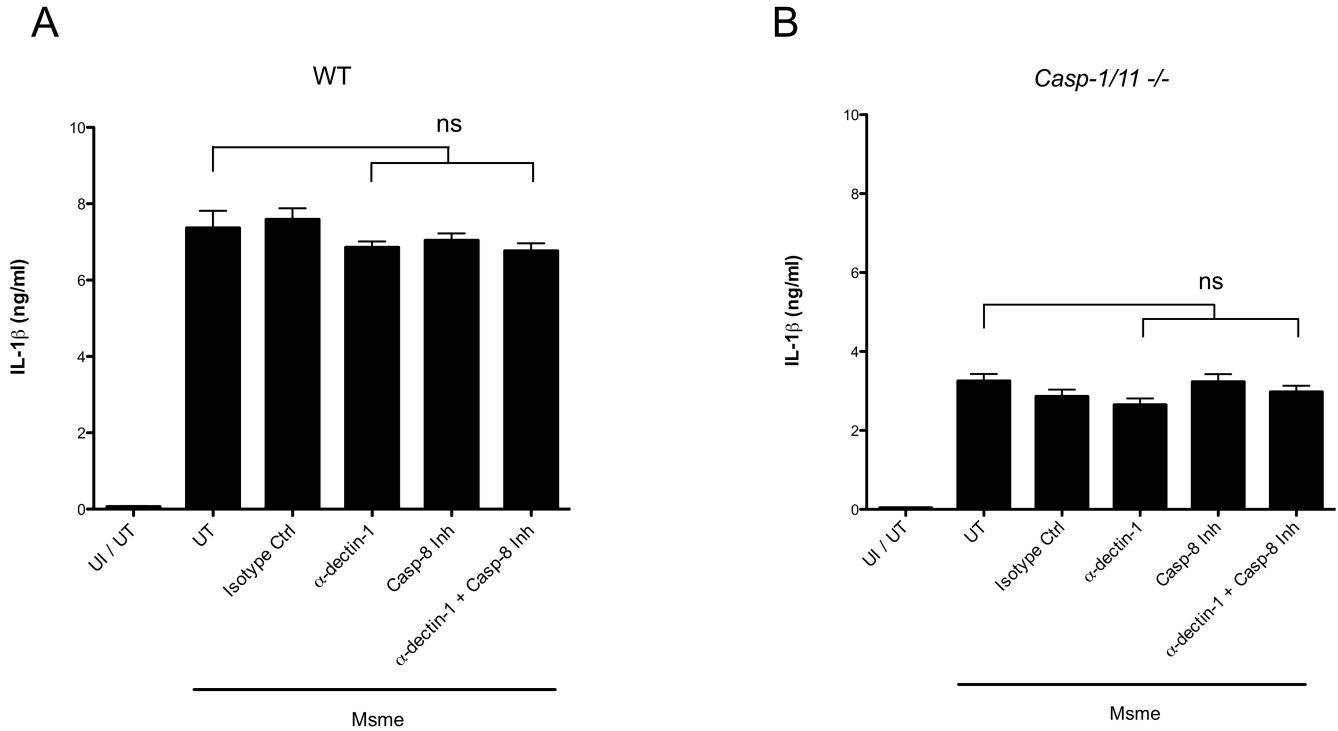


Figure 19. The caspase-1-independent IL-1 β secretion is independent of dectin-1. WT (A) and *caspase-1/11*^{-/-} (B) BMDCs were pre-treated with mouse anti-dectin-1 neutralizing antibody (α -dectin-1), caspase-8 inhibitor (casp8 inh), both inhibitors, or left untreated (UT) then infected with *Msme* at MOI 10:1 for duration of two hours. The untreated cells were also left uninfected (UT /UI). Cells were then washed and incubated in infection medium with gentamicin for an additional 20 hours while maintaining the same concentration of inhibitors in the media. The amounts of secreted IL-1 β were measured in the supernatant after 20 hours by ELISA. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

production of the cytokine. Therefore, we pre-treated WT and *caspase-1/11* deficient BMDCs with anti-dectin-1 neutralizing and *caspase-1/11* deficient BMDCs with anti-dectin-1 neutralizing antibody (α -dectin-1), caspase-8 inhibitor, both α -dectin-1 and caspase-8 inhibitor, inactive chemical analog, or left them untreated for 4 hours then infected them with *Msmc* as previously described for duration of 2 hours followed by 20 hours incubation in infection medium with gentamicin and same concentrations of neutralizing antibodies and inhibitory chemicals. WT BMDCs infected with *Msmc* showed similar levels of secreted IL-1 β among all treatment conditions (approx. 7-7.8 ng/ml) compared to the untreated (UT) *Msmc*-infected cells (8.2 ng/ml) (Figure 19A). None of the treatments showed a significant decrease in IL-1 β secretion compared to the UT control ($p > 0.05$) (Figure 19A). Concurrently, *Casp1/11*^{-/-} *Msmc*-infected BMDCs secreted similar amount of IL-1 β , except that the amounts of cytokine were overall decreased (approx. 3-3.4 ng/ml) across all conditions compared to WT BMDCs (Figure 19B). This decrease in IL-1 β secretion was expected since IL-1 β secretion is partially dependent on caspase-1/11 as was previously demonstrated (Figure 13A). These results failed to reveal any role of caspase-8 or dectin-1 in the secretion of IL-1 β whether it is caspase-1-dependent or -independent. Our previously described results showed that BMDCs deficient in *Asc* were completely defective in IL-1 β secretion upon infection with *Msmc* (Figure 13A). Indeed, we confirmed that all IL-1 β processing and secretion in *Msmc*-infected BMDCs were dependent on inflammasome activation, specifically ASC-dependent inflammasome activation. We also demonstrated that the IL-1 β secretion is only partially dependent on NLRP3 in *Msmc*-infected BMDCs (Figure 13A). In order to further characterize the caspase-1/11-

