

SecA2 of *Mycobacterium tuberculosis* contributes to intracellular survival, immune modulation, and surface properties of the bacillus

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ABSTRACT

SHERRY LYNN KURTZ: SecA2 of *Mycobacterium tuberculosis* contributes to intracellular survival, immune modulation, and surface properties of the bacillus

(Under the direction of Dr. Miriam Braunstein)

SecA2 is part of a specialized protein export pathway of *Mycobacterium tuberculosis* that is important to virulence. We present evidence that a $\Delta secA2$ mutant of *M. tuberculosis* is defective for replication in macrophages. In addition, macrophages infected with the $\Delta secA2$ mutant produce more proinflammatory TNF- α , IL-6, and RNI than those infected with wild type *M. tuberculosis*. These host factors are induced by *M. tuberculosis* through TLR/MyD88 signaling pathways. The attenuated phenotype of the $\Delta secA2$ mutant in macrophages was dependent on the signaling adaptor MyD88 and TNF- α . Our data suggests that the role of SecA2 in virulence is to export factors which limit the host immune response, and thereby protects the bacillus against MyD88/TNF- α dependent defenses. Finally, we reveal a role for SecA2 in assembling the cell surface structure of *M. tuberculosis*, a finding that may have implications on the biology of the organism, and pathogenesis.

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LIST OF ABBREVIATIONS AND SYMBOLS

aa	amino acid
ADS	Albumin-Dextrose-Catalase supplement
BMDM	bone marrow derived macrophage
CBA	cytometric bead array
CFU	colony forming unit
DMEM	Dulbecco's modified eagle medium
ELISA	enzyme linked immunosorbent assay
gly	glycerol
HIV	human immunodeficiency virus
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factor
iNOS/Nos2	inducible nitric oxide synthase
IFN- γ	interferon-gamma
IL-6	interleukin-6
kb	kilobase
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary response gene 88
NF- κ B	nuclear factor kappa B
qRT-PCR	quantitative Real-Time PCR
PBS	phosphate buffered saline
PCR	polymerase chain reaction

rhIFN- γ	recombinant human IFN- γ
rmIFN- γ	recombinant murine IFN- γ
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the means or scanning electron microscopy
TIR	Toll/Interleukin-1 receptor
TIRAP	MyD88/TIR domain-containing adaptor protein
TLR	toll-like receptor
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF/TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor-inducing interferon- β
TNF- α	tumor necrosis factor alpha
Tw80	Tween 80
Δ	deletion

CHAPTER 1

INTRODUCTION

Mycobacterium tuberculosis and the disease it causes has plagued mankind for centuries, and it still remains as much of a global health threat today as ever before. Signs of tuberculosis and its symptoms have been described in skeletons from several archeological sites dating back to the Bronze Age and Iron Age more than 4000 years ago (79, 119). Greek historians from 500-700 B.C. write of a wasting disease called phthisis, marked by chest pain, coughing, and blood in the sputum. Epidemics of tuberculosis (TB) have arisen throughout the course of time. In the first half of the 19th century, it is estimated that up to 25% of Europeans died from tuberculosis (108). Yet it was not until the later part of the 19th century that Robert Koch was able to identify *M. tuberculosis* as the causative agent of tuberculosis, a discovery announced on March 24, 1882 (59). The anniversary of this report each year is marked as World TB Day (115). Since that initial discovery, scientists have learned much about the disease itself, as well as about the organism, and yet today we are still faced with the highest global burden of tuberculosis ever. The theme of World TB Day 2007, “TB anywhere, is TB everywhere”, gives us

perspective on the pandemic, that unless the disease is eradicated in all countries, it will continue to be a global health burden.

Disease Process and Epidemiology

Mycobacterium tuberculosis is a rod shaped acid-fast bacillus. The primary route of transmission of *M. tuberculosis* is via aerosols person to person. When an individual comes into contact with the aerosols, the aerosol droplets containing bacilli are taken up into the lungs. The bacilli are phagocytosed in the lungs by alveolar macrophages, or to a lesser extent are ingested by alveolar type II pneumocytes (10, 69). If the macrophage has been preactivated, it will be contain the bacilli and the patient will not develop disease. However, the majority of macrophages are non-activated and are permissive for bacterial replication. When the bacilli enter these resting macrophages they are able to combat the defense mechanisms of the macrophage, enabling them to survive and replicate. Once infected, these macrophages secrete chemoattractants, recruiting lymphocytes, neutrophils and monocytes to the site of infection (123)(Fig. 1.1). These cells will begin to form a barrier around the infected monocytes, producing a granulomatous lesion, and large macrophage derived foamy cells will form at the center of the granuloma. As macrophages and dendritic cells present *M. tuberculosis* antigens to CD4⁺ and CD8⁺ T cells, a Th1 cell mediated immune response is mounted (39). This results in amplification of antigen specific T cells, which play a crucial role in inhibiting the infection by secreting IFN- γ , which then in turn activates macrophages to control the growth of the bacteria. CD8⁺ T cells can also inhibit mycobacterial growth

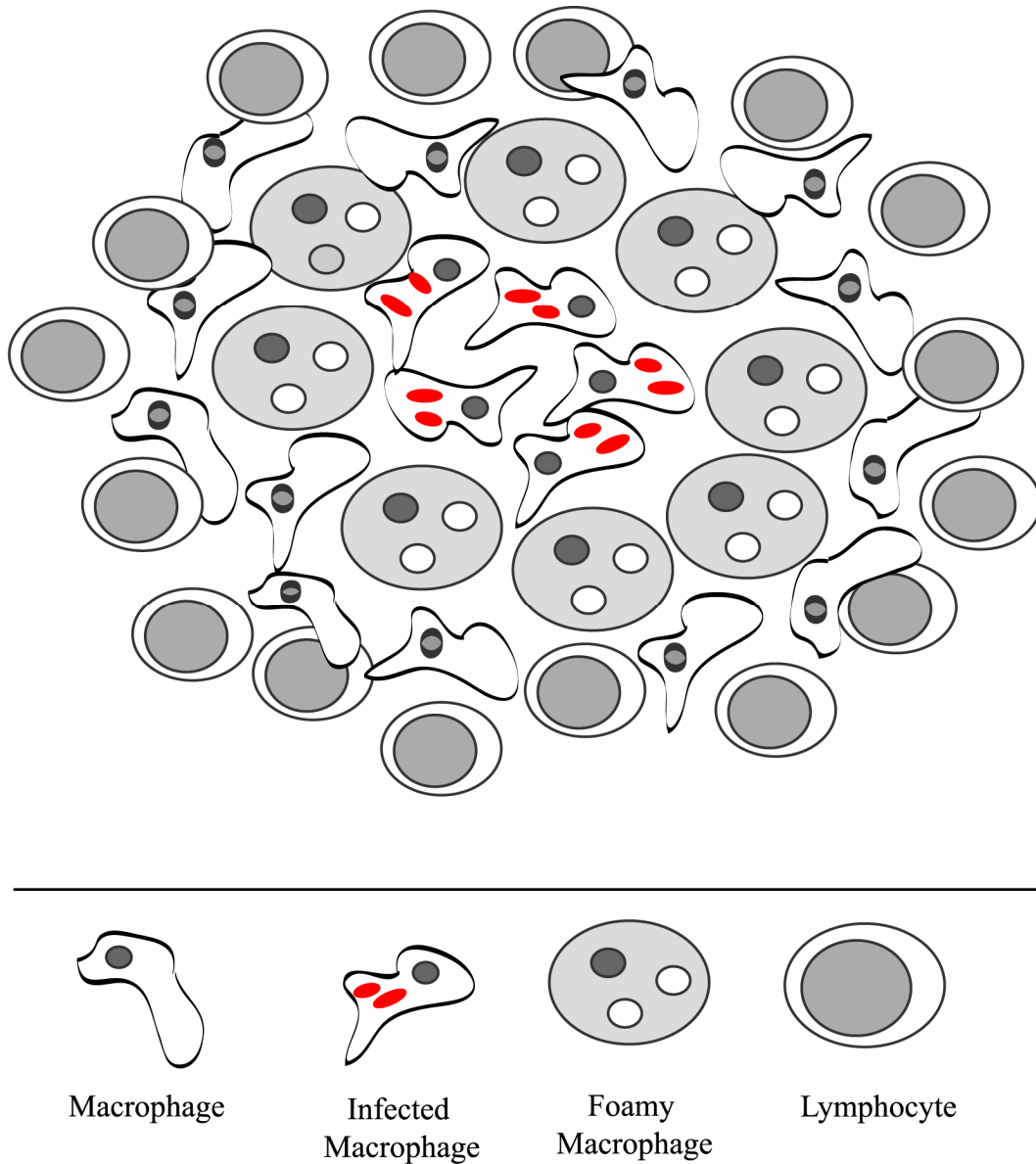


Fig. 1.1. The structure of a granuloma. Infected macrophages secrete chemoattractants, recruiting lymphocytes, neutrophils and monocytes to the site of infection. These cells will begin to form a barrier around the infected monocytes, producing a granulomatous lesion, and large macrophage derived foamy cells will form at the center of the granuloma. A Th1 cell mediated immune response is mounted as macrophages and dendritic cells present M. tuberculosis antigens to CD4⁺ and CD8⁺ T lymphocytes. The antigen specific T cells will secrete IFN- γ , which activates macrophages to control the growth of the bacteria.

by lysing infected host cells (22, 113). Often the bacteria are held in check at this stage, and the infection is considered “latent”. Individuals with a healthy immune system can remain latently infected for years, showing no signs of disease. Individuals who are latently infected have a 10% chance in their lifetimes of having the bacilli reactivating to cause active disease, while some will resolve the infection entirely. A decrease in immune system function from several factors contributes to disease reactivation, and these factors include age, immunosuppressive drugs, malnutrition, and HIV (70). The global pandemics of HIV and tuberculosis are having a deadly interplay. Someone infected with HIV, who also has a latent tuberculosis infection, has a 10% per year chance of developing an active case of tuberculosis (124). Tuberculosis kills 1.6 million people each year, an average of 4400 per day. Of these, approximately 200,000 are those with HIV/AIDS, making tuberculosis the leading killer of people with HIV/AIDS (124). When tuberculosis becomes reactivated, or when the initial infection is not controlled, the individual has active disease and is infectious.

One of the biggest challenges in the fight against tuberculosis is the rise of drug resistant forms of the bacterium. Drug resistance arises when chemotherapeutics are used improperly, either due to misprescribed treatments by healthcare workers, patient non-compliance for completing a drug regimen, poor accessibility to treatment medications, or receiving a poor quality treatment (70). The history of tuberculosis and chemotherapy dates back to the very first therapeutics used. Streptomycin was the first antibiotic discovered for human use in 1943, and was used to treat tuberculosis by 1944 (24). Within months of use, the first strains of *M. tuberculosis* resistant to streptomycin began to appear (24). Isoniazid (1952) and rifampin (1963) joined streptomycin as the standard

of treatment for tuberculosis, and are still used as frontline treatments today. However, resistant strains of *M. tuberculosis* exist for each of these drugs. The concerns about mono-resistant strains have now given way to even greater concerns with the emergence of multi-drug resistant (MDR), and the newest extensively drug resistant (XDR). MDR-TB strains are resistant to at least the two front line drugs isoniazid and rifampin, and represent 20% of isolates tested from 2000-2004 (125). The newly described XDR-TB strain is an MDR-TB strain that is also resistant to any fluoroquinolone and is resistant to at least one of the second line injectable drugs (capreomycin, kanamycin, amikacin). XDR-TB represents 2% of all isolates tested, and 4% of the MDR-TB isolates tested in the United States (125).

Vaccine Development

Robert Koch initiated the first studies to find a vaccine against tuberculosis. His initial attempts to use purified protein and cell wall components from *M. tuberculosis* to vaccinate against infection was unsuccessful in terms of protection against disease, but yielded a diagnostic tool for the detection of tuberculosis that is still in practice today (100). *M. bovis*, a close relative of *M. tuberculosis*, was passaged by Calmette and Guerin to create the attenuated strain BCG (100). Since the 1920s, BCG has been used as a live, attenuated vaccine to prevent *M. tuberculosis*, with more than four billion doses being administered world wide (70). However, this vaccine has demonstrated varying degrees of efficacy in different clinical trials. BCG immunization has a proven efficacy in preventing extrapulmonary tuberculosis in children (122). However, in adults clinical trials demonstrate anywhere from 70% effectiveness to no protection, as seen in the

largest scale retrospective analysis done in India (70). Why this vaccine shows such varied protection is unclear. One hypothesis is in certain endemic areas, individuals may be more readily exposed to environmental mycobacteria prior to vaccination (27, 83). The prior exposure may lead to a greater recognition of BCG by the immune system, and clearance of the vaccine before it can confer protection. Given the variable protection of BCG and continued world wide presence of tuberculosis, a better vaccine must be found. Many different approaches toward this end are being taken, and include the development of subunit vaccines, DNA vaccines, manipulation of BCG to increased efficacy, and use of other recombinant mycobacteria to express *M. tuberculosis* antigens (70). A better understanding of the biology of the organism itself and its relationship with the host immune response will help these efforts.

Effectors of the Host Response Against *M. tuberculosis*: Cytokines

To understand how *M. tuberculosis* evades the immune system, we must first understand the host immune effectors capable of controlling mycobacterial infection. IFN- γ is an essential cytokine in the defense against *M. tuberculosis*. As described, IFN- γ secretion by T cells is important for mediating granuloma formation. But perhaps more important, is the role that IFN- γ plays in limiting mycobacterial growth through the activation of macrophage antimicrobial responses (38, 97). IFN- γ , in concert with TNF- α , activates macrophages to inhibit the intracellular growth of mycobacteria, primarily through the action of inducible nitric oxide synthase (iNOS) (30, 38). Mice that are deficient in the production of IFN- γ are highly susceptible to an *M. tuberculosis* infection, and the bacteria replicate uncontrollably until the mice succumb to disease (40). In the

murine system, iNOS and the nitrosative burst are one of the most potent host defense mechanisms against *M. tuberculosis* (65).

However, there are other IFN- γ related loci in the mouse that are key to the defense program against mycobacterium. MacMicking *etal* first reported a novel IFN- γ induced host mechanism for controlling *M. tuberculosis*, the IFN- γ inducible guanosine triphosphatase family LRG-47 (36, 66). Mice lacking LRG-47 are highly susceptible to *M. tuberculosis* and succumb rapidly to disease. The action of LRG-47 is independent of iNOS, and seems to be directed toward promoting proper phagosome maturation and phagosome/lysosome fusion. Together, iNOS and LRG-47 combine to form the majority of the host IFN- γ induced antimycobacterial activity (66).

The importance of IFN- γ in host protection against tuberculosis has also been seen in the clinical setting of human disease. Patients with mutations in the IL-12/IL-23/IFN- γ pathways of Type I cytokines or in IFN- γ receptors show an increased susceptibility to infection by *M. tuberculosis* and non-tuberculosis mycobacterium (NTM), and an increased severity of disease symptoms (70, 80). Given its potent role in protection against intracellular pathogens, IFN- γ is being investigated as a clinical treatment of infections, including *M. tuberculosis* (95).

The proinflammatory cytokine TNF- α is also essential for productive immune response against TB. Early studies demonstrated this importance using TNF- α knockout mice. These mice are unable to control the replication of the bacteria, which grow uncontrollably, resulting in a rapid mortality in the mice (5, 12, 41). TNF- α is critical for granuloma formation and maintenance, and inhibition of TNF- α results in reactivation of latent infection in a murine model (72). There is now data from human subjects and the

importance of TNF- α . A new class of drugs developed to treat chronic inflammation from arthritis is specifically targeted to neutralize TNF- α . Patients treated with these drugs are at greater risk for reactivation of latent tuberculosis (70, 101, 112).

As described, TNF- α works in concert with IFN- γ to activate the macrophage antimicrobial effector iNOS (19, 30, 38). However, if and how TNF- α in the absence of IFN- γ inhibits growth of mycobacteria at the cellular level is unclear. Some studies suggest that there are TNF- α dependent mechanisms independent of IFN- γ that either inhibit the growth or kill mycobacteria (6, 21, 34, 58, 103). TNF- α alone can trigger iNOS induction and mycobacterial growth inhibition through the production of RNI. However, there may also be TNF- α dependent, but iNOS independent, mechanisms of growth inhibition (6). In contrast, there are opposing studies that would suggest that TNF- α may even enhance the growth of virulent mycobacteria (17, 34, 92). The discrepancies in these results remain to be resolved and may come from differences in cell types and protocols used amongst the labs.

TNF- α may also exert antimicrobial effects against *M. tuberculosis* via the induction of apoptosis, an effector mechanism that is independent of iNOS and IFN- γ . Several reports have shown an apoptosis induced killing or growth inhibition of *M. tuberculosis* (29, 56, 78). Consistent with this, some avirulent strains of mycobacteria induce apoptosis or are incapable of blocking the apoptotic pathway, as shown by several of the apoptotic pathway signaling intermediates such as caspases 3, 8, 9, and 10 being upregulated during infection. The levels of apoptosis are related to the levels of bioactive TNF- α , and strains inducing higher levels of TNF- α or TNF- α bioactivity induce more apoptosis (4, 55, 56, 61, 96, 112). Data in the literature demonstrates that virulent *M.*

tuberculosis evades apoptosis (4, 55, 110). Taken together, this data suggests that TNF- α may exert antimicrobial effects on *M. tuberculosis* by the induction of host cell death, a mechanism that is independent of iNOS and IFN- γ .

Effectors of the Host Response Against *M. tuberculosis*: Innate Immunity

Cytokine production by host cells is triggered as part of an innate host response. Key components of innate immunity defense mechanism against *M. tuberculosis* are the mammalian pattern recognition receptors, such as Toll-like receptors (TLR), which specifically recognize microbial ligands. TLR are a family of transmembrane receptors that contain an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain (68). Upon ligand binding, TLR recruit TIR-domain containing adaptor protein MyD88, and the accessory adaptor MyD88/TIR domain-containing adaptor protein (TIRAP) to the receptor (Fig 1.2). MyD88 is an essential signaling component of signaling for all TLR receptors, except TLR3 (91). A MyD88 independent signaling pathway using TIR domain-containing adaptor-inducing interferon- β (TRIF) is engaged by TLR3, and is important for interferon regulatory factors-3 (IRF-3) mediated activity (91). TLR4 can also utilize this TRIF dependent, MyD88 independent pathway, in addition to its signaling through MyD88 (91). Activation of MyD88 leads to the formation of the IL-1 receptor associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) complexes, which activate NF- κ B, leading to expression of proinflammatory cytokines and regulatory molecules, such as TNF- α and IL-12. TLR activation also activates macrophage antimicrobial effector mechanisms, such as inducible nitric oxide synthase (iNOS), and modulate the

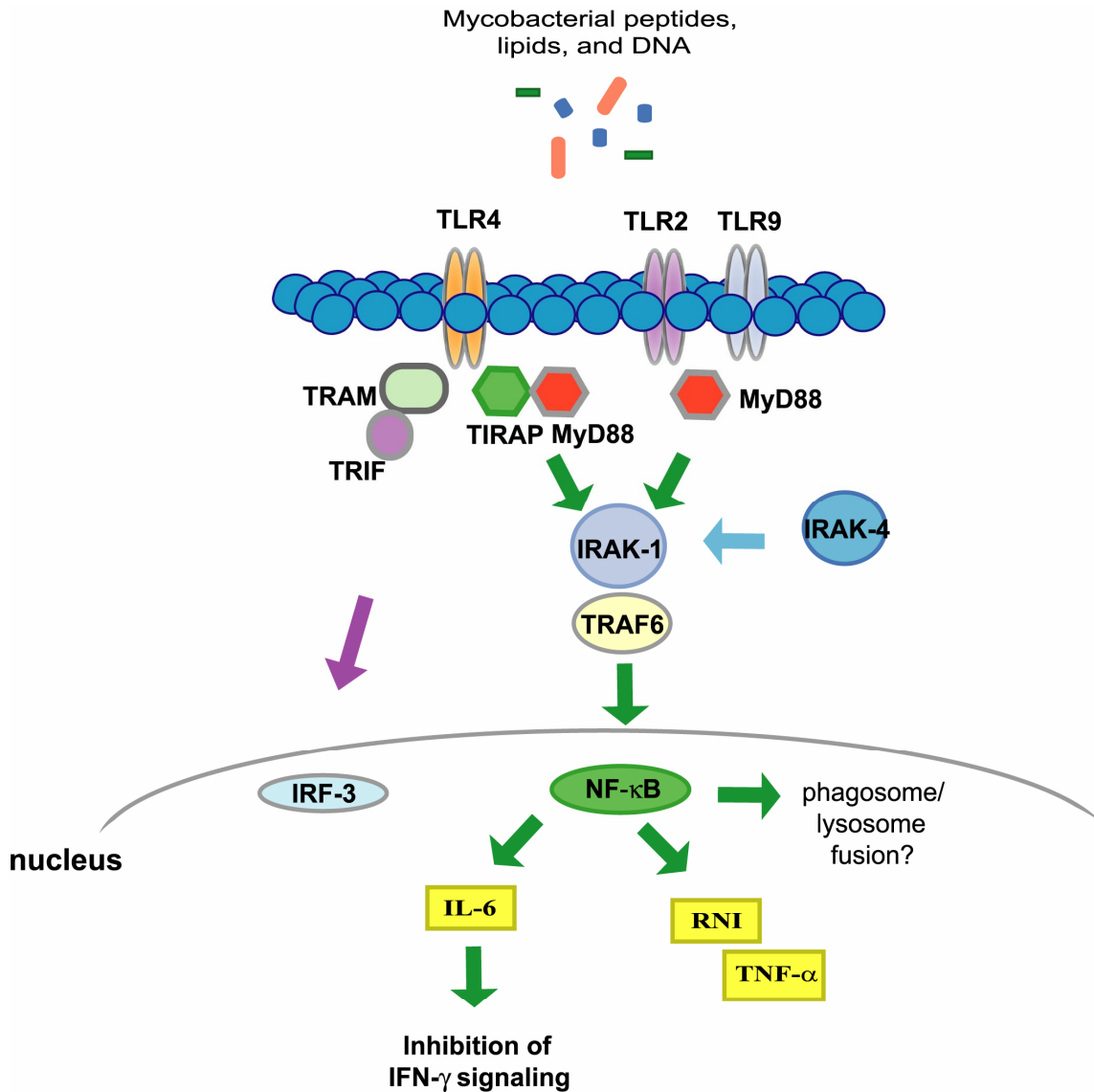


Fig. 1.2. Toll signaling pathways. Mycobacterial peptides, lipids, and DNA interact with Toll-like receptors. These receptors contain intracellular signaling domains which interact with signaling adaptors, including MyD88 and downstream, IRAK-1 and TRAF6. TRAF6 activates nuclear factor κ B (NF- κ B), a transcription factor which upregulates the expression of such host response factors as IL-6, TNF- α and iNOS (RNI). IRAK-4 is needed to activated IRAK-1. TLR/MyD88 signaling may also trigger pathogen induced phagosome/lysosome fusion. TLR4 can also signal through an alternate adaptor protein, TIRAP in conjunction with MyD88, or can use a MyD88 independent pathway which utilizes TRAM and TRIF to upregulate IRF- β signalling.

developing adaptive immune response by influencing dendritic cell (DC) maturation and T-cell activation (31). Furthermore, TLR signaling may play a role in the regulation of phagosome maturation and subsequent antigen presentation, although there is some controversy surrounding this model (11).

M. tuberculosis is able to activate cells through interactions with TLR2, TLR4, and TLR9 to produce host effectors such as TNF- α , IL-6 and RNI via the adaptor protein MyD88, and progress has been made in identifying *M. tuberculosis* surface molecules that are TLR ligands [Reviewed in (50, 52, 91, 114)]. These TLR agonists include lipids, such as lipomannan (LM) and lipoarabinomannan (LAM), lipoproteins, and protein molecules. The TLR agonists of *M. tuberculosis* are found both associated with the surface of the bacterium, as well as exported into the extracellular space. The *M. tuberculosis* agonists signal primarily via TLR2, but TLR4 also plays a role. In addition, *M. tuberculosis* DNA is recognized by TLR9. TLR pathways play an important role in the host response to several pathogens, and studies of the specific role(s) of this system in host protection against *M. tuberculosis* are underway. Studies using mice deficient in either TLR2, TLR4, TLR9 or MyD88, or having multiple deficiencies, have been tested for their susceptibility to *M. tuberculosis* infection. While the roles of TLR2 and TLR4 are unclear, mice missing MyD88 or TLR9/TLR2 have a drastically increased susceptibility to *M. tuberculosis*, supporting a role for these TLR pathways in host protection (1, 3, 13, 31, 37, 44, 51, 54, 75, 94, 99, 104, 106, 116, 121). This also suggests that there is redundancy in the function of the TLR, in that the TLR9/TLR2 double knockout has a more dramatic phenotype than either of the individual mutants. The data

using the MyD88 knockout mice shows that TLR signaling through this adaptor is important for host defense against *M. tuberculosis*.

Effectors of the Host Response Against *M. tuberculosis*: Adaptive Immunity

In addition to an innate immune response against mycobacteria, an infected host must also mount an adaptive immune response to control infection. The protective immune response to *M. tuberculosis* is a cell-mediated Th1 response involving both CD4⁺ and CD8⁺ T cells (18, 42, 71, 109, 118). As described, CD4⁺ cells are recruited to the site of a nascent granuloma and represent an important source of IFN- γ , essential for the control of *M. tuberculosis*. CD8⁺ T cells can also inhibit mycobacterial growth by lysing infected host cells (22, 113). Depletion of CD4⁺ T cells results in the reactivation of tuberculosis in both humans and mice, showing the importance of these immune effector cells in controlling *M. tuberculosis* (105).

In addition to a T cell response, *M. tuberculosis* infection elicits a humoral response. Although the antibody response to *M. tuberculosis* is not likely to contribute to the control of infection, antigens recognized by host antibodies could be the basis of serodiagnostic techniques for tuberculosis (46, 48, 53).

Intracellular Survival Mechanisms of Mycobacteria

In prior sections, we described the host immune responses involved in controlling a mycobacterial infection. However, *M. tuberculosis* is a successful pathogen, which has evolved mechanisms to thwart many of the antimicrobial host responses. When it first enters the lungs of a nascent host, the bacterium must invade and replicate within host

cells. Here we will discuss four mechanisms by which *M. tuberculosis* survives in the non-activated host cell. This first step in the infection process is important, as without intracellular growth the infection cannot continue and there will be no disease. *M. tuberculosis* is phagocytosed by monocytes a process triggered via several host receptors, including the complement receptors, scavenger receptor and mannose receptor (35). During the normal process of phagocytosis, a particle or organism is taken up into a phagosome. As the phagosome matures it acquires markers such as Rab5 and EEA1, and these markers direct fusion of the phagosome with endosomal vesicles. Acquisition of the late markers Rab7 and LAMP1 promote subsequent fusion with lysosomal vesicles (60). Fusion with the lysosome delivers hydrolytic enzymes, such as cathepsin D, which digest the bacteria. During maturation the phagosome also acidifies as the vacuolar H⁺-ATPase is recruited. The resultant low pH is harmful to the bacteria contained within the cell, and also activates antimicrobial protease activity (60, 98). Mycobacteria alter the normal phagosome maturation pathway, and this is proposed to be an important mechanism for survival. Once in the phagosome, *M. tuberculosis* is able to block acquisition of the vacuolar H⁺-ATPase, thus preventing acidification of the phagosome (Reviewed in (28)). The phagosome is also blocked at other stages of maturation, and does not acquire late endosomal markers Rab 7 and LAMP1, and does not fuse with lysosomes (60). This prevents the delivery of lysosomal hydrolytic enzymes to the *M. tuberculosis* phagosome. Several recent reports have suggested the importance of the inhibition of phagolysosome fusion to the pathogenesis of *M. tuberculosis*. Studies have uncovered mutants of *M. tuberculosis* that are defective for this process, and are attenuated for growth in macrophages (63, 87). However, there was also a subset of

mutants in this screen that did not inhibit phagolysosome fusion and ended up in mature phagolysosomes, and yet grew to wild type levels. Clearly there is complexity to the survival of *M. tuberculosis* in the context of the phagosome, but this remains one possible mechanism of intracellular survival for *M. tuberculosis*.

The second survival mechanism is the ability of *M. tuberculosis* to combat the toxic effects mediated by reactive nitrogen and reactive oxygen intermediates produced by the host cells. Enzymes that can detoxify these radicals are an important defense mechanism. In *M. tuberculosis*, there is a precedent for the importance of such enzymes, including the superoxide dismutases SodA (secreted) and SodC (surface localized), and the catalase-peroxidase KatG (secreted), in pathogenesis and protection against the oxidative burst of macrophages (32, 33, 67, 74, 88). Reactive nitrogen intermediates are a potent antimycobacterial effector in the murine host, and mice defective for iNOS have a dramatically increased susceptibility to mycobacterial infection (65). *M. tuberculosis* can limit its sensitivity to RNI. One such protective mechanism is via the production of a proteosome (25). The proteosome and proteosome associated proteins Mpa and PafA function to maintain the levels of enzymes required for fatty acid and polyketide synthesis, and these enzymes are important for pathogenesis and mycobacterial metabolism (85). The role of the proteosome in protecting against RNI is likely the degradation of proteins damaged by the nitrosative burst.

A third survival mechanism of *M. tuberculosis* is the acquisition of nutrients inside host cells. Inside macrophages nutrients are sequestered away from the invading organism, so nutrient acquisition and alternative metabolic pathways are important mechanisms to promote intracellular. Metals such as iron and magnesium are essential to

life, and intracellular pathogens must produce molecules to scavenge these away from the host. *M. tuberculosis* produces MgtC, a magnesium transporter, and Mbt, an iron scavenging siderophore operon, which are both essential for the full virulence of the organism (16, 26, 90). When growing inside a host, *M. tuberculosis* preferentially shifts its metabolism to pathways that use fatty acids versus carbohydrates. Thus, enzymes involved in the fatty acid metabolism pathways of *M. tuberculosis* are important for virulence, such as the isocitrate lyase enzymes (Icl1/Icl2). *M. tuberculosis* strains with mutations these enzymes are attenuated for growth in macrophages, supporting the idea that *M. tuberculosis* must alter its metabolic pathways inside the host cells in order to survive Munoz-Elias, 2005 #1309}.

The fourth mechanism employed by *M. tuberculosis* is its ability to inhibit macrophage activation. As has been discussed, IFN- γ mediated host responses are critical to controlling *M. tuberculosis*. *M. tuberculosis* has evolved mechanisms to inhibit a subset of IFN- γ induced pathways (60). *M. tuberculosis* interferes with the signaling pathway downstream of the IFN- γ receptor, preventing the activation of transcription factors, and subsequently inhibiting the production of a subset of host responses, including cytokines, chemokines, and components of the MHC class II antigen presentation pathway (43, 57, 81, 82, 120). *M. tuberculosis* inhibits IFN- γ stimulation of MHC class II molecules, which are an integral component of the antigen presentation pathway to MHC class II restricted CD+4 T cells (43, 57, 81, 82, 120). Lipoproteins of *M. tuberculosis*, such as the well characterized 19kDa lipoprotein, are important for inhibition of IFN- γ pathways via prolonged signaling through TLR2 (43, 45, 47, 57, 76, 77, 81, 82, 86, 120). However, it is still unclear if *M. tuberculosis* inhibition of MHC

class II antigen presentation results in a diminished T cell response in a whole animal model.

