Recognition of *Mycobacterium tuberculosis* by the Host Inflammasome

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ABSTRACT

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The NLR gene family mediates host immunity to various acute pathogenic stimuli but its role in chronic infection is not known. This thesis addressed the role of NLRP3 (NALP3), its adaptor protein ASC, and caspase-1 during infection with *Mycobacterium tuberculosis* (*Mtb*). *Mtb* infection of macrophages in culture induced IL-1β secretion, and this requires the inflammasome components ASC (also PYCARD, TMS1), caspase-1, and NLRP3. However *in vivo Mtb* aerosol infection of *Nlrp3*$^{-/-}$, *Casp-1*$^{-/-}$, and WT mice showed no differences in pulmonary IL-1β production, bacterial burden, or long-term survival. In contrast, a significant role was observed for *ASC* in host protection during chronic *Mtb* infection, as shown by an abrupt decrease in survival of *ASC*$^{-/-}$ mice. Decreased survival of *Mtb*-infected *ASC*$^{-/-}$ animals was associated with defective granuloma formation and reduced CD11c^{+} CD11b$^{mid/low}$ cells. ASC is known to bind the non-inflammasome forming protein Nlrp12in an artificial expression system. Following *Mtb* infection, Nlrp12$^{-/-}$ mice had similar survival, bacterial burden, and

cytokine levels compared to the wild-type controls, indicating that Nlrp12 does not protect the host during *Mtb* infection. These data demonstrate that ASC exerts a novel inflammasome-independent role during chronic *Mtb* infection.

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LIST OF ABBREVIATIONS AND SYMBOLS

AFB	acid fast bacteria
AIM-2	Absent in melanoma 2
ANOVA	Analysis Of Variance
Apaf-1	apoptotic protease activating factor 1
ASC	adaptor molecule apoptotic speck-like protein containing a CARD
Atg	antigen
ATP	adenosine tri-phosphate
BAL	bronchoalveolar lavage
BCG	bacillus Calmette-Guérin
Blimp-1	B lymphocyte−induced maturation protein-1
BMDM	bone-marrow derived macrophages
CAPS	Cryopyrin-associated periodic syndromes
CARD	caspase activation and recruitment domain
CATERPILLER	CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats family
CD	cluster of differentiation
CDC	Centers for Disease Control and Prevention
CFU	colony-forming unit
CIITA	MHC class II transactivator
CINCA	chronic infantile, neurological, cutaneous, and articular syndrome
C-terminus	carboxy-terminus
DAMP	danger-associated molecular patterns

DC	dendritic cells
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
ERK	extracellular-signal-regulated kinases
ESAT-6	early secreted antigen target 6 kilodalton
FBS	fetal bovine serum
FCAS	familial cold autoinflammatory syndrome
FIIND	function to find domains
HIV	Human Immunodeficiency Virus
i.p.	intra-perteritoneal
IACUC	Institutional Animal Care and Use Committee
ICE	IL-1β converting enzyme
IFN	interferon
IGRA	interferon release assay
IL	interleukin
iNOS	nitric oxide synthase
IP-10	interferon-gamma-induced protein
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
JNK	c-Jun N-terminal kinases
kDa	kilodalton
LAM	lipoarabinomannan

LRR	leucine rich repeat
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling
MCP-1	monocyte chemotactic protein-1
MDP	muramyl dipeptide
MDR	multi-drug resistant
MHC	major histocompatibility complex
MLN	mediastinal lymph node
MMP	matrix metalloproteinases
MOI	multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
MWS	Muckle-Wells syndrome
MyD88	myeloid differentiation primary response gene 88
NBD	nucleotide binding domain
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLR	nucleotide-binding domain, leucine rich repeats containing family
NOMID	neonatal-onset multisystem inflammatory disease
N-terminus	amino-terminus
OVA	ovalbumin
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline

PE/PPE	Pro-Glu/Pro-Pro-Glu
PGN	peptidoglycan
PMA	phorbol myristate acetate
PPD	purified protein derivative
PRR	pattern recognition receptors
PYHIN	pyrin and HIN domain-containing protein
R	resistance
RANTES	regulated on activation normal T cell expressed and secreted
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RSV	respiratory syncytial virus
SCID	Severe Combined Immunodeficiency
sh	short hairpin
SPF	specific pathogen free
ssRNA	single-stranded RNA
TB	Tuberculosis
T_H	T helper
TIR	Toll/IL-1 receptor
TLR	Toll-like receptors
TMS1	identified target of methylation-induced silencing
TNF	tumor necrosis factor
TRAF	TNF Receptor Associated Factor

WHO	World Health Organization
XDR	extensively drug resistant
ZN	Ziehl-Neelsen
α	alpha
β	beta
γ	gamma
κ	kappa

CHAPTER 1

INTRODUCTION

Tuberculosis in humans

Mycobacterium tuberculosis (*Mtb*) is the causative agent of the disease tuberculosis. One-third of the world's population is infected with *Mtb,* including 9 million newly infected people and 2 million deaths per year (CDC, 2009). Tuberculosis (TB) is an immense health threat of global significance today, but the bacterium has also been a human pathogen throughout recorded history. TB was widespread among people of the ancient world. The earliest evidence of TB comes from bone fragments unearthed during archeological excavations dated to 5000 BC. Bone deformities indicate that the person had extrapulmonary TB (Arriaza et al., 1995). Due to new DNA sequencing techniques, *Mtb* DNA has been dated from such diverse locations as Egypt, Europe, and pre-Colombian North and South America since 2000 BC (Morell, 1994; Nerlich et al., 1997; Salo et al., 1994).

Before germ theory, there were many ideas for how TB is spread. The Greek philosopher Hippocrates believed children inherited TB from their parents since parents with TB tended to have children with TB. By the Renaissance, an

association had been drawn between those infected with TB and transmission to others via direct contact. People of the time believed correctly that TB was caused by something outside the human body. In the mid-1800s people with active TB infection were quarantined in open-air sanatoriums where fresh air and high altitude were used to treat TB. This method of treatment is not as far-fetched as it seems. From a public health standpoint, quarantining infected individuals prevents the spread of *Mtb* to others. Recent epidemiological studies correlate TB susceptibility with low vitamin D levels. Increased sun exposure is one way to elevate the amount of bioavailable vitamin D in the body (Holick et al., 1980; Liu et al., 2006; Ustianowski et al., 2005). In the absence of antibiotics, sanatoriums were a reasonable way to treat and prevent the spread of TB infection.

In 1882, Heinrich Hermann Robert Koch uncovered the etiology of TB. He used many techniques he developed including microphotography, cell staining, and growth of the organism on solid media to identify and grow *Mtb*. Koch revealed that *Mtb* was present in the granulomas of TB patients. Within the granulomas, *Mtb* was found intracellularly within macrophages (Kaufmann, 2003). It was not until 1944, however, that the first chemotherapy for TB became available with the identification of streptomycin by Selman A. Waksmann. Streptomycin was followed by other anti-tuberculosis drug treatments: isoniazid (1952), pyrazinamide (1954), cycloserine (1955), efhambutol (1962), and rifampicin (1963). Several additional drugs were developed in the years that

followed, but unfortunately, no new drugs have been introduced to treat TB in decades (Kaufmann, 2008).

Standard “short course” therapy for patients diagnosed with active TB is treatment with two of the following first-line anti-TB drugs, isoniazid, rifampicin, pyrazinamide, and ethambutol, for two months followed by isoniazid and rifampicin alone for an additional four months. Patients with latent TB are treated with isoniazid alone for 6-9 months. More aggressive drug treatment is needed to cure children, HIV-infected individuals, and those with drug resistant strains of *Mtb* (CDC, 2009). Besides the long duration of antibiotic treatment, many drugs used to treat *Mtb* have toxic side effects. Both of these factors reduce drug compliance and increase the chance that patients will develop drug-resistant strains of *Mtb*. While TB is primarily a disease of the third world, multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *Mtb* are a growing threat worldwide. MDR strains of *Mtb* are defined as those resistant to at least isoniazid and rifampin. XDR strains are resistant to isoniazid and rifampin plus any fluoroquinolone and at least one of three injectable second-line drugs (CDC, 2009). This makes some XDR TB strains virtually untreatable. In 2007, half a million new cases of MDR TB occurred within the year. Especially troubling is the fact that most of these cases had *de novo* drug resistance and were not caused by treatment failure. XDR TB has been found in 55 countries. This is likely an under estimation due to the fact that any third world countries do not have the capacity to test for XDR TB. The magnitude of XDR TB is unknown and threatens to bring us back to the days before the development of anti-TB

antibiotics (WHO, 2009a). These statistics on MDR and XDR TB highlight the pressing need for new drug treatments which act on different biochemical targets or block different stages of the *Mtb* growth cycle.

The first TB vaccine was developed by Albert Calmette, a French bacteriologist, and his assistant, Camille Guérin, a veterinarian. The vaccine was based on the successful model of cow pox vaccination used as a vaccine to prevent small pox infection. Unlike cow pox, *M. bovis* is a virulent human pathogen, so Calmette and Guérin grew and passaged the *M. bovis* on potato slices soaked in ox gall to achieve attenuation. After thirteen years and over 230 passages, they found that *M. bovis* was indeed attenuated and named their strain *M. bovis* bacilli Calmette Guérin (BCG). Calmette and Guérin used animal models to validate the vaccine, and in 1921, they vaccinated the first human newborn with BCG. Studies have since shown that the BCG vaccine successfully protects against childhood TB. Unfortunately, it is ineffective against the most prevalent form of disease, adult pulmonary tuberculosis (Kaufmann, 2008; Sterne et al., 1998).

At the time of Koch's discovery, 15 percent of deaths worldwide were attributed to TB. Despite a century of research, the BCG vaccine, and antibiotics, in the year 2000 *Mtb* is still responsible for 4.4 percent of deaths worldwide (WHO, 2003). Of the two billion people infected with *Mtb*, 90-95 percent have a latent form of the disease. The ability to maintain latent infection for decades demonstrates that the majority of immunocompetent patients can control *Mtb* infection. Of those infected only 5-10 percent will develop active

disease during their lifetime. In comparison, immunocompromised patients with HIV have a 5-10 percent chance of developing active TB each year. Latent TB is clinically defined as an *Mtb* infection without symptomatic or other clinical evidence of active disease. Those infected with latent TB have immunological evidence of *Mtb* exposure—either by a positive test for purified protein derivative (PPD^+) or *Mtb*-specific interferon-γ (IFN-γ) release assay (IGRA)—along with a normal or abnormal but unchanging chest radiograph.

The concept of latent TB was first discovered by collecting lymph nodes at autopsy from patients that had not shown signs of TB while they were alive. After inoculation of the lymph nodes into guinea pigs, twelve percent of the guinea pigs developed TB (Loomis, 1890; Wang, 1917). The same procedure was followed with lung tissue from asymptomatic patients and, as seen with the lymph nodes, a high percentage of guinea pigs developed TB (Opie, 1927). These and many additional studies led scientists to conclude that *Mtb* is capable of persisting in human tissues without causing overt disease.

Primary progressive TB—cases in which the patients are infected with *Mtb* and immediately proceed to active TB—is the most common form of the disease in children. In contrast, reactivation of latent TB is the major source of active TB in adults. The causes of reactivation are an area of active investigation, but they correlate strongly with age or an immunocompromised health status brought on by conditions such as cancer chemotherapy treatment, diabetes, and human immunodeficiency virus (HIV). *Mtb* and HIV form a co-epidemic that fosters *Mtb* reactivation, faster progression of disease, and increased prevalence of drug-

resistant strains of *Mtb*. Due to these factors, *Mtb* is the leading cause of death among those infected with HIV (WHO, 2009b).

The lack of an effective vaccine and new drug treatments caused the World Health Organization (WHO) to declare TB a global health emergency in 1993. The Stop TB Partnership was formed in 1998 and set forth the following goals to eliminate TB worldwide: [1] by 2005, 70% of people with infectious TB will be diagnosed and 85% of those cured, [2] by 2015, the global burden of TB disease (deaths and prevalence) will be reduced by 50% relative to 1990 levels, and [3] by 2050, the global incidence of TB disease will be less than 1 in a million. The Stop TB Partnership plans to achieve their goals by increasing patient access to health care and increasing rates of diagnosis, treatment, and cure. They also advocate the development and implementation of new preventive, diagnostic, and therapeutic tools and strategies to stop TB. The Stop TB Partnership hopes that with these strategies TB will be eradicated as a human pathogen (WHO, 2010).

Innate immune response to *Mycobacterium tuberculosis*

Mtb is spread from person to person via aerosol. The lung is constantly bombarded with inhaled debris and pathogens, most of which are removed through cilliary action of epithelial cells present in the upper airways. Small particles can travel deeper into the lung to the alveolar spaces where they are phagocytosed by patrolling alveolar macrophages (Hirsch et al., 1994). Foreign material is taken up into phagosomes which mature and fuse with lysosomes.

Inside the lysosome, foreign debris and pathogens are destroyed and degraded. *Mtb* is phagocytosed by alveolar macrophages, but it has defense mechanisms that block phagosome-lysosome fusion and this is widely believed to allow the bacteria to survive and replicate intracellularly within the macrophage phagosome (Brown et al., 1969; Russell, 2007).

Infection of alveolar macrophages with *Mtb* leads to an innate immune response. Toll-like receptors (TLRs) are a family of 11 transmembrane receptors that are responsible for some of the innate immune response to pathogens. TLRs contain an intracellular Toll/IL-1 receptor (TIR) domain and an extracellular leucine rich repeat (LRR) domain (Pasare and Medzhitov, 2005). TLRs are pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns (PAMPs) of bacteria, virus, fungi, and foreign DNA. Members of the TLR family are located on the plasma membrane or within endosomes of host immune cells. During infection, TLRs recognize microbial ligands through their LRR domains, initiating recruitment of the adaptor protein myeloid differentiation primary response gene 88 (MyD88) which is essential to all TLR signaling with the exception of TLR3. MyD88 is part of a signaling cascade involving interleukin-1 receptor-associated kinase 1 (IRAK-1) and TNF Receptor Associated Factor 6 (TRAF-6) which results in nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation and cytokine production. In addition TLR stimulation initiates mitogen-activated protein (MAP) kinase and interferon regulatory factor 3 (IRF-3) signaling pathways. Activation

of NFκB and MAPK result in transcriptional changes that effect immunity, mitosis, differentiation, proliferation, and cell survival.

Mtb contains many known TLR agonists including secreted proteins, lipids, and deoxyribonucleic acid (DNA). *Mtb* is recognized by TLR2, TLR4, and TLR9 in cell culture, but *in vivo* the results are less clear (Heldwein and Fenton, 2002; Quesniaux et al., 2004; Stenger and Modlin, 2002). Human polymorphisms in TLR2 are associated with increased susceptibility to *Mtb* (Ben-Ali et al., 2004; Ogus et al., 2004). Mice deficient in TLR9/TLR2 or the TLR adaptor MyD88 have drastically reduced survival following *Mtb* infection (Bafica et al., 2005; Feng et al., 2003; Fremond et al., 2004). These data indicate a clear role for TLRs in host defense against *Mtb.* However, the individual contributions of TLR2 and TLR4 are less clear in a mouse model of *Mtb* infection, but they may play a supporting role in host protection (Abel et al., 2002; Drennan et al., 2004; Shim et al., 2003).

Cytokine secretion is critical for macrophage activation and recruitment of immune cells to the site of infection. In response to *Mtb* infection, resting macrophages secrete pro-inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and type I interferon (IFN) (Kaufmann, 2008). *Mtb*-induced secretion of these cytokines results in the activation of proximal macrophages. Activated macrophages provide a less conducive intracellular environment for *Mtb* and therefore are able to limit its growth (Chan et al., 1992; Flesch and Kaufmann, 1990; Rook et al., 1986). Cytokines are not the only important immune regulators during *Mtb* infection.

IFN-γ induces transcription of nitric oxide synthase (iNOS), which allows the generation of reactive nitrogen intermediates (RNI), which contribute to control of *Mtb* growth (Xie et al., 1992). The culmination of macrophage activation, immune cell recruitment, and generation of ROI and RNI ultimately aid in controlling *Mtb* infection.

Host adaptive immune response to *Mtb*

Small animal models are commonly used to study the adaptive immune response during *Mtb* infection. We utilize a low-dose aerosol method to infect mice with *Mtb* because it mimics the route of human transmission. During aerosol infection, bacteria are taken up by resident alveolar macrophages and immature dendritic cells (DCs) in the lungs. *Mtb* infection induces macrophages and DCs to secrete cytokines and chemokines to recruit inflammatory cells into the lung. During early infection, the majority of immune cells recruited to the lung are macrophages and neutrophils. DCs are antigen-presenting cells that are crucial for initiation of the adaptive immune response. *Mtb*-infected DCs or those that have encountered *Mtb* proteins migrate from the lung to the draining lymph node where they present *Mtb* antigens to naïve T cells. Antigen-specific $CD4^+$ T cells migrate back to the lung where they secrete interferon-gamma (IFN-γ) and activate macrophages to kill intracellular bacteria (Chan et al., 1992; Rook et al., 1986). At this stage, the number of lung neutrophils decrease indicating that they act primarily as a first line of defense and are only effective during the initial phase of infection (Tsai et al., 2006).

Adaptive immunity is critical for control of *Mtb*. $CD4^+$ T cells recognize *Mtb* antigens presented by major histocompatibility complex (MHC) class II on the surface of antigen-presenting cells. $CD4^+$ T cells are the dominant adaptive immune cell population in the lung during *Mtb* infections. Severe combined immunodeficiency (SCID) mice that lack functional T and B cells succumb to *Mtb* infection within 5 weeks (Sambandamurthy et al., 2002). Patients with HIV are at high risk of dying from *Mtb* infection partly because they have reduced $CD4^+$ T cells and cannot control bacterial growth during chronic *Mtb* infection. In addition to production of IFN-γ for macrophage activation, $CD4^+$ T cells also produce perforin and granulysin which have cytolytic and microbicidal effects on mycobacteria. Mice lacking $CD4^+$ T cells succumb to *Mtb* infection sooner than wild-type infected mice, but not as quickly as SCID mice (Saunders et al., 2002). $CD8^+$ T and natural killer (NK) T cells both produce IFN-γ upon activation and may partially compensate for the loss of $CD4^+$ T cells. These data indicate that $CD4^+$ T cells are essential but not sufficient for mounting an effective immune response against *Mtb*.

CD8+ T cells are also important for controlling bacteria during the chronic phase of *Mtb* infection. Upon recognition of *Mtb* ligands via MHC class I presentation, CD8+ T cells are cytolytic through the release of perforin, granzymes, and granulysin (Lewinsohn et al., 1998; Ochoa et al., 2001). Similar to $CD4^+$ T cells, $CD8^+$ T cells produce IFN-γ when activated. $CD8^+$ T cells preferentially recognize heavily infected cells while $CD4^+$ T cells do not discriminate between lightly and heavily infected cells (Lewinsohn et al., 2003).

Similar to $CD4^+$-deficient mice, mice lacking $CD8^+$ T cells succumb to *Mtb* infection sooner than wild-type infected mice, but not as quickly as SCID mice (Saunders et al., 2002; van Pinxteren et al., 2000). This indicates that while both $CD4^+$ and $CD8^+$ T cells are important, neither cell type can mount an effective adaptive immune response against *Mtb* alone. This suggests that other IFN-γ producing cells including NK cells compensate for the loss of $CD4^+$ and $CD8^+$ T cells. It is hypothesized that during the early stages of *Mtb* infection $CD4^+$ T cells are important for control and local containment of infection while $CD8^+$ T cells are important for granuloma maintenance during chronic infection (Gonzalez-Juarrero and Orme, 2001; Gonzalez-Juarrero et al., 2001; Tsai et al., 2006).

B-cells do not play a prominent role within the granuloma during chronic *Mtb* infection. B-cells are present during chronic infection, but B cell-deficient mice do not succumb to *Mtb* infection sooner than wild-type mice (Bosio et al., 2000). B-cell deficient mice have less severe lung pathology compared to wild-type mice, and it is hypothesized B cells play a minor role in recruitment and retention of lymphocytes during chronic *Mtb* infection.

During human *Mtb* infection B-cells produce anti-mycobacterial antibodies against different groups of antigens depending on the stage of disease. *Mtb*-specific antibody levels are nearly undetectable during the early and latent phases of human *Mtb* infection. Those present bind *Mtb* and block bacterial binding to mannose receptors, inhibiting bacterial uptake into macrophages (Diaz-Silvestre et al., 2005). There is also evidence that complement is important for killing *Mtb*, as in mice lacking certain complement components are

more susceptible to *Mtb* infection (Daniel et al., 2006). Upon primary progressive TB or reactivation of TB antibody levels rise significantly. Upon reactivation, the host has already been exposed to surface-associated and secreted proteins from the initial infection. Major antigenic proteins are secreted proteins in the Pro-Glu (PE)/Pro-Pro-Glu (PPE), and early secretory antigenic target (ESAT) families, Antigen 85 (Atg85) complex, and 38 kDa protein as well as heat shock proteins and 19 kDa lipoprotein and lipoarabinomannan (LAM) surface proteins (Brightbill et al., 1999; Kaufmann, 2008). The antibody response against active *Mtb* is defined by multiple targets and varies greatly between individuals, making it difficult to utilize for diagnostic purposes (Lyashchenko et al., 1998).

Granuloma formation

Chronic *Mtb* infection is controlled by granuloma formation. A granuloma is an organized group of immune cells and extracellular matrix that form around a pathogen. Granulomas occur when the immune system attempts to wall off something it senses is foreign, but which it is unable to eliminate. In this way *Mtb* is contained, but not eliminated within the body (Ulrichs and Kaufmann, 2006). *Mtb*-induced granulomas consist of a central core of infected macrophages surrounded by successive waves of activated macrophages, giant multinucleated cells, epithelioid cells, T and B cells, fibroblasts and dendritic cells. A subset of granulomas undergo caseous necrosis, a form of biological tissue death results in a cheese-like appearance due to proteinaceous dead cell mass present at the center of the granuloma. This is a location hypothesized to be favorable for

extracellular *Mtb* growth (Ulrichs and Kaufmann, 2006). After *Mtb* infection, mice form granulomas that mimic human granulomas with the exceptions that they do not form caseous necrotic centers and are slightly less organized. In the C57BL/6 mouse, $CD4^+$ T cells congregate around infected macrophages, initiating the early stages of granuloma formation. The granuloma is mainly comprised of activated macrophages and $CD4^+$ T cells with CD8+ T cells present around the periphery of the granuloma. In the absence of either $CD4^+$ or $CD8^+$ T cells, few lymphocytes are recruited to the granuloma with macrophages and neutrophils being the dominant cell types. Dendritic and B cells are also present in mouse granulomas during chronic *Mtb* infections (Rhoades et al., 1997). DCs can be infected by *Mtb,* but the intracellular environment is not conducive to bacterial replication (Gonzalez-Juarrero and Orme, 2001). DCs are know to migrate out of the granuloma and home to the draining lymph node (Schreiber and Sandor, 2010). By this route *Mtb* infect DCs within the granuloma and use them as a Trojan horse to spread infection to other parts of the body. It is also hypothesized that DCs may sample mycobacterial antigens within the granulomas and migrate to the draining lymph nodes providing continuous T-cell priming (Schreiber and Sandor, 2010). Granulomas are essential to chronic *Mtb* infection. They contain *Mtb* and sequester it from the host immune response, but they are unable to eliminate bacteria (Ulrichs and Kaufmann, 2006). In the absence of factors important for granuloma formation and maintenance, granuloma integrity is compromised resulting in escape of *Mtb* and reactivation of disease (Flynn et al., 1995).

Proinflammatory cytokines and chemokines

Cytokines and chemokines are vital to control *Mtb* infection. Pro-inflammatory cytokines are important during the early immune response to *Mtb* as well as for host defense during chronic *Mtb* infection. They establish gradients which direct migration of inflammatory cells, activate cells, and are vital for normal granuloma formation. *In vivo* assessments of *IL-1α/β*$^{-/-}$, *IL-1R*$^{-/-}$, and *IL-18*$^{-/-}$ mice have shown that these cytokines play a role in limiting bacterial lung burden, regulating other cytokines, nitric oxide production, and forming organized granulomas (Juffermans et al., 2000; Sugawara et al., 2001; Sugawara et al., 1999). Likewise, mice deficient in pro-inflammatory cytokines TNFα and IL-6 are highly susceptible to *Mtb* infection, succumbing to infection 30 and 100 days post infection, respectively (Bean et al., 1999; Ladel et al., 1997). TNFα is important for granuloma formation and maintenance in the mouse (Flynn et al., 1995). Patients treated with anti-TNFα therapies to combat autoimmune diseases have a high incidence of TB, indicating that TNFα is important for controlling latent *Mtb* and that in its absence, TB reactivates from latent to active disease (Meya and McAdam, 2007; Saliu et al., 2006; Stenger, 2005). Chemokines also play an important role in controlling *Mtb* infection. Human TB patients have elevated levels of multiple chemokines including monocyte chemotactic protein-1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), and interferon-gamma-induced protein (IP-10) in their blood serum and BAL fluid compared to uninfected patients (Algood et al., 2003). Therefore, cytokine and

cytokine regulation are critical to combat *Mtb* infection during both innate and chronic infection.

T cell activation by cytokines is important for controlling *Mtb* during chronic infection. During *Mtb* infection, macrophages secrete the cytokine IL-12 to recruit and activate $CD4^+$ T cells. Activated T cells secrete IFN-γ which activate macrophages. The cytokines that are involved in positive feedback loop are important for control of *Mtb* infection in mice and humans. Mice lacking either IFN-γ or IL-12 succumb to infection 30-45 days post-*Mtb* infection (Cooper et al., 1993; Cooper et al., 1997; Flynn et al., 1993). Humans and mice with defects in the IFN-γ signaling pathway are more susceptible to mycobacterial infections (Jouanguy et al., 1999). This highlights the importance of macrophage and CD4+ T cell activation in controlling *Mtb* infection.