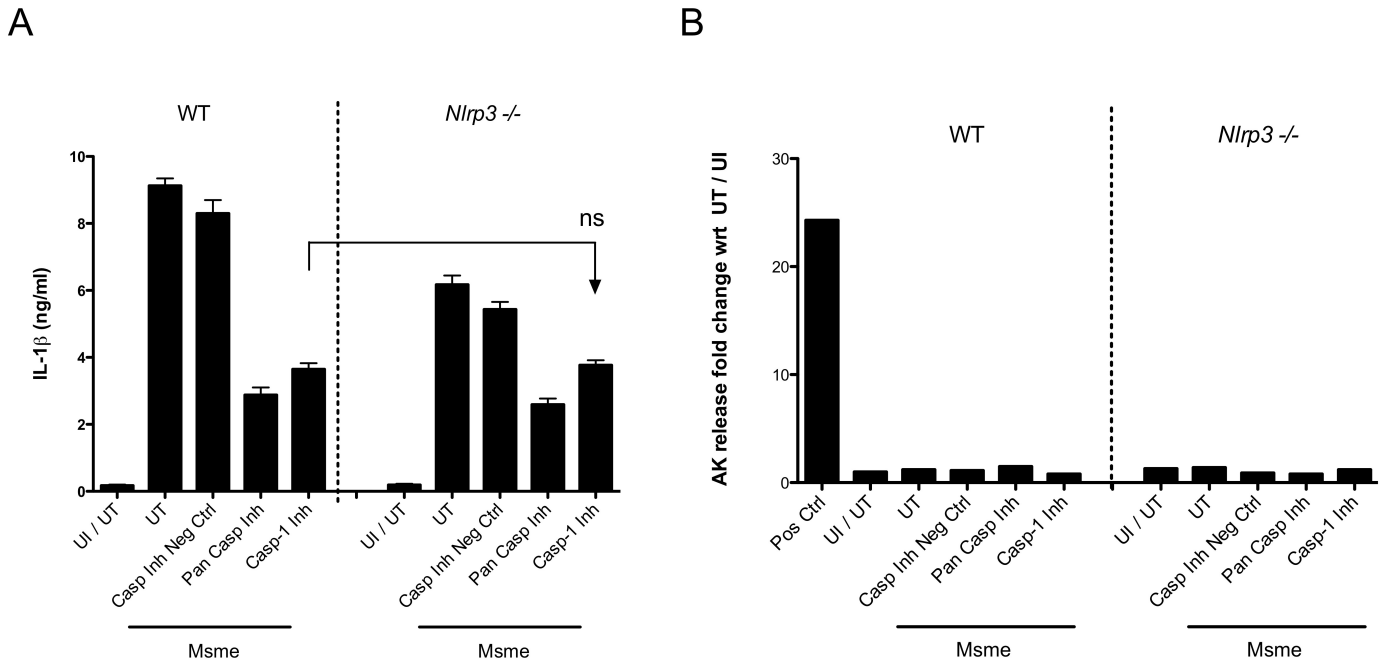


Figure 20. Characterization of the caspase-1/11-independent IL-1 β secretion in dendritic cells. WT and *Nlrp3*^{-/-} BMDCs were pre-treated with capase-1 inhibitor (casp 1 inh), general caspase inhibitor (pan casp inh), inactive chemical analog (Casp Inh Neg Ctrl) or left untreated (UT) then infected with *Msme* for duration of 2 hours. The untreated cells were also left uninfected (UI /UI). The cells were then washed and incubated in media supplemented with gentamicin and the same concentrations of inhibitors. Culture supernatants were collected 20 hours later and assayed for the amounts of secreted IL-1 β by ELISA. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

-independent IL-1 β secretion, we used WT and *Nlrp3*^{-/-} BMDCs and pre-treated them with caspase-1 inhibitor, pan-caspase inhibitor (z-vad-fmk) or left them untreated, then infected them with *Msmc*. Both *Msmc*-infected WT and *Nlrp3*^{-/-} cells treated with caspase-1 inhibitor secreted the same amounts of IL-1 β (3.8 ± 0.2 ng/ml and 3.9 ± 0.1 ng/ml respectively) (Figure 20). These results demonstrate that the caspase-1/11-independent IL-1 β production in *Msmc*-infected BMDCs is independent of NLRP3.

3.3.9 *Msmc*-induced IL-1 β secretion in dendritic cells is partially dependent on AIM2 but independent of NLRP6, NLRP10, NLRP12, and IPAF.

We have shown that IL-1 β secretion in *Msmc*-infected BMDCs was only partially-dependent on NLRP3 inflammasome component (Figure 13A and 21A). However, IL-1 β Secretion was strictly dependent on ASC (Figure 13A and 21A). The indispensable involvement of ASC suggests that there is another inflammasome involved in the production of IL-1 β since ASC is a major adaptor protein that is common to several NLR family member inflammasomes (NLRP1, NLRP2, NLRP3, IPAF, NLRP6, NLRP10, NLRP12) as well as the PYHIN family member AIM2 [210]. In order to examine the involvement of another inflammasome other than NLRP3 in the secretion of IL-1 β , we used BMDCs from *Asc*^{-/-}, *Nlrp3*^{-/-}, *Ipafl*^{-/-}, *Nlrp6*^{-/-}, *Nlrp10*^{-/-}, *Nlrp12*^{-/-}, *Aim2*^{-/-}, and WT mice and infected them with *Msmc* as previously described. The amounts of IL-1 β secreted in the supernatant were measured via ELISA. The levels of IL-1 β secreted by BMDCs deficient in IPAF (12.1 ± 0.6 ng/ml), NLRP6 (9.5 ± 0.6 ng/ml), NLRP10 (11.1 ± 1.4 ng/ml), and NLRP12 (9.9 ± 1.1 ng/ml) were similar to the level of IL-1 β secreted by WT BMDCs (11 ± 0.7 ng/ml) ($p > 0.05$, Figure 21A). The secretion of IL-1 β by *Msmc*-infected *Nlrp3*