In addition to inhibiting IFN- γ mediated signaling pathways, *M. tuberculosis* has also been shown to inhibit other cytokine responses of macrophages. It was previously demonstrated that virulent *M. tuberculosis* could inhibit an IL-12 response elicited by *Escherichia coli* if the bacteria were coinoculated (73). The importance of immune modulation has also been demonstrated with several hypervirulent strains of *M. tuberculosis*, which elicit decreased levels of proinflammatory cytokines. This phenomenon has been described for the hypervirulent HN878 strain of *M. tuberculosis* and the hypervirulent *mceI* mutant of *M. tuberculosis* (67, 93, 107). The hypervirulent strain HN878 produces a polyketide-derived phenolic glycolipid (PGL) (93). The production of PGL dampens the host immune response, resulting in hypervirulence. Disruption of PGL biosynthesis results in an increase in cytokine induction and in a loss of hypervirulence (93). *MceI* is a surface associated protein of *M. tuberculosis* (20). When *mceI* is disrupted, the resultant *M. tuberculosis* mutant induces less proinflammatory cytokines and is hypervirulent in a murine infection (107).

In addition to surface associated factors of *M. tuberculosis* that can modulate the immune response, there is a recent report that secreted proteins of *M. tuberculosis* also function to dampen the host response. It was recently shown that ESAT-6, an antigen exported by *M. tuberculosis* by a specialized *esx-1* export pathway, is able to bind TLR2. Once bound, ESAT-6 can suppress downstream TLR signaling by activating the kinase Akt, which in turn inhibits the interaction of MyD88 and its catalytic partner IRAK4, thus abrogating the signaling of MyD88 (84). ESAT-6 was able to block the induction of

cytokines such as IL-12 and TNF- α via its interaction with TLR2 (84). An ESAT-6 mutant of *M. tuberculosis*, or mutants in the ESAT-6 export pathway, show increased cytokine induction compared to the wild type strain (64, 111), which would result from the loss of the TLR inhibitory actions of ESAT-6.

Taken together, these reports demonstrate that surface and secreted molecules of *M. tuberculosis* can modulate the host immune response, a process that may promote the virulence of the organism.

Protein export and the accessory SecA2 export pathway

The proper localization of proteins is not only essential for the viability of all organisms, but contributes to the virulence of many pathogens. To understand how *M. tuberculosis* is able to survive in the host, we must first have a basic understanding of the biology of the bacterium, including protein export pathways. Proteins are synthesized in the cytoplasm. While some proteins remain in the cytoplasm, other must be transported out and localized to other subcellular compartments for proper function.

The essential process of protein translocation in bacteria is mediated by the Sec protein export pathway. The Sec pathway is ubiquitous in all bacteria and is essential for bacterial viability. The Sec pathway is comprised of several components including SecA, an ATPase, and SecYEG, integral membrane proteins which form the protein export channel. SecA functions as a dimer, and interacts with the signal peptide of a nascent preprotein and SecYEG to promote protein export across the inner membrane (Fig. 1.1). The ATPase function of SecA is essential to this process, and SecA is essential for the viability of all bacteria.

In Gram-negative organisms, there are alternate export pathways that localize proteins across the outer membrane of the bacteria, and often directly translocate virulence factors into host cells. These alternate protein export pathways represent a powerful virulence mechanism for Gram-negative pathogens (23, 89, 102). While the specialized secretion systems of Gram-negative bacteria have been well characterized, we are now only beginning to uncover the specialized secretion systems for Gram-positive organisms and mycobacterium species.

One of the first such specialized protein export systems to be identified is the SecA2 pathway. Recent genome sequences have uncovered multiple copies of SecA in the genomes of several *Mycobacterium* species, as well as in several Gram positive organisms. Among the Gram-positive species that have multiple SecA loci there are several pathogens, including *Listeria monocytogenes*, *Streptococcus gordonii*, and *Streptococcus parasanguis*. In *M. tuberculosis* and *M. smegmatis*, there is one additional copy of SecA. As reported by Braunstein *etal* (14), in *M. smegmatis*, SecA1 is the “housekeeping” SecA that is essential for viability of the bacterium, as it can not be deleted from the bacteria without an additional copy being provided. SecA2 is the “accessory” SecA protein, which is non-essential and can be deleted from *M. smegmatis* and *M. tuberculosis* (15). SecA1 and SecA2 can not functionally complement each other, suggesting that they have independent roles in the bacterium. The $\Delta secA2$ mutant of *M. smegmatis* has several altered phenotypes when compared to the wild type organisms, including hypersensitivity to sodium azide, an inhibitor of ATPase activity, and a growth defect in rich growth medium (14). This demonstrates that SecA2 is functional in *M. smegmatis*. For the other examples of Gram-positive pathogens with multiple copies of

SecA, it has also been shown that one copy represents the essential, “housekeeping” SecA, while the other SecA is non-essential. We hypothesized that the accessory SecA2 of *M. tuberculosis* would function in virulence. The investigations into this hypothesis are discussed later.

Substrates of the accessory SecA2 export pathway

There is evidence that SecA2 promotes the export of a specific subset of mycobacterial proteins. Gibbons *etal* used a proteomic approach to identify SecA2 dependent proteins in the cell wall of *M. smegmatis* (49). Comparing the protein profiles of cell wall and cell membrane preparations from wild type *M. smegmatis* and a $\Delta secA2$ mutant using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), revealed two proteins whose export to the cell surface is dependent on SecA2. Proteomics identified these proteins to be two lipoproteins. Homology predicts that these are sugar-binding lipoproteins. These lipoproteins also have traditional Sec signal sequences. These proteins have no homologs in *M. tuberculosis*, yet when they are expressed in *M. tuberculosis*, they are recognized by SecA2 (49). It is important to note that there are many lipoproteins in both *M. smegmatis* and *M. tuberculosis* that are exported by SecA2 independent mechanisms, and only a small subset depend on SecA2 for export, indicating specificity in SecA2 dependence.

A similar proteomics study by Braunstein *etal* (15), examined the proteins released into the culture filtrate in wild type *M. tuberculosis* and in a $\Delta secA2$ mutant by 2D-PAGE. Three proteins show diminished export in a $\Delta secA2$ mutant, including the antioxidant enzymes superoxide dismutase (SodA) and catalase-peroxidase (KatG).

Antioxidant enzymes function in virulence to prevent bacteria from the harmful effects of the oxidative burst. In contrast to the SecA2 dependent lipoproteins found in *M. smegmatis*, SodA and KatG of *M. tuberculosis* are secreted via SecA2 but lack any recognizable signal peptide (15). Interestingly, *L. monocytogenes* also exports a superoxide dismutase (MnSod) lacking a signal peptide in a SecA2 dependent manner (2). The MnSod of *L. monocytogenes* is also an important virulence factor (2). Given that the identified SecA2 substrates of *M. tuberculosis* have a potential role in pathogenesis, we hypothesized that SecA2 of *M. tuberculosis* would also be important for virulence. We investigate the role of SecA2 in the pathogenesis of *M. tuberculosis* in Chapter 2.

The SecA2 dependent proteins identified in *M. tuberculosis* and *M. smegmatis* demonstrate heterogeneity in substrates, some that contain traditional signal peptides and some that do not. While some of the factors that act in concert with SecA2 to promote protein export have been described for at least one organism, how SecA2 works in mycobacteria and what factors it interacts with remain to be elucidated. In the Gram-positive pathogen *Streptococcus gordonii*, SecA2 works with an accessory copy of SecY, SecY2, to promote export (8, 117). However, in mycobacteria there are no accessory copies of SecY, SecE, or SecG. We propose several potential models for the role of SecA2 in protein export, which are depicted in Fig. 1.3. As mentioned, SecA functions as a dimer. The first model is one in which SecA2 also functions as a homodimer, to promote the export of a subset of proteins independently of SecA1. In a second model, SecA2 would interact with SecA1 to form a heterodimer, and this complex would recognize a specific subset of proteins. In the third model, SecA2 combines with a set of

General Secretion (Sec) Pathway

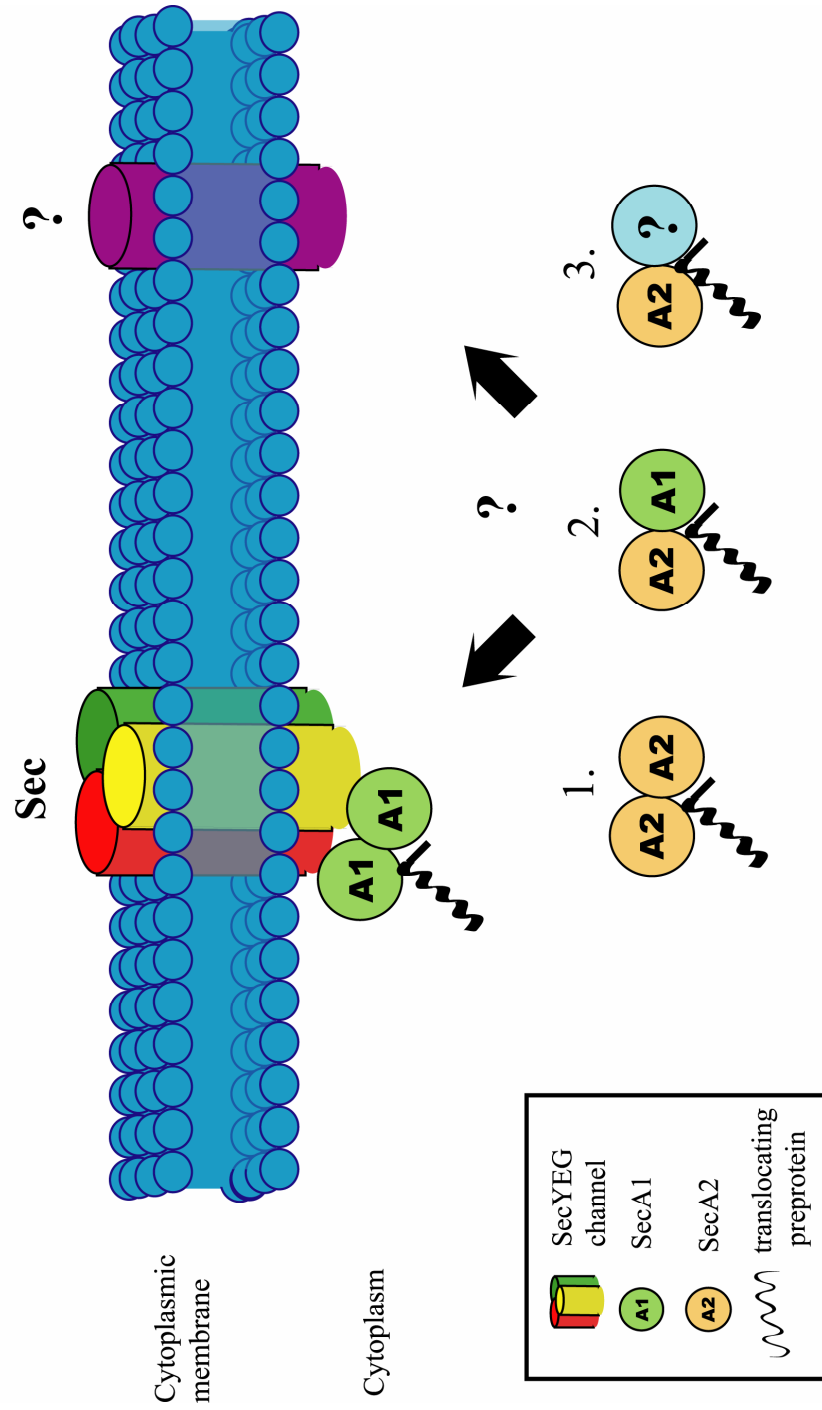


Fig. 1.2. The general (Sec) and accessory secretion pathways. The Sec pathway is comprised of the membrane bound SecYEG channel, through which preproteins are transported. The ATPase SecA1 functions as a dimer to bind and translocate the preprotein through SecYEG. The location and function of SecA2 is unclear. SecA2 may function as 1) a homodimer, 2) as a heterodimer with SecA1, or 3) in concert with as yet undefined factors to promote protein export. SecA2 may interact with the general SecYEG pore, or may direct protein export through an alternative channel.

as yet undescribed proteins to promote export. It is also unknown whether or not SecA2 and any complexes it forms interact with the SecYEG channel, or with a novel membrane complex to facilitate export.

There may in fact be multiple roles for SecA2 in promoting protein export: one in which SecA2 promotes the export of a subset of signal-peptide containing proteins, such as those identified in *M. smegmatis*, and another in which SecA2 promotes the export of substrates which lack a conventional signal peptide, such as those identified in *M. tuberculosis*. SecA2 may work in conjunction with SecA1 or SecYEG to promote a subset of proteins that contain traditional signal peptides. Given that there is only a small subset of signal-sequence containing proteins that are SecA2 dependent, proteins may span a continuum from being exclusively SecA1 dependent to exclusively SecA2 dependent. Those in the middle of the spectrum would be proteins dependent on both SecA1 and SecA2 for export. For the non-signal peptide bearing proteins, SecA2 may function to export a signal-peptide containing protein to the cell envelope, which is itself a component of a novel secretion system. There must be an undefined characteristic of either the signal peptide, the mature protein domain, or in the combination of the two that determines SecA2 dependence. For the SecA2 substrate GspB of *S. gordonii*, several of these SecA2 targeting determinants have been described, but for the substrates of SecA2 in mycobacteria they still have yet to be determined (7, 9). Studies currently underway in our laboratory hope to elucidate the mechanism by which SecA2 promotes export and interacting proteins.

The SecA2 export pathways of the Gram-positive pathogens *L. monocytogenes*, *S. gordonii*, and *S. parasanguis*, and of *M. tuberculosis* and *M. smegmatis* represent a

highly specialized route of protein export. In each of these organisms there is a very small subset of proteins that are exported via SecA2, ranging from 17 known substrates in *L. monocytogenes* to one, GspB, in *S. gordonii* (8, 62, 117). These SecA2 dependent proteins include examples of those with signal peptides and those without signal peptides, as well as include examples localized to the bacterial cell and examples that are secreted.

The role of the *M. tuberculosis* SecA2 pathway in virulence

SecA2 plays a distinct role in the virulence of *L. monocytogenes* (62). SecA2 may also contribute to the pathogenesis of *S. parasanguinis* and *S. gordonii*, given that the identified substrates of the SecA2 export pathway in these organisms are predicted to function in virulence (62). In *M. tuberculosis*, we are just beginning to understand how SecA2 promotes virulence. Braunstein *et al* (15) demonstrated that an *M. tuberculosis* $\Delta secA2$ mutant is attenuated for virulence in a whole animal model of infection using tail vein injection.

Summary of Thesis Findings

In this work, we expanded studies on the role of SecA2 in the virulence of *M. tuberculosis*. In Chapter 2, we will present evidence that the $\Delta secA2$ mutant of *M. tuberculosis* is defective for replication in macrophages. Compared to macrophages infected with wild type *M. tuberculosis*, cells infected with the $\Delta secA2$ mutant show a more robust immune response, as demonstrated by increased production of the proinflammatory cytokines TNF- α and IL-6, and RNI. These host factors are controlled

by TLR signaling through MyD88. Our data suggests that the role of SecA2 in virulence is to export factors which modulate the host immune response, therefore promoting virulence. In Chapter 3, we demonstrate that the attenuated phenotype of the $\Delta secA2$ mutant in macrophages depends on host defense mechanisms controlled by the host signaling adaptor MyD88 and by the proinflammatory cytokine TNF- α . Wild type *M. tuberculosis* is resistant to host effects mediated by MyD88/TNF- α , and the $\Delta secA2$ mutant is more susceptible to these effects. This suggests that the role of SecA2 in promoting virulence in *M. tuberculosis* is to protect the bacterium against these MyD88/TNF- α dependent host immune effectors. In Chapter 4, we reveal a role for SecA2 in promoting the proper cell surface structure of *M. tuberculosis*, a finding that may have implications on the biology of the organism. Many cell surface associated factors of *M. tuberculosis* have a role in immune modulation and promoting virulence. Perhaps the altered surface of the $\Delta secA2$ mutant contributes to its attenuation due to mislocalization of a SecA2 dependent-TLR/MyD88 agonist.

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CHAPTER 2

The SecA2 secretion factor of *Mycobacterium tuberculosis* promotes growth
in macrophages and inhibits the host immune response

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The SecA protein is present in all bacteria, and it is a central component of the general Sec-dependent protein export pathway. An unusual property of *Mycobacterium tuberculosis* is the presence of two SecA proteins: SecA1, the essential “housekeeping” SecA, and SecA2, the accessory secretion factor. Here we report that a $\Delta secA2$ mutant of *M. tuberculosis* was defective for growth in the early stages of low dose aerosol infection of C57BL/6 mice, a time during which the bacillus is primarily replicating in macrophages. Consistent with this *in vivo* phenotype, we

found that the $\Delta secA2$ mutant was defective for growth in macrophages from C57BL/6 mice. The $\Delta secA2$ mutant was also attenuated for growth in macrophages from $phox^{-/-}$ mice and from $NOS2^{-/-}$ mice. These mice are defective in the reactive oxygen intermediate (ROI) generating phagocyte oxidase or the reactive nitrogen intermediate (RNI) generating inducible nitric oxide synthase, respectively. This indicated a role for SecA2 in the intracellular growth of *M. tuberculosis* that is independent of protecting against these ROI or RNI. Macrophages infected with the $\Delta secA2$ mutant produced higher levels of TNF- α , IL-6, RNI and IFN- γ induced MHC class II. This demonstrated a function for *M. tuberculosis* SecA2 in suppressing macrophage immune responses, which could explain the role of SecA2 in intracellular growth. Our results provide another example of a relationship between *M. tuberculosis* virulence and inhibition of the host immune response.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is one of the most successful bacterial pathogens of all time. The global burden of tuberculosis is at its highest level ever and includes an increasing number of multiple drug resistant *M. tuberculosis* infections (74). Novel anti-tuberculosis prevention and treatment measures are needed to control this health crisis, and a complete understanding of *M. tuberculosis* pathogenesis will help achieve this goal.

M. tuberculosis is inhaled and taken up by unactivated host macrophages where it is able to replicate and survive. Much remains to be learned about how *M. tuberculosis* avoids host defenses in these macrophages. The processes involved are likely complex and multifactorial. Among the properties implicated in *M. tuberculosis* survival in macrophages is the ability of the bacillus to

block acidification of the phagocytic vacuole, to block phagosome-lysosome fusion, and to resist reactive oxygen and nitrogen intermediates (ROI and RNI) (34, 67).

The early innate immune response to *M. tuberculosis* includes Toll-like receptor (TLR) stimulation of macrophages (35, 55). TLR signaling leads to the secretion of RNI and pro-inflammatory cytokines that include TNF- α and IL-6. Later in infection, activation of host macrophages by an adaptive TH1 immune response occurs leading to the inhibition of intracellular growth and control of *M. tuberculosis* infection. IFN- γ is an important mediator of this response; it upregulates antimycobacterial processes and antigen presentation by macrophages (22). One of the antimycobacterial effectors induced by IFN- γ is RNI, which is produced by the inducible nitric oxide synthase (NOS2), although other IFN- γ -dependent effector mechanisms exist (13, 39, 40). TNF- α is another cytokine that plays an integral role in host control of *M. tuberculosis* infection (6, 8, 23). Among the many properties reported for TNF- α is that it can synergize with IFN- γ to induce potent antimycobacterial activity of macrophages (13, 21, 22)

It is increasingly apparent that *M. tuberculosis* can limit the host immune response and macrophage activation, and that this inhibition is likely to be important to survival in macrophages (34). Although dependent on experimental conditions, there are several reports of macrophages infected with more virulent *M. tuberculosis* producing lower levels or activities of TNF- α and/or RNI in comparison to macrophages infected with less virulent or attenuated mycobacteria (2, 7, 20, 58, 65). In a more direct fashion *M. tuberculosis* has been shown to suppress expression of *Escherichia coli* induced pro-inflammatory IL-12 (45). *M. tuberculosis* is also known to inhibit IFN- γ upregulation of genes including the major histocompatibility complex (MHC) class II genes (25, 43, 50, 53). Inhibition of MHC class II reduces antigen presentation which has implications for development of an effective TH1 response *in vivo*.

A common theme in bacterial pathogenesis is the importance of protein export and secretion pathways of the pathogen to virulence. These pathways export proteins beyond the cytoplasm to the cell surface or secrete proteins out of the bacterium. Both surface and secreted proteins are exposed to the external environment. As a result, these proteins are ideally positioned to protect the bacterium from macrophage attack or to modify the host immune response to the bacillus (14, 36). Like all bacteria, *M. tuberculosis* has the conventional Sec pathway and Sec proteins for exporting proteins beyond the cytoplasm (36). More unusual is the presence of two SecA proteins (SecA1 and SecA2) in *M. tuberculosis* (9). We have shown that the SecA2 protein of *M. tuberculosis* is an accessory secretion factor that promotes secretion of a subset of proteins that include superoxide dismutase (SodA) and catalase-peroxidase (KatG) (10). Both of these enzymes detoxify oxygen radicals: SodA converts superoxide to hydrogen peroxide and oxygen and KatG converts hydrogen peroxide to water and oxygen. KatG is also able to break down peroxynitrite which is a dangerous reaction product of superoxide and nitric oxide (72). SecA2 contributes to the virulence of *M. tuberculosis* as shown previously using a $\Delta secA2$ mutant of *M. tuberculosis* in a high-dose murine model of tuberculosis (10).

Here, we describe our studies of the $\Delta secA2$ mutant of *M. tuberculosis* in an aerosol model of murine infection and in murine bone marrow derived macrophages. Our results with the two models were consistent and revealed a role for SecA2 in promoting *M. tuberculosis* growth early in murine infection and in unactivated macrophages. Using macrophages from $phox^{-/-}$ and $NOS2^{-/-}$ mice we further showed that the role of SecA2 is not simply explained by a failure to resist ROI produced by the phagocyte oxidase or RNI produced by the inducible nitric oxide synthase. In comparing macrophage responses to infection with the $\Delta secA2$ mutant or parental H37Rv *M. tuberculosis*, we discovered that macrophages infected with the $\Delta secA2$ mutant were more

activated on the basis of several criteria: increased TNF- α , IL-6, RNI, and IFN- γ regulated MHC class II. This indicated a role for SecA2 in *M. tuberculosis* inhibition of the immune response which is likely important to survival in the host and pathogenesis.

Materials and Methods

Bacterial Strains and Growth Conditions

The *Mycobacterium tuberculosis* strains used in this study H37Rv, mc²3112 (Δ secA2 mutant, stock derived from a single colony) and mc²3116 (Δ secA2, attB::secA2) (10) were grown in Middlebrook 7H9 broth (Difco) with 0.2% glycerol, 1X ADS (albumin dextrose saline) and 0.05% Tween 80 (Tw). When appropriate, the media was supplemented with the antibiotic kanamycin (20 μ g/ml).

Animals

Female C57BL/6 mice were purchased from Charles River Laboratories. p47^{phox}^{-/-} mice were generated and maintained as described previously (4). gp91^{phox}^{-/-} mice and NOS2^{-/-} mice were purchased from Jackson Laboratories. All mice were housed in sterile microbarrier cages and were given autoclaved food and water *ad libitum*. Both strains of phox^{-/-} mice were maintained on an antibiotic oral suspension of sulfamethoxazole (200mg/5ml) and trimethoprim (40mg/5ml) (Hi-Tech Pharmacal) in their water to prevent opportunistic infections.

Aerosol Infection

Female C57BL/6 mice ages 38-45 days were used for the aerosol studies. The *M. tuberculosis* strains were cultured to mid-log phase ($OD_{600} \sim 0.5-1.0$). The cultures were washed one time and resuspended in phosphate buffered saline (PBS) with 0.05% Tween 80 (PBS-Tw) to a concentration of 1×10^7 CFU (Colony Forming Units)/ml. The bacterial suspension was placed into the nebulizer jar of a whole body exposure aerosol chamber (Mechanical Engineering Workshop, Madison, WI). Mice were exposed for 15 minutes with a chamber purge time of 20 minutes. At specific timepoints, four mice were sacrificed from each group by CO₂ asphyxiation and the lungs, livers and spleens removed and homogenized in PBS-Tw with 100ng/ml cycloheximide and 50 μ g/ml carbenicillin. The homogenates were plated onto 7H10 ADS glycerol plates with 10mg/ml cycloheximide for CFU enumeration.

Macrophage Infections

Bone marrow derived macrophages were prepared from C57BL/6, p47^{phox-/-}, gp91^{phox-/-}, and NOS2^{-/-} mice as follows. Mice were sacrificed by CO₂ asphyxiation and the femurs were removed. The cells were flushed out of the femurs using supplemented DMEM (Sigma) containing 10% heat-inactivated fetal bovine serum (HI-FBS), 2mM glutamine and 1X non-essential amino acids (NEAA). Cells were washed twice and cultured in supplemented DMEM for 6 days in the presence of 20% L929 conditioned medium (LCM). After 6 days, the cells were removed using 5mM EDTA (in PBS), washed twice with supplemented DMEM, and resuspended in supplemented DMEM with 10% LCM. The cells were then seeded into the wells of either an 8-well chamber slide (2×10^5 macrophages/well), or a 24-well plate (8×10^5 macrophages/well) and were allowed to adhere overnight before infection. In some experiments, the macrophages were pretreated for 24 hours with 10ng/ml recombinant murine IFN- γ (rmIFN- γ) (Chemicon) before

infection. *M. tuberculosis* strains were taken from mid-log growth phase and washed one time in PBS-Tw. Cells were resuspended in PBS-Tw and further diluted to the appropriate CFU/ml in supplemented DMEM. The bacteria were added at the appropriate concentration to the cell monolayers to achieve a multiplicity of infection (MOI) of 1 or 10. Macrophages were infected for 4 hours, at which time the monolayers were washed three times with supplemented DMEM to remove any non-cell associated bacteria, and then fresh media with 10% LCM was added back. At various timepoints, the media was removed from the wells and the cells were lysed with 0.1% Tween 80 and vigorous pipetting. Lysates were diluted in PBS-Tw and plated onto 7H10 ADS glycerol 0.05% Tw plates for enumeration of CFU.

RNI measurements

To measure RNI we used the Griess Reagent (Molecular Probes) and followed the manufacturer's protocol. Briefly, supernatants from infected macrophage monolayers were filter-sterilized twice using a 0.2µm low-protein binding filter, and mixed 1:1 with Griess reagent. Samples were measured at 548nm, and the values converted to µM nitrite using a standard curve generated with sodium nitrite.

In Vitro Sensitivity Assays

Bacteria were grown to mid-log phase in 7H9 ADS glycerol Tw, washed, and diluted to a density of approximately 1×10^6 CFU/ml in PBS-Tw. Percent killing was determined for each strain by comparing the number of CFU from treated samples to untreated samples. The following treatments were employed: heat shock (53°C for 45 minutes) and acid pH (pH4.0 for 24 hours). *In vitro* ROI killing assays were performed using various compounds. We tested sensitivity to

hypoxanthine/xanthine oxidase (0 and 3 hours): (250 μ M hypoxanthine) (Sigma)/xanthine oxidase (0.1U/ml xanthine oxidase) (Roche) in the presence or absence of catalase (1U/ml) (Sigma), added to detoxify hydrogen peroxide generated (16, 54). Other ROI generating compounds tested include hydrogen peroxide (0, 5, 10mM for 4 hours), plumbagin (0.2mM for 3.5, 7.5, 10.5 hours), pyrogallol (2mM for 0, 1, and 4 hours), and cumene hydroperoxide (0.5mM for 0, 1 and 4 hours) (Sigma). *In vitro* sensitivity to RNI was tested using spermidine nonoate (SPER/NO) (200 μ M for 0 and 4 hours)(Alexis Biochemicals). All *in vitro* ROI and RNI assays were performed at 37°C.

Cytometric Bead Array (CBA)

Infection of bone marrow derived macrophages was performed as described above, infecting cells at MOI of 1. Supernatants were collected from the infected macrophages at 24 hours post infection and filtered twice through a 0.22 μ m low-protein binding filter and supernatants were stored at -80°C. CBA was performed using the Mouse Inflammation CBA Kit (BD Biosciences) according to the manufacturer's protocol. Samples were analyzed on a BD Biosciences FACSCalibur flow cytometer. The triplicate samples for each strain were averaged and the data is shown as the mean \pm standard deviation.

Quantitative Real Time-PCR (qRT-PCR) to measure IFN- γ responses of infected macrophages

Murine bone marrow derived macrophages were prepared and infected as described above at an MOI of 10 for 3 hours, at which time unincorporated bacteria were washed off and fresh media added back to the monolayers. After 21 hours post-infection, the media was removed from the monolayers and replaced with fresh medium containing 2ng/ml rmIFN- γ . After 15 hours of

rmIFN- γ treatment, the supernatants were removed, and the monolayers lysed with TRIzol reagent (Invitrogen). The human monocytic cell line THP-1 (ATCC TIB-202) was maintained in supplemented RPMI with 10% HI-FBS. To prepare the THP-1 cells for infection, they were washed twice in fresh RPMI with FBS, resuspended in RPMI with FBS containing 50ng/ml phorbol myristate acetate (PMA) (Sigma), and seeded into 6-well tissue culture plates at 2×10^6 cells/well. After 24 hours PMA treatment, the cells were infected at an MOI of 10 in the presence of 200ng/ml recombinant human IFN- γ (rhIFN- γ) (Pierce) for 4 hours. After uptake, the cells were washed and fresh media with rhIFN- γ was added. At 24 hours post-infection, the supernatant was removed from the infected monolayers and the cells were lysed with TRIzol. TRIzol lysates were processed for RNA extraction. RNA samples were reverse transcribed and Real-time PCR was performed using the Taq-Man sequence detection system with Absolute QPCR Mix (Applied Biosystems). Values were calculated based on standard curves generated for each gene. Normalization of samples was determined by dividing copies of MHC class II mRNA by copies of 18S rRNA. 18S probe (5'-TAMRA- CAAATTACCCACTCCCGACCG-3') and primers F (5'-GCTGCTGGCACCAGACTT-3') and R (5'- CGGCTACCACATCCAAGG-3'); murine MHC class II (I-Ab) probe (5'-FAM-CGCAGCGCATACGATATGTGACCA-TAMRA-3') and primers Rev (5'-CTGTCGTAGCGCACGTACT-3') and For (5'-GGCGAGTGCTACTTCACCA-3'); human MHC class II (HLA-DRA) probe (5'-FAM-CTGGACCCTTTGCAAGAACCCTTCCC-TAMRA-3') and primers F (5'-TCCAATGAACGGAGTATCTTGTGT-3') and R (5'-TGAGATGACGCATCTGTTGCT-3') (27, 72).

Results

The $\Delta secA2$ mutant is attenuated in the murine low-dose aerosol infection model. Previously, we demonstrated that an in-frame and unmarked $\Delta secA2$ deletion mutant of *M. tuberculosis* is attenuated in mice following high-dose intravenous administration (10). To better characterize the phenotype of the $\Delta secA2$ mutant, we used a more natural low-dose aerosol exposure model infecting C57BL/6 mice with approximately 200 bacilli to the lungs, and included earlier time points than previously analyzed. Following infection with the $\Delta secA2$ mutant or the virulent *M. tuberculosis* parent H37Rv, groups of mice were sacrificed at times post-infection and the bacterial burden in the lungs, livers and spleens was enumerated by plating homogenates for colony forming units (CFU). We observed a typical growth pattern for H37Rv in the lungs of the mice: significant growth during the early phase of infection followed by a period of persistence in which the number of bacilli remained constant over time (52) (Fig. 2.1A). The transition between the growth and persistence phases correlates with the establishment of the TH1 response, a central feature of which is macrophage activation by IFN- γ (49, 52). The $\Delta secA2$ mutant was defective in the early growth phase of the infection when compared to H37Rv. As early as nine days post-infection, the $\Delta secA2$ mutant had one log fewer bacilli in the lungs in comparison to H37Rv (Fig. 2.1A). This difference was maintained throughout the experiment, although both strains continued to grow to the day 16 time point. We also observed fewer CFU for the $\Delta secA2$ mutant in livers and spleens (0.5-1.0 log less) when compared to H37Rv (data not shown). During the latter phase of infection, the $\Delta secA2$ mutant resembled H37Rv in persisting although at the final 80 day time point the CFU burden in lungs of the $\Delta secA2$ mutant infected mice had dropped slightly. In addition to reduced bacillary load, mice infected with the $\Delta secA2$ mutant exhibited an increase in length of survival (mean survival of 372 ± 29 days) in comparison to mice infected with H37Rv (mean survival of 173 ± 16 days) (Fig. 2.1B).

