ABSTRACT

Title of Document: FUNCTIONAL ANALYSIS OF ESAT-6 AND ESPB, TWO VIRULENCE PROTEINS SECRETED BY THE ESX-1 SYSTEM IN *MYCOBACTERIUM MARINUM*

Jennifer Ann Smith, Doctor of Philosophy, 2010

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Mycobacterium tuberculosis (*Mtb*) and *Mycobacterium marinum* (*Mm*) are able to persist inside host cell macrophages by modulating the phagosome environment. ESX-1 is a specialized secretion system that is required for virulence. Two of the proteins secreted by ESX-1 are ESAT-6 and EspB. They are codependent for secretion and are important virulence effectors, though their specific functions are not known. *Mm* is able to escape from the phagosome into the host cell cytosol where it can initiate actin-based motility. *Mm* escape is dependent on a functional ESX-1 system. I show that the ESAT-6 protein is able to form pores in host cell membranes which may play a role in *Mm* escape from the phagosome. I also dissect the *Mm* EspB protein and show that cleavage of EspB is required for growth inside RAW cells, virulence in zebrafish, and for modulating ESAT-6 secretion. The resulting C-terminal 11 kDa fragment is sufficient for the codependent secretion of ESAT-6; while the 50 kDa N-terminal fragment seems to be somewhat dispensable for ESAT-6

secretion but is definitely required for virulence. When EspB is expressed as a full-length protein, the highly conserved WXG motif in the N-terminal fragment is involved in the codependent secretion of the two proteins since secretion is reduced when this motif is mutated. Interestingly, when the N-terminal fragment is expressed without the C-terminal fragment it can secrete independent of the ESX-1 system, indicating that the C-terminus confers specificity for EspB secretion through ESX-1. I show that the virulence function of the EspB N-terminal fragment is dependent on the secretion of ESAT-6, and EspB must be expressed in its full-length form in order to be fully functional. These results indicate that EspB function is dependent on a close association with ESAT-6. It is possible that the N-terminus is translocated into the host cell cytosol through the ESAT-6 formed pore.

FUNCTIONAL ANALYSIS OF ESAT-6 AND ESPB, TWO VIRULENCE PROTEINS SECRETED BY THE ESX-1 SYSTEM IN *MYCOBACTERIUM MARINUM*

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2010

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Foreward

This dissertation is a culmination of research done by Jennifer Smith over the course of her graduate career under the guidance of Dr. Lian-Yong Gao at the University of Maryland in College Park, Department of Cell Biology and Molecular Genetics. I served as Chair of her examining committee. Chapter two is an adaptation of a manuscript that was previously published in the December, 2008 issue of *Infection and Immunity*, entitled, "Evidence of Pore Formation in the Host Cell Membranes by ESX-1 Secreted ESAT-6 and Its Role in *Mycobacterium marinum* Escape from the Vacuole" (Smith et al., 2008). Permission to reuse this manuscript was given on October 22, 2010 by the American Society for Microbiology (License Number: 253421007792). Jennifer is a first-author on this manuscript and her examining committee has determined that she made substantial contributions to the research content. Some of the figures were contributions from other members of Dr. Gao's lab and those figures have been cited as such. The manuscript was co-written in collaboration with Dr. Gao. All other chapters are the research and writings of Jennifer alone.

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October 26, 2010

Dedication

For Jason

“I love you not only for what you are but for what I am when I am with you.”

~ Elizabeth Barrett Browning

These words ring even truer today than they did the day that I married you.

Acknowledgements

First and foremost I'd like to thank my advisor, Dr. Lian-Yong Gao. Your excitement for the importance of our work was incredibly contagious. You always made yourself available to help me and I've learned so much from you about research and scientific thinking. I appreciate your commitment to my education and my project; even when circumstances led you on a new and exciting career path you sacrificed so much of your time to make sure that I was able to learn and grow as a scientist and student. Not only that, but you ensured that I'd be able to graduate and for that I owe you a whole heap of gratitude. I hope you know how much I appreciate all you've done for me.

Along those lines I also want to thank my co-advisor, Dr. Volker Briken. I can't express my gratitude for the effort you've made to make sure I was able to succeed and graduate. You took responsibility for my project and our lab even when you had so many other things pressing on your time. You've always been very patient and I appreciate having your fresh and intelligent perspective on my project and my goals. I will never forget that I wouldn't be here if not for you, Dr. Briken and I sincerely appreciate it.

I'd also like to thank the rest of my committee, Dr. Stein, Dr. Frauwirth, and Dr. Julin; I really appreciate your support these past years. Your invaluable insight helped to make my project a lot more meaningful to me in the broader sense. I gained a lot from our meetings, and appreciate how constructive and helpful they always were.

I'd like to thank Dr. Junjie Xu who, as a post doc in our lab, was very patient to train me and teach me all the technical things that I needed to know in order to get started as a scientific researcher. When you left, our lab just wasn't the same! I'd also like to thank lab-mates Dandan Li, Swati Shah, and Lalitha Srinivasan. We've been through a lot together! Having your support, suggestions, assistance, and commiseration through it all has really been a vital sanity-saver for me. You've been a lot of fun to work with and I am happy to call you my friends. I've also enjoyed getting to know the other members of the Briken lab during my last year. Thank you Gaya, Ben, Serdar, and Hana for always including me at your parties and meetings. Being up there all alone, I really appreciated that support. I also can't forget to thank some members of my CBMG cohort; Beth, Courtney, Megan, and Kathryn, looking back at our first year here, I just can't imagine getting through it without having you guys for constant commiseration. I've enjoyed continuing that tradition with you on all subsequent years and look forward to future commiserating as lifelong friends!

I'd also like to thank a few collaborators; Dr. Nicole LaRonde-LeBlanc and Dr. Agnieszka Szyk, Department of Biochemistry at the University of Maryland, for helping with protein purification studies which were invaluable to my research. Also, Dr. Jim Du at the University of Maryland, Biotechnology Institute for providing zebrafish and expertise; and also for providing funding during my final semester! Your kindness is much appreciated!

Last but certainly not least, I'd like to thank my family. Jason, words cannot express how much I appreciate you and all that you've done and continue to do for me each and every day. I guess I can't say that I wouldn't have been able to do it without you,

but I can definitely say that I never would have *wanted* to do it without you! Without having your immeasurable support, this challenge would have been exponentially more overwhelming! I'd also like to thank my parents; Mom and Dad, thanks for laying the groundwork and for giving me all the love and support that I've always needed from you, today and all my days before. Finally, thank you, POTUS for keeping me company and giving me the time I needed to finish before making your highly anticipated debut!

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List of Abbreviations

AEBSF	4-(2-Aminoethyl)-benzenesulfonyl-fluoride-hydrochloride
ATP	Adenosine Triphosphate
BCG	The vaccine strain Bacillus Calmette-Guérin
Bla	β-lactamase
BMDM	Bone Marrow Derived Macrophage
BSA	Bovine Serum Albumin
BSL	Biosafety Level
cAMP	cyclic Adenosine Monophosphate
CCCP	Carbonyl Cyanide 3-Chlorophenylhydrazone
CD4 or 8	Cluster of Differentiation 4 or 8
CFP-10	Culture Filtrate Protein – 10 kDa
CFU	Colony Forming Units
Cya	Adenylate Cyclase
DMEM	Dulbecco Modified Eagle Medium
ESAT-6	Early Secreted Antigenic Target – 6 kDa protein
EspA,B,C,F	ESX-1 Secretion Protein A, B, C, or F
ESX-1	ESAT-6 Secretion System – 1
GSK	Glycogen Synthase Kinase
HIV	Human Immunodeficiency Virus
HRP	Horseradish-Peroxidase
IFN-γ	Interferon – gamma
IL-1, 6, 12	Interleukin-1, 6, or 12

iNOS	Nitric Oxide Synthase
IRAK1 or 4	Interleukin-1 Receptor Associated Kinase 1 or 4
kDa	Kilo Dalton
LAM	Liparabinomannan
LAMP	Lysosomal Associated Membrane Protein
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MCV	Mycobacterium Containing Vacuole
MDR-TB	Multi-Drug Resistant Tuberculosis
MHC I or II	Major Histocompatibility Complex I or II
Mm	*Mycobacterium marinum*
MOI	Multiplicity of Infection
Mtb	*Mycobacterium tuberculosis*
MyD88	Myeloid Differentiation Primary Response gene – 88
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor - Kappa light-chain enhancer of activated B cells
NO	Nitrous Oxide
NOX2	Subunit of the NADPH oxidase
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PE	Proline-Glutamic Acid proteins
PEG	Polyethylene Glycol
PI3P	Phosphatidylinositol-3-Phosphate

PknG	Protein Kinase G protein
PPE	Proline-Proline-Glutamic Acid proteins
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene Fluoride
RD1	Region of Difference 1
RNI	Reactive Nitrogen Intermediates
ROI	Reactive Oxygen Intermediates
SNARE	Soluble NSF Attachment Receptor protein
Snm1, 2, 4	Secretion in Mycobacteria 1, 2, or 4
T3SS	Type III Secretion System, or IV, or VII
TACO	Tryptophan-Aspartate Containing Coat protein
Tat	Twin-Arginine Transporter
TB	Tuberculosis disease
TIR	Toll/Interleukin-1 Receptor
TLR-2 or -4	Toll-Like Receptor 2 or 4
TNF-α	Tumor Necrosis Factor - alpha
TRAF6	TNF Receptor Associated Factor 6 protein
WT	Wild Type
WXG	Tryptophan-Variable-Glycine motif
XDR-TB	Extensively-Drug Resistant Tuberculosis

Chapter 1: Introduction

Mycobacterium tuberculosis (*Mtb*) is the causative agent of the devastating disease, tuberculosis (TB), which infects one third of the world's population and is responsible for killing two million people each year (World Health, 2009). The only available vaccine, Bacillus Calmette-Guérin (BCG) is an attenuated strain isolated through passage of *Mycobacterium bovis*. Unfortunately, this vaccine is ineffective at preventing disease in the majority of cases. Alternatively, there are several drug therapies available to treat TB disease, but treatment is intensive and requires taking several different antibiotics over the course of many months. Incomplete treatment has caused an increase of multi-drug and extensively drug resistant strains of *Mtb*. This further intensifies the need for new drugs. Because active TB disease is more prevalent in immune-compromised patients, there has been a huge surge of TB-induced death in persons co-infected with HIV. It is clear that newer and better TB drugs and a more effective vaccine would be an enormous benefit to the health of the global population. However, there is still a lot to learn about the pathogenesis of *Mtb* in order to design effective drugs and vaccines.

1.1 The history of *Mycobacterium tuberculosis*

TB has been plaguing humans since ancient times. It has co-evolved with humans, making it an extremely well adapted pathogen. Skeletal remains dating back to ~9000 BC revealed that even prehistoric humans were infected with TB (Hershkovitz et al., 2008). Around 460 BC, Hippocrates, a physician from ancient Greece, clearly recognized the clinical presentation in young adults of "phthisis", as TB disease was

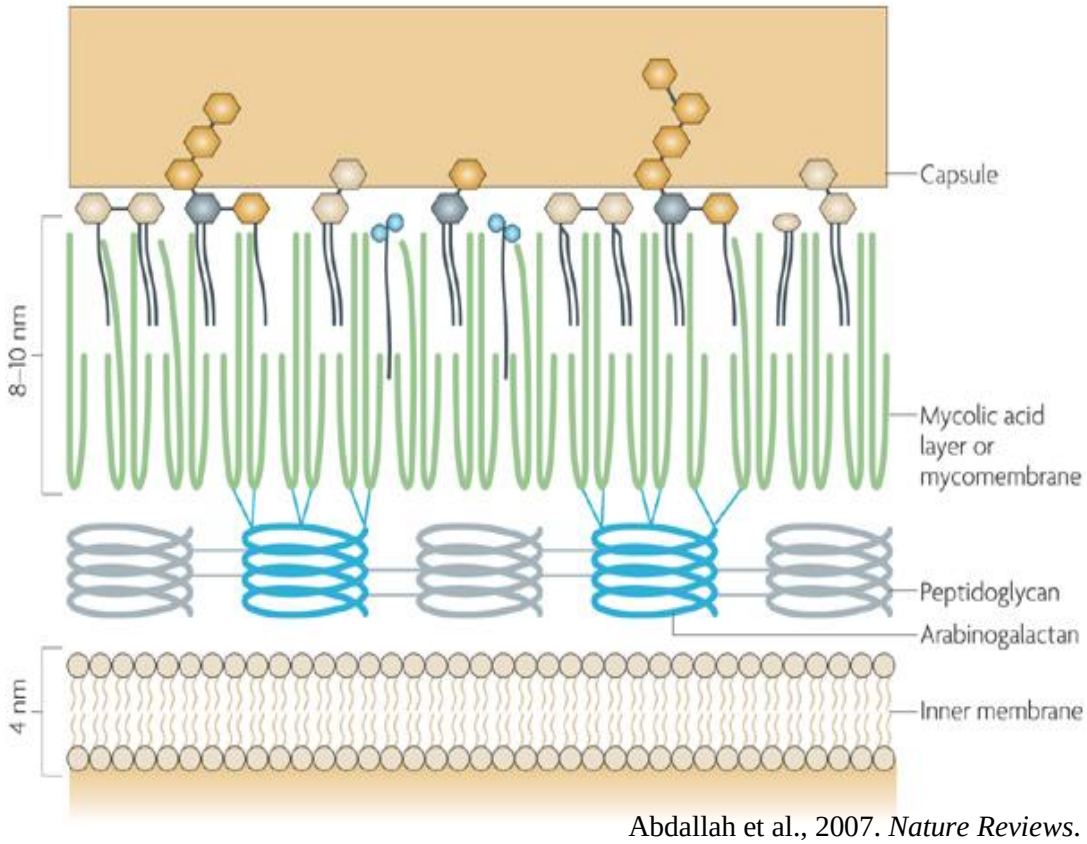
called there. He described it as the most prevalent disease of the time. Another Greek physician, Clarissimus Galen wrote about TB disease in 174 AD and recommended that treatment include "fresh air, milk, and sea voyages" (Daniel, 2006). The symptoms of TB have prompted several names that describe the disease. The paleness and long periods of relentless wasting associated with the active form of TB makes it seem as though patients are being consumed from within. For this reason the disease has been called "consumption", "wasting disease", and "white plague". TB is also sometimes referred to as "Koch's disease" because Dr. Robert Koch was the first to identify the *Mycobacterium tuberculosis* bacillus that causes TB disease. He won the Nobel Prize for his discovery in 1905 (Daniel, 2006). Dr. Koch also developed a glycerin extract of the tubercle bacilli that he called "tuberculin". He proposed that it be used as a TB vaccine but it was ineffective. However, tuberculin is useful as a diagnostic for determining if a person is infected with *Mtb*.

1.2 *Mycobacterium tuberculosis* and its transmission

Mtb is a small, bacillus-shaped member of the Actinomycetes family of bacteria that have a high G+C ratio in their genome (Embley and Stackebrandt, 1994). They are technically Gram-positive because they do not have an outer membrane. However, they do have a thick cell wall that is composed of dual layers of peptidoglycan and arabinogalactan. The cell wall is covalently linked to a layer of branched-chain fatty acids called mycolic acids. This provides the bacteria with a protective hydrophobic, waxy coat, called a mycomembrane that is much less penetrable than most Gram-positive cell walls (Brennan and Nickaido, 1995; Abdallah et al., 2007). Beyond the mycomembrane they have a capsule that is composed primarily of polysaccharides,

mainly glucan and arabinomannan, but also contains proteins and a small amount of lipid, namely lipoarabinomannan (LAM) (Daffe and Etienne, 1999) [Fig. 1]. Because of this complex cell wall, mycobacteria are difficult to Gram-stain, and are characterized as acid-fast because they resist the dilute acid de-colorization stain used in typical staining procedures. To identify them, the Ziehl-Neelson staining procedure is readily used instead (Glickman and Jacobs, 2001). To date there have been 85 different species of *Mycobacterium* identified. *Mtb* is among the slow growing of the genus, dividing every 15-20 hours. Unlike most *Mycobacterium* species, *Mtb* has never been found outside of its host. *Mtb* requires high levels of oxygen which is why they thrive so well in the lung alveoli of humans. However, exposure to *Mtb* does not always result in disease even though the infectious dose is relatively low, usually only about 10 bacteria. This is because co-evolution of *Mtb* with humans has allowed for the latent survival of the bacteria without causing duress in the host.

Typically, an uninfected person becomes infected with *Mtb* when a person suffering from active TB disease coughs or sneezes. They expel tiny aerosol droplets that contain the bacteria. When the uninfected person inhales these droplets they too become infected and then there are three possible outcomes; 1) the host immune system will clear the bacteria, 2) the person develops active TB disease, or 3) the person develops latent TB disease which may or may not reactivate at a later time. Latent TB disease is by far the most common response to infection. The bacteria



Abdallah et al., 2007. *Nature Reviews.*

Figure 1. The mycobacterial cell envelope. Beyond the mycobacterial inner membrane is the cell wall which is composed of peptidoglycan, arabinogalactan, and covalently linked mycolic acids. The mycolic acid layer with intercalated lipids, creates a hydrophobic barrier sometimes called a mycomembrane. The mycomembrane is much less penetrable than the typical Gram-positive cell wall, and the lipids are generally mycobacteria specific. Beyond the mycomembrane, mycobacteria have what is often referred to as a capsule that is composed mainly of polysaccharides.

enter the lungs and are taken up by alveolar macrophages and dendritic cells. *Mtb* is able to live inside macrophages and reside in a dormant sort of state for many, many years. In fact, a person with latent *Mtb* infection has only a 10% lifetime risk of progressing to active TB disease. Compare this with persons co-infected with HIV, who have an 8-10% *annual* risk of developing active disease (Selwyn et al., 1989). When a person's immune system is compromised, as is the case with HIV infection, people taking immunosuppressive drugs, or the elderly, it causes a break in the infection-protection balance and the bacteria progresses to active disease (Wells et al., 2007). The symptoms of active disease include the incessant, often bloody cough that is typically thought of when there is mention of tuberculosis disease. Also, it can cause a loss of appetite leading to weight loss, fever and night sweats, and chest pain. If left untreated, pulmonary TB kills about 50% of patients. *Mtb* can also disseminate in the blood stream throughout the body. This more severe form of disease most often occurs in children, and is called miliary tuberculosis. If left untreated, miliary TB kills nearly 100% of patients (Sharma et al., 2005).

1.3 Modulation of immune defenses by *Mycobacterium tuberculosis*

Upon infection with *Mtb*, the host innate immune response is the first defense. The bacteria are taken up by the phagocytic cells that reside in the lungs. The primary host cell for *Mtb* is the macrophage, though *Mtb* can also live inside dendritic cells (Giacomini et al., 2001). Because *Mtb* is phagocytosed by cells rather than invading the cell cytoplasm, it is considered an extracellular pathogen. Typically, extracellular pathogens induce the macrophage or dendritic cell to produce pro-inflammatory cytokines, such as Interleukin-12 (IL-12) and Tumor Necrosis Factor-α (TNF-α).

These cytokines are sensed by surrounding T-cells, which secrete more TNF-α and Interferon-γ (IFN-γ) to activate the macrophage and make it a more efficient killer. The pathogen-containing phagosome then matures along the endocytic pathway where it fuses with various endosomes. Lysosomes fuse, forming a phagolysosome and release degradative enzymes that destroy the invading pathogen and cause a decrease in the relative pH to around 4.5. The pathogen is processed into its specific antigens which bind Major Histocompatibility Complex Class-II (MHC-II) molecules and are brought to the cell surface for presentation to $CD4^+$ T-cells that recognize MHC-II molecules. Antigens of intracellular pathogens, such as viruses that invade the host cell cytoplasm, are presented via another pathway. They are processed in the proteosome and transported to the endoplasmic reticulum where they bind MHC-I. Then they are transported to the cell surface for presentation to $CD8^+$ T-cells which recognize MHC-I molecules. Sometimes, antigens from extracellular bacteria can be presented to $CD8^+$ T-cells via cross-presentation.

Once activated, macrophages employ another defense against extracellular bacteria by upregulating production of two enzymes, Nitric Oxide Synthase (iNOS) which generates Nitric Oxide (NO) and other Reactive Nitrogen Intermediates (RNI) and a subunit of the NADPH oxidase, NOX2 which generates Hydrogen Peroxide (H_2O_2) and other Reactive Oxygen Intermediates (ROI) (Ehrt and Schnappinger, 2009). ROI and RNI are toxic to bacteria.

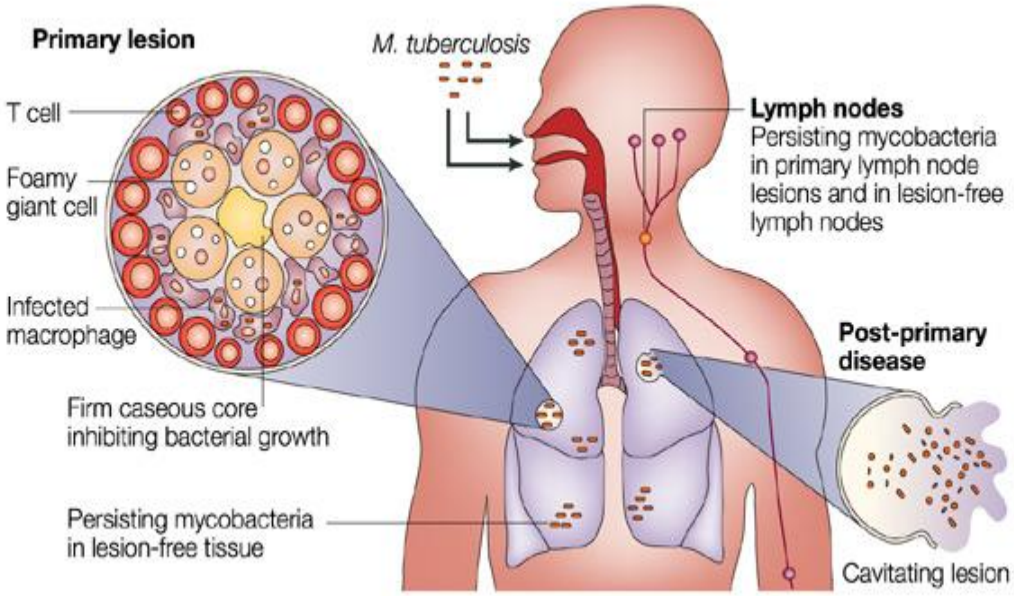
Though $CD8^+$ T-cells can be activated by extracellular pathogens, the primary response is that of $CD4^+$ T-cells. $CD4^+$ T-cells are helper T-cells that secrete cytokines to activate phagocytic cells to kill the invading pathogen. This cell

mediated immunity is required for controlling the infection (Flynn and Chan, 2001). $CD8^+$ T-cells are cytotoxic T-cells. They can also activate macrophages, but their primary role is to induce cell death, or apoptosis, in cells that are infected with intracellular pathogens. Interestingly, CD8+ T-cells are also required for controlling *Mtb* infection; however it is not entirely known how they gain access to *Mtb* antigens.