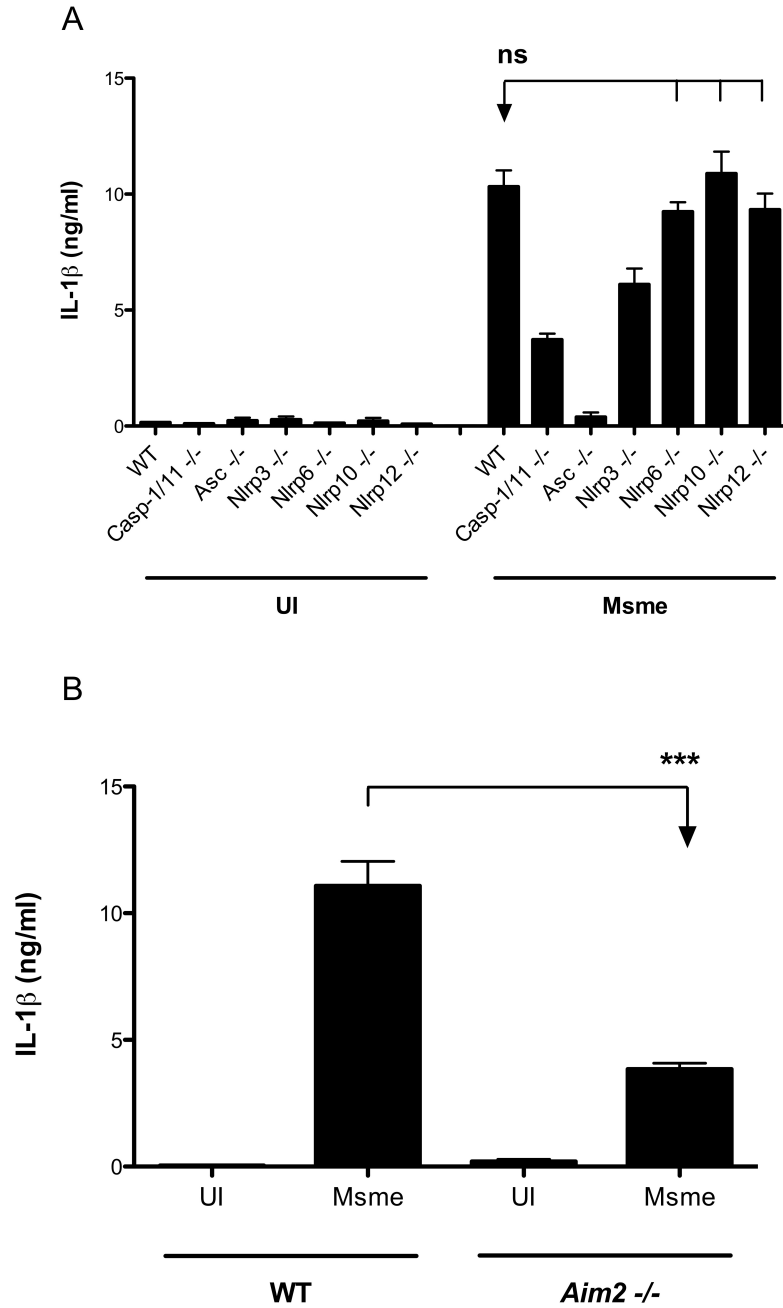


Figure 21. *Msme*-induced IL-1 β secretion is partially dependent on AIM2 but independent of NLRP6, NLRP10, and NLRP12. BMDCs from *Asc*^{-/-}, *Nlrp3*^{-/-}, *Ipadf*^{-/-}, *Nlrp6*^{-/-}, *Nlrp10*^{-/-}, *Nlrp12*^{-/-}, WT mice (A), and *Aim2*^{-/-} BMDCs (B) were infected with *Msme* or left uninfected (UI) for two hours then washed and incubated in media supplemented with gentamicin. The supernatants were collected 20 hours later and assayed for the amounts of secreted IL-1 β by ELSA. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

deficient BMDCs was expectedly decreased (6.1 ± 0.9 ng/ml) and completely abolished in *Asc* deficient BMDCs since no significant amounts of IL-1 β were secreted compared to the uninfected control (UI) as was observed in previous experiments (Figure 13A). These results eliminate NLRP6, NLRP10, NLRP12, and IPAF from the pool of possible NLR family inflammasomes that might have been involved in the secretion of IL-1 β in BMDCs.

Interestingly, the absence of AIM2 had a very significant effect on the levels of *Msm*-induced secretion of IL-1 β in BMDCs. As shown in Figure 21B, *Aim2*^{-/-} BMDCs secreted only $3.7 \pm$ ng/ml IL-1 β upon infection with *Msm* compared to WT cells, which secreted 12.3 ± 1.3 ng/ml IL-1 β . This significant decrease in cytokine secretion demonstrates the involvement of AIM2 inflammasome in the secretion of IL-1 β in *Msm*-infected BMDCs. All previous studies only reported the involvement of NLRP3 in the inflammasome response against *Mtb* in macrophages and dendritic cells [104] [220]. This is the first time that AIM2 is implicated in the inflammasome activation in response to infection with a mycobacterial species in dendritic cells.

3.3.10 AIM2 plays a role in the IL-1 β secretion by dendritic cells infected with non-pathogenic and attenuated but not virulent mycobacteria.

Thus far, our analysis of *Msm*-induced IL-1 β production in mouse BMDCs has shown that IL-1 β is abundantly secreted and that this secretion was indispensable of ASC, and partially dependent on NLRP3 and caspase-1/11. The caspase-1/11-independent secretion of IL-1 β was also shown to be independent of NLRP3. Our analysis revealed the partial involvement of AIM2 inflammasome component in the secretion of mature IL-1 β in *Msm*-infected BMDCs. This involvement of AIM2 has not been examined *in vitro* or *in*

vivo. This is the first time that AIM2 is implicated in the secretion of IL-1 β in response to infections with a mycobacterial species. Several studies addressed the capacity of different mycobacterial species to activate the inflammasome and induce the secretion of IL-1 β in macrophages but no detailed analysis was performed on DCs. Studies involving *Mtb* infections have shown that virulent *Mtb* H37Rv and attenuated *Mtb* H37Ra induced NLRP3, ASC, and caspase-1-dependent inflammasome activation and subsequent IL-1 β secretion in human macrophage cell line THP-1 and in primary mouse BMDMs [104] [220]. These studies have also shown that ASC but not NLRP3 nor caspase-1 are important for the host defense against *Mtb in vivo* despite their importance *in vitro* [218]. Other studies showed that the NLRP3/ASC inflammasome contributes to caspase-1 activation and IL-1 β secretion during *Mkan* infection of THP-1 cells [220]. Facultative-pathogenic BCG fails to induce the secretion of mature IL-1 β in macrophages and DCs due to its inability to process pro- IL-1 β to its mature form [257]. We therefore examined the involvement of AIM2 in the production of IL-1 β in response to infections with a multitude of virulent, attenuated, and non-pathogenic mycobacterial species in DCs. We used *Aim2*^{-/-} and WT BMDCs and infected them with virulent *Mtb* H37Rv, attenuated *Mtb* H37Ra, *Mkan*, *Mfort*, *Msme*, and *F. tularensis* Live Vaccine Strain (LVS). *F. tularensis* was shown to induce the secretion of IL-1 β via AIM2-dependent pathway in macrophages and DCs [217] [258]. Cells were infected for 2 hours then washed and incubated in media with gentamicin for additional 20 hours as previously described. The amounts of secreted IL-1 β were quantified by ELISA. Interestingly, All mycobacterial species, except virulent *Mtb* H37Rv, showed a significant decrease in the secretion of IL-1 β compared to the WT (Figure 22). As expected, the *Francisella* control (LVS) did not induce any significant