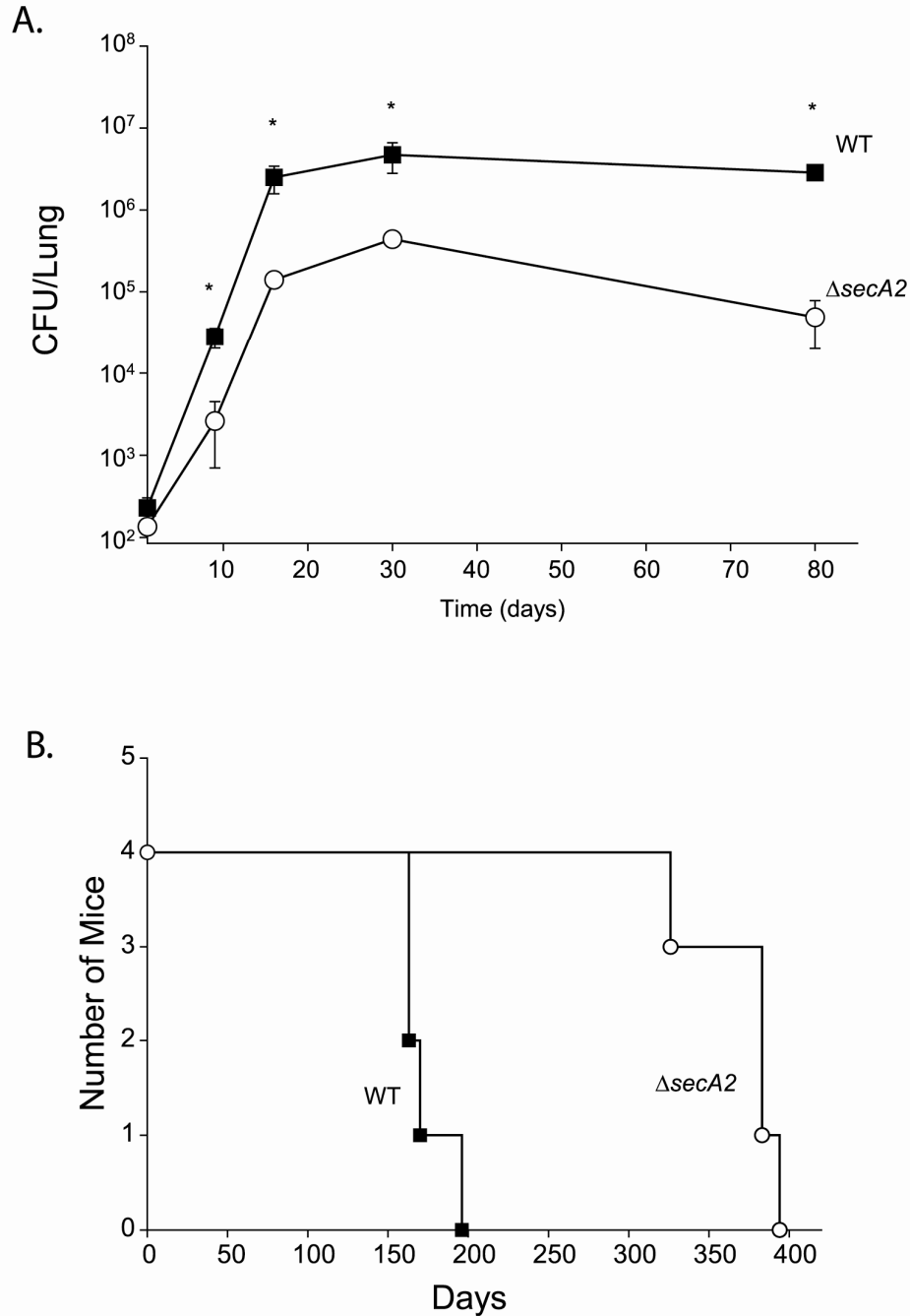


Fig. 2.1. The $\Delta secA2$ mutant of *M. tuberculosis* is attenuated in a low dose aerosol infection of C57BL/6 mice. Immunocompetent C57BL/6 mice were exposed in a whole body aerosol chamber to either *M. tuberculosis* H37Rv (■) or the $\Delta secA2$ mutant (○), each mouse receiving approximately 200 CFU (Colony Forming Units) per lung. (A) Mice were sacrificed at various timepoints post-infection and the growth of the strains assessed by plating for CFU from lung homogenates. The results are representative of two experiments. * p values compared to the $\Delta secA2$ mutant are $p < 0.005$ by Student's t test. (B) Groups of 4 mice infected with either strain were monitored for survival.

The $\Delta secA2$ mutant has an attenuated phenotype in unactivated macrophages but not in activated macrophages. The observed *in vivo* phenotype of the $\Delta secA2$ mutant indicated a role for SecA2 in the early phase of infection, during which *M. tuberculosis* is growing in macrophages. We hypothesized that the $\Delta secA2$ mutant is defective for growth in unactivated macrophages. We tested this hypothesis by comparing the ability of the $\Delta secA2$ mutant and H37Rv to replicate within unactivated bone marrow derived macrophages from C57BL/6 mice. The macrophages were infected *ex vivo*, and after four hours uptake the monolayers were washed and fresh media added back. The growth of each strain was evaluated over a five day period by plating macrophage lysates for viable CFU. The $\Delta secA2$ mutant showed diminished growth in the macrophages and displayed up to one log less CFU by Day 5 (Fig. 2.2A). The intracellular growth defect of the $\Delta secA2$ mutant was complemented by introduction of a wild-type copy of *secA2* on an integrating plasmid, which indicated that the mutant phenotype was due to the absence of *secA2* (Fig. 2.2B).

In contrast to unactivated macrophages which are permissive for *M. tuberculosis* growth, activated macrophages inhibit *M. tuberculosis* growth due to enhanced antimycobacterial activities (13, 21, 22). We compared the $\Delta secA2$ mutant and H37Rv in murine bone marrow derived macrophages activated by 24 hours pretreatment with recombinant murine IFN- γ (rmIFN- γ). In the IFN- γ treated macrophages, H37Rv failed to grow but survived over time, and the $\Delta secA2$ mutant behaved similarly (Fig. 2.3A). Infection of unactivated macrophages was performed in parallel and again revealed an intracellular growth defect of the $\Delta secA2$ mutant (Fig. 2.3B).

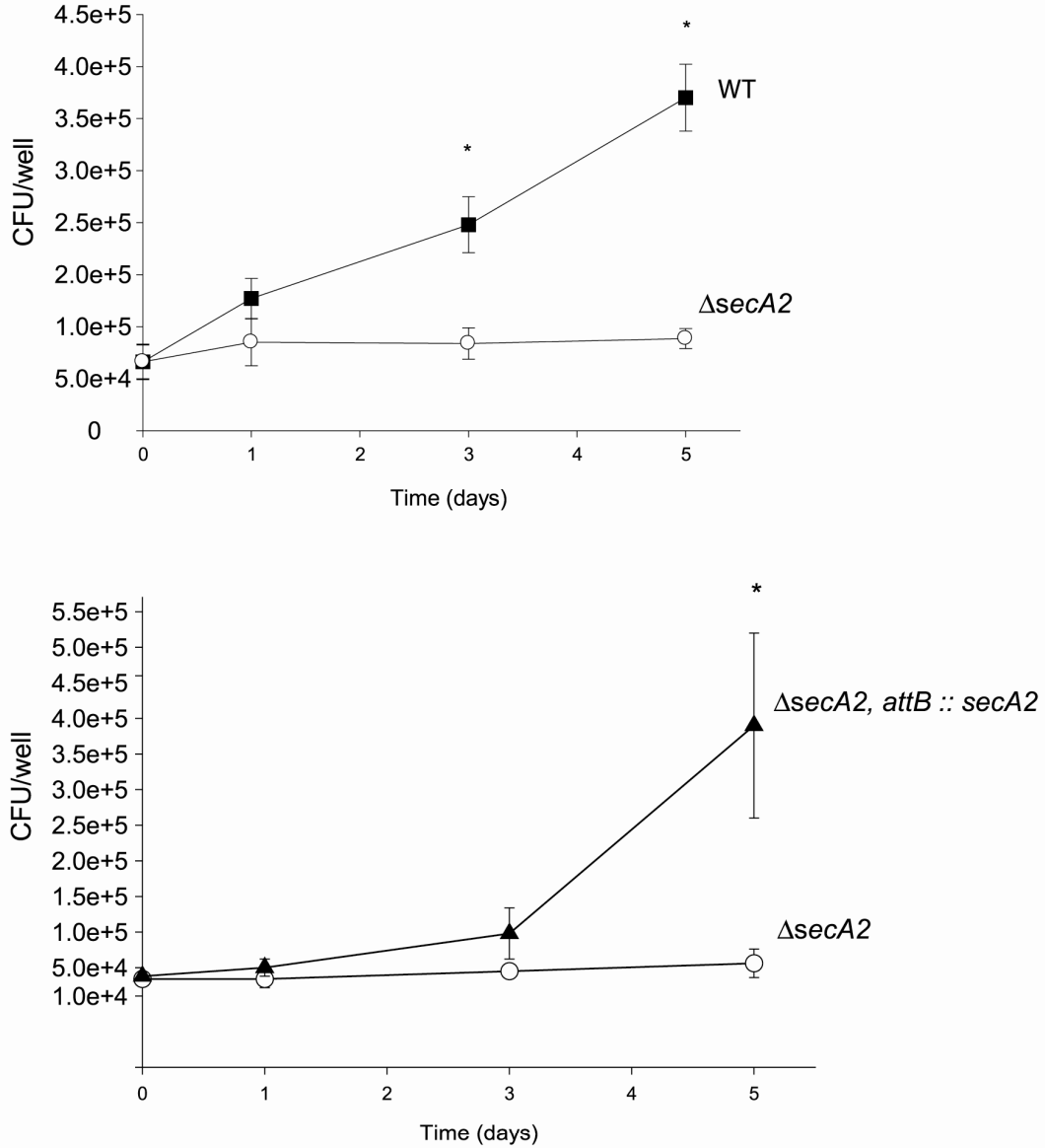
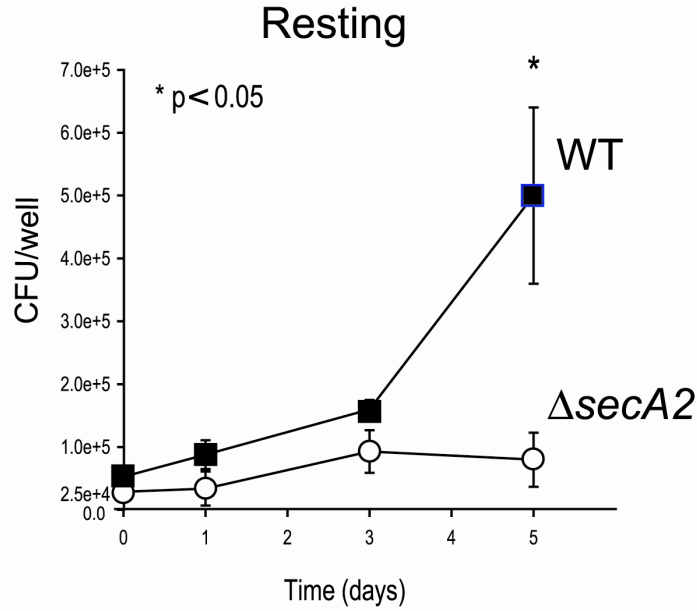


Fig. 2.2. The $\Delta secA2$ mutant of *M. tuberculosis* is attenuated in unactivated murine bone marrow derived macrophages. Unactivated murine bone marrow derived macrophages from C57BL/6 mice were infected at MOI=1 with (A) *M. tuberculosis* H37Rv (■) or the $\Delta secA2$ mutant (○) and (B) the $\Delta secA2$ mutant (○) or the $\Delta secA2$ complemented mutant ($\Delta secA2, attB::secA2$) (▲). CFU were determined by plating macrophage lysates at various timepoints post-infection. The infection was performed with triplicate wells for each strain per timepoint, and the error bars represent mean \pm standard deviation for the triplicate wells. Data shown are representative of more than 20 experiments for wild-type and the $\Delta secA2$ mutant, and five experiments for the complemented strain. * p values compared to $\Delta secA2$ are $p < 0.05$ by Student's t test.

A.



B.

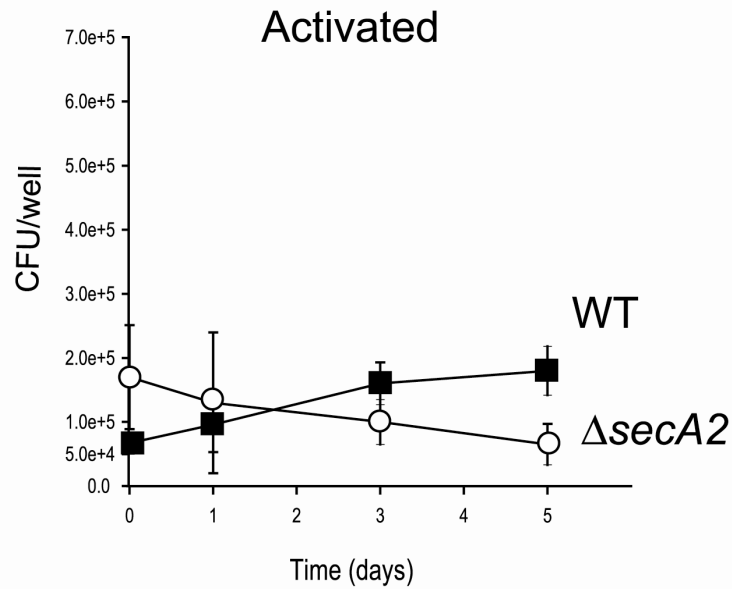


Fig. 2.3. Survival of the $\Delta secA2$ mutant in IFN- γ treated murine bone marrow derived macrophages. In parallel experiments we examined survival and growth of the $\Delta secA2$ mutant and H37Rv in (A) activated and (B) unactivated murine bone marrow derived macrophages from C57BL/6 mice. Macrophages were activated by 24 hour pretreatment with 10ng/ml rmIFN- γ . The macrophages were infected at MOI=1 with H37Rv (■) or $\Delta secA2$ mutant (○) as described. Graphs are shown as the average of 5 experiments \pm SEM. * p values compared to $\Delta secA2$ mutant are $p < 0.05$ by Student's t test.

These results revealed a role for SecA2 in promoting *M. tuberculosis* growth in unactivated macrophages but no apparent role for SecA2 in *M. tuberculosis* survival in activated macrophages. They are consistent with the pattern of growth *in vivo*, where the $\Delta secA2$ mutant exhibited reduced growth in the lungs of infected mice in the early growth phase of infection and then behaved similarly to H37Rv after the onset of the TH1 immune response and concomitant macrophage activation.

The $\Delta secA2$ mutant has an attenuated phenotype in oxidative burst deficient macrophages.

We previously reported that the $\Delta secA2$ mutant of *M. tuberculosis* is defective in secreting the antioxidant enzymes SodA and KatG (10). *M. tuberculosis* is relatively resistant to ROI (13) which could be an important property for intracellular survival in the face of the macrophage oxidative burst, and the SecA2-dependent secreted SodA and KatG are among the *M. tuberculosis* molecules implicated in this resistance (67). We tested whether the sole role of SecA2 in macrophage growth is to protect the bacillus from the oxidative burst during intracellular infection. Bone marrow derived macrophages from p47^{phox}^{-/-}, gp91^{phox}^{-/-}, and C57BL/6 mice were prepared and infected in parallel with the $\Delta secA2$ mutant or H37Rv. These phox^{-/-} mice lack different components of the phagocyte oxidase complex, which is responsible for the oxidative burst of macrophages (46). In these oxidative burst deficient macrophages, the $\Delta secA2$ mutant showed the same growth defect as observed in wild-type C57BL/6 macrophages (Fig. 2.4A and 2.4B). This indicated that SecA2 contributes to intracellular growth even in the absence of an oxidative burst. Consequently, SecA2 must have a function in intracellular growth other than just protecting the bacillus from ROI generated during the oxidative burst.

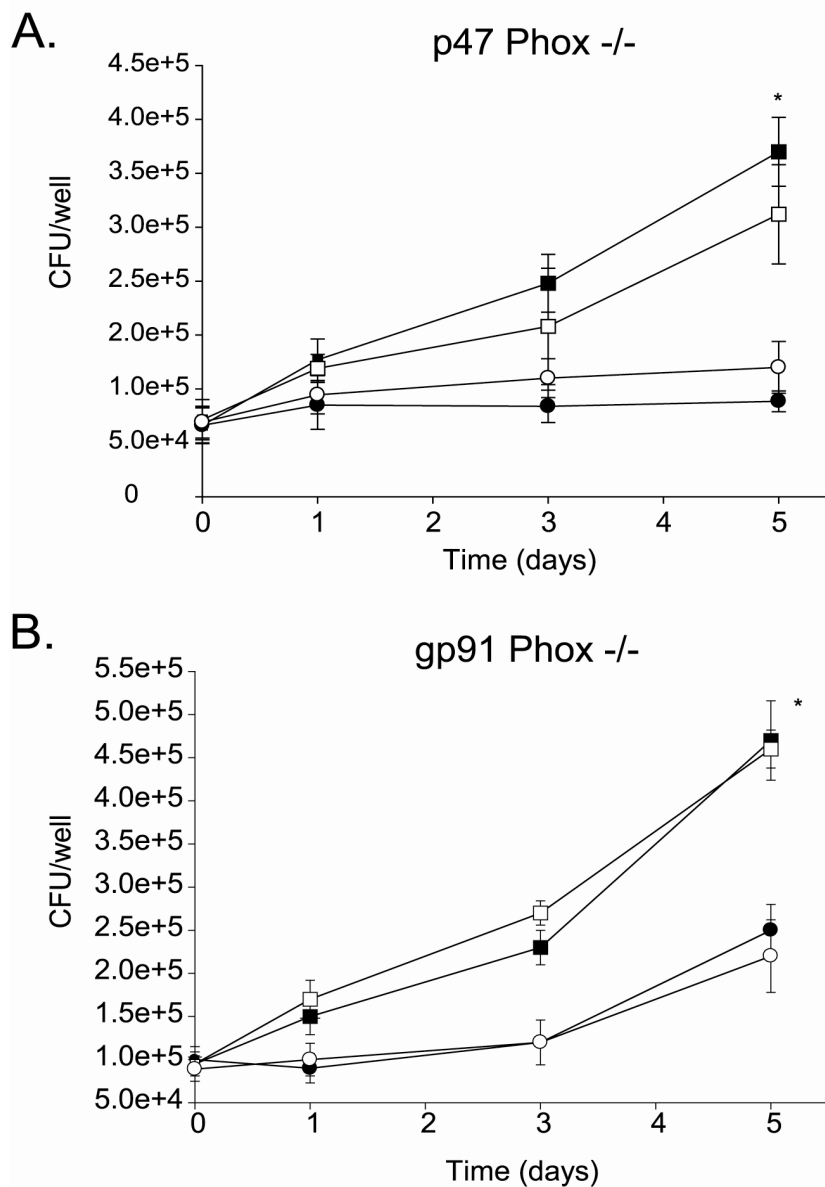


Fig. 2.4. The $\Delta secA2$ mutant is attenuated in bone marrow derived macrophages from mice defective in components of the phagocyte oxidase (phox $-/-$). Unactivated bone marrow derived macrophages from C57BL/6 mice and from either (A) p47phox $-/-$ or (B) gp91phox $-/-$ were infected in parallel at MOI=1 with *M. tuberculosis* H37Rv (in C57BL/6 macrophages= ■ ; in phox $-/-$ macrophages □) and the $\Delta secA2$ mutant (in C57BL/6 macrophages= ● ; in phox $-/-$ macrophages ○). Graphs are shown as the average of multiple experiments (six for p47phox $-/-$, three for gp91phox $-/-$) \pm standard error of the means (SEM). * p values compared to $\Delta secA2$ mutant are p < 0.05 by Student's t test.

As an independent assessment of the role of SecA2 in protecting against oxygen radicals, we compared *in vitro* sensitivity of the $\Delta secA2$ mutant and H37Rv to a variety of ROI species. Among the compounds tested were pyrogallol and hypoxanthine/xanthine oxidase; both of these treatments generate extracellular superoxide. Plumbagin (cytoplasmic superoxide generator), hydrogen peroxide, and cumene hydroperoxide were also tested. In all cases, the ROI treatments showed an equivalent degree of killing for the $\Delta secA2$ mutant and H37Rv (data not shown). We further tested the strains for their sensitivity to other stresses including acid pH and heat shock and again saw no differences in killing. The unaltered *in vitro* sensitivities of the $\Delta secA2$ mutant in combination with the data from $phox^{-/-}$ macrophages suggested that the role of SecA2 in promoting growth in macrophages is not solely to protect the bacteria from damaging ROI and involves other mechanisms.

Macrophages infected with the $\Delta secA2$ mutant release higher levels of pro-inflammatory cytokines and RNI. To determine if the SecA2-dependent secretion pathway contributes to *M. tuberculosis* inhibition of host immune responses, we compared cytokine production by macrophages infected with the $\Delta secA2$ mutant or H37Rv. Bone marrow derived macrophages from C57BL/6 mice were infected as before and 24 hours post-infection supernatants were assayed for cytokine production using the Cytometric Bead Assay (BD Biosciences). Macrophages infected with the $\Delta secA2$ mutant produced more TNF- α and IL-6 than those infected with H37Rv (Fig. 2.5), with an average difference of 2.5 fold for TNF- α and 3.5 fold for IL-6. Introduction of an integrated plasmid expressing wild-type *secA2* complemented the increased cytokine production phenotype of the $\Delta secA2$ mutant.

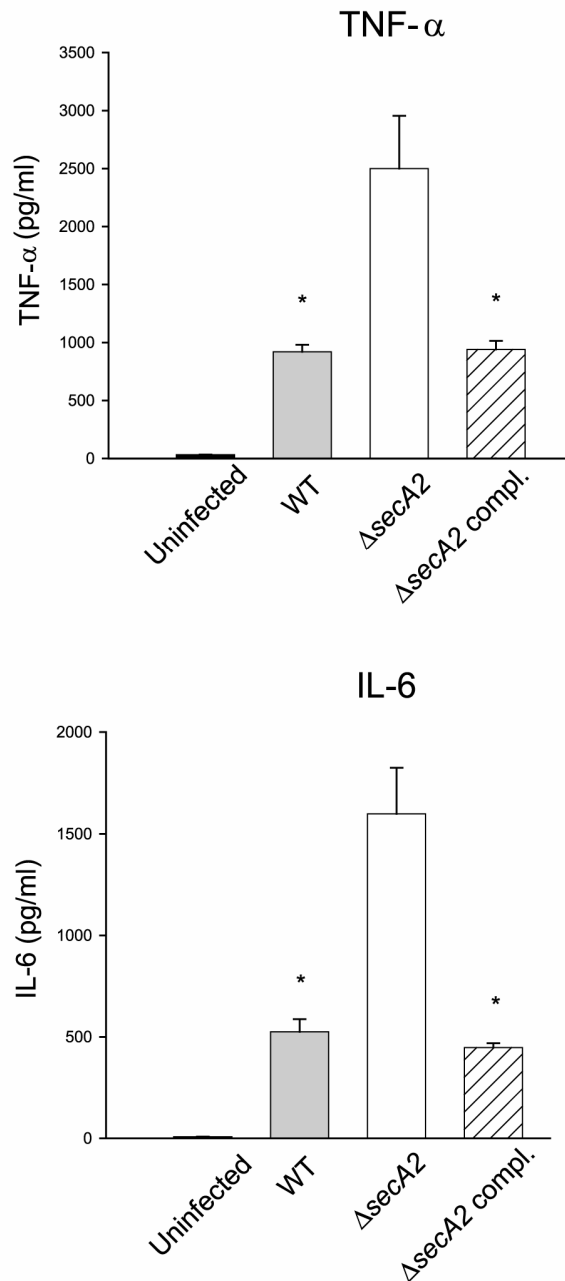


Fig. 2.5. Cytokine release from infected bone marrow derived macrophages. Cytokines were measured using the Cytometric Bead Array kit (BD Biosciences). Bone marrow derived macrophages from C57BL/6 mice were infected at MOI=1 in triplicate. Supernatants were collected at 24 hours post-infection and assayed for cytokines. Graphs present levels of cytokine for (A) TNF- α or (B) IL-6 in uninfected macrophages (black bar), or those infected with H37Rv (grey bar), the Δ secA2 mutant (white bar), or the Δ secA2 complemented strain (hatched bar), with error bars depicting standard deviation (SD) of triplicate samples. Data shown are representative of eight experiments. * p values compared to Δ secA2 mutant are p < 0.05 by Student's t test.

We also assayed production of RNI in the supernatants of infected macrophages using the Griess Reagent which measures nitrite, the oxidation product of nitric oxide. Measurements were taken at 48, 72 and 96 hours post-infection. The macrophages infected with the $\Delta secA2$ mutant produced more RNI at all time points compared to macrophages infected with H37Rv (Fig. 2.6A, data shown for 72 hours), with an average difference of 1.5 fold. In the same experiments at 0, 4 and 24 hours post-infection, we also measured the levels of ROI in the cells, and saw no differences in level of ROI between cells infected with the $\Delta secA2$ mutant or H37Rv (data not shown). We also tested the level of RNI production by infected cells pretreated with IFN- γ 24 hours prior to infection which upregulates RNI production. As expected, there was an overall increase in RNI production in cells pretreated with IFN- γ compared to untreated cells. At 24 hours post-infection macrophages infected with the $\Delta secA2$ mutant again exhibited higher levels of RNI in the supernatant (Fig. 2.6B). The increased RNI production observed following infection with the $\Delta secA2$ mutant in the absence and presence of IFN- γ was complemented by introduction of a wild-type copy of *secA2*.

It is important to note that in these experiments the number of CFU in macrophage lysates following the 4 hour infection period was equivalent for both strains. The above results suggest that, in comparison to H37Rv, the $\Delta secA2$ mutant is defective in the ability to inhibit macrophage production of immunostimulatory molecules. The end result being more highly activated macrophages upon infection with the $\Delta secA2$ mutant.

The $\Delta secA2$ mutant has an attenuated phenotype in NOS2^{-/-} macrophages. Given the antimycobacterial activity of RNI, we considered the possibility that the increased levels of RNI observed were alone responsible for the growth defect of the $\Delta secA2$ mutant in macrophages. To

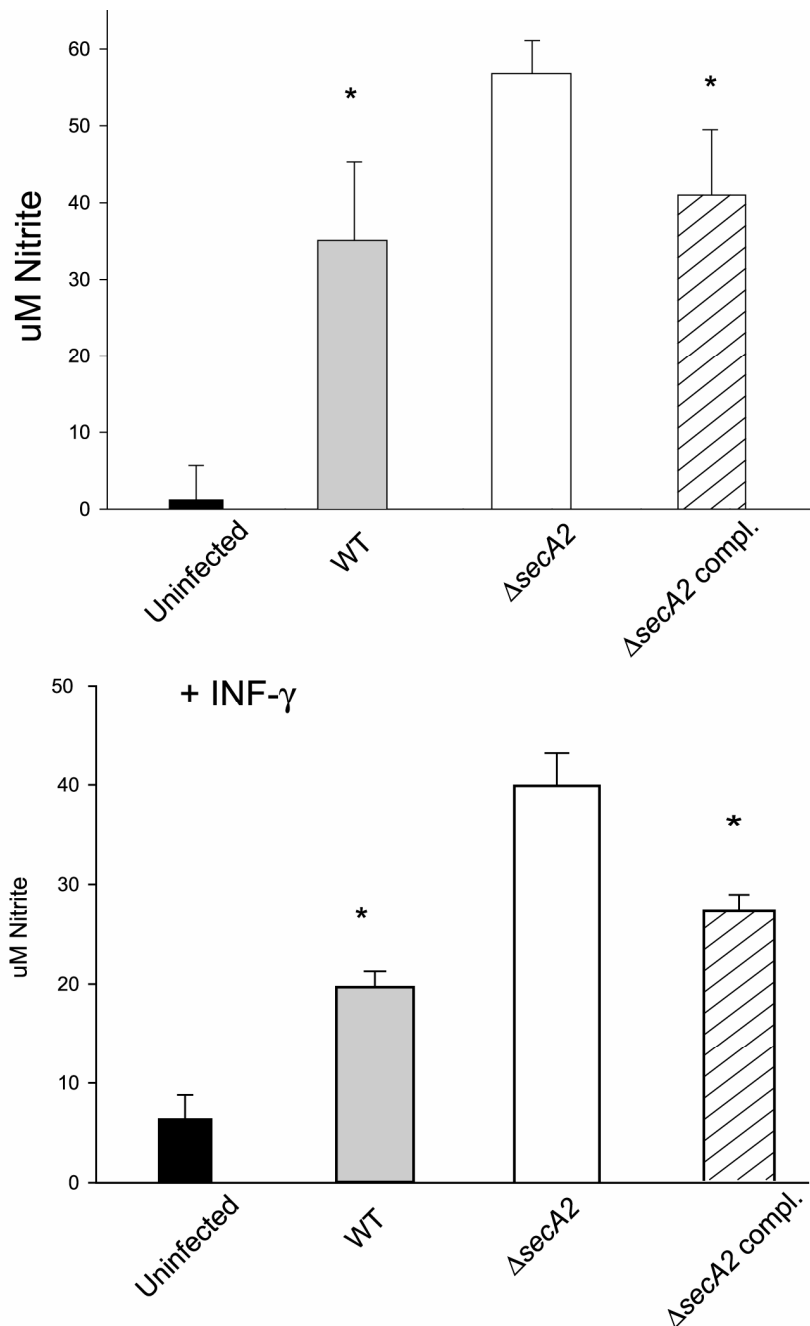


Fig. 2.6. Reactive Nitrogen Intermediates (RNI) produced by infected bone marrow derived macrophages. RNI was measured using the Griess reagent. Infections were performed at MOI=10 and samples were collected at 72 hours post-infection for unactivated macrophages (A) and 24 hours post-infection for IFN- γ activated macrophages (B). Bars show the average of triplicate samples for uninfected cells (black bars), or those infected with H37Rv (grey bars), the Δ secA2 mutant (white bars), or the Δ secA2 complemented strain (hatched bars), with error bars depicting standard deviation (SD) of triplicate samples. Data shown are representative of three experiments. * p values compared to secA2 mutant are p < 0.05 by Student's t test.

test this idea, we examined the growth of the $\Delta secA2$ mutant and H37Rv in unactivated macrophages from NOS2^{-/-} mice, which lack the inducible nitric oxide synthase and are defective for the nitrosative burst (38). The $\Delta secA2$ mutant still exhibited a growth defect in NOS2^{-/-} macrophages that was equivalent to the defect observed in macrophages from C57BL/6 mice (Fig. 2.7). This data indicated that the role of SecA2 in intracellular growth involves functions other than protecting against reactive nitrogen intermediates.

As was done for ROI, we also assayed *in vitro* sensitivity of the $\Delta secA2$ mutant and H37Rv to NO generated by spermadine nonoate (SPER/NO). Treatment with SPER/NO led to an equivalent degree of killing of $\Delta secA2$ mutant and H37Rv (data not shown). The *in vitro* sensitivity result taken together with the observed growth defect of the $\Delta secA2$ mutant in NOS2^{-/-} macrophages suggests that the role of SecA2 in intracellular growth is not solely to protect against RNI.

The $\Delta secA2$ mutant is associated with higher IFN- γ induced MHC class II expression.