$CD4^+$ and $CD8^+$ T-cells, as well as natural killer (NK) T-cells and B-cells, migrate to the site of *Mtb* infection, driven by the cytokines and chemokines that are released from the infected macrophages. The infected cells are thus "walled-off", surrounded by foamy macrophages rich in lipid droplets, giant macrophages that are multinucleated, and lymphocytes (Russell, 2007). These granulomas are advantageous for the host because the infection is contained pretty much indefinitely unless the immune system becomes compromised; at which point the granuloma caseates and the bacteria are released and free to invade other cells, progressing to active TB disease [Fig. 2]. Granulomas are also advantageous to the bacteria. They allow the bacteria to persist and eventually transmit to other cells. In fact, *Mtb* has been shown to actually induce granuloma formation via secreted proteins and cell wall lipids (Russel, 2007).

1.3.1 *Mycobacterium tuberculosis* can resist phagolysosome fusion

Typically when a macrophage engulfs a pathogen the infected phagosome will fuse with lysosomes and the hydrolytic enzymes within the lysosomes will kill the pathogen. Lysosomes are the most acidic organelle in a cell, with a pH of 4.5-5.0 and this pH is transferred to the phagosome. The fusion of phagosome with lysosomes is



Stewart et al., 2003. *Nature Reviews*.

Figure 2. *Mtb* transmission and persistence. *Mtb* is transmitted to non-infected persons through aerosol droplets. The *Mtb* are taken up by alveolar macrophages in the lungs, which release cytokines that signal the recruitment of neighboring lymphocytes. During the persistence stage, these lymphocytes effectively "wall off" the infected cells in a caseous granuloma that prevents *Mtb* growth and dissemination. This containment of *Mtb* bacilli is the hallmark of latent infection, however viable *Mtb* have been found in granuloma-free regions of the lung and lymph nodes in asymptomatic patients. If an infected person becomes immune-compromised the granuloma may no longer hold its integrity. In which case it cavitates and infection progresses to an active disease stage where the bacteria are released and are free to infect surrounding tissues.

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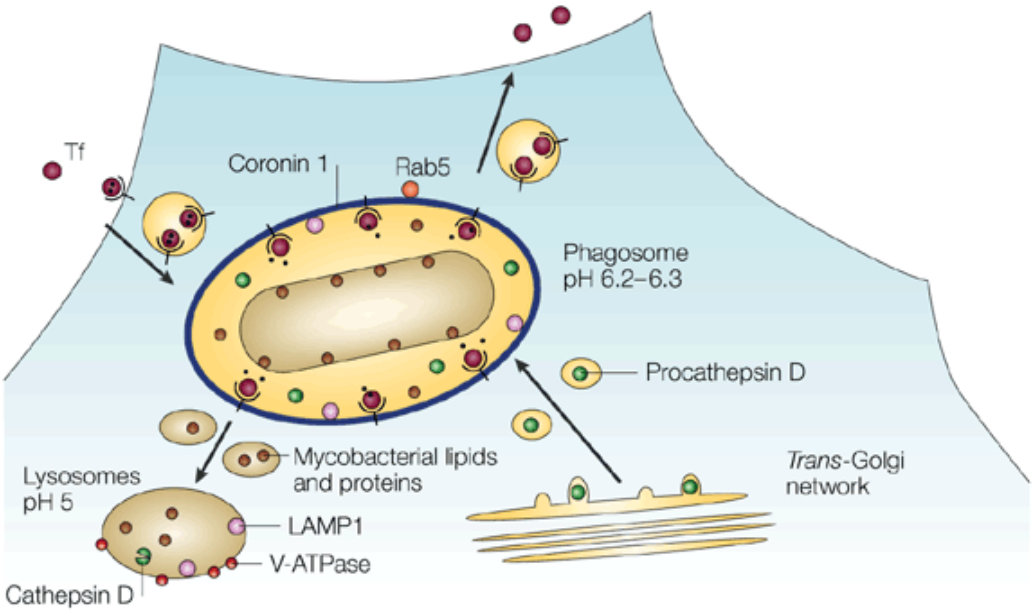
a dynamic process that can be tracked by the markers present on the membrane over time. During the early stages of phagocytosis the membranes contain markers that are present on the plasma membrane of the macrophage. The small GTPase, rab5 is present from the earliest time point, as well as mannose, Fc, and transferrin receptors. However, the mannose, Fc, and transferrin receptors disappear relatively quickly and get recycled back to the plasma membrane. The rab5 marker remains associated and plays an important role in the biogenesis of phagolysosomes (Dejardines, 1994). At later time points, the late endosome marker, rab7 is present and plays a role in regulating the timing and specificity of fusion events along the phagocytic pathway. Eventually the phagosome fuses with terminal lysosomes which are enriched in Lysosome Associated Membrane Proteins (LAMP). The various endocytic vesicles that fuse with the phagosome move along microtubules, where rab and Soluble NSF Attachment Receptor (SNARE) proteins are required to bring the phagosome in contact with the various endocytic vesicles along the pathway. Protein phosphorylation and G-protein signaling are also important for phagolysosome fusion (Dejardines, 1995).

Mtb are able to resist phagolysosome fusion and grow inside modified vesicles that represent earlier time point endosomes (Raja et al., 2004). It's not known how the bacteria are able to inhibit phagosome maturation, but they do play an active role. When infected with dead bacteria, it only takes an hour for the vacuole to mature to a late phagolysosome (MacGurn and Cox, 2007). Vacuoles containing live *Mtb* however, are transferrin-accessible and only mildly acidic (pH 6.3), even at very late time points (MacGurn and Cox, 2007). The phagosome retains rab5, which plays a

role in early endocytic interaction, but does not have rab7, which is a later endosomal membrane marker. Therefore, mycobacterial inhibition of phagolysosome fusion occurs sometime before the events that lead to the retention of rab7. Interestingly, lysosome-associated membrane protein-1 (LAMP-1) and cathepsin D are also present on the *Mtb*-containing phagosome. Cathepsin D, however is in an immature form which indicates that it is not acquired through fusion with lysosomes. Somehow it is delivered directly from the trans-Golgi network (Russel, 2001) [Fig. 3].

In addition to inhibiting phagolysosome fusion, *Mtb* is able to exclude Vacuolar Proton-ATPases (V-ATPase) (Sturgill-Koszycki et al., 1994), which are released from endosomes and pump protons across phagosomal membranes to acidify and kill the enclosed bacteria. The mycobacterial cell wall lipid, lipoarabinomannan (LAM) and the secreted enzyme, SapM have been implicated in the inhibition of phagosome maturation by acting on phosphatidylinositol-3-phosphate (PI3P). PI3P is a cell membrane associated, phospholipid that plays an essential role in membrane trafficking (Deretic et al., 2006). A specialized secretion system, called ESX-1 has also been shown to be required for inhibition of phagolysosome fusion and this will be discussed further in section 1.5 (MacGurn and Cox, 2007).

One interesting study by Ferrari et al., 1999, found that the Tryptophan-Aspartate Containing Coat (TACO) protein is retained on phagosomes infected with live Mycobacteria. TACO is homologous to coronin, an actin-binding protein involved in cytoskeletal rearrangements. TACO is relocalized to the phagosome membrane during the early stages of phagocytosis. However, if the phagosome contains dead



Russell, 2001. *Nature Reviews*.

Figure 3. The mycobacteria-containing vacuole. When *Mtb* is taken up by a macrophage they are able to survive for long periods of time inside an immature phagosome. The mycobacteria-containing vacuole (MCV) acquires transferrin at the cell surface and remains transferrin accessible. The vacuole also acquires the small GTPase, rab5 and is able to maintain a comfortable pH by inhibiting fusion with lysosomes and excluding vacuolar ATPases. Interestingly, the MCV does have some lysosome-associated markers such as LAMP1 and cathepsin D. Cathepsin D is in an immature form that suggests it is delivered directly from the Golgi rather than through fusion with lysosomes. Another interesting feature of the MCV is that it retains TACO, or coronin. TACO is an actin-binding protein that is actively retained by *Mtb* and may play a role in inhibiting phagosome maturation.

bacteria, the association usually only lasts a couple of hours; whereas TACO remains associated on the surface of phagosomes containing live mycobacteria, suggesting that the bacteria is actively able to retain the TACO protein. Inhibition of phagolysosome fusion only occurs with phagosomes containing live mycobacteria. Dead bacteria-containing phagosomes go to lysosomes. Perhaps retaining TACO is how mycobacteria are able to inhibit phagolysosome fusion. It is interesting that TACO is not present in Kupffer cells, which are macrophages that reside in the liver. This is perhaps a reason why the liver is one organ that remains relatively resistant to *Mtb* infection (Flynn et al., 2001).

Not only does *Mtb* inhibit phagosomal maturation, it has also evolved ways to avoid the harmful effects of ROI and RNI. The catalase-peroxidase (KatG) enzyme is released from *Mtb* to break down H_2O_2 into water and oxygen. *Mtb* lacking KatG are much more susceptible to the harmful effects of H_2O_2 (Ng et al., 2004). *Mtb* also releases Superoxide Dismutase enzymes, SodA and SodC which break down superoxide anions into H_2O_2. SodA and KatG are SecA2 dependent for secretion and SecA2 mutants have been shown to have growth defects in both macrophages and mice (Kurtz et al., 2006). *Mtb* also expresses an NADH-dependent peroxidase and peroxynitrite reductase that can detoxify RNI and ROI.

1.3.2 *Mycobacterium tuberculosis* alters macrophage signaling.

Mtb can manipulate the macrophage signaling required for the production of immunostimulatory cytokines and effectors (Beltan et al., 2000; Falcone et al., 1994). It actively suppresses the transcriptional induction of the p40 subunit of IL-12, which

is a cytokine that is critical for control of mycobacterial infection (Giacomini et al., 2001; Nau et al., 2002). Macrophages also produce the pro-inflammatory cytokine, TNF-α and the antimicrobial effector, NO to control *Mtb* infection. Non-virulent mycobacteria induce an increase of TNF-α and NO, suggesting that virulent mycobacteria are able to suppress these responses (Beltan et al., 2000).

1.3.3 *Mycobacterium tuberculosis* can inhibit TLR-2 signaling.

Toll-Like Receptors (TLR) on the surface of host cells are one way that extracellular pathogens can be recognized. These receptors recognize pathogen associated molecular patterns (PAMPs). For instance, TLR-4 can recognize LPS from Gram-negative bacteria, and TLR-2 can recognize the mycobacterial cell wall extract, lipomannan. Once a molecular pattern is recognized a signaling cascade ensues, activating an immune response. TLRs have an extracellular domain, a transmembrane domain, and an intracellular domain. Intracellularly, the TLR has a TIR domain that changes conformation once the extracellular domain comes in contact with its ligand. This new conformation allows for the recruitment of adaptor molecules which begins a whole signaling cascade. MyD88 is one such adaptor molecule. Once it is bound to the TIR domain it recruits IRAK4, IRAK1, and TRAF6 to form a complex. Further signaling events lead to the activation of the transcription factor, NF-κB and its recruitment to the nucleus. In the nucleus, NF-κB binds to DNA and activates the genes necessary to illicit an immune response. This includes the release of cytokines and the activation of T-cells.

In 2007, Patnak et al. discovered that the C-terminus of the secreted mycobacterial protein, ESAT-6 binds directly to the extracellular domain of TLR-2 and inhibits TLR-2 signaling. This interaction interferes with the recruitment of IRAK4 to the MyD88 complex, thus inhibiting the recruitment of NF-κB to the nucleus. This leads to a reduction in cytokine expression, including IL-6, TNF-α, and IL-12 (Patnak et al., 2007).

1.3.4 *Mycobacterium tuberculosis* can inhibit apoptosis.

Apoptosis, or programmed cell death, can be used by host cells as a defense mechanism against invading intracellular pathogens. Apoptosis is a highly-controlled form of cell suicide in which the cell compacts, its chromatin is degraded, then the cell breaks apart into contained blebs that are phagocytosed by other cells and destroyed. This process is anti-inflammatory which is counter to another form of cell death that is highly inflammatory, called necrosis. Necrosis is caused by damage of the host cell membrane and the contents of the cell spill out uncontained. Apoptosis can be induced via two separate pathways. The extrinsic pathway is induced when there is an extracellular signal, such as cytokines released from T-cells in response to cellular infection. Receptors on the surface of the apoptotic cell bind with their ligand (i.e. the Fas receptor binds the Fas ligand, or the TNF receptor binds TNF). This leads to conformational changes in the intracellular death domains of these receptors and signals the activation of the cysteine protease, caspase 8. The intrinsic pathway is induced when there is an intracellular stress on the cell such as a high concentration of cytosolic Ca^{2+} or DNA damage. In response to these stresses, members of the Bcl-2 family of pro-apoptotic proteins, such as bak and bax, bind to

the mitochondrial membrane leading to the release of cytochrome-c and the activation of caspase 9. From there the two pathways, extrinsic and intrinsic, converge and activate caspase 3, 6, and 7 which leads to apoptosis of the cell.

There is an obvious benefit to a phagocytosed pathogen if they are able to inhibit apoptosis; namely survival. If an infected macrophage undergoes apoptosis, the blebs taken up by other professional phagocytes process those antigens and present them to MHC-I molecules, which go on to prime $CD8^+$ T-cells. Virulent mycobacteria have demonstrated the ability to inhibit apoptosis by both the intrinsic and extrinsic pathway (Briken, 2008). Several proteins have been identified that play a role in *Mtb* inhibition of apoptosis, though the mechanism of inhibition is not yet known. These include NuoG, a subunit of the NADH dehydrogenase, NDH-1 (Velmurugan et al., 2007); SodA, which, as discussed in section 1.3.1, is secreted in response to ROIs released by the macrophage (Hinchey et al., 2007); and PknE, a member of the serine-threonine protein kinase family which is expressed in response to RNIs released by the macrophage (Jayakurmar et al., 2007).

1.4 Treatment and prevention of tuberculosis

The first and only widely-used vaccine against *Mtb* was developed by the French bacteriologist, Albert Calmette and veterinarian, Camille Guérin in 1906 through several years of passaging a virulent culture of *Mycobacterium bovis*, a close relative of *Mtb* that infects cows. Eventually they isolated an attenuated strain that did not cause disease in the animal model. They called it the Bacillus of Calmette and Guérin (BCG) and it was first used as a human vaccine in France in 1921. Since then, and to

this day it has been used routinely in nearly all countries of the world. Unfortunately BCG has variable efficacy in humans. Its usefulness depends on age as well as geography. In children, BCG can be very effective at protecting against miliary TB, but it is not so effective at protecting against pulmonary TB in adults. Unfortunately, it is pulmonary TB that is by far the most prevalent form of TB infection. BCG's effectiveness against pulmonary TB depends greatly on geographical region. The reasons for this variability are poorly understood. In the United Kingdom, clinical trials have shown efficacy in up to 80% of cases, whereas in India the efficacy is negligible. This could be due to differences in the environmental mycobacteria species that might mask the effect of BCG, or it could be due to variations in the BCG strains being used or even the genetic pool of the population living in these different regions (Andersen and Doherty, 2005).

Drug therapy against TB disease started with the discovery of Streptomycin in 1946. Because *Mtb* is covered in a waxy coat and thrives inside immune cells, drug penetration is difficult and effective drug therapy has to proceed for 6-9 months; sometimes up to two years. Current treatment of active TB disease requires administration of four first-line drugs for at least two months; Isoniazid, Rifampin, Pyrazinamide, and Ethambutol, followed by at least another four months of just Isoniazid and Rifampin. When the drugs are not taken as prescribed, the chance of recovery is reduced and there is a higher risk that the bacteria will become resistant. Multi-drug resistant tuberculosis (MDR-TB), when the bacteria is resistant to Isoniazid and Rifampin, and extensively drug resistant tuberculosis (XDR-TB), when the bacteria is also resistant to three second-line drugs, is becoming an increasingly

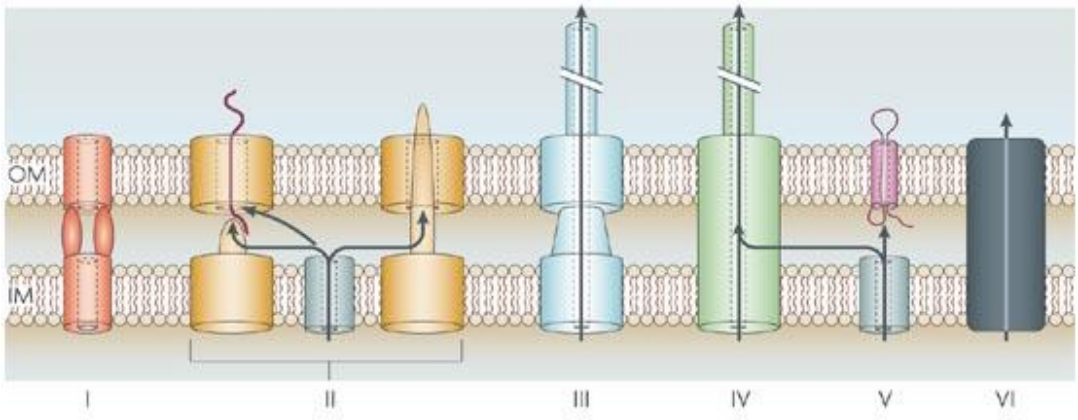
alarming problem in the world today. Also, there has been a reemergence of active TB disease around the world because of the high incidence of HIV co-infection, which has not only increased mortality of *Mtb* infected patients, but has also led to more MDR and XDR-TB strains (Wells et al., 2007). This emphasizes the dire need for new drugs and a better vaccine against *Mtb* (MacKenzie, 2007).

1.5 A specialized secretion system in mycobacteria

Because of the nature of their unique cell wall, it is more challenging for mycobacteria to export their bacterial products than it is for typical classes of Gram-positive bacteria. Like all other bacteria, mycobacteria have a Sec secretion system for general secretion of unfolded proteins across the cytoplasmic membrane. These proteins are transported by chaperones right off the ribosome to the membrane channel. They have conserved N-terminal signal sequences that are recognized by the SecA channel ATPase, which drives secretion (DiGiuseppe Champion and Cox, 2007). Mycobacteria also have a twin-arginine transporter (Tat) system for transporting folded proteins across the cytoplasmic membrane. These proteins also have a signal sequence that is conserved and predictable (DiGiuseppe Champion and Cox, 2007). In most Gram-positive bacteria the secretion of proteins across the cytoplasmic membrane is a one step process because the proteins are able to traverse the cell wall without having to pass through an outer membrane. In Gram-negative bacteria, these proteins need another way out of the cell because they cannot cross the outer membrane on their own. In addition to Sec and Tat, they have evolved several, more specialized systems that function to transport their bacterial products out of the cell, and in some cases directly into the cytoplasm of their host. These specialized

secretion systems are classed as Type I-VI [Fig. 4]. Type II and Type V Secretion Systems transport Sec and Tat proteins across the outer membrane. Type I Secretion Systems form a continuous channel and transport proteins across both membranes. Type III, Type IV, and Type VI Secretion Systems form a continuous channel across both membranes *and* form a pore in the host cell to effectively translocate their effector proteins (Saier, 2006).

Because mycobacteria have a thick and less penetrable cell wall it seems likely that they too would need a second step process to transport proteins out of the cell, though no system has yet been found. For the majority of time that BCG has been used as a vaccine it was not known what caused its attenuation. In 1996, Mahairas et al. used subtractive hybridization to compare the BCG genome with that of wild type *Mycobacterium bovis.* They found three regions of difference; of these, the Region of Difference 1 (RD1) is the only region that is conserved in all virulent strains of *M. bovis* and *Mtb*, and is absent from all BCG substrains, dating back to the original BCG isolation (Mahairas et al., 1996). Other groups compared the BCG genome with *Mtb* and found 16 large deletions (Behr et al., 1999; Gordon et al., 2001). Of these, only five were unique to BCG and only RD1 was absent from all BCG vaccine strains being used in the present day. Complementation of RD1 back to BCG partially restored its virulence (Gordon et al., 2001). In 2003, Hsu et al. deleted RD1 from several *Mtb* strains and found them all to be attenuated. They also found that virulence was restored upon complementation. What's more, the *Mtb* RD1 mutant offers similar protection as BCG in *Mtb* challenged mice (Hsu et al., 2003).



Abdallah et al., 2007. *Nature Reviews.*

Figure 4. Type I-VI bacteria secretion systems. Gram-negative bacteria have evolved several types of secretion systems to transport proteins across both their inner (IM) and outer membranes (OM). Type I secretion is a one-step process in which proteins with C-terminal signal sequences are transported across both membranes. It is a simple channel made up of an ATPase-binding cassette protein in the IM, a membrane fusion protein in the periplasmic space, and an outer membrane channel in the OM. Type II secreted proteins contain an N-terminal signal sequence to cross the IM via the Sec or Tat system (shown as a grey IM channel in the figure). In the periplasmic space, the proteins fold and then cross the OM through a complex known as the secreton which has an OM pore and a pilus-like structure in the IM. Type III secreted proteins are translocated through one continuous channel across both the IM and OM, and then they are "injected" directly into the host cell. The Type III injectisome is homologous to the basal body of bacterial flagella. The Type IV secretion system also spans both the IM and OM, and translocates substrates into the host cell. Type IV systems are homologous to the bacterial conjugation machinery and the process of translocation requires a pilus rather than an injectisome. The Type V system is a two-step process in which Sec proteins that cross the IM (shown as a grey IM channel in the figure) have an autotransporter β-barrel domain that inserts in the OM and secretes the rest of the protein across. Type VI secretion systems are not as well studied. The substrates do not have Sec or Tat secretion signals and so it is believed that the Type VI channel crosses both the IM and the OM.

It is now known that RD1 encodes the majority of genes that make up a novel secretion system. The first indication that *Mtb* has a specialized secretion system was the discovery of secreted proteins that lack a Sec secretion signal (Sørensen et al., 1995). Namely, the Early Secreted Antigenic Target – 6 kDa protein (ESAT-6, sometimes called EsxA) and the Culture Filtrate Protein – 10 kDa (CFP-10, sometimes called EsxB). Both are encoded within the RD1 region and both are known to be major antigenic targets of the host immune system. Many of the other genes within and surrounding the extended RD1 region (extRD1) are required for ESAT-6 and CFP-10 secretion (Tekaia et al., 1999; Gey Van Pittius, 2001; Pallen et al., 2002), and so this novel secretion system is called ESAT-6 Secretion System – 1 (ESX-1).

There are actually five ESX systems in *Mtb*, which likely evolved by gene duplication. It is believed that ESX-4 is the original system and it contains the least number of genes. From ESX-4, duplication events likely lead to the addition of ESX-1, then ESX-3, then ESX-2, and finally ESX-5 (Abdallah et al., 2007). ESX-5 is only present in the slow-growing species of mycobacteria, so its presence divides the genus between fast-growers (colonies form within 7 days) and slow-growers, of which *Mtb* and most other pathogenic species are a member (Abdallah et al., 2006; Gey van Pittius et al., 2006). The expansion of a family of proteins, which are unique to mycobacteria and make up 10% of the coding capacity in *Mtb* (Cole et al., 1998), have been linked to the expansion of ESX clusters (Gey van Pittius et al., 2006). These proteins are characterized by a conserved proline-glutamic acid (PE) or proline-proline-glutamic acid (PPE) motif within their N-terminus (Cole et al., 1998).