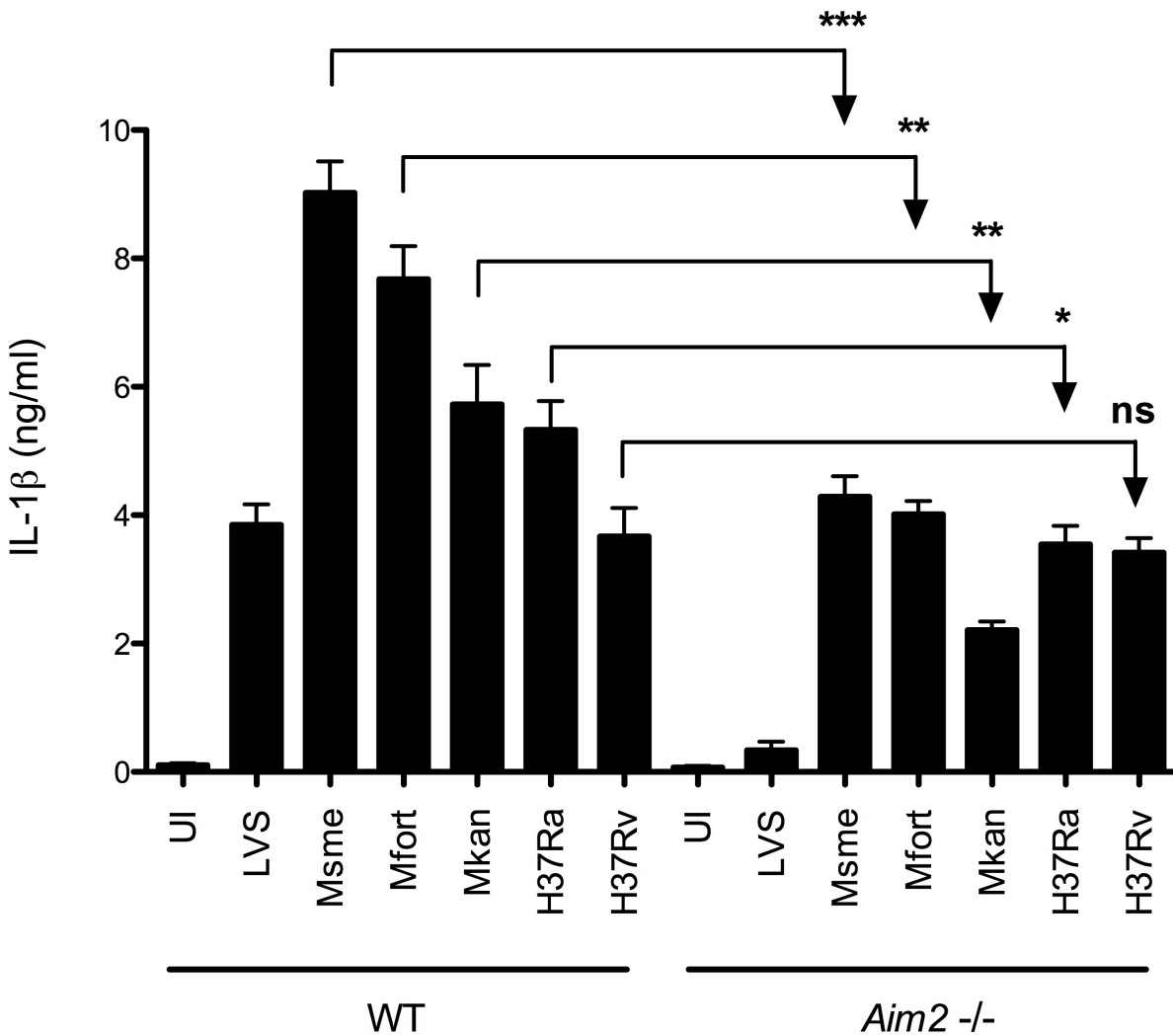


Figure 22. AIM2 plays a role in the IL-1 β secretion by dendritic cells infected with non-pathogenic and attenuated but not virulent mycobacteria. WT and *Aim2*^{-/-} BMDCs were infected with *Francisella* Live vaccine strain (LVS), *Msme*, *Mfort*, *Mkan*, *Mtb* H37Rv, *Mtb* h37Ra, or left uninfected (UI) for duration of 2 h then washed and incubated in media with gentamycin. The supernatants were collected 20 h later and assayed for the amounts for secreted IL-1 β by ELISA. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

secretion of IL-1 β (Figure 22) since it depends primarily on the AIM2 inflammasome for the production of mature IL-1 β [211] [258]. The capacity of virulent *Mtb* H37Rv strain to induce the secretion of IL-1 β in BMDCs was not undermined by the lack of AIM2 as was expected since a recent study in our lab demonstrated that the secretion of IL-1 β in virulent *Mtb*-infected BMDCs indispensably requires NLRP3 [259]. In this study, *Nlrp3*^{-/-} BMDCs infected with virulent *Mtb* failed to secrete any significant amounts of IL-1 β . In our analysis, *Mtb* H37Rv induced the secretion of approximately 3.8 ng/ml IL-1 β in WT BMDCs compared to a similar 3.7 ng/ml secretion in *Aim2*^{-/-} BMDCs (Figure 22). The attenuated *Mtb* H37Ra-induced IL-1 β secretion in BMDC showed the least dependence on AIM2 among all examined facultative-pathogenic and non-pathogenic mycobacteria since the secretion of IL-1 β induced by H37Ra only decreased from 5.3 \pm 0.6 ng/ml in WT to 2.9 \pm 0.3 ng/ml in *Aim2*^{-/-} BMDCs compared to higher decreases in other mycobacterial species (Figure 22). Our results suggest an evolutionary role for AIM2-dependent inflammasome activation and IL-1 β secretion in mycobacteria-infected DC where non-pathogenic and attenuated species of mycobacteria are capable of engaging another inflammasome in addition to NLRP3 upon infection of host DCs.

3.3.11 IFN- β is important for the AIM2-dependent *Msmc*-induced IL-1 β secretion in dendritic cells.

AIM2 inflammasome activation was shown to be dependent on type I interferon family member interferon- β (IFN- β) in BMDMs but this dependence was only partial in BMDCs [217]. This was reported in studies where *Francisella*-infected BMDMs and BMDCs deficient in type I interferon receptor (*Ifnr*^{-/-}) showed total reduction in the