Having demonstrated that macrophages infected with the $\Delta secA2$ mutant produced higher levels of cytokine and RNI in comparison to H37Rv infected macrophages, we set out to see if the inhibition of other macrophage responses was altered by the $\Delta secA2$ mutation. Multiple laboratories have demonstrated that *M. tuberculosis* inhibits the expression of several IFN- γ regulated genes of the host. Among these genes are the IFN- γ induced MHC class II genes required for antigen presentation (43, 50, 53). To test for a role of SecA2 in *M. tuberculosis* inhibition of IFN- γ responses, we employed two different systems previously used to study this IFN- γ inhibition: primary murine macrophages and the human monocytic cell line THP-1. Murine bone marrow derived macrophages were pre-infected with the $\Delta secA2$ mutant, H37Rv, or the

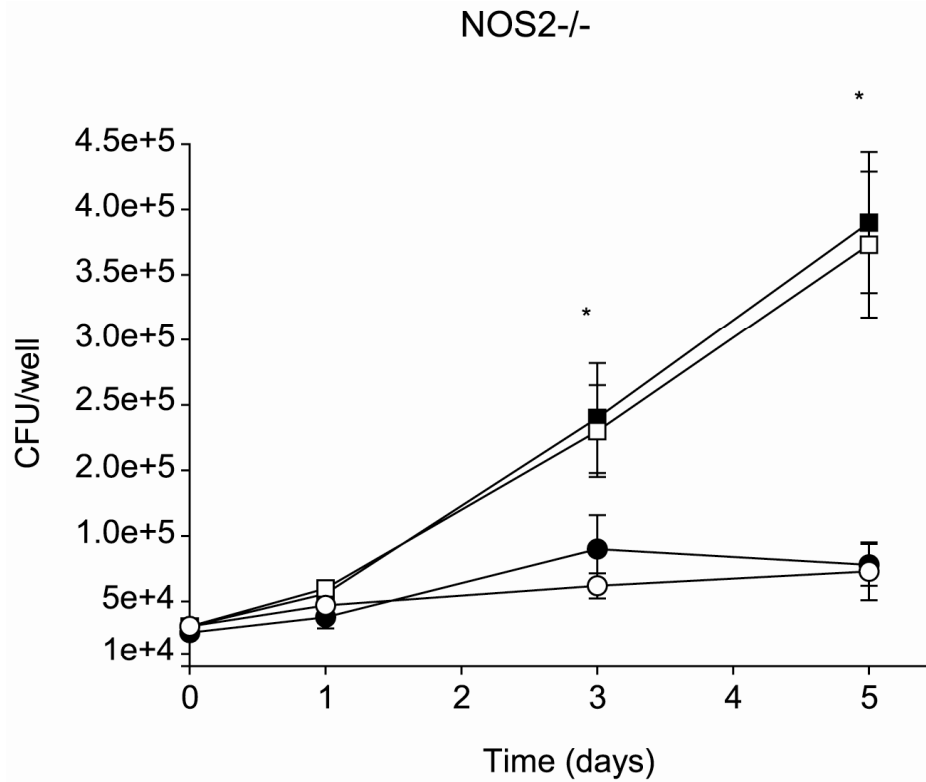


Fig. 2.7. The $\Delta secA2$ mutant is attenuated in bone marrow derived macrophages from mice defective in inducible nitric oxide synthase (NOS2). Unactivated bone marrow derived macrophages from C57BL/6 mice and from NOS2^{-/-} mice were infected in parallel at MOI=1 with *M. tuberculosis* H37Rv (in C57BL/6 macrophages= ■ ; in NOS2^{-/-} □) and the $\Delta secA2$ mutant (in C57BL/6 macrophages= ●; in NOS2^{-/-} macrophages ○) as described above. Graphs are shown as the average of four experiments \pm standard error of the means (SEM). * p values compared to $\Delta secA2$ are $p < 0.05$ by Student's t test.

complemented strain at MOI 10 for 24 hours followed by stimulation with rmIFN- γ . After 15 hours of IFN- γ treatment, expression of MHC class II (IA-b) transcript was measured by quantitative Real Time PCR (qRT-PCR). As expected, IFN- γ treatment of uninfected cells increased MHC class II transcript and H37Rv inhibited the IFN- γ induction of MHC class II. In contrast, infection with the $\Delta secA2$ mutant did not reduce the level of MHC class II to the same degree as H37Rv (Figure 2.8A). This difference between the $\Delta secA2$ mutant and H37Rv in the level of IFN- γ induced MHC class II was at least 2.0 fold greater with the $\Delta secA2$ mutant in five separate experiments ($p=0.008$ by Mann-Whitney test). Similar experiments were performed with human THP-1 cells. In these experiments rhIFN- γ was added to the cells at the time of infection with either the $\Delta secA2$ mutant, H37Rv, or complemented strain. Expression of MHC class II (HLA-DR) transcript was measured by qRT-PCR on samples collected at 24 hours post-infection. Again, infection with the $\Delta secA2$ mutant did not reduce the level of MHC class II to the same degree as H37Rv (Figure 2.8B). This difference between the $\Delta secA2$ mutant and H37Rv in the level of IFN- γ induced MHC class II was reproducible with the $\Delta secA2$ mutant exhibiting an average 1.5 fold difference in HLA-DR transcript level over five independent experiments ($p=0.00003$ by Student's t test). The phenotypes of the $\Delta secA2$ mutant in both murine macrophages and THP-1 cells were complemented by the introduction of a wild-type copy of *secA2*. This data provided another example of SecA2 functioning in the process(es) of *M. tuberculosis* inhibition of host immune responses.

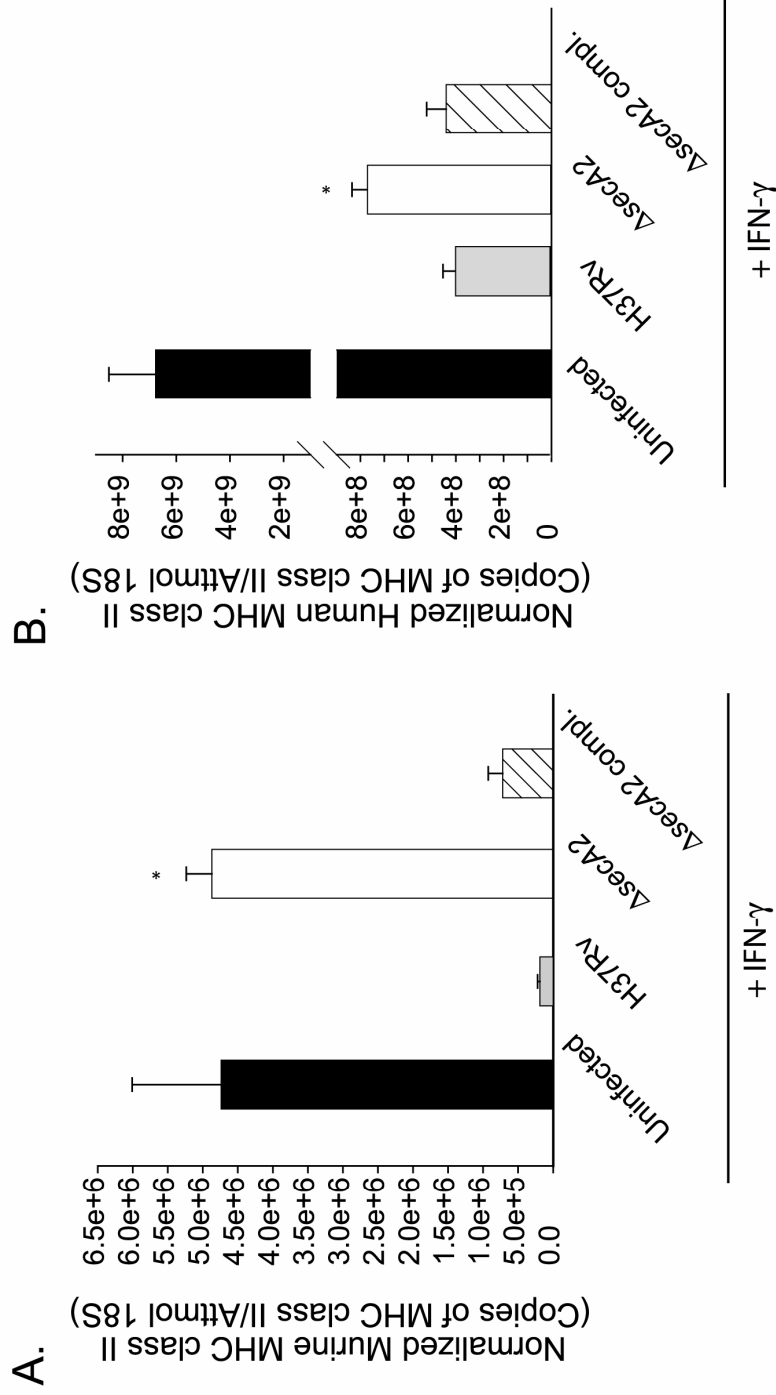


Fig. 2.8. Inhibition of MHC class II expression measured by quantitative Real-Time PCR. Host mRNA expression was determined by qRT-PCR of RNA made from murine bone marrow derived macrophages or THP-1 cells. Cells were infected with *M. tuberculosis* strains at MOI=10 and stimulated with rIFN- γ , and uninfected cells were similarly treated with rIFN- γ . For murine macrophages the cells were infected with *M. tuberculosis* strains and let sit for 21 hours prior to addition of 2ng/ml rIFN- γ . RNA was sampled at 15 hours post rIFN- γ addition. For THP-1 cells, the cells were simultaneously infected with *M. tuberculosis* and treated with 200ng/ml rIFN- γ . RNA was sampled at 24 hours post-infection. The qRT-PCR samples were normalized to an internal 18S control. Bars represent the normalized MHC class II levels of triplicate infections performed on a single day with error bars showing standard deviation. Data is shown as a representative of five experiments for each (A) murine bone marrow derived macrophages and (B) THP-1 cells. Samples are shown as uninfected (black bars), or cells infected with H37Rv (grey bars), the Δ secA2 mutant (white bars), or the complemented strain (hatched bars). * p values compared to wild type and complemented are $p < 0.003$ by Student's t test.

Discussion

The accessory secretion factor SecA2 contributes to *M. tuberculosis* virulence. Here we showed, with a natural low-dose aerosol model of infection, that as early as nine days post-infection the $\Delta secA2$ mutant had a lower bacterial burden in the lungs of mice in comparison to the parental *M. tuberculosis* H37Rv. The attenuated phenotype of the mutant occurred during the early growth phase of infection, when *M. tuberculosis* is primarily growing in unactivated macrophages. Consistent with this result, we showed that the $\Delta secA2$ mutant had a growth defect in unactivated murine bone marrow macrophages in comparison to H37Rv. It should be noted that the $\Delta secA2$ mutant does not display any significant growth defect when grown in broth media (10). In contrast to the phenotype in unactivated murine macrophages, the $\Delta secA2$ mutant did not exhibit a phenotype in IFN- γ activated macrophages. Our experiments *in vitro* with primary murine macrophages appear to reflect the phenotypes observed *in vivo* in the murine model of infection.

Since SecA2 is an accessory secretion factor, the role of SecA2 could be the proper secretion or surface localization of *M. tuberculosis* proteins required for intracellular growth. Previously, we identified SodA and KatG as proteins whose secretion into culture media depends on SecA2 (10). Since both of these proteins are antioxidants, we considered the simple hypothesis that SecA2 promotes detoxification of ROI and thereby protects the bacillus from the oxidative burst of macrophages to promote intracellular growth. Macrophage generated ROI could have direct antimicrobial effects or indirect effects involving alterations in host cell signaling pathways (44, 69). There is a precedent for exported superoxide dismutases contributing to virulence in

bacterial pathogens (5), and various experiments suggest roles for *M. tuberculosis* SodA, KatG, and a surface localized superoxide dismutase, SodC, in pathogenesis and protection against the oxidative burst of macrophages (17, 18, 41, 47, 54). However, when we tested the $\Delta secA2$ mutant in $phox^{-/-}$ macrophages, it exhibited a growth defect equivalent to that observed in wild-type C57BL/6 macrophages. In addition, our efforts to detect altered *in vitro* sensitivity of the $\Delta secA2$ mutant to ROI species, including extracellularly generated superoxide, were unsuccessful. Taken together, these experiments do not reveal a role for SecA2 in protecting against the oxidative burst and they indicate an alternate function of SecA2 in intracellular growth.

Yet, we can not entirely rule out the possibility that SecA2 contributes to ROI resistance. A role in protecting against ROI in macrophages and *in vitro* could have been masked by the other function(s) of SecA2 in intracellular growth and/or redundant ROI resistance mechanisms of *M. tuberculosis*. Furthermore, even in the absence of phagocyte oxidase there are still reactive oxygen species generated in the cell through mitochondrial electron transport. Our experiments can not rule out the possibility that mitochondrial generated ROI contribute to the phenotypes of the $\Delta secA2$ mutant.

In our studies, we also assessed the role that SecA2 plays in modulating the immune response. It is becoming evident that virulent *M. tuberculosis* inhibits innate and adaptive immune responses. In regard to innate immune responses, *M. tuberculosis* has been shown to inhibit macrophage production of IL-12 in a co-infection assay (45). In addition, there is an emerging trend that virulent *M. tuberculosis* strains elicit reduced levels of pro-inflammatory cytokines (including TNF- α and IL-6) and RNI from macrophages than less virulent *M. tuberculosis* strains. This phenomenon has been described for the hypervirulent HN878 strain of *M. tuberculosis* and the hypervirulent *mce1* mutant of *M. tuberculosis* (42, 58, 65). There are also reports of virulent *M.*

tuberculosis eliciting lower levels or activities of cytokine and RNI from macrophages than attenuated and avirulent mycobacteria (2, 7, 20, 26). However, it must be noted that for TNF- α a correlation between increased mycobacterial virulence and reduced cytokine production has not been a universal finding (12, 19, 57, 66). In regard to adaptive responses, *M. tuberculosis* inhibits macrophage responses to IFN- γ stimulation, such as the upregulation of MHC class II (25, 43, 50, 53). MHC class II and presentation of mycobacterial antigens to CD4⁺ T cells is an essential component of the adaptive immune response to *M. tuberculosis* (22).

In this study, we found that macrophages infected with the $\Delta secA2$ mutant produced significantly greater levels of TNF- α , IL-6 and RNI than macrophages infected with H37Rv. This suggested a function for SecA2 in inhibiting the innate immune response. We were unable to detect IL-12 production by our infected macrophages. The increased level of IFN- γ induced MHC class II message observed with the $\Delta secA2$ mutant versus H37Rv suggested an additional role for SecA2 in suppressing the adaptive immune response. To our knowledge this is the first report of a *M. tuberculosis* mutant with a defect in the inhibition of IFN- γ responses.

These immunomodulatory activities of SecA2 are likely important to *M. tuberculosis* virulence. All of the host molecules we identified as being regulated by SecA2 are demonstrated to contribute to the control of tuberculosis in the mouse model. Mice deficient in TNF- α , IL-6, NOS2, or CIITA (MHC class II transcriptional regulator) are all more permissive for *M. tuberculosis* infection as shown by increased bacterial burden in organs and decreased length of survival in comparison to infection of wild-type mice (6, 23, 39, 60, 62). Immunosuppression by SecA2 may influence the course of *M. tuberculosis* infection in many ways including the establishment of a permissive environment for intracellular growth in macrophages and limiting antigen presentation in the host. By infecting NOS2^{-/-} macrophages with the $\Delta secA2$ mutant, we

demonstrated that suppression of RNI was not the only important factor regulated by SecA2 for promoting intracellular growth. We believe the attenuated phenotype of the $\Delta secA2$ mutant in macrophages and mice is a reflection of an imbalance in multiple cytokines and effector mechanisms, possibly including molecules we have yet to identify.

Interestingly, all four of the molecules we found upregulated by macrophages infected with the $\Delta secA2$ mutant are associated with Toll like receptor 2 (TLR2) and Myeloid differentiation factor 88 (MyD88) signaling pathways in host cells. To varying degrees TNF- α , IL-6 and RNI are induced by mycobacterial stimulation of TLR2-dependent MyD88-dependent pathways (11, 30, 32, 48, 59, 63, 64, 70). Thus, an increased ability of the $\Delta secA2$ mutant to signal through TLR2-MyD88-dependent pathways could account for the increased macrophage responses. However, there are alternate explanations given the complexities of cell signaling. Interestingly, there is also a role for TLR2 in the process of *M. tuberculosis* inhibition of IFN- γ induced MHC class II (25, 27, 51). However, our observation that infection with the $\Delta secA2$ mutant led to higher IFN- γ induced MHC class II message than infection with H37Rv is opposite to what would be predicted by increased TLR2 signaling. TLR2-independent pathways of *M. tuberculosis* inhibition of IFN- γ responses have also been identified in which SecA2 could be involved (25, 55). Alternatively, increased TNF- α levels generated by the $\Delta secA2$ mutant could synergize with IFN- γ to promote the observed increase in MHC class II (71).

Modification of the immune response is a strategy used by other bacterial pathogens to survive in the host, and the bacterial factors involved are often secreted and surface proteins localized by specialized secretion systems (14). Currently, there are two known specialized secretion systems in *M. tuberculosis*: the SecA2-dependent system and the ESAT-6/Snm system (36). Interestingly, the ESAT-6/Snm system which localizes the small ESAT-6 and CFP-10

proteins has also been reported to contribute to immunosuppression (37, 68). *M. tuberculosis* mutants lacking the *snm4* (Rv3877) and *snm9* (Rv3615c) components of the ESAT-6 system elicit increased levels of TNF- α , RNI and IL-12 by macrophages. Further, *M. tuberculosis snm* mutants exhibit a growth defect in unactivated macrophages and an early growth phenotype in mice (24, 29, 31, 37, 68). There is no immediate explanation for the similarity in *snm* and $\Delta secA2$ mutant phenotypes, since the $\Delta secA2$ mutant secretes ESAT-6 (data not shown).

Because SecA2 is a secretion factor, its role in immunomodulation is most likely related to proper secretion or cell wall localization of an immunosuppressive factor. However, all of the proteins localized by SecA2, particularly cell wall proteins, are not yet known. The mycobacterial cell wall is a complex structure that contains many diverse molecules with reported immunomodulatory properties (33). These include several surface molecules reported to influence the production of inflammatory TNF- α , IL-6 and/or RNI such as surface lipoproteins, lipoarabinomannan (LAM) and its precursor lipomannan (LM), phthiocerol dimycocerosates (PDIMs or DIMs), and modified trehalose dimycolate (1, 3, 11, 15, 35, 55-57, 61). The increased immune response elicited by the $\Delta secA2$ mutant could be explained by different amounts of immunoregulatory molecules in the cell wall or an altered cell wall structure with greater exposure of stimulatory molecules that interact with host receptors, such as TLR2. Thus, we are considering the possibility that the immunosuppressive effect of SecA2 is due to a role in localization of cell wall synthetic enzymes that influence cell wall architecture. A final possibility is that the $\Delta secA2$ mutant phenotypes actually represent increased release of a stimulatory molecule (10). Future efforts will be aimed at identifying the SecA2-dependent factors that suppress innate and adaptive immune responses.

Acknowledgements

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Attributions

All experiments in this work were performed and analyzed by myself with one exception, Karen McKinnon assisted with FACS data collection and analysis. Dr. Marschall Runge and Dr. Jenny Ting contributed reagents and expertise.

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CHAPTER 3

Determining the role of host signaling pathways in the attenuation of the $\Delta secA2$ mutant
of *M. tuberculosis*

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SecA2 is an accessory secretion factor of *M. tuberculosis* involved in the export of a subset of proteins. A $\Delta secA2$ mutant of *M. tuberculosis* is attenuated for growth in non-activated murine bone marrow derived macrophages and in the early stages of murine infection. Macrophages infected with the $\Delta secA2$ mutant produce increased levels of TNF- α , IL-6 and RNI compared to those infected with the wild type. These host factors are known to be induced by *M. tuberculosis* via the host adaptor protein MyD88, an integral component of the TLR pathway, and they were all shown to function in host control of *M. tuberculosis*. The more robust cytokine responses induced by the $\Delta secA2$ mutant suggests that the role of SecA2 is to promote virulence of *M. tuberculosis* by dampening the immune response. Given that the altered responses we observed are controlled by MyD88, we hypothesized that MyD88 would have a critical role in

controlling the growth of the $\Delta secA2$ mutant. We directly tested this hypothesis by comparing the growth of the $\Delta secA2$ mutant in macrophages derived from wild type C57BL/6 mice in parallel with cells derived from MyD88 deficient mice (MyD88^{-/-}). We observed that MyD88 is important for the attenuation of the $\Delta secA2$ mutant, by demonstrating that the $\Delta secA2$ mutant grows to wild type levels in MyD88^{-/-} macrophages, but not in C57BL/6 macrophages. This supports the hypothesis that increased MyD88 responses can control intracellular growth of the $\Delta secA2$ mutant. More importantly, this suggests that MyD88 responses have potential to control *M. tuberculosis*, but that virulent *M. tuberculosis* evades this protective response. TNF- α is an important host factor in controlling mycobacterial infection, and cells infected with the $\Delta secA2$ mutant produce more of this proinflammatory cytokine. We hypothesized that the antimycobacterial effects mediated by MyD88 may depend on TNF- α . Consistent with that notion, we observed that TNF- α also contributes to the intracellular growth inhibition of the $\Delta secA2$ mutant, as the mutant grows to wild type levels in TNF- α ^{-/-} macrophages. TNF- α induction of cytotoxicity via apoptosis of host cells has been described as a mechanism to inhibit the growth of *M. tuberculosis*. However, we found that the $\Delta secA2$ mutant induced less overall cytotoxicity than the wild type during macrophage infection. Therefore, cytotoxicity does not account for the attenuated phenotype. This data, in combination with our previously published data, demonstrates that there is a MyD88/TNF- α dependent growth inhibition of the $\Delta secA2$ mutant that is independent of RNI/ROI/cytotoxicity. We believe this represents a novel mechanism for intracellular growth inhibition of *M. tuberculosis*.

Introduction

Mycobacterium tuberculosis, like many other successful human pathogens, has a complex relationship with the host immune response. *M. tuberculosis* needs host cells, notably the immune effector cells macrophages and dendritic cells, as sites for replication. The bacterium protects itself within these cells from the deleterious effects of the host response, which include the acidification of the phagosomal compartment and maturation of the phagosome, production of reactive nitrogen intermediates (RNI), and production of reactive oxygen intermediates (ROI). *M. tuberculosis* also down modulates several of the host mechanisms that drive the adaptive response, including the upregulation of MHC class I and class II molecules. Some of the *M. tuberculosis* factors that contribute to the host immune modulation have been found, and yet still many remain undiscovered.

The arsenal of cytokines and immune effector molecules that the host employs against *Mycobacterium tuberculosis* is diverse. TNF- α and INF- γ are two of the most important cytokines contributing to the host response against *M. tuberculosis*. Mice deficient in either of these cytokines do not mount an effective adaptive response or form granulomas. The bacteria replicate uncontrollably and mice succumb to disease rapidly (6, 11, 30, 31).

While the role that TNF- α plays in the control of an *M. tuberculosis* infection in the whole animal model is undisputable, the exact mechanisms by which TNF- α controls growth of the bacteria in the host is complex and not fully understood. How TNF- α affects *M. tuberculosis* in a cell autonomous system is a complicated story, and reports

range from TNF- α inhibiting the growth of *M. tuberculosis* in cultured macrophages to promoting intracellular growth (7, 17, 21, 25, 44, 66). In the reports where TNF- α promotes intracellular killing of mycobacteria, the exact mechanism is still also yet to be determined. TNF- α has been shown to stimulate iNOS and growth inhibition of mycobacteria by iNOS dependent mechanisms. In addition, TNF- α dependent, iNOS independent mechanisms have also been described (7, 19, 29). One proposed TNF- α dependent, iNOS independent mechanism of growth inhibition is via induction of cytotoxicity by apoptosis. It is proposed that TNF- α promotes apoptosis and that the intracellular replication of mycobacteria can be inhibited by apoptosis (32, 40, 52, 55). Avirulent strains of *M. tuberculosis* have been shown to induce higher levels of apoptosis than virulent strains, by a mechanism linked by several groups to the levels of bioactive TNF- α , although this has not been a universal finding (1, 5, 22, 40, 41, 56, 59, 63, 64, 73, 74). However, it is likely that other mechanisms of TNF- α growth inhibition also exist.

The innate host response, including the induction of TNF- α , is controlled by a complex network of signaling molecules. An important innate signaling pathway is the Toll-like receptor (TLR) response pathway (18). There are eleven described TLR surface receptors, which recognize a variety of pathogen associated molecular patterns (PAMPs). Once engaged, the TLRs signal through a series of intracellular adaptor proteins, including MyD88 (18). MyD88 is a central signaling component of all TLR, with the exception of TLR3. TLR2, TLR4, TLR9 and MyD88 have all been implicated in the host response to *M. tuberculosis* (reviewed by Jo *et al*, (38)). Mice with deficiencies in TLR2, TLR4, TLR9 or MyD88 show varying degrees of increased susceptibility to *M. tuberculosis* infection, with MyD88 showing the most dramatically increased

susceptibility, demonstrating the importance of these innate signaling molecules in the host response against *M. tuberculosis* (2, 4, 12, 23, 27, 34, 36, 39, 54, 62, 65, 67, 70, 79, 80). There is an ever growing list of mycobacterial factors that engage the receptors of the TLR pathway, including an array of lipids, lipoproteins, and proteins (38). Significant to our studies, the host induction of IL-6, RNI and TNF- α in response to *M. tuberculosis* have all been linked to signaling through TLR via MyD88 (15, 36, 37, 54, 62, 68, 70, 81).

The $\Delta secA2$ mutant of *M. tuberculosis* is attenuated for growth in a murine model of infection during the first three weeks of infection, a time during which mycobacteria are replicating in macrophages (14, 46). Supporting this, a $\Delta secA2$ mutant is also attenuated for growth in non-activated macrophages (46). The macrophages infected with the $\Delta secA2$ mutant have a more robust innate immune response to infection compared to those infected with the wild type strain, and produce increased levels of TNF- α , IL-6 and RNI (46). These cytokines all have a demonstrated role in controlling *M. tuberculosis* in a murine model (31, 47, 51). As described, these proinflammatory molecules have all been shown to be triggered by signaling through MyD88. Given the importance of these cytokines to the host immune response against *M. tuberculosis* and the central role that MyD88 plays in controlling these responses, we sought to investigate the role of MyD88 in controlling the growth of the $\Delta secA2$ mutant. In addition, given the potential for TNF- α to be an antimycobacterial effector, we also sought to determine the role this cytokine plays in attenuating the growth of the mutant. We tested the $\Delta secA2$ mutant for growth in macrophages from both MyD88 and TNF- α deficient mice and showed that both of these host immune regulators had an antimicrobial effect on the

$\Delta secA2$ mutant. This suggests that there are MyD88/TNF- α dependent antimicrobial mechanisms against *M. tuberculosis*, but that wild type *M. tuberculosis* is able to combat these host effectors through the actions of SecA2.

Materials and Methods

Bacterial Strains and Growth Conditions

The *Mycobacterium tuberculosis* strains used in this study H37Rv, mc²3112 ($\Delta secA2$ mutant, stock derived from a single colony), H37Ra (ATCC 25177), (13) were grown in Middlebrook 7H9 broth (Difco) with 0.2% glycerol, 1X ADS (albumin dextrose saline) and 0.05% Tween 80 (Tw). When appropriate, the media was supplemented with the antibiotic hygromycin (50 μ g/ml).

Animals

Female C57BL/6 mice were purchased from Charles River Laboratories. The MyD88^{-/-} mice were a kind gift of Dr. Shizuo Akira (Osaka University, Osaka, Japan), Dr. Mark Heise (University of North Carolina, Chapel Hill, USA), and Dr. Stephen Clarke (University of North Carolina, Chapel Hill, USA) and were generated and maintained as described previously (3). These mice were backcrossed ten times on a C57BL/6 background. TNF- α ^{-/-} mice were a kind gift from Dr. Jonathon Sedgwick (Eli Lilly and Co., Indianapolis, USA) and Dr. Jenny P.Y. Ting (University of North Carolina, Chapel Hill, USA) were generated and maintained as described previously (45). These mice were generated using direct gene targeting of C57BL/6 embryonic stem cells (45).. All

mice were housed in sterile microbarrier cages and were given autoclaved food and water *ad libitum*.

Macrophage Infections

Bone marrow derived macrophages were prepared from C57BL/6, MyD88^{-/-} and TNF- α ^{-/-} mice as follows. Mice were sacrificed by CO₂ asphyxiation and the femurs were removed. The cells were flushed out of the femurs using supplemented DMEM (Sigma) containing 10% heat-inactivated fetal bovine serum (HI-FBS), 2mM glutamine and 1X non-essential amino acids (NEAA). Cells were washed twice and cultured in supplemented DMEM for 6 days in the presence of 20% L929 conditioned medium (LCM). After 6 days, the cells were detached using 5mM EDTA (in PBS), washed twice with supplemented DMEM, and resuspended in supplemented DMEM with 10% LCM. The cells were then seeded into the wells of either an 8-well chamber slide (2 x 10⁵ macrophages/well), or a 24-well plate (8 x 10⁵ macrophages/well) and were allowed to adhere overnight before infection. In some experiments, the macrophages were pretreated for 24 hours with 10ng/ml recombinant murine IFN- γ (rmIFN- γ) (Chemicon) before infection. *M. tuberculosis* strains were taken from mid-log growth phase and washed one time in PBS 0.05%Tw. Cells were resuspended in PBS-Tw and further diluted to the appropriate CFU/ml in supplemented DMEM. The bacteria were added at the appropriate concentration to the cell monolayers to achieve a multiplicity of infection (MOI) of 1 or 10. Macrophages were infected for 4 hours, at which time the monolayers were washed three times with supplemented DMEM to remove any non-cell associated bacteria, and then fresh media with 10% LCM was added back. At various timepoints,

the media was removed from the wells and the cells were lysed with 0.1% Tween 80 and vigorous pipetting. Lysates were diluted in PBS-Tw and plated onto 7H10 ADS glycerol 0.05% Tw plates for enumeration of CFU.

RNI measurements

To measure RNI we used the Griess Reagent (Molecular Probes) and followed the manufacturer's protocol. Briefly, supernatants from infected macrophage monolayers were filter-sterilized twice using a 0.2 μ m low-protein binding filter, and mixed 1:1 with Griess reagent. Samples were measured at 548nm, and the values converted to μ M nitrite using a standard curve generated with sodium nitrite.

TNF- α ELISA

Infections were performed as described above using differentiated murine bone marrow derived macrophages. Supernatants were harvested at 24hr and 96hr post-infection, double filtered using a 0.2 μ m filter to remove any bacteria and cell debris, and frozen at -80°C until use. TNF- α levels were measured using the BD OptEIA murine TNF ELISA kit (Becton Dickinson) as described by the manufacturer's protocol.

Lactate dehydrogenase (LDH) Assay

Murine bone marrow derived macrophages infections were performed as described above at MOI=1 and supernatants harvested at 24hrs and 96hrs post-infection. Maximal cell death and LDH release is determined by making a lysate from a control set of uninfected cells using 0.1% Tween 80. LDH levels in the supernatants were determined using the

CytoTox 96 kit from Promega. The values obtained from the unknown samples were divided by the values obtained for the control lysate to give percent cytotoxicity.