In the model species, *Mycobacterium marinum* (*Mm*), ESX-5 has been shown to be important for secreting several PE and PPE proteins (Abdallah et al., 2009), one of which is the PPE41 protein (Rv2430c) (Abdallah et al., 2006), which is able to induce a strong B-cell response (Choudhary et al., 2003). ESX-5 has been shown to play a role in the modulation of macrophage secretion of proinflammatory cytokines, and it seems likely that members of the PE/PPE family of proteins are involved (Abdallah et al., 2008).

The ESX-3 secretion system is conserved in all known mycobacterial genomes and is essential for optimal *Mtb* growth (Serafini et al., 2009; Siegrist et al., 2009). It has been demonstrated that mycobacteria require this specialized secretion system for the optimal uptake of iron and zinc (Serafini et al., 2009; Siegrist et al., 2009).

ESX-4 and ESX-2 have ESAT-6 family members in their gene clusters, though no secretion of these proteins has been detected. It is possible that they are not functional secretion systems (Abdallah et al., 2007). Interestingly, ESX systems have even been identified in low G+C Gram-positive bacteria, including the *yuk*A-*yuk*E gene cluster in *Bacillus subtilis* (Pallen, 2002). Since both pathogenic and non-pathogenic bacteria have these ESX systems, they cannot be solely attributed to a virulence adaptation.

ESX-1 is clearly required for virulence *in vivo* and for growth in macrophages (Stanley et al., 2003; Guinn et al., 2004). Without a functional ESX-1, *Mtb* infected phagosomes are unable to inhibit fusion with lysosomes (MacGurn et al., 1996; 2007; Tan et al., 2006). It is also likely that ESX-1 is a key reason why the attenuated BCG

vaccine does not offer better protection against *Mtb*, since an intact ESX-1 secretion system is able to secrete more potent T-cell antigens. ESX-1 is required for activation of the NALP3 inflammasome and release of the IL-1β and IL-18 pro-inflammatory cytokines that are involved in granuloma formation and dissemination of infection (Koo et al., 2008; Volkman et al; 2004). ESX-1 has also been shown to inhibit macrophage expression of IL-12 and TNF-α, and is required for cytotoxicity of host cells (Stanley et al., 2003; Gao et al., 2004).

The ESX-1 system has many important similarities to Type IV secretion systems (T4SS) in Gram-negative bacteria; the secreted proteins lack a Sec-secretion signal, and instead have a signal at the C-terminus which is recognized by coupling proteins (Abdallah et al., 2007). The secretion signal of CFP-10 has been identified as the last seven amino acids of its C-terminus and has been shown to be sufficient to direct the secretion of the non-related, cytoplasmic yeast ubiquitin protein (DiGiuseppe Champion et al., 2006). It has also been shown that the C-terminus of ESAT-6 and another ESX-1 secreted substrate, EspB are required for secretion (Xu et al., 2007). Similar to T4SS, ESX-1 utilizes coupling proteins in the SpoIIIE/FtsK family of ATPases to drive secretion through the secretion channel. Also, many of the ESX-1 substrates are targeted for secretion as a substrate pair. In T4SS, substrates have chaperones that are often small proteins with an acidic pI, are encoded by a gene adjacent to the gene that encodes the secreted substrate, and binds its substrate with high affinity; which are all characteristics of ESAT-6 and CFP-10. What's more, T4SS are homologous to the conjugation pilus. Interestingly, the ESX-1 system in the non-pathogenic species, *Mycobacterium smegmatis*, has been shown to be involved in

the conjugation of DNA transfer rather than virulence as it is in pathogenic species (Coros et al., 2008).

In T4SS, proteins are translocated directly into the host cell cytosol. Translocation of ESX-1 secreted proteins has not yet been demonstrated; however there is evidence to suggest that this is a very real possibility. Since mycobacteria reside within a membrane-bound vacuole, MHC Class I molecules can gain access to antigens one of two ways; either the antigens are generated in a vesicular compartment and loaded onto recycled MHC, or the antigens enter the cytosol and undergo proteasome processing. It does appear that *Mtb* antigens are able to enter the cytosol (Mazzaccaro et al., 1996; Lewinsohn et al., 2006). Translocation of proteins via ESX-1 would explain how $CD8^+$ T-cells gain access to these cytosolic *Mtb* antigens. One report showed that secretion of CFP-10 is absolutely required to prime $CD8^+$ T-cells (Woodsworth et al., 2008), which suggests that CFP-10 may be translocated into the cytosol. Another report used fractionation and immunogold labeling to detect the Protein Kinase G (PknG) protein in the host cytosol (Walburger et al., 2004), and our lab has preliminary data to show that PknG is ESX-1 dependent for secretion (unpublished results).

The clear correlations between ESX-1 and T4SS in Gram-negative bacteria, lends support to the hypothesis that mycobacteria require a specialized secretion system to secrete proteins across the mycomembrane. Therefore, it has been proposed that ESX systems be designated as Type VII secretion systems, in line with Gram-negative nomenclature (Abdallah et al., 2007).

1.6 Molecular components of the ESX-1 secretion system

1.6.1 Machinery

There is still much to learn about the ESX-1 machinery. Many who study ESX-1 believe that the system forms a channel through both the cytosolic membrane and also the cell wall, though the cell wall channel protein(s) has not yet been discovered. It is also not yet known if the channel through the cytosolic membrane and cell wall is continuous or a separate two-step process. It is thought that ESX-1 may form a continuous channel through the mycomembrane (Abdallah et al., 2007; DiGiuseppe Champion and Cox, 2007), similar to T4SS. It's even tempting to think that ESX-1 could possibly form a pore in the host cell membrane in order to translocate virulence effectors. All of these assumptions are speculative based on some phenotypic evidence, however the involved proteins have not yet been identified, there are no proteins within the extRD1 region that have predictable domains involved in such functions, and the ESX-1 secretion system has not yet been visualized.

The proteins known to be involved in ESX-1 secretion are summarized in Table 1. There are three proteins that are well accepted as being part of the ESX-1 machinery. Using *Mtb* H37Rv nomenclature, these three proteins are Rv3870 (also sometimes called Snm1), Rv3871 (Snm2), and Rv3877 (Snm4). Mutating any of these three proteins completely abolishes secretion of all of the known ESX-1 substrates, and these mutants show the same growth defect and altered immune response as a complete RD1 deletion (Stanley et al., 2003; Guinn et al., 2004). All five of the *Mtb* ESX systems have homologs to these three proteins, though the homolog of Rv3871

Table 1. ESX-1 proteins

Mtb H37Rv Nomenclature	*Mm* homolog	Common Name	Putative Function and Protein Interactions
Rv0757	Mh0757	PhoP	A virulence protein that is potentially responsible for the attenuation of the avirulent strain, H37Ra. It has been shown to negatively regulate the secretion of EspA.
Rv3614c	Mh3614c	EspA	An ESX-1 secreted protein that is encoded outside the extRD1 region in a three-gene operon with EspC and Rv3616c. EspA is codependent for secretion with other ESX-1 substrates and its secretion is negatively regulated by EspR and PhoP.
Rv3615c	Mh3615c	EspC	An ESX-1 secreted protein with a C-terminal secretion signal that associates with the cytosolic AAA ATPase, Rv3868. This targeting is required for secretion of EspA.
Rv3849	Mh3849	EspR	A DNA-binding protein that activates transcription of the EspA operon. Secretion of EspR negatively regulates EspA secretion which in turn affects the secretion of other ESX-1 substrates.
Rv3864	Mh3864		An ESX-1 secreted protein that is cell wall associated. It has been shown to interact with EspC.
Rv3865	Mh3865	EspF	An ESX-1 secreted protein that is expressed more abundantly in *Mm* than *Mtb* and has been shown to interact with EspC and Rv3868.
Rv3868	Mh3868		A cytosolic AAA ATPase that has been shown to interact with EspC and EspF.
Rv3870	Mh3870	Snm1	An FtsK/SpoIIIE-like ATPase that associates with Rv3871 and is believed to provide the energy for secretion.
Rv3871	Mh3871	Snm2	An FtsK/SpoIIIE-like ATPase that associates with Rv3870 and is believed to provide the energy for secretion. Snm2 associates with the C-terminal secretion signal of CFP-10 and also Rv3879c.
Rv3874	Mh3874	CFP-10 or EsxB	An ESX-1 secreted virulence protein that is highly antigenic and forms a tight 1:1 heterodimer with ESAT-6. CFP-10 has a C-terminal secretion signal that associates with Snm2.
Rv3875	Mh3875	ESAT-6 or EsxA	An ESX-1 secreted virulence protein that is highly antigenic and forms a tight 1:1 heterodimer with CFP-10. The C-terminus of ESAT-6 has been shown to bind TLR-2, and ESAT-6 associates with liposome membranes.
Rv3877	Mh3877	Snm4	A 12-transmembrane domain protein that likely forms the cytosolic membrane channel.
Rv3879c	Mh3879c		A cytosolic protein that is required for EspB secretion. It has been shown to interact with EspB and Snm2.
Rv3881c	Mh3881c	EspB	An ESX-1 secreted virulence protein that associates with ESAT-6 and Rv3879c. It is cleaved by MycP1 in the periplasmic space and likely plays a role in the inhibition of phagolysosome fusion
Rv3883c	Mh3883c	MycP1	A serine protease that likely associates with the ESX-1 machinery. It anchors to the cytoplasmic membrane with its protease domain present in the periplasmic space. MycP1 has been shown to cleave EspB and this cleavage negatively regulates the secretion of other ESX-1 substrates.

and Rv3870 is sometimes encoded as one gene (Abdallah et al., 2007). In addition, all five systems have a homolog to the serine protease, Rv3883c protein (called Mycosin-1 (MycP1)), and to Rv3869, which is not well studied. There are also ESAT-6 family member proteins present in all five systems. ESAT-6 and CFP-10, mentioned above, are secreted proteins but it is possible that they also contribute to the secretion machinery. Because these six proteins are conserved and present in all five ESX systems, it is likely that they are the core components that make up the secretion machinery (Abdallah et al., 2007).

Rv3877 has a 12-transmembrane-domain, it is not secreted, and because it is essential for a functional ESX-1 secretion system, it likely forms the cytosolic membrane channel. Rv3870 and Rv3871 form an FtsK/SpoIIIE-like ATPase which likely act as coupling proteins to drive secretion through the Rv3877 channel. Protein interaction studies have shown that these two proteins interact with each other (Stanley et al., 2003). What's more, Rv3871 interacts with the C-terminal secretion signal of the secreted substrate, CFP-10. It has also been shown to interact with Rv3879c which binds to another ESX-1 secreted substrate, EspB, though it itself is not secreted. In the current model it is believed that Rv3879c and CFP-10 act as chaperones that deliver their cognate substrates to the secretion channel. This is a common feature of secretion systems, as the T4SS in Gram-negative bacteria also utilize FtsK/SpoIIIE coupling proteins to drive secretion.

Rv3883c (MycP1), is another protein believed to associate with the ESX-1 machinery. This protein is likely a member of the subtilase class of serine proteases, because it has a propeptide and a catalytic triad of aspartic acid, histidine, and serine

residues. It has a transmembrane domain which anchors to the cytoplasmic membrane while the protease domain is believed to be present in the periplasmic space between the cytoplasmic membrane and the cell wall (Brown et al., 2000). Mutating MycP1 results in ESX-1 defects that are similar to those seen in Rv3877 mutants where no known substrates are secreted. Recently, in 2010, Ohol et al. reported that the ESX-1 secreted substrate, EspB is cleaved by MycP1 near its C-terminus. They created a protease defective mutant that allows for a functional ESX-1 system but can no longer cleave EspB. Interestingly, this mutant showed a substantial increase in the secretion of other ESX-1 substrates like ESAT-6 and CFP-10. The fact that the MycP1 mutant completely abolishes ESX-1 secretion, which is not due to a defect in protease activity, points toward a hypothesis that MycP1 interacts with the ESX-1 machinery and is important for complex formation (Ohol et al., 2010). Because the protease deficient mutant showed an *increase* in secretion of ESX-1 substrates, Ohol et al. hypothesized that MycP1 also plays a role in regulation. This will be discussed further in section 1.6.3.

1.6.2 Substrates

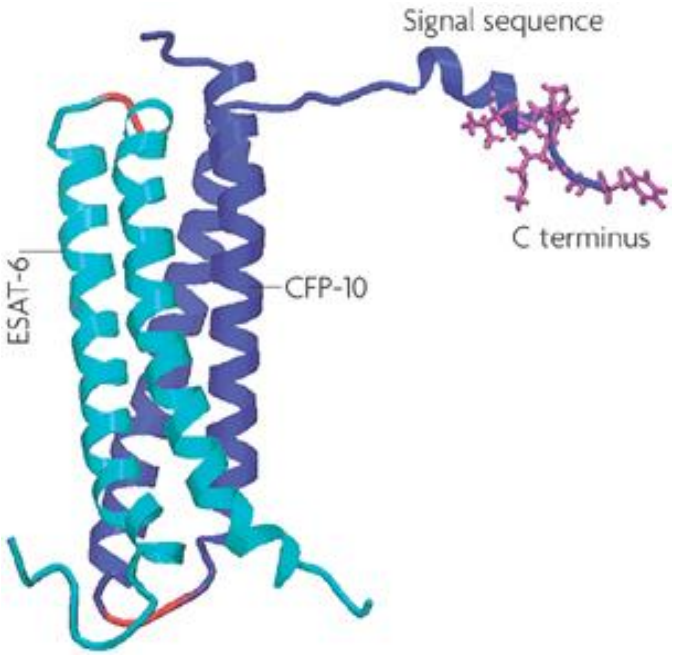
ESAT-6 and CFP-10:

By far, the two most studied proteins known to be secreted by the ESX-1 system are Rv3875, commonly known as ESAT-6 (or sometimes EsxA), and Rv3874, commonly known as CFP-10 (or sometimes EsxB). Both are members of the WXG100 superfamily, characterized by their small size of around 100 amino acids, the presence of a conserved tryptophan-variable-glycine (WXG) motif, and also their tendency to be near other WXG100 proteins in the genome (Pallen, 2002). A

sensitive search for ESAT-6-like proteins was done in an attempt to gather some idea as to function of these very important *Mtb* virulence proteins. It was found that WXG100 proteins are present in a wide range of Gram-positive bacteria including *Bacillus subtilis* and *Staphylococcus aureus*, though sequence similarity is very low, save the three features described previously. Most also possess coiled-coil domains and none of them were found to have a predictable secretion signal (Pallen, 2002).

Not all WXG100 proteins are involved in virulence since they are present in both pathogenic and non-pathogenic species; however, the ESAT-6 and CFP-10 proteins, secreted by the ESX-1 system in *Mtb*, are very clearly involved in virulence. In fact, the search for a specialized secretion system in mycobacteria began with the identification of these potent immunodominant antigens that lack a Sec secretion signal (Sørensen et al., 2005). *Mtb* virulence *in vivo* and growth in macrophages is dependent on the secretion of ESAT-6 and CFP-10. These proteins play a role in the inhibition of phagosome maturation and modulation of cytokine signaling (Stanley et al., 2003; Hsu et al., 2003; Pathak et al., 2007). As discussed in section1.3.3, the C-terminus of ESAT-6 was shown to directly bind TLR-2. This interaction interferes with the recruitment of IRAK4 to the Myd88 complex and leads to a reduction in pro-inflammatory cytokine expression (Patnak et al, 2007).

ESAT-6 and CFP-10 are codependent for secretion; that is, ESAT-6 mutants do not secrete CFP-10 and CFP-10 mutants do not secrete ESAT-6. In 2002, Renshaw et al. discovered that ESAT-6 and CFP-10 form a tight 1:1 heterodimer. The structure of both proteins is that of a helix-turn-helix, and they associate via hydrophobic interactions to form an antiparallel four-helix bundle [Fig. 5]. The WXG motif of



Abdallah et al., 2007. *Nature Reviews*.

Figure 5. Ribbon structure of the ESAT-6/CFP-10 heterodimer. ESAT-6 (light blue) and CFP-10 (dark blue) form a helix-turn-helix structure that associates with high affinity through hydrophobic interactions to form a 1:1 heterodimer. Their association is antiparallel across their helices, leaving the hairpins, which contain the highly conserved WXG motif (red), free to associate with other proteins. The unstructured C-terminus of CFP-10 (purple) contains a seven amino acid signal sequence that is sufficient to secrete the cytosolic yeast protein, ubiquitin.

both proteins is present in the hairpin turn and is not involved in this interaction. CFP-10 has an unstructured C-terminus that was shown to interact with the channel ATPase, Rv3871 and the last seven amino acids are required for secretion and are sufficient to secrete the cytosolic yeast protein, ubiquitin (DiGiuseppe Champion et al., 2006). The association of ESAT-6 and CFP-10 *in vitro* has high affinity both in the cell lysate and the culture filtrate; however, they have been shown to dissociate at low pH, such as that typically encountered in the phagosome (de Jonge et al., 2007). de Jonge et al., also found that ESAT-6 is able to associate with liposome membranes, but only when it is not in complex with CFP-10. Their hypothesis is that CFP-10 acts as a chaperone for ESAT-6 until environmental factors cause the complex to dissociate, which allows ESAT-6 to carry out its virulence function. In addition to ESAT-6 and CFP-10, four other *Mtb* proteins have been identified that are ESX-1 dependent for secretion; Rv3614c (EspA) (Fortune et al., 2005; MacGurn et al., 2005), Rv3881c (EspB) (Xu et al., 2007; McLaughlin et al., 2007), Rv3615c (EspC) (DiGiuseppe Champion et al., 2009), and Rv3849 (EspR) (Raghavan et al., 2008). In addition, Carlsson et al., 2009 identified another ESX-1 secreted protein using the *Mycobacterium marinum* (*Mm*) model (discussed below in section 1.7); Mh3864 (for *marinum* homolog of Rv3864). Another protein, Mh3865 (EspF) was shown to be ESX-1 dependent for secretion in *Mm*, however its expression in *Mtb* is much reduced (DiGiuseppe Champion et al., 2009). The function of these secreted proteins and their role in *Mtb* pathogenesis remains to be elucidated, but several have been shown to be involved in virulence, and all display some form of codependence in their requirement for secretion.

EspA:

In 2005, Fortune et al. compared culture filtrates from H37Rv and an H37Rv RD1 mutant strain to find proteins besides ESAT-6 and CFP-10 that might be secreted by ESX-1. They identified Rv3614c (EspA) which is encoded outside the ESX-1 region in a three gene operon with Rv3615c and Rv3616c. EspA is a 40 kDa protein with no predicted signal sequence or transmembrane domain. It is 31% identical to Rv3864 which lies within the ESX-1 region. EspA is codependent for secretion with ESAT-6 and CFP-10.

At the same time, MacGurn et al., 2005 also discovered that the EspA protein is required for ESX-1 secretion. They showed that EspA interacts directly with Rv3882c. They also showed that this operon is important for combating the host response during early infection. The operon is upregulated in low iron (Rodriguez et al., 2002) and low pH (Fisher et al, 2002), which are features of a macrophage phagosome.

EspB:

Two groups simultaneously discovered that Mh3881c (*Marinum* homolog of Rv3881c), or EspB is secreted by ESX-1 (Xu et al., 2007; McLaughlin et al., 2007). This 61 kDa protein is encoded within the ESX-1 region in a 2-gene operon with Mh3880c. It shows 68% identity and 80% similarity with Rv3881c and Rv3881c is able to complement the secretion phenotype when expressed in the *Mm esp*B::tn mutant (Xu et al., 2007). This indicates that the protein is functionally conserved in *Mtb*. EspB is codependent for secretion with ESAT-6 and CFP-10 (Xu et al., 2007)

and it is cleaved upon secretion (Xu et al., 2007; McLaughlin et al., 2007). Inside the cytosol, EspB is expressed in its full-length form, however in the culture filtrate two fragments are detected; an N-terminal 50 kDa fragment and a C-terminal 11 kDa fragment. When only the N-terminal 50 kDa protein is expressed it is secreted normally; however ESAT-6 and CFP-10 are not secreted (Xu et al., 2007). Therefore, the C-terminus is required for codependent secretion. In fact, the cellular stability of ESAT-6 is diminished in the C-terminal mutant due to the fact that the C-terminus of EspB was shown to interact directly with ESAT-6 in the cytosol (Xu et al., 2007). The N-terminus of EspB was shown to interact directly with Mh3879c, and Mh3879c interacts with the channel ATPase, Mh3871 (McLaughlin et al., 2007). Perhaps Mh3879c acts as a chaperone to bring EspB to the channel for secretion. The two groups showed that secretion of EspB is required for cytotoxicity and intracellular growth inside macrophages (Xu et al., 2007; McLaughlin et al., 2007), virulence in zebrafish (McLaughlin et al., 2007), and the inhibition of phagolysosome fusion (Xu et al., 2007).

EspC:

Another protein, Rv3615c (EspC) was found to be codependent for secretion with ESAT-6 and CFP-10 and also requires Rv3870, Rv3871, and Rv3877 (MacGurn et al., 2005). The C-terminus of EspC has a signal sequence that is similar to the C-terminal signal sequence of CFP-10 (DiGiuseppe Campion et al., 2006); however, these two signal sequences are not interchangeable. CFP-10 expressing the C-terminal 7 amino acids of EspC is not secreted; demonstrating that this secretion signal is required for EspC secretion but is not sufficient (DiGiuseppe Champion et

al., 2009). This is likely because the C-terminus of EspC was found to interact with a different ATPase than CFP-10. While CFP-10 interacts with Rv3871, EspC interacts with Rv3868, a cytosolic AAA ATPase that is also required for ESX-1 secretion, though its role in secretion is unknown (Gao et al., 2004; Brodin et al., 2006). When EspC expresses the C-terminal 14 amino acids of CFP-10 this hybrid protein does interact with Rv3871, demonstrating the targeting specificity of the C-terminal signal sequence of CFP-10 to the Rv3871 ATPase (DiGiuseppe Champion et al., 2009). However EspC expressing the C-terminus of CFP-10 is not secreted, so other protein interactions must be required for secretion. The targeting of CFP-10 and EspB to Rv3871 is independent of the targeting of EspC to Rv3868, however secretion of EspA requires EspC targeting to Rv3868.

EspF:

Further protein interaction studies revealed that ESAT-6 interacts with another protein, Rv3865 (EspF). EspF was shown to be an *Mtb* secreted protein (Bahk et al., 2004) and is paralogous to EspC. It is therefore likely that EspF is also an ESX-1 secreted substrate, and this was shown to be true in *Mm* (DiGiuseppe Champion et al., 2009). However, EspF is expressed much more abundantly in *Mm* than *Mtb* and so it was not possible for DiGiuseppe Champion et al., to conclusively show that EspF is an ESX-1 secreted substrate in *Mtb*. Yeast two-hybrid analysis of *Mtb* proteins did show that EspF interacts directly with EspC and Rv3868, however. In a previous study, it was shown that the Mh3864 protein specifically interacts with EspC (Carlsson et al., 2009), but was not shown to directly interact with other known ESX-1 components and substrates.