Mtb induction of cell death

Macrophages, the primary target of *Mtb* infection, have been the focus of the majority of research on *Mtb*-induced cell death. Attenuated strains of mycobacteria elicit more macrophage apoptosis than virulent strains (Keane et al., 1997). Additional studies have confirmed that virulent strains of *Mtb* actively suppress macrophage apoptosis by inhibition of TNFα signaling and upregulation of host anti-apoptotic proteins (Balcewicz-Sablinska et al., 1998; Sly et al., 2003; Spira et al., 2003). A myriad of studies support a model in which cells undergo apoptosis to contain and kill bacteria which benefits the host. In contrast, cells undergo necrosis, which induces bacterial escape from the cell and spread to

uninfected cells, therefore benefiting the bacterium (Fratazzi et al., 1997; Keane et al., 2000; Lee et al., 2006; Molloy et al., 1994; Oddo et al., 1998; Riendeau and Kornfeld, 2003; Velmurugan et al., 2007). Mice with extreme susceptibility to *Mtb* infection were found to have a susceptibility allele (designated $sst1^S$) within the Ipr1 gene (Pan et al., 2005). Macrophages from mice containing $sst1^S$ predominantly underwent necrosis, in contrast to mice with a resistant allele ($sst1^R$) which primarily undergo apoptosis. After *in vivo Mtb* infection $sst1^S$ containing mice had massive pulmonary necrosis that was absent in $sst1^R$ mice. These data suggest that apoptosis is a process initiated by the host. When apoptosis is inhibited by *Mtb*, necrosis predominates.

NLR proteins in immunity

The nucleotide-binding domain, leucine rich repeats containing family, known as the NLRs, includes genes important in immune regulation, inflammation, and autoimmunity. In 2002, the Ting lab identified a group of proteins with similar domain structure to the MHC class II transactivator (CIITA). These proteins share a tripartite structure with a variable N-terminal effector domain, a central nucleotide-binding domain, and a series of C-terminal leucine-rich repeats (Ting and Davis, 2005). A schematic representation of NLR protein domains can be found in **Figure 1.1**. With the aid of a near complete human genome sequence, the lab determined that this family had at least 22 members and named it the CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats, or CATERPILLER gene family (Harton et al., 2002). Although

prior to this others have identified some of the structural domains of this family (Martinon et al., 2001; Pawlowski et al., 2001), this report represents the first to identify this group of proteins based on structural homology. Around the same time several other research laboratories discovered the same group or partial group of proteins (Inohara and Nunez, 2003; Martinon et al., 2001; Pawlowski et al., 2001; Tschopp et al., 2003; Wang et al., 2002). Each gave the proteins a different name, leading to confusion within the field. Standard nomenclature for the NLR gene family was implemented in 2008 (Ting et al., 2008a). Names consist of "NLR", followed by a letter designation for the effector domain of the protein. Because there are multiple NLRs with N-terminal pyrin and CARD effector domains, each is followed with a number. A list of standardized gene names and aliases can be found in **Table 1.1**.

NLRs share both functional and structural similarity with plant resistance (R) proteins, part of the plant immune system. Functionally, both NLRs and R protein families recognize specific molecules produced by diverse classes of pathogens including bacteria, viruses, and fungi. Structurally, the majority of R proteins contain a nucleotide binding domain (NBD) and LRR domain which are the two protein domains shared by all NLR proteins. Each R protein recognizes a specific pathogen unlike NLRs which have been shown to recognize multiple pathogens. The plant *Arabidopsis* has approximately 150 R proteins in its genome to accommodate this one-on-one recognition while the promiscuous NLR gene family is significantly smaller (Ausubel, 2005; Nimchuk et al., 2001).

NLR family proteins and disease associations

The initial discovery of NLR proteins was partly due to mutations in these genes being associated with human immunological and inflammatory disorders. Mutations in *CIITA* result in a recessive condition called bare lymphocyte syndrome. Patients with this condition do not present MHC class II on the surface of antigen-presenting cells. Consequently, recognition of antigen by T cells is impaired leading to a defective adaptive immune response. Patients with bare lymphocyte syndrome have increased susceptibility to pathogens (Ting and Davis, 2005).

Mutations in human *nucleotide-binding oligomerization domain containing 2* (*NOD2)* correlate with a subset of patients that develop the inflammatory bowel disease, Crohn's disease (Hugot et al., 2001; Hugot et al., 1996; Ogura et al., 2001). Other *NOD2* mutations are associated with granulomatous inflammatory disorders including early onset sarcoidosis and Blau syndrome (Miceli-Richard et al., 2001). In each case, these mutations lead to impaired immunity in patients that are unable to eliminate harmful pathogens (Kobayashi et al., 2005). In addition, NOD1 mutations are linked to increased susceptibility to asthma and atopic eczema (Fritz et al., 2006; Geddes et al., 2009).

Mutations in *NLRP1* and *NLRP12* cause autoimmune diseases. Vitiligo is caused by human *NLRP1* variants. Patients with vitiligo have areas of patchy depigmented skin caused by the destruction of skin melanocytes. In addition, patients with this disease are at increased risk for other autoimmune diseases including rheumatoid arthritis, diabetes, lupus, and thyroid disease (Jin et al.,

2007). The mechanism by which *NLRP1* variants affect skin melanocytes is unknown. Mutations in *NLRP12* have been linked to Familial Mediterranean Fever, a hereditary inflammatory disorder (Jeru, 2008, PNAS). A polymorphism in NLRP12 has been loosely associated with atopic dermatitis, although it is unclear how this intronic polymorphism affects disease pathology (Macaluso et al., 2007).

In humans, NLRP3 was first identified by its disease association with hereditary periodic fever syndromes (Hoffman et al., 2001). Cryopyrin-associated periodic syndromes (CAPS) are a group of autoinflammatory syndromes caused by autosomal dominant mutations in human *NLRP3*. All of the 84 known human *NLRP3* mutations are located within exon 3, which encodes the central NBD. These gain of function mutations in human *NLRP3* cause the NLRP3 inflammasome to be activated in the absence of stimulation (Agostini et al., 2004; Gattorno et al., 2007). This result is a hyperinflammatory state due to overproduction of active IL-1β within the body in the absence of infection. CAPS encompass a range of disease severity. At the mild end of the spectrum is familial cold autoinflammatory syndrome (FCAS) which presents with fever, cold-induced urticaria (hives) and mild arthralgia (joint pain) (Hoffman et al., 2001). At the intermediate stage is Muckle-Wells syndrome (MWS) in which patients have fever, spontaneous urticaria that is not cold-induced as well as sensorineural hearing loss (sensory hearing loss), arthralgia, and sometimes renal amyloidosis (Dode et al., 2002). The most severe form of CAPS is chronic infantile, neurological, cutaneous, and articular syndrome (CINCA) also known as

neonatal-onset multisystem inflammatory disease (NOMID). CINCA/NOMID patients have deforming arthropathy (joint disease) and chronic aseptic meningitis in addition to all the other symptoms of MWS patients (Aksentijevich et al., 2002). CAPS patients are often treated with the IL-1 receptor antagonist Anakinran to decrease inflammation (Shinkai et al., 2008).

Inflammasome formation

A subset of NLR proteins—NLRP1, NLRP3, NLRC4, and NAIP5—are able to form multi-protein complexes known as inflammasomes in response to stimuli (Martinon et al., 2002). When triggered, an inflammasome-forming NLR protein comes together to form a multi-protein complex consisting of the adaptor molecule apoptotic speck-like protein containing a CARD (ASC) and the inactive preprotein pro-caspase-1. The adaptor molecule ASC contains a N-terminal pyrin domain and a C-terminal CARD domain. This allows ASC to bind the N-terminal pyrin effector domain of inflammasome-forming NLRs and bind the N-terminal CARD domain of pro-caspase-1. Formation of the inflammasome results in autocleavage of pro-caspase-1 to its active form. Caspase-1 then recruits inactive forms of the cytokines IL-1β and IL-18 and processes them to their active forms (Srinivasula et al., 2002). These cytokines are then rapidly secreted into the extracellular environment where they cause inflammation and drive recruitment of cells to the site of infection (**Figure 1.2**).

The functional arrangement of the inflammasome was based on human apoptotic protease activating factor 1 (Apaf-1) formation of a multiprotein

structure called the apoptosome. Apaf-1 is not an NLR protein but contains similar domain homology to the NLR family. Apaf-1 contains an N-terminal CARD domain and central NBD similar to the NLRs, but has C-terminal WD40 repeats rather than LRRs. During times of cellular stress, cytochrome c is released from mitochondria and recognized by Apaf-1. Upon detection, Apaf-1 monomers come together and form a ringed structure named the apoptosome, which is involved in cell apoptosis. The inflammasome structure is hypothesized to form a similar ringed structure consisting of multimers of NLR, ASC, and pro-caspase-1 proteins (Fritz et al., 2006; Zou et al., 1999).

ASC has been associated with apoptosis, cancer, and inflammation. It was discovered as a cytoplasmic protein in promyeloleukemic cell line HL-60 that formed an insoluble “speck” during retinoic acid-induced apoptosis (Masumoto et al., 1999). By confocal and electron microscopy, the structure of the ASC speck resembles a hollow ball composed of smaller balls and spikes protruding from the outer surface (McConnell and Vertino, 2004). Cloning revealed that ASC is a 195 amino acid protein consisting of an N-terminal pyrin domain and C-terminal CARD domain, the latter of which is characteristic of many apoptosis-signaling proteins (Masumoto et al., 1999).

A second paper identified the target of methylation-induced silencing (TMS1), later identified as ASC, as a protein involved in apoptosis during cancer. *TMS1* was identified from a screen of genes silenced due to aberrant methylation. *TMS1* was aberrantly methylated and silenced in human breast cancer cells and these findings were confirmed in forty percent of primary breast

tumor samples. This paper also confirmed a role for TMS1 in apoptosis, concluding that aberrant methylation and subsequent gene silencing of TMS1 promotes breast tumorigenesis by allowing tumor cells to escape from host-induced apoptosis (Conway et al., 2000). Further research has shown that methylation-induced silencing of TMS1 is not specific to breast cancer, but can also be found in a percentage of gastric carcinomas, small cell and non-small cell lung carcinomas, malignant melanomas, and primary glioblastomas (McConnell and Vertino, 2004). Aberrant methylation and silencing of TMS1 is cancer specific, as corresponding normal tissue lacks TMS1 methylation. Induced expression of ASC has been proposed as a cancer treatment strategy as methylation-induced silencing of ASC has caused resistance to p53-mediated chemosensitivity (Ohtsuka et al., 2006).

ASC is most abundantly expressed in human epithelial cells and leukocytes (Masumoto et al., 2001a). The murine ortholog of ASC (mASC) is a 193 amino acid protein that with a well conserved N-terminal pyrin and C-terminal CARD domains with human ASC (hASC). It has retained a similar expression profile as well as the ability to self-associate and aggregate during apoptosis similar to hASC and Apaf-1 (Masumoto et al., 2001b).

The structure of ASC was used to link the protein to a role in inflammation. It was hypothesized that the pyrin and CARD domains of ASC could be sites of protein-protein interaction with other proteins containing pyrin and CARD domains. Indeed, ASC was found to associate with NLRP1, NLRP3, NLRP12, and NLRP6 through pyrin-pyrin domain interactions (McConnell and Vertino,

2004). Overexpression of ASC with and these individual NLR proteins has been linked to increased NFκB activation and apoptosis mediated through the pyrin domain. These studies contained much conflicting data, but the discrepancies were thought to be due to differences in expression levels of the overexpressed proteins. To combat the problems caused by overexpression systems, the Ting lab used THP-1 cells with reduced expression of ASC by shRNA to show that ASC is indeed an activator of NFκB signaling (Taxman et al., 2006). Other studies focused on the CARD domain of ASC. They found that ASC binds pro-caspase-1 through CARD-CARD interactions, resulting in caspase-1 activation and subsequent processing of pro-IL-1β to its active form (Srinivasula et al., 2002). Together these reports revealed that ASC, along with other NLR proteins participated in caspase-1 dependent IL-1β cleavage and activation of NFκB signaling.

Due to high ASC expression in macrophages and myeloid cells, many studies have used this cell type to determine the specific stimuli that activate a NLR inflammasome. Martinon *et al.* used LPS-stimulated THP-1 human monocyte cell line for the initial description of the inflammasome. This report found that the inflammasome consisted of a multi-protein complex containing ASC, NLRP1, caspase-1, and caspase-5 and that the inflammasome processed caspase-1 (Martinon et al., 2002). Since then ASC has been identified as an adaptor protein for several additional NLR proteins that form inflammasomes including NLRP3, NLRC4, and NAIP5. Although it makes sense that the NLR proteins with N-terminal CARD domains do not absolutely require ASC for

caspase-1 recruitment, the presence of ASC often enhances inflammasome activation. This is the case with NAIP5 and NLRC4 inflammasomes (Ye and Ting, 2008). In addition to these inflammatory functions, ASC association with NLR proteins is required for pyroptotic and pyronecrotic cell death (Fernandes-Alnemri et al., 2007; Ting et al., 2008b).

Caspase-1 was originally named IL-1β converting enzyme (ICE) (Cerretti et al., 1992). Caspase-1 is an essential component of all inflammasome complexes formed by NLR proteins. Indicated by its name, caspase-1 regulates inflammatory responses by processing pro-inflammatory cytokines IL-1β and IL-18 from inactive precursor proteins to active cytokines. IL-1β and IL-18 do not contain signal peptides and are secreted from the cell in an unconventional manner independent of the Golgi apparatus and endoplasmic reticulum. Caspase-1 may be a carrier for secretion of these leaderless cytokines (Keller et al., 2008). In addition to IL-1β and IL-18, a proteomic study has identified over 70 substrates cleaved by caspase-1 including chaperones, cytoskeletal and translation machinery, immune proteins, and components of the glycolysis pathway (Keller et al., 2008; Shao et al., 2007). Although caspase-1 is considered an “inflammatory” caspase, it plays a role in apoptotic and pyroptotic cell death (Ting et al., 2008b). Together inflammasome-forming NLRs protect the host against PAMPs and danger-associated molecular patterns (DAMPs).

NLRP1 inflammasome

NLRP1 was the first identified inflammasome-forming NLR. The human NLRP1 protein consists of an N-terminal pyrin domain, a central NBD domain, and C-terminal LRRs. The C-terminus of NLRP1 also contains two additional domains, a function to find domains (FIIND) and a CARD domain. The NLRP1 inflammasome is activated by a small group of identified stimuli including *Bacillus anthracis* lethal toxin and muramyl dipeptide (MDP). Early studies in human cells indicated that the NLRP1 inflammasome consists of NLRP1, ASC, caspase-1, and caspase-5. The NLRP1 inflammasome was the first discovered to involve caspase-1 activation (Martinon et al., 2002). The role of caspase-5 in human inflammatory diseases has been difficult to study because it is not present in mice. In the NLRP1 inflammasome, ASC is not essential for NLRP1 inflammasome activation, but does enhance caspase-1 processing (Faustin et al., 2007). There is some evidence to suggest that NLRP1 and another NLR protein, nucleotide oligomerization domain 2 or NOD2, oligomerize in the presence of *B. anthracis* lethal toxin and MDP, although there is nothing to suggest either NLR is binding the stimuli directly (Hsu et al., 2008)

NLRC4 inflammasome

The NLRC4 inflammasome is stimulated by flagellin and flagellated pathogens including *Salmonella typhimurium* (Franchi et al., 2006; Mariathasan et al., 2004; Miao et al., 2006) and *Legionella pneumophila* (Amer et al., 2006). In the case of *S. typhimurium* cytosolic bacterial flagellin is delivered to the cell directly through a type III secretion system (Miao et al., 2006). Additionall, the

NLRC4 is activated by non-flagellated pathogens *Shigella flexneri* (Suzuki et al., 2007) and *Pseudomonas aeruginosa* (Sutterwala et al., 2007).

NLRC4 contains a C-terminal CARD domain, a central NBD, and C-terminal LRRs. Upon stimulation, the CARD domain of NLRC4 can directly associate with the CARD domain of pro-caspase-1 to induce cleavage to active caspase-1 (Mariathasan et al., 2004; Poyet et al., 2001). Although there is no direct evidence showing ASC bound to NLRC4, in the absence of ASC, IL-1β secretion is impaired in response to many NLRC4-stimulating bacteria (Mariathasan et al., 2004; Sutterwala et al., 2007; Suzuki et al., 2007). This indicates that the NLRC4 inflammasome requires ASC or that the bacterial pathogens are additionally stimulating another inflammasome forming NLR that requires ASC.

NLRP3 inflammasome

NLRP3 is the most characterized of all the NLR inflammasome-forming proteins. It is a cytoplasmic protein expressed in macrophages, monocytes, granulocytes, dendritic cells, some epithelial cells, osteoblasts, and eroepithelial cells (Feldmann, J, 2002, Am J Hum Genet). Structurally NLRP3 consists of an N-terminal pyrin domain, central NBD domain, and C-terminal LRRs. The pyrin domain of NLRP3 allows the protein to bind with other pyrin-containing proteins through homotypic pyrin-pyrin interactions. This is how NLRP3 binds the inflammasome adaptor ASC. The NLRP3 NBD domain is hypothesized to allow oligomerization of NLRP3 monomers and inflammasome formation. The NLRP3

LRR domain is hypothesized to function in recognition of PAMPs and DAMPs, based on the LRR domain of TLR proteins being the site of PAMP recognition (Bell et al., 2003). How NLRs are sensing pathogens is still unknown, and there is no evidence to suggest the LRR domains of NLR proteins are directly recognizing pathogens (Pedra et al., 2009).

The NLRP3 inflammasome is activated by a broad range of pathogens and pathogen products such as LPS, lipooligosaccharide, muramyl dipeptide (MDP), nucleic acids, toxins including nigericin from *Streptomyces hygroscopicus*, maitotoxin from murine dinoflagellates, and α-hemolysin produced by *Staphylococcus aureus*. Additionally, NLRP3 is stimulated by cellular danger signals like ATP, uric acid crystals, hyaluronan and heprin sulfate, and amyloid-β, as well as environmental dangers such as asbestos, silica, and alum adjuvant. A list of inflammasome activators and references can be found in **Table 1.2**.

Reconstitution of the NLRP1 inflammasome revealed that inflammasome is activated by a two step process (Faustin et al., 2007). Signal one functions as a priming step to ready the cell for inflammasome activation by activation of NFκB signaling. This results in the upregulation of pro-IL-1β and Nlrp3 transcripts, necessary for inflammasome function. TLR signaling is often involved in activation of signal one, as it causes potent activation of thee NFκB signaling pathway (Bauernfeind et al., 2009). Only after a specific trigger is the inflammasome activated resulting in recruitment and cleavage of caspase-1 and subsequent IL-1β activation and secretion (Ghiringhelli et al., 2009). To prevent

aberrant activation of the inflammasome, the presence of multiple checkpoints may play a role in limiting inflammasome activation in the absence of a host assault.

Other Inflammasome forming NLR proteins

Several other NLR proteins are capable of forming inflammasomes when stimulated by specific stimuli. The NLR apoptosis-inhibitory protein 5 (NAIP5) contains an N-terminal baculovirus inhibitor of apoptosis repeat (BIR) domain, a central NBD, and C-terminal LRRs. Similar to NLRC4, NAIP5 is a sensor of cytoplasmic flagellin. Co-immunoprecipitation studies have shown that NAIP5 can associate with NLRC4, indicating they may come together form an inflammasome in response to *Legonella pneumophilia* flagellin (Zamboni et al., 2006). NAIP5 does not contain a CARD domain, so it most likely needs NLRC4 to recruit and process caspase-1 to its active form.

Absent in melanoma 2 (AIM-2) is a cytoplasmic protein that functions as a double stranded DNA (dsDNA) receptor and activator of antiviral and inflammatory responses. AIM2 is a member of the pyrin and HIN domain-containing protein (PYHIN) family of proteins. Although AIM-2 is not an NLR, it directly binds dsDNA through its HIN200 domain and associates with ASC through its pyrin domain. This allows AIM2 to activate the inflammasome in the absence of an NLR protein. Upon activation, the AIM2 inflammasome results in pro-caspase-1 processing and subsequent IL-1β activation. Stimulation of AIM2

can also activate NFκB signaling (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).

Very recently, another NLR has been shown to regulate immune signaling pathways. NLRC5 is the largest member of the NLR family with a predicted size of 230 kDa. The Ting lab has identified NLRC5 as a putative inflammasome forming protein (Davis and Ting, unpublished data; (Cui et al., 2010). Two recent papers have identified a role for NLRC5 in NFκB and IFN signaling pathways in response to viral stimulations. Currently there is some discrepancy about the protein function with one report that NLRC5 is an inhibitor of NFκB and type I IFN signaling and the other stating that NLRC5 is a positive regulator of IFN-γ during viral infection (Kuenzel, JI, 2010; Cui, Cell, 2010). It is possible that both reports could be correct under different circumstances.

Non-inflammasome forming NLRs

A number of NLRs do not form inflammasomes are still associated with immune regulation, inflammation, and autoimmunity (Ting et al., 2010). CIITA is expressed constitutively in immune cells where it functions as a transcription factor and master regulator of MHC class II expression (Steimle et al., 1994; Ting and Trowsdale, 2002). CIITA is essential to mount an effective adaptive immune response against pathogens. Patients with a loss of function mutations in human CIITA have a defective T helper (T_H)-dependent immune response (Reith and Mach, 2001; Steimle et al., 2007). *CIITA*$^{-/-}$ mice cannot limit *Mtb* growth and they succumb to infection approximately 60 days postinfection compared to 300 days

postinfection for wild-type mice. The phenotype of *Mtb* infected *CIITA*$^{-/-}$ mice was due to a severely depleted $CD4^+$ T-cells and reduced IFN-γ levels (Repique et al., 2003). Although not tested *in vivo*, one would presume that the reduction in INF-γ would result in fewer activated macrophages which are important for limiting intracellular *Mtb* growth.

In contrast to the inflammasome-forming NLRs, NLRP12 acts as an anti-inflammatory NLR in response to stimulation (Williams et al., 2003). NLRP12 negatively regulates both the canonical and non-canonical NFκB signaling pathways. During canonical NFκB signaling NLRP12 prevents IRAK-1 hyper-phosphorylation which dampens NFκB activation (Williams et al., 2005). In the non-canonical NFκB signaling pathway, NLRP12 induces inducing NIK degradation which inhibits NFκB activation (Lich et al., 2007). The inhibitory function of NLRP12 is driven by ATP hydrolysis by its central NACHT domain (Ye et al., 2008). In cell culture, NLRP12 is downregulated after stimulation with TLR agonists, *Mtb*, and cytokines TNF-α and IFN-γ (Williams et al., 2005). This is in contrast to the vast majority of studied NLRs which have pro-inflammatory functions after PAMP and DAMP stimulation. However more recent studies increasingly show evidence for NLRs that bear negative regulatory functions (Cui et al., 2010). It is hypothesized that downregulation of NLRP12 after pathogen exposure allows the host to mount an appropriate immune response. After the pathogen is eliminated, NLRP12 prevents overzealous inflammation and allows the cell to return it a resting state in the absence of pathogens.

NLRX1 is a negative regulator of the immune response in response to viral stimuli. The N-terminal X domain of this protein bears similarity to both CARD and PYRIN yet can not be categorized either (Moore et al., 2008). The X domain contains a mitochondrial targeting sequence which together with the NBD domain is necessary to mediate protein interaction, which confers its functional regulatory property. At the mitochondria, viral ribonucleic acid (RNA) induces type I interferon signaling through a signaling pathway involving the adaptor protein, mitochondrial antiviral signaling (MAVS). NLRX1 binds MAVS and is a negative regulator of IFN signaling (Moore et al., 2008). NLRX1 is also reported to increase reactive oxygen species in the mitochondria, which amplifies NFκB and c-Jun N-terminal kinases (JNK) signaling pathways (Tattoli et al., 2008).

NOD1 and NOD2 are positive regulators of inflammation through activation of signaling pathways. These NLR proteins are cytosolic sensors of the bacterial cell wall component peptidoglycan (PGN) (Girardin et al., 2003a; Girardin et al., 2003b). When activated NOD1 and NOD2 upregulate NFκB signaling leading to increased pro-inflammatory cytokine production (Chin et al., 2002). In addition to NFκB signaling, NOD2 and the adaptor protein CARD9 upregulate MAPK signaling pathways JNK and p38 (Hsu et al., 2007). NOD proteins have functions beyond PGN recognition. Recently, NOD2 has been implicated in regulation of IRF3 signaling and type I IFN production in response to the single-stranded RNA (ssRNA) respiratory syncytial virus (RSV) (Sabbah et al., 2009).

The role of NOD2 during both the innate and adaptive immune response to *Mtb* has also been studied. *Mtb*-infected macrophages and dendritic cells from *NOD2*-deficient mice have reduced pro-inflammatory cytokines and NO compared to wild type. However, *NOD2*$^{-/-}$ mice infected with *Mtb* control bacterial growth and survive as long as wild-type mice measured up to eight weeks postinfection (Gandotra et al., 2007). Another paper by Divangahi *et al*. (year) studied *NOD2*$^{-/-}$ mice during chronic *Mtb* infection and found that *NOD2*$^{-/-}$ mice have higher lung bacterial burden six months postinfection and reduced survival due to impaired T cell function (Divangahi, JI, 2008). The second paper implicates NOD2 in resistance to *Mtb* infection via the adaptive immune response. This study demonstrates the importance of following *Mtb* infections late into the chronic phase of infection, as the first paper missed the role of NOD2 in host protection against *Mtb* infection.