secretion of IL-1 β from infected BMDMs but only partial reduction of the cytokine in infected BMDCs of [211]. IFN- β was also shown to act upstream of AIM2 leading to activation of the AIM2 inflammasome and subsequent activation of caspase-1 and processing of pro- IL-1 β into the mature secreted form [258] [260] [261]. Conversely, IFN- β was not shown to have any effect on other ASC dependent inflammasomes such as NLRP3, NLRP6, and NLRP12 [221]. IFN- β was also shown to play a role in the inhibition of *Il-1 β* gene expression in THP-1 cells. *Mtb*-induced IL-1 β production was shown to be suppressed by IFN- β in THP-1 cells but not monocytes [221]. This suppression of mature IL-1 β production was shown to be due to lower levels of pro- IL-1 β as a result of IFN- β induced inhibition of *Il-1 β* gene expression [221]. These studies of IFN- β inhibition of *Il-1 β* expression only involved macrophages and did not examine this newly found role of IFN- β in DCs. Therefore, we examined the involvement of IFN- β and its role in AIM2-mediated secretion of IL-1 β in BMDCs upon infection with virulent *Mtb* and non-pathogenic *Msmc*. We pre-treated WT and *Aim2*^{-/-} deficient BMDCs with anti-mouse IFN- β neutralizing antibody (10 μ g/ml) or left untreated (UT) for duration of 4 hours, then infected them with *Mtb*, *Msmc*, and *Francisella* LVS vaccine strain (positive control) for two hours as previously described. We then washed the cells and incubated them in infection media supplemented with gentamicin and the same amount of anti-mouse IFN- β . The amounts of secreted IL-1 β in the supernatants were measured using ELISA (Figure 22). The anti-mouse IFN- β (Abcam # ab24324) has been reported in published studies to be specific and effective for the neutralization of mouse IFN- β in primary macrophages and RAW cells [262]. The lack of AIM2 in *Mtb*-infected BMDCs (*Aim2*^{-/-}) did not have any effect on the secretion of IL-1 β confirming that the production of IL-1 β in response to *Mtb*

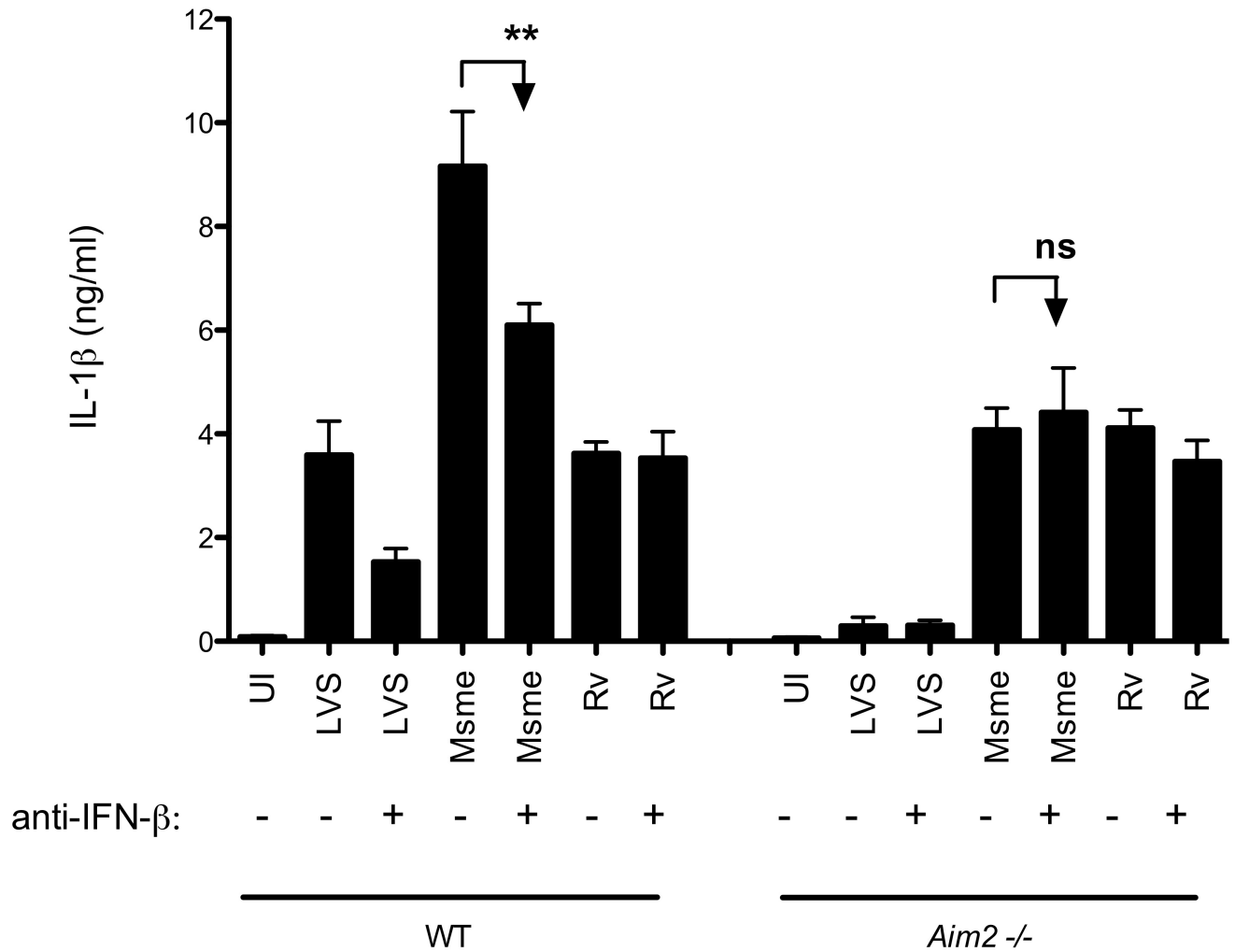


Figure 23. IFN- β is important for the AIM2-dependent *Msme*-induced IL-1 β secretion in BMDCs. WT and *Aim2*^{-/-} BMDCs were pre-treated with anti-mouse IFN- β neutralizing antibody (10 μ g/ml) or left untreated for duration of 4 hours then infected them with *Francisella* Live vaccine strain (LVS), *Msme*, virulent *Mtb* H37Rv (Rv), or left uninfected (UI) for duration of two hours then washed and incubated in media with gentamycin. The supernatants were collected 20 hours later and assayed for the amounts for secreted IL-1 β by ELSA. Shown are means and standard deviation of triplicate measurements of one representative experiment out of three.

infections of DCs does not involve the AIM2 inflammasome (Figure 22). Indeed, the blocking of IFN- β in *Mtb*-infected WT and *Aim2*^{-/-} BMDCs had no effect on the production of IL-1 β (Figure 23). However, our results indicated that the blocking of IFN- β in *Msmc*-infected cells significantly but only partially reduced the amount of secreted IL-1 β in WT BMDCs (9.1 ± 0.9 ng/ml without anti-IFN- β and 6 ± 0.3 ng/ml with anti IFN- β (Figure 23). *Msmc* infection of *Aim2*^{-/-} BMDCs treated with anti-IFN- β antibody or left untreated (UT), induced the secretion of similar levels of IL-1 β (approx. 3.9 ng/ml and 4.3 ng/ml, Figure 23), which demonstrates that IFN- β inhibition does not have any additive effect on *Msmc*-infected *Aim2*^{-/-} BMDCs. These results agree with previous studies in *Francisella* where infected macrophages and DCs showed that IFN- β is only involved in the activation of AIM2 inflammasome but not NLRP3, NLRP6, and NLRP12 inflammasomes. Our results underscore the important role of IFN- β in the AIM2-dependent IL-1 β secretion in BMDCs upon infection with non-pathogenic mycobacterial species *Msmc*.