Results

The $\Delta secA2$ mutant induces a more robust cytokine response that is sustained during the course of macrophage infection. We previously demonstrated that the $\Delta secA2$ mutant of *M. tuberculosis* is defective for growth in non-activated murine bone marrow derived macrophages (46). Concomitant with the growth defect, macrophages infected with the $\Delta secA2$ mutant produce increased levels of the proinflammatory cytokines TNF- α , IL-6, and increased production of reactive nitrogen intermediates (RNI) compared to macrophages infected with the parental wild type H37Rv (46). Our previous cytokine measurements were done at 24hrs post-infection, so we sought to determine if the more robust macrophage responses induced by the $\Delta secA2$ mutant are transient, or if the macrophages maintain heightened cytokine production. We measured cytokine levels at 24hrs as before, and also measured levels at 96hrs post-infection. At 96hrs, the macrophages infected with the $\Delta secA2$ mutant are still showing a comparably heightened level of TNF- α production as compared to 24hrs post-infection, as shown in a representative of four individual experiments ($p \leq 0.001$ at 24hrs and 96hrs by Student's *t* test) (Fig. 3.1). We were unable to test the complemented strain in these experiments, but have previously demonstrated that the increased cytokine production induced by the $\Delta secA2$ mutant can be complemented by the addition of a wild type copy of *secA2* (46). We also saw a similar sustained response at 96hrs for RNI production (data not shown).

This demonstrates that the elevated levels of TNF- α and RNI induced by the $\Delta secA2$ mutant are sustained throughout the course of the infection. We also tested for IL-6 production at 96hrs, but saw inconsistent results in the ability of the mutant to induce a sustained increase in IL-6 production versus cells infected with the wild type strain. We can not explain this discrepancy.

MyD88 dependent antimicrobial responses control the growth of the $\Delta secA2$ mutant. MyD88 is an integral signaling adaptor in the TLR pathway. Molecules from mycobacterium signal through TLRs and MyD88 to induce TNF- α , IL-6 and RNI (Reviewed in (38)). These cytokines all have a demonstrated role in controlling *M. tuberculosis* in a murine model (31, 47, 51). The macrophages infected with the $\Delta secA2$ mutant have a more robust immune response to infection compared to those infected with the wild type strain, and produce increased levels of TNF- α , IL-6 and RNI (46). Given the importance of these cytokines to the host immune response against *M. tuberculosis* and the central role that MyD88 plays in controlling these responses, we sought to investigate the role of MyD88 in inhibiting the growth of the $\Delta secA2$ mutant. We compared growth of H37Rv and the $\Delta secA2$ mutant in macrophages derived from mice deficient in MyD88 (3). Bone marrow derived macrophages from MyD88^{-/-} mice and the

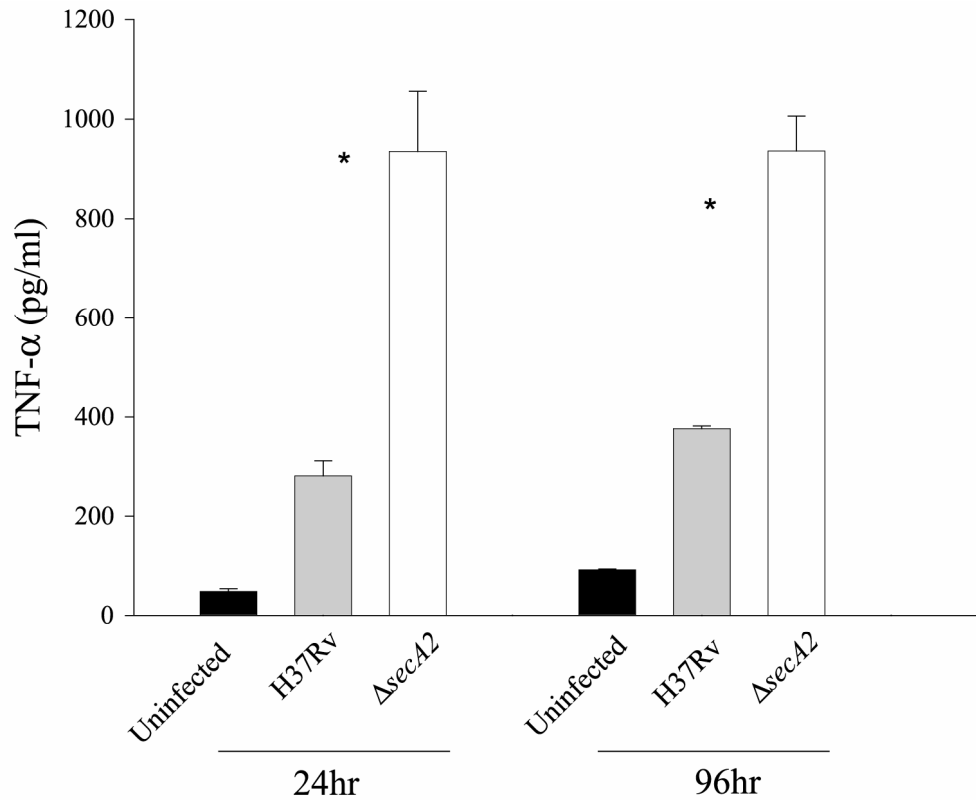


Fig. 3.1. The $\Delta secA2$ mutant induces a heightened cytokine response as late as 96hrs post-infection. Cytokines were measured using the Murine TNF- α ELISA kit (BD Biosciences). Bone marrow derived macrophages from C57BL/6 mice were infected at MOI=1 in triplicate. Supernatants were collected at 24hrs and 96hrs post-infection and assayed for cytokines. Graphs present levels of cytokine for TNF- α in macrophages left uninfected (black bars) or infected with H37Rv (grey bars), or those infected with the $\Delta secA2$ mutant (white bars), with error bars depicting standard deviation (SD) of triplicate samples. Data shown are representative of four experiments. * p values for H37Rv compared to $\Delta secA2$ mutant are $p < 0.001$ by Student's t test.

corresponding wild type C57BL/6 mice were prepared and infected in parallel with the $\Delta secA2$ mutant or H37Rv. As we had demonstrated previously, the $\Delta secA2$ mutant was attenuated for growth in C57BL/6 macrophages, presented as the average of six independent experiments each performed with triplicate samples for each strain ($p=0.001$) (Fig. 3.2). In stark contrast, the $\Delta secA2$ mutant replicated in the MyD88^{-/-} macrophages and was no longer statistically different from the wild type ($p=0.09$ vs H37Rv). The mutant grew four-fold over five days in the MyD88^{-/-} macrophages, compared to no growth observed for the mutant in C57BL/6 macrophages ($p=0.002$). The wild type itself displayed an equivalent growth pattern in both C57BL/6 and MyD88^{-/-} macrophages ($p=0.45$).

Characterization of the attenuated strain H37Ra. In the literature, there are reports of other attenuated mutants of *M. tuberculosis* that also induce increased levels of proinflammatory cytokines, such as mutants in ESAT-6 or the ESAT-6 secretion system (50, 75). These data raise the question whether there is induction of a more robust immune response to certain mutants of *M. tuberculosis*, or whether it is a general property of all attenuated mutants. Supporting the idea that it is not a general phenotype of all mutants, there is a report that the *pca* mutant of *M. tuberculosis* is attenuated, and yet does not induce increased levels of TNF- α (61). In fact, the attenuated *pca* mutant induces lower levels of cytokines compared to its parental wild type strain (61). Mutants in the acid induced operon Rv3083-Rv3089 of *M. tuberculosis* are also attenuated, yet induce lower levels of cytokines, including IL-6, than the parental wild type (20). These reports demonstrate that there is complexity to the host cytokine response to *M.*

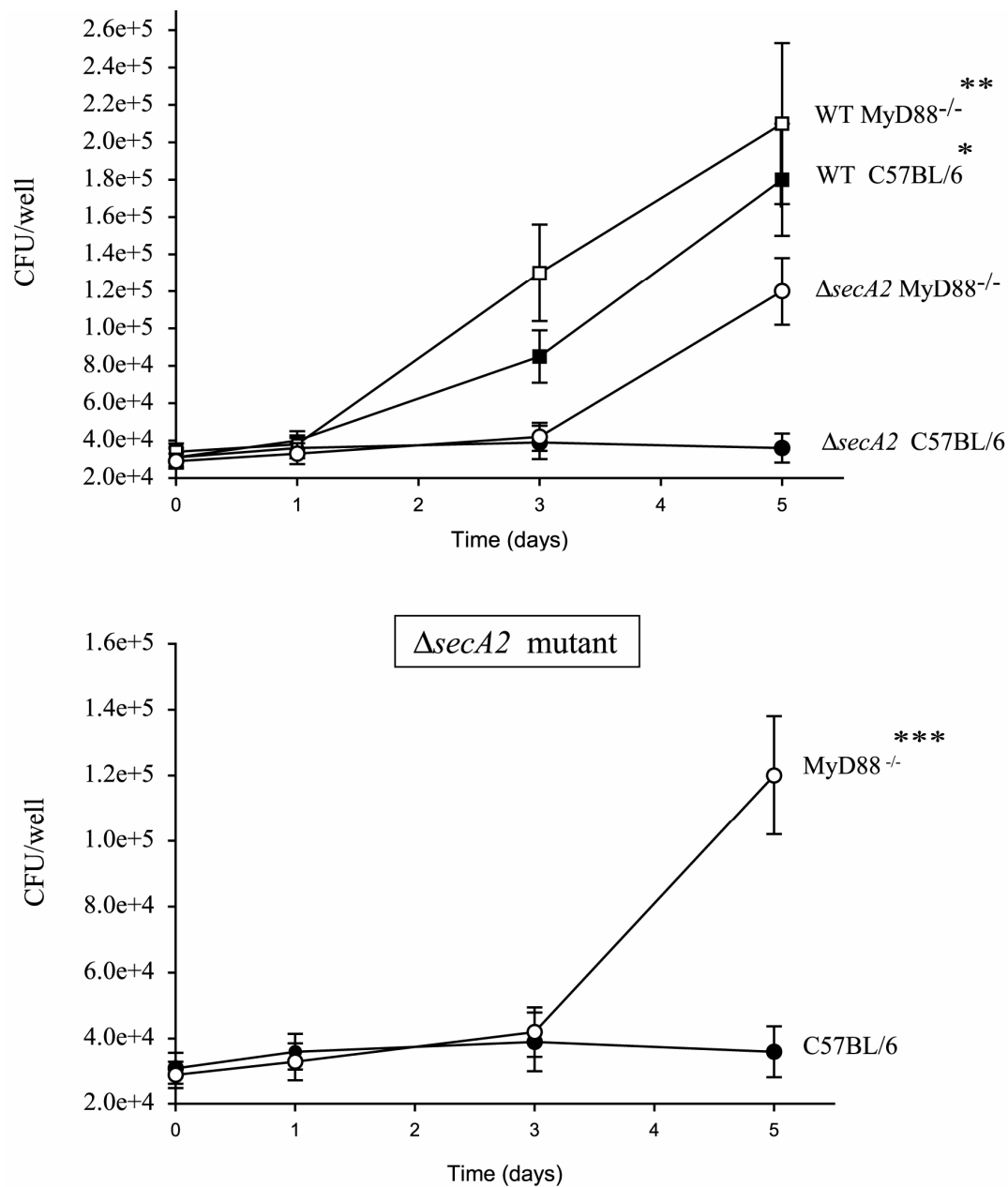


Fig.3.2. The attenuation of the $\Delta secA2$ mutant is alleviated in bone marrow derived macrophages from mice defective in myeloid differentiation factor 88 (MyD88). Fig. 3A) Unactivated bone marrow derived macrophages from C57BL/6 mice and from MyD88^{-/-} mice were infected in parallel at MOI=1 with *M. tuberculosis* H37Rv (in C57BL/6 macrophages=■; in MyD88^{-/-} □) and the $\Delta secA2$ mutant (in C57BL/6 macrophages=●; in MyD88^{-/-} macrophages ○) as described above. Fig 3B) Illustrates the $\Delta secA2$ mutant samples alone. Graphs are shown as the average of six experiments \pm standard error of the means (SEM). * p values as determined by Student's *t* test for H37Rv compared to $\Delta secA2$ in C57BL/6 macrophages are $p=0.001$ at Day 5; ** $p=0.09$ in MyD88^{-/-} macrophages; *** p value for the $\Delta secA2$ mutant in C57BL/6 vs MyD88^{-/-} =0.002

tuberculosis and that there does not seem to be a universal correlation between the levels of TNF- α and the growth outcome of a particular strain.

H37Ra is an attenuated strain of *M. tuberculosis* used by many research groups to study the immunology of *M. tuberculosis* infection. H37Ra, like our parental wild type strain H37Rv, was generated from the classical *M. tuberculosis* strain H37, and H37Ra was attenuated through multiple passages on culture media (76, 77). While several studies have been undertaken to determine the loci in H37Ra that are responsible for the attenuation, the full extent of the contributing mutations still remain to be elucidated (16, 35, 49, 57). Several groups have reported that this strain induces increased levels of TNF- α compared to the wild type H37Rv; however, this finding has not been universal (5, 26, 33, 40, 71). Discrepancies in these reports could be due to differences in experimental design. We compared H37Ra to the $\Delta secA2$ mutant and H37Rv for its ability to induce TNF- α in non-activated murine bone marrow derived macrophages in our experimental system. As demonstrated previously, the $\Delta secA2$ mutant induced an increased level of TNF- α compared to parental H37Rv ($p=0.001$) (Fig.3.3). H37Ra induced heightened macrophage responses in our system, inducing two fold more TNF- α than wild type ($p=0.0006$), and even exhibited increased levels of TNF- α compared to the $\Delta secA2$ mutant ($p=0.003$).

These data demonstrated that like the $\Delta secA2$ mutant, H37Ra also induces a more robust TNF- α response. As will be discussed in Chapter 4, H37Ra has an altered colony morphology on plates similar to that of the $\Delta secA2$ mutant. Given the similarities between these two strains, and the fact that the strain background of H37Ra and its

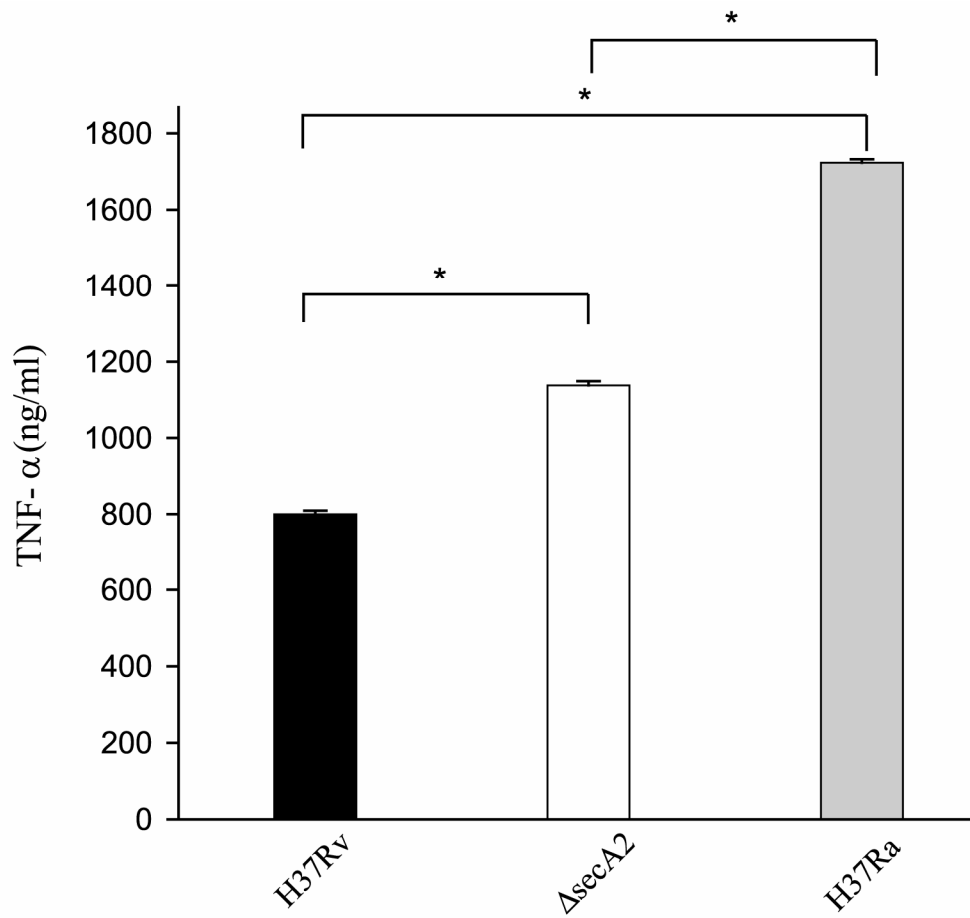


Fig. 3.3. H37Ra elicits an increased TNF- α response in murine bone marrow derived macrophages. Cytokines were measured using the Murine TNF- α ELISA kit (BD Biosciences). Bone marrow derived macrophages from C57BL/6 mice were infected at MOI=1 in triplicate. Supernatants were collected at 24 hours post-infection and assayed for cytokines. Graphs present levels of cytokine for TNF- α in macrophages infected with H37Rv (black bar), or those infected with the $\Delta secA2$ mutant (white bar), or H37Ra (grey bar), with error bars depicting standard deviation (SD) of triplicate samples. Data shown are representative of four experiments. * p values for H37Rv compared to $\Delta secA2$ mutant and H37Ra, and for $\Delta secA2$ mutant compared to H37Ra are $p < 0.003$ by Student's *t* test.

mutations are largely uncharacterized, we sought to determine if there were any alterations in *secA2* in H37Ra. We sequenced the *secA2* gene of H37Ra to ensure that there were no mutations, and found the H37Ra sequence to be identical to H37Rv (data not shown). We also performed a Western blot analysis to confirm expression of SecA2 in H37Ra and observed production of SecA2 under standard growth conditions (N. Rigel, data not shown). This confirmed that phenotypes observed with H37Ra were not due to *secA2* mutations. This data demonstrated that in our cell autonomous system, H37Ra is another attenuated mutant of *M. tuberculosis* that induces a more robust immune response. We will use this strain to assess the specificity of the $\Delta secA2$ mutant phenotypes below.

H37Ra is attenuated even in the absence of MyD88. Given that H37Ra, like $\Delta secA2$ mutant, induces a more robust immune response, we sought to compare the role of MyD88 in the attenuation of this strain. For this reason, we compared the growth H37Ra in parallel to wild type H37Rv and the $\Delta secA2$ mutant in C57BL/6 and MyD88^{-/-} macrophages, in a set of three independent experiments. As we had seen, the wild type replicated to a similar level in C57BL/6 and MyD88^{-/-} macrophages, and the $\Delta secA2$ mutant was attenuated in the C57BL/6 macrophages and not in the MyD88^{-/-} macrophages (Fig 3.4, data not shown). H37Ra was attenuated for growth in the C57BL/6 macrophages compared to wild type ($p=0.0008$ for Day 5). In contrast to the $\Delta secA2$ mutant, H37Ra was as attenuated in the MyD88^{-/-} macrophages as it was in the C57BL/6 macrophages and showed no statistical growth difference in the two types of macrophages ($p=0.65$ for C57BL/6 vs MyD88^{-/-} at Day 5).

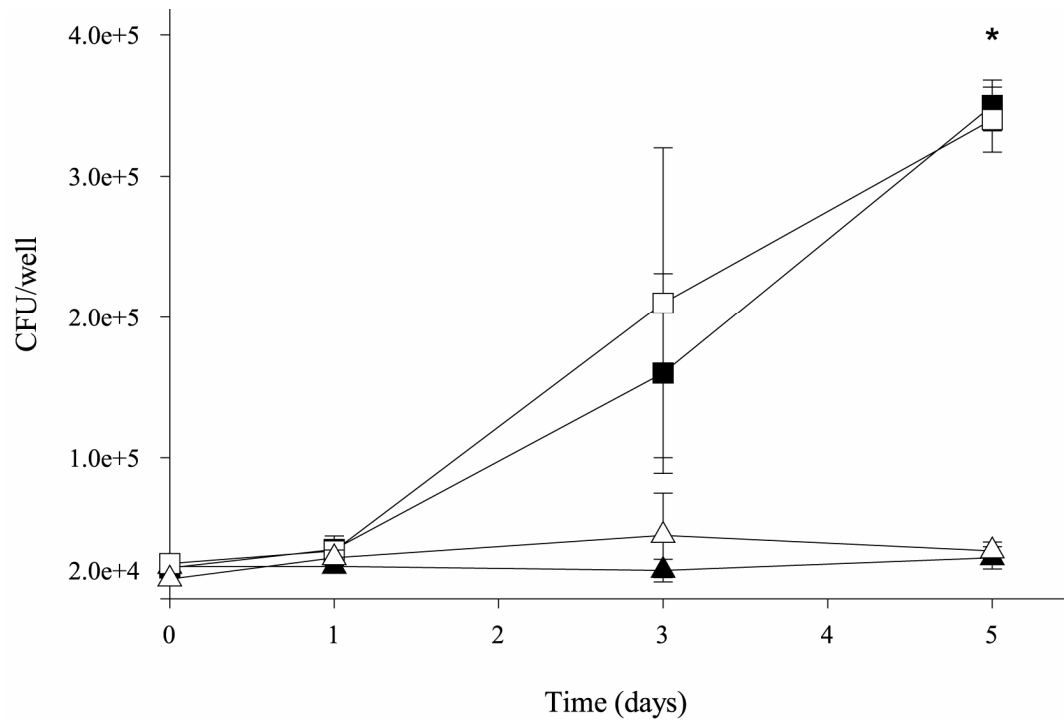


Fig. 3.4. H37Ra is equally attenuated in bone marrow derived macrophages from C57BL/6 mice and mice defective in MyD88 (MyD88^{-/-}). Unactivated bone marrow derived macrophages from C57BL/6 mice and from MyD88^{-/-} mice were infected in parallel at MOI=1 with *M. tuberculosis* H37Rv (in C57BL/6 macrophages=■; in MyD88^{-/-} macrophages □) and H37Ra (in C57BL/6 macrophages=▲; in MyD88^{-/-} macrophages △). Graphs are shown as the average of three experiments ± standard error of the means (SEM). * p values compared to H37Ra in both C57BL/6 and MyD88^{-/-} macrophages are $p < 0.0002$ by Student's *t* test.

MyD88^{-/-} cells have been shown to have diminished phagocytosis, however, this has not been a universal finding with all bacteria (9, 10, 24, 72). We confirmed that after the bacterial uptake period of our infection, there were equivalent numbers of bacteria in the MyD88^{-/-} macrophages and in C57BL/6 macrophages ($p \geq 0.05$) (Fig. 3.2 and Fig. 3.4), and that equivalent numbers of cells were infected (data not shown). In our system, there does not seem to be any phagocytosis impairment in the MyD88^{-/-} macrophages.

Taken together, these data show that the attenuation of the $\Delta secA2$ mutant is due to a MyD88-dependent antimicrobial response, but that these cells are not permissive for the growth of any attenuated strain. To our knowledge, this exciting result represents the first example of MyD88 dependent antimicrobial response against *M. tuberculosis* in a cell autonomous system.

As we have mentioned previously, published studies demonstrate that the production of *M. tuberculosis* induced immune effectors, such as TNF- α , IL-6 and RNI is dependent on MyD88. Our hypothesis is that the $\Delta secA2$ mutant is able to grow in MyD88^{-/-} macrophage due to the lack of a MyD88 directed response. We tested in our system the possibility that *M. tuberculosis* induction of TNF- α and RNI were MyD88 dependent. We examined the production of TNF- α in macrophages derived from wild type C57BL/6 and MyD88^{-/-} mice when uninfected, or infected with the wild type H37Rv or $\Delta secA2$ mutant. As we had seen previously, the $\Delta secA2$ mutant induced increased levels of TNF- α in C57BL/6 macrophages when compared to H37Rv ($p=0.003$) (Fig. 3.5). In contrast to C57BL/6 macrophages, the MyD88^{-/-} cells were unable to produce

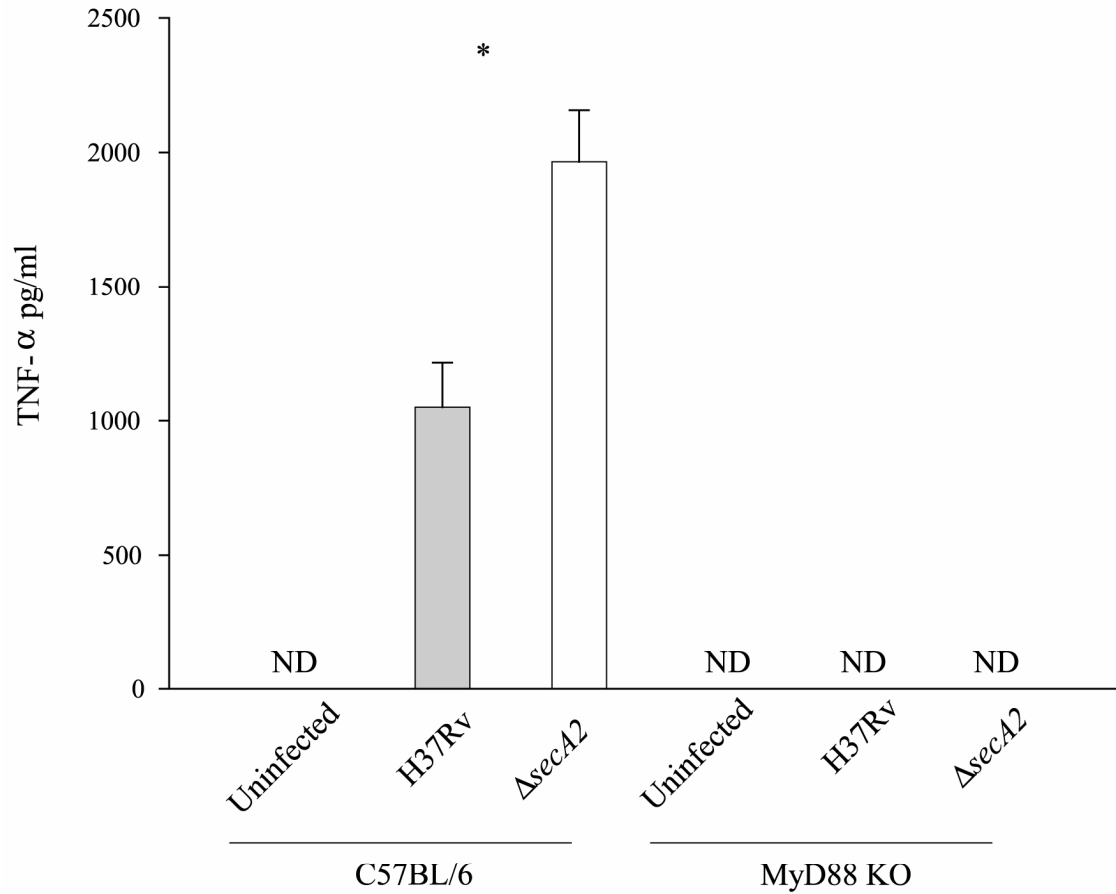


Fig. 3.5. TNF- α release from infected bone marrow derived macrophages is dependent on MyD88. Cytokines were measured using the Murine TNF- α ELISA kit (BD Biosciences). Bone marrow derived macrophages from C57BL/6 and MyD88^{-/-} mice were infected at MOI=1 in triplicate. Supernatants were collected at 24 hours post-infection and assayed for cytokines. Graphs present levels of cytokine for TNF- α in macrophages infected with H37Rv (grey bar), or those infected with the $\Delta secA2$ mutant (white bar), with error bars depicting standard deviation (SD) of triplicate samples. Data shown are representative of two experiments. * p values for H37Rv compared to $\Delta secA2$ mutant in C57BL/6 < 0.003 by Student's t test.

detectable levels of TNF- α when infected with either strain (Fig. 3.5). RNI levels were also undetectable in the infected MyD88^{-/-} macrophages (data not shown). We also observed similar results for RNI production being dependent on MyD88 (data not shown). We did not measure IL-6 levels, but expect that IL-6 production in infected cells would have also been dependent on MyD88.

TNF- α plays a role in the attenuation of the $\Delta secA2$ mutant. TNF- α is an important proinflammatory molecule that has pleiotropic effects over many signaling pathways within the host (78). While the importance of TNF- α in controlling *M. tuberculosis* infection in the whole animal model is clear, the exact role that TNF- α plays in controlling *M. tuberculosis* in a cell autonomous system is still undetermined. When used in synergy with IFN- γ , TNF- α exerts antimicrobial activity against mycobacteria, which is mediated by RNI (7, 19, 28). Although, when TNF- α alone is used to stimulate macrophages, its role in antimicrobial defense is not as clear.

Given the increase of TNF- α during infection with the $\Delta secA2$ mutant, we tested the role of this cytokine in the attenuation of the mutant. We infected macrophages derived from TNF- α ^{-/-} mice and the corresponding wild type C57BL/6 mice in parallel in five independent experiments. In our system, we saw that the wild type displayed similar growth kinetics in macrophages from both mice (Fig. 3.6). The growth of the wild type is somewhat elevated at Day 5 in the TNF- α ^{-/-} cells, however it is not statistically different (p=0.13). When we examined the growth of the $\Delta secA2$ mutant, it again showed attenuation in C57BL/6 macrophages at Day 3 and Day 5 post-infection (p ≤

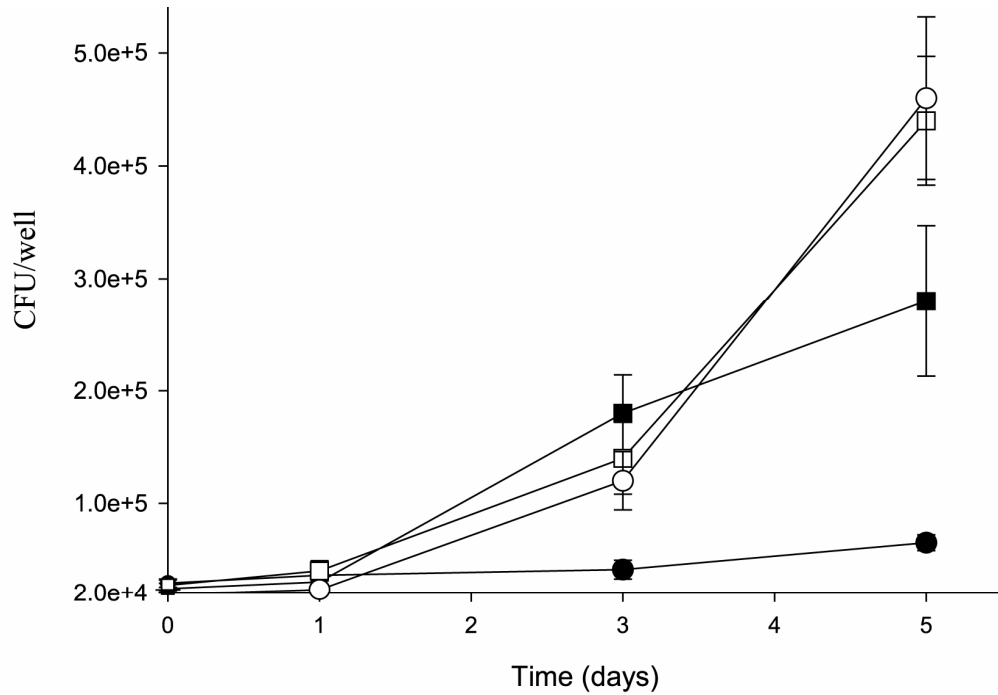


Fig. 3.6. The attenuation of the $\Delta secA2$ mutant is alleviated in bone marrow derived macrophages from mice defective in tumor necrosis factor alpha (TNF- α). Unactivated bone marrow derived macrophages from C57BL/6 mice and from TNF- $\alpha^{-/-}$ mice were infected in parallel at MOI=1 with *M. tuberculosis* H37Rv (in C57BL/6 macrophages= ■ ; in TNF- $\alpha^{-/-}$ macrophages= □) and the $\Delta secA2$ mutant (in C57BL/6 macrophages= ● ; in TNF- $\alpha^{-/-}$ macrophages= ○) as described above. Graphs are shown as the average of five experiments \pm standard error of the means (SEM). * p values for H37Rv compared to $\Delta secA2$ in C57BL/6 macrophages are $p < 0.008$ at Day 3 and Day 5, and $p > 0.15$ in TNF- $\alpha^{-/-}$ macrophages by Student's *t* test.