Summary

Immense interest in the role of NLRs in host immunity has led to the study of inflammasome complexes in response to many pathogens. Much of the research has been in human monocytic cell lines or in mouse macrophages. Chapter 2 of this thesis focuses on the role of the NLRP3 inflammasome during *Mtb* infection in cultured macrophages and in a mouse model. We find that the NLRP3 inflammasome is activated by *Mtb* in cultured human monocytes and primary mouse macrophages. Because the role of NLR stimulation in one cell type is not representative of the role of NLRs in the context of a whole organism,

we used a mouse model of *Mtb* infection to determine the importance of the NLRP3 inflammasome *in vivo*. We found that in contrast to our *in vitro* results, only murine *ASC* helps protect the host from death during chronic *Mtb* infection while the effects of *Casp-1* and *Nlrp3* were negligible. The mouse model of *Mtb* infection allows us to study the role of the NLRP3 inflammasome during the chronic phase of *Mtb* infection which cannot be modeled *in vitro*. Next we focused on granuloma formation and immune cells in the lungs during chronic infection. We determined that *ASC*$^{-/-}$ mice have a decrease in CD11c^{+} CD11b$^{mid/low}$ lung cells and reduced number of lung granulomas compared to wild-type lungs. The inability of *ASC*$^{-/-}$ mice to form organized granulomas indicates a breakdown in host defense against *Mtb*. In chapter 3 we investigated the role of a non-inflammasome forming NLR, NLRP12, in a mouse model of *Mtb* infection. NLRP12 has been shown to bind ASC, thus we hypothesized it may be important for the host response to *Mtb*, similar to ASC. We found that Nlrp12 did not play a prominent role in host protection against *Mtb*. Thus, we identify ASC as a critical protein involved in host response to *Mtb* infection in an inflammasome-independent manner.

FIGURES

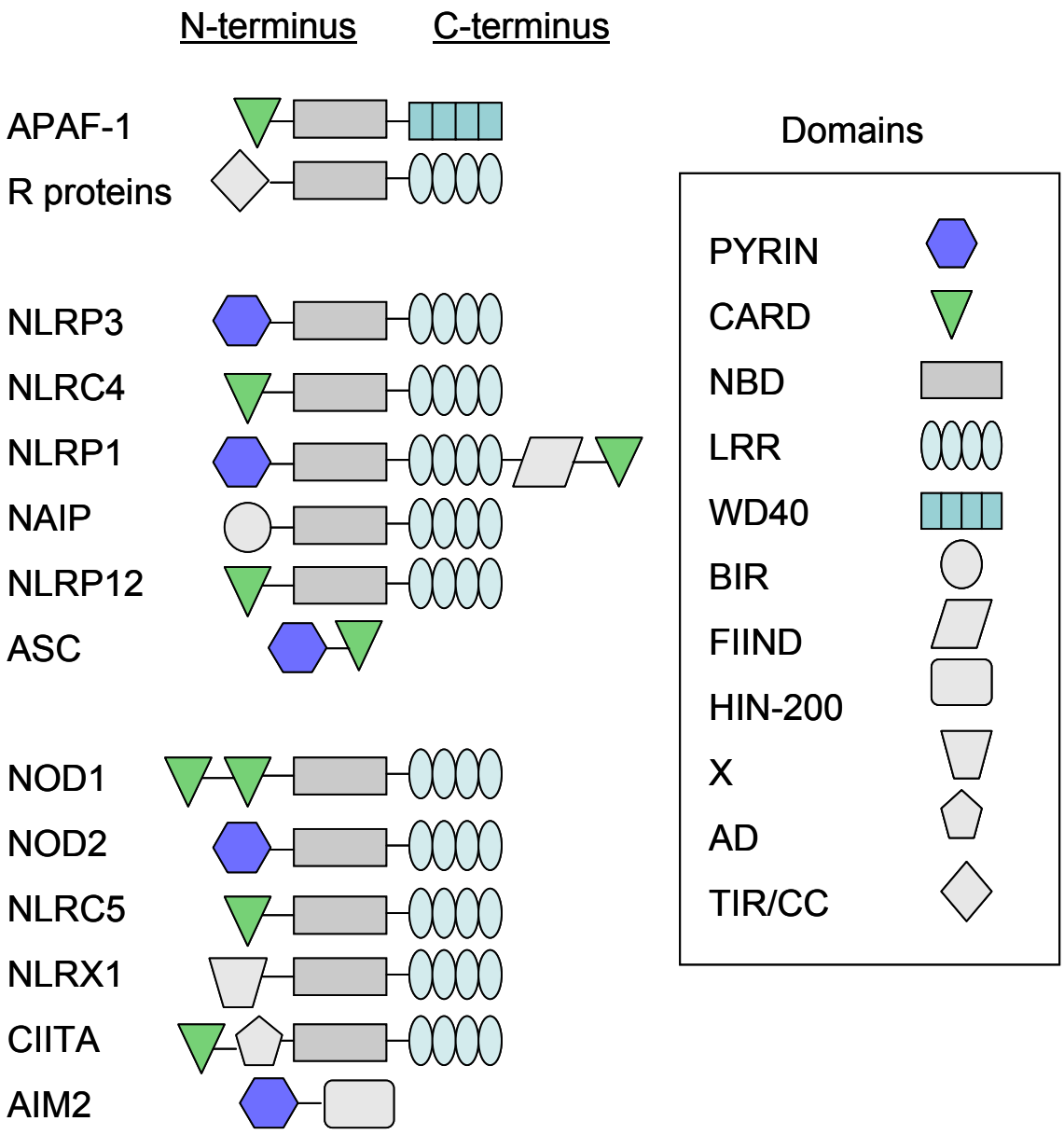

Figure 1.1 Structural domains of NLR proteins

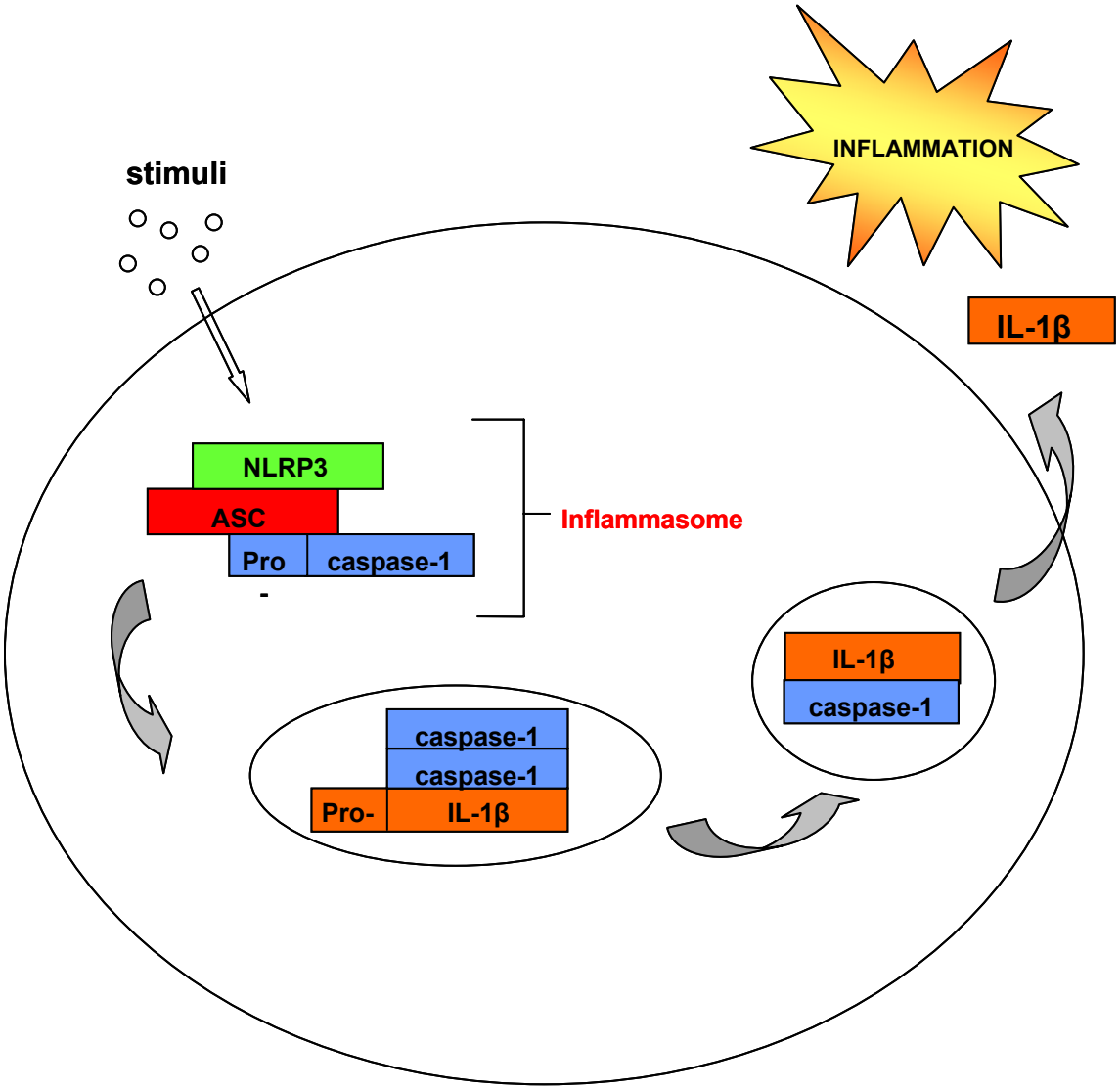

Figure 1.2 NLRP3 Inflammasome activation

CIITA	*NLRA*; *MHC2TA*; *C2TA*
NAIP	*NLRB1*; *BIRC1*; *CLR5.1*
NOD1	*NLRC1*; *CARD4*; *CLR7.1*
NOD2	*NLRC2*; *CARD15*; *CD*; *BLAU*; *IBD1*; *PSORAS1*; *CLR16.3*
NLRC4	*CARD12*; *CLAN*; *CLR2.1*; *IPAF*
NLRC5	*NOD27*; *CLR16.1*
NLRP1	*NALP1*; *DEFCAP*; *NAC*; *CARD7*; *CLR17.1*
NLRP3	*CIAS1*; *PYPAF1*; *Cryopyrin*; *CLR1.1*; *NALP3*
NLRP12	*NALP12*; *PYPAF7*; *Monarch1*; *RNO2*; *PAN6*; *CLR19.3*
NLRX1	*NOD9*; *CLR11.3*
ASC	*PYCARD; TMS1*

Table 1.1 Standardized NLR names and aliases

NLR	Stimuli	References
NLRP3	*Neisseria gonorrhoeae*	(Duncan et al., 2009)
	Listeria monocytogenes	Wu et al., 2010; Kim et al., 2010
	Listeria monocytogenes	Kim et al., 2010; Meixenberger et al., 2010
	Streptococcus pyogenes streptolysin O	Harder et al., 2009
	Klebsiella pneumoniae	Willingham et al., 2009
	Shigella flexneri	Willingham et al., 2007
	Chlamydia trachomatis	Abdul-Sater et al., 2009
	Chlamydia pneumoniae	He et al., 2010
	Aspergillus fumigatus	Said-Sadier et al., 2010
	Candida albicans hyphae	Joly et al., 2009
	Staphylococcus aureus α-hemolysin	Craven et al., 2009; Munoz-Planillo et al., 2009
	Pathogenic *Vibrio* species	Toma et al., 2010
	Mycobacterium species	Koo et al., 2008; Masters et al., 2008; Mishra et al., 2010; Meyer-Barber et al., 2010; Carlsson et al., 2010
	Porphyromonas gingivalis	Huang et al., 2009; Bostanci et al., 2009
	Anaplasma phagocytophilum	Pedra et al., 2007
	Influenza virus	Allen et al., 2009; Ichinohe et al., 2009; Thomas et al., 2009
	Sendai virus	Kanneganti et al., 2006
	Vaccinia virus	Delaloye et al., 2009
	Malarial hemozoin	Shio et al., 2009
	Fungal zymosan and mannan	Lamkanfi et al., 2009
	LPS	Kanneganti et al., 2006; Sutterwala et al., 2006; Martinon et al., 2006
	MDP	Marina-Garcia et al., 2008
	Viral dsRNA	Kanneganti et al., 2006
	Bacterial RNA	Kanneganti et al., 2006
	Microbial DNA	Muruve et al., 2008
	Nigericin and maitotoxin	Mariathasan et al., 2006
	ATP	Mariathasan et al., 2006
	Gout-associated crystals	Martinon et al., 2006
	Danger signals	Shi et al., 2003
	Amyloid-β	Halle et al., 2008
	Asbestos and silica	Dostert et al., 2008; Perkins et al.

		1993
	Hyaluronan	Scheibner et al., 2006
	Adjuvents	Li et al., 2008; Eisenbarth et al., 2008
	Necrosis	Li et al., 2009; Iyer et al., 2010
	Cholesterol-dependent cytolysins	Chu et al., 2009
NLRC4	*Legionella pneumonphila*	Amer et al., 2006
	Salmonella typhimurium	Amer et al., 2006
	Shigella felxneri	Suzuki et al., 2007
	Pseudomonas aeruginosa	Miao et al., 2008; Sutterwala et al., 2007
	flagellin	Miao et al., 2006
NLRP1	MDP	Faustin et al., 2007
	Bacillus anthracis	Hsu et al., 2008
NAIP5	*Legionella pneumonphila*	Lightfield et al., 2008
	flagellin	Lightfield et al., 2008

Table 1.1 NLR inflammasomes activators

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CHAPTER 2

Granuloma formation and host defense in chronic *Mycobacterium tuberculosis* infection requires ASC but not NLRP3 and caspase-1

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ABSTRACT

The NLR gene family mediates host immunity to various acute pathogenic stimuli but its role in chronic infection is not known. This paper addresses the role of NLRP3 (NALP3), its adaptor protein ASC, and caspase-1 during infection with *Mycobacterium tuberculosis* (*Mtb*). *Mtb* infection of macrophages in culture induced IL-1β secretion, and this requires the inflammasome components ASC (also PYCARD, TMS1), caspase-1, and NLRP3. However *in vivo Mtb* aerosol infection of *Nlrp3*$^{-/-}$, *Casp-1*$^{-/-}$, and WT mice showed no differences in pulmonary IL-1β production, bacterial burden, or long-term survival. In contrast, a significant role was observed for *ASC* in host protection during chronic *Mtb* infection, as shown by an abrupt decrease in survival of *ASC*$^{-/-}$ mice. Decreased survival of *ASC*$^{-/-}$ animals was associated with defective granuloma formation and reduced lung dendritic cell populations. These data demonstrate that ASC exerts a novel inflammasome-independent role during chronic *Mtb* infection by containing the bacteria in granulomas.

INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis, a disease affecting one-third of the world's population and killing 1.7 million people each year (2007). *Mtb* is spread by aerosol droplets from persons with active infection. Upon inhalation, *Mtb* travels to the lung where it infects resident alveolar macrophages (Leemans et al., 2001). This initial infection leads to an innate immune response, which includes stimulation of Toll-like receptors (TLRs) that recognize pathogens and are located on the plasma membrane and within endosomes of host cells. *Mtb* is

specifically recognized by TLRs 2, 4, and 9 (Quesniaux et al., 2004). TLR activation upregulates transcription of proinflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor alpha (TNFα), and interleukin-6 (IL-6), which are essential for the recruitment of immune cells to the site of infection and controlling *Mtb* infection (Bean et al., 1999; Ladel et al., 1997; Yamada et al., 2000).

In addition to TLR recognition a newly discovered class of intracellular danger sensing proteins, the nucleotide binding domain, leucine rich repeats-containing family proteins known as NLRs, sense pathogens and pathogen products in the cell cytoplasm (Ting et al., 2008a). With more than twenty members, the NLRs function in host protection against a broad range of danger signals. Several NLRs function in immunity through the formation of a mutli-protein complex known as an inflammasome (Martinon et al., 2002). When activated by a specific danger signal, the inflammasome forms and results in recruitment and processing of pro-caspase-1, which in turn processes IL-1β and IL-18 to their active forms for secretion from macrophages.

NLRP3 is the most characterized of all the NLR inflammasome-forming proteins due to its abundant expression in macrophages and activation in response to the largest number of identified stimuli. In humans, a gain of function mutation in *NLRP3* is associated with hyperinflammatory hereditary periodic fever syndromes with symptoms ranging from mild rash to severe joint swelling (Hoffman et al., 2001).

Apoptotic speck-like protein containing a CARD (ASC, also PYCARD) was originally identified as a speck-forming protein during apoptosis of HL-60 cells (Masumoto et al., 1999). ASC has also been recognized as an adaptor protein that interacts with NLR proteins forming a protein inflammasome structure resulting in

caspase-1 processing and subsequent IL-1β and IL-18 activation (Srinivasula et al., 2002). ASC has been identified as an adaptor protein for NLRP3 and NLRP1, and it is functionally required for the NAIP5 and NLRC4 inflammasomes (Ye and Ting, 2008). Each inflammasome responds to a specific set of stimuli, although there is some redundancy between NLRs. Together, ASC, NLRP3, and caspase-1 are essential for macrophage IL-1β maturation in response to a broad range of stimuli including bacteria (Mariathasan et al., 2006; Willingham et al., 2007) and viruses (Allen et al., 2009; Ichinohe et al., 2009; Kanneganti et al., 2006; Muruve et al., 2008). In addition to these inflammatory functions, ASC association with NLR proteins is required for pyroptotic and pyronecrotic cell death (Fernandes-Alnemri et al., 2007; Ting et al., 2008b). Although the host's innate immune response to *Mtb* infection is critical for the initial defense against bacteria, the adaptive immune response is ultimately required for containment of the infection in the chronic stage of disease. Adaptive immunity to *Mtb* infection is characterized by the appearance of antigen specific $CD4^+$ T-cells that secrete interferon-gamma (IFN-γ), which is responsible for activating macrophages to kill intracellular bacteria (Chan et al., 1992). CD8+ T-cells are also important for controlling bacteria during the chronic phase of *Mtb* infection (van Pinxteren et al., 2000). Chronic *Mtb* infection is controlled by granuloma formation which contains, but does not eliminate bacteria (Ulrichs and Kaufmann, 2006). Granulomas consist of a central core of *Mtb*-infected macrophages surrounded by successive waves of activated macrophages, giant multinucleated cells, epithelioid cells, lymphocytes, fibroblasts and dendritic cells. A subset of granulomas undergoes central caseous necrosis due to proteinaceous dead cell mass. Mice form slightly different granulomas that do not form

caseous necrotic centers, but otherwise possess the same cell types and similar granuloma organization to humans (Rhoades et al., 1997).

Pro-inflammatory cytokine regulation can be critical to long-term survival of *Mtb* infection. *In vivo* assessments of *IL-1α/β*$^{-/-}$, *IL-1R*$^{-/-}$, and *IL-18*$^{-/-}$ mice have shown that these cytokines play a role in limiting bacterial lung burden, regulating other cytokines, nitric oxide production, and forming organized granulomas (Juffermans et al., 2000; Sugawara et al., 2001; Sugawara et al., 1999). Likewise, mice deficient in pro-inflammatory cytokines IL-6 and TNFα have increased mortality during *Mtb* infection (Bean et al., 1999; Ladel et al., 1997). TNFα is important for granuloma formation and maintenance (Flynn et al., 1995). Therefore, these cytokines are not only important in the innate immune response to *Mtb*, but also in host defense during chronic *Mtb* infection.

High interest in the role of NLRs in host immunity has led to the study of inflammasome complexes in response to many pathogens. The research thus far has almost exclusively focused on the acute effects of pathogens and other NLR stimuli. Consequently, the *in vivo* role of the NLR inflammasome during chronic infection has not been studied. *Mtb* infection exemplifies a chronic infection of paramount public health interest. *Mtb* infects macrophages where it must thwart the host immune response to survive and replicate. Nlrp3 inflammasome proteins are expressed in macrophages, thus we hypothesized that *Mtb* infection would induce inflammasome activation. Here we show that *Mtb* induced IL-1β secretion in human and mouse macrophages *in vitro* and this process was dependent on ASC, caspase-1, and NLRP3, but not NLCR4. *In vivo,* murine *ASC* helps protect the host from death during chronic

Mtb infection while the effects of *Casp-1* and *Nlrp3* were negligible. The inability of *ASC*$^{-/-}$ mice to form organized granulomas and the reduced presence of lung dendritic cells indicates a breakdown in host defense against *Mtb*. Thus, we identify ASC as a critical protein involved in host response to *Mtb* infection in an inflammasome-independent manner.

RESULTS

Virulent and attenuated *Mtb* require ASC, NLRP3, and caspase-1 for IL-1β secretion by cultured human cells

During human *Mtb* infection, bacteria travel to the alveolar spaces where they infect and replicate inside macrophages. Secretion of IL-1β by macrophages requires pro-IL-1β processing by caspase-1. Caspase-1 interaction with several NLR forming inflammasomes results in caspase-1 processing, a prerequisite for IL-1β activation (Ye and Ting, 2008). To determine if host detection of *Mtb* involves inflammasome activation, we used a used a panel of human monocytic THP-1 cell lines with reduced expression of inflammasome genes due to shRNA targeting sequences (**Table 2.1, Figure 2.1)**. During infection with the virulent *Mtb* strain H37Rv, THP-1 cells with shASC or shNLRP3 secreted significantly less IL-1β than their scrambled controls (**Figure 2.2 A**). This indicates that *Mtb* activates the NLRP3 inflammasome. IL-18 is an inflammatory cytokine which induces cell-mediated immunity and causes T-cells to secrete IFN-γ (Okamura et al., 1995). Similar to IL-1β, it also requires caspase-1 cleavage for activation. IL-18 secretion from *Mtb* infected THP-1 cells was dependent on ASC and NLRP3 (**Figure 2.2 B**). These data demonstrated that both IL-1β and IL-

18 were processed during *Mtb* infection and that each required the NLRP3 inflammasome formation for activation in the THP-1 human monocytic cell line.

As seen with H37Rv, the attenuated *Mtb* H37Ra strain induced IL-1β secretion from THP-1 cells in a ASC- and NLRP3-dependent manner. Induction of IL-1β by H37Ra was dependent on the NLRP3 inflammasome (**Figure 2.2 C**). We consistently see more IL-1β secretion in THP-1 cells infected with H37Ra, although the difference is not statistically significant. Thus, the NLRP3 inflammasome is activated by both virulent and attenuated *Mtb.* Chemically inhibiting caspase-1 with caspase-1-specific inhibitor Y-VAD significantly reduced IL-1β secreted by THP-1 cells, confirming the necessity of caspase-1 cleavage for IL-1β secretion (**Figure 2.2 D**).

Production of IL-1β in *Mtb* infected primary mouse macrophages is *ASC, Nlrp3*, and *Casp-1* dependent

To confirm our THP-1 data that the inflammasome is necessary for IL-1β processing, we infected primary mouse macrophages from gene depletion mice. We infected either bone-marrow derived (BMDM) or thioglycolated elicited macrophages with *Mtb*. *Mtb* induced IL-1β secretion in wild type macrophages, confirming the results we obtained with THP-1 cells. *Mtb* infected BMDM from *ASC*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Casp-1*$^{-/-}$ mice had significantly reduced IL-1β secretion, indicating that the Nlrp3-inflammasome is necessary for *Mtb* induced IL-1β secretion in macrophages. *Nlrc4*$^{-/-}$ BMDM secreted IL-1β at the same or higher levels compared to wild type, indicating this inflammasome is not involved in the host response to *Mtb* (**Figure 2.3 A**). This demonstrates congruent findings using THP-1 cells and primary mouse macrophages.

Induction of the NLRP3 inflammasome was not limited to virulent *Mtb* H37Rv infection, but also occurred with attenuated *Mtb* H37Ra (**Figure 2.3 B**). Wild type BMDM infected with *Mtb* H37Ra secreted abundant amounts of IL-1β while *ASC*$^{-/-}$ and *Nlrp3*$^{-/-}$ macrophages did not. Our findings were repeated in primary thioglycolate elicited macrophages. These highly activated macrophages had a significant reduction in IL-1β secretion in the absence of *ASC* and *Nlrp3* (**Figure 2.3 C**), reinforcing our data from human THP-1 cells and BMDM infected with virulent and attenuated *Mtb*. IL-18 secretion from both BMDM and thioglycolate elicited macrophages was below the level of detection for all samples tested. Taken together, our data show that the ability of *Mtb* to induce IL-1β secretion was ASC, NLRP3, and caspase-1 dependent in human THP-1 cells as well as primary mouse macrophages. In contrast, NLRC4 did not affect IL-1β production, indicating that *Mtb* induces a host response through the NLRP3, but not the NLRC4, inflammasome.

ASC but not *Nlrp3* protects the host against virulent *Mtb* infection

Lack of IL-1β *in vivo* has been previously shown to be important for response to *Mtb* infection resulting in increased lung bacterial burden, differential regulation of cytokines, and defects in granuloma formation (Sugawara et al., 2001; Yamada et al., 2000). Given our data from infecting cultured cells, we hypothesized that mice lacking the *ASC* or *Nlrp3* would be unable to process IL-1β resulting in an inability to control *Mtb in vivo*. To test this hypothesis, wild type, *ASC*$^{-/-}$, and *Nlrp3*$^{-/-}$ animals were infected with aerosolized *Mtb* H37Rv. Each mouse received between 250-350 cfu per lung. During the first four-and-a-half months, survival of wild type and *ASC*$^{-/-}$ mice was similar.

However, *ASC*$^{-/-}$ mice had a survival defect thereafter and they died precipitously between 130 and 142 days postinfection with a mean survival of 137 days (**Figure 2.4 A**). By comparison, wild type mice infected with *Mtb* had a mean survival of 214 days. These results were confirmed in a second (**Figure 2.4 B**) and third experiment (not shown). Death of *ASC*$^{-/-}$ mice during *Mtb* infection was not due to advanced age as we routinely keep *ASC*$^{-/-}$ breeder pairs for up to a year with no unexplained deaths. This strong survival phenotype shows that *ASC* is important for host defense against virulent *Mtb* infection *in vivo*.