EspR:

In 2008, Raghavan et al. identified Rv3849 (EspR) as another protein secreted by the ESX-1 system. In this mutant, ESAT-6 secretion levels are reduced and a growth defect is observed in mice. The authors found that EspR is able to bind DNA and activate transcription of the EspA operon. They conclude that this protein is an ESX-1 regulator and thus will be discussed further in the next section. It appears that secretion of EspR negatively regulates the ESX-1 system, potentially to benefit the bacteria by allowing it to hide from the host immune system during later stages of infection (Raghavan et al., 2008).

Mh3864:

Using the *Mm* model Carlsson et al., 2009 found that Mh3864 is secreted by ESX-1 and is cell wall associated. They also found that the ESX-1 secretion apparatus localizes primarily to the bacterial poles. *Mm* is able to polymerize actin and spread from cell to cell (Gao et al., 2004), which is further discussed in section 1.7. This study found that ESX-1 is preferentially localized to the poles that polymerize actin. Mh3864 is not required for actin tail formation but it is possible that other substrates of the ESX-1 system are required. Actin polymerization has not yet been demonstrated in *Mtb*.

Because of the codependent nature of the ESX-1 secreted substrates and the data showing multiple interactions in the cytosol, a model is emerging of a large complex that forms in the cytosol to transport substrates to their cognate ATPases, either Rv3871 or Rv3868 (DiGiuseppe Champion et al., 2009). This model supports the

hypothesis that ESX-1 secretion has to be coordinated. All of the substrates must be present in the cytosol for secretion to occur. Then, regulation of a few substrates can determine secretion of the entire complex [Fig. 6].

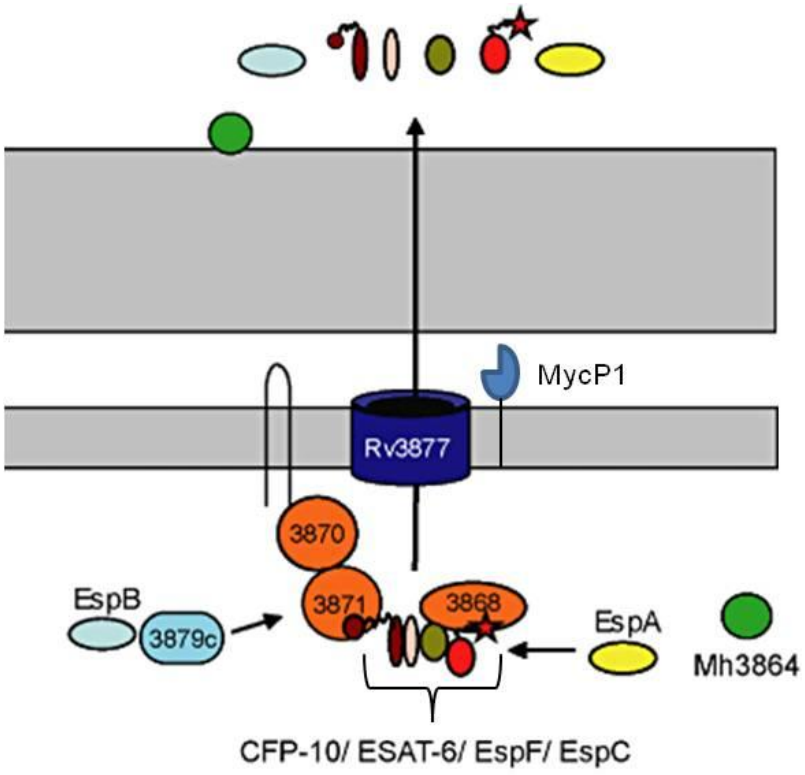
1.6.3 Regulation

EspR:

In 2008, Raghavan et al. identified Rv3849 (EspR) as a novel secreted substrate of ESX-1. Their EspR transposon mutant induces high levels of IL-12 which is common of ESX-1 secretion mutants. Without EspR, ESAT-6 secretion is ablated. Likewise, in the ESAT-6 mutant, EspR secretion is ablated, indicating a codependent relationship for secretion.

EspR has similar structure as the *Bacillus subtilis* transcription factor SinR which is a helix-turn-helix DNA-binding protein. As it turns out, the EspR mutant downregulates transcription of the Rv3616c-Rv3614c operon which includes the secreted substrate, EspA that is required for ESX-1 secretion (Raghavan et al., 2008). EspR is induced early during infection upon phagocytosis by macrophages and Raghavan et al. found that the N-terminus of EspR binds directly to the Rv3616c promoter to activate transcription. The C-terminus is also important for ESX-1 function, perhaps as a secretion signal.

Raghavan et al., also found that when the Rv3616c-Rv3614c operon is constitutively expressed in the EspR mutant, ESX-1 secretion is restored to WT levels. Therefore, the function of EspR appears to be the transcriptional activation of this operon and it is not required for subsequent secretion. This distinguishes it from other co-



Adapted from Ohol et al., 2010. *Cell Host Microbe*.

Figure 6. The ESX-1 secretion system. A current model of ESX-1 secretion predicts that a complex of secreted substrates form in the cytosol. This is based on extensive protein-protein interaction studies. In the figure the CFP-10/ESAT-6/EspF/EspC complex is shown, however EspB, which has been shown to interact with ESAT-6, and Mh3864, which has been shown to interact with EspC, may also make up a part of the complex. EspB has also been shown to interact with Rv3879c. Rv3879c is not itself secreted, but interacts with the channel ATPase, Rv3871 and may target EspB to the secretion channel. EspA is another ESX-1 secreted protein that is encoded outside the extended RD1 genomic region. It too is codependent for secretion with several other ESX-1 substrates; therefore it is possible that EspA interacts with the cytosolic substrate complex through protein interactions with unidentified ESX-1 proteins. The complex is targeted to ATPase proteins at the membrane channel (i.e. Rv3871 or Rv3868). Rv3871 and Rv3870 are coupling ATPases that associate and likely provide the energy to drive secretion across the membrane. It is thought that Rv3877, a 12-transmembrane protein, forms the inner membrane channel. The MycP1 protein is thought to be present in the periplasmic space, anchored to the inner membrane. MycP1 is required for a functional ESX-1 system and also cleaves the EspB protein upon secretion. This cleavage is thought to inhibit secretion of the other substrates in order to downregulate ESX-1 when it is beneficial for the bacteria to do so. It is not yet known how substrates are able to cross the mycomembrane channel.

dependent ESX-1 substrates; the codependence observed for secretion of EspR with ESAT-6 is likely because the normal function of EspR is required for EspA secretion, which in turn is required for ESAT-6 secretion. In an EspR mutant that is defective at secretion but still binds DNA, the intracellular protein levels increased and there was a corresponding increase in Rv3615c expression; therefore, secretion of EspR likely functions to limit its intracellular activity.

PhoP:

Frigui et al., 2008 compared the single nucleotide polymorphisms (SNPs) between H37Rv and its avirulent counterpart strain, H37Ra. They found 13 non-synonymous SNPs, one of which is at position S219L in the two-component regulator PhoP which is involved in *Mtb* virulence (Pérez et al., 2001). This S219L mutation was simultaneously discovered by Lee et al., 2008. Complementation of H37Ra with wild type *pho*P partially restores virulence, so the S219L mutation in the *pho*P gene is one factor contributing to the attenuation of the H37Ra strain. They found that a fully functional PhoP is important for generating T-cell responses against ESAT-6 and CFP-10, and also for the expression and secretion of EspA. Possibly, a fully functional PhoP is required to regulate the secretion of EspA and thus affects the secretion of ESAT-6 and subsequent T-cell responses against that antigenic target. Expression of the Rv3614c-Rv3616c operon does show differences when cultured in roller bottles compared to shake flasks, so PhoP/PhoR may regulate these proteins dependent on environmental conditions (Gao Q et al., 2004). Both BCG and H37Ra are attenuated for reasons linked to loss of ESAT-6 function which emphasizes the importance of the ESX-1 system.

MycP1:

Rv3883c (MycP1) likely interacts with the secretion machinery as discussed in section 1.6.1. In the same 2010 study by Ohol et al., MycP1 was also shown to be a regulator of ESX-1 secretion. It is the serine protease responsible for cleavage of the secreted protein, EspB. In the MycP1 mutant, no secretion of ESX-1 substrates is observed, however Ohol et al. created a protease deficient mutant of MycP1 that can still be secreted but is no longer able to cleave EspB. Interestingly, this mutant actually showed an increase in secretion of ESX-1 substrates. Therefore, cleavage of EspB somehow limits the secretion of other ESX-1 substrates. Ohol et al. also showed that the protease activity of MycP1 is important for virulence during the chronic stage of infection. The periplasmic location of MycP1 (Ohol et al., 2010; Dave et al., 2002), anchored to the cytosolic membrane, gives it a prime location for sampling the extracellular environment. Therefore, MycP1 regulation of EspB cleavage may allow the bacteria to downregulate secretion of immune stimulating proteins later during infection to avoid detection by the immune system. This supports the idea that ESX-1 secretion must form a delicate balance between initiation of virulence and avoiding host defense mechanisms (Ohol et al., 2010).

1.7 The *Mycobacterium marinum* model

Mycobacterium marinum (*Mm*) has become an important model system for studying mycobacterial pathogenesis. *Mm* is genetically closely related to *Mtb* and causes chronic, systemic TB-like disease in its natural host, fish and frogs. The optimal growth of *Mm* is between 25-35°C. It grows poorly at 37°C, and therefore is only an opportunistic human pathogen. Human infection with *Mm* is localized to the cooler

extremities and forms what is often referred to as “fish tank granulomas” since fish handlers are the humans most likely to become infected with *Mm*. Because it is not a natural human pathogen, work with *Mm* can be done under Biosafety Level-2 (BSL-2) conditions, whereas *Mtb* work must be done under BSL-3 conditions. What’s more, the generation time of *Mm* is only four hours, compared to the 16-20 hour generation time that is typical of *Mtb* strains. *Mm* colonies form on agar plates within seven days, whereas *Mtb* colonies don’t form for several weeks. These are all reasons why *Mm* has become a useful model for which to study TB disease (Stamm and Brown, 2004).

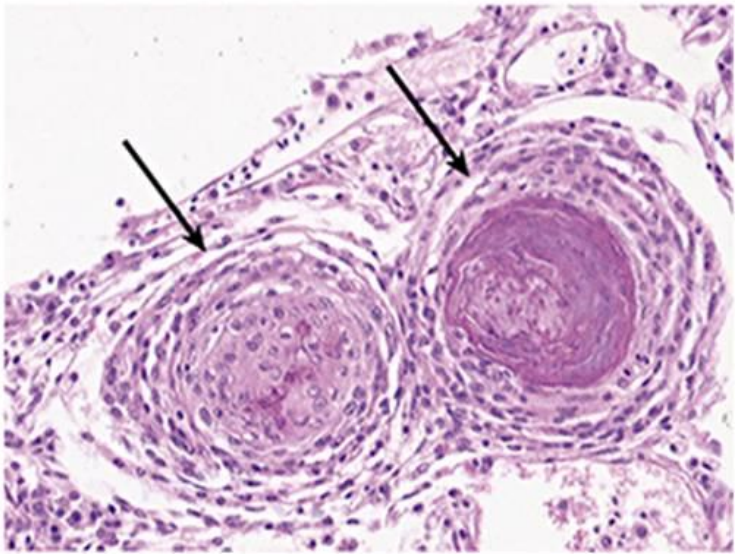
In addition to being a useful model, *Mm* is proving to be a very reliable model from which to understand the pathogenesis of mycobacterial infection (Swaim et al., 2006; Davis et al., 2002; Gao et al., 2004). *Mm* and *Mtb* share many important features. The *Mm* genome is larger than *Mtb*, but this difference is likely a product of the diverse environments and hosts that *Mm* can inhabit compared to *Mtb*, which is restricted to growth inside its human host. The orthologous regions however, show 85% identity between the two species (Stinear et al., 2008). What’s more, the virulence proteins discovered in *Mm* can generally be complemented by their *Mtb* orthologs, indicating a conservation of function (Tobin and Ramakrishnan, 2008).

Pathogenic mycobacteria exhibit a cording phenotype where the bacilli aggregate end-to-end, giving them a cord-like appearance. Cording is observed in both *Mtb* and *Mm*. It has even been shown that *Mm* cording is correlated with virulence as demonstrated in *kas*B and *iip*A mutants that exhibit altered cell wall structure (Gao et al., 2003b; 2006).

The model organism most widely used to study *Mm* is the zebrafish (*Danio rerio*). Even though the immune system of zebrafish is not as well understood as it is in mammals, several important components appear to be conserved; including phagocytes, TLRs, and some cytokines (Stamm and Brown, 2004). Fish do not have lungs, but even so, the caseating granulomas formed by *Mm* in the fish spleen and liver are morphologically very similar to those formed by *Mtb* in the human lung (Tobin and Ramakrishnan, 2008) [Fig. 7]. Conversely, the mouse is the most widely used model organism for studying *Mtb* infection. Unlike the *Mtb*-induced granulomas in human lungs, lung granulomas in mice are aggregates of disorganized macrophages and lymphocytes that display non-caseous centers (Flynn et al., 2006). *Mm*-induced granulomas have been shown not to recruit as many lymphocytes as *Mtb*-induced granulomas; however fish lacking B- and T-cells are much more sensitive to *Mm* infection. This indicates that lymphocytes are also important for control of *Mm* infection (Swaim et al., 2006).

Like *Mtb*, *Mm* resides inside host macrophages in non-acidified phagosomes that inhibit phagolysosome fusion and do not acquire vacuolar proton ATPases (Barker et al., 1997). Importantly, *Mm* has a functional ESX-1 system that exhibits the same mutant phenotype as in *Mtb*; that is, an altered modulation of cytokine secretion by macrophages and the inability to inhibit phagolysosome fusion (Stanley et al., 2003; 2007; Gao et al., 2004; Guinn et al., 2004; Tan et al., 2006).

One significant difference between *Mm* and *Mtb* is the ability of *Mm* to escape the phagosome. *Mtb* is generally thought not to escape the phagosome (Clemens et al., 2002; Russell, 2001), though there is some controversy to that claim. In 2007, van



Tobin and Ramakrishnan, 2008. *Cell Microbiol*.

Figure 7. The *Mycobacterium marinum*-induced granuloma. Haematoxylin and eosin stain of two *Mm*-induced granulomas in zebrafish, 6-weeks post infection. Arrows point to outermost edge of granuloma. The non-caseating granuloma on the left has nucleated cells at its core, while the granuloma on the right is caseating, similar to what is seen in *Mtb*-induced granulomas of the human lung.

der Wel et al., showed via electron microscopy of infected dendritic cells that *Mtb* is able to escape to the cytosol. *Mm* is also able to polymerize actin and spread from cell-to-cell once it gains access to the host cytosol (Stamm et al., 2003). Actin-based motility has never been demonstrated in *Mtb*. One hypothesis is that *Mm* uses actin based motility and escape from the phagosome as a feature of its transmission in ectotherms (Stamm and Brown, 2004). Since *Mtb* transmits differently than *Mm*, these abilities may not be a useful mechanism for *Mtb*. However, the similarities during the persistence stage involving granuloma formation and the inhibition of phagolysosome fusion are clear between these two species.

The research presented in this dissertation is carried out using the *Mm* model. What has been learned may one day be studied in *Mtb* for the benefit of better drug targets or a more protective vaccine.

Chapter 2: ESAT-6 is a pore-forming toxin and may play a role in *Mycobacterium marinum* escape from the vacuole.

This chapter is adapted from a manuscript previously published in the December, 2008 issue of *Infection and Immunity* entitled, "Evidence for pore formation in host cell membranes by ESX-1 secreted ESAT-6 and its role in *Mycobacterium marinum* escape from the vacuole" (Smith et al, 2008). I was a first author on this manuscript though some figures were contributed by other members of Dr. Gao's lab and those figures have been cited. Permission to reuse this manuscript was given by the American Society for Microbiology on October 22, 2010 (License Number: 253421007792).

2.1 Introduction

As discussed in Chapter 1, ESX-1 plays a critical role in the virulence of *Mtb* and *Mm* *in vitro* and *in vivo* (Fortune et al., 2005; Gao et al., 2004; Guinn et al., 2004; Hsu et al., 2003, Stanley et al., 2003; Swaim et al., 2006), but the precise molecular and cellular mechanisms are not clearly defined. Our lab previously reported that *Mm* is able to escape from the *Mycobacterium*-containing vacuole (MCV) into the host cell cytosol, where it is able to polymerize host cell actin and spread from cell to cell (Stamm et al., 2003). However, the *Mm* genes involved in mycobacterial escape from the MCV and actin polymerization have not been characterized. Because mutations in various ESX-1 genes abolish *Mm* cell-to-cell spreading (Gao et al., 2004), we hypothesized that the ESX-1 secretion system could secrete a pore-forming protein into the MCV to compromise the integrity of the vacuole membrane and facilitate the escape of *Mm* into the host cell cytosol. In this study, we show that ESX-1 plays an essential role in the escape of *Mm* from the MCV. We provide evidence that ESX-1 secretion and secreted ESAT-6 play a critical role in causing pore formation in host cell membranes. These results suggest that ESAT-6 secretion by ESX-1 may cause

membrane pore formation in the MCV, facilitating *Mm* escape from the vacuole and spreading.

2.2 Results

2.2.1 ESX-1 is essential for *M. marinum* to escape from the vacuole.

Mm is able to escape from the MCV into the host cell cytosol, polymerize actin, and spread from cell to cell (Gao et al, 2004; Stamm et al, 2003; Stamm et al, 2005). Because mutations in various ESX-1 genes abolish *Mm* spreading (Gao et al, 2004), we hypothesized that the ESX-1 secretion system may play a role in either the escape of *Mm* from the MCV or initiation of actin polymerization. We first determined if ESX-1 is involved in *Mm* escape from the vacuole. We examined the association of WT or ESX-1 mutant bacteria with the vacuole membranes in live macrophages by using a fluorescent membrane dye, DiI. DiI is frequently used to label the live cell membranes and has worked well in previous studies labeling MCV membranes (Stamm et al., 2003). As shown in Fig. 8A to C and Table 2, at 72 h postinfection only a small fraction (18%) of the WT bacteria colocalized with DiI, suggesting that the majority of the bacteria entered the cytosol. In sharp contrast, for all of the nine ESX-1 mutants examined, the majority of the bacteria (≥80%) colocalized with DiI [Fig. 8D to F and Table 2], indicating that they reside predominantly within MCV membranes. To confirm the above observations, we used transmission electron microscopy to examine in greater detail the association of *Mm* with vacuole membranes. This study shows that a fraction of the WT bacteria are bound with MCV membranes (36%) while most are free of the membrane (64%) [Fig. 9A and Table 2].

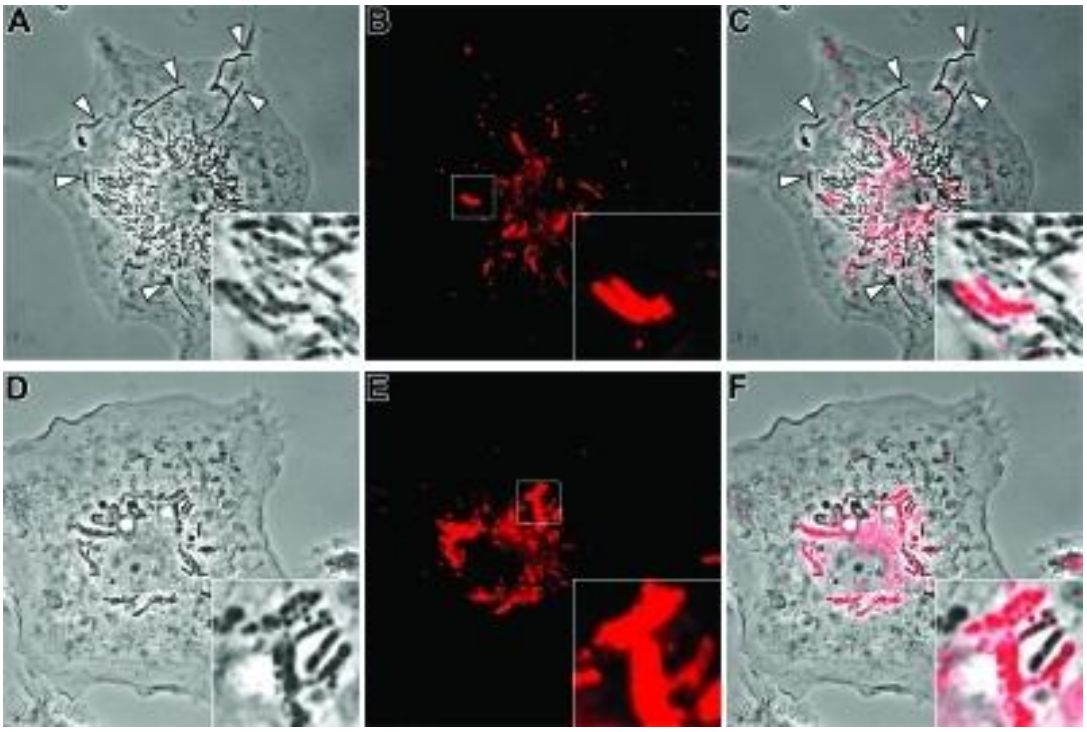


Figure contributed by members of the Gao lab. Smith et al., 2008. *Infect Immun*.

Figure 8. ESX-1 secretion plays an essential role in the escape of *Mm* from the vacuole. BMDMs were infected with WT *Mm* or the ESX-1 mutants at an MOI of 2. At 72 h postinfection, the cells were stained with DiI. For the ESX-1 mutants, only the results for *mh*3868::tn are shown; the results for the rest of the mutants are shown in Table 1. The top panels show the phase (A), DiI fluorescence (B), and merge (C) images from a representative macrophage infected by WT *Mm*. The bottom panels show the phase (D), DiI fluorescence (E), and merge (F) images from a representative macrophage infected by *mh*3868::tn. The insert in the lower right corner is an enlarged section of the indicated area in each panel. Arrowheads indicate *Mm* bacteria on the tips of actin stalks, which show no colocalization with DiI, suggesting their cytosolic localization. Information on duplication of experiments, the number of cells examined, and statistical analyses is shown in Table 2.

Table 2. Analysis of WT *M. marinum* and the ESX-1 mutants for colocalization with MCV membranes and polymerization of actin

M. marinum strain	% Membrane localization by DiI			% Membrane localization by TEM[g]			% Actin polymerization		
	Mean[b]	SD[c]	n_1[d]	Mean	SD	n_2[e]	Mean	SD	n_1
WT	18.2	2.9	100	35.5	1.4	20	34.4	2.3	100
*mh*3866::tn	79.6	2.7	100	ND[f]			0.7	0.6	100
*mh*3867::tn	82.7	2.0	100	ND			0.8	0.5	100
*mh*3868::tn	85.2	2.7	100	97.6	0.8	20	0.7	0.5	100
*mh*3871::tn	95.6	2.5	100	ND			0.0		100
Δ*cfp*-10+*esat*-6	95.1	2.8	100	100	0.0	20	0.0		100
Δ*esat*-6	97.3	2.0	100	98.5	0.9	20	0.0		100
*mh*3876::tn	85.2	2.5	100	ND			0.8	0.6	100
*mh*3877::tn	95.5	2.8	100	ND			0.0		100
*esp*B::tn	96.9	1.8	100	100	0.0	20	0.0		100
*esp*B::tn-C[a]	21.8	3.1	100	ND			32.6	3.1	100

[a] *esp*B::tn-C indicates the *esp*B::tn mutatn complemented with both the *esp*B and *mh*3880c genes (17).