3.3.12 Discussion.

IL-1 β is a very important proinflammatory cytokine whose production and secretion play an important role in the host IR against bacterial and viral pathogens [207] [212] [218] [237]. Mice lacking the *Il-1 β* or IL-1 receptor (*Il-1R*) genes are more susceptible to infections with *Mtb* [116] [117] [122] [218] [219]. Inflammasome activation leads to secretion of IL-1 β , which is well established as an important player in the host defense against *Mtb* infections in macrophages *in vitro* [104]. This inflammasome-dependent IL-1 β secretion is not required for the production of IL-1 β *in vivo* [218]. This difference in IL-1 β production requirements between *in vivo* and *in vitro* macrophage infections suggests that

cell types other than macrophages are involved in the processing and secretion of IL-1 β . Our analysis focused on IL-1 β secretion in response to mycobacterial infections of DCs, which to date are poorly studied in terms of inflammasome activation and IL-1 β secretion. We primarily used the non-pathogenic mycobacteria *Msmc* in our study since *Msmc* induces a stronger innate and proinflammatory IR in host macrophages and DCs when compared to a weaker host IR against facultative pathogenic mycobacteria as was demonstrated in our previous results (Figures 5, 6, and 11).

Our analysis in BMDCs revealed that *Msmc* induces the secretion of IL-1 β via an ASC-dependent and a NLRP3-caspase-1/11-partially independent pathway (Figure 13). *Msmc*, as shown in our results, as well as *Mtb* [207], have the capacity to trigger both signals that are required for the production of IL-1 β (section 1.5.1.2) without the need for additional triggers.

Furthermore, the partial dependence of *Msmc*-induced secretion of IL-1 β on caspase-1/11 (Figure 13A) was also demonstrated in *Mtb*-infected BMDCs [259]. These findings suggest the involvement of other protease(s) in the processing and secretion of IL-1 β . This caspase-dependent but caspase-1-independent secretion of IL-1 β is shown to be independent of NLRP3 (Figure 20). The capacity of a general caspase inhibitor to further reduce the amount of secreted IL-1 β in *Casp-1/11*^{-/-} BMDCs (Figure 17) suggests that such protease is a member of the caspase family. Our extended analysis further eliminated the involvement of caspases-3, -8, -9, dectin-1, as well as CatB, CatL, CastS, and Calpain in the *Msmc*-induced secretion of IL-1 β in BMDCs (Figures 18 and 19). Dectin-1 was reported to induce the secretion of IL-1 β via a non-canonical caspase-8 inflammasome-dependent pathway, with dectin-1 acting as an extracellular sensor for pathogens [257].

This non-canonical inflammasome activation requires extracellular pathogen recognition, is independent of pathogen internalization, and does not require caspase-1 for the processing of pro- IL-1 β [257].

The processing of pro- IL-1 β into the mature form in DCs was dependent on canonical inflammasome activation and caspase-1 activity for *Mtb*-infected cells, but dependent on processing via caspase-8 in *M. leprae* infections [257]. This suggests that different mycobacterial species invoke different pathways for the induced secretion of IL-1 β in the host. Our results demonstrated that infection of BMDCs with *Msmc*, similar to *Mtb* infections [104], does not depend on dectin-1 mediated activity of caspase-8. RIP3 was suggested to be required for optimal NLRP3-caspase-1 inflammasome-dependent and – independent IL-1 β processing. NLRP3-caspase-1-dependent processing of IL-1 β was shown to rely on RIP3 kinase activation in response to the inhibition of inhibitors of apoptosis (IAP) in macrophages and DCs [255]. In addition, RIP3 signaling can promote mitochondrial ROS production. This ROS generation is proposed to facilitate NLRP3-dependent IL-1 β processing. We have shown that ROS generation and accumulation is high in *Msmc*-infected BMDCs (Figure 12) in contrast with *Mtb*, which is reported to negatively affect ROS generation and accumulation in host cells [163]. This increased ROS accumulation provides one explanation for the high levels of IL-1 β secretion in *Msmc*-infected BMDCs. Furthermore, the inability of non-pathogenic mycobacteria such as *Msmc* to inhibit host cell apoptosis as demonstrated in Figures 11 and 13B could provide another explanation for this increased IL-1 β secretion in DCs. Therefore, we hypothesized that *Msmc* does not induce the expression of inhibitors of apoptosis (IAP) in infected cells, which in turn leads to increased RIP3 kinase activity and subsequent optimal NLRP3-

caspase-1 inflammasome-dependent and –independent IL-1 β processing. In this hypothesis, IAPs would act upstream of caspase-3-dependent apoptosis induction since *Msmc*-induced apoptosis in BMDCs and BMDMs was shown to be dependent on caspase-3 (Figure 8). We were unable to unveil the other caspase(s) that could be involved in the processing of IL-1 β . Further studies using RNA silencing approach (siRNA) to knockdown individual caspases in BMDCs are needed to check the involvement of these caspases in IL-1 β secretion in *Msmc*-infected BMDCs. Our inability to knockdown genes in BMDCs for technical reasons was a limiting factor in our analysis.

We also demonstrated that *Msmc* induces host DCs cell death via apoptosis but not necrosis, pyroptosis, or pyronecrosis (Figures 11, 13B, and 13C). This caspase-3-dependent apoptotic response was not dependent on any inflammasome component (NLRP3, ASC, caspase-1, and AIM2) (Figure 13B), and the secretion of IL-1 β in *Msmc*-infected BMDC does not depend on caspase-3. These findings suggest that there is no direct link between inflammasome activation / IL-1 β secretion and apoptosis induction.

The NLRP-partially-dependent but –ASC-dependent IL-1 β secretion by *Msmc*-infected BMDCs (Figure 13A) suggested the involvement of another PRR that can interact with ASC leading to the processing of IL-1 β . Our analysis involving a series of ASC-interacting NLRs (NLRP6, NLRP10, NLRP12, and IPAF) and PYHIN (AIM2) proteins revealed the involvement of AIM2 but not the aforementioned NLRs in the secretion of IL-1 β in *Msmc*-infected BMDCs (Figure 21). This is first time that the recently described AIM2 inflammasome is implicated in mycobacteria-induced IL-1 β secretion in dendritic cells. AIM2 is a member of the PYHIN protein family, a family of IFN-inducible proteins [208] [211] [217], and is localized in the cytoplasm. AIM2 is a cytosolic PRR that binds