ASC is an adaptor protein that is required for several NLR proteins to form inflammasomes. Since our *in vitro* data indicated NLRP3 was necessary for *Mtb* induction of IL-1β in macrophages, we also investigated survival of *Nlrp3*$^{-/-}$ mice during aerosolized *Mtb* infection. Although NLRP3 is important for IL-1β secretion from cultured macrophages, there was no difference in survival of *Nlrp3*$^{-/-}$ mice *in vivo* (**Figure 2.4 C**). Overall, *Nlrp3*$^{-/-}$ mice had a mean survival of 211 days compared to 214 days for wild type mice. This data was also confirmed in a second experiment **(Figure 2.4 D**). In a separate experiment, we tested the ability of another closely related NLR gene, *Nlrc4,* to protect the host from *Mtb*. *Nlrc4*$^{-/-}$ mice had a similar survival profile to paired wild type controls, with a mean survival of 190 days compared to 198 days for wild type controls (**Figure 2.4 E**). These data indicate that elimination of *ASC*, but not *Nlrp3* nor *Nlrc4*, resulted in a reduced ability of the host to defend against *Mtb* infection.

ASC and *Nlrp3* do not significantly affect *Mtb* bacterial burden

Prior *in vivo* studies show that *Mtb* has two phases of infection (Orme, 1994). In the first three weeks following aerosol infection *Mtb* grows logarithmically in the lungs. After three weeks of infection, an effective T_H1 adaptive immune response is established and the exponential growth of *Mtb* ends. *Mtb* bacterial burden in the lungs, spleen, and liver persist at the same level for the remainder of the infection (Kaufmann, 2008). We assessed bacterial burden in the lungs of *ASC*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice in two separate infections, with each compared to paired C57BL/6 controls. Animals lacking *ASC* and *Nlrp3* have similar levels of bacteria in the lungs during the growth and persistence phases of *Mtb* infection compared to wild type (**Figure 2.5 A, B**). The last time points were taken as *ASC*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice neared death. We saw no statistical difference between the bacterial burden of lungs from *ASC*$^{-/-}$, *Nlrp3*$^{-/-}$, or wild type mice. The bacterial burden in the liver and spleen of the *ASC*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice was also similar to that found in wild type mice, indicating that *ASC* and *Nlrp3* do not affect bacterial dissemination (**Figure 2.5 C-F**). Thus a change in bacterial burden and dissemination cannot explain the different survival rate of *ASC*$^{-/-}$ compared to WT or *Nlrp3*$^{-/-}$ mice.

Pro-inflammatory cytokines are produced at similar levels in the lungs of wild type, *ASC*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice following *Mtb* H37Rv aerosol infection

Our earlier studies led us to hypothesize that less IL-1β would be produced in the lungs of *ASC*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice compared to wild type because the gene deletion mice would not be able to cleave pro-IL-1β to its active form in response to *Mtb* infection. To test this, cytokines were measured from tissue free homogenized lung extract by

enzyme-linked immunosorbent assay (ELISA). Surprisingly, we found all three groups of animals produced IL-1β in similar amounts. IL-1β levels increased during the logarithmic growth phase of infection and were still high during the persistence phase of infection (**Figure 2.6 A**). By week 16, levels of IL-1β decreased slightly. There was no significant reduction in IL-1β secretion between *ASC*$^{-/-}$ and *Nrp3*$^{-/-}$ mice compared to wild type at any of the time points we measured. Because of the mechanical nature of lung homogenization, cells are broken open in the process which could result in release of both pro and cleaved IL-1β in the lung homogenate extracts. We performed IL-1β western blots in addition to ELISAs to ensure we were measuring the amount of cleaved IL-1β produced in the lungs of *Mtb*-infected animals *in vivo.* At one week postinfection, as expected, only pro-IL1β was present (**Figure 2.6 B, top panel**). At 16 weeks postinfection, *ASC*$^{-/-}$ and *Nlrp3*$^{-/-}$ animals produced comparable if not enhanced levels of cleaved IL-1β compared to wild type mice (**Figure 2.6 B, bottom panel**). The amount of IL-1β was reproducible between mice of the same genotype and correlates with the ELISA data. These data show that differences in IL-1β production cannot explain differences in the survival rate of *ASC*$^{-/-}$ compared to WT or *Nlrp3*$^{-/-}$ animals. We also measured pro-inflammatory cytokines IL-6 and TNFα. TNFα is of particular interest as it is important for granuloma formation and the control of infection in *Mtb*-infected animals and humans (Bean et al., 1999; Flynn et al., 1995). IL-6 has also been shown to be an important host response against *Mtb* (Ladel et al., 1997). Furthermore, a previous report showed that human ASC is required for TNFα and IL-6 expression upon bacterial infection (Taxman et al., 2006). Both cytokines followed similar trends as IL-1β with total cytokine levels rising during early infection and then dropping off significantly by

week 16 (**Figure 2.6 C, D**). *Nlrp3*$^{-/-}$ mice had a modest but statistically significantly increase in IL-6 and TNF-α cytokines at 2 weeks (p=0.026 and p=0.028, respectively) and 5 weeks postinfection (p=0.004, p=0.001, respectively), but all differences were diminished by 16 weeks postinfection. There were no significant differences in lung cytokine levels between *ASC*$^{-/-}$ and wild type mice at any time points analyzed. Proinflammatory cytokines in serum and bronchoalveolar lavage fluid were below the level of detection. These data indicate that reduced TNFα and IL-6 are not correlated with increased death in *Mtb*-infected *ASC*$^{-/-}$ mice.

Caspase-1 is not required for survival, bacterial containment, or the production of proinflammatory cytokines in *Mtb* infected mice

Caspase-1 recruits and cleaves IL-1β to its active form and is common to all NLR inflammasomes. A previous report has shown that at a low infectious unit of influenza virus, viral-induced host response is mediated by an *Nlrp3*-independent but *ASC*/*Casp-1*-dependent inflammasome (Ichinohe et al., 2009). To assess if Caspase-1 and ASC together mediate host protection in the context of inflammasome activation, we investigated the contribution of Caspase-1 during *Mtb* infection. Infection of *Casp-1*$^{-/-}$ mice with *Mtb* produced no difference in survival compared to wild type mice (**Figure 2.7 A**). Measurement of bacterial burden showed that *Casp-1*$^{-/-}$ and wild type mice had similar levels of bacteria in the lungs over the course of *Mtb* infection (**Figure 2.7 B**). *Casp-1*$^{-/-}$ and wild type mice also had similar bacterial burden in the liver and spleen, indicating that bacterial dissemination and growth in these organs are comparable between genotypes (**Figure 2.7 C**). Overall, these data indicate that Caspase-1 does

not play a prominent role in host protection during *Mtb* infection. Hence this work describes a novel *ASC*-dependent but *Casp-1*-independent form of host immunity.

Caspase-1 cleaves pro-IL-1β to its biologically active form, however our earlier results demonstrated that cleaved IL-1β levels were similar in *ASC*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Nlrc4*$^{-/-}$ lungs. An analysis of *Casp-1*$^{-/-}$ mice show that rather than diminished IL-1β, they had increased lung IL-1β in their lungs compared to wild type as measured by ELISA (**Figure 2.8 A**). We utilized Western blots to measure both the pro- and cleaved forms of IL-1β from lung homogenate extracts collected one week and 20 weeks post infection. At one week post infection only low levels of pro-IL-1β were present in the *Casp-1*$^{-/-}$ and wild type lungs (**Figure 2.8 B, top panel**). At 20 weeks post infection pro- and cleaved IL-1β were present in both *Casp-1*$^{-/-}$ and wild type mouse lungs with more found in the former (**Figure 2.8 B, bottom panel**). These data demonstrate that *Casp-1*$^{-/-}$ mice have compensatory mechanisms of cleaving IL-1β in the absence of caspase-1. Levels of IL-6 and TNF-α also were unchanged between *Casp-1*$^{-/-}$ and wild type lungs (**Figure 2.8 C-D**). Overall our *in vivo* characterization of the *Casp-1*$^{-/-}$ mouse reveals that Caspase-1 does not play a prominent role in host protection against *Mtb*. This supports the conclusion that protective host immunity to *Mtb* is independent of the inflammasome.

ASC$^{-/-}$ macrophages show a modest reduction of signal transduction

ASC has been associated with NFκB and AP1 signaling independent of NLRP3 or caspase-1 function (Hasegawa et al., 2009; Stehlik et al., 2002; Taxman et al., 2006). To determine if NFκB activation is affected in the absence of ASC we infected BMDM

with *Mtb* and assessed phosphorylation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) and NFκB p65. In all cases we saw modest but reproducible decreases in NFκB signaling proteins in the *ASC*$^{-/-}$ macrophages compared to wild type macrophages. We also measured activation of MAP kinases extracellular-signal-regulated kinases (ERK), JNK, and p38 which are known to be upregulated during *Mtb* infection (Roach and Schorey, 2002). We found that ERK and JNK phosphorylation was modestly decreased in *ASC*$^{-/-}$ macrophages compared to wild type, but p38 showed no reduction in activation (**Figure 2.9).**

ASC$^{-/-}$ mice have functional T-cells, but a reduced CD11c^{+} CD11b$^{mid/low}$ lung cell population during chronic *Mtb* infection

T-cell function is essential to mount an adaptive immune response against *Mtb*. We collected lymphoid cells from wild type and *ASC*$^{-/-}$ mice infected with *Mtb* for 95 days and identified the cell populations by flow cytometry. Overall, *ASC*$^{-/-}$ mice had higher percentages of CD4^{+} and CD8^{+} T-cells in their lung, spleen, and mediastinal lymph node (MLN) (**Figure 6A-F**). To test T-cell function, we measured the ability of T-cells from *Mtb* infected mice to be activated by a general CD3/CD28 stimulation or *Mtb*–specific PPD, shown by production of intracellular IFN-γ. In all organs tested inherent T-cell function in *ASC*$^{-/-}$ mice was comparable to wild type after 5 hours of stimulation (**Figure 2.10 A-F**). The total number of cells recovered from each organ was equivalent between *ASC*$^{-/-}$ and wild type mice (**Figure 2.11 A**). These data indicate that T-cells are not the cause of *ASC*$^{-/-}$ susceptibility to *Mtb* infection. To further explore the mechanism by which *ASC* enhances survival, we investigated myeloid cell populations present in

the lungs of chronically infected mice. *ASC*$^{-/-}$ mice have a significantly reduced CD11c^{+} CD11b$^{mid/low}$ cell population compared to wile type mice (p=0.000015) (**Figure 2.11 B-C**). During the chronic phase of infection CD11c^{+} cells are located in both human and mouse granulomas, present from 2 weeks post infection and progressively increasing over the course of infection (Tsai et al., 2006; Uehira et al., 2002). Reduced dendritic cells in the lungs of *ASC*$^{-/-}$ provide plausible mechanisms to explain the decreased lifespan observed in these animals during chronic *Mtb* infection.

ASC$^{-/-}$ mice have increased GR1^{+} lung cell population during chronic *Mtb* infection

Following whole lung digest of mice infected with *Mtb* for 95 days, we found an increased percentage of GR1^{+} cells in the lungs of *ASC*$^{-/-}$ mice when compared to wild-type controls (**Figure 2.12 A-B**). GR1^{+} cells, which are classically defined as neutrophils, are recruited to the lungs of wild type mice during initial infection, peaking at two weeks post infection, after which the number of neutrophils decline (Kaufmann, 2008). It is unusual for neutrophils to be present within the granuloma during chronic infection. We found that wild-type lungs contain approximately one percent of GR1^{+} cells during chronic *Mtb* infection (**Figure 2.12 A-B**). Increased numbers of GR1^{+} cells within the lungs of *ASC*$^{-/-}$ mice may indicate an inability by the host to effectively control *Mtb* during chronic infection.

ASC$^{-/-}$ mice form fewer granulomas

To further explore the mechanism by which *ASC* enhances survival in chronically infected mice, we examined the histopathology of *Mtb* infected mouse lungs. We

examined granuloma structure because of its role in containing bacteria during chronic *Mtb* infection (Ulrichs and Kaufmann, 2006) and because *ASC*$^{-/-}$ mice exhibit an abrupt drop in survival during the chronic phase of infection. We assessed lung inflammation 16 weeks post infection by measuring the amount of inflamed tissue from each mouse lung (see experimental procedures). Low magnification images showed that the alveolar spaces of wild type, *ASC*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Casp-1*$^{-/-}$ mice were obstructed by an influx of immune cells into the lung. We found the lungs of all our gene depletion and wild type mice to be highly inflamed, ranging from 90-98 percent of the total lung tissue (**Figure 2.13 A-B**). In all infected mice we observed dense areas of immune cells, indicative of granulomatous lesions. Strikingly, the *ASC*$^{-/-}$ animals formed significantly fewer granulomas per lung compared to wild type, *Nlrp3*$^{-/-}$, and *Casp-1*$^{-/-}$ lungs (**Figure 2.13 C-D**). Examination under higher magnification showed that wild type and *Nlrp3*$^{-/-}$ mice formed similar sized granulomas, hence *ASC* affected the formation of granulomas, but once they were formed, granuloma size was not affected by this gene (**Figure 2.13 E-F**). *Casp-1*$^{-/-}$ mice formed larger granulomas, although this did not affect the outcome of infection. To further assess the lung granuloma defect in *ASC*$^{-/-}$ mice we stained mouse lung sections with Ziehl Neelsen stain to identify *Mtb*. Earlier data showed that the overall lung bacterial burden was similar in wild type and *ASC*$^{-/--}$ mice by bacterial plating of homogenized lungs (**Figure 2.5 A-B**). In contrast, analysis of acid fast staining revealed striking differences in bacterial localization in wild type and *ASC*$^{-/-}$ lungs. Acid fast staining of wild type lungs shows very little *Mtb* located in non-granulomatous lung tissue. In contrast, *ASC*$^{-/-}$ mice contain bacteria throughout the lung and have extremely high amounts of bacteria in tissue that is not associated with

granulomas (**Figure 2.14 A**). The amount of bacteria located outside of the granuloma was quantified by using a scale of 0-4 based on the amount of bacteria present in lung tissue that was not associated with granulomas (see experimental procedures) (**Figure 2.14 H**). *ASC*$^{-/-}$ mice form significantly fewer lung granulomas than wild type mice. The acid-fast staining data demonstrate that *ASC*$^{-/-}$ animals were unable to contain bacteria within granulomas, shown by massive amounts of *Mtb* located in non-granulomatous lung tissue compared to wild type mice. Reduced CD11c^{+} CD11b$^{mid/low}$ cells and granulomas in the lungs of *ASC*$^{-/-}$ provide plausible mechanisms to explain the decreased lifespan observed in these animals during chronic *Mtb* infection.

DISCUSSION

In this report, we focus on the ability of virulent *Mtb* to stimulate inflammasome activation and the role of the inflammasome in host defense against *Mtb*. Prior studies have implicated the inflammasome in mycobacterium infection *in vitro*. Consistent with our data, one report showed that *M. marinum*, a mycobacterium that naturally infects fish and amphibians, activates IL-1β production in a ASC, NLRP3, and caspase-1-dependent manner *in vitro*, but no *in* vivo investigation was conducted (Koo et al., 2008). Another report showed that *M. bovis* BCG, the vaccine strain of *Mtb*, can limit caspase-1 and IL-1β activation due to a putative protease Zmp1. In this latter study, however, there was no analysis of *ASC, Nlrp3* or *Nlrc4* to directly implicate the inflammasome components (Master et al., 2008). A third report showed that *Mtb* infected THP-1 human monocyte cell line secrete IL-1β in a ASC and NLRP3-

dependent process, but did not investigate the role of the inflammasome *in vivo* (Mishra et al., 2010).

Our study showed that virulent *Mtb* H37Rv, along with the attenuated derivative *Mtb* H37Ra, induced NLRP3, ASC, and caspase-1-dependent inflammasome activation in a human macrophage cell line and in primary mouse macrophages. However these *in vitro* results do not predict the outcome in mice. Most importantly, this study showed that *ASC* is important for host protection during chronic infection with virulent *Mtb* infection *in vivo*, demonstrated by decreased survival of *ASC*$^{-/-}$ mice compared to wild type controls. *ASC* did not confer a survival advantage through the production of IL-1β. Rather it functions to prolong host survival through a novel inflammasome independent role likely through appropriate presence of $CD11c^{+}$ $CD11b^{mid/low}$ cells in the lung and granuloma formation during chronic *Mtb* infection. Surprisingly, neither *Nlrp3* nor *Casp-1* played a prominent role in host protection during *Mtb* infection *in vivo* despite its importance in culture. The inconsistencies between the *in vitro* and *in vivo* role of *Nlrp3* and *Casp-1* underscores the absolute necessity for *in vivo* validation in relating host immune genes to the outcome of microbial infections.

IL-1β cleavage is a hallmark of inflammasome activation and we observed significant *ASC, Nlrp3,* and *Casp-1*-dependent inflammasome activation during *in vitro* infection of cultured cells. However in our *in vivo* studies, we observed mature IL-1β in the lungs of *Mtb* infected wild type, *ASC*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Casp-1*$^{-/-}$ mice. This, along with mouse survival data, indicates that ASC host protection during *Mtb* infection *in vivo* is inflammasome independent. The similar survival profiles of *Nlrp3*$^{-/-}$ and *Casp-1*$^{-/-}$ mice compared to wild type mice may be due to compensatory mechanisms of IL-1β

processing. It is important to note that the inflammasome is not the only host mechanism for cleaving pro-IL-1β to its active form. Through less well established, caspase-1-independent IL-1β cleavage can be carried out by several other host proteases including granzyme A, chymase, chymotrypsin, and matrix metalloproteinases as well as bacterial enzymes (Black et al., 1988; Irmler et al., 1995; Mizutani et al., 1991; Schonbeck et al., 1998). Meanwhile *ASC*$^{-/-}$ mice lacked an additional mechanism to form or maintain granulomas, thus compromising their resistance to *Mtb* infection.

During chronic *Mtb* infection we found that lungs of *ASC*$^{-/-}$ mice had a dramatically reduced CD11c^{+} CD11b$^{mid/low}$ cell population compared to wild type lungs. CD11c^{+} cells are present in both human and mouse granulomas, and progressively increase in the lung during chronic *Mtb* infection (Tsai et al., 2006; Uehira et al., 2002). Despite a decrease in the CD11c^{+} CD11b$^{mid/low}$ cell population, T cells from *ASC*$^{-/-}$ mice are not inherently defective in T-cell function after stimulation with CD3/CD28 or *Mtb*-specific PPD.

To investigate the mechanism further, we show that *ASC*$^{-/-}$ mice have fewer lung granulomas compared to wild type, *Nlrp3*$^{-/-}$, or *Casp-1*$^{-/-}$ mice, despite having the same amount of inflamed lung tissue. Acid-fast staining for *Mtb* localization within the lung demonstrated that *ASC*$^{-/-}$ mice are defective in containing *Mtb* with in granulomas, as shown by large amounts of bacteria found in non-granulomatous lung tissue. In comparison, infected wild type mice have almost no bacteria found outside of granulomas. Our data show that overall the lungs of *ASC*$^{-/-}$ and wild type mice have the same amount of bacteria, however the localization of *Mtb* within the lung is radically

different. These data support a working hypothesis that ASC affects proper DC accumulation during chronic *Mtb* infection and promotes proper granuloma formation.

Recently, another group implicated an inflammasome-independent role for ASC in host protection against *Mtb in vivo,* but did not investigate the mechanism by which this was occurring (Mayer-Barber et al.). In contrast to our data, this paper found that *Casp-1* deficient mice had decreased survival during *Mtb* infection. This may be due to differences in the amount of bacteria delivered to the mice or mouse housing conditions. Nonetheless, both their and our papers come to the same conclusion: that ASC has a role in host protection independent of Nlrp3.

In summary, this work demonstrates that ASC promotes survival during the late phase of *Mtb* infection independent of inflammasome activation. This result also highlights the importance of ASC in the process of granuloma formation during chronic *Mtb* infection. Considering the high rates of chronic *Mtb* infection worldwide and the increasing number of multi-drug resistant and extensively drug resistant tuberculosis cases, our study suggests that understanding the role of ASC during *Mtb* infection may lead to new and more effective therapies to treat chronic *Mtb* infection. In contrast, the lack of a role for NLRP3 and caspase-1 in the *in vivo* containment of *Mtb* is encouraging for the development of inhibitors for autoinflammatory disorders. These results suggest that anti-NLRP3 and caspase-1 strategies are not likely to cause the inadvertent activation of latent *Mtb* infection in patients.

EXPERIMENTAL PROCEDURES

Generation of shRNA knockdown THP-1 cell lines. shRNA knockdown and control vectors for ASC and NLRP3 have been described previously (Taxman et al., 2006). All shRNA hairpins were confirmed by sequencing (**Table 2.1**). Lentiviral packaging and transduction of THP1 cells has been previously described (Moore et al., 2008). Verification by RT-PCR indicated a significant reduction in ASC and NLRP3 expression (**Figure 2.1 A-B**). Functionally, shASC and shNLRP3 had reduced IL-1β production in response to LPS (**Figure 2.1 C**).

Bacterial strains. *Mtb* H37Rv and H37Ra were obtained from the. Bacteria were grown to log phase in Middlebrook 7H9 broth (Difco) with 0.2% glycerol, 1× albumin dextrose saline, and 0.05% Tween 80. Inoculum was assessed by plating infection media on Middlebrook 7H10 agar plates supplemented with glycerol and ADS as described above. Colony forming units (cfu) were counted after 21 days of incubation.

***Mtb* infection of cultured cells.** Cell lines were cultured in RPMI (Gibco) with 10% FBS (Hyclone). Bone marrow derived macrophages were harvested from 6- to 8-week-old mouse femurs and cultured for 6 days in DMEM supplemented with L-glutamine, non-essential amino acids, 10% fetal bovine serum, and 20% L929 conditioned media. Thioglycolate elicited macrophages were obtained by peritoneal lavage 4 days after intraperitoneal injection with 3% thioglycolate and cultured in DMEM supplemented as above. All cells were infected under BSL3 conditions with *Mtb* H37Rv or H37Ra at an MOI of 10 and incubated for 8 hours at 37°C with 5% CO_2. Cell free supernatants were

harvested, double filtered with 0.2μ filters, and assayed for cytokines by ELISA and western blot.

***Mtb* H37Rv aerosol infection of mice.** Generation of *ASC*$^{-/-}$, *Nlrp3*$^{-/-}$, *Casp-1*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice (Kuida et al., 1995; Mariathasan et al., 2004; Sutterwala et al., 2006) have been described previously and were backcrossed onto C57BL/6 (Jackson Lab) background for a minimum of 9 generations. Female *ASC*$^{-/-}$, *Casp-1*$^{-/-}$ *Nlrp3*$^{-/-}$,and *Nlrc4*$^{-/-}$ mice and age matched C57BL/6 female controls were infected via aerosol as previously described (Kurtz et al., 2006). Mice received 250-350 colony forming units per lung, determined by sacrificing a subset of mice 1d post infection as described by others (Kurtz et al., 2006). Bacterial organ burden was quantified by plating serial dilutions of lung, liver, and spleen homogenates on 7H10 plates containing cycloheximide (1μg/ml) and carbenicillin (50μg/ml) to minimize contamination. Animal infections and organ harvests were carried out under BSL3 conditions. All studies were conducted in accordance with the National Institutes of Heath Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of North Carolina at Chapel Hill.

Cytokine determination. Cytokines were measured from infected cell supernatants with human or mouse BD OptEIA IL-1β, TNFα, and IL-6 ELISA Sets (BD Biosciences) and IL-18 ELISA (MBL International). *In vivo* cytokines were measured from lung homogenate extracts by centrifuging homogenized lung tissue to create a tissue free supernatant. Amounts of pro- and cleaved IL-1β in lung homogenate extracts were

determined by Western blot. Immunoblots were probed with goat anti-mouse IL-1β primary antibody (R&D Biosystems). Bands were visualized by Super Signal Chemiluminescence (Pierce).

Histopathology. Lungs were fixed in 10% buffered formalin and stained with H&E to evaluate airway inflammation and identify granulomas. The percent of inflamed lung tissue was determined by dividing the inflamed areas by the total lung area. Granuloma frequency was determined by counting the number of granulomas present in the total lung section. Granuloma size was measured by defining the granuloma borders for 26 lesions using Image J software and determining the average. Ziehl-Neelsen (ZN) staining was used to determine bacterial localization. The entire *Mtb* infected mouse lung was scored on a scale from 0-4: 0=no bacteria found outside of granulomas, 1=1-5 bacteria found outside of granulomas, 2=6-20 bacteria found outside of granulomas, 3=approximately half of each field contains 20-50 bacteria outside of granulomas, and 4= greater than half of each field contains greater than 50 bacteria outside of granulomas. H&E lung sections were blindly scored by E.M., I.C.A., M.S., and P.H. ZN stained lung sections were blindly scored by M.S. and P.H. For all histology quantification 4-8 mouse lungs per genotype were analyzed.

Whole lung digest and T-cell restimulation assays. Lungs were digested in dispase at room temperature for 45 minutes. Immune cells were removed and stained with CD11b (M1/70) and CD11c (N418) antibodies (eBioscience). Splenocytes and MLN cells were harvested from the same mice and, along with lung cells, were incubated for

5 hours with Brafeldin A (7ug/ml) alone or in combination with CD3 (145-2C11)/CD28 (37.51)(2.5ug/ml each) or PPD (1/40 dilution)(Synbiotics). Lung, spleen, and MLN cells were stained with CD4 (L3T4), CD8 (Ly-2), and intracellular IFN-γ (XMG1.2) antibodies (eBiosciences). Samples were analyzed by flow cytometry on FACSCalibur.

Statistics. Data are presented as the means +/- standard deviation (SD) unless otherwise noted. Analysis Of Variance (ANOVA) followed by Tukey-Kramer HSD for multiple comparisons was performed on complex data sets. Statistical significance for single data points was assessed by the Student's two-tailed t-test. Survival curves were generated utilizing the product limit method of Kaplan and Meier and comparisons were made using the log rank test. In all cases, a p-value of less than 0.05 was considered statistically significant.

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currently an American Cancer Society Postdoctoral fellow. The authors have no conflicting financial interests.

FIGURES

Construct	Target sequence
shASC	**GCTCTTCAGTTTCACACCA**
control ASC	**GCTCTTCctggcCACACCA**
shNLRP3	**GGATGAACCTGTTCCAAAA**
control NLRP3	**GGATGAACgcaTTCCAAAA**

Table 2.1. Target sequences for shRNA THP-1 cell lines. Target sequences for human *ASC* and *NLRP3* shRNA hairpins used to construct stable knockdown THP-1 cell lines.