0.008). In contrast, the mutant grew to the same levels as wild type in the TNF- $\alpha^{-/-}$ macrophages, and demonstrated increased levels of growth at Day 3 and Day 5 compared to $\Delta secA2$ mutant in C57BL/6 macrophages ($p \leq 0.03$). The mutant showed no statistical difference from the wild type in TNF- $\alpha^{-/-}$ cells at Day 3 or Day 5 ($p \geq 0.15$). This data demonstrates that a TNF- α dependent host response is required for the attenuation of the $\Delta secA2$ mutant. In our system, TNF- α has an antimicrobial against *M. tuberculosis* that is able to inhibit the growth of the $\Delta secA2$ mutant. Wild type *M. tuberculosis* can resist this antimicrobial mechanism in a SecA2 dependent manner.

TNF- $\alpha^{-/-}$ macrophages are deficient in the production of immune effectors. We have demonstrated that TNF- α mediates an antimicrobial response against the $\Delta secA2$ mutant using macrophages from TNF- $\alpha^{-/-}$ mice. As described, TNF- α is a potent host signaling molecule, and one of the known effectors modulated by TNF- α is RNI (7, 19, 29). We sought to confirm that there was an abrogation of TNF- α dependent responses in the TNF- $\alpha^{-/-}$ macrophages, by measuring levels of RNI. We measured RNI production in macrophages derived from TNF- $\alpha^{-/-}$ mice and compared the levels to those induced in C57BL/6 macrophages. When non-activated macrophages were infected with either the wild type or $\Delta secA2$ mutant at MOI of 10, we saw that the levels of RNI in TNF- $\alpha^{-/-}$ macrophages are reduced below the level of detection, compared to those measured in C57BL/6 macrophages (Fig. 3.7A). This is in agreement with published data that TNF- α is required RNI production (7, 19). We saw that the $\Delta secA2$ mutant induces increased levels of RNI in untreated C57BL/6 macrophages as we have described previously ($p \leq$

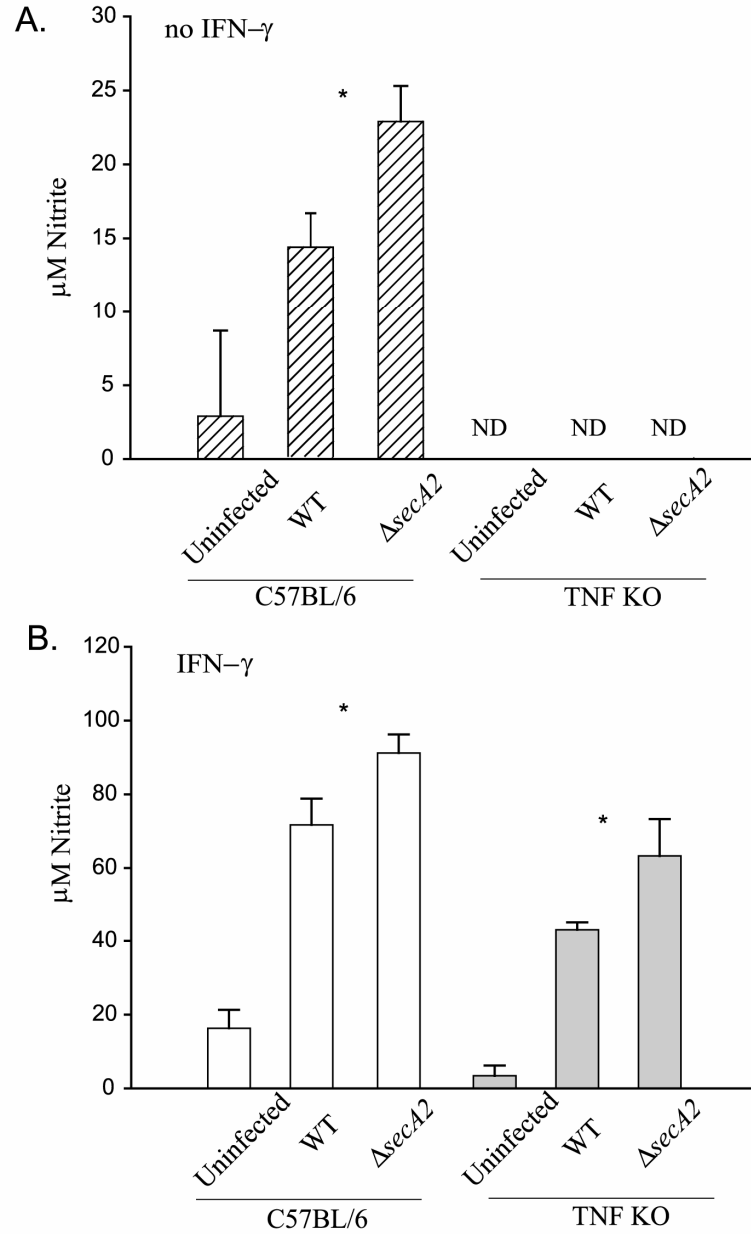


Fig. 3.7. The production of Reactive Nitrogen Intermediates (RNI) by infected bone marrow derived macrophages is dependent on TNF- α . RNI was measured using the Griess reagent. Infections were performed at MOI=10 and samples were collected at 72 hours post-infection for unactivated macrophages (A) and 24 hours post-infection for IFN- γ activated macrophages (B). Bars show the average of triplicate samples for uninfected cells, or those infected with wild type or the ΔsecA2 mutant with error bars depicting standard deviation (SD) of triplicate samples. Bars represent sample from C57BL/6 macrophages (white), either IFN- γ treated (plain) or untreated (hatch marks); or samples from TNF KO macrophages (grey). ND=not detectable . * p values compared to ΔsecA2 mutant are $p < 0.02$ by Student's t test.

0.02) (46). When macrophages from TNF- $\alpha^{-/-}$ or C57BL/6 mice were treated with IFN- γ , we again saw that there were overall lower levels of RNI produced in the TNF- $\alpha^{-/-}$ macrophages (Fig. 3.7B). We also saw that the $\Delta secA2$ mutant induced increased levels of RNI compared to the wild type strain in both IFN- γ treated C57BL/6 and TNF- $\alpha^{-/-}$ macrophages ($p \leq 0.02$) (Fig. 3.7B). This data demonstrated that in IFN- γ treated cells, the increased RNI production observed with the mutant is not dependent on TNF- α , since the difference between the wild type and mutant is still present in the TNF- $\alpha^{-/-}$ macrophages. This supports the idea that increased RNI production observed in the presence of the $\Delta secA2$ mutant is due to increased TLR signaling, and not to a downstream effect of increased TNF- α . Overall, this data confirmed that in our experimental system, there is an abrogation of TNF- α dependent host effectors in the TNF- $\alpha^{-/-}$ macrophages, by measuring one such effector RNI. However, our previous data demonstrates that the $\Delta secA2$ mutant is equally attenuated in non-activated murine bone marrow derived macrophages from wild type and iNOS-/- mice (46). Therefore, we hypothesize that the TNF- $\alpha^{-/-}$ macrophages are deficient in an as yet undetermined host factor that controls the growth of the $\Delta secA2$ mutant. Alternatively, TNF- α may function to activate an effector, and the TNF- $\alpha^{-/-}$ macrophages are defective for this activation.

The $\Delta secA2$ mutant triggers less cell death than wild type *M. tuberculosis*. What is the TNF- α dependent, RNI independent antimicrobial effector mechanism? As described, there are reports that some attenuated strains of *M. tuberculosis* induce more apoptosis than virulent strains, and that the induction of apoptosis may be a TNF-

α dependent antimicrobial mechanism against *M. tuberculosis* (32, 40, 52, 55). We sought to investigate if the TNF- α dependent antimicrobial mechanism contributing to the attenuation of the $\Delta secA2$ mutant was via host cell death. We measured levels of LDH release at 24hrs and 96 hrs post-infection as a marker of host cell death (43, 48). During the course of the experiment, we saw an increase in cytotoxicity under all conditions at 96hrs versus 24hrs (Fig. 3.8). At 24hrs, we saw a decrease in cell death in the wild type infected cells compared with uninfected. There was no difference in the level of cell death measured between the uninfected cells or wild type infected cells at 96hrs. In the cells infected with the $\Delta secA2$ mutant, there was less cell death at both timepoints than in uninfected cells ($p \leq 0.01$). The $\Delta secA2$ mutant also induced less cell death than H37Rv at both timepoints ($p \leq 0.002$ at 96hrs). We also measured cell death using the redox dye Alamar Blue (Invitrogen), and again saw that $\Delta secA2$ mutant induced less cell death than wild type H37Rv (J. McCann, data not shown). This data demonstrates that $\Delta secA2$ mutant does not cause increased levels of host cell death. The host mechanism acting to inhibit the growth of the $\Delta secA2$ mutant must be independent of host cytotoxicity.

Taken together, our data demonstrates a role for MyD88 in the production of an antimicrobial response against the $\Delta secA2$ mutant *M. tuberculosis* in our cell autonomous system. TNF- α , a MyD88 dependent host effector, is also involved in the antimicrobial effect controlling the $\Delta secA2$ mutant. Give our previously published results, in conjunction with data presented in this report, we propose the existence of a novel antimicrobial host response against *M. tuberculosis*, which is acting to control the growth of the $\Delta secA2$ mutant. This novel defense mechanism is dependent on MyD88 and TNF-

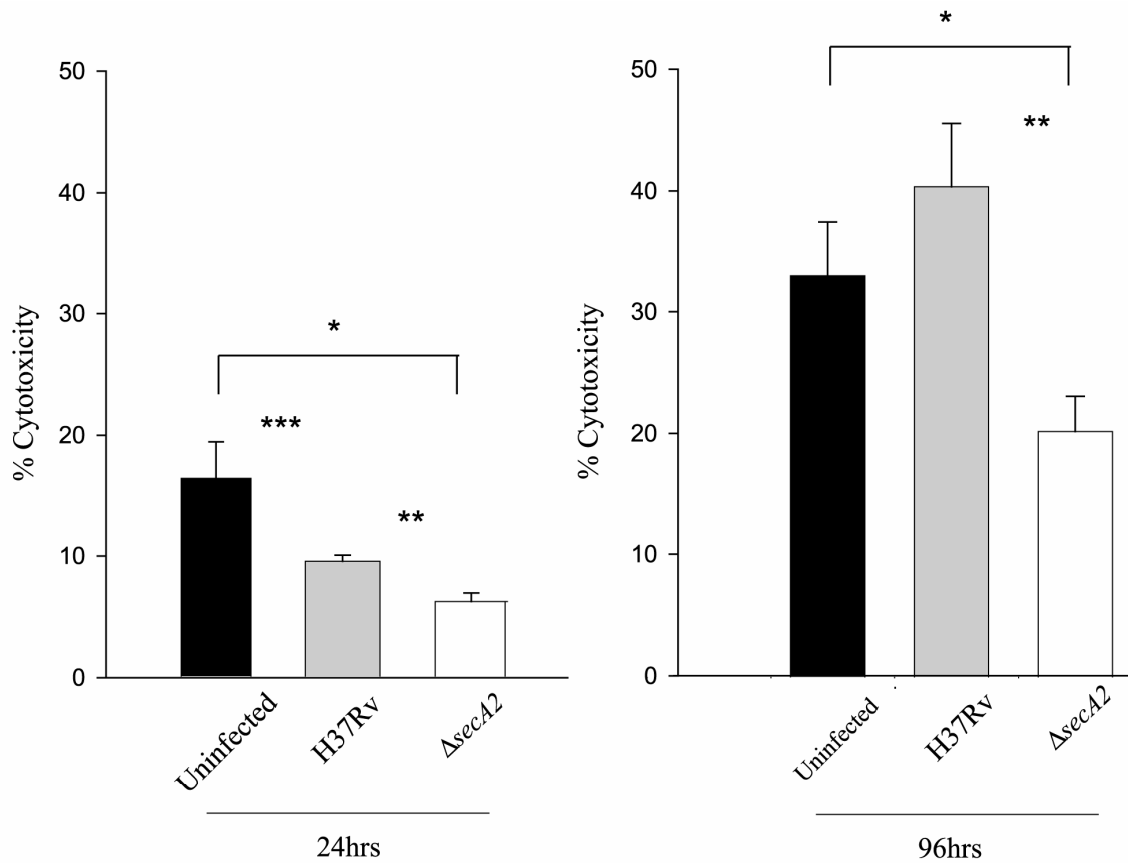


Fig 3.8. The $\Delta secA2$ mutant induces less cell death than wild type *M. tuberculosis*. Murine bone marrow derived macrophages were left untreated (black bars) or infected at an MOI=1 with wild type H37Rv (grey bars) or the $\Delta secA2$ mutant (white bars) in triplicate, and supernatants were harvested at 24hrs and 96hrs post-infection. LDH release was measured using the CytoTox96 Kit from Promega. A cell lysate was used as the positive control for 100% LDH release, and all values are represented as the unknown LDH/value for 100% LDH release. Graph is the average \pm SEM of 7 experiments for 24hrs and 4 experiments for 96hrs. * $p \leq 0.01$ for uninfected vs $\Delta secA2$ mutant; ** $p \leq 0.002$ for H37Rv vs $\Delta secA2$ mutant; *** $p \leq 0.04$ for H37Rv vs uninfected. p values were determined by Student's t test.

α , yet independent of RNI, ROI, or host cell death. We believe that SecA2 plays a role in protecting *M. tuberculosis* from this novel host defense mechanism.

Discussion

We previously demonstrated that the accessory secretion factor SecA2 contributes to the virulence of *M. tuberculosis* (46). We previously tested the role for RNI and ROI in non-activated macrophages in controlling the growth of the $\Delta secA2$ mutant, and found that the $\Delta secA2$ mutant was still attenuated in the absence of these effectors (46). This indicates that there is an ROI/RNI independent host mechanism controlling the growth of the $\Delta secA2$ mutant. Macrophages infected with the $\Delta secA2$ mutant produce a more robust host immune response compared to that elicited by the wild type H37Rv, including elevated levels of TNF- α , IL-6 and RNI (46). We hypothesized that the role of SecA2 was to modulate the host immune response, and that the immune dysregulation was responsible for the attenuation of the mutant. As described, the elevated host molecules that we identified have all been linked to signaling by the host adaptor MyD88. We hypothesized that the altered macrophage responses controlled by MyD88 were contributing to the attenuation of the $\Delta secA2$ mutant. In this work we tested this hypothesis, by comparing the growth of the $\Delta secA2$ mutant in macrophages from wild type C57BL/6 mice and in MyD88^{-/-} mice. The $\Delta secA2$ mutant of *M. tuberculosis* is attenuated for growth in wild type C57BL/6 macrophages. However, the $\Delta secA2$ mutant replicates to wild type levels in MyD88 deficient macrophages. This suggests that there

is a MyD88 dependent antimicrobial response against *M. tuberculosis*, that the $\Delta secA2$ mutant is sensitive to the effects, and that wild type H37Rv is relatively resistant.

MyD88 is an indispensable component of the host immune response to *M. tuberculosis*. Mice deficient in this adaptor protein are unable to control the replication of *M. tuberculosis* and rapidly succumb to disease within 30-40 days, although one report said that MyD88 played a lesser role (34, 67, 68, 79). Surprisingly, there has been no reported role for antimicrobial MyD88 in a cell autonomous macrophage infection model. One previous report demonstrates that wild type *M. tuberculosis* displays similar growth kinetics in macrophages from C57BL/6 mice and MyD88^{-/-} mice (69). This is consistent with our model that wild type *M. tuberculosis* is resistant to effects mediated by MyD88, as it grows equally well in the presence or absence of this signaling adaptor (Fig. 3.2).

However, we demonstrated that the $\Delta secA2$ mutant is more sensitive to a MyD88 dependent antimicrobial response, a novel finding. This suggests that virulent wild type *M. tuberculosis* is able to protect itself against this MyD88 dependent antimicrobial mechanism. In contrast to $\Delta secA2$ mutant, the attenuated strain H37Ra still demonstrates a growth defect in MyD88^{-/-} macrophages. This is a demonstration that the MyD88 macrophages are still capable of inhibiting the growth of certain attenuated strains of mycobacteria, and that the growth of the $\Delta secA2$ mutant in these cells is not due to a pleiotropic deficiency of all innate responses. It also indicates that the basis of attenuation between H37Ra and $\Delta secA2$ mutant is different.

How does MyD88 contribute to controlling the growth of the $\Delta secA2$ mutant? We have demonstrated that the attenuation of $\Delta secA2$ mutant is not due to host mechanisms of ROI/RNI/cytotoxicity. We demonstrated that the more robust host

responses induced by the *ΔsecA2* mutant are not transient, and continue until the late stages of the macrophage infection. This demonstrates that the cells infected with the *ΔsecA2* mutant are subjected to prolonged stimuli and to the potential downstream signaling effects of TNF- α . As described, TNF- α induction by *M. tuberculosis* is MyD88 dependent. We hypothesized that this host effector may be mediating an antimicrobial response against the *ΔsecA2* mutant. We demonstrated that TNF- α is mediating antimicrobial effects against the *ΔsecA2* mutant, and that wild type *M. tuberculosis* is resistant to TNF- α mediated growth inhibition.

What are the inhibitory effects mediated by TNF- α ? A previous report demonstrated that TNF- α can trigger growth inhibition of mycobacteria in an iNOS dependent manner (7). However, this report also described a TNF- α dependent, iNOS independent mechanism of growth inhibition. This was corroborated by another report, which demonstrated that the attenuated strain H37Ra was unable to replicate in macrophages from wild type, iNOS^{-/-}, or IFN- γ ^{-/-} mice, but replicated to wild type levels in macrophages from TNF- α ^{-/-} mice (33). Our data using the *ΔsecA2* mutant of *M. tuberculosis* mirrors these previously published reports, in that the *ΔsecA2* mutant is attenuated for growth in macrophages from wild type and iNOS^{-/-} mice, but grows to wild type levels in macrophages from TNF- α ^{-/-} mice (this work, (46)). Bekker *et al* suggest that the TNF- α dependent/iNOS independent inhibitory pathway could be via the induction of ROI through *phox* (7). However, we have previously tested the growth of the *ΔsecA2* mutant in macrophages derived from two strains of mice deficient in different components of *phox*, and the *ΔsecA2* mutant was as attenuated in these cells as in those

derived from wild type mice (46). This suggests, at least in the case of the *ΔsecA2* mutant, that there must be another mechanism of inhibition at work. One potential effector of the iNOS-independent mechanism is β -chemokines, whose expression are modulated by TNF- α and have been shown to play a role in inhibiting *M. tuberculosis* intracellular growth, in an as yet undetermined fashion (66). At this point, we have not proven the TNF- α mediated effect on the mutant is due to the increased levels of TNF- α induced by the *ΔsecA2* mutant. Alternatively, the *ΔsecA2* mutant may be defective in inhibiting a TNF- α dependent antimicrobial response.

The ability of *M. tuberculosis* to replicate in host cells is due to its ability to modulate the intracellular environment to favorable growth conditions, including modulating the cell death pathways triggered during infection. It has been demonstrated that the intracellular replication of mycobacteria can be inhibited by the induction of apoptosis (32, 40, 52, 55). Avirulent strains of *M. tuberculosis* have been shown to induce higher levels of apoptosis than virulent strains, by a mechanism linked by several groups to levels of bioactive TNF- α (1, 5, 22, 40, 41, 59, 63, 64, 73, 74). However, Park *et al* compared strains of *M. tuberculosis* for their ability to induce TNF- α and cell death by apoptosis or necrosis (56). They demonstrate that strains inducing increased levels of TNF- α also induce higher levels of cell death, but that the cytotoxicity was resultant of necrotic death, versus apoptosis (56). The differences in experimental design, types of cells used, and strains used are all variables that may contribute to the disparity in findings. In our experimental system, we tested the wild type *M. tuberculosis* and *ΔsecA2* mutant for their ability to induce cytotoxicity. We found that the *ΔsecA2* mutant

in fact caused less cytotoxicity than the wild type, and even found less overall cell death than in uninfected cells.

It has been demonstrated that cells *in vitro* stimulated with exogenous TNF- α alone do not produce an antimicrobial response against *M. bovis* (19, 29). We hypothesize that in our system, increased levels of TNF- α alone may not be causing an antimicrobial effect, that it is the synergy of TNF- α and an as yet undetermined factor, that invoke an antimycobacterial response.

To explain our data, we proposed that *M. tuberculosis* limits MyD88 signaling, thereby inhibiting MyD88 dependent host antimicrobial responses. We have demonstrated that MyD88 mediates an antimicrobial response against *M. tuberculosis*, as shown by the MyD88 dependent growth inhibition of the $\Delta secA2$ mutant. Therefore, altering the MyD88/TLR host signaling cascade may promote the survival of *M. tuberculosis*. A recent report demonstrates the first evidence that to support the model that *M. tuberculosis* can suppress MyD88/TLR signaling through an inhibition event (58). ESAT-6 is an antigen exported by *M. tuberculosis* by the specialized *esx* export pathway. To inhibit the TLR response, ESAT-6 binds to TLR2, and suppresses downstream TLR signaling by inhibiting interaction of MyD88 and its catalytic partner IRAK4. This abrogates the signaling of MyD88, and blocks the induction of cytokines such as IL-12 and TNF- α (58). This is consistent with published phenotypes of an ESAT-6 mutant, or mutants in the *esx* export pathway, which induce more robust TNF- α and RNI responses during infection of murine macrophages and are attenuated for growth (50, 75). This is likely due to the loss of the TLR inhibitory actions of ESAT-6. ESAT-6 mutants and $\Delta secA2$ mutant of *M. tuberculosis* both induce a more robust cytokine

response and are attenuated. However, we do not believe the phenotypes observed with the $\Delta secA2$ mutant are due to a loss of ESAT-6, as we observe no decrease in ESAT-6 export in the $\Delta secA2$ mutant (data not shown). It is interesting to speculate however, that there is a SecA2 dependent molecule that has a function similar to that of ESAT-6.

An alternative to SecA2 having a direct inhibitory role for TLR/MyD88 signaling is the possibility that it has a role in assembling the cell envelope in a way to make it less stimulatory to the host cells. This role may be to mask a TLR ligand, or to modify a ligand in a way that it is no longer recognized by TLR. The $\Delta secA2$ mutant may be missing a factor that alters the surface of the bacterium, resulting in $\Delta secA2$ mutant inducing a more robust immune response. There are examples of surface modifications in *M. tuberculosis* that have roles in modulating the immune response.

Lipoarabinomannan (LAM) is a component of the mycobacterial cell wall exposed to the host environment. In the slow growing, pathogenic mycobacterium species *M. tuberculosis* and *M. leprae*, LAM is modified with a mannose, yielding manLAM. Fast-growing, non-pathogenic mycobacteria have unmodified LAM, termed araLAM (42). Modified manLAM is less stimulatory than the unmodified araLAM, and each has a differential signaling effect on the TLR pathway, as reviewed by (60). It will be important to compare the cell wall components of the $\Delta secA2$ mutant to those of wild type. If LAM or other surface molecules are altered, they may be altering the interaction with the immune response.

Recent evidence suggests that the TLR/MyD88 pathway may be a mechanism by which phagosome maturation is triggered. The model proposed for this action begins with the phagocytosis of a pathogen containing TLR ligands, and is reviewed by Blander

(8). TLRs, and subsequently MyD88 are recruited to the vacuole and engage downstream signaling molecules. Triggering of this signaling cascade results in a rapid maturation of the phagosome and fusion with lysosomes, which results in growth inhibition of *E. coli* (9). In the absence of MyD88, there is a delay in phagosome/lysosome fusion and killing of *E. coli* (9). However, other reports demonstrate that MyD88^{-/-} macrophages have comparable, or even increased levels of antimicrobial activity to wild type macrophages, indicating pathogen specific effects (10, 53, 82). If the $\Delta secA2$ mutant is altered in a way that it has an increased signaling through TLR/MyD88, then perhaps in addition to triggering an increased cytokine and RNI responses, the mutant is also engaging a heightened degree of phagosome/lysosome fusion through this signaling pathway, resulting in growth inhibition. Yates *et al* (83) also demonstrate that MyD88 is important for phagosome/lysosome fusion; however, they suggest the function is independent of TLR engagement. A role for MyD88 in phagosome/lysosome fusion may help explain the MyD88 dependent nature of the attenuation of $\Delta secA2$ mutant. It is an attractive possibility that the $\Delta secA2$ mutant induces increased signaling through MyD88, which results in increased phagosome/lysosome fusion, inhibiting the growth of the mutant.

While the exact mechanism is still undetermined, we have demonstrated that there is a MyD88 dependent antimicrobial response against *M. tuberculosis*, the first finding of its kind in a cell autonomous macrophage infection system. We also know that the inhibitory effects of MyD88 on the growth of the $\Delta secA2$ mutant are dependent on TNF- α , but independent of ROI/RNI/cell death. We propose a model, in which SecA2 of wild type *M. tuberculosis* promotes survival in the presence of a MyD88 dependent, TNF- α

dependent antimicrobial response. SecA2 may export factors that limit signaling or actively inhibit the antimicrobial response. When SecA2 is missing, the bacteria can no longer modulate the MyD88 response, resulting in increased levels of TNF- α , and an increased antimicrobial effect. However, without further investigation, more complex possibilities can not be ruled out. Our findings have revealed a potentially novel mechanism by which host cells are able to inhibit the growth of *M. tuberculosis*, however, wild type *M. tuberculosis* is able to evade this response.

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CHAPTER 4

M. tuberculosis $\Delta secA2$ mutant has an altered colony morphology
on plates with Tween 80

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The $\Delta secA2$ mutant of *Mycobacterium tuberculosis* displays an altered colony morphology (Smooth) when compared to that of the wild type (Rough) on agar plates in the presence of Tween 80. The Smooth $\Delta secA2$ colonies are able to give off Rough variants at a relatively high frequency. The Rough phenotype does not seem to switch back to Smooth. We hypothesize that the change in colony morphology is due to an alteration in the cell envelope of the mutant. The alteration is likely due to a defect in exporting a protein to the cell surface or due a defect in exporting a biosynthetic enzyme important for lipid modifications. This alteration in the surface structure of the $\Delta secA2$ mutant could be linked to its growth defect in macrophages or altered immunomodulatory properties. Understanding the colony phenotype may give us a better understanding of the role of SecA2 in virulence and physiology. This may also help us to better understand the role of SecA2 in the basic biology of *M. tuberculosis*, specifically to delineate more SecA2-dependent exported proteins.

Introduction

The architecture of bacterial envelopes is complex, and the composition of the cell envelope of *M. tuberculosis* is no exception to this. As reviewed by P. Brennan (10), the mycobacterial cell envelope is comprised of the cell wall and cell membrane subcellular components. The cell wall itself is a unique layered structure including peptidoglycan, arabinogalactan, mycolic acids, protein and other longer chain fatty acids. Several of the components found in the cell envelope are unique to mycobacteria. As is found in other bacteria, there are many components of the cell envelope of mycobacteria that are important for virulence (2). It is also not surprising that, mutations in proteins that are localized to the cell envelope often alter the surface phenotype of the bacterium, resulting in an altered colony morphology when the strains are grown on solid medium (4, 5, 13-15).

There is a long history of alterations in the colony morphology of *M. tuberculosis* being associated with changes in virulence. A 1947 report by Gardner Middlebrook *et al* (25) investigated the colony morphologies of virulent H37Rv and avirulent H37Ra and *M. bovis* BCG strains of mycobacterium using plates containing various concentrations of Tween 80. As shown in the reproduction in Fig. 4.1A, they documented the appearance of colonies from all strains, virulent or attenuated, on plates that did not contain Tween as being rough, with a serpentine looking structure. On plates with high concentrations of Tween, they also observed that colonies from both avirulent and virulent strains had a similar morphotype, and looked smooth and domed. However, plates with an intermediate concentration of Tween revealed a differential colony

morphology between virulent and avirulent strains. On these intermediate Tween plates, the virulent H37Rv strain looked rough and serpentine, much as it had on plates without Tween. In contrast, at the intermediate Tween concentrations the avirulent strains H37Ra and *M. bovis* BCG looked smooth and domed, similar to the morphology seen on plates with high Tween concentrations. The overall observation was that the more avirulent the strain, the more smooth it looked on intermediate concentrations of Tween. However, on plates with no Tween or high Tween concentrations, the avirulent strains were indistinguishable from the virulent one.

The SecA2 alternate secretion pathway in *M. tuberculosis* plays a role in the export of a small set of proteins into the culture filtrate (9). This pathway contributes to the virulence of *M. tuberculosis*, as a $\Delta secA2$ mutant is attenuated in a whole animal model of infection, as well as in macrophages (9, 22). The $\Delta secA2$ mutant induces a more robust immune response compared to the wild type *M. tuberculosis*, and macrophages infected with the $\Delta secA2$ mutant produce increased levels of reactive nitrogen intermediates (RNI) and proinflammatory cytokines such as TNF- α and IL-6 than those infected with the wild type (22).

As we will demonstrate, the $\Delta secA2$ mutant of *M. tuberculosis* has an altered colony morphology when cultured on plates supplemented with Tween 80, and colonies look smooth and domed (Smooth), compared to the wild type colonies which are rough and serpentine (Rough). The phenotype of the $\Delta secA2$ mutant is similar to the altered colony phenotype described by Middlebrook *et al* in 1947 for the attenuated strains *M. bovis* BCG and H37Ra (25). This interesting finding suggested that SecA2 promotes the proper cell envelope architecture of *M. tuberculosis*. Also, given the similarity between

the altered morphology of the $\Delta secA2$ mutant to that of the avirulent strains H37Ra and *M. bovis* BCG, we hypothesized that the changes in the cell envelope of the $\Delta secA2$ mutant may be playing a role in its attenuation. For these reasons, we performed experiments to begin characterization of the surface phenotypes of the $\Delta secA2$ mutant.

The Smooth phenotype of the mutant can revert back to Rough, and we have examined the selective pressures that Tween 80 play in the reversion. The altered colony morphotype of the $\Delta secA2$ mutant can be complemented by adding back an additional copy of *secA2*, demonstrating that the surface alteration is directly linked to SecA2. In an attempt to elucidate the biochemical basis for the surface alteration, we have also isolated cosmid clones containing non-*secA2* genes that can suppress the Smooth phenotype of the mutant.

The relationship between *M. tuberculosis* and the host response is a complex one, and many surface and secreted lipids, proteins and lipoproteins interact with the host immune system (20). It is interesting to speculate that the surface changes of the $\Delta secA2$ mutant may be contributing to the attenuation and altered stimulation of the host immune response described in previous chapters. SecA2 may contribute to pathogenesis by promoting a cell envelope structure that limits immunostimulation.

Materials and Methods

Bacterial Strains and Growth Conditions

The *Mycobacterium tuberculosis* strains used in this study H37Rv, mc²3112 Sm#2 and MBTB6 (individually isolated $\Delta secA2$ mutants, stocks derived from a single colony for each), mc²3111 (merodiploid strain used to generate the $\Delta secA2$ mutants), mc²3116 ($\Delta secA2$, *attB::secA2*) (9), the attenuated strain H37Ra (ATCC 25177), and *Mycobacterium bovis* BCG strain Pasteur (from Dr. W.R. Jacobs, Jr.) (Statens Serum Institute vaccine strain) were grown in Middlebrook 7H9 broth (Difco) with 0.2% glycerol, 1X ADS (albumin dextrose saline), and 0.05% Tween 80 (Fisher). When appropriate, the media was supplemented with the antibiotic kanamycin (20µg/ml) or hygromycin (50µg/ml). For colony morphology experiments, the strains were plated onto Middlebrook 7H10 plates with 0.2% glycerol, 1X ADS (albumin dextrose saline), supplemented with various concentrations of Tween 80.

Colony Photomicrographs

Strains were plated onto 7H10 ADS Gly plates with various concentrations of Tween 80 (Fisher), and incubated for 3-4 weeks at 37⁰C. An Olympus DP70 Digital Microscope camera system (a kind gift from Dr. Janne Cannon) was set-up in a biological safety cabinet. Photomicrographs were taken of plates in the safety cabinet with the lids removed.