[b] Mean indicates the average of the percentages for the number of cells (*n*) examined.

[c] SD indicates the standard deviation of the mean for the number of cells (*n*) examined.

[d] n_1 indicates the sum of cell numbers from duplicate experiments, 50 cells for each. All bacteria in each of the cells were scored, and percentages were calculated.

The mean is the average of the percentages for the 100 cells examined.

[e] n_2 indicates the sum of cell numbers from duplicate experiemnts, 10 cells for each. All bacteria in the representative areas of each cell were scored, and percentages were calculated. The mean is the average of the percentages for the 20 cells examined.

[f] ND, not determined.

[g] TEM, transmission electron microscopy.

On the other hand, for the four ESX-1 mutants examined, more than 98% of the bacteria are surrounded by MCV membranes [Fig. 9B and Table 2]. The mutants used in the above assays contain mutations in either the ESX-1 secretion apparatus (such as *mh*3877::tn and *mh*3871::tn) or the secreted substrates (such as Δ*esat-6*, Δ*cfp-10+esat-6*, and *esp*B::tn). Because the two groups of mutants showed similar phenotypes [Table 2], these results indicate that the ESX-1 secretion system plays a critical role in the escape of *Mm* from the MCV.

2.2.2 M. marinum escape from the vacuole is required for the polymerization of actin.

Next, we determined if ESX-1 is required for *Mm* to initiate actin polymerization. At 72 h postinfection, 34% of the WT bacteria showed actin polymerization at one pole of the bacterium, forming the "actin comet tail" [Fig. 10A to C and Table 2], similar to previous observations (Stamm et al, 2003; Stamm et al, 2005). In contrast, none of the nine ESX-1 mutants was able to polymerize actin [Fig. 10D to F and Table 2].

One possible explanation for the above results is that an ESX-1-secreted protein is directly involved in the recruitment and polymerization of actin. Alternatively, an ESX-1-secreted protein could be involved in compromising the integrity of MCV membranes to facilitate *Mm* escape into the host cell cytosol. To distinguish between these two possibilities, we treated the infected macrophages with a hypotonic solution to artificially deliver the ESX-1 mutant bacteria into the cytosol and then reexamined actin polymerization. In 1982, Okada and Rechsteiner showed elegantly that hypotonic shock treatment causes lysis of pinocytic/endocytic vesicle membranes without disrupting the plasma membrane. This finding supports that the hypotonic

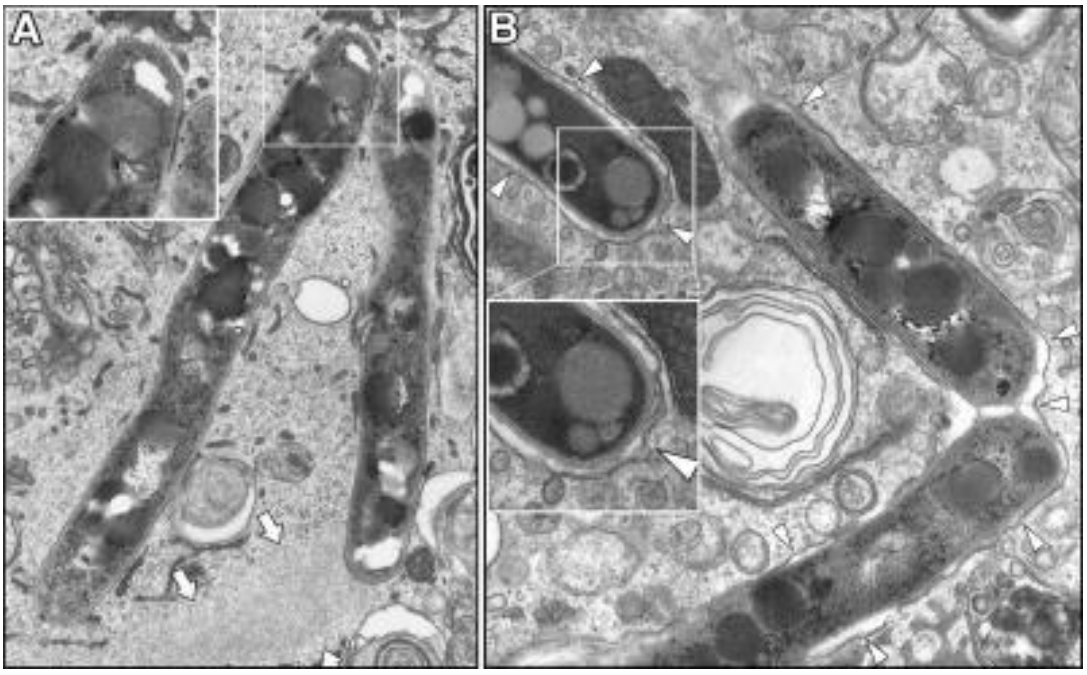


Figure contributed by members of the Gao lab. Smith et al., 2008. *Infect Immun.*

Figure 9. Transmission electron microscopy confirming the role of ESX-1 in *Mm* escape from the vacuole. BMDMs were infected with WT *Mm* or the ESX-1 mutants at an MOI of 2. At 72 h postinfection, the cells were processed for electron microscopy. For the ESX-1 mutants, only the results for *mh*3868::tn are shown; the results for the rest of the mutants are shown in Table 1. Panel A shows a section of a representative macrophage infected by WT *Mm*. The upper left insert shows an enlarged area of the cell. No host cell membranes are visible surrounding the bacteria. Note that a bacterium on the right shows an actin tail (indicated by arrows). Panel B shows a section of a representative macrophage infected by *mh*3868::tn. Almost all of the mutant bacteria are surrounded by vacuole membranes (indicated by arrowheads). The insert on the mid-left shows an enlarged area of the cell. Information on duplication of experiments, the number of cells examined, and statistical analyses is shown in Table 2.

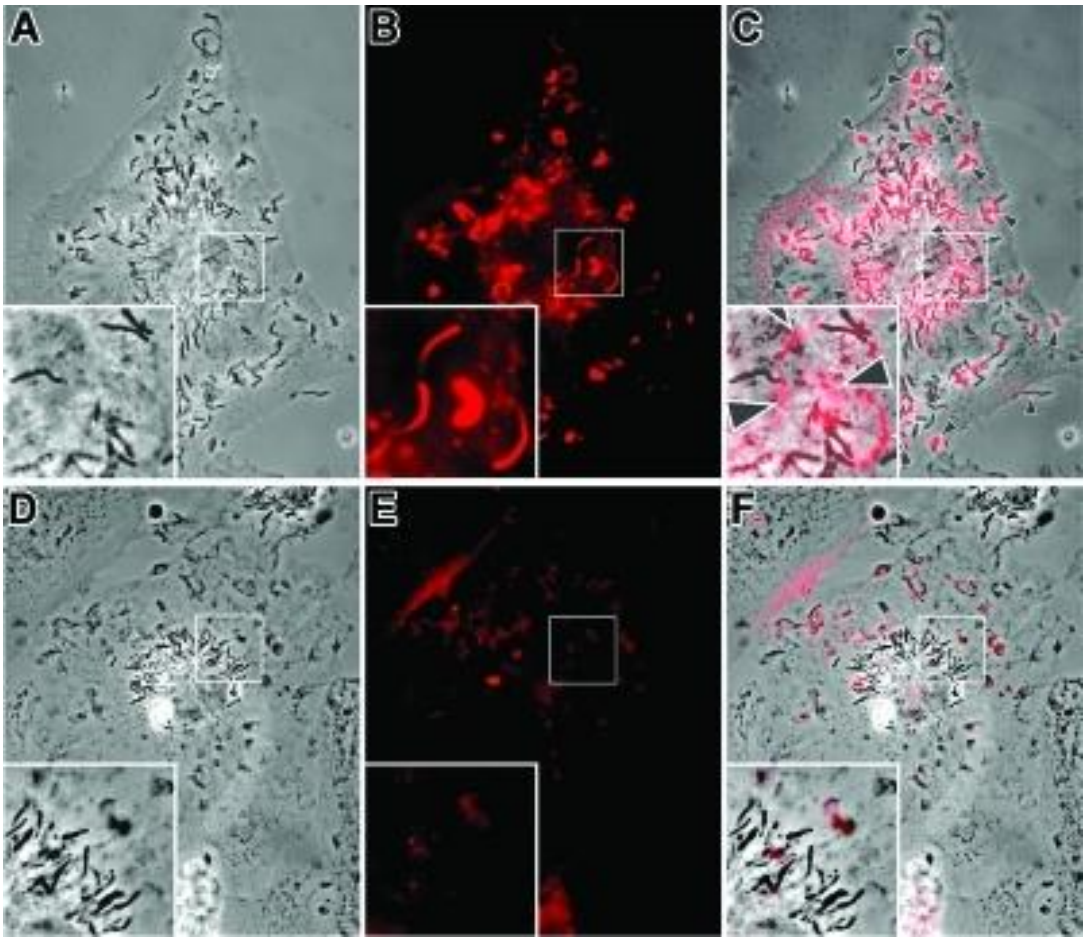


Figure contributed by members of the Gao lab. Smith et al., 2008. *Infect Immun.*

Figure 10. ESX-1 secretion plays an essential role in polymerization of actin by *Mm* in macrophages. BMDMs were infected with WT *M. marinum* or the ESX-1 mutants at an MOI of 2. At 72 h postinfection, the cells were stained with Alexa Fluor phalloidin to detect F-actin. For the ESX-1 mutants, only the results for *mh*3868::tn are shown; the results for the rest of the mutants are shown in Table 1. The top panels show the images of phase (A), F-actin (B), and merge (C) from a representative macrophage infected by WT *Mm*. The bottom panels show the images of phase (D), F-actin (E), and merge (F) from a representative macrophage infected by *mh*3868::tn. The insert in the lower left corner is an enlarged section of the area indicated in each panel. Arrowheads indicate actin tails. Information on duplication of experiments, the number of cells examined, and statistical analyses is shown in Table 2.

shock treatment could facilitate entry of the ESX-1 mutant bacteria into the host cell cytosol. Indeed, as shown in Fig. 11, after the treatment, *mh*3868::tn gained the ability to polymerize actin. Similar results were observed with *esp*B::tn (data not shown). These results indicate that the defect in the ability of the ESX-1 mutants to polymerize actin is due to their inability to escape from the vacuole rather than a deficiency in initiation of actin polymerization. They also provide direct evidence that *Mm* initiates actin polymerization only after it enters the host cell cytosol. Moreover, they suggest that ESX-1 is involved in secreting a pore-forming protein that may compromise the integrity of the MCV membranes to facilitate the escape of *Mm*.

2.2.3 Evidence for membrane pore formation by *M. marinum* ESX-1.

Listeria monocytogenes represents a group of bacteria that are able to disrupt the vacuole membranes to enter the host cell cytosol by the secretion of pore-forming proteins (Schnupf and Portnoy, 2007). We hypothesized that the ESX-1 secretion system may play a similar role to facilitate *Mm* escape into the host cell cytosol. A direct test of this hypothesis would require the analysis of pore formation in the MCV membranes of infected cells, which is technically challenging. In an attempt to address this hypothesis, we took an alternative approach. We incubated *Mm* with host cells at a relatively high MOI and examined pore formation in the cell plasma membranes. Membrane pore formation was determined by an osmoprotection assay (Scherrer and Gerhardt, 1971) which has been used in a number of studies to demonstrate membrane pore formation and estimate pore size. This assay has been used to detect membrane pores and estimate the size of pores induced by pathogens such as *Gardnerella vaginalis* (Moran et al., 1992), *Legionella pneumophila* (Kirby et

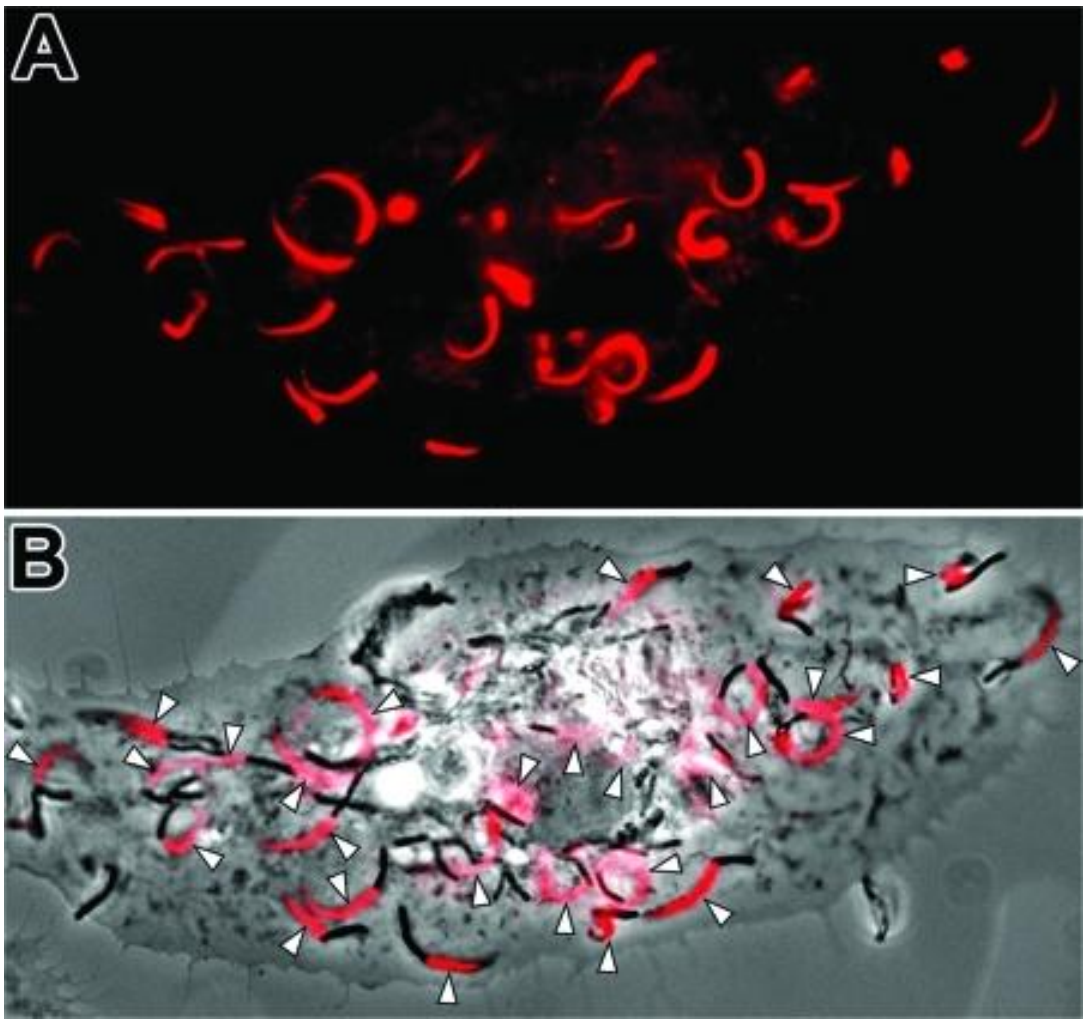


Figure contributed by members of the Gao lab. Smith et al., 2008. *Infect Immun*.

Figure 11. ESX-1 mutants acquire the ability to polymerize actin after being delivered into the macrophage cytosol by hypotonic shock treatment. BMDMs were infected by *mh*3868::tn at an MOI of 2. At 48 h postinfection, the cells were treated with a hypotonic solution (see Materials and Methods) to lyse the vacuole membranes without disrupting the plasma membranes (Okada and Rechsteiner, 1982). This treatment is expected to facilitate the escape of *Mm* into the macrophage cytosol. At 24 h after hypotonic shock treatment, the cells were processed for F-actin staining with Alexa Fluor phalloidin. Panel A shows F-actin staining, and panel B shows a merged phase and F-actin staining image. Actin tails are indicated by arrowheads. With one hypotonic shock treatment, more than 10% of the infected macrophages show actin polymerization by the mutant bacteria. Within these macrophages, an average of 46% of the bacteria show actin polymerization. Duplicate experiments were performed in which a total of 100 cells were observed.

al., 1998), *Shigella flexneri* (Blocker et al., 1999), and *Pseudomonas aeruginosa* (Dacheux et al., 2001). We first determined if the hemolysis of red blood cells induced by *Mm* could be blocked by polyethylene glycol (PEG) of various molecular weights. The lysis of red blood cells (hemolysis) by pore-forming proteins occurs through osmotic shock, which can be prevented by osmoprotectants that have larger sizes than the membrane pores (Blocker et al., 1999; Dacheux et al., 2001; Kirby et al., 1998; Menestrina et al., 1994). As shown in Fig. 12A, WT *Mm* caused contact-dependent hemolysis of red blood cells, which was blocked completely by PEG8000 (6.4 nm in diameter) and 60% by PEG6000 (5.0 nm) but not blocked by PEG3350 (3.8 nm). Importantly, the blocking effect of PEG8000 is reversible, as indicated by the recovery of hemolysis after the removal of PEG8000 [Fig. 12A]. We then examined pore formation in macrophage cell membranes with a similar assay in which different-size PEGs were used to block the release of lactate dehydrogenase (LDH) from infected macrophages. We found that WT *Mm* caused the release of LDH from macrophages, which was blocked completely by PEG8000 but not by PEG3350. Together, the results of both assays suggest that *Mm* causes contact-dependent pore formation in host cell membranes.

To estimate the size of the membrane pores produced by *Mm*, PEG of different sizes were used at various concentrations to determine the osmolarity required for each PEG to provide 50% protection from hemolysis (Kirby et al., 1998; Scherrer and Gerhardt, 1971). Fig. 12B shows the osmolarity of each PEG that provides 50% protection as a function of its Einstein-Stokes molecular diffusion radium, R_{ES} (Scherrer and Gerhardt, 1971). The response curve is hyperbolic and approaches a

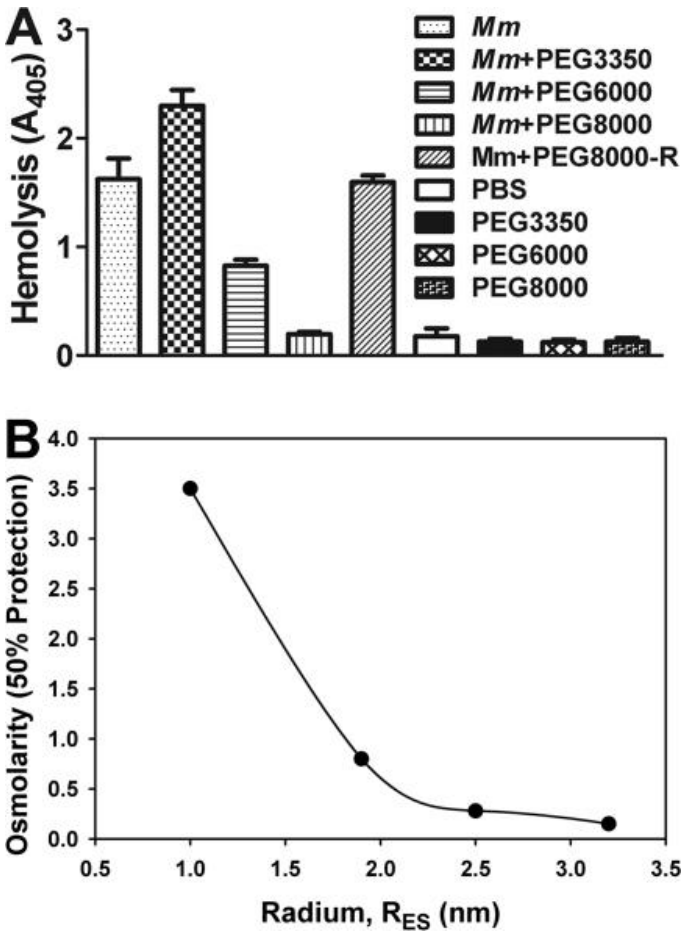


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Figure 12. *Mm* induces pore formation in red blood cell membranes. (A) Pore formation in red blood cell membranes as determined by an osmoprotection assay with different-sized PEGs to prevent hemolysis. PEG8000-R indicates recovery of hemolysis after the removal of PEG8000. The bacterium-to-red blood cell ratio is 25:1. *Mm* indicates *M. marinum*. (B) Estimation of the size of the pores induced by *Mm*. The osmolarity of each of the PEGs (PEG1000, PEG3350, PEG6000, and PEG8000) required to provide 50% protection from hemolysis is plotted as a function of its Einstein-Stokes molecular diffusion radium, R_{ES} (Scherrer and Gerhardt, 1971). The R_{ES} value for each PEG is as follows: PEG1000, 1.0 nM; PEG3350, 1.9 nM; PEG6000, 2.5 nM; PEG8000, 3.2 nM. The graphs are the summation of two independent experiments, each performed in duplicate, and error bars indicate standard deviations.

membrane stabilization limit asymptotically. By using a similar method developed by Scherrer and Gerhardt, we estimated pore size by extrapolating to the zero abscissa the linear regression between PEG1000 and PEG3350, and the intercept at R_{ES} of 2.25 is believed to represent the radius of the membrane pore.

We then determined if ESX-1 plays a role in membrane pore formation. As shown in Fig. 13A and B, mutations in either the ESX-1 secretion apparatus (*mh*3877::tn and *mh*3871::tn) or the secreted substrate (Δ*esat-6*) completely abolished the ability of *Mm* to induce membrane pore formation, demonstrating that ESX-1 secretion plays an essential role in this process. To determine if continuous ESX-1 secretion or predeposition of ESX-1-secreted proteins on the bacterial surface is necessary to cause pore formation, we treated WT *Mm* with carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a membrane deenergizer that blocks energy-dependent pathways including ESX-1. Fig. 13C shows that the CCCP treatment abolished hemolysis completely and in a reversible manner, indicating that continuous energy-dependent ESX-1 secretion is required for *Mm* to induce membrane pore formation.