dsDNA via HIN200 domain interaction and engages the adapter protein ASC via PYD-PYD domains interaction. ASC then recruits and activates caspase-1 via CARD-CARD domain interaction leading to the processing of pro-IL-1 β into the mature form [211]. It is reported that AIM2 regulates caspase-1 activation and IL-1 β secretion in response to dsDNA from a variety of viruses, bacteria, and even from the host [211]. It has also been shown that AIM2 is activated in response to *Francisella* infections [217]. *Francisella* escapes into the cytosol of infected dendritic cells where AIM2 senses its dsDNA. This recognition leads to the activation of AIM2 inflammasome via ASC-dependent pathway leading to activation of caspase-1, which leads to rapid release of proinflammatory IL-1 β and rapid cell death via pyroptosis [217]. Our assessment of the involvement of AIM2 in IL-1 β secretion in DCs demonstrated that AIM2 is partially involved in the production of IL-1 β in *Msmc*-infected BMDCs (Figures 22 and 23). Indeed, the involvement of AIM2 in the production of IL-1 β in response to infection with a multitude of virulent, attenuated, and non-pathogenic mycobacterial species also showed a role for AIM2 in the secretion of IL-1 β induced by *Mfort*, *Mkan* and attenuated *Mtb* H37Ra in DCs. AIM2 was shown to have no importance in virulent *Mtb*-induced secretion of IL-1 β in DCs (Figure 22). This AIM2-independent *Mtb*-induced IL-1 β secretion in BMDCs agrees with a recently published study in our lab [259], which demonstrated that the production of IL-1 β in *Mtb*-infected BMDCs is strictly dependent on NLRP3. The attenuated *Mtb* H37Ra induced IL-1 β secretion in BMDC showed the least dependence on AIM2 among all tested facultative-pathogenic and non-pathogenic mycobacteria. Our results suggest an evolutionary role for AIM2-dependent inflammasome activation and IL-1 β secretion in mycobacteria-infected DCs, whereas non-pathogenic and attenuated species of mycobacteria are capable of

engaging another inflammasome in addition to NLRP3 upon infection of host DCs. This high inflammatory response involving the activation of more than one inflammasome type is beneficial to the host making it more resistant to such infections. In contrast, virulent *Mtb* is suggested to have evolved to possess the capacity to prevent the activation of AIM2 during infection of DCs. A very recent report implicated a critical role of AIM2 in *Mtb* infections [221]. *In vivo*, mice deficient in *Aim2* were highly susceptible to *Mtb* infections. *In vitro*, *Aim2* deficient peritoneal macrophages infected with *Mtb* or transfected with *Mtb* DNA were shown to have defective caspase-1 activation and impaired IL-1 β secretion [221]. Our findings disagree with these *in vitro* reports [221]. However, it is important to note that our analysis was done in dendritic cells and not macrophages suggesting that the AIM2-dependent *Mtb*-induced IL-1 β production depends on the type of host cells.

The activation of AIM2 inflammasome requires sensing of bacterial dsDNA in the cytoplasm [211] suggesting that mycobacterial DNA should be released in the cytosol of infected cells in order to mount an AIM2-dependent IL-1 β secretion. *Mtb* resides within phagosomes of host macrophages and DCs. The escape of mycobacteria from the phagosome into the cytosol of infected cells has been a subject of debate for many years since translocation of *Mtb* into the cytosol has been observed by several groups [263], [264], while other groups failed to observe such a phenotype [264]. *M. marinum* has been shown to escape from the phagosome into the cytoplasm via actin-based motility, which depends on the region of difference 1 (RD1) [264]. *Mtb* and *M. leprae* were also shown to escape the phagosome and translocate into the cytoplasm of myeloid cells in a RD1-dependent fashion [264]. Our results support the hypothesis that *Mtb* does not translocate to the cytosol of DCs since *Mtb* infection of BMDCs did not lead to the activation of the

AIM2 inflammasome (Figure 22), which is activated by cytosolic bacterial dsDNA. However, attenuated *Mtb* H37Ra as well facultative pathogenic *Mkan* and non-pathogenic *Msmc* and *Mfort* were able to induce the secretion of IL-1 β via AIM2-dependent pathway (Figure 22) suggesting that these mycobacterial strains can somehow escape to the cytoplasm or have their DNA exposed in the cytosol of host DCs and interact with AIM2. It is therefore interesting to determine how mycobacterial DNA becomes exposed in the cytoplasm of host cells and is sensed by AIM2.

The activation of AIM2 inflammasome was shown to be dependent on IFN- β in macrophages and partially dependent on IFN- β in DCs [217]. IFN- β has been suggested to act upstream of AIM2 [221]. First, the sensing of bacteria in the cytoplasm via an unidentified process leads to the secretion of IFN- β and type I interferon receptor (IFNR) signaling. Second, the recognition of bacteria in the cytoplasm via a PRR that leads to activation of the inflammasome and subsequent IL-1 β processing if the first signal from the IFNR was received [260]. Our assessment of the involvement of IFN- β in AIM2-dependent mycobacteria-induced IL-1 β secretion demonstrated that IFN- β plays an important role in the AIM2-dependent secretion of IL-1 β in *Msmc*-infected but not *Mtb*-infected DCs. Our results showing that blocking of IFN- β in *Msmc*-infected BMDCs results in a decreased secretion of IL-1 β (Figure 23) support the hypothesis that *Msmc* induces secretion of IFN- β in DCs. IFN- β was reported to increase AIM2 protein levels and inflammasome activity in response to *Francisella* infection [258]. However, it is important to note that this IFN- β /AIM2 inflammasome activation is not the only inflammasome pathway leading to the production of IL-1 β since *Msmc* still induces the secretion of mature IL-1 β in DCs via NLP3-dependent pathway as we have demonstrated.

CHAPTER 4. SUMMARY AND FUTURE DIRECTIONS

Non-pathogenic mycobacteria are strongly attenuated and do not cause disseminating disease even in immunocompromised individuals [59]. We hypothesized that this attenuation is due to their capacity to induce a strong innate IR via induction of apoptosis and proinflammatory cytokine secretion in host cells. Our results demonstrated that non-pathogenic mycobacterial species *Msmc* and *Mfort* induce rapid and elevated host cell apoptosis, as well as increased TNF and IL-12 secretion levels seen in host macrophages and DCs compared to facultative pathogenic BCG and *Mkan*. We have characterized this apoptotic response as dependent on TNF and caspase-3 signaling pathways in addition to being dependent on the host's genotype; only BALB/c, but not C57Bl/6, macrophages showed increased apoptosis when infected with non-pathogenic mycobacteria. The comparison of macrophages to DCs revealed that the apoptotic response in the latter is stronger upon infection with non-pathogenic mycobacteria. Our finding that non-pathogenic *Msmc* induced the accumulation of high levels of ROS only in the phagosome of infected DCs, but not macrophages, supported the hypothesis that DCs are more susceptible to infection-induced apoptosis. This may be due to their capacity to generate high levels of ROS, which can lead to a sustained JNK activation and shift the TNF signaling pathway towards cell death via apoptosis [163]. Research in our lab has shown that the accumulation of ROS in DCs, but not macrophages, was reported to be due to NOX2 activity in DCs that is sustained compared to the rapid induction and inactivation of NOX2 in macrophages [163]. We have also shown in published data that the cell wall component PI-LAM, purified from non-pathogenic *Msmc* and *Mfort*, induced increased inflammatory cytokine secretion and apoptosis response when compared to Man-LAM of

facultative-pathogenic mycobacteria [71].