Cording Assay

The strains were cultured in 7H9 ADS Gly 0.05% Tween 80 to mid-logarithmic phase. The optical density of the cultures was determined, and the concentration of the strains was adjusted to 1×10^8 colony forming units (CFU)/ml in culture media with 0.05% Tween 80. A 5 μ l sample of each strain was pipetted into a well of a tissue culture treated 2-well chamber slide (Lab-Tek), spread onto the slide and allowed to air dry. When dry, 1ml of culture media containing 0.05% Tween 80 was gently pipetted onto the well. The inoculated chamber slides were enclosed in a Tupperware container and incubated at 37°C without shaking for 2-3 weeks in a 5% CO₂ incubator. After incubation, the media was pipetted off the slides, and the slides were allowed to dry before being fixed in 10% formalin. After fixation, the slides were stained using the Difco TB Fluorescent Stain kit and visualized using a Nikon Eclipse E600 fluorescence microscope.

Sodium dodecyl sulfate (SDS) Sensitivity Assay

Bacteria were grown in 7H9 ADS Gly 0.05% Tween 80 to mid-logarithmic phase, washed and resuspended in PBS 0.05% Tween 80 to 1×10^7 CFU/ml. SDS was added to a final concentration of 0.05%, and samples were incubated at 37°C with shaking. Each strain was tested in triplicate, and samples were plated immediately after the addition of SDS and 24 hours later for enumeration of CFU.

Scanning Electron Microscopy (SEM)

Single colonies of H37Rv and the $\Delta secA2$ mutant were resuspended in 300 μ l of PBS 0.05% Tw80, then an equal volume of EM fixative (2% glutaraldehyde in 0.1M sodium cacodylate, pH 7.4) was added for one hour. Fixed samples were processed by the

University of North Carolina Microscopy Services Laboratory and mounted for Scanning Electron Microscopy. Samples were visualized over a range of magnifications on a Cambridge S200 SEM.

Southern Blot

Genomic DNA was prepared from H37Rv, $\Delta secA2$ mutant, and each of the 28 Rough library clones as described (6). A Southern blot was performed using the same conditions and *secA2* probe described in Braunstein *etal* (9). The DNA was digested with EcoRI/HindIII and probed with plasmid pMB146, which contains *secA2*.

Plasmids and cosmid library

The H37Rv cosmid library was a kind gift from Dr. William R. Jacobs Jr. and was generated as described in (1). Plasmid pMB162, a single copy vector that integrates at the *attB* site, was generated by Braunstein *etal* (9) and contains a kanamycin resistance cassette.

Retrieval and sequencing of cosmids from chromosomes of Rough suppressing clones

Cosmids were isolated from the genomes of the Rough clones as described by Pascopella *etal* (26), with some modification. Genomic DNA from Rough clones was isolated and digested with PacI to release the cosmid insert (6). This digested DNA mixture was ligated to PacI digested pYUB412 based cosmid arms containing *cos* sites. This ligation was then packaged into lambda phage heads using the Stratagene GigaPack kit and

recovered by transduction in *Escherichia coli* as hygromycin resistant cosmids. Primers used to sequence the cosmid ends: pYUB412out (5' TGCCACCTGACGTCTAAGAA 3') and Hygout (5'GCATGCAAGCTCAGGATGTC 3')

Cosmid subcloning

Cosmid DNA was digested with MluI. Individual cosmid fragments were purified from an agarose gel and ligated into MluI digested pMB198. pMB198 is an integrating single copy vector that does not contain a promoter and is marked with a hygromycin resistance cassette(7).

Colony replating assay to determine the role of Tween 80 in selecting for Rough revertants

This assay is described in detail in the figure legend for Figure 4.5. All plates used in this experiment were 7H10 ADS Gly plates. When listed, Tween 80 (Fisher) was added to the plates at a final concentration of 0.05% Tw80. To resuspend colonies and patches, a sterile wooden applicator stick was used to remove the colony/patch from the plate.













Bacteria were resuspended in 500µl PBS 0.05% Tw80 in a microcentrifuge tube by mechanical disruption and vortexing. All serial dilutions for plating were done in PBS 0.05% Tw80.

Results

The $\Delta secA2$ mutant of *M. tuberculosis* has an altered colony morphology on plates with Tween 80. When culturing the $\Delta secA2$ mutant on 7H10 plates containing 0.05% Tween 80, referred to as an intermediate Tween concentration in Fig. 4.1B, we observed the majority of colonies were smooth and domed (Smooth). However, reproducibly a small number of colonies were rough and serpentine (Rough). The Rough colonies resembled the morphology of the parental wild type H37Rv. We wanted to confirm that the Smooth phenotype that we were observing was due to the mutation in *secA2*, and not a second site mutation. The $\Delta secA2$ mutant of *M. tuberculosis* used in all our studies has an in-frame, complete deletion of *secA2*. The morphology phenotype was complemented by adding back an wild type copy of *secA2* on plasmid pMB162 (9). This demonstrated that the altered phenotype is due to the mutation in *secA2* (Fig. 4.1B). In addition, we also used the original single-crossover merodiploid strain, mc²3111 to generate a new $\Delta secA2$ mutant. Of seven clones generated by a double-crossover event, four clones displayed the Smooth colony phenotype and three displayed the Rough phenotype. All four Smooth clones were determined by Southern Blot to be $\Delta secA2$ mutants, and the three Rough clones were determined to be wild type recombinants (data not shown). This new $\Delta secA2$ mutant clone was named MBTB6. This again demonstrated that the altered colony phenotype was due to the mutation in $\Delta secA2$.

Given the similarity of the colony phenotype of the $\Delta secA2$ mutant to the phenotypes described by Middlebrook *etal* in 1947 (Fig. 4.1A), we sought to replicate that experiment to see if our strains behaved similarly. Cultures of wild type H37Rv, $\Delta secA2$ mutant, the complemented mutant, and the attenuated strain H37Ra were grown to mid-logarithmic phase, diluted in PBS with 0.05% Tween 80, and plated onto

Fig. 4.1A Tween 80: None Intermediate High

	No Tween	0.005% Tw	0.01% Tw	0.02% Tw
H37Rv				
BCG (low virulence)				
H37Ra (avirulent)				

Gardner Middlebrook *etal*, *J. Exp. Med.* 1947. 86:175-183
(reproduced with permission from *J. Exp. Med.*)

Fig. 4.1B No Tween 80 Intermediate Tween 80 High Tween

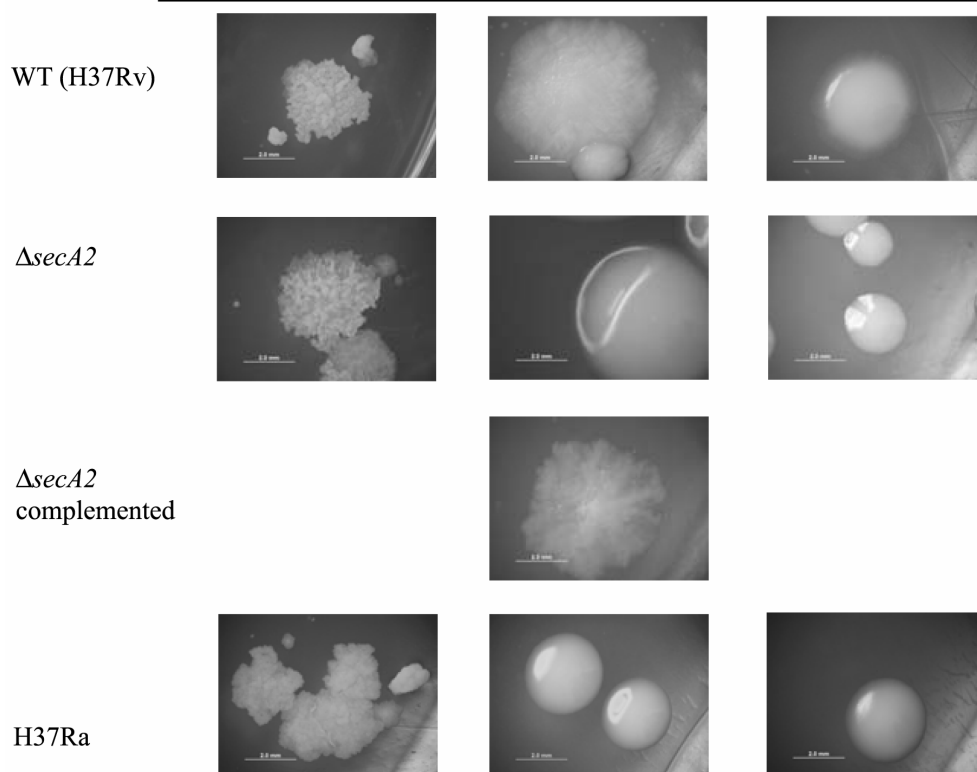


Fig 4.1. The $\Delta secA2$ mutant of *M. tuberculosis* shows an altered colony morphology on Tween 80 similar to that described for attenuated strains in 1947 by Middlebrook *etal*. Fig 4.1A) A reproduction on the original drawings of the Tween 80 dependent morphotypes described by Middlebrook *etal* in 1947 (*J. Exp. Med.*, 86:175-183). Fig 4.1B) Wild type H37Rv, the $\Delta secA2$ mutant, the complemented mutant, and attenuated strain H37Ra were plated onto Middlebrook 7H10 plates supplemented with various concentrations of Tween 80 (Intermediate Tween=0.01-0.05%; High Tween= 0.1-0.5%).

Middlebrook 7H10 plates with various Tween 80 concentrations. All strains displayed a similar colony phenotype on plates with no Tween; they were brittle looking and rough, and showed serpentine like cords on the colony surface (Fig. 4.1B). On plates with intermediate Tween 80 concentrations (0.01-0.05%), we again saw that the $\Delta secA2$ mutant colonies were Smooth, compared to the Rough colonies of the wild type and the complemented strain. The attenuated strain H37Ra, also one of the strains tested by Middlebrook, looked much like the $\Delta secA2$ mutant producing smooth and domed colonies. Interestingly, we have not observed Rough revertants produced by H37Ra. At plates with high concentrations of Tween 80 (0.1-0.5%), we see that all strains are smooth and domed, with even the wild type colonies resembling those of the $\Delta secA2$ mutant and H37Ra.

H37Ra is strain with multiple attenuations, many of them still unknown (11, 23, 26, 27). Given the similarities in colony morphotype, we sought to determine if the Smooth colony morphology in H37Ra could be linked to a mutation in *secA2*. We transformed H37Ra with the *secA2* complementing plasmid pMB162 (9), and cultured the transformants on 7H10 plates with 0.05% Tween 80. The transformants were still Smooth, suggesting that the phenotype in H37Ra is not directly linked to SecA2 (data not shown). We also sequenced the *secA2* gene in H37Ra and found 100% sequence identity to the H37Rv *secA2* coding region (data not shown), and have observed the expression of SecA2 protein by Western blot (data not shown). We conclude that the altered colony phenotype of H37Ra is not directly linked to a *secA2* mutation.

The $\Delta secA2$ mutant is more sensitive to killing by detergent. The glossy, Smooth appearance of the $\Delta secA2$ mutant colonies in the presence of Tween 80 suggested that the alterations in the cell envelope are making it more permeable to the disruptive detergent effect of Tween 80. We hypothesized that the cell envelope of the mutant may be more sensitive to the harmful effects of detergent. We tested the sensitivity of the wild type, the $\Delta secA2$ mutant, and the complemented strain to another detergent, sodium dodecyl sulfate (SDS). The strains were grown in broth to mid-logarithmic phase, washed and resuspended in PBS 0.05% Tween, and SDS was added to a final concentration of 0.05%. The samples were incubated at 37°, and the viability of the strains was determined immediately after added SDS and at 24hrs by plating. The wild type viability decreased by approximately ten fold after 24hrs (Fig 4.2). The $\Delta secA2$ mutant was more sensitive to killing by SDS, and showed a fifty fold reduction in CFU ($p=0.01$ vs wild type). The increased sensitivity of the $\Delta secA2$ mutant was complemented by adding back a wild type copy of *secA2*. This data demonstrates that the cell envelope of the $\Delta secA2$ mutant is less resistant to the effects of detergent.

The $\Delta secA2$ mutant is defective for cording. Mycobacteria under standing culture conditions have the ability to form serpentine cords as the bacteria align themselves in long rope like structures. The ability of strains to cord has long been associated as a property of virulent, but not avirulent, mycobacteria (12, 18). The avirulent strain H37Ra was directly compared to H37Rv for the ability to cord, and H37Ra was found to be defective for this process (16). Other *M. tuberculosis* mutants with alterations in cell envelope metabolic pathways also have defects in cording, including the *pca* and *kasB*

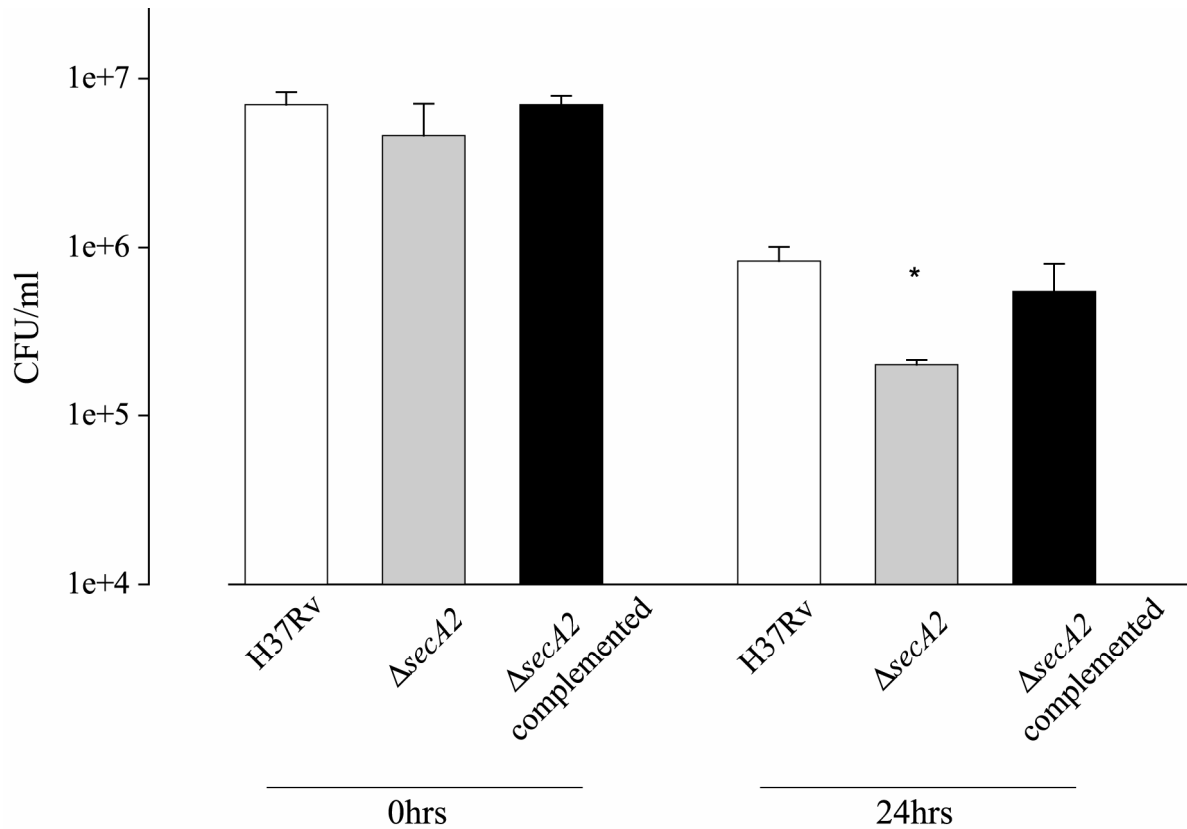


Fig 4.2. The $\Delta secA2$ is more sensitive to killing by SDS. Bacteria were washed and resuspended in PBS 0.05% Tween to 1×10^7 CFU/ml. SDS was added to a final concentration of 0.05%, and samples were incubated at 37°C with shaking. Each strain was inoculated in triplicate, and samples were plated immediately after the addition of SDS and 24hrs later for enumeration of CFU. Bars represent the mean of the triplicate samples with error bars representing SD for wild type H37Rv (white), the $\Delta secA2$ mutant (grey) and complemented mutant (black). This is a representative experiment of four. * $p < 0.01$ for the $\Delta secA2$ mutant compared to wild type and the complemented strain as determined by the Student's t test.

mutants (17); (5). Cording has also been linked to the ability for mycobacteria to form biofilms (19). Given the similarity in altered colony morphotypes of the *ΔsecA2* mutant and H37Ra, we sought to determine if the *ΔsecA2* mutant had an altered cording phenotype. Wild type, *ΔsecA2* mutant, and the complemented strain were inoculated into chamber slides and allowed to grow without shaking at 37°C for 3 weeks. When the slides were fixed and stained, we observed that the wild type had formed highly ordered aggregates and had an abundance of serpentine cords (Fig 4.3). However, the *ΔsecA2* mutant bacilli were found in predominantly loose aggregates of only a few bacteria (Fig 4.3). The cording defect was restored by adding back a wild type copy of *secA2* (Fig 4.3). The altered colony morphology of the *ΔsecA2* mutant and the defect in cording is consistent with the *ΔsecA2* mutant having an altered cell envelope structure that prohibits it from proper cell to cell interactions.

There are no changes in bacterial organization of the *ΔsecA2* mutant visible by Scanning Electron Microscopy (SEM). The demonstrated alterations in the surface structure of the *ΔsecA2* mutant colonies led us to examine the bacteria at the level of a single cell, to see if there was an altered aggregation or obvious surface defect present on the *ΔsecA2* mutant. Single colonies from both the *ΔsecA2* mutant and wild type H37Rv were harvested from plates containing Tw80, resuspended in PBS 0.05% Tw80 and EM fixative, prepared and examined by SEM. As shown in Fig 4.4, bacteria were examined at 3650X and 16000X magnification. There were no obvious differences in the organization of the bacteria for either strain, the bacilli were of similar length and showed no other obvious alterations in the shape and morphology of the bacteria.

Cording Assay

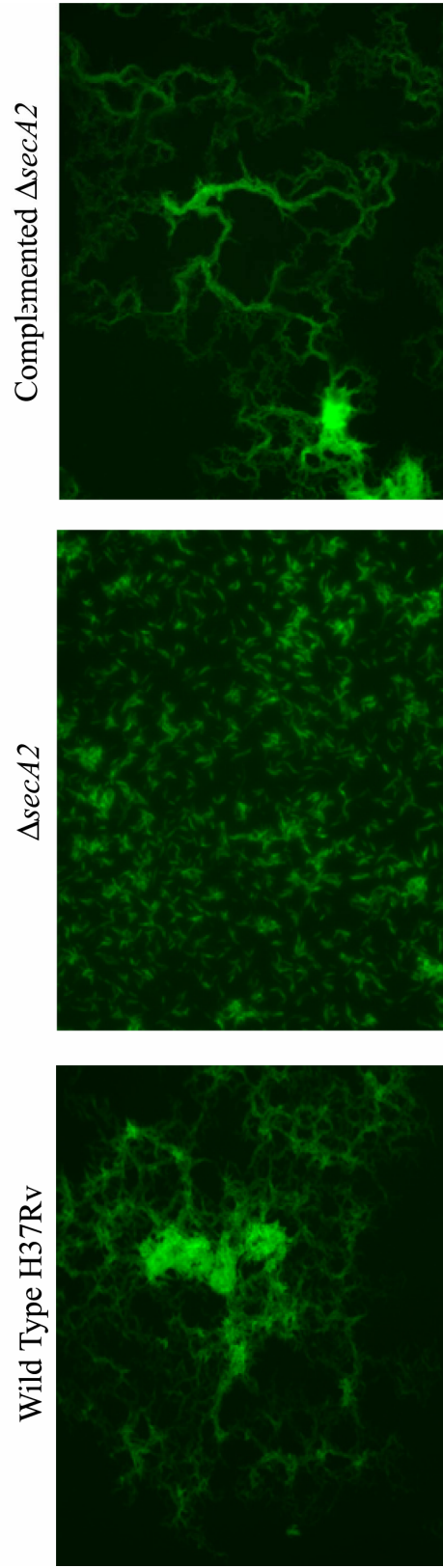
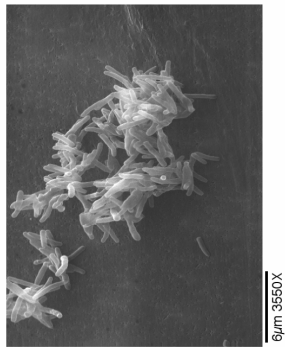


Fig 4.3. The $\Delta secA2$ mutant exhibits a cording defect. Strains were grown in 7H9 ADS Gly media with 0.05% Tw80. Strains were washed and diluted in PBS 0.05% Tw80 to a density of 1×10^8 CFU/ml. 5 μ l of each strain was spread onto a well of a 2-well chamber slide and allowed to dry. 1ml of 7H9 ADS Gly media with 0.05% Tw80 was added to each well. Slides were incubated at 37°C for 2-3 weeks. To harvest the slides, media was removed from the wells, slides were allowed to dry and were stained using the Difco TB Stain kit.

Wild Type



$\Delta secA2$

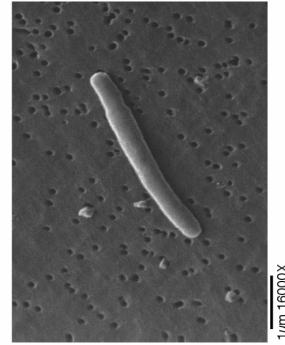
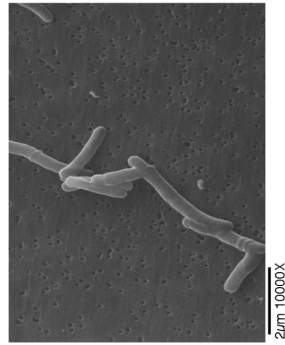
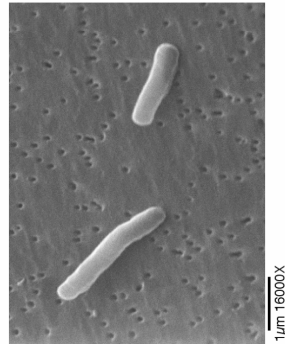
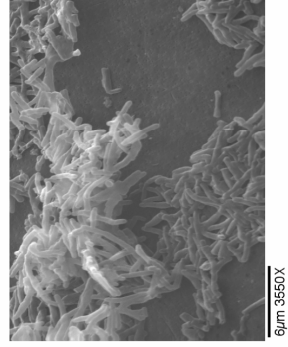


Fig 4.4. Scanning Electron Micrographs (SEM) of wild type *M. tuberculosis* and the $\Delta secA2$ mutant. Samples were prepared as follows: a single colony from a 7H10 plate containing 0.05% Tw80 was resuspended in PBS 0.05% Tw80, fixed and processed for SEM. Photos shown are at 3650x and 16000x magnification.

The frequency of the Smooth to Rough reversion on plates. As mentioned, the Smooth colony phenotype of the $\Delta secA2$ mutant can revert back to the Rough morphotype, as seen on plates with intermediate Tween 80 concentrations. We typically observe 0-10% Rough clones in a given population on plates. Interestingly, the frequency of Rough reversion seems to be dependent on the density of colonies on the plates. We observe that there is an inverse relationship between the number of Rough revertants and the density of colonies, such that on high density plates (500-1000 CFU/plate), there is a lower percentage of Rough clones than on low density plates (20-100 CFU/plate). Plates with an intermediate density have an intermediate frequency of Rough reversion. We employed several different methods to determine the rate at which the Smooth $\Delta secA2$ mutant was reverting to Rough. For an initial examination, we took two individual Smooth colonies from 7H10 plates containing 0.05% Tween 80, resuspended them in PBS 0.05% Tween and plated them onto 7H10 0.05% Tween plates. For the first single colony, there were 102 Rough out of 19,000 total colonies examined total, for a frequency of 0.054%. The second colony yielded a frequency of 456 Rough out of 17000 total CFU examined, a frequency of 2.7%. This high frequency is similar to what is observed for phase variation (29), much higher than what would be expected for the rate of spontaneous mutation (less than ~ 1 out of 10^6) (24). However, phase variation refers to a reversible switch, and out of approximately 70,000 colonies we have not observed any Rough clones reverting back to Smooth (29).

Given the high frequency of reversion, we hypothesized that growth of the $\Delta secA2$ mutant on plates with Tween 80 was selecting for Rough revertants. A second possibility was that the Tween 80 was simply revealing a phenotypic reversion that is

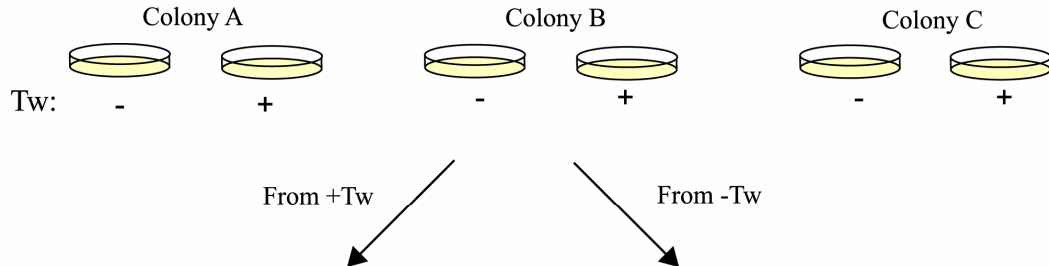
selected for by Tween 80 independent mechanisms. We sought to investigate the selective pressures of Tween 80 on the reversion of the Smooth phenotype to Rough. To determine whether Tween 80 is a selective pressure to enhance phenotypic reversion, we preformed a series of passages on media without Tween 80, as described in Fig. 4.5.

Step 1: A single Smooth *ΔsecA2* mutant colony was isolated from a 7H10 ADS Gly plates with 0.05% Tw80, resuspended in PBS 0.05% Tween 80 and replated onto plates with 0.05% Tw80. This process was repeated a second time to achieve a purified clone. Step 2: After the purification, three individual Smooth colonies (A,B,C) were selected, resuspended and plated onto plates with and without Tw80. The plates with Tw80 were to determine the starting frequency of the Smooth to Rough reversion. As shown in Fig. 4.5, the starting frequency of the clones on high density plates ranged from 0-4.2% Rough. As previously mentioned, the frequency of Rough revertants seems to be dependent on the density of colonies on the plate. In this experiment, we observed at least two-fold higher Rough revertants on low density plates than on high density plates. As an example, using the data from all three colonies replated in Step 2, the frequency of revertants for the high density plates (approximately 1000 CFU/plate), was at least two fold lower than for low density (20-100CFU/plate). Step 3: From each of the original colonies replated (A, B, C), 75 colonies from the plates without Tw80 were taken and patched onto plates with and without Tw80. The patches on the plates containing Tween would allow us to confirm the Smooth phenotype of the clone before proceeding, so that no Rough clones would be replated in large scale. The Smooth and Rough phenotypes are distinguishable at the patch level. All clones patched were Smooth on the

Step 1: Single Colony Purification



Step 2: Replate 3 Smooth Colonies (A,B,C) onto +/- Tw80 plates



Frequency of Rough Colonies
determined from plates with 0.05%
Tween 80:

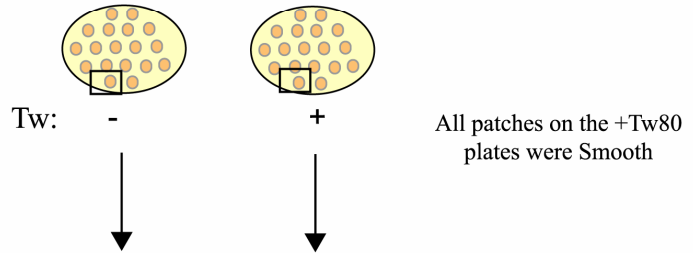
All Plates:

	Rough/Total	Percent Rough
Colony A:	11/676	0.6%
Colony B:	67/830	8.3%
Colony C:	29/1873	1.5%

High density plates (500-1000 CFU/plate):

	Rough/Total	Percent Rough
Colony A:	4/1200	0.3%
Colony B:	20/480	4.2%
Colony C:	0/1200	0

Step 3: Patch onto +/- Tw80



Step 4: Replate corresponding patches from each +Tw80 and - Tw80 plate pair onto +Tw80 plates

Number of Rough Clones:

	Patches from: no Tween		+ 0.05% Tween 80	
	Rough/Total	Percent Rough	Rough/Total	Percent Rough
Colony A	0/~20000	ND	16/~20000	0.08%
Colony B	0/~20000	ND	18/~20000	0.09%
Colony C	0/~20000	ND	12/~20000	0.06%

ND=none detected

Fig 4.5 Replating assay to determine selective properties of Tween 80 on the Rough reversion of the $\Delta secA2$ mutant

Step 1: A single Smooth colony was picked, resuspended in PBS 0.05% Tw80, and plated onto *7H10 ADS Gly 0.05% Tw80 plates. This process was repeated, giving two rounds of single colony purification.

Step 2: After colony purification, three individual Smooth colonies were picked and processed throughout the remainder of the experiment in parallel. Each of the three colonies (A,B,C) was homogenized in PBS 0.05% Tw80 and replated onto plates with and without 0.05% Tw80.

Step 3: From the plates without Tween 80 from Step 2, 75 colonies were patched onto plates with and without 0.05% Tw80 for each of the original colonies replated (A, B, C)

Step 4: Corresponding patches from plates with Tw80 and without Tw80 were taken from each of the original colonies replated (A,B,C), and were homogenized and plated onto plates with 0.05% Tw80 to assess frequency of Rough; ~20,000 colonies were examined for each condition at a density of approximately 500-1000 CFU/plate

+Tw80 plates. We then took a patch from a plate without Tw80, and the corresponding patch from the plate with Tw80, and resuspended the patch and plated on a large scale, onto plates with Tw80. Patches from each of the original clones A, B, C were tested, giving a total of six patches replated. The suspensions were plated at a high density of approximately 500-1000 CFU/plate for a total of approximately 20,000 CFU derived per patch replated. Of all patches replated from plates without Tw, no Rough clones were found of the 60,000 CFU examined. A second experiment using another independently derived Smooth colony also yielded no Rough clones out of an additional 20,000 CFU screened. The patches replated from plates with Tw80 yielded Rough clones at a frequency slightly lower than the starting frequency, ranging from 0.06-0.09% Rough. This data suggests that Tween is a selective pressure on plates for the reversion of a Smooth colony to Rough. The results of this experiment suggest that propagating the strain on plates in the absence of Tween 80 will avoid selecting for Rough revertants. This will help to obtain a more pure colony population for future experiments.

Testing the selective pressure of Tween 80 in liquid culture. Given the high rate of Rough reversion on plates, we investigated the frequency of Smooth and Rough morphologies from broth grown cultures, which by necessity contain 0.05% Tween 80, to see if the Rough morphotype would accumulate in broth over multiple serial passages. The $\Delta secA2$ mutant was cultured in triplicate in 7H9 broth containing 0.05% Tween 80, and each week 0.5ml of culture was diluted into 10ml of fresh media. The $\Delta secA2$ mutant was passaged thirty times over the course of thirty weeks. The cultures were plated on the first passage, and then plated during the course of the experiment to

evaluate the frequency of rough variants. The frequency of Rough revertants did not show a statistical increase over time (Table 4.1). This suggests that although selection for the revertants occurs on plates containing Tween 80, and does not occur in liquid culture in the presence of Tween. These experiments suggest that the procedure to achieve a homogeneous Smooth population would be to take a colony from a plate without Tween 80, to patch that colony onto plates without Tween 80, then to take that patch and inoculate into liquid media. Several rounds of patching on plates without Tween 80 may also be beneficial.