2.2.4 Evidence for membrane pore formation by ESX-1-secreted ESAT-6.

Three known ESX-1-secreted proteins, ESAT-6, CFP-10, and EspB, are codependent for secretion (Xu et al, 2007). To determine the relative role of each individual protein in pore formation, we compared the hemolysis levels induced by different *Mm* strains producing various amounts of these proteins. We have shown previously that EspB is cleaved during secretion to produce an N-terminal 50-kDa and a C-terminal

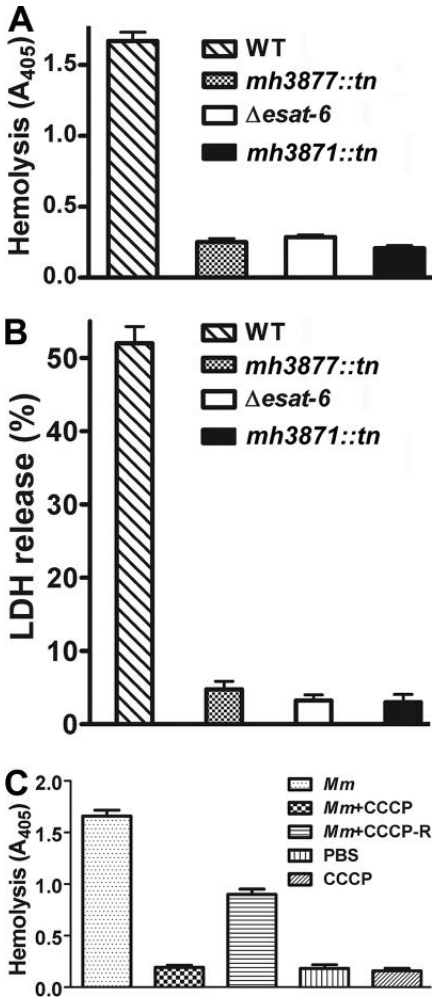
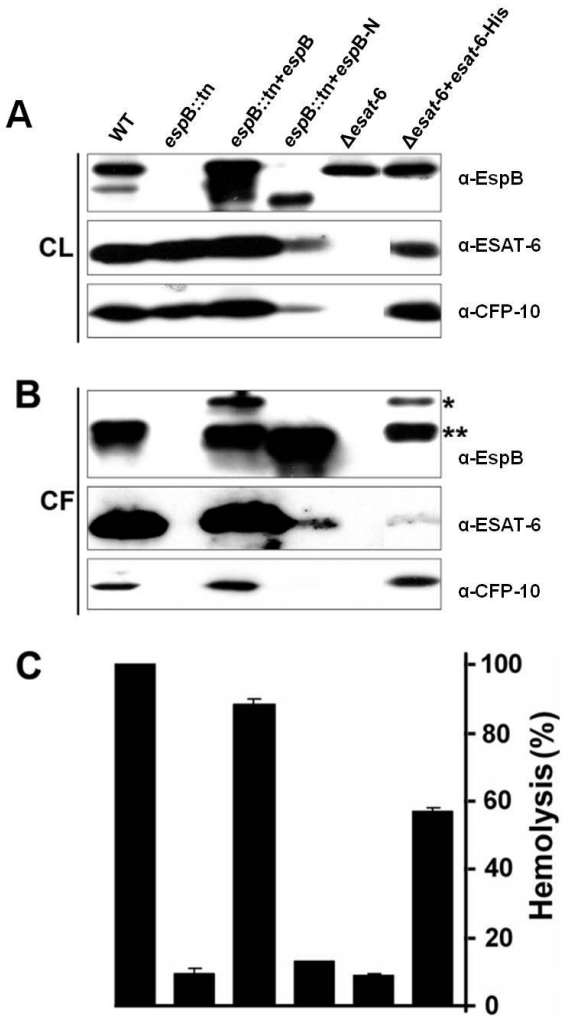


Figure contributed by members of the Gao lab. Smith et al., 2008. *Infect Immun*.

Figure 13. ESX-1 secretion plays an essential role in the induction of pore formation in host cell membranes by *Mm*. (A) ESX-1-dependent induction of pore formation in red blood cell membranes. WT *Mm* or the ESX-1 mutants were mixed with red blood cells at a ratio of 25:1, centrifuged to allow close bacterium-cell contact, and incubated for 2 h before measurement of hemolysis. (B) ESX-1-dependent induction of pore formation in macrophage cell membranes. WT *M. marinum* or the ESX-1 mutants were incubated with Raw264 murine macrophages at a ratio of 50:1. Pore formation in macrophage cell membranes was detected by release of LDH and its blockage by different-sized PEGs. LDH release (percent) is the percentage of LDH released by the infected macrophages with respect to the total amount of LDH produced by lysis of the macrophages with H_2O. (C) Induction of membrane pore formation by *M. marinum* requires continuous energy-dependent secretion. WT *Mm* was left untreated or treated with CCCP prior to incubation with red blood cells. CCCP-R indicates recovery of pore formation after removal of CCCP. The values are means of two independent experiments, each performed in duplicate, and error bars indicate standard deviations.

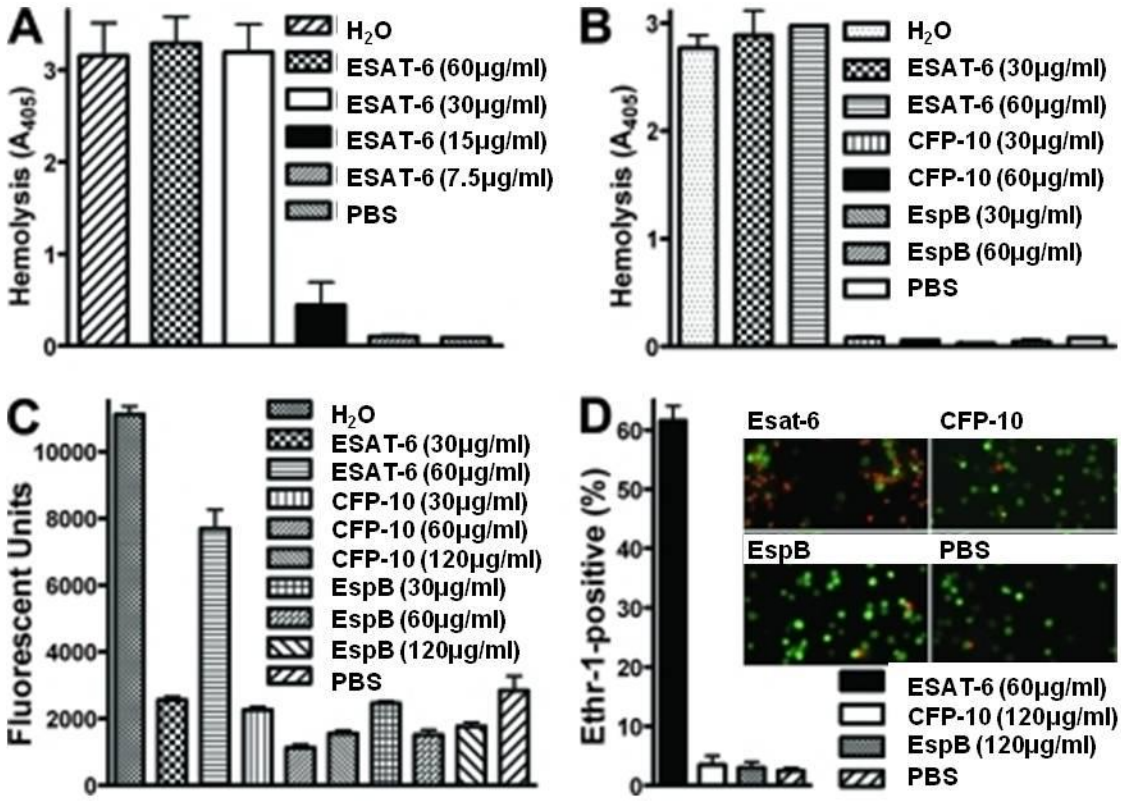
11-kDa fragment (apparent molecular masses) [Fig. 14, lane 1] (Xu et al., 2007). The 50-kDa fragment is relatively stable and present at abundant levels in the culture supernatant, while the majority of the 11-kDa fragment is degraded proteolytically (Xu et al, 2007; data not shown). As shown in Fig. 14, *esp*B::tn fails to secrete these three proteins and is defective in pore formation [lane 2]. Both the secretion and pore formation defects of this mutant are almost fully restored by expression of the WT *esp*B gene [lane 3]. On the other hand, when only the N-terminal 50-kDa fragment of EspB was expressed in this mutant, it was secreted by the mutant at levels comparable to those produced by WT bacteria, but ESAT-6 secretion was not detected and CFP-10 secretion was minimal [lane 4]. Because this strain shows a complete defect in pore formation, the data suggest that EspB, or at least the 50-kDa fragment, does not contribute directly to pore formation. To determine if the secretion of ESAT-6 or CFP-10 plays a role, we examined pore formation by Δ*esat-6* and the complementation strain. Δ*esat-6* fails to secrete these three proteins and is defective in pore formation (lane 5). Expressing *esat-6-His*$_{(6)}$ in Δ*esat-6* substantially restored the secretion of ESAT-6 and EspB, although CFP-10 secretion was only recovered by a marginal level [lane 6]. The reason that we used *esat-6-His*$_{(6)}$ instead of *esat-6* was to express this protein in a form that is exactly the same as the recombinant protein used in other assays (see below). Because this strain shows a substantial increase in hemolysis, which correlates with the much increased ESAT-6 secretion, the results suggest that ESAT-6 secretion plays an important role in pore formation.



Panels A and B contributed by members of the Gao lab. Smith et al., 2008. *Infect Immun.*

Figure 14. Membrane pore formation induced by *Mm* correlates with ESAT-6 secretion. (A) The steady-state cellular levels of EspB, CFP-10, and ESAT-6 in the WT, *espB*::tn, Δ*esat-6*, and complemented strains. *espB*::tn plus *esp*B indicates the *esp*B::tn mutant complemented with the WT *esp*B gene. *esp*B::tn plus *esp*B-N indicates the *esp*B::tn mutant complemented with only the N-terminal 50-kDa fragment of EspB. Δ*esat-6* plus *esat-6*-His indicates the Δ*esat-6* mutant complemented with *esat-6-His*$_{(6)}$. CL, cell lysate. (B) Secretion of EspB, CFP-10, and ESAT-6 by the WT, *esp*B::tn, r*esat-6*, and complemented strains. The lane order is the same as in panel A. The single asterisk indicates full-length EspB with an apparent molecular mass of 61 kDa; the double asterisks indicate the N-terminal fragment of EspB with an apparent molecular mass of 50 kDa. CF, culture filtrate. (C) Membrane pore formation (measured by hemolysis) induced by the WT, *esp*B::tn, Δ*esat-6*, and complemented strains. The hemolysis level obtained with the WT bacteria is normalized to 100%. The bar order is the same as the lane order in panel A.

To determine if ESAT-6 has a direct role in membrane pore formation, we examined the pore-forming activity of recombinant ESAT-6 [rESAT-6-$His_{(6)}$] purified from *Escherichia coli* and compared it to that of rCFP-10-$His_{(6)}$ or rEspB-$His_{(6)}$. These recombinant proteins were affinity purified and free of detergents and had endotoxins removed (see Materials and Methods for details). As shown in Fig. 15A, rESAT-6-$His_{(6)}$ from *Mtb* produced dose-dependent hemolysis after a 2-h incubation, i.e., partial hemolysis at 15 μg/ml and complete hemolysis at 30 μg/ml, almost equivalent to lysis with H_2O. In contrast, neither rCFP-10-$His_{(6)}$ from *Mtb* nor rEspB-$His_{(6)}$ from *Mm* caused hemolysis, even at a higher concentration of 60 μg/ml [Fig. 15B] or 120 μg/ml (data not shown). The combination of rESAT-6-$His_{(6)}$ with rCFP-10-$His_{(6)}$ or with rEspB-$His_{(6)}$ produced hemolysis at levels similar to those produced by rESAT-6-$His_{(6)}$ alone (data not shown). Hemolysis induced by rESAT-6-$His_{(6)}$ was not due to the residual endotoxins present in the recombinant protein preparations (≤0.24 ng/mg protein), since lipopolysaccharide at a concentration of 0.007 ng/ml [equivalent to the level of endotoxins present in rESAT-6-$His_{(6)}$ at a concentration of 30 μg/ml] failed to induce a detectable level of hemolysis (data not shown). Consistent with the hemolysis results, rESAT-6-$His_{(6)}$ at 60 μg/ml, but not rCFP-10-$His_{(6)}$ or rEspB-$His_{(6)}$ (even at 120 μg/ml), caused release of LDH from macrophages [Fig. 15C]. Permeation of macrophage cell membranes was similarly observed by microscopic detection of penetration of ethidium homodimer-1 across the plasma membrane into the cytosol to stain the nuclei red [Fig. 15D]. These results together suggest that ESAT-6 may play a direct role in membrane pore formation. To provide a more direct demonstration that ESAT-6 by itself can induce pore formation in cell membranes,



Panel D contributed by members of the Gao lab. Smith et al., 2008. *Infect Immun*.

Figure 15. Purified ESAT-6, but not CFP-10 or EspB, plays a direct role in causing pore formation in host cell membranes. (A) rESAT-6-$His_{(6)}$ induces dose-dependent pore formation in red blood cell membranes. The protein was incubated with red blood cells for 2 h before measurement of hemolysis. (B) rESAT-6-$His_{(6)}$, but not rCFP-10-$His_{(6)}$ or rEspB-$His_{(6)}$, induces pore formation in red blood cell membranes. The proteins were incubated with red blood cells for 2 h before measurement of hemolysis. (C) rESAT-6-$His_{(6)}$, but not rCFP-10-$His_{(6)}$ or rEspB-$His_{(6)}$, induces pore formation in macrophage cell membranes. J774 macrophages were incubated with the proteins in cell culture medium for 2 h before measurement of LDH release. H_2O was used to obtain complete lysis of the macrophages. (D) Detection of ESAT-6-induced membrane pore formation by fluorescence microscopy. J774 macrophages were incubated with rESAT-6-$His_{(6)}$, rCFP-10-$His_{(6)}$, or rEspB-$His_{(6)}$ for 2 h before detection of penetration of ethidium homodimer-1 across the macrophage cell membranes to stain the nuclei red. The bar graph shows the quantification of the representative images. Error bars indicate standard deviations of data from two experiments, with each performed in duplicate.

we performed an osmoprotection assay similar to that described above. As shown in Fig. 16A, the hemolysis induced by rESAT-6-$His_{(6)}$ was blocked completely by 30 mM PEG8000 and PEG6000, ~40% by PEG3350, and to a small extent by PEG1000. The fact that hemolysis is induced by rESAT-6-$His_{(6)}$ and that it can be blocked by PEG of increasing sizes indicate that ESAT-6 indeed plays a direct role in membrane pore formation. It was noticed that the membrane pores induced by rESAT-6-$His_{(6)}$ are somewhat smaller than those produced by the bacteria (see discussion). We further characterized the ESAT-6-induced membrane pores by determining the kinetics of hemolysis produced by rESAT-6-$His_{(6)}$. Figure 16B shows that rESAT-6-$His_{(6)}$ at 30 μg/ml caused 75% hemolysis after a 5-min incubation, which increased to 93% after 10 min. This is almost equivalent to the hemolysis produced by H_2O, which caused 95% and complete hemolysis after 5 and 20 min of incubation, respectively.

2.2.5 Hemolysis can be used as a technique to study ESAT-6 secretion.

As shown in Figure 14, hemolysis of RBCs correlates with ESAT-6 secretion. WT, *esp*B+Complement, and Δ*esat*-6+Complement all secrete a normal amount of ESAT-6 and show the highest levels of hemolysis. Alternatively, *esp*B::tn and Δesat-6 do not secrete any ESAT-6 and show very little hemolysis. I perfomed this hemolysis assay on several truncation mutants of EspB and found that it was a good technique to determine quickly if the mutation affects ESAT-6 secretion. Truncating full-length EspB at either the N- or C-terminus leads to a reduction in hemolysis and presumably ESAT-6 secretion [Fig 17]; therefore, it looks like both fragments are important for the codependent secretion of these two proteins. In subsequent Western blot analysis, I found that the correlation isn't completely linear; that is, a reduction in hemolysis

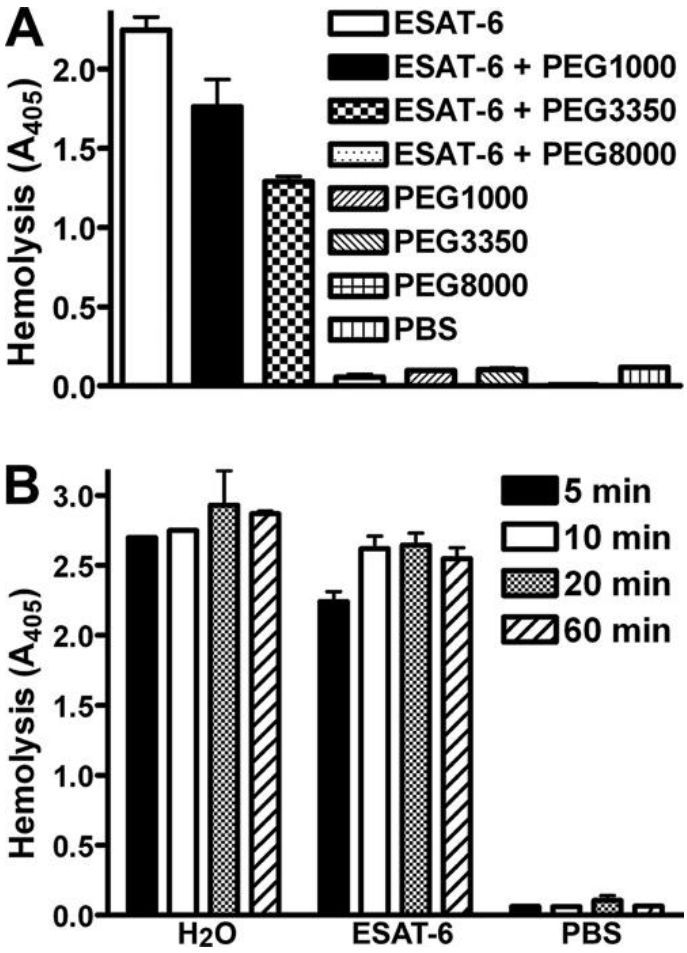


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Figure 16. Purified ESAT-6 induces pore formation in red blood cell membranes. (A) Membrane pores induced by rESAT-6-$His_{(6)}$ were blocked by PEGs of appropriate sizes in an osmoprotection assay. Hemolysis was also completely blocked by 30 mM PEG6000 (data not shown). (B) The kinetics of membrane pore formation induced by ESAT-6. The concentration of rESAT-6-$His_{(6)}$ was 30 μg/ml for both panels. Error bars indicate standard deviations of data from two or three independent experiments, with each performed in duplicate.

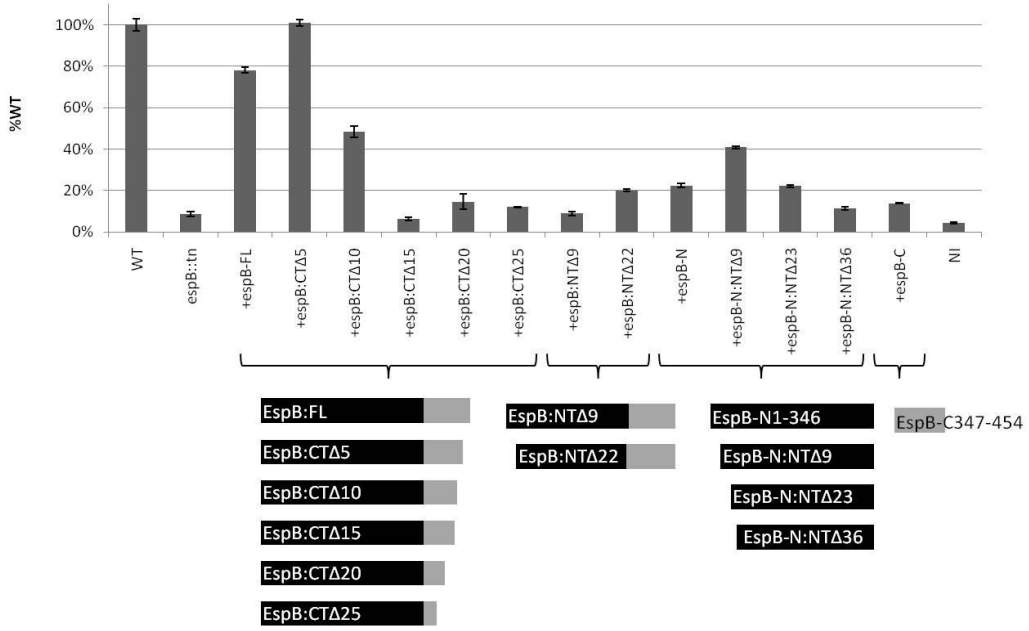

Figure 17. Red blood cell hemolysis induced by EspB truncation mutants. *Mm* WT, *esp*B::tn, and several *esp*B mutants episomally expressed in *esp*B::tn were mixed with red blood cells at a ratio of 25:1, centrifuged to allow close bacterium-cell contact, and incubated for 2 hours. Hemolysis was measured by taking the OD of the red blood cell supernatant which contains lysed cell hemoglobin. OD is represented as a percentage of WT. Mutants include full-length EspB with truncations at the C-terminus (1st set), or N-terminus (2nd set), expression of just the N-terminal 1-346 amino acids fragment with truncations at the N-terminus (3rd set), or expression of just the C-terminal 347-454 amino acids fragment (4th set). NI is a negative control of red blood cells without bacteria added.

does not necessarily indicate the same reduction in ESAT-6 secretion. Really, any mutation that even just reduces the amount of ESAT-6 that is secreted shows a relatively severe drop in hemolysis; indicating that the hemolysis assay is not as sensitive at determining ESAT-6 secretion. It is, however a quick assay for gathering information that will help to design further experiments.

2.3 Materials and Methods

2.3.1 Bacteria and media.

Mm strain M was cultured and maintained as described previously (Gao et al, 2003b). The *Mm* ESX-1 mutants were produced as described previously (Gao et al, 2004). The *esp*B::tn mutant was recently isolated from an *Mm* transposon mutant library (Gao et al, 2003a). The *esp*B::tn mutant and its complementation were described previously (Xu et al, 2007).

2.3.2 Generation of *M. marinum* Δ*esat-6* mutant.

Δ*esat-6* mutant *Mm* was generated by allelic exchange. The left flanking fragment was amplified by PCR with primers DelESAT-F1 (5′CCGCTCTAGACCTGGTTGCAGACCGCCTCGAC3′) and DelESAT-R1 (5′GCCCGAATTCAGAAGCCCATTTGCGAGGACAGCGC3′). The right flanking fragment was amplified by PCR with primers DelESAT-F2 (5′CGGGAATTCGCGTAGAATACCGAAGCACGAGATCGGG3′) and DelESAT-R2 (5′CCGCAAGCTTCTAGATTCATGCCGGTTTGGCGTGGC3′). The left flanking fragment was digested with XbaI and EcoRI, and the right flanking fragment was digested with EcoRI and HindIII. The left and right flanking fragments and a

kanamycin resistance cassette (*kan*^{r}) (cut with EcoRI from pUC4K) were ligated into pBluescript. The entire sequence containing the flanking sequences and *kan*^{r} was cut from the pBluescript clone and ligated into pLYG304.zeo (Gao et al, 2006) to generate the *esat-6* knockout plasmid. The plasmid was electroporated into wild-type (WT) *Mm*, and homologous recombinants were selected as described previously (Gao et al, 2006). Confirmation of the Δ*esat-6* mutation was carried out by PCR with two primer pairs. One pair confirms recombination within the left flanking sequence, in which a primer that anneals to a sequence upstream from the flanking sequence (5′CGTGGACCGGAGGCGGCAGCGAGAAAG3′) and another that anneals to a sequence in *kan*^{r} (5′CACCTTCTTCACGAGGCAGACCTCAGCGCC3′) were used. The other pair confirms recombination within the right flanking sequence, in which a primer that anneals to a sequence downstream from the flanking sequence (5′GGATTCAGCCTCCGGTGGCCCTGGAG3′) and another that anneals to a sequence in *kan*^{r} (5′GGCAATGTAACATCAGAGATTTTGAGACACAACGTGGC3′) were used.