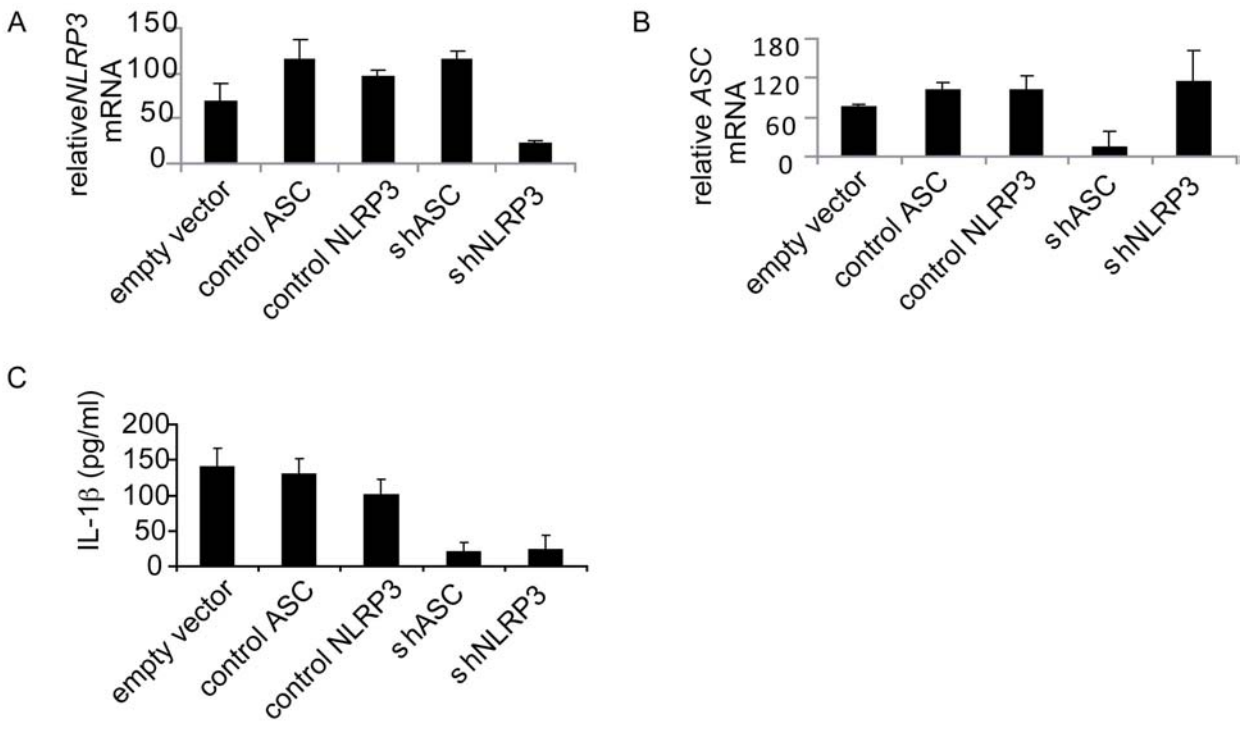

Figure 2.1. Validation of shRNA THP-1 cell lines. A-B. To ensure shRNA THP-1 cell lines had reduced gene expression we measured human *NLRP3* **(A)** and *ASC* **(B)** gene transcript by RT-PCR. **C.** Functional knockdown was shown by reduced IL-1β production in shASC and shNLRP3 THP-1 cell lines after LPS stimulation.

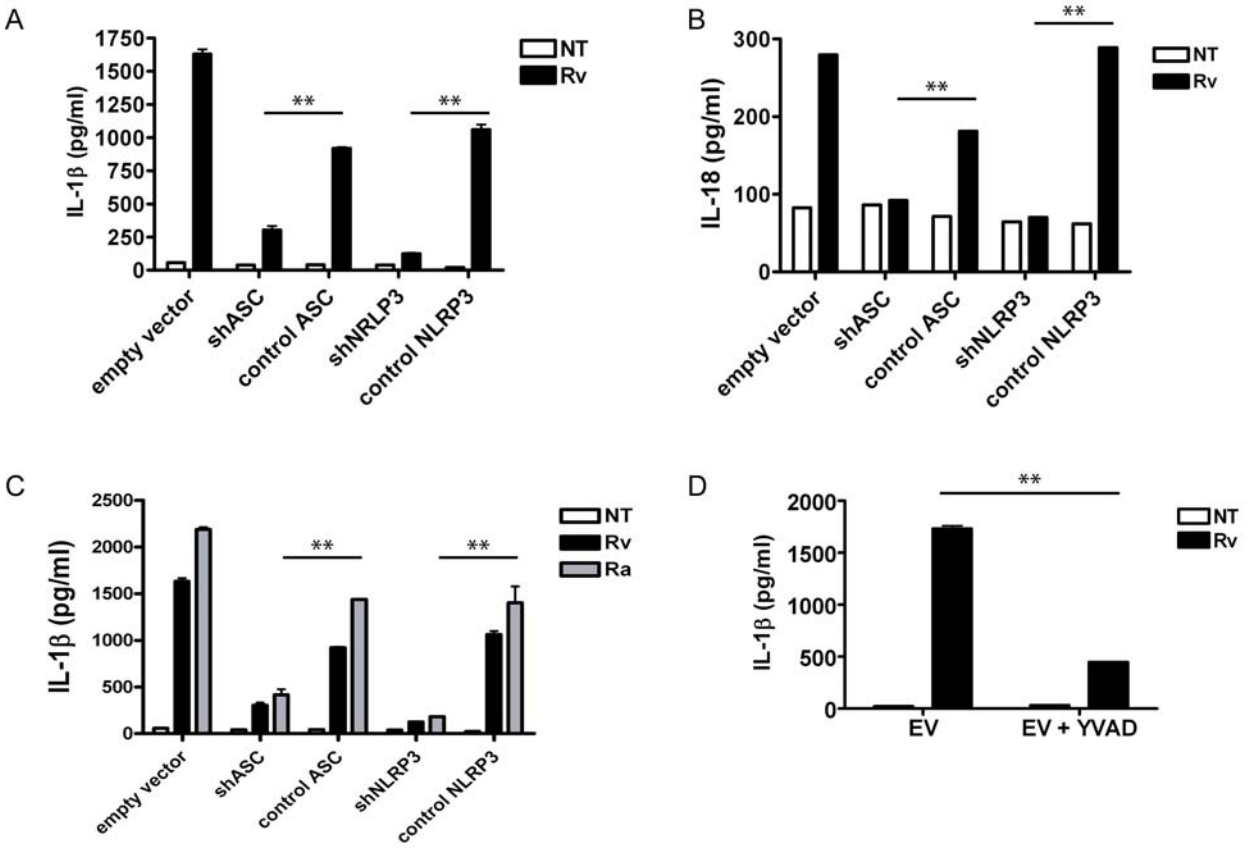

Figure 2.2. *Mycobacterium tuberculosis* induced IL-1β in a human monocytic cell line required ASC and NLRP3. A. Virulent *Mtb* H37Rv induced IL-1β release was decreased in THP-1 cells stably transduced with ASC and NLRP3-specific shRNAs (shASC and shNLRP3, respectively) but not in cells transduced with an empty vector (EV) or scrambled shRNAs (mut) or untreated (NT) cells. **B.** *Mtb* induced IL-18 secretion was observed in EV and mut controls but not with THP-1 cells transduced with shASC and shNLRP3. **C.** Attenuated *Mtb* H37Ra, similar to virulent *Mtb* H37Rv, induced ASC and NLRP3-dependent IL-1β release. **D.** Caspase-1 specific inhibitor (YVAD-CHO, 100 μM) blocked *Mtb* induced IL-1β. In THP-1 cells expressing an empty vector (EV). For all experiments non-adherent THP-1 cells were infected with *Mtb* at MOI 10 for 8 hours. IL-1β and IL-18 secretion were determined by ELISA. Error bars represent SD of a representative experiment. All experiments were repeated a minimum of three times. ** $p<0.001$

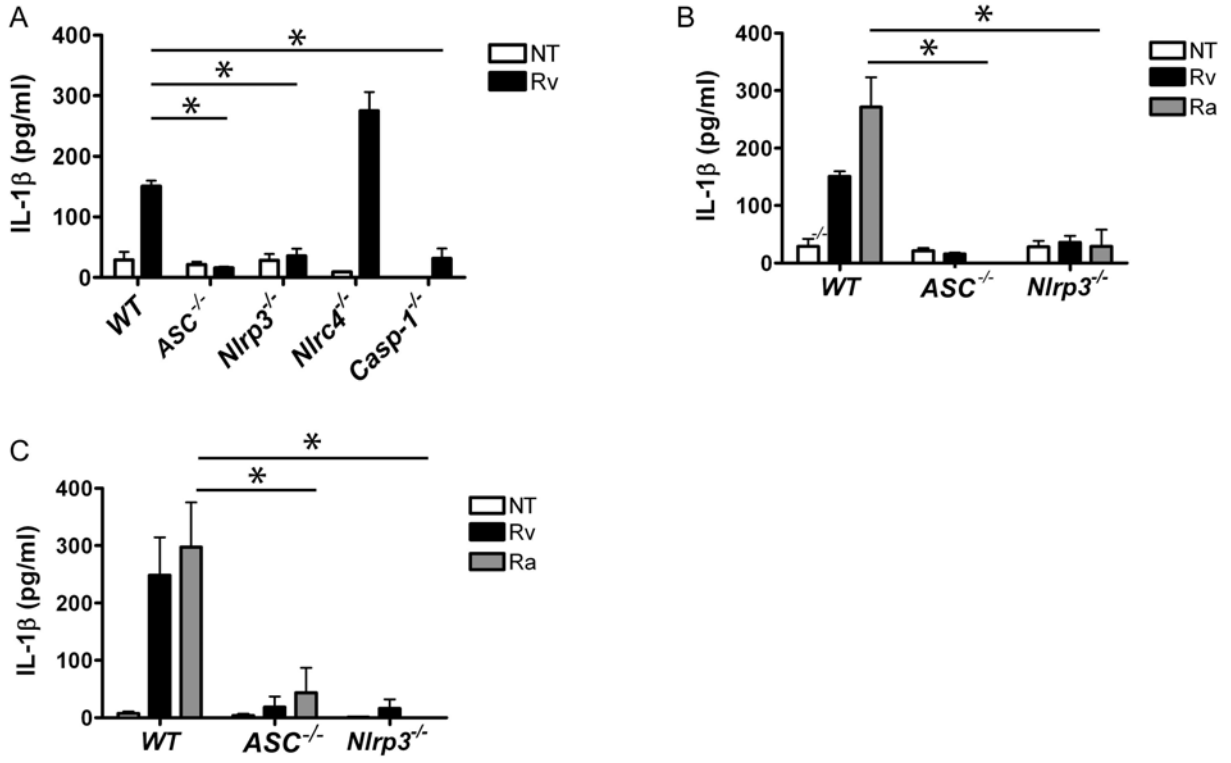

Figure 2.3. *Mycobacterium tuberculosis* induced *ASC, Nlrp3*, and *Casp-1* dependent IL-1β release in primary macrophages. A. *ASC*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Casp-1*$^{-/-}$ BMDM have decreased levels of IL-1β in response to *Mtb* H37Rv infection compared to WT or untreated (NT) BMDM. **B.** Attenuated *Mtb* H37Ra, similar to virulent *Mtb* H37Rv, induced *ASC* and *Nlrp3*-dependent IL-1β release. **C.** Thioglycolate elicited macrophages infected with *Mtb* H37Rv and *Mtb* H37Ra induced *ASC* and *Nlrp3*-dependent IL-1β release. Error bars represent SD of a representative experiment. Each experiment was repeated a minimum of three times. * $p<0.05$

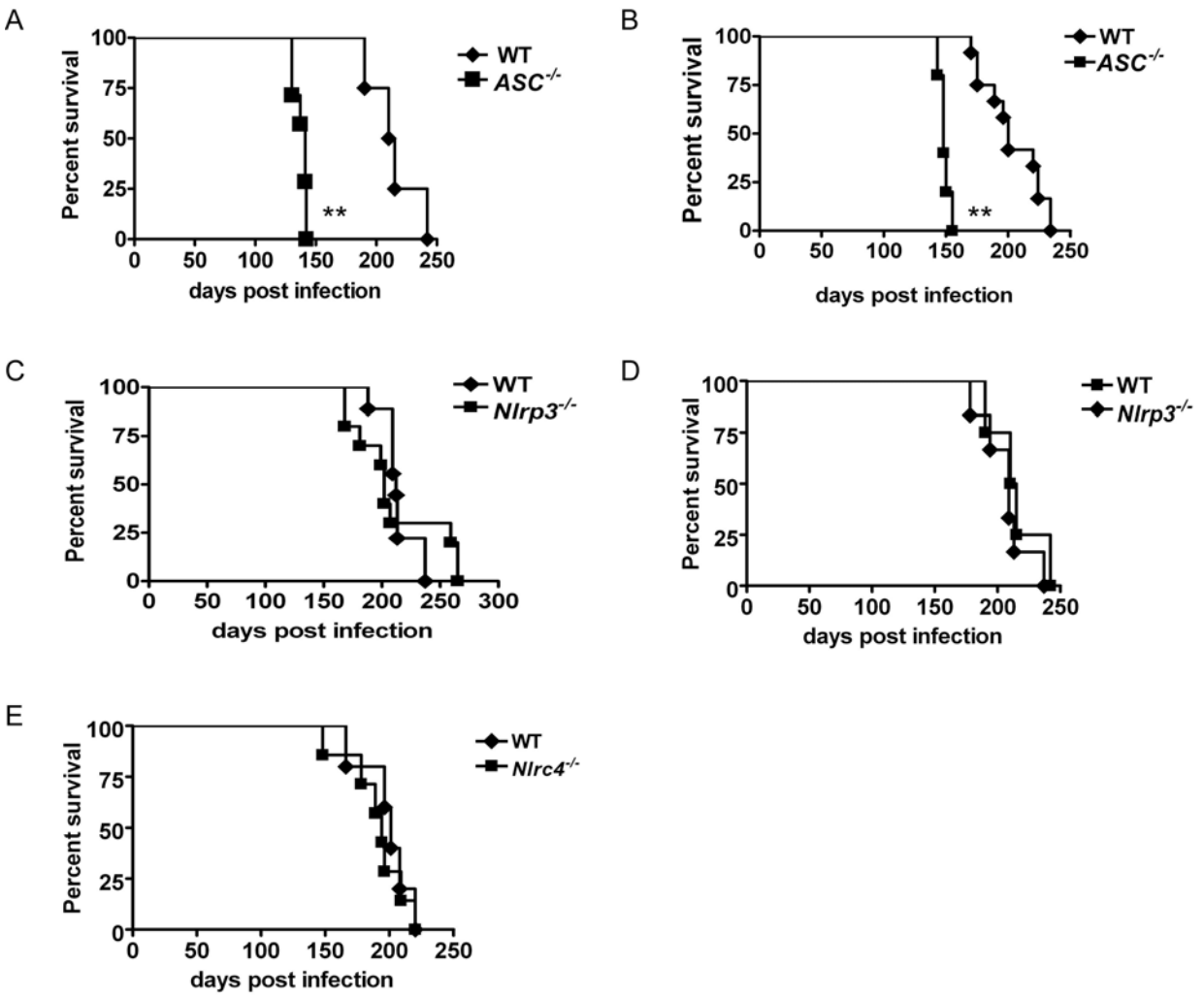

Figure 2.4. *ASC*$^{-/-}$ mice have significantly increased mortality following *Mtb* H37Rv aerosol infection. Mice were infected by aerosol with each lung receiving 250-350 cfu. **A.** *ASC*$^{-/-}$ mice infected with *Mtb* H37Rv die significantly earlier than age and sex matched wild type mice (**p=0.0011; log rank). Mean survival *ASC*$^{-/-}$ =137 vs. WT=214 days post infection. **B.** Repeat infection of *ASC*$^{-/-}$ mice and wild type mice confirmed survival phenotype with mean survival *ASC*$^{-/-}$ =148 vs. WT=203 days post

infection ($p<0.0001$; log rank). **C.** No significant difference in survival was observed between *Nlrp3*$^{-/-}$ and WT mice after *Mtb* H37Rv infection. Mean survival *Nlrp3*$^{-/-}$ =211 vs. WT=214 days post infection. **D.** Repeat of *Nlrp3*$^{-/-}$ survival confirmed no difference between *Nlrp3*$^{-/-}$ and WT mice. Mean survival *Nlrp3*$^{-/-}$ =206 vs. WT=214 days post infection. **E.** *Mtb* infection of *Nlrc4*$^{-/-}$ mice showed no significant difference in survival compared to wild type mice. Mean survival *Nlrc4*$^{-/-}$ =190 vs. WT=198 days post infection.

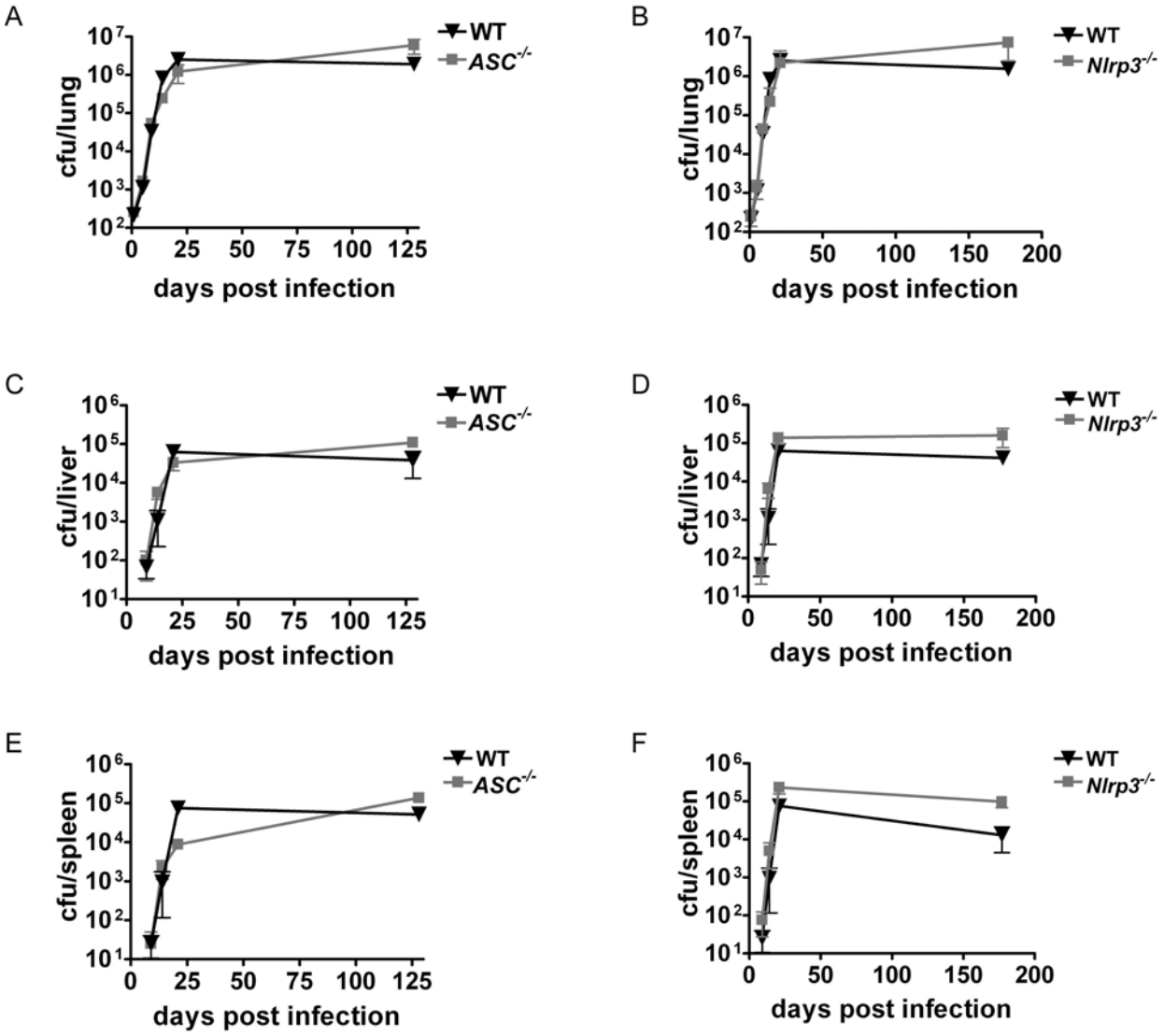

Figure 2.5. Bacterial burden of *ASC*$^{-/-}$ mice is not increased following *Mtb* H37Rv aerosol infection. A-F. Bacterial organ burden. (**A-B**) Lungs, (**C-D**) liver, and (**E-F**) spleen of *Mtb* infected *ASC*$^{-/-}$ (**A,C,E**) and *Nlrp3*$^{-/-}$ (**B,D,F**) mice. Each gene-deletion mouse was individually controlled by concurrent infection of wild type mice. Error bars represent SD of a representative experiment. Organ burden data were obtained from two independent experiments each containing at least three mice per genotype.

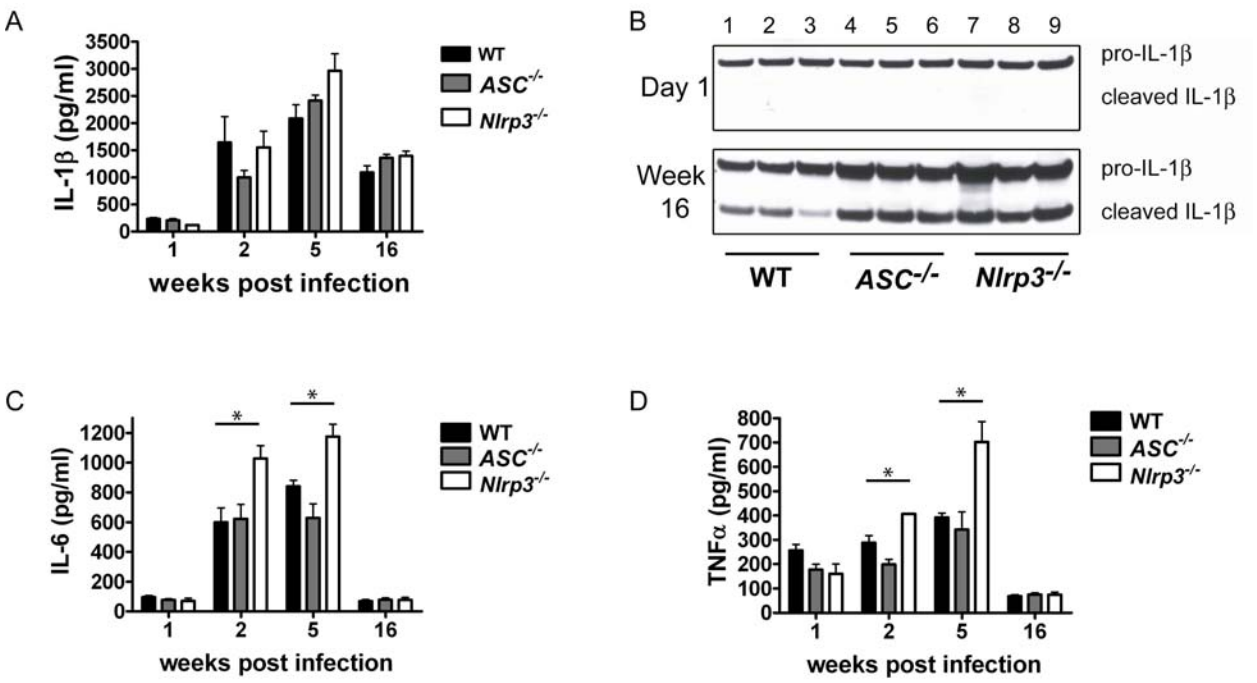

Figure 2.6. During *Mtb* infection *ASC*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice produce similar levels of mature IL-1β when compared to wild type animals. Lung homogenates were centrifuged and the extract collected to assess the levels of proinflammatory cytokines in the lung at each indicated time point. **A-B.** Cytokine measurements of IL-1β by ELISA (**A**) and western blot detection of pro- and cleaved IL-1β in lung homogenates one week (**B, top panel**) and 16 weeks (**B, bottom panel**) post aerosol infection. IL-1β levels in *ASC*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were higher than wild type animals. Each number represents a different mouse. **C-D.** Cytokine measurements of IL-6 (**C**) and TNFα (**D**) increased during acute infection, but IL-6 and TNFα returned to base line level by week 16. IL-6 and TNFα were modestly increased in *Nlrp3*$^{-/-}$ mice when compared to *ASC*$^{-/-}$ and WT controls. Cytokine measurements were taken from at least three mice per genotype in two independent experiments. *p <0.05

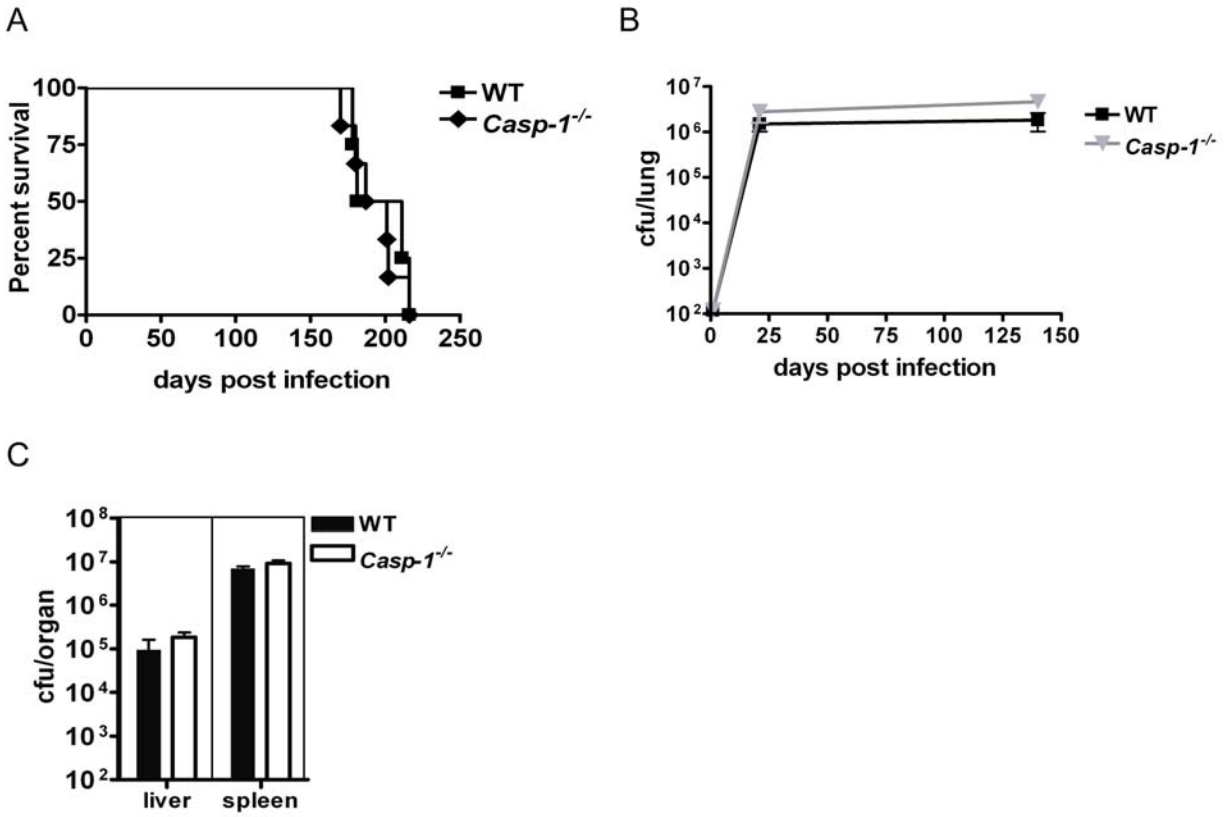

Figure 2.7. *Casp-1* is not protective during *Mtb* infection.