Based on our findings, we propose that the different mycobacterial species can be organized into the following three functional groups: 1) Nonpathogenic mycobacteria that have no mechanisms to inhibit the host IR and have a multitude of mechanisms to induce a strong host IR, 2) facultative-pathogenic mycobacteria that have few if any mechanisms to inhibit host cell IR but have evolved to mask some of their PAMPs so they do not induce a strong innate IR, and finally 3) highly adapted virulent mycobacteria that can mask their PAMPs and have mechanisms to inhibit host immune responses. We therefore hypothesize that mycobacteria in the non-pathogenic functional group possess genes involved in the production and modification of PAMPs that interact with the host's PRRs leading to the induction of a strong IR. For future directions, we propose an experimental strategy to identify new genes in the non-pathogenic *Msmc* that are involved in the production and/or modification of PAMPs. This analysis would involve the construction of a transposon mutants library in *Msmc* and then screening of this library for mutants that are defective in the induction of innate IR. We propose to mass infect THP-1 cells with the *Msmc* transposon mutants and screen for mutants that are defective in their capacity to induce rapid host cell apoptosis. We would then test individual screened mutants for their ability to induce apoptosis in BMDMs and BMDCs. Once identified, the genomic DNA of these mutants would be isolated, digested, transformed into *E. coli*, and sequenced for the determination of the site of transposon insertion and identification of the gene of interest. Once identified, the gene(s) of interest would be transformed into the mutant *Msmc* for complementation. We would then use the mutant, the complemented, and the WT *Msmc* strains to characterize the gene product's location via myc tagging and analysis of

subcellular fractions and culture filtrates. We also propose to test the viability of the mutant(s) *in vitro* and *in vivo* by infecting BMDMs and BMDCs as well as SCID mice, respectively, followed by organ histopathology and detection of host cell apoptosis.

In addition, our analysis of the induction of inflammasome activation and IL-1 β secretion in macrophages and DCs demonstrated that the *Msmc*-induced IL-1 β secretion in DCs is dependent on ASC but partially independent of NLRP3 and caspases-1/11. This caspase-1/11- partially independent IL-1 β secretion was also shown in *Msmc*-infected macrophages. Further analysis of the *Msmc*-induced IL-1 β secretion revealed that this secretion is independent of caspases-3, -8, -9, as well as cathepsins (B, S, and L), calpain, and dectin-1. Nevertheless, we demonstrated that the secretion of IL-1 β in *Msmc*-infected BMDCs involves a caspase(s) other than caspase-1 as the treatment of IL-1 β ^{-/-} *Msmc*-infected BMDCs with a general caspase inhibitor further decreased the secretion of IL-1 β . A recent study has implicated the involvement of caspase-8 and dectin-1 in the secretion of IL-1 β independently of inflammasome activation in macrophages and DCs [255]. Conversely, our analysis did not agree with these reports and caspase-8 was not shown to be involved in the secretion of IL-1 β in infected BMDCs. It is therefore important to identify the potential caspase(s) involved in the secretion of the cytokine. As future directions we propose to use a siRNA approach to knockdown several individual caspases such as caspase-2 and caspase-10 in BMDCs and check the effect of their silencing on the secretion of *Msmc*-induced IL-1 β secretion.

Our results also implicated, for the first time, the involvement of the AIM2 inflammasome in the secretion of IL-1 β in mycobacteria-infected DCs. Our comparative analysis of a wide range of mycobacterial species demonstrated that only non-pathogenic

and attenuated mycobacterial species but not virulent *Mtb* involve AIM2 in the secretion of IL-1 β in DCs. It is reported that AIM2 regulates caspase-1 activation and IL-1 β secretion in response to dsDNA from a variety of viruses, bacteria, and even from the host [211]. The activation of AIM2 inflammasome requires sensing of bacterial dsDNA in the cytoplasm [211] suggesting that mycobacterial DNA should be released in the cytosol of infected cells in order to mount an AIM2-dependent IL-1 β secretion. Despite the contradicting reports about the ability of mycobacteria to escape from the phagosome into the cytosol of host cells [263] [264], there has been no clear evidence that *Mtb* and *Msmc* can escape into the cytosol. It is therefore important to understand how non-pathogenic and attenuated mycobacteria can activate the AIM2 inflammasome. We propose, as future directions, to check for the presence of mycobacteria and mycobacterial DNA in the cytosol as well as their proximity to AIM2 proteins of infected cells. For this purpose we propose to use immunofluorescence confocal microscopy of BMDCs infected with *Msmc* and stained with antibodies detecting endogenous AIM2 and *Msmc* in the cytosol. We would also pre-label *Msmc* and virulent *Mtb* with Hoechst 33342 nucleic acid stain and use them to infect BMDCs in order to visualize the spatial localization of mycobacterial DNA and AIM2 via fluorescence microscopy. We would expect *Msmc* but not virulent *Mtb* remnants and/or DNA to colocalize with AIM2 in the cytosol. Such results would provide one explanation for the capacity of *Msmc* to activate the AIM2 inflammasome, in contrast to virulent *Mtb*, which is not able to do so.

We also characterized the AIM2-dependent IL-1 β secretion as dependent on IFN- β , which was reported to be important for the *Francisella*-induced AIM2 activation and IL-1 β secretion in macrophages and DCs [217] [258]. Our results showing that blocking of IFN- β

in *Msmc*-infected BMDCs results in a decreased secretion of IL-1 β (Figure 23) support the hypothesis that *Msmc* strongly induces the secretion of IFN- β in DCs compared to virulent *Mtb*, which does not induce such cytokine secretion. IFN- β was reported to increase AIM2 protein levels and inflammasome activity in response to *Francisella* infection [258]. However, it is important to note that this IFN- β /AIM2 inflammasome activation is not the only inflammasome pathway leading to the production of IL-1 β since *Msmc* still induces the secretion of mature IL-1 β in DCs via NLP3-dependent pathway. To further test this hypothesis, we propose, as future directions, to check the levels of IFN- β secreted by BMDCs upon infection with the wide range of non-pathogenic, attenuated, and virulent mycobacterial species like in Figure 23. We would expect the non-pathogenic and attenuated mycobacteria, but not virulent *Mtb*, to induce the secretion of large amounts of IFN- β by host DCs. Such results would provide one explanation for the capacity of virulent *Mtb* to inhibit the activation of the AIM2 inflammasome.

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