2.3.3 Complementation of *M. marinum* Δ*esat-6* mutant.

To complement the Δ*esat-6* mutant with both the *esat-6* and *cfp-10* genes together, a fragment containing both genes was amplified by PCR from the *Mm* genome with primers 5′CAGAGATGAAGACCGATGCCGCTACCCTCG3′ and 5′GGCCGGATCCTTAGTGATGGTGATGGTGATGAGCAAACATCCCCGTGAC GTTGCC C3′. The reverse primer contains a six-His tag fused to the C terminus of ESAT-6. The PCR product was cloned into pLYG206.Zeo (Gao et al., 2003b) to

generate the complementation plasmid. This plasmid was electroporated into Δ*esat-6* to obtain the complementation strain.

2.3.4 Macrophages.

J774.A1 (ATCC TIB67) or Raw264.7 (ATCC TIB-71) murine macrophage-like cells were cultured and maintained as described previously (Gao et al., 2003b). Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice as previously described (Roach et al., 1998). Cells were harvested 8 to 10 days after plating and allowed to adhere to fibronectin-coated coverslips (Becton Dickinson) for infection with *Mm* the next day.

2.3.5 DiI labeling of MCV membranes.

BMDMs on glass coverslips were infected with *Mm* at a multiplicity of infection (MOI) of 2 for 2 h, followed by three washes with phosphate-buffered saline (PBS) and 1 h of incubation with 200 μg/ml amikacin to kill the extracellular bacteria. At the end of the antibiotic incubation, the cells were washed two times with PBS and incubated at 32°C in 5% CO_2 for 48 to 72 h. CM-DiI (Molecular Probes) was added to the cells at a 2 μM final concentration and incubated for 1 h. The cells were then washed two times with PBS to remove excess DiI, incubated for 1 h in the cell culture medium, and fixed with 4% paraformaldehyde. The fixed cells were washed three times with PBS, mounted on a glass slide with Prolong Antifade (Molecular Probes), and imaged.

2.3.6 Fluorescent labeling of F-actin.

BMDMs on glass coverslips were infected at an MOI of 2 as described above. At 48 to 72 h postinfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, stained with Alexa Fluor phalloidin (Molecular Probes), and imaged. To artificially deliver the ESX-1 mutant bacteria into macrophage cytosol, the cells at 48 h postinfection were treated for 5 min with a hypotonic solution (4 parts phenol red-free Dulbecco modified Eagle medium [DMEM] to 1 part H_2O), incubated in cell culture medium at 32°C in 5% CO_2 for an additional 24 h, and then processed for actin staining. The procedures for the hypotonic shock treatment were similar to those described by Okada and Rechsteiner, 1982.

2.3.7 Electron microscopy.

BMDMs were infected at an MOI of 2 as described above. At 72 h after infection, the cells were processed for electron microscopic analysis as described previously (Taunton et al, 2000).

2.3.8 Detection of pore formation in red blood cell membranes.

Induction of pore formation in red blood cell membranes by *Mm* was detected by hemolysis assay as previously described (Gao and Kwaik, 1999; Gao et al, 2004). Briefly, *Mm* grown in 7H9 medium to mid-log phase was washed twice with PBS. A volume of 1.3 ml of *Mm* suspension (containing 2.5×10^9 bacteria) was mixed with 400 µl of sheep red blood cells (sRBC; Quad Five) (containing 1×10^8 cells) in a microcentrifuge tube and centrifuged at 8,000 × *g* for 2 min. The tubes were

incubated at 32°C for designated periods of time. The pellets were then resuspended and centrifuged, and the A_{405} of the supernatants was measured. To examine the role of energy-dependent secretion in membrane pore formation, *Mm* WT bacteria were pretreated for 15 min with 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to uncouple the proton motive force and then incubated with sRBC in the presence of CCCP for 2 h. To observe the reversibility of CCCP in hemolysis, the pellet containing *M. marinum* and red blood cells was resuspended in PBS and then repelleted and further incubated for 2 h before measurement of hemolysis.

In the polyethylene glycol (PEG) osmoprotection experiment, PEG1000, PEG3350, PEG6000, and PEG8000 were resuspended in PBS and added to red blood cells with or without *Mm* to a final concentration of 30 mM. Hemolysis was measured after 2 h of incubation at 32°C. To determine if protection from hemolysis by PEG8000 is reversible, the pellet containing *Mm* and red blood cells was resuspended in PBS and then repelleted and further incubated for 2 h before measurement of hemolysis. To estimate membrane pore size, the various PEGs were resuspended in PBS at the following ranges of osmolarities: PEG1000, 1.0, 2.0, 3.0, and 4.0 M; PEG3350, 0.2, 0.4, 0.6, 0.8, and 1.0 M; PEG6000, 0.2, 0.3, 0.4, and 0.5 M; PEG8000, 0.1, 0.2, and 0.3 M. The osmolarity required for each PEG to provide 50% protection from hemolysis was determined and plotted against the Einstein-Stokes molecular diffusion radium, R_{ES} (Scherrer and Gerhardt, 1971).

2.3.9 Expression and purification of recombinant ESX-1 proteins.

The rESAT-6-$His_{(6)}$, rCFP-10-$His_{(6)}$, and rEspB-$His_{(6)}$ proteins were affinity purified and had endotoxins removed. The rESAT-6-$His_{(6)}$ and rCFP-10-$His_{(6)}$ proteins were obtained from Colorado State University under the NIH TB Research Materials Contract. The inclusion bodies containing rESAT-6-$His_{(6)}$ were solubilized with 6 M urea, and the protein was purified with the His-Bind resin (Novagen). Endotoxins were removed by washing the column with 10 mM Tris-HCl, followed by 0.5% ASB-14. The protein was eluted with 10 mM Tris-HCl containing 1 M imidazole. The eluted protein was dialyzed against 10 mM ammonium bicarbonate. The residual concentration of endotoxins was ≤0.24 ng/mg protein. Two lots were obtained, one produced in 2001 and the other in 2007. Both lots were used for the analysis of membrane pore formation, and similar results were observed. We expressed and purified the rEspB-$His_{(6)}$ protein by using procedures similar to those described above.

2.3.10 Detection of pore formation by purified ESX-1 proteins.

To determine the ability of ESX-1-secreted proteins to induce membrane pore formation, the above-described purified rESAT-6-$His_{(6)}$, rCFP-10-$His_{(6)}$, or rEspB-$His_{(6)}$ in a 50-μl volume was mixed with 100 μl of sRBC (containing 1×10^9 cells) in a microcentrifuge tube. The tubes were incubated at 32°C for designated periods of time. The cells were then resuspended and centrifuged at 4,000 rpm for 7 min. The supernatants were transferred to corresponding wells in a 96-well plate, and the A_{405}

was measured. In the PEG osmoprotection experiment, PEG1000, PEG3350, PEG6000, and PEG8000 were resuspended in PBS and added to the red blood cell-protein mixture at a final concentration of 30 mM. Hemolysis was measured after 2 h of incubation at 32°C.

2.3.11 Detection of pore formation in macrophage cell membranes.

Mm strains grown to mid-log phase were washed twice with DMEM, added to macrophage monolayers at an MOI of 50, centrifuged for 10 min at 1,500 × *g* to allow immediate bacterium-cell contacts, and incubated at 32°C in 5% CO_2 for designated periods of time. The release of lactate dehydrogenase (LDH) by the infected and noninfected cells was measured with a CytoTox-One Homogeneous Membrane Integrity Assay kit (Promega) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Pore formation in macrophage cell membranes was determined by an osmoprotection assay with various PEGs at a final concentration of 30 mM. To examine the induction of pore formation in macrophage cell membranes by rESAT-6-$His_{(6)}$, rCFP-10-$His_{(6)}$, or rEspB-$His_{(6)}$, the proteins were dissolved in DMEM and added individually to Raw264 cells in a 96-well plate at designated concentrations. After 2 h of incubation at 32°C in 5% CO_2, the release of LDH was determined.

To detect membrane pore formation by a microscopy method, infected or noninfected macrophages were incubated with ethidium homodimer-1 (Molecular Probes) and penetration of the cell by ethidium homodimer-1 across the membranes was detected by fluorescence microscopy. In brief, macrophages were incubated for 40 min in

phenol red-free culture medium containing ethidium homodimer-1 (4 μM) and calcein AM (2 μM), followed by imaging. Ethidium homodimer-1 only penetrates permeabilized cell membranes and stains the nuclei red. Calcein AM permeates every cell membrane and is only metabolized by live cells to produce green fluorescence.

2.3.12 Preparation of *M. marinum* short-term culture filtrate and cell lysate.

Preparation of *Mm* short-term culture filtrate and cell lysate was carried out as previously described (Xu et al, 2007). In brief, WT and mutant *Mm* cells were first grown in 7H9 medium to mid-log phase. The bacteria were then washed and diluted 10 times in Sauton's medium and cultured for 2 days to reach mid-log phase. The bacteria were washed again and diluted 10 times in Sauton medium and cultured for another 2 days. The culture supernatant was collected, filtered through a 0.2-μm filter, and concentrated 100 times with a Centricon centrifugal filter with a molecular weight cutoff of 3,000. The cell lysate was obtained by bead beating the bacterial pellet.

2.3.13 Western blotting.

Proteins were separated by 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking with 2% bovine serum albumin, the membrane was incubated with a primary antibody diluted in 2% bovine serum albumin overnight, followed by incubation for 1 h with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). The membrane was developed by enhanced chemiluminescence (Pierce) and exposed to films. The following primary antibodies were used at the dilutions

indicated: anti-ESAT-6 (monoclonal antibody HYB 76-8; Abcam), 1:3,000; anti-CFP-10 (Colorado State University, NIH contract NO1-AI-75320), 1:2,000; anti-EspB (Michael Lodes, Corixa Corporation, Seattle, WA), 1:5,000.

2.4 Discussion

The ESX-1 (type VII) secretion system plays an important role in the virulence of *Mtb* and *Mm*, but the precise molecular and cellular mechanisms by which it enhances virulence are not clearly defined. This chapter describes a comprehensive study of these mechanisms which has led to several important observations. Firstly, by examining nine *Mm* ESX-1 mutants and the WT by fluorescence and electron microscopy detecting MCV membranes, this study demonstrates conclusively that ESX-1 plays an essential role in the escape of *Mm* from the MCV. The role of vacuole escape in mycobacterial pathogenesis is not clearly understood. The observations that *Mm c*an polymerize actin inside the host cell cytosol and spread from cell to cell (Gao et al., 2004; Stamm et al., 2003; 2005) suggest that vacuole escape may play a role in mycobacterial spreading.

Secondly, we show that the ESX-1 mutant bacteria are able to polymerize actin after being delivered into the host cell cytosol by hypotonic shock treatment. This result suggests that the defect in the ability of the ESX-1 mutants to polymerize actin during normal cell infection is due to their inability to escape from the MCV rather than a deficiency in initiation of actin polymerization. This conclusion helps to redirect future research efforts aimed at identifying the mycobacterial molecules directly responsible for initiating actin polymerization. In addition, since this assay clearly

shows that *Mm* induces actin polymerization only after it enters the cytosol, it suggests that actin tails can be used as a readout of cytosolic localization for *Mm.*

Thirdly, this study demonstrates for the first time that *Mm* utilizes ESX-1 to produce membrane pores ~4.5 nm in diameter in red blood cells and macrophages. Importantly, purified ESAT-6 by itself at a concentration of 30 μg/ml is sufficient to cause pore formation in cell membranes. These observations are a significant advancement of the previously published works. For example, *Mtb* (Hsu et al., 2003) and *Mm* (Gao et al., 2004) have been shown to induce the permeation of cell membranes, and here we demonstrate that the cause of this permeation is pore formation. In addition, earlier studies show that purified ESAT-6 can cause permeation of liposome membranes (de Jonge et al., 2007; Hsu et al., 2003), and here we show that it causes pore formation in cell membranes. Our study suggests that ESAT-6 secreted by *Mm* ESX-1 could play a direct role in causing pore formation in MCV membranes to facilitate mycobacterial escape from the vacuoles.

We have noticed that purified ESAT-6 induces membrane pores that are somewhat smaller than those produced by bacteria. We hypothesize that the membrane pores could be formed by the insertion of multimers of ESAT-6, exposing their hydrophobic surface to the lipid bilayer and their hydrophilic surface to the center of the pore. This model suggests that the number of ESAT-6 molecules inserted to form a pore could determine the size of the pore. It is possible that ESAT-6 secreted by *Mm* ESX-1 at the bacterium-cell contact site could have optimal insertion and/or multimerization to produce pores larger than those produced by the protein alone. Alternatively, it is possible that certain bacterial surface structures or molecules or

some other secreted molecules could enhance membrane pore formation. For example, the secretion of phospholipases C by *Listeria* plays a role in enhancing membrane pore formation by the pore-forming toxin listeriolysin O (Goldfine et al., 1995). *Mm* and *Mtb* contain multiple copies of phospholipase C (Cole et al., 1998; Stinear et al., 2008), and *Mtb* has been shown to secrete phospholipase C (Raynaud et al., 2002). The involvement of phospholipase C in membrane pore formation is worthy of further investigation.

A topic related to the above is whether ESAT-6 alone or ESAT-6 in complex with CFP-10 or the other ESX-1-secreted proteins forms the membrane pores during mycobacterial infection. Thus far, the published studies have assigned the membrane destabilization activity to ESAT-6 alone (de Jonge et al., 2007; Hsu et al., 2003). Our results are consistent with these studies in that ESAT-6 alone causes pore formation. Then, the question comes if the pore-forming activity of ESAT-6 is biologically plausible, considering that some studies show that ESAT-6 and CFP-10 can interact with each other and can form a tight 1:1 complex (Renshaw et al., 2005; 2002). However, our studies suggest that not all of the ESAT-6 and CFP-10 molecules have to be held in an exact 1:1 complex, and this does not contradict the published results. We have shown that EspB forms a complex with ESAT-6 (Xu et al., 2007). When EspB is pulled down from *M. marinum* cell lysate, ESAT-6 coprecipitates with EspB; however, no CFP-10 is detected in the precipitate (Xu et al., 2007). In addition, as shown in Figure 14, the Δ*esat-6* complementation strain produces a much more abundant level of ESAT-6 than CFP-10 in the culture supernatant. Both of our studies suggest that during *Mm* infection at least some of the ESAT-6 molecules could be

targeted to the host cell membranes not in a complex with CFP-10. It remains a possibility that EspB facilitates the pore-forming activity of ESAT-6, which warrants future investigation.

A recent study showed that ESAT-6 in a concentration range of 2 to 5 μg/ml can induce apoptosis of macrophages after overnight incubation (Derrick and Morris, 2007). This result does not conflict with our observation in the present study. By using this concentration range of ESAT-6, we were not able to detect apparent membrane pore formation in red blood cells and macrophages [Fig. 15]. It is possible that the insertion and multimerization of ESAT-6 in cell membranes also depend on the concentration of ESAT-6 (indeed, we have observed a dose-dependent induction of membrane pore formation by ESAT-6 [Fig. 15A]), and 2 to 5 μg/ml might be below a critical concentration of ESAT-6 required to induce pore formation. On the other hand, since this sub-pore-forming concentration of ESAT-6 was shown to induce cell apoptosis (Derrick and Morris, 2007), it suggests that ESAT-6 might use one domain to induce pore formation and another to induce apoptosis. In this regard, it has recently been shown that the C-terminal six amino acids of ESAT-6 can bind to macrophage surface Toll-like receptor 2 to modulate host cell signaling (Pathak et al., 2007). Whether this interaction plays a role in the induction of apoptosis remains elusive and deserves further investigation. It should also be noted that the time course in the induction of apoptosis is very different from that in pore formation. While apoptosis is observed after an overnight incubation (Derrick and Morris, 2007), pore formation is detected in minutes (this study). This analysis suggests that ESAT-6 may change its mode of action quite dramatically when present at different concentrations:

either apoptotic or pore forming. Further studies on the structure and function of ESAT-6 will not only provide insight into the virulence mechanisms of mycobacterial infection but also promote the development of important tools based on ESAT-6 for research in broad areas of cell biology and immunobiology studies.

Finally, several studies have observed that *Mtb* can also escape from vacuoles into the host cell cytosol (McDonough et al., 1993; van der Wel et al., 2007) and that ESX-1 plays a role in this process (van der Wel et al., 2007), although this has not been consistently observed by some other researchers (Clemens et al., 2000; Deretic et al., 2006; Russell, 2007). In these studies that have observed *Mtb* escape from the vacuole, most of the observations were made by detailed electron microscopic examinations. One argument in the field is that during sample processing for electron microscopy, some of the cell membranes may not be preserved well, causing false-positive results. Therefore, using alternative methods for detection of MCV membranes is crucial and should provide additional confirmation of the electron microscopic observations. In this regard, in this study we have used three additional methods besides electron microscopy to confirm the association of *Mm* with MCV membranes. One method detects fluorescent labeling of MCV membranes by using the DiI stain in live cells, which avoids the perturbation of membranes that could be generated by the other detection methods. The second method detects polymerization of actin by *Mm* by fluorescence microscopy. Since WT *Mm*, but not the ESX-1 mutants, is able to polymerize actin, the data indicate that WT bacteria, but not the ESX-1 mutants, is able to escape from the vacuole. The third method uses hypotonic shock treatment to artificially deliver the ESX-1 mutant bacteria into the host cell

cytosol, and then actin polymerization by the mutant bacteria is determined. We show that the ESX-1 mutants acquire the ability to polymerize actin after hypotonic shock treatment, confirming that the mutant bacteria reside within MCV membranes before the treatment but enter the cytosol after. However, actin polymerization has not been observed for *Mtb* in host cells (van der Wel et al., 2007), which makes this method unavailable for the detection of escape of *Mtb* from the vacuole. Nonetheless, as several recent studies show that *Mtb* can cause lysis of host cell plasma membranes *in vitro* and *in vivo* (Dobos et al., 2000; Hsu et al., 2003; Junqueira-Kipnis et al., 2006), these results suggest that *Mtb* could compromise vacuole membranes at a certain step of infection. This analysis suggests that escaping into the host cell cytosol could be a common strategy of pathogenic mycobacteria, while it occurs frequently and evidently in *Mm* infection. The reproducibility of observations and ease of manipulation make *Mm* an ideal system for studying these cellular processes important for the pathogenesis of mycobacteria.

Chapter 3: The molecular properties and virulence role of EspB

3.1 Introduction

As discussed in Chapter 1, there have been several substrates identified that are ESX-1 dependent for secretion. The best studied is ESAT-6, a strong antigenic target of the host immune system and absolutely required for virulence (Sørensen et al., 2005; Stanley et al., 2003; Hsu et al., 2003; Pathak et al., 2007). In Chapter 2, I demonstrated that the ability of *Mm* to lyse host cells is dependent on ESAT-6 and I also showed that the purified ESAT-6 protein is sufficient for inducing pores in macrophage membranes. It is not known how the pore-forming ability of ESAT-6 is used by the bacteria, though one possibility is that the pores allow for the bacteria to escape the phagosome (Smith et al., 2008). Phagosome escape has only been widely accepted as a function in *Mm* (Stamm et al., 2003; Gao et al., 2004), and still remains a controversial issue in *Mtb* (Clemens et al., 2002; Russel et al., 2001; van der Wel et al., 2007). Yet, the *Mtb* ESAT-6 protein has been shown able to complement the Δ*esat*-6 mutant in *Mm*, indicating a conservation of function (Gao et al., 2004). Another possibility is that the pores formed by ESAT-6, at least during the persistence stage of both *Mm* and *Mtb*, allow for the translocation of other ESX-1 substrates into the host cell in order to mediate bacterial survival inside the phagosome.

EspB is another protein that is ESX-1 dependent for secretion (Xu et al., 2007; McLaughlin et al., 2007). The function of this protein is unknown and it is hard to determine function based on the phenotype of the mutant because the EspB, ESAT-6,

and CFP-10 proteins are codependent for secretion (Xu et al., 2007). The EspA protein is also codependent for secretion of ESAT-6 (MacGurn et al., 2005), so without EspB the secretion of several substrates is disrupted and the phenotype observed cannot be attributed solely to the function of the EspB protein.

The EspB mutant is attenuated and EspB is required for normal functioning of the ESX-1 system. Whether its role is strictly to aid the secretion of other substrates that go on to have a virulence function, or whether EspB itself plays a direct role in virulence is unknown and the question is a difficult one to differentiate given the secretion codependence issues. However, there are studies that indicate that EspB has an additional function required for virulence besides helping aid ESAT-6 and CFP-10 secretion. In *Mm*, the *espB*::tn mutant is defective at intracellular growth and shows a more profound defect in preventing phagosome maturation than the ΔCFP-10/ESAT-6 mutant (Xu et al., 2007). Also, the *esp*B::tn mutant is more severely attenuated in zebrafish than the ΔCFP-10/ESAT-6 mutant, and shows significantly lower CFU in zebrafish livers (Gao et al., 2004). Therefore, EspB is particularly important for growth *in vivo*. EspB is also required for cell-to-cell spread during *Mm* infection of macrophage (Gao et al., 2004).

There are no structural motifs in the predicted EspB protein that would indicate its role in virulence. The EspB protein has been shown to be cleaved upon secretion by the serine protease, MycP1, and this cleavage is important for modulating the secretion of other ESX-1 substrates (Ohol et al., 2010). The result is a 50 kDa N-terminal fragment and an 11 kDa C-terminal fragment secreted into the culture filtrate (Xu et al., 2007; McLaughlin et al., 2007). ESAT-6 secretion is dependent on the C-

terminus of EspB since a C-terminally truncated protein is unable to secrete ESAT-6 (Xu et al., 2007). The EspB N-terminus binds to the Mh3879c protein, and Mh3879c was shown to interact with the Mh3871 ATPase at the membrane channel (McLaughlin et al., 2007). Presumably, Mh3879c acts as a chaperone to target the EspB/ESAT-6 complex to the ESX-1 channel for secretion. When the EspB N-terminus is expressed without the C-terminal fragment, it is still secreted, though ESAT-6 and CFP-10 are not (Xu et al., 2007; McLaughlin et al., 2007). What then is the function of the N-terminus? If this fragment can secrete independent of other ESX-1 substrates, does it go on to carry out a virulence function, or is EspB only functional in its full-length form to regulate ESX-1 secretion?