Also, the reversion to a Rough phenotype does not seem to be selected for intracellularly in either murine bone marrow derived macrophages or the human monocytic cell line THP-1, as the percentage of Rough variants during the course of an infection does not vary (data not shown). This may suggest that there is no survival advantage for one variant versus another in the macrophages. Although, given the short time course of the experiments of six days (or 3-4 doublings), perhaps there is not enough time to select for an enriched population.

Complementing the Smooth colony phenotype using an *M. tuberculosis* cosmid library. The altered colony phenotype of the $\Delta secA2$ mutant is likely due to diminished export of a cell envelope factor. We hypothesized that if we were able to add an additional copy of the unknown factor, that increased levels of that protein might be able to suppress the $\Delta secA2$ mutant phenotype. We transformed a *M. tuberculosis* H37Rv genomic cosmid library (1) into the $\Delta secA2$ mutant, in hopes of identifying genes that, when over-expressed in the $\Delta secA2$ mutant background, would suppress the Smooth

Table 4.1: Percentage of Rough variants after multiple rounds of passage

Passage:	1	15	30
% Rough	4.6 \pm 2.2	10.3 \pm 4.1	8.7 \pm 8.6

Table 4.1: The $\Delta secA2$ mutant was serially passaged in triplicate cultures in 7H9 ADS Gly media with 0.05% Tw80. At various timepoints, each culture was serially diluted in PBS 0.05% Tw80 and plated in triplicate onto 7H10 ADS Gly plates with 0.05% Tw80. For each plating, the total number of colonies was counted, the number of Rough determined, and divided by the total to determine the percent Rough. Values represent the average and standard deviation of the triplicate samples. All p values for subsequent passages versus Passage 1 are > 0.05 as determined by the Student's t test.

phenotype back to Rough. These factors would then lead us to the types of proteins that contribute to the Rough morphotype. The cosmid library used has an average insert size of 30-40kb and integrates in single copy into the *M. tuberculosis* genome (1).

Transformants were plated onto 7H10 plates supplemented with 0.05% Tween and hygromycin to select for the cosmids, and the colony phenotypes examined. Out of 4648 colonies, we isolated 28 Rough clones. A control transformation using the cosmid backbone vector pYUB412 showed no Rough revertants out of 4500 clones. The genome of *M. tuberculosis* is approximately 4Mb, so given the average cosmid insert size of 35kb, we examined 40X genome equivalents represented by our 4648 cosmid clones. We generated genomic DNA from these 28 clones and performed Southern Blot analysis to determine if any of the Rough complementing cosmids contained *secA2*, using a strategy designed by Braunstein *etal* (9). The *secA2* probe hybridizes to an 11.4kb *secA2*-containing fragment in wild type, and a 3.5kb fragment in the $\Delta secA2$ mutant (Fig. 4.6). Any Rough complementing clone that contains a cosmid with *secA2* would have two bands: the $\Delta secA2$ 3.5kb fragment and an 11.4kb wild type fragment generated from the cosmid. Two Rough complemented clones, Cosmid 23 and Cosmid 25, were found to contain an additional wild type copy of *secA2* (Fig. 4.6).

We next rescued the cosmids from the genomes by digesting genome DNA from the Rough clones, ligating the mixture to cosmid arms, and packaging the ligation into lambda phage. The phage were used to transduce *E. coli*, and the cosmid clones were selected on hygromycin. The ends of the retrieved cosmids were sequenced to determine the boundaries of the inserts. To date, we have extracted the cosmids from 13 clones, and have sequenced the ends of inserts from 12 clones using primers which hybridize to the

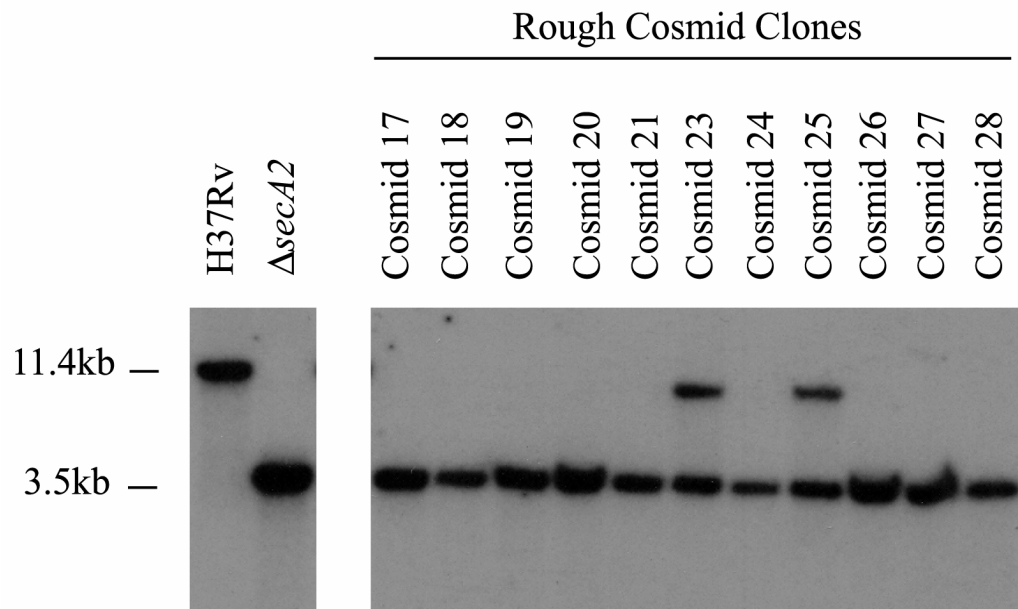


Fig 4.6. Two rough complementing cosmids contain *secA2*. A Southern blot of EcoRI/HindIII digested genomic DNA probed with *secA2*. A wild type *secA2* band is 11.4kb, the $\Delta secA2$ band is 3.5kb. Genomic DNA from wild type H37Rv and the $\Delta secA2$ mutant was used as a control. Band sizes are depicted to the left.

vector (Table 4.2). The sequence obtained from the cosmids suggests that there are several groups of cosmids that may share overlap in insert sequence. However, given the nature of the cosmid library construction, there is a possibility that a single cosmid may contain more than one insert, or that the insert is scrambled. Our sequence analysis determined that this was the case, and that at least five of our cosmid clones contained scrambled inserts (Table 4.2). Cosmids 7 and 9 shared identical sequence overlap, demonstrating that these represented a single clone that was duplicated in the library or during outgrowth after library transformation. Several cosmids were transformed back into the $\Delta secA2$ mutant to confirm that the cosmid could complement the colony phenotype back to Rough, proving that the original cosmid clones were not spontaneously Rough. Cosmids 6, 13, 11 and 21 were able to complement the $\Delta secA2$ mutant phenotype to the parental Rough.

Sequence analysis and restriction digest mapping of Cosmid 21 demonstrated that the insert was 38kb, and may share sequence overlap with Cosmid 20. The chromosomal region contained in the insert of Cosmid 21 is depicted in Fig. 4.7A. To ascertain the gene responsible for complementation, Cosmid 21 was subcloned into smaller fragments, and the smaller subclones were tested for their ability to suppress the $\Delta secA2$ mutant Smooth phenotype to Rough. On Cosmid 21, the locus that was able to confer the Rough phenotype to the $\Delta secA2$ mutant was identified as Rv3271c, and the suppressing fragment is represented in Fig. 4.7B. Rv3271c was contained on a 1.5kb MluI fragment cloned into the integrating vector pMB198, which does not contain a constitutive fragment reveals several potential promoter regions. The suppressing fragment also

Table 4.2 : Cosmids conferring the Rough colony morphotype to the *M. tuberculosis* Δ secA2 mutant

Cosmid Clone # ¹	Cosmid Sequence Forward ^{2,3}	Beginning gene? ⁴	Cosmid Sequence Reverse	Ending Gene?	Potential sequence overlap with other cosmids? ⁵	Notes ⁶
1	3411663 (F)	Rv3050c				
3	3545572 (R)	Rv3177				
4	395073(R)	Rv0329c			Cos6	
6	407119 (R)	Rv0339c			Cos4	
7	281904 (R)	Rv0235c	1603104 (F)	Rv1427c	Cos9/ Hygout sequence may overlap with Cos13	Cos7 and Cos9 are the exact same cosmid clone, and the cosmid is scrambled
9	281904 (R)	Rv0235c	1603104 (F)	Rv1427c	Cos7/ Hygout sequence may overlap with Cos13	Cos7 and Cos9 are the exact same cosmid clone, and the cosmid is scrambled
11	1090025 (R)	Rv0976c	4202077 (R)	Rv3756c	Hygout may overlap with Cos14	cosmid is scrambled
13	726609 (R)	Rv0631c	255333	Rv0213c	Hygout may overlap with Cos7/Cos9	
14	3658440 (F)	Rv3276c	4201382 (R)	Rv3755c	Cos11	
16	3755534 (R)	Rv3349c	1297895 (R)	Rv1166		cosmid is scrambled
17	893542 (R)	Rv0800	222342 (R)	Rv0190	Hygout sequence may overlap with Cos18	cosmid is scrambled
18	222342 (F)	Rv0191				
19	1772933 (F)	Rv1565c				
20			3673602(R)	Rv3292	Insert may overlap with Cos21/Cos28	
21	3636275(F)	Rv3257c			Insert may overlap with Cos20/Cos28	Cosmid subcloned
23						Contains <i>secA2</i>
24	355888 (F)	Rv0292				
25						Contains <i>secA2</i>
28	3642809 (F)	Rv3262	3682376 (R)	Rv3298c	Insert may overlap with Cos21	

¹These represent the Rough complementing clones for which sequence information is known

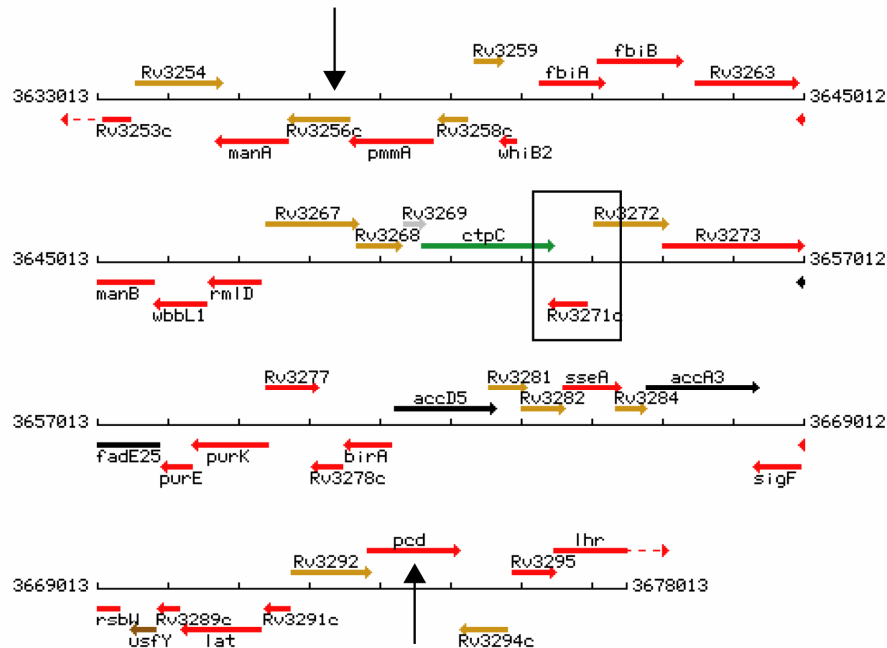
² The rescued cosmids were sequenced using primers derived from the pYUB412 cosmid backbone, Forward (412out1) and Reverse (Hygout). The number represents the location on the H37Rv genome (Tuberculist) where the sequence alignment begins

³ The directionality of the sequence as read from the pYUB412 backbone, to determine the orientation of the insert

⁴ The open reading frame on the H37Rv genome where sequence alignment begins

⁵ If the cosmid sequence suggests potential overlap with another cosmid clone, that clone is listed in this column. Given the potential for scrambled inserts, there may be only partial overlap

Region of Cosmid 21 suppressing the Smooth colony phenotype



Subcloned Cosmid 21 fragment that suppresses the Smooth Phenotype

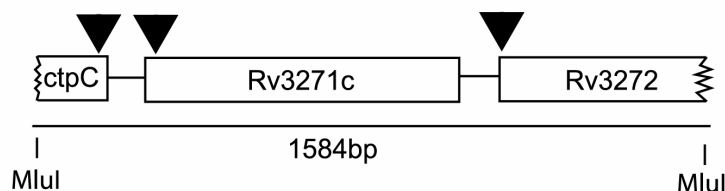


Fig 4.7. Region from Cosmid 21 suppressing the Smooth colony phenotype. Sequence and restriction digest analysis from Cosmid 21, which suppresses the Smooth phenotype of the $\Delta secA2$ to Rough, contained an insert of approximately 38kb of overlap, as shown in Fig. 4.7A. Arrows depict the approximate ends of the cosmid insert. The cosmid was subcloned into smaller fragments, and the individual reading frame responsible for complementation, Rv3271c, was isolated on a 1584bp MluI fragment, as shown in Fig. 4.7B, and contained within the box in Fig. 4.7A. The fragment contains the first 19 amino acids (aa) of *ctpC* and the first 253 aa of Rv3272. Arrowheads indicated the start of the open reading frames.

contained two other truncated proteins, including the first 19aa of CtpC and the first 253aa (of 349aa total) of Rv3272, however Rv3271c was the only complete reading frame contained within the fragment cloned. This open reading frame (orf) corresponds to a protein annotated in the *M. tuberculosis* H37Rv genome as a probable conserved integral membrane protein. *In silico* analysis of Rv3271c reveals six transmembrane helices, but no cleavable signal peptide. There have been no functional studies to date on this protein, however, homology searches show similarity to cation efflux proteins. A saturating transposon mutagenesis of *M. tuberculosis* identified this gene as one that is likely to be essential for the optimal growth of *M. tuberculosis* (28).

Taken together, our data supports that there is an altered cell surface of the $\Delta secA2$ mutant of *M. tuberculosis* by multiple evaluations. We have demonstrated that the $\Delta secA2$ mutant of *M. tuberculosis* displays an altered colony phenotype, perhaps due to an increased sensitivity of the $\Delta secA2$ mutant cell envelope to the detergent properties of Tween 80. The $\Delta secA2$ mutant has altered cell to cell interaction, as shown by the defect in cording. We have uncovered an integral membrane protein, Rv3271c, which can suppress the altered morphotype. Further examination of the other complementing cosmids may lead to a better understanding of the changes in the cell envelope of $\Delta secA2$ mutant, as well as a direct examination of the cell envelope and its properties. Achieving a better understanding of the behavior and biology of the $\Delta secA2$ mutant may give us a better understanding of SecA2 dependent factors important for the normal processes and cell envelope maintenance of the bacterium, but also those factors important for virulence.

Discussion

The $\Delta secA2$ mutant of *M. tuberculosis* has an altered colony phenotype on plates supplemented with Tween 80, a phenotype similar to that described for the attenuated strains H37Ra and *M. bovis* BCG in 1947 (25). Interestingly, the Smooth phenotype observed in the $\Delta secA2$ mutant is unstable, and clones revert back to the wild type Rough morphotype at a relatively high frequency when plated on agar with Tween 80. Our experiments demonstrating the selective pressure of Tween 80 on the reversion, as well as the increased sensitivity of the $\Delta secA2$ mutant to SDS, suggests that there are alterations in the cell envelope of the $\Delta secA2$ mutant that make it more sensitive to the disruptive properties of detergent. Perhaps the instability of the cell envelope is driving the selection of a more stable, Rough revertant. As mentioned previously, we observe a higher frequency of Rough revertants on plates with a lower density of colonies. This may suggest that Tween 80 is having a toxic effect on the Smooth bacteria, and that at higher densities the toxic effects of Tween 80 are titrated away by the bacteria, perhaps by a secreted factor that is able to breakdown the Tween. More studies will have to be done to determine what the effects of Tween 80 are.

As there were no visible differences in the morphology of the individual bacilli, it raises the questions as to what the altered cell envelope properties of the $\Delta secA2$ mutant are. We hypothesize that SecA2 exports a cell envelope associated factor, or a biosynthetic enzyme that modifies the cell envelope. We attempted to isolate sequences of the genome that could suppress the Smooth morphotype of the $\Delta secA2$ mutant. We hypothesized that if we were able to add an additional copy of the unknown factor,

increased levels of that factor would be able to suppress the $\Delta secA2$ mutant phenotype. We isolated several unique clones that are able to restore the wild type Rough morphotype. Subcloning from one of the Rough cosmids yielded a single locus that we were able to identify, Rv3271c, is annotated as an essential, putative integral membrane protein. In silico analysis suggests that Rv3271c is an integral membrane protein, supporting idea that alterations to the membrane can cause the phenotypic reversion. When an additional copy of this gene is expressed under the control of its own promoter, it may alter the surface or protein interactions of the cell envelope. Given the nature of this system, we may simply be screening for genes that, when presented in an extra copy change the surface composition. Further studies will be needed to determine if the genes we isolated that suppress the Smooth phenotype are SecA2 specific. We hypothesize that there may be several categories of factors that can suppress the Smooth phenotype. There may be membrane and secreted proteins that are directly involved with cell envelope structure. Alternatively, there may be biosynthetic enzymes that modify cell envelope associated lipids. There may be multiple factors that contribute to the Smooth/Rough phenotype. Isolation of the other loci responsible for suppression may give more insight to the nature of the cell envelope perturbation in the $\Delta secA2$ mutant.

The $\Delta secA2$ mutant of *M. tuberculosis* is attenuated in a murine model of infection (9, 22). In murine macrophages, the $\Delta secA2$ mutant is attenuated for growth, and hyperactivates the macrophages to produce increased levels of proinflammatory cytokines TNF- α and IL-6 and reactive nitrogen intermediates (RNI) (22). The alteration of the cell envelope in the $\Delta secA2$ mutant may contribute to its attenuation by changing the interaction of $\Delta secA2$ mutant with the immune system. Given the instability of the

colony morphotype of the $\Delta secA2$ mutant, it is important to establish the virulence of the Rough revertants in the mutant background. The factors in the $\Delta secA2$ mutant that are inducing the more robust immune response discussed in Chapter 3 and Chapter 4 may be related to its altered cell wall. If so, then the Rough revertants may have reestablished some of the wild type characteristics, and may no longer induce a more robust response. As we have discussed in Chapter 4, this more robust response may be directly related to the attenuation of the $\Delta secA2$ mutant. Therefore, if a given population of $\Delta secA2$ mutant culture has more Rough revertants, it may be more virulent than a pure Smooth population, and may be confounding our studies. However, as mentioned previously there does not seem to be an accumulation of the Rough revertants over time in macrophages cultures. Nonetheless, our results have demonstrated a way that the $\Delta secA2$ mutant can be propagated in order to give the most pure Smooth population. If the strain is cultured on plates without Tween 80, and subsequently inoculated into liquid media from the plates without Tween, this should minimize the level of Rough revertants. It is still important to determine the virulence properties of these Rough revertants.

The proper localization of cell envelope components is important for virulence in many organisms, including *M. tuberculosis*, and the alterations in the cell envelope of the $\Delta secA2$ mutant may be contributing to its attenuation. There are several mutants of *M. tuberculosis* that demonstrate an altered colony morphology and that are attenuated for virulence. KasB is an enzyme required for the biosynthesis of mycolic acids, an integral component of the cell envelope of *M. tuberculosis*. A *kasB* mutant is defective for cording as mentioned previously, and has an altered colony morphotype (5). This mutant is highly attenuated in mice (5). Phthiocerol dimycocerosates (PDIM) is a cell envelope-

associated lipid found only in pathogenic mycobacteria. A screen of a transposon mutant library for attenuated mutants of *M. tuberculosis* revealed three mutants to be avirulent, in *pps*, *FadD28* and *mmpL7*, that were each involved in the biosynthesis and localization of PDIM (13). These mutants also had altered colony morphotypes when compared to the wild type strain. Proteins localized to the surface of the bacterium can also contribute to the overall cell envelope architecture of the bacterium. The exported repetitive protein (Erp) is surface exposed in *M. tuberculosis* and *M. bovis* BCG (4, 14, 15). When *erp* is disrupted, the resulting strain of *M. tuberculosis* is attenuated (4, 14). The Δ *erp* strain also has an altered colony morphology compared to the parental wild type (4, 14, 15). However, the altered colony morphotypes of these mutants done by visual comparison of published colony micrographs, do not resemble those of the Δ *secA2* mutant, suggesting that the alterations in the Δ *secA2* mutant may be unrelated to these described cell envelope components. It may be worthwhile to examine these described mutants in parallel to the Δ *secA2* mutant to confirm that they do not share similar characteristics.

The colony and cording phenotypes of the Δ *secA2* mutant most closely resemble those of the attenuated strain H37Ra (25). Our studies indicate that the altered phenotype of H37Ra is independent of *secA2*. One group examined gene expression profiles of H37Rv and H37Ra grown under several conditions in which the wild type showed the cording phenotype and H37Ra did not (16). Under these conditions, there were 22 genes consistently downregulated in H37Ra, a significant population of which represented genes involved in lipid metabolism and cell membrane processes.

Also in *Mycobacterium kansasii*, there are similar Rough and Smooth colony morphotypes when grown on plates containing Tween 80 (3). Analysis of cell envelope

components showed that the lipooligosaccharide of the Smooth strain contained trehalose, while that of the Rough strain did not. The Smooth morphotype of *M. kansasii* was less virulent than the Rough strain, which was able to persist longer in mice. Given the similarities in the phenotypes of these strains, these may be attractive candidates to investigate in the $\Delta secA2$ mutant.

We have presented data that the $\Delta secA2$ mutant of *M. tuberculosis* induces a more robust immune response. Because SecA2 is a secretion factor, its role in immunomodulation is most likely related to proper secretion or cell wall localization of an immunosuppressive factor. Alternatively, SecA2 may secrete an enzyme that modifies a cell wall factor to make it less immunogenic. However, all of the proteins localized by SecA2, particularly cell wall proteins, are not yet known. The mycobacterial cell wall is a complex structure that contains many diverse molecules with reported immunomodulatory properties (21). The increased immune response elicited by the $\Delta secA2$ mutant could be explained by different amounts of immunoregulatory molecules in the cell wall or an altered cell wall structure with greater exposure of stimulatory molecules that interact with host receptors. Thus, we are considering the possibility that the immunosuppressive effect of SecA2 is due to a role in localization of cell wall synthetic enzymes that influence cell wall architecture. A final possibility is that the $\Delta secA2$ mutant phenotypes actually represent increased release of a stimulatory molecule (8).

Overall, we are just beginning to understand the role that SecA2 plays in the establishment of the cell envelope architecture and how that may affect the virulence of

the organism. Achieving a better understanding of the SecA2 dependent factors that alter the cell wall may give insight to the SecA2 dependent factors that promote virulence.

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We would like to thank Victoria Madden in the UNC Microscopy Services lab for her assistance in preparing SEM samples. We would also like to thank Dr. Janne Cannon for helpful insight and discussions in designing the modified fluctuation test. We would also like to thank Dr. Janne Cannon for the kind gift of her colony microscope.

Attributions

All experiments presented were performed by myself with the following exceptions; Nathan W. Rigel passaged the $\Delta secA2$ mutant over the course of 30 weeks and plated passages to observe the frequency of colony morphotype over time. He also performed a western blot analysis on H37Ra to confirm production of SecA2 in that strain.

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CHAPTER 5

DISCUSSION

An estimated 1.6 million people died from tuberculosis in 2005, an equivalent of 4400 people per day (3). The continuing tuberculosis pandemic burdens the global health care system, and a better vaccine and improved anti-mycobacterial therapies are needed to control this health crisis. It is essential that we achieve a greater understanding of the biology of *M. tuberculosis* and how it combats the host immune response to help eradicate this disease.

For many pathogens, the full virulence of the organism is dependent on accessory secretion systems and secreted virulence factors. For mycobacteria and some Gram positive pathogens, the roles that these alternate secretion systems play in pathogenesis are only beginning to be uncovered. In Chapter 2, we present data that demonstrated the accessory SecA2 secretion system of *M. tuberculosis* to be essential to the full virulence of the organism. We further showed that a $\Delta secA2$ mutant of *M. tuberculosis* is attenuated for growth in non-activated murine bone marrow derived macrophages. The ability of *M. tuberculosis* to replicate in macrophages relies on a complex array of virulence traits. These include *M. tuberculosis* overcoming the toxic effects of reactive oxygen (ROI) and reactive nitrogen intermediates (RNI) in order to survive, as reviewed in (2). However, the $\Delta secA2$ mutant phenotype is not due to an inability to resist these

host defense mechanisms. The $\Delta secA2$ mutant is equally attenuated in macrophages from $phox^{-/-}$ or $iNOS^{-/-}$ mice, which are deficient for ROI and RNI respectively, as it is in cells from wild type mice. We also demonstrated that macrophages infected with the $\Delta secA2$ mutant produce a more robust immune response than those infected with the parental wild type *M. tuberculosis*. This led to our hypothesis that SecA2 promotes virulence of *M. tuberculosis* by modulating the host immune response.

In Chapter 3, we investigated alternate host factors that might be contributing to the attenuation of the mutant. Macrophages infected with the $\Delta secA2$ mutant produced increased levels of the proinflammatory cytokines IL-6 and TNF- α , as well as increased RNI. *M. tuberculosis* induces the production of these proinflammatory molecules through TLR/MyD88 dependent signaling cascade, as reviewed by (1). The role of SecA2 may be to inhibit the TLR/MyD88 dependent immune response. We hypothesized that if the mutant is stimulating a more intense TLR response, perhaps the host factors induced by this pathway control the growth of the mutant. We tested this hypothesis by examining the growth of the $\Delta secA2$ mutant in macrophages derived from mice deficient in MyD88. We observed that the attenuation of the $\Delta secA2$ mutant is reversed in the $MyD88^{-/-}$ macrophages. This data proposes a role for the TLR/MyD88 signaling pathway in the growth inhibition of the $\Delta secA2$ mutant. More specifically, this data suggests that there is a MyD88 dependent mechanism that inhibits the growth of *M. tuberculosis*. However, virulent *M. tuberculosis* is able to overcome this host defense mechanism to grow intracellularly. MyD88 is a downstream signaling adaptor for the individual TLRs, therefore it will be interesting to investigate the contribution of the individual TLR molecules to the attenuation of the mutant.

Given the increase of TNF- α during infection with the $\Delta secA2$ mutant, we tested the role of this cytokine in the attenuation of the mutant. Our data demonstrates that a TNF- α dependent host response is required for the attenuation of the $\Delta secA2$ mutant. In our cell autonomous macrophage infection, TNF- α directs an antimicrobial mechanisms against $\Delta secA2$ mutant. However, wild type *M. tuberculosis* is able to overcome this TNF- α dependent response.

There are several potential mechanisms used by the host to control the growth of *M. tuberculosis*. We have demonstrated that the $\Delta secA2$ mutant is attenuated by a host response mechanism that is dependent on MyD88 and TNF- α , yet independent of ROI, RNI, or host cell cytotoxicity. Further investigations will be needed to uncover this novel MyD88/TNF- α dependent antimicrobial mechanism.

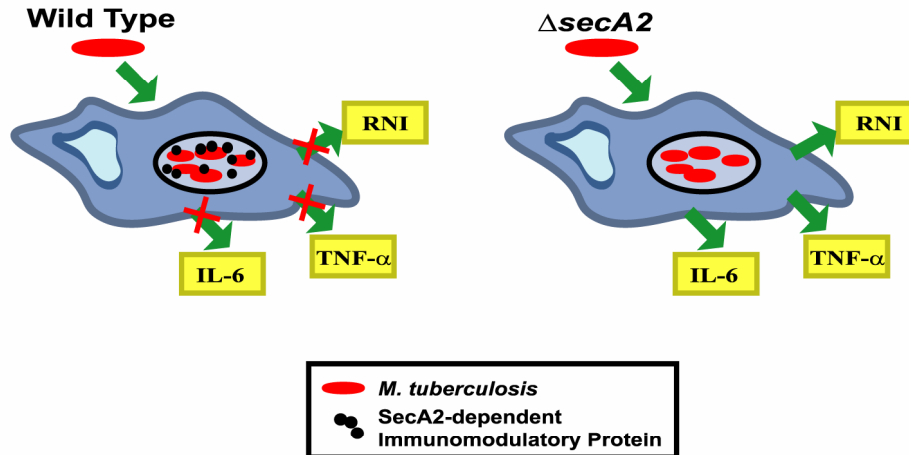
We propose two models for the role of SecA2 in promoting the growth of *M. tuberculosis* in host macrophages. In the first model (Fig. 5.1), *M. tuberculosis* produces immunomodulatory proteins that are exported via SecA2. The $\Delta secA2$ mutant is deficient for the export of these immunomodulatory proteins, and therefore can not properly alter the host cell environment into one favorable for replication

The second proposed model of the role of SecA2 in modulating the host immune response is one where the surface exposed components of the cell envelope of *M. tuberculosis* contain modifications that decrease its ability to induce a TLR response, or mask exposure of a TLR ligand (Fig. 5.1). In this model, the surface modifications would be dependent on factors localized by the SecA2 export pathway. In the $\Delta secA2$ mutant these factors are absent, and the altered surface of the $\Delta secA2$ mutant is more stimulatory.

Macrophages infected with the $\Delta secA2$ mutant release more immunostimulatory molecules

Model 1

In macrophages:



Model 2

In macrophages:

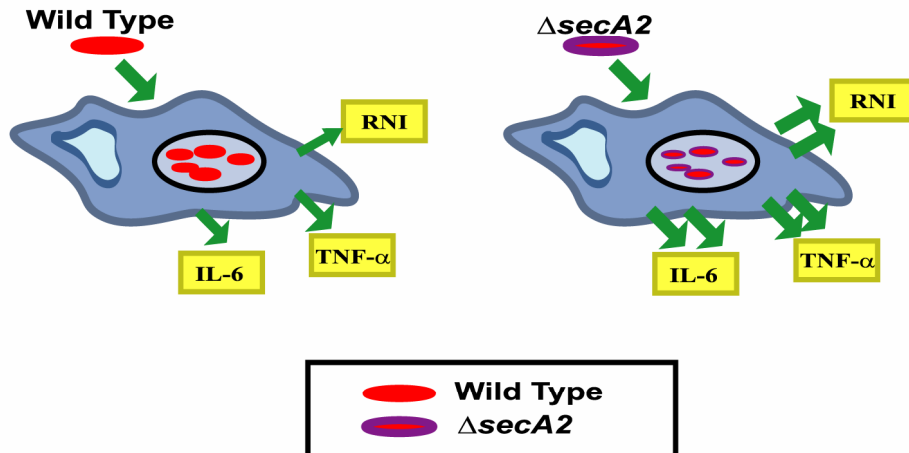


Fig. 5.1 Two Models for the role of SecA2 in modulating the immune response

In *M. tuberculosis* there are examples of exported factors that would support either SecA2 model of immunomodulation. Further investigation is needed to determine which model is correct.

In Chapter 4, we demonstrated that the $\Delta secA2$ mutant of *M. tuberculosis* has an altered colony morphology (Smooth) on plates containing Tween 80 and is defective for cording. This data suggests that there is some alteration in the cell envelope of the $\Delta secA2$ mutant. We also saw that the $\Delta secA2$ mutant is able to revert back to a wild type colony morphotype (Rough). This reversion is dependent on the presence of the Tween 80 in the plates. The altered surface structure of the $\Delta secA2$ mutant may correlate with our second model of how SecA2 functions to modulate the host immune response, by altering the surface of the bacterium to make is less stimulatory. We have proposed a method for culturing the $\Delta secA2$ mutant in order to minimize the potential for Rough reversion. It will be important to analyze the cell envelope fractions of the $\Delta secA2$ mutant to determine the factors contributing to the altered morphotype, as these may be factors that are also important for virulence.

References

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2. **Smith, I.** 2003. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin Microbiol Rev* **16**:463-96.
3. **WorldHealthOrganization** 2007, posting date. WHO Information tuberculosis fact sheet. [Online.]