A. *Casp-1*$^{-/-}$ mice infected with *Mtb* did not have a difference in survival compared to wild type mice. **B-C.** Bacterial burden in the lungs, liver, and spleen was comparable between *Casp-1*$^{-/-}$ and wild type mice.

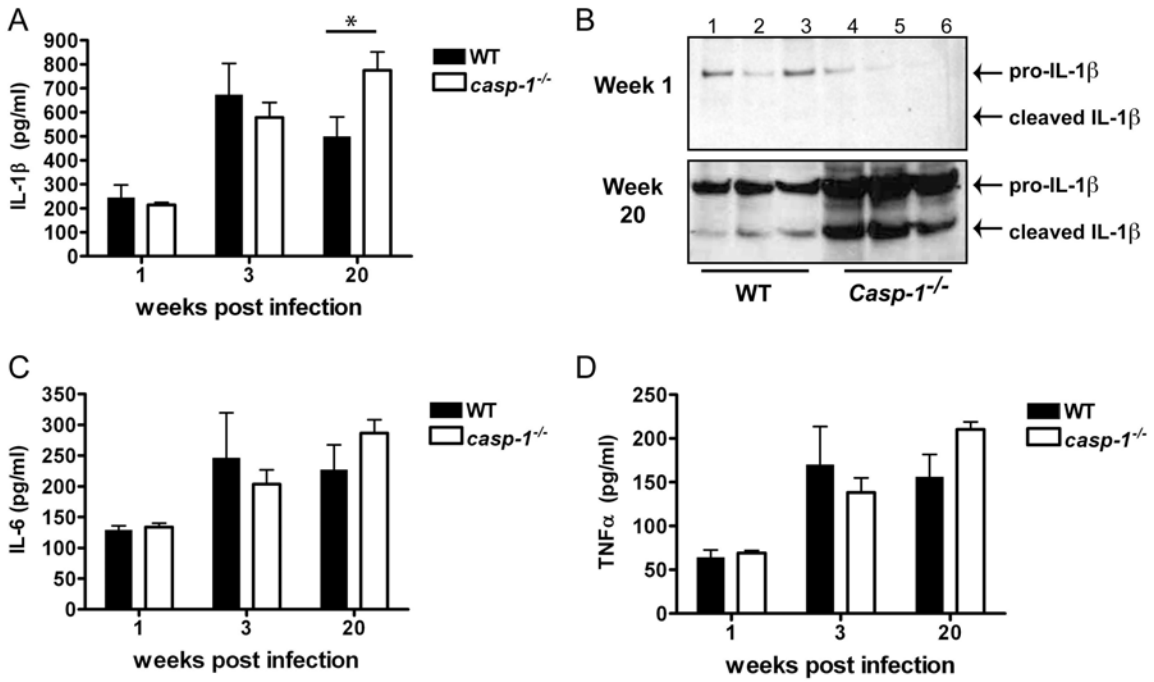

Figure 2.8. During *Mtb* infection *Casp-1*$^{-/-}$ mice produce similar levels of mature IL-1β when compared to wild type animals. A. *Casp-1*$^{-/-}$ lungs contained significantly more mature IL-1β than wild type lungs by ELISA (* $p<0.05$). **B.** Western blot detection of pro- and cleaved IL-1β in lung homogenates one week (**B, top panel**) and 20 weeks (**B, bottom panel**) post aerosol infection indicate that both pro and cleaved IL-1β are increased during chronic infection in both wild type and *Casp-1*$^{-/-}$ lungs. **C-D.** Cytokine measurements of IL-6 (**C**) and TNFα (**D**) increased during acute infection and plateaued during the chronic phase of *Mtb* infection. Cytokine measurements were taken from at least three mice per genotype in two independent experiments.

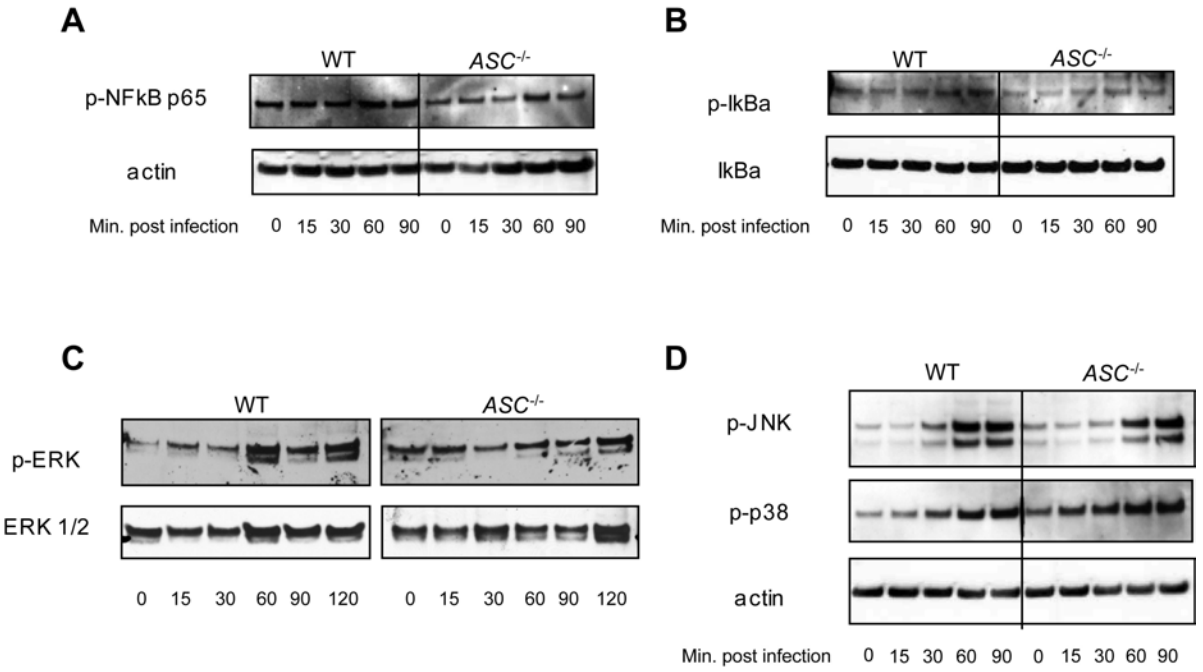

Figure 2.9. ASC does not affect MAP kinase or NFκB signaling pathways during *Mtb* infection. A-B. BMDM from *ASC*$^{-/-}$ mice showed a modest decrease in activation of the NFκB signaling pathway compared to wild type following *Mtb* infection measured by phosphorylation of IκBα (**A**) and NFκB p65 (**B**). **C-D.** Slight differences were also found in activation of MAPK proteins shown by phosphorylation of signaling proteins ERK (**C**), and JNK, but not p38 (**D**).

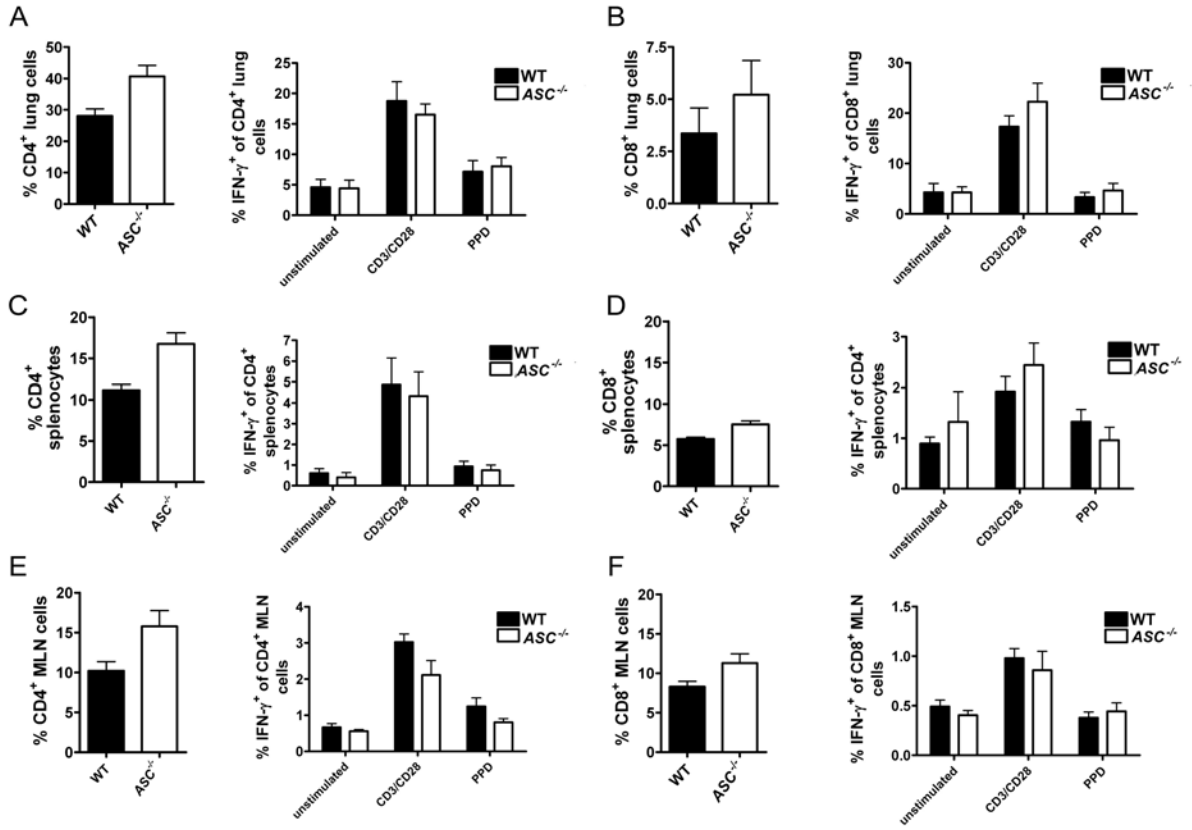

Figure 2.10. ASC$^{-/-}$ mice produce equal numbers of *Mtb*-antigen specific T-cells to wild-type mice during chronic *Mtb* infection. A-F. Lung cells (**A-B**), splencytes (**C-D**) and MLN cells (**E-F**) from the same mice that were left unstimulated or restimulated with CD3/CD28 or PPD for 5 hours showed no difference in CD4 and CD8 T-cell function, demonstrated by IFN-γ production.

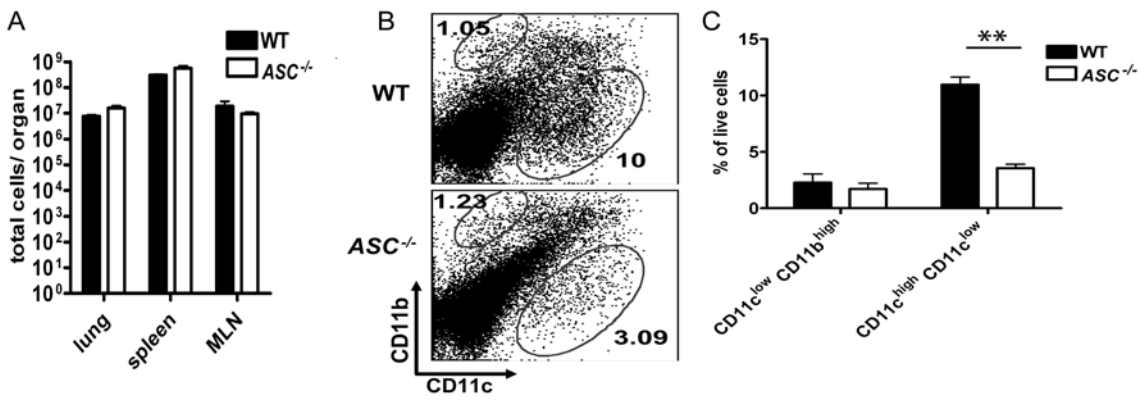

Figure 2.11. ASC$^{-/-}$ mice have fewer CD11c^{+} CD11b$^{mid/low}$ cells during chronic *Mtb* infection. A. Lungs from ASC$^{-/-}$ and wild type mice had similar levels of total cells 90 days post *Mtb* infection. **B-C.** Flow cytometry analysis showed that the lungs of ASC$^{-/-}$ mice have a reduced CD11c^{+} CD11b$^{mid/low}$ cell population compared to wild type (n=6 per genotype, **p=0.000015)

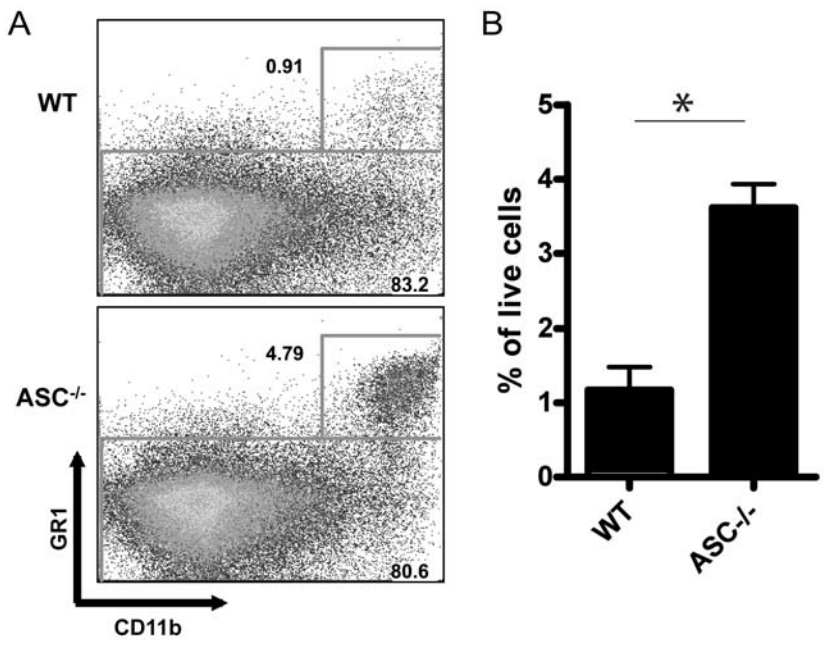

Figure 2.12. ASC$^{-/-}$ mice increased GR1^{+} lung cells during chronic *Mtb* infection. **A.** Lungs from ASC$^{-/-}$ and wild type mice were harvested 90 days after *Mtb* infection and immune cell populations were identified by flow cytometry. The lungs of ASC$^{-/-}$ mice have an increased GR1^{+} cell population compared to wild type (n=5 per genotype, ** p=0.000383)

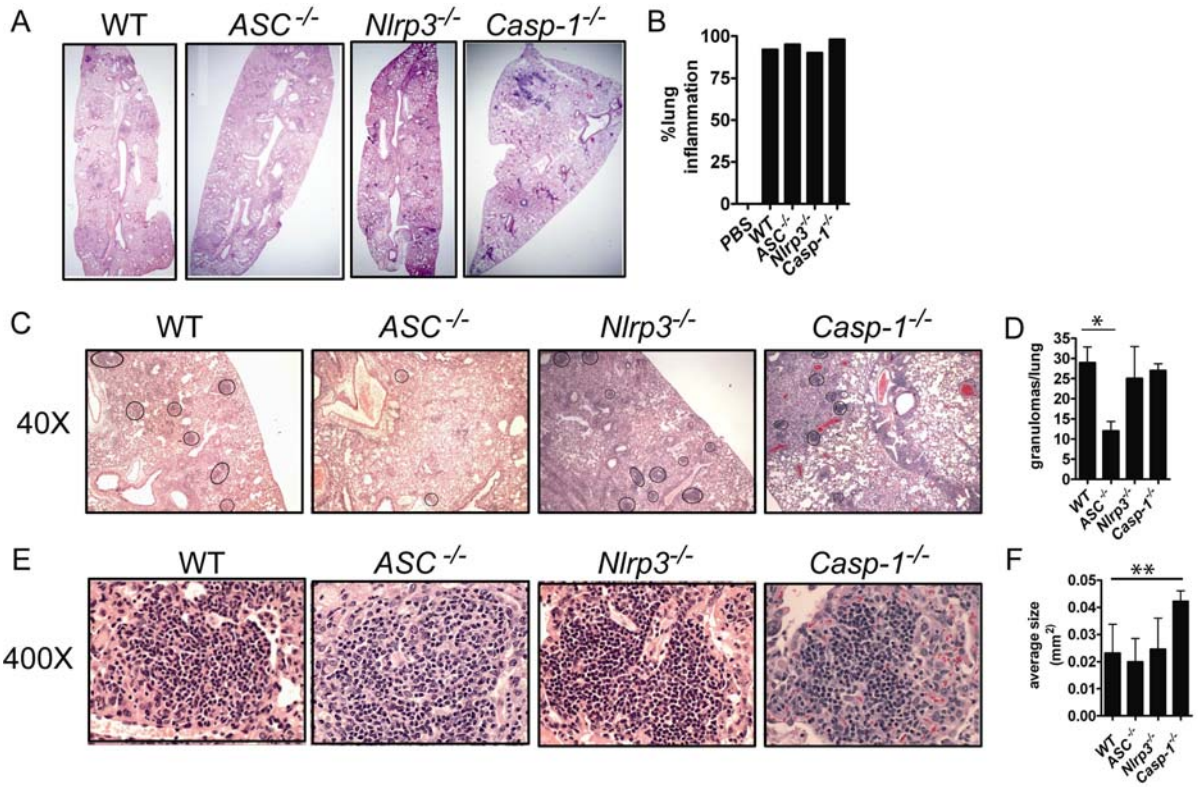

Figure 2.13. *ASC*$^{-/-}$ mice have fewer lung granulomas during chronic *Mtb* infection. Lungs were harvested 16 weeks postinfection, inflated, and fixed for histological sectioning. **A-B.** Whole lung images of *Mtb* infected lungs show similar percentages of inflamed lung tissue for all *Mtb* infected mice. **C-D.** 40X images demonstrate that *ASC*$^{-/-}$ mice form significantly fewer granulomas compared to wild type, *Nlrp3*$^{-/-}$, and *Casp-1*$^{-/-}$ mice (* $p<0.05$). **E-F.** 400X images demonstrate wild type, *ASC*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice form similarly sized granulomas while *Casp-1*$^{-/-}$ mice form significantly larger granulomas (** $p<0.001$). Lung histopathology was analyzed from at least three mice per genotype in two independent experiments. Quantification was performed on sections from 4-8 mouse lungs per genotype.

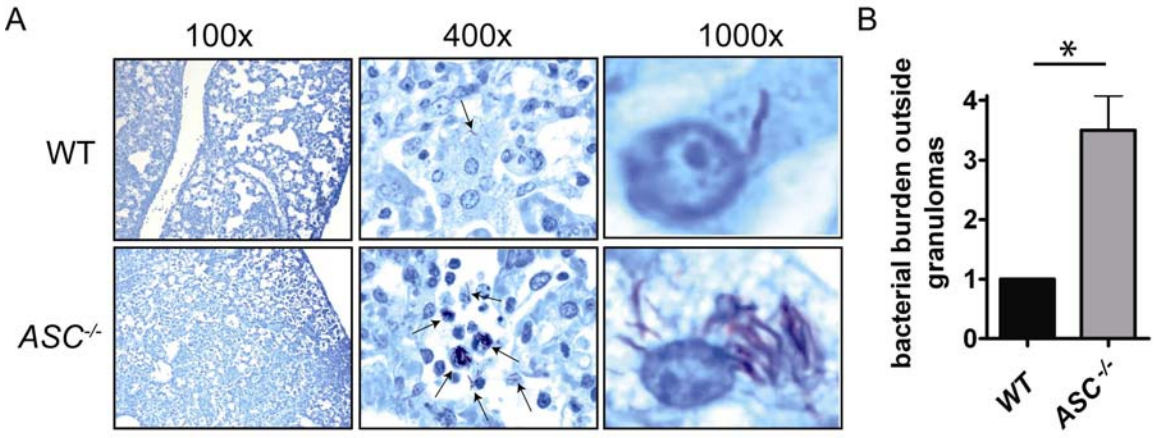

Figure 2.14. *ASC*$^{-/-}$ mice are unable to contain bacteria within granulomas. A-B. Acid-fast staining of *Mtb* infected lung sections illustrate dramatic differences in bacterial localization within *ASC*$^{-/-}$ lungs. Different images of non-granulomatous regions of *Mtb* infected lung tissue taken at 100x and 400x demonstrate that wild type lungs contain very little *Mtb* in non-granulomatous tissue in contrast with *ASC*$^{-/-}$ mice which contain great numbers of *Mtb* throughout the lung (*$p<0.01$). To view bacteria within a single macrophage 1000x images were selected, cropped, and enlarged. Lung histopathology was analyzed from at least three mice per genotype in two independent experiments. Quantification was performed on sections from 4-8 mouse lungs per genotype.

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CHAPTER 3

The role of NLRP12 in a mouse model of *Mycobacterium tuberculosis* infection

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ABSTRACT

The NLR gene family mediates host immunity to various acute pathogenic stimuli. Here we investigated the role of NLRP12 during *Mtb* infection. Previous data found that NLRP12 played an anti-inflammatory role during *Mtb* infection of a human monocytic cell line, occurring through inhibition of canonical and non-canonical NFκB signaling. We hypothesized that NLRP12 would be protective against *Mtb* infection, resulting in increased lung cytokines following *Mtb* infection. In contrast to our hypothesis, *Nlrp12* deficient mice did not have increased lung cytokines in response to *Mtb* infection. We found no difference in survival of *Nlrp12*$^{-/-}$ mice compared to wild-type controls. Bacterial burden of the lungs as well as *Mtb* dissemination to the liver and spleen was also similar between *Nlrp12*$^{-/-}$ and wild-type mice. These data indicate that Nlrp12 does not play a prominent role in the host response to *Mtb* infection *in vivo*.

INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis, a disease affecting one-third of the world's population and resulting in 1.7 million deaths per year (2007). *Mtb* is transmitted via aerosol from person to person. Upon inhalation, *Mtb* travels to the alveolar spaces of the lung where it infects its primary targeted cell type, the alveolar macrophage (Leemans et al., 2001). The innate immune response to *Mtb* occurs through stimulation of TLRs that recognize pathogens and are located on the plasma membrane and within endosomes of host cells. *Mtb* is specifically recognized by TLRs 2, 4, and 9

(Quesniaux et al., 2004). TLR activation upregulates transcription of proinflammatory cytokines IL-1β, TNFα, and IL-6, which are essential for the recruitment of immune cells to the site of infection and controlling *Mtb* infection (Bean et al., 1999; Ladel et al., 1997; Yamada et al., 2000). Pro-inflammatory cytokine regulation is critical to long-term survival during *Mtb* infection. Mice deficient in pro-inflammatory cytokines TNFα and IL-6 have increased mortality during *Mtb* infection (Bean et al., 1999; Ladel et al., 1997). TNFα is important for granuloma formation and maintenance (Flynn et al., 1995). Therefore, these cytokines are not only important in the innate immune response to *Mtb*, but also in host defense during chronic *Mtb* infection.

NLRP12 (also Monarch-1) is a member of the nucleotide binding domain, leucine rich repeats-containing family proteins (NLR) which sense pathogens and pathogen products in the cell cytoplasm (Ting et al., 2008). The NLR family proteins are increasingly associated with immune regulation, inflammation, and autoimmunity. Mutations in *NLRP12* have been linked to Familial Mediterranean Fever and recently a polymorphism in NLRP12 has been loosely associated with atopic dermatitis (Jeru et al., 2008; Macaluso et al., 2007).