In this chapter I dissect the *Mm* EspB protein to see if mutations can lend insight into the important role that it plays in secretion and virulence. I show that the C-terminal fragment is all that is needed to secrete ESAT-6, whereas the N-terminal fragment can secrete independent of ESX-1 altogether. Neither the C- nor N-terminal fragment is able to complement the intracellular growth defect of *esp*B::tn, indicating that the full-length protein is required for virulence. I also show that mutating an EspB cleavage site leads to an increase in ESAT-6 secretion, supporting what Ohol et al., 2010 hypothesized; that MycP1 cleavage of EspB acts to regulate ESX-1 secretion. I also show that the EspB cleavage mutant is defective at intracellular growth in macrophages and is attenuated in zebrafish, indicating that cleavage of EspB is required for virulence function. Finally, I mutated a conserved WXG motif in the N-terminus of EspB and show that this motif plays a role in the codependent secretion of EspB and ESAT-6. These data support the hypothesis that full length EspB is

important for secretion and virulence. The N-terminus may play a more direct role in virulence but it cannot carry out this function without the tightly controlled secretion of other ESX-1 substrates.

3.2 Results

3.2.1 The role of EspB N- and C-terminal sequences in secretion and virulence

As already discussed, EspB is cleaved upon secretion near the C-terminus, resulting in a 50 kDa N-terminal fragment and an 11 kDa C-terminal fragment [Fig. 18]. The C-terminus of the full length protein binds to ESAT-6 and is required for ESAT-6 secretion (Xu et al., 2007; McLaughlin et al., 2007). What then, is the purpose of the N-terminal fragment? It is known that cleavage of EspB occurs at multiple sites (Ohol et al., 2010; our lab, unpublished). Using mass spectroscopy of the secreted N-terminal fragment, our lab discovered that one of the cleavage sites is between Leu346 and S347, resulting in a 50 kDa N-terminal fragment and an 11 kDa C-terminal fragment. Mass spectroscopy results of the C-terminal fragment indicate that this fragment is not stable; it undergoes degradation at multiple sites, eventually leading to a stable 5.5 kDa C-terminal fragment (data not shown). Ohol et al., 2010 discovered that the serine protease, MycP1 is responsible for EspB cleavage in at least two locations.

Because EspB is cleaved upon secretion and the C-terminus is required for the codependent secretion of ESX-1 substrates, my hypothesis is that the N-terminus plays a more direct role in virulence. This is a difficult hypothesis to support because

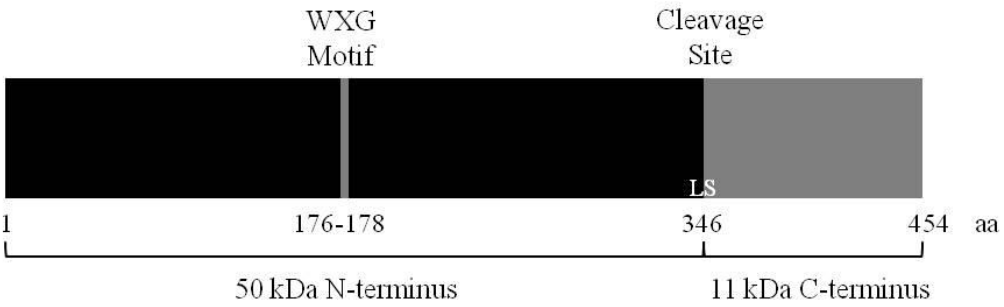

Figure 18. Diagram of the EspB protein. The *Mm* EspB protein in its full-length form is 454 amino acids long, but is cleaved upon secretion between leucine 346 and serine 347. The result is a 50 kDa N-terminal fragment and an 11 kDa C-terminal fragment. The C-terminal fragment plays a role in the codependent secretion of the ESAT-6 protein, whereas the N-terminus appears to have a virulence function that is dependent on expression of the full-length protein and close association with ESAT-6. Cleavage at L346 is important for the modulation of substrates secreted from the ESX-1 system. At position tryptophan 176 through glycine 178, EspB also has a highly conserved WXG motif that appears to play a role in secretion.

of the codependency issues already discussed. Determining EspB function independent of ESAT-6 cannot be examined in *esp*B::tn because both EspB and ESAT-6 secretion is abolished in this mutant (Xu et al., 2007). When the N-terminus is expressed without the C-terminus in *esp*B::tn, it is detected in the culture filtrate, even though there is no secretion of ESAT-6 or CFP-10 (Xu et al., 2007; McLaughlin et al., 2007). I expressed the N-terminal fragment in the *mh*3877::tn channel mutant and found that it is secreted independent of ESX-1 altogether [Fig. 19, lane 11]. It has already been demonstrated that the full length protein requires a functional ESX-1 channel for secretion (McLaughlin et al., 2007). This N-terminal fragment however, is also able to secrete through some other unknown channel, indicating that the C-terminus confers specificity of EspB secretion through ESX-1. I decided to take advantage of the unique nature of the EspB N-terminus to look at the role that it plays in virulence.

I expressed the first 346 amino acids of the EspB N-terminus in *esp*B::tn (*esp*B::tn +pEspB:N1-346) and confirmed that it is abundantly secreted, though ESAT-6 secretion is ablated [Fig 19, lane 8]. I also expressed the last 106 amino acids of the EspB C-terminus in the *esp*B::tn mutant (*esp*B::tn+pEspB:C347-454) to see if ESAT-6 is secreted. Our EspB polyclonal antibody recognizes a 100 amino acid sequence in the N-terminal fragment (amino acids 234-333); therefore I cannot detect the C-terminal fragment of the EspB protein. I was able to detect ESAT-6 secretion, albeit less than WT [Fig. 19, lane 9], indicating that the C-terminus is indeed present and that the N-terminus is somewhat dispensable for the secretion of ESAT-6.

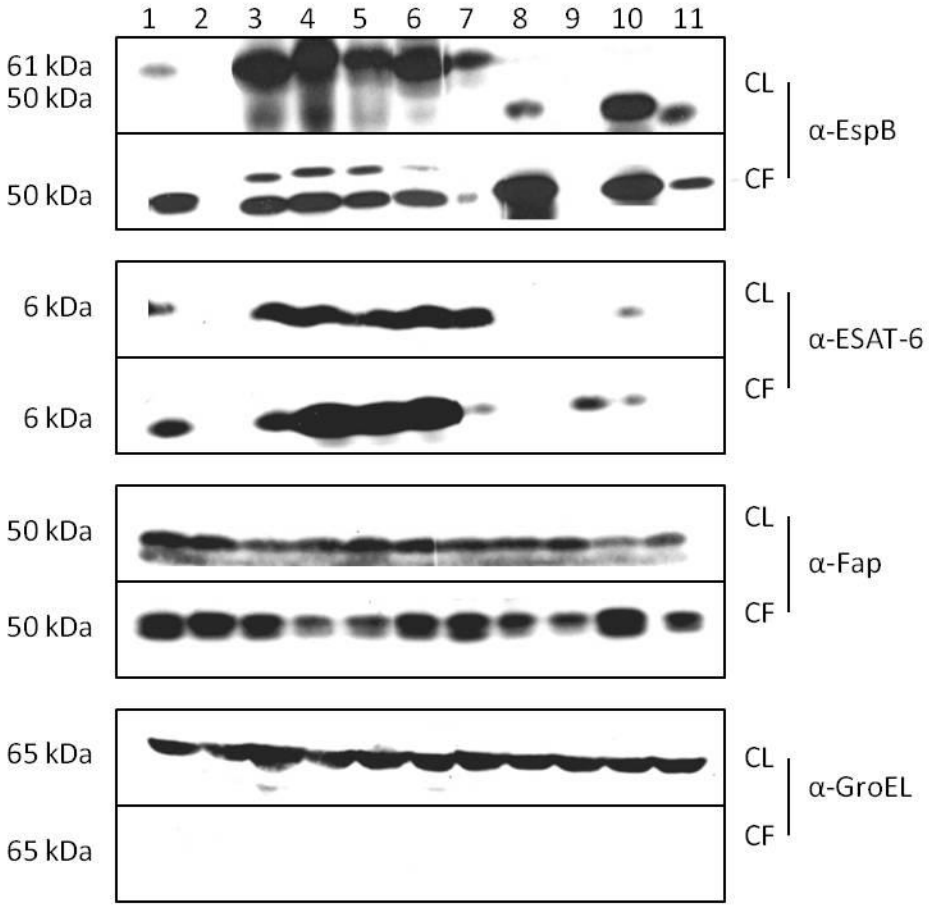

Figure 19. Western blots of EspB mutants. Culture filtrate (CF) and cell lysate (CL) proteins of *Mm* WT (lane 1), *esp*B::tn (lane 2), *esp*B::tn+Complement (lane 3), *esp*B::tn+pEspB:ΔL346-S347 (lane 4), *esp*B::tn+pEspB:ΔL346 (lane 5), *esp*B::tn+pEspB:ΔS347 (lane 6), *esp*B::tn+pEspB:W176A (lane 7), *esp*B::tn+pEspB:N1-346 (lane 8), *esp*B::tn +pEspB:C347-454 (lane 9), *esp*B::tn+pEspB:N1-346/C347-454 (lane 10), *mh*3877::tn+pEspB:N1-346 (lane 11). Loading adjusted according to the expression and secretion of the Sec-secreted Fap protein. The cytosolic GroEL protein is used to show that CF profiles are not due to lysis. For EspB, 61 kDa is the molecular weight of the full-length protein and 50 kDa is the molecular weight of the N-terminal cleaved fragment.

Interestingly, ESAT-6 expression was not detected in the cell lysate, but this could be due to reduced stability of the complex; since there is a reduced amount of ESAT-6, nearly all of it is secreted and there is no accumulation in the cell.

Since the N-terminus is able to secrete independent of ESX-1, and doesn't appear to play a major role in secretion of ESAT-6, I wanted to see if this fragment alone could complement the defective virulence phenotype of *esp*B::tn. This would indicate that the EspB N-terminal fragment does have a direct role in virulence that is not dependent on the secretion of other ESX-1 substrates. Alternatively, I wanted to see if expressing the C-terminus alone, which allows for ESAT-6 secretion, could complement *esp*B::tn (*esp*B::tn+pEspB:C347-454). This would indicate that the N-terminus does not play a direct role in virulence; rather, its only function is to regulate ESX-1 secretion. I infected RAW cells, a murine macrophage cell line, with the individual mutants and found that they were both defective at intracellular growth compared to the mutant expressing the full length protein (*esp*B::tn+Comp) [Fig. 20A], though growth was slightly better than the *esp*B::tn mutant.

The N-terminal fragment also was not able to fully complement *esp*B::tn for virulence in zebrafish [Fig. 21], indicating that the full length protein is required for virulence function; though this experiment also showed a slight increase in virulence over *esp*B::tn. In the zebrafish study, fish infected intraperitoneally with the *esp*B::tn+EspB complement strain began to die after only a couple of weeks. The N-terminal mutant however, persisted much longer. These fish did eventually succumb to infection, as did the fish infected with *esp*B::tn. This may be due to too high of a

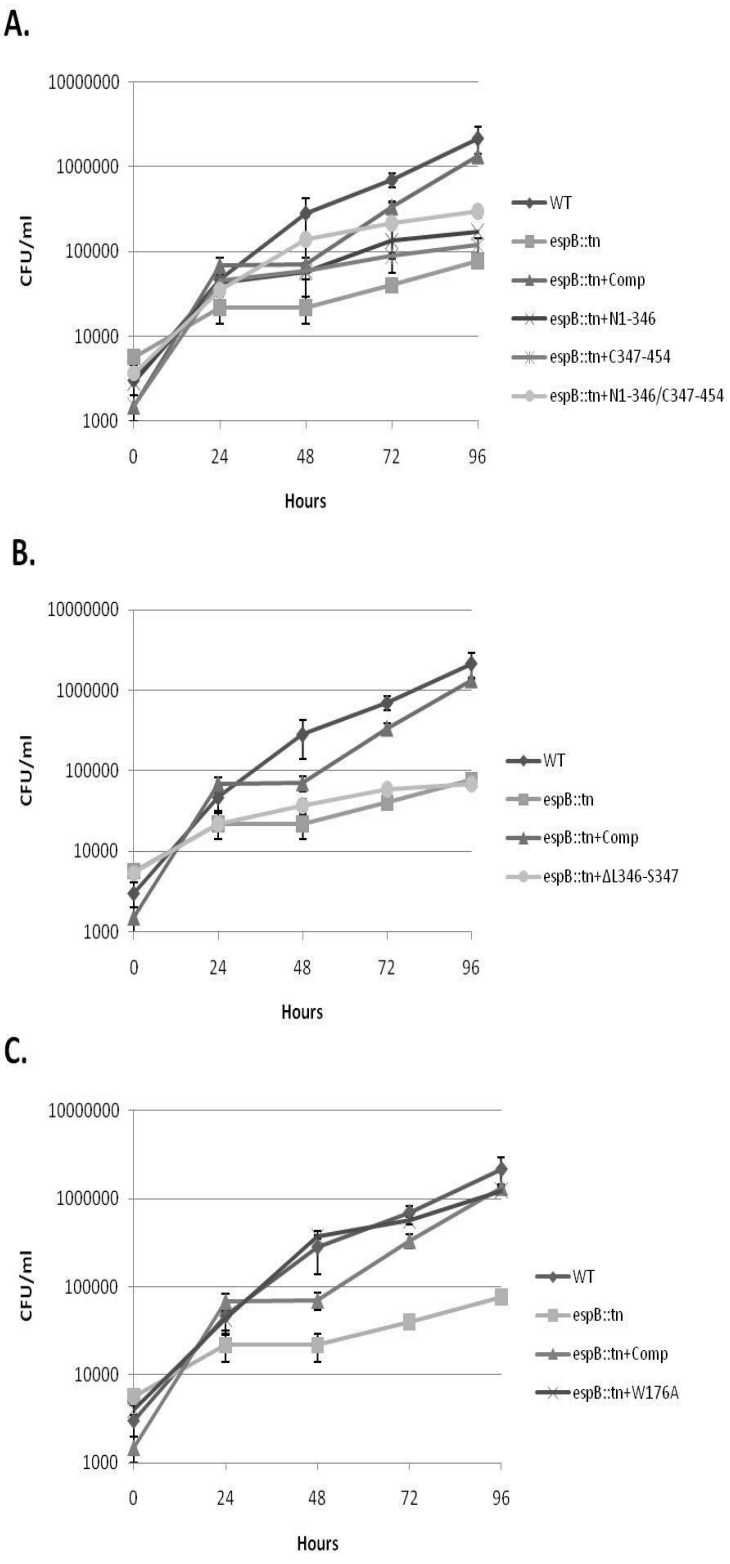

Figure 20. *Mm* EspB mutants and their affect on intracellular growth inside RAW cells. RAW cells infected at an MOI of 2. The CFUs were evaluated at 0, 24, 48, 72, and 96 hours post infection. Error bars indicate standard deviation of data collected in triplicate. WT is wild type. The mutants are episomally expressed in *esp*B::tn. Panel A) N1-346 is the EspB N-terminal fragment, C347-454 is the EspB C-terminal fragment, N1-346/C347-454 is the EspB N- and C-terminus expressed as separate fragments. Panel B) ΔL346-S347 is EspB with a deletion of the cleavage site, L346-S347. Panel C) W176A is EspB with a mutation of the WXG motif.

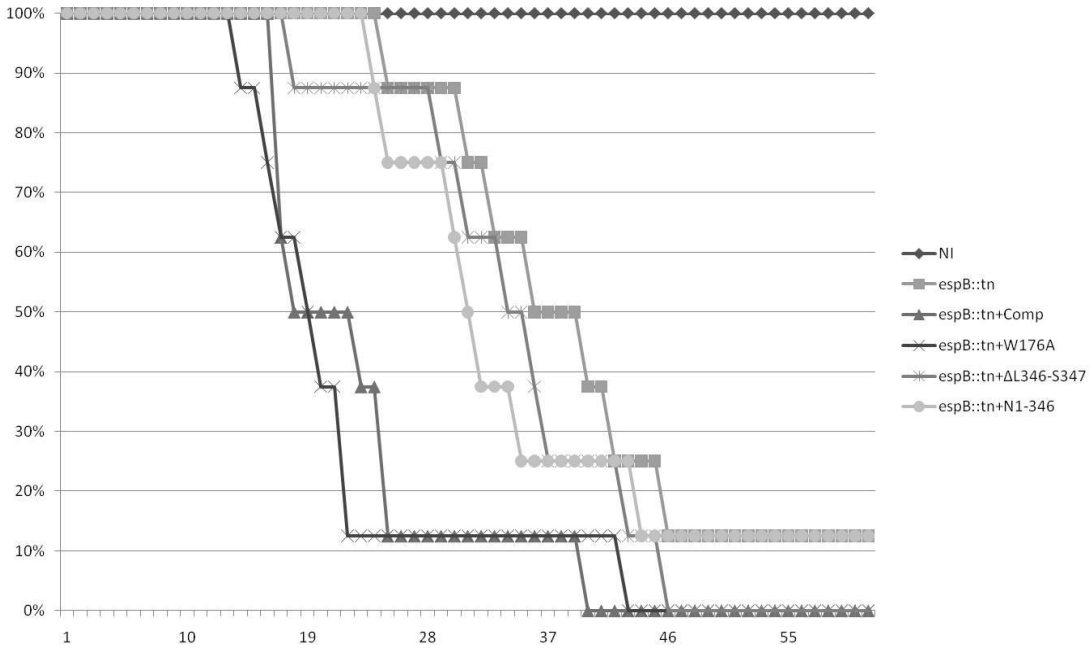

Figure 21. *Mm* EspB mutants and their affect on virulence in zebrafish. Survival of 8 zebrafish following intraperitoneal infection of 5 µl of PBS containing $2x10^4$ *Mm* bacteria per strain over the course of 10-weeks. NI, non-infected, is injection of 5 µl PBS used as a negative control. The mutants are episomally expressed in *esp*B::tn; Comp is full lenghth EspB, W176A is EspB with a mutation of the WXG motif, ΔLS is EspB with a deletion of the L346-S347 cleavage site, N is the EspB N-terminal 1-346 amino acids fragment.

bacterial burden in the fish; however, it is clear that the mutant is less virulent than the complement strain and the fish survive longer. These results indicate that the full-length protein is required for full virulence; however the individual N- and C-terminal fragments may each play a partial role.

In order to confirm that the bacterial intracellular growth differences observed in RAW cells were not due to differences in growth rates among the various strains, I also determined CFUs of bacteria growing in culture. All of the cultures were started at the same concentration of bacteria that was used to infect RAW cells and I took the culture and infection CFUs at the same time points. I found that all of the strains used in this study grew at similar growth rates [Fig. 22]. The growth rate for the *esp*B::tn+pEspB:C347-454 mutant was a little slower in the beginning, but by 48 and 96 hours it was growing at the same CFU/ml as all the others. Therefore, intracellular growth defects can be attributed to the various mutations.

I then created a mutant that expresses both the N- and C-terminal fragments separately on different regions of the same plasmid. This plasmid was episomally expressed in the *esp*B::tn mutant (*esp*B::tn+pEspB:N1-346/C347-454). In this mutant, both fragments are present in the cell, but they are not one single protein. The C-terminus is available for interaction with ESAT-6 and the N-terminus is free to secrete independent of ESX-1. I could detect expression and secretion of the N-terminal fragment via Western blot, and also secretion of ESAT-6, indicating that the C-terminus is expressed [Fig. 19, lane 10]. This mutant could result in one of two phenotypes: 1) expressing both fragments separately could fully or partially complement virulence, indicating that the N-terminus does have a direct role in

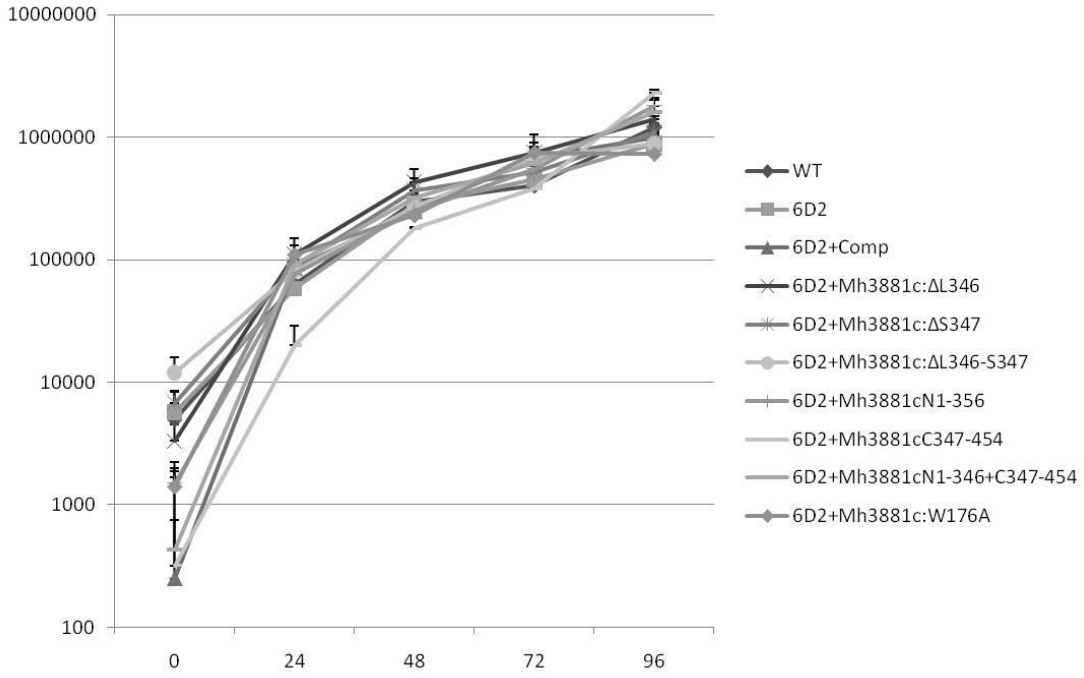

Figure 22. *In vitro* growth rates of EspB mutants. Bacterial cultures growing in DMEM RAW cell infection medium for 0, 24, 48, 72, and 96 hours. At each time point the cultures were serially diluted and plated onto 7H10 plates for the enumeration of CFU. Cultures are *Mm* WT, *esp*B::tn, and EspB mutants episomally expressed in *esp*B::tn. Comp is full length EspB; ΔL346, ΔS347, and ΔL346-S347 are EspB cleavage site mutants; N1-346 is expression of the EspB N-terminal fragment, C347-454 is expression of the EspB C-terminal fragment, N1-346/C347-454 is expression of the EspB N- and C-terminus as separate fragments; W176A is EspB with a mutation of the WXG motif. Error bars indicate the standard deviation of cultures plated in triplicate.

virulence that is independent of its secretion through the ESX-1 system; however C-terminal association of ESAT-6 is necessary for it to accomplish this function; or 2) the full-length protein is required for virulence and expressing both separately inside *Mm* would not complement virulence. In the second scenario, no conclusions could be made as to a direct role of the N-terminus in virulence. Its role could be direct or it could just be in regulating ESX-1 secretion.

My results showed that expressing the N- and C-terminus together but separate does not fully complement the *esp*B::tn mutant in intracellular growth