Nlrp12 is highly expressed in human myeloid cells including monocytes, granulocytes, and eosinophils (Williams et al., 2003). NLRP12 is able to bind ASC in an artificial overexpression system (McConnell and Vertino, 2004). Studies in the human monocytic cell line THP-1 identified NLRP12 is a negative regulator of TLR and TNF receptor signaling (Williams et al., 2005). In addition, *Mtb* infection of THP-1 cells with reduced NLRP12 expression via shRNA

resulted in increased levels of pro-inflammatory cytokines. This demonstrates that NLRP12 is an anti-inflammatory protein, downregulating cytokines through suppression of NFκB signaling (Lich et al., 2007; Williams et al., 2005). In addition to NLRP12, NLRX1 and NLRC5 have recently been identified as anti-inflammatory NLRs through inhibition of type I IFN and NFκB signaling pathways (Cui et al., 2010; Moore et al., 2008). Consistent with the anti-inflammatory role of NLRP12 in cultured macrophages, we hypothesized that mice lacking *Nlrp12* will have increased lung cytokines after aerosol *Mtb* infection. We also hypothesize that survival of *Nlrp12* deficient mice may be reduced following *Mtb* infection, similar to the phenotype we observed in $ASC^{-/-}$ mice.

Tight regulation of Nlrp12 is important for initiation and termination of the host inflammatory response. NLRP12 is highly expressed in human monocytes and macrophages, the primary target of *Mtb*. In response to stimulation with TNF-α, IFN-γ, or *Mtb* *NLRP12* expression is downregulated to allow the host to mount an inflammatory response (Williams et al., 2005). B lymphocyte−induced maturation protein-1 (Blimp-1), a protein that regulates terminal differentiation of B cells and cells of the myeloid lineage, is partially responsible for the reduced expression of *NLRP12* under inflammatory conditions (Chang et al., 2000; Lord et al., 2009). This suggests a model in which during *Mtb* infection Blimp-1 inhibits NLRP12 expression. In the absence of NLRP12 the host is able to mount an immune response through increased NFκB signaling, resulting in an increase in pro-inflammatory cytokines. To prevent an overzealous immune response, NLRP12 expression increases and inhibits NFκB signaling to

downregulate the host immune system. This allows the host return to equilibrium following initiation of inflammatory signaling.

In this work, we examined the role of Nlrp12 in Mtb infection. We find that Nlrp12 deficient mice have similar levels of pro-inflammatory cytokines during early and chronic *Mtb* infection. *Nlrp12* does not play a prominent role in host protection against *Mtb*, shown by survival and bacterial organ burden similar to wild-type mice.

MATERIALS AND METHODS

Bacterial strains. *Mycobacterium tuberculosis* H37Rv and H37Ra were obtained from the American Type Culture Collection (ATCC). Bacteria were grown to log phase in Middlebrook 7H9 broth (Difco) with 0.2% glycerol, 1× albumin dextrose saline, and 0.05% Tween 80. Inoculum was assessed by plating infection media on Middlebrook 7H10 agar plates supplemented with glycerol and ADS as described above. Colony forming units (cfu) were counted after 21 days of incubation.

Mice. *Nlrp12*$^{-/-}$ mice kindly supplied by Millenium Inc. Mice were obtained backcrossed for 5 generations and were further backcrossed by the Ting Lab onto the C57BL/6 (Jackson Lab) background until reaching 9 generations. All studies were conducted in accordance with the National Institutes of Heath Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and

Use Committee (IACUC) guidelines of the University of North Carolina at Chapel Hill.

***Mtb* H37Rv aerosol infection of mice.** Female *Nlrp12*$^{-/-}$ mice and age matched C57BL/6 female controls were infected via aerosol as previously described (Kurtz et al., 2006). Mice received 250-350 colony forming units per lung, determined by sacrificing a subset of mice 1d post infection as described by others (Kurtz et al., 2006). Bacterial organ burden was quantified by plating serial dilutions of lung, liver, and spleen homogenates on 7H10 plates containing cycloheximide (1µg/ml) and carbenicillin (50µg/ml) to minimize contamination. All animal infections and organ harvests were carried out under BSL3 conditions.

Cytokine determination. To measure cytokines *in vivo* we assayed lung homogenate extracts by centrifuging homogenized lung tissue to create a tissue free supernatant. Cytokines were measured by BD OptEIA Mouse IL-1β, TNFα, and IL-6 ELISA Sets (BD Biosciences)

Immunoblotting. Amounts of pro- and cleaved IL-1β in lung homogenate extracts were determined by Western blot. Immunoblots were probed with goat anti-mouse IL-1β primary antibody (R&D Biosystems).

Statistics. Data are presented as the means +/- standard deviation (SD) unless otherwise noted. Analysis Of Variance (ANOVA) followed by Tukey-Kramer HSD

for multiple comparisons was performed on complex data sets. Statistical significance for single data points was assessed by the Student's two-tailed t-test. Survival curves were generated utilizing the product limit method of Kaplan and Meier and comparisons were made using the log rank test. In all cases, a p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

NLRP12 is known to be an anti-inflammatory NLR during *Mtb* infection cultured macrophages (Williams et al., 2005). We envisioned three models in which Nlrp12 could play a role during *Mtb* infection *in vivo*. In the first model, we hypothesized that the absence of *Nlrp12* would cause an overzealous immune response leading to the destruction of lung tissue and increased inflammation. In this environment aberrant cytokine secretion may cause disregulation of immune cell migration into and within the lung, preventing *Nlrp12*$^{-/-}$ mice from forming proper granulomas, and subsequently containing *Mtb* within granulomas. This would result in increased bacterial organ burden and reduced survival of *Nlrp12*$^{-/-}$ mice compared to wild-type mice following *Mtb* infection. In a second possible model, Nlrp12 interacts with ASC and together these proteins protect the host during *Mtb* infection. In this scenario *Nlrp12*$^{-/-}$ mice would have the same phenotype that was observed in *ASC*$^{-/-}$ mice including reduced survival and fewer granulomas during chronic infection. In contrast to these models, a third model was envisioned in which lack of *Nlrp12* leads to increased cytokines. Instead of causing destruction of host tissue, increased cytokines increased bacterial killing

and resulted in limited bacterial growth and reduced bacterial spread. In this scenario we predict that *Nlrp12*$^{-/-}$ mice would have increased survival, living longer than their wild-type controls.

Nlrp12$^{-/-}$ and wild-type mice have similar pro-inflammatory lung cytokines following *Mtb* infection.

To test these possible models, *Nlrp12*$^{-/-}$ and wild-type mice were infected with *Mtb* via aerosol. Time points were taken at 1, 14, 26, and 177 days postinfection to evaluate bacterial burden and cytokines during both acute and chronic *Mtb* infection. Pro-inflammatory lung cytokines IL-1β, TNFα, and IL-6 were measured from cleared lung homogenates of *Nlrp12*$^{-/-}$ and wild-type mice. Surprisingly, proinflammatory cytokines were not increased during *Mtb* infection (**Figure 3.1 A-C**). The amount of lung IL-1β increased in *Nlrp12*$^{-/-}$ mice as *Mtb* infection progressed. TNFα and IL-6 levels were comparable between the lungs of *Nlrp12*$^{-/-}$ mice compared to wild-type controls at one day and postinfection as well as during chronic infection. At day 14, surprisingly, we found that *Nlrp12*$^{-/-}$ lungs had reduced TNFα and IL-6 compared to wild-type that neared significance ($p=0.056$ and $p=0.06$, respectively). This suggests in contrast to the anti-inflammatory role NLRP12 played in human monocytes, in an *in vivo* model of *Mtb* infection Nlrp12 plays an inflammatory role. Similar to our data from *Nlrp3* and *Casp-1* deficient mice, infection of macrophages in culture was not predictive of the role of *Nlrp12* in a mouse model of *Mtb* infection.

Nlrp12 does not protect the host during *Mtb* infection *in vivo*

Nlrp12$^{-/-}$ mice and wild-type control mice were infected with *Mtb* via aerosol infection. Mouse survival was monitored postinfection and *Nlrp12*$^{-/-}$ mice were found to live as long as wild-type mice, but not longer (**Figure 4.2 A**). This result indicates that Nlrp12 does not play a prominent role in host protection during *Mtb* infection.

Bacterial lung burden and bacterial dissemination are unaffected by *Nlrp12*

To determine if Nlrp12 has an effect on bacterial growth during *in vivo* infection we measured bacterial burden within the lungs at various time points post *Mtb* infection. Lungs of *Nlrp12*$^{-/-}$ and wild type mice were harvested from mice during acute and chronic time points following *Mtb* infection. In contrast to both proposed models, we saw no difference in the amount of *Mtb* within the lungs of *Nlrp12*$^{-/-}$ or wild type mice at any time point postinfection (**Figure 4.2 B**). In combination with data indicating that the amount of pro-inflammatory cytokines in the lung were unchanged in the absence of *Nlrp12*, we conclude that *Nlrp12* does not play a significant role in attenuating *Mtb* during infection, nor does it create an environment where *Mtb* can more easily replicate and spread.

We also measured the ability of bacteria to disseminate to secondary sites of infection within mice. Bacterial dissemination to the liver and spleen occurs by 14 days postinfection. There were no differences between the bacterial burden of the liver and spleen when we compared Nlrp12$^{-/-}$ and wild type animals

(**Figure 3.2 C-D**). These results indicate that Nlrp12 does not play a role in *Mtb* dissemination or growth at these secondary sites of infection.

Previous results indicated that ASC played a role in host protection during *Mtb* infection independent of the inflammasome. NLRP12 is a non-inflammasome forming NLR that suppresses cytokine production through inhibition of NFκB signaling (Williams et al., 2005). Two additional NLRs, NLRC5 and NLRX1, are anti-inflammatory in response to viral pathogens resulting in suppression of NFκB and type I IFN signaling. NLRP12 is able to bind ASC in an artificial overexpression system (McConnell and Vertino, 2004). We hypothesized that NLRP12 may play a role in host protection through association with ASC. In addition we proposed two additional scenarios in which Nlrp12 could play a role following *Mtb* infection *in vivo*. Nlrp12 did not affect lung cytokine production, mouse survival, or bacterial burden in an *in vivo* model of *Mtb* infection. This result demonstrates the importance of validating findings from cell culture in mouse models. We also did not find a role for NLRP12 during infection with other bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* (unpublished observation). However, in inflammatory models such as colitis, NLRP12 is found to attenuate inflammation in the gut (*Allen IC et al.,* in preparation). Furthermore, *Nlrp12*$^{-/-}$ macrophages also show a reduction in non-canonical NF-kB activation (Lich et al., 2007). Thus NLRP12 does appear to attenuate inflammation, but does not appear to play a detectable role during *Mtb* infection.

FIGURES

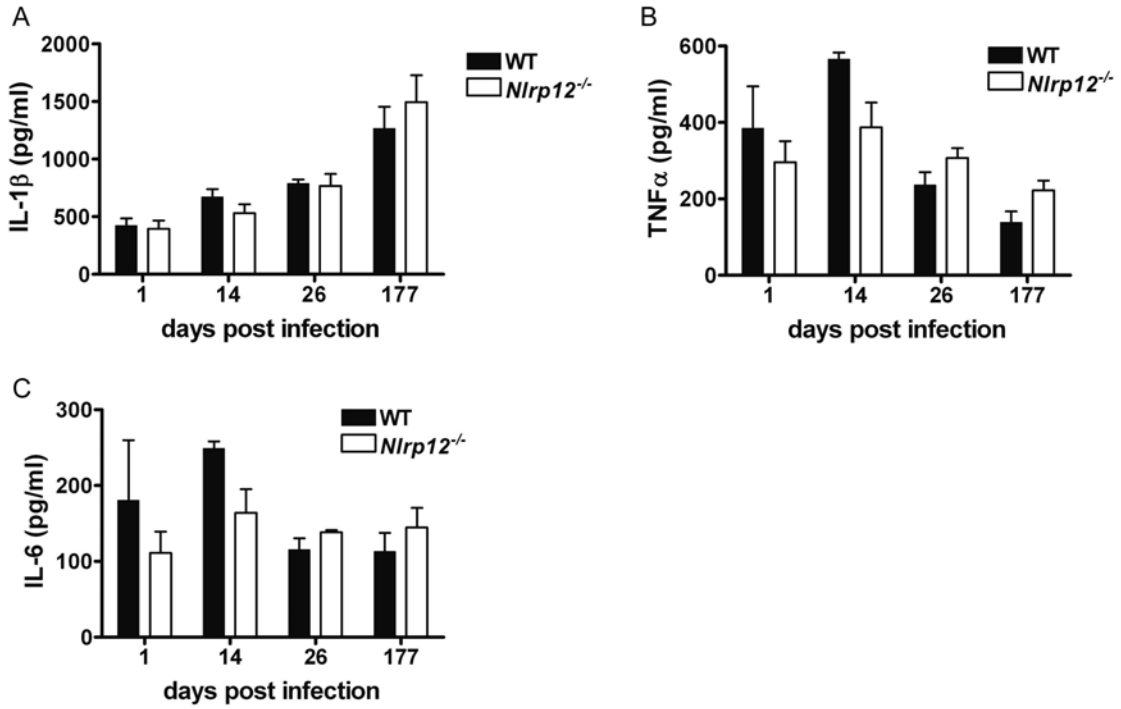

Figure 3.1. During *Mtb* infection *Nlrp12*$^{-/-}$ mice produce similar levels cytokines compared to wild type animals. A. *Nlrp12*$^{-/-}$ lungs similar amounts of IL-1β compared to wild-type lungs at both early and chronic phases of *Mtb* infection. **B-C.** Cytokine measurements of TNFα (**B**) and IL-6 (**C**) showed that *Nlrp12*$^{-/-}$ and wild-type lungs had similar amounts of cytokines at one day postinfection and during chronic *Mtb* infection. At 14 days postinfection, *Nlrp12*$^{-/-}$ lungs had reduced cytokines compared to wild-type lungsthat neared significance (p= 0.056 and p=0.06, respectively). Cytokine measurements were taken from at least three mice per genotype.

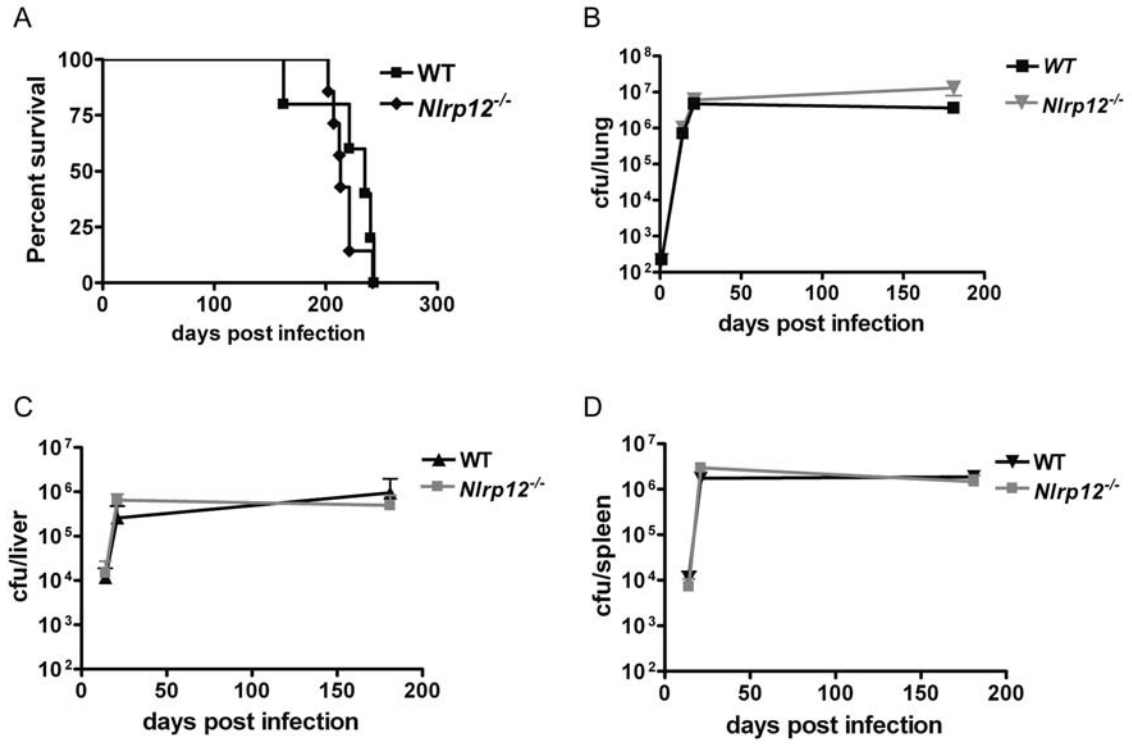

Figure 3.2. *Nlrp12* is not protective during *Mtb* infection

A. *Nlrp12*$^{-/-}$ mice infected with *Mtb* did not have a difference in survival compared to wild type mice. **B-D.** Bacterial burden in the lungs, liver, and spleen was comparable between *Nlrp12*$^{-/-}$ and wild type mice.

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CHAPTER 4

DISCUSSION

Mtb infects one-third of the world's population resulting in 9 million newly infected people and 2 million deaths per year (CDC, 2009). The current BCG vaccine is not effective against adult pulmonary TB. Resistant strains of *Mtb* are on the rise, and no new antibiotics have been developed since the 1960s. A greater understanding of the interaction between *Mtb and* the host may lead to new and more effective vaccines and therapies to treat *Mtb* infection.

The overall goal of this thesis was to identify host proteins important for protection during *Mtb* infection. We chose to study the NLR family, a group of proteins that function in immune regulation, inflammation, and autoimmunity (Ting and Davis, 2005). We specifically chose to study the protein NLRP3, which detects a broad range of pathogens and pathogen products (**Table 1.2**). Upon stimulation, NLRP3 forms an inflammasome with the adaptor protein ASC and pro-caspase-1 which results in caspase-1 processing and recruitment and cleavage of cytokines pro-IL-1β and pro-IL-18 to their active forms. In chapter 2, we discovered that in cultured macrophages *Mtb* stimulates the NLRP3 inflammasome in an ASC and caspase-1-dependent manner. To determine if the NLRP3 inflammasome was important for host protection *in vivo*, we infected

mice lacking individual NLRP3 inflammasome genes *Nlrp3, ASC,* and *Casp-1* with *Mtb* via aerosol. Mice lacking IL-1α/β, IL-1R, and IL-18 play a role in limiting bacterial lung burden, regulating other cytokines, nitric oxide production, and forming organized granulomas (Juffermans et al., 2000; Sugawara et al., 2001; Sugawara et al., 1999). We hypothesized that mice lacking any of the Nlrp3 inflammasome components would have reduced survival following an *in vivo Mtb* infection, primarily due to lack of cleaved IL-1β in these mice. In contrast to our hypothesis, we found that only ASC played a role in host protection. More specifically, we discovered ASC to be important during the chronic phase of *Mtb* infection. The absence of either *Nlrp3* or *Casp-1* had no effect on mouse survival. Another surprise in these studies was that mice lacking the Nlrp3 inflammasome genes did not have reduced cleaved IL-1β in their lungs. These data indicate that ASC protects the host in an inflammasome-independent manner during *Mtb* infection. To further understand the host response to *Mtb* we investigated the role of ASC during chronic infection. *ASC*$^{-/-}$ mice have a significantly reduced CD11c^{+} CD11b$^{mid/low}$ cell population that is present in the lungs of wild-type mice. In addition, *ASC*$^{-/-}$ mice have fewer lung granulomas during the chronic phase of infection. In *ASC*$^{-/-}$ mice, *Mtb* is spread throughout the lung instead of being contained within granulomas as is the case in wild-type mice. The inability of *ASC*$^{-/-}$ mice to contain bacteria within granulomas constitutes a breakdown in host defense in response to *Mtb* infection. Chapter 3 focuses on the role of another NLR, Nlrp12, during an *in vivo* model of *Mtb* infection. NLRP12 is an anti-inflammatory and non-inflammasome forming NLR

that is shown to bind ASC *in vitro* (Lich et al., 2007; Williams et al., 2005). We hypothesized that Nlrp12$^{-/-}$ mice may have reduced survival similar to ASC$^{-/-}$ if Nlrp12 and ASC are interacting during *Mtb* infection. Following *Mtb* infection Nlrp12$^{-/-}$ mice did not have reduced survival compared to wild-type mice. This indicates that Nlrp12 does not play a prominent role in the host response against *Mtb* in an *in vivo* mouse model of disease. Taken together, our data supports a novel, inflammasome-independent role for ASC in host protection against *Mtb* infection since neither *Nlrp3* nor *Casp-1* had reduced survival or defective granuloma formation after *Mtb* infection. In addition, cleaved IL-1β was present in the lungs of all mice lacking Nlrp3 inflammasome components, including *ASC*$^{-/-}$ mice. In this discussion, I will be addressing the future direction of the project and speculation as to the role of ASC in host protection following *Mtb* infection.

Mtb stimulates the NLRP3 inflammasome in cultured macrophages

While our research was underway, three papers were published that report similar findings of Nlrp3 inflammasome activation occurring during *Mtb* infection *in vitro.* The authors of the first study found that BMDM infection with *M. marinum*, a mycobacterium species that naturally infects fish and amphibians, resulted in IL-1β and IL-18 secretion (Koo et al., 2008). Secretion of IL-1β and IL-18 was dependent on the NLRP3-inflammasome components Nlrp3 and ASC*,* but independent of the closely related inflammasome-forming NLR Nlrc4. In the second report, the authors infected a murine macrophage cell line with *M. bovis* BCG, the attenuated vaccine strain of *Mtb* (Master et al., 2008). Infection

resulted in Caspase-1 and IL-1β cleavage which is indicative of inflammasome activation. However, in this paper the authors only measured caspase-1 cleavage and did not investigate the role of any inflammasome components directly (Master et al., 2008). The authors further reported that a mutation in a putative protease, Zmp-1, resulted in increased Caspase-1 activation compared to the wild-type strain of *M. bovis* BCG. Master *et al*. (2008) conclude that mycobacteria contain proteins that modulate host defense mechanisms including some that limit caspase-1 activation. Finally, a paper from Mishra *et al.* (2010) showed that *Mtb*-infected THP-1 cells secrete IL-1β in an Nlrp3 and ASC-dependent manner. The conclusions of all three of these studies correlate with our findings that *Mtb* activates the NLRP3 inflammasome in cultured macrophages and monocytes. In agreement with our hypothesis, IL-1β secretion from these cells following *Mtb* infection is dependent on NLRP3, ASC, Caspase-1, but independent of NLRC4.

The role of the NLRP3 inflammasome in an *in vivo* mouse model of *Mtb* infection

Many pathogens stimulate the NLRP3 inflammasome in cell culture, but very few studies have investigated the role of NLR proteins *in vivo.* To determine the role of the NLRP3 inflammasome during *in vivo* infection, we exposed mice lacking Nlrp3, ASC, and Casp-1 along with wild-type controls to *Mtb* via an aerosol route of infection. During our cultured cell experiments, inflammasome activation was measured by secretion of IL-1β into the culture medium by ELISA

assay. *In vivo,* cytokines were measured by ELISA assay from tissue-free lung homogenates. The homogenization process causes loss of cellular integrity; therefore, we additionally measured IL-1β by western blot to distinguish between inactive pro-IL-1β and cleaved IL-1β within the mouse lung.

Data from cultured macrophages demonstrated that *Mtb* stimulates the NLRP3 inflammasome and cleavage of IL-1β was dependent on inflammasome proteins NLRP3, ASC, and caspase-1. We hypothesized that IL-1β would not be cleaved in mice lacking Nlrp3 inflammasome components during an *in vivo* mouse model of *Mtb* infection. Contrary to our hypothesis, we found that cleaved IL-1β was present in the lungs of both Nlrp3- and ASC-deficient mice after *Mtb* infection. Initially after this surprising result, we theorized that other NLRs were being activated by *Mtb in vivo* and were forming an inflammasome with Caspase-1 resulting the observed in IL-1β cleavage. To investigate the source of cleaved IL-1β in the lungs of *Nlrp3* and *ASC* mice we investigated the role of Caspase-1 during *in vivo Mtb* infection.

The initial characterization of *Casp-1*$^{-/-}$ mice found that they were resistant to endotoxic shock and were unable to process pro-IL-1β and pro-IL-18 to their active forms after *in vivo* LPS exposure (Li et al., 1995). Our *in vitro* studies determined that *Casp-1* was essential for IL-1β cleavage in BMDM infected with *Mtb.* Surprisingly, since *Casp-1* is directly responsible for cleaving pro-IL-1β, we did not observe the same result in *Mtb* infected *Casp-1*$^{-/-}$ mice. We detected wild-type levels of cleaved IL-1β in the lungs of *Casp-1*$^{-/-}$ mice following *Mtb* infection. This unexpected result may be explained by additional lung proteases

that are able to cleave pro-IL-1β in the absence of Caspase-1. The most thoroughly studied of these proteases are chymases which are released from mast cells upon degranulation (Mizutani et al., 1991). Another protease that may play a role in cleaving pro-IL-1β include granzyme A, which is a serine protease produced by $CD8^+$ T cells and NK cells that is released by cytoplasmic granules (Irmler et al., 1995). Additionally, the matrix metalloproteinases (MMPs) are a family of membrane-associated and secreted endopeptidases, and MMP-2, -3, and -9 have been shown to process pro-IL-1β to its active form with varying kinetic efficiencies (Schonbeck et al., 1998). Finally, the proteolytic enzyme chymotrypsin also processes pro-IL-1β into its active form (Black et al., 1988).

To explain why *Casp-1*$^{-/-}$ mice have cleaved IL-1β in their lungs during chronic *Mtb* infection, we propose a model in which dying cells release pro-IL-1β protein into the extracellular environment where the protein is cleaved to its active form by extracellular proteases in the absence of Caspase-1. We speculate that caspase-1 is a more efficient protease than other extracellular proteases and is therefore preferred by the host for cleavage of pro-IL-1β. In the absence of Caspase-1, the activity of other extracellular proteases may provide a compensatory mechanism for pro-IL-1β cleavage, resulting in cleaved IL-1β in the lungs of *Casp-1*$^{-/-}$ mice. It is possible that this compensation is inefficient and requires additional time to obtain sufficient levels of cleaved IL-1β. The duration of chronic *Mtb* infection may allow sufficient time for compensatory mechanisms of pro-IL-1β cleavage to be activated in the absence of Caspase-1. Our results show that caspase-1-independent mechanisms of IL-1β cleavage are sufficient to

produce levels of IL-1β in mice lacking caspase-1 similar to that found in wild-type during *in vivo Mtb* infection unlike in a cell autonomous culture system.

Nlrp3-, *ASC*-, and *Casp-1*-deficient BMDM did not secrete IL-1β during *Mtb* infection, but during *in vivo Mtb* infection, these mice produced as much cleaved IL-1β in their lungs as wild-type mice. However, it is important to note that these *in vivo* results do not rule out the possibility that cleaved IL-1β is specifically reduced within the granulomas of the *Nlrp3*, *ASC*, and *Casp-1* deficient mice. Differences in IL-1β within granulomas may be masked in the context of a whole lung homogenate. In the future, immunohistochemistry could be used to identify the location of cleaved IL-1β in paraffin-embedded lung tissue of *Nlrp3*$^{-/-}$, *ASC*$^{-/-}$, and *Casp-1*$^{-/-}$ mice during chronic *Mtb* infection. We hypothesize that the amount of cleaved IL-1β may be at least partially reduced within the granulomas of *Nlrp3*$^{-/-}$, *ASC*$^{-/-}$, and *Casp-1*$^{-/-}$ mice compared to wild-type mice. This is due to the fact that granulomas contain many macrophages which we have shown in cell culture models do not secrete IL-1β in the absence of Nlrp3 inflammasome components following *Mtb* infection. Despite possible differences in the amount of IL-1β secreted within granulomas, these differences are unlikely to have a dramatic effect during *Mtb* pathogenesis. This is indicated by the fact that mice lacking *Nlrp3* and *Casp-1* form the same number of granulomas as wild-type mice, suggesting that a reduction in IL-1β levels within granulomas was not responsible for the formation of fewer granulomas in the lungs of *ASC*$^{-/-}$ mice. Reduced quantities of IL-1β in granulomas may lead to

differences in the lung immune cell composition of *Nlrp3*$^{-/-}$ and *Casp-1*$^{-/-}$ mice compared to wild-type mice, which we did not measure.

While our research was underway, an independent study was published and it confirmed our results that *ASC*$^{-/-}$ mice have reduced survival following *Mtb* infection (Mayer-Barber et al., 2010). While we concluded that Caspase-1 did not play a role in host protection during *Mtb* infection, in the study of Mayer-Barber *et al.* (2010), they report that *Casp-1*$^{-/-}$ mice have reduced survival compared to wild-type controls. The difference results with *Casp-1*$^{-/-}$ mice may be explained by the housing conditions of *Casp-1*$^{-/-}$ mice following *Mtb* infection. We infected *Casp-1*$^{-/-}$ mice with *Mtb* in two independent experiments. During the first experiment, we obtained results similar to Mayer-Barber *et al.* (2010), in which *Casp-1*$^{-/-}$ mice had reduced survival compared to wild-type mice. The survival curve was biphasic with half the *Casp-1*$^{-/-}$ mice succumbing to infection approximately 40 days postinfection and the remaining mice persisting until 130 days postinfection. For comparison, wild-type mice in this experiment survived for approximately 200 days postinfection. After completion of this experiment, we discovered that the *Casp-1*$^{-/-}$ mice were being housed in non-specific pathogen free (SPF) conditions. The infection was repeated with *Casp-1*$^{-/-}$ mice housed under SPF conditions. We found that the *Casp-1*$^{-/-}$ mice lived as long as wild-type mice under SPF conditions, indicating that Casp-1 is not required for host defense during *Mtb* infection. Our data suggests that the reduced survival of *Casp-1*$^{-/-}$ mice compared to wild-type mice seen in Mayer-Baber *et al.* (2010) and our first experiment may have been due to *Mtb* infection in combination with

other pathogens. Caspase-1 has over 70 substrates, and it is possible that one or more could be playing a role in host resistance to pathogens making *Casp-1*$^{-/-}$ mice more susceptible to a range of animal pathogens (Keller et al., 2008; Shao et al., 2007). Due to concern over housing conditions, we repeated the *Mtb* aerosol infection of *ASC*$^{-/-}$ mice and housed them under SPF conditions. Importantly, we observed the same survival phenotype of the ASC$^{-/-}$ mice under SPF and non-SPF conditions, allowing us to be confident that reduced survival of the ASC$^{-/-}$ mice was due solely to *Mtb* infection.

Bacterial localization in the lungs of *ASC*$^{-/-}$ mice

As determined by plating for viable bacteria, our initial studies showed no difference in the bacterial burden of the lungs, liver, or spleen of ASC$^{-/-}$ mice compared to wild-type mice. This result was somewhat unexpected as it is common for reduced survival to correlate with increased *Mtb* replication. Histopathological analysis of lung sections showed that ASC$^{-/-}$ mice contain fewer lung granulomas during chronic *Mtb* infection than to wild-type mice. Through acid-fast staining, we observed that *Mtb* was spread throughout the lungs of ASC$^{-/-}$ mice. This contrasts with wild-type mice in which *Mtb* is almost entirely contained within granulomas. These data demonstrate that although the total bacterial burden is similar in the lungs of ASC$^{-/-}$ and wild-type mice, ASC$^{-/-}$ mice have a difference in bacterial localization within the lung.

Granuloma formation vs. granuloma maintenance

ASC$^{-/-}$ mice also have fewer granulomas than wild-type mice during chronic *Mtb* infection. It is unknown if the reduced number of granulomas is due to a defect in granuloma formation or granuloma maintenance. To address this question, we obtained lung tissue from ASC$^{-/-}$ and wild-type mice infected with *Mtb* for 21 days and analyzed it for signs of granuloma formation. Perivascular accumulation of immune cells, a precursor to granuloma formation, was present 21 days postinfection, but granulomas had not yet formed. Histopathology of lungs 50 days after *Mtb* infection showed that ASC$^{-/-}$ and wild-type lungs had a similar number of granulomas. However, these data cannot distinguish between the possibility of a defect in granuloma formation or maintenance. If ASC$^{-/-}$ mice have stopped forming granulomas at 50 days postinfection while wild-type mice continue to form additional granulomas, we would conclude that ASC$^{-/-}$ mice have a defect in formation. Alternatively, wild-type mice could have formed all their granulomas by 50 days postinfection and from this point on the number of granulomas in the ASC$^{-/-}$ mice will decline, indicating a defect in granuloma maintenance. To further investigate this question, we would need to perform a more thorough study of the kinetics mouse granuloma formation. This information may lead to an increased understanding of the role of ASC during granuloma formation.

Differences in the lungs of ASC$^{-/-}$ and wild-type mice during chronic *Mtb* infection

ASC$^{-/-}$ mice have reduced survival following *Mtb* infection compared to wild-type mice. Although the mechanism by which ASC protects the host is not yet clear, we amassed several clues during the course of our research. Using whole lung digests, we identified immune cell populations within the lung during chronic *Mtb* infection. We found that *ASC*$^{-/-}$ lungs have a greatly reduced CD11c^{+}, CD11b$^{mid/low}$ cell population compared to wild-type mice. Different papers report that CD11c^{+}, CD11b$^{mid/low}$ cells are DCs, alveolar macrophages, or a hybrid of the two cell types (Gonzalez-Juarrero et al., 2003; Hall et al., 2008; Matthews et al., 2007).

DCs are the main antigen-presenting cell of the body, bridging the innate and adaptive immune system. During acute infection, DCs are highly migratory, a trait *Mtb* exploits by infecting DCs and using them for dissemination to other areas of the body. DCs play an important role in antigen presentation to naïve T cells. We have shown that despite the absence of lung CD11c^{+} CD11b$^{mid/low}$ cells, *ASC*$^{-/-}$ mice have similar numbers of *Mtb* antigen-specific T-cells in their lungs, mediastinal lymph nodes, and spleens compared to wild-type mice. DCs are present in both mouse and human granulomas during chronic *Mtb* infection, but their role within the granuloma is unknown (Tsai et al., 2006; Uehira et al., 2002). We theorize that CD11c^{+} CD11b$^{mid/low}$ cells may play a structural role within the granuloma. In the absence of this population we hypothesize that granuloma integrity is compromised, a phenotype seen in *ASC*$^{-/-}$ mice. Because *ASC*$^{-/-}$ mice had similar numbers of antigen-specific T cells compared to wild-type mice, we speculate that CD11c^{+} CD11b$^{mid/low}$ cells do not play a prominent role in

activation of naïve T cells, or their role is compensated by other antigen-presenting cells.

$CD11c^{+}$ $CD11b^{mid/low}$ cells may also represent an intermediate cell type between dendritic cells and macrophages (Matthews et al., 2007). These hybrid cells retain some characteristics of each cell type. This was demonstrated in a report in which $CD11c^{+}$ $CD11b^{-}$ cells were able to phagocytose OVA, but were unable to process and present OVA antigen. We envision a model in which these hybrid cells are recruited to the granuloma where they are infected by *Mtb* or take up *Mtb* proteins. Within the granuloma, the hybrid cells are unable to process and present antigen (Matthews et al., 2007). In addition, they do not upregulate activation markers required for migration out of the lung. Similar to our hypothesis for DCs, we suggest that these cells may provide structure within the granuloma, and in their absence granuloma integrity may be compromised, indicative of the phenotype present in $ASC^{-/-}$ mice.

Alveolar macrophages are another cell population classified as $CD11c^{+}$, $CD11b^{mid/low}$. Similar to DCs, they are present within lung granulomas. A recent study observed that the number of pulmonary macrophages increased during *Mtb* infection as detected by histology, but the number of macrophages recovered in BAL fluid does not increase during infection (Redente et al., 2010) This indicates that large numbers of macrophages are recruited to the lungs but these cells are not migrating out of the lungs. Redente *et al*. (2010) speculate that retention of macrophages within the lung may be due to their tight adherence properties. Complete depletion of alveolar macrophages during *Mtb* infection

results in defective granuloma formation (Leemans et al., 2001). Due to alveolar macrophages being the primary cell type infected by *Mtb* during initial infection, their role in chronic infection alone is difficult to surmise from this model. These examples allow us to hypothesize that alveolar macrophages within *Mtb*-induced granulomas comprise the infected core as well as surrounding uninfected macrophages. Within granulomas, alveolar macrophages may be important for providing structure due to the adherent properties of macrophages.

Whether the $CD11c^{+}$ $CD11b^{mid/low}$ cell population consists of DCs, alveolar macrophages, or both cell types, we are interested in determining their function during chronic *Mtb* infection. We hypothesize that $CD11c^{+}$, $CD11b^{mid/low}$ cells may play a role in granuloma structure, either directly by providing scaffolding similar to Langerhans cells, or indirectly by regulating chemokine gradients and co-stimulatory molecules which recruit and direct immune cells within the *Mtb*-infected lung. In the absence of $CD11c^{+}$, $CD11b^{mid/low}$ cells, $ASC^{-/-}$ mice have fewer granulomas than wild-type mice and are unable to contain *Mtb* within granulomas. To further study this cell population, we could repeat whole lung digests of $ASC^{-/-}$ and wild-type mice during chronic *Mtb* infection and perform flow cytometry analysis using additional antibodies to further classify $CD11c^{+}$, $CD11b^{mid/low}$ cells. F4/80 antibody could be used to differentiate between alveolar macrophages ($F4/80^{+}$) and DCs ($F4/80^{-}$). In addition, we would stain cells with antibodies for activation markers (CD40, CD86,80, MHC class II) on macrophages and DCs. Determining the activation state of macrophages and DCs would provide hints to their functional role during chronic *Mtb* infection. In

addition we could perform immunohistochemistry on paraffin-embedded lung tissue sections to identify localization of $CD11c^{+}$ $CD11b^{mid/low}$ within *ASC*$^{-/-}$ and wild-type lungs. Cytokine and chemokine staining that colocalizes with areas of $CD11c^{+}$ cells may provide hints as to what these cells are doing within specific areas of the lung, something not possible to detect by flow cytometry. Additionally, we could identify chemokines that $CD11c^{+}$ $CD11b^{mid/low}$ cells migrate towards, and then see if these chemokines are reduced in *ASC*$^{-/-}$ mice during *Mtb* infection. We could confirm the importance of the chemokine by either challenging a gene depletion mouse for the specific chemokine with *Mtb* or by neutralizing the chemokine in a wild-type mouse during chronic *Mtb* infection. In both cases we hypothesize that the mice would have reduced granulomas similar to the phenotype we observed in *ASC*$^{-/-}$ mice.

In addition to differences in the $CD11c^{+}$, $CD11b^{mid/low}$ cell population, our whole lung digests identified an increase in $GR1^{+}$ cells within the lungs of $ASC^{-/-}$ mice. Neutrophils are usually present during the first two weeks of infection after which their numbers drop within the lungs (Gonzalez-Juarrero et al., 2001). We hypothesize that the increase $GR1^{+}$ cells is a result of *Mtb* located throughout the lungs of *ASC*$^{-/-}$ mice rather than contained within granulomas as in wild-type mice. We hypothesize that *ASC*$^{-/-}$ mice may constantly recruit $GR1^{+}$ cells to the lung to combat *Mtb,* thereby creating a lung environment that constantly mimics initial *Mtb* infection. It is possible that increased lung $GR1^{+}$ cells are destructive to the lung tissue, directly resulting in reduced survival of $ASC^{-/-}$ mice following *Mtb* infection. To test this hypothesis, we could use neutralizing antibodies

against $GR1^+$ during chronic *Mtb* infection to determine if $ASC^{-/-}$ mice live as long as wild-type controls.

Functions of ASC independent of the inflammasome

A key finding in our studies is that ASC plays an inflammasome-independent role in host defense against *Mtb*, as Nlrp3 and Casp-1 deficient mice did not exhibit reduced survival compared to wild-type mice in a mouse aerosol model infection. The non-inflammasome forming NLR, NLRP12, is known to bind ASC (McConnell and Vertino, 2004). NLRP12 has been shown to suppress cytokine production through inhibition of NFκB signaling in a human monocytic cell line following *Mtb*, LPS, and TNFα stimulation (Williams et al., 2005). We hypothesized that NLRP12 could also be playing an important role in host protection against *Mtb* through an interaction with ASC. In contrast to this hypothesis, we found that Nlrp12 does not play a prominent role in host protection against *Mtb*, shown by survival and bacterial organ burden similar to wild-type mice. Unexpectedly, Nlrp12 did not have an anti-inflammatory effect during *Mtb* infection. In the absence of Nlrp12 we observed reduced TNFα and IL-6, indicating that Nlrp12 has an inflammatory effect during *Mtb* infection. Similar to our NLRP3 data, this underscores the importance of transitioning results found in cell culture into *in vivo* models of infection.

Our findings were not the first to indicate an inflammasome-independent role for ASC. ASC has been associated with NFκB and AP1 signaling independent of NLRP3 or caspase-1 function (Hasegawa et al., 2009; Stehlik et

al., 2002; Taxman et al., 2006). Recently a microbial pathogen-induced mechanism for ASC in activating chemokine expression through MAPK activation was discovered which occurred independent of the inflammasome (Taxman *et. al*, manuscript submitted). Two recent reports have implicated a role for ASC in the absence of the inflammasome using *in vivo* models of arthritis, but they provide little mechanism to explain their findings (Ippagunta et al., 2010; Kolly et al., 2009). Mechanistic insight gained while studying the role of ASC in the host response to *Mtb* may be informative for a myriad of chronic diseases.

We propose the following model to explain the reduced survival of $ASC^{-/-}$ mice following *Mtb* infection (**Figure 4.1**). Through yet undescribed processes possibly involving changes in MAPK on NFκB signaling and chemokines regulation, ASC is involved in recruitment of $CD11c^{+}$ $CD11b^{mid/low}$ to the granuloma where they play a role in granuloma structure. In the absence of ASC, granuloma integrity is compromised and bacteria are not contained with the granulomas, but are instead spread throughout the lung. *Mtb* outside of the granuloma mimics the initial stages of *Mtb* infection, a process during which $GR1^{+}$ cells are recruited to the lung. In wild-type mice, the number of $GR1^{+}$ cells in the lung peak at two weeks postinfection after which the numbers recede. In the case of the $ASC^{-/-}$ mice, $GR1^{+}$ cells are continually recruited to the lung throughout the chronic phase of *Mtb* infection. The antimicrobial functions of $GR1^{+}$ cells can damage host cells as well pathogens (Smith, 1994). It is possible that the constant influx of $GR1^{+}$ cells into the lung causes extensive damage to the lung tissue, which could result in the premature death of $ASC^{-/-}$ mice.

Cellular composition of granulomas in ASC$^{-/-}$ and wild-type lungs

Our studies have identified an absent lung cell populations during chronic *Mtb* infection that correlates with reduced granulomas in the lung. This suggests that the absent cell population may play a role in granuloma formation and/or maintenance during chronic *Mtb* infection. Ultimately, it is important to determine what cells are present within the *Mtb*-induced granuloma. Experiments are underway to determine the immune cell composition of ASC$^{-/-}$ and wild-type granulomas isolated from their surrounding environment. To bypass the challenge of isolating granulomas from mouse lungs under BSL3 conditions, we are using an approach developed by our collaborator Dr. Matyas Sandor at the University of Wisconsin-Madison. His method uses intra-perteritoneal (i.p.) delivery of the *Mtb* vaccine strain *M. bovis* BCG to induce the formation of liver granulomas. The benefits of this model are the use of a BSL2 pathogen and the rapid formation of liver granulomas with the latest time point needed to be tested being 10 weeks postinjection. Granulomas are isolated, disrupted, and the immune cell composition is identified by flow cytometry analysis. We will use the same cell surface antibodies as were used in our whole lung digests—CD4 and CD8 to identify lymphocyte populations along with CD11b, CD11c, and F4/80 for monocyte-derived populations. In addition, we will use LFA and CD40L, markers of T cell activation, as well as CD40, CD86/80, and MHC class II, activation markers on antigen-presenting cells. We are optimistic that this experiment will identify differences in the immune cell composition between ASC$^{-/-}$ and wild-type

granulomas. It is our hope that these data corroborate and expands upon our findings with whole lung digests and provide insights into differences in lung granulomas between ASC$^{-/-}$ and wild-type mice.

Future studies

The future direction of this project involves the continued study of the lungs of ASC$^{-/-}$ mice to determine mechanisms by which ASC is impacting survival during chronic *Mtb* infection. We have identified differences in the CD11c^{+} CD11b$^{mid/low}$ and GR1^{+} cell populations of *ASC*$^{-/-}$ lungs compared to wild-type lungs. To determine the immune cell composition of isolated granulomas, in contrast to the entire lung, we are isolating *M. bovis* BCG-induced liver granulomas and analyzing their immune cell composition through flow cytometry. To continue this project, we would confirm any findings in our liver granuloma model by isolating virulent *Mtb*-induced lung granulomas and analyzing their cellular composition.

Secondly, we want to identify cell signaling pathways that differ within the cells of *ASC*$^{-/-}$ and wild-type mice due to the inflammasome-independent role of ASC during chronic *Mtb* infection. These experiments will allow us to better understand the mechanisms by which ASC protects the host during chronic *Mtb* infection. There are several ways to identify expression differences in transcripts within granulomas of *ASC*$^{-/-}$ and wild-type mice. Using laser capture microscopy, we could extract RNA from granulomatous tissue within paraffin-embedded lung tissue of *Mtb*-infected *ASC*$^{-/-}$ and wild-type mice. RNA could then be quantified

by either real-time PCR or by microarray. Alternatively, RNA could be isolated from liver granulomas for transcriptional analysis. Identifying cytokine, chemokines, or cell signaling differences in *ASC*$^{-/-}$ mice may provide insight into the role of CD11c^{+} CD11b$^{mid/low}$ cells in *ASC*$^{-/-}$ lungs or clarify the role of ASC in granuloma formation. Ultimately, they could aid us in understanding the role of ASC in host protection against *Mtb* infection.

Summary

Our initial hypothesis was that the NLRP3 inflammasome would be activated by *Mtb* in cultured macrophages, and that activation would be dependent on NLRP3, ASC, and caspase-1. Our hypothesis was correct in cultured macrophages and monocytes. We believed that the NLRP3 inflammasome would thus be important for host resistance to *Mtb* using an *in vivo* mouse model of infection. We predicted that *Nlrp3*, *ASC*, and *Casp-1* deficient mice would result in reduced survival after *Mtb* infection due to decreased IL-1β within the lungs of the mice compared to wild-type. In contrast to our hypothesis, we found that ASC alone was important for host protection during chronic *Mtb* infection while *Nlrp3*$^{-/-}$ and *Casp-1*$^{-/-}$ mice survived as long as wild-type mice. In addition, cleaved IL-1β was present in the lungs of *Nlrp3*$^{-/-}$, *ASC*$^{-/-}$, and *Casp-1*$^{-/-}$ mice. Identification of ASC as an important protein for host protection during *Mtb* infection—occurring independently of the inflammasome—represents an unexpected and novel finding. Although two other *in vivo* reports

from the arthritis field have identified an inflammasome-independent role for ASC, very little is known about the mechanism by which this occurs.

With high rates of *Mtb* infection worldwide and an increasing number drug resistant TB cases, it is our hope that increased understanding of the interaction between *Mtb* and the host immune system will lead to new and more effective anti-TB therapies. Our data suggest that overexpression of ASC may lead to increased host protection against *Mtb* infection through increased granuloma stability and therefore through bacterial containment within granulomas. We propose that this treatment could be used to prevent TB reactivation in immunocompromised individuals.

Also, the lack of a role for NLRP3 during an *in vivo* model of *Mtb* infection has important implications for treatment of autoinflammatory disorders. The TNFα inhibitor Remicade is used to treat patients with arthritis, Crohns disease, ulcerative colitis, and other autoimmune diseases. TNFα is important for granuloma maintenance in humans, and treatment with TNFα inhibitors often lead to reactivation of TB. Our data suggests that NLRP3 could developed as a drug target to treat inflammatory diseases, and inhibition of NLRP3 is not likely to cause reactivation of TB as is the case with TNFα inhibitors.

FIGURES

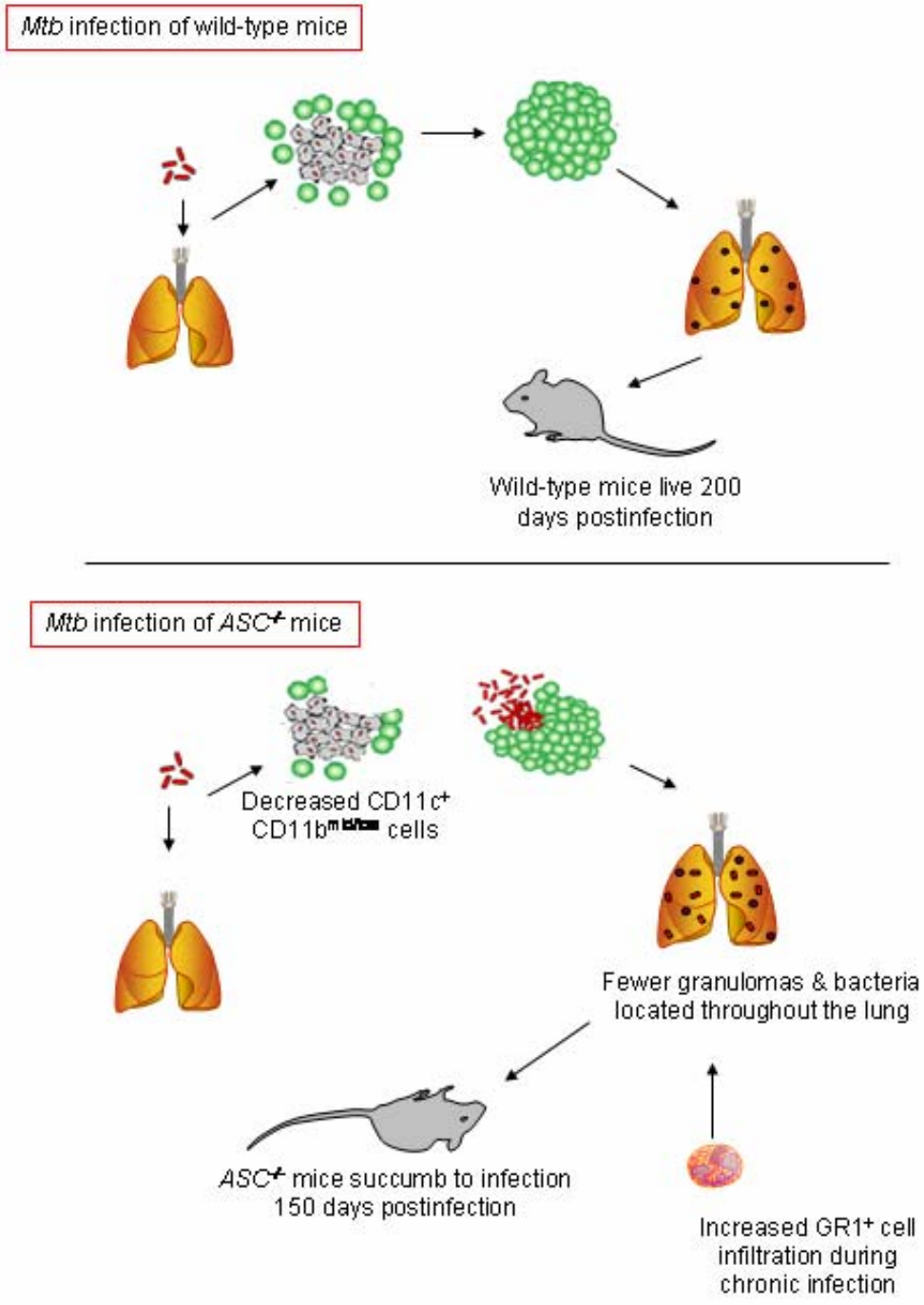
Mtb infection of wild-type mice
Wild-type mice live 200 days postinfection
Mtb infection of *ASC*$^{-/-}$ mice
Decreased CD11c^{+} CD11b$^{mid/low}$ cells
Fewer granulomas & bacteria located throughout the lung
ASC$^{-/-}$ mice succumb to infection 150 days postinfection
Increased GR1^{+} cell infiltration during chronic infection

Figure 4.1. Working model that demonstrates why ASC-/- mice are more susceptible to *Mtb* infection. Figure adapted from New York Academy for Sciences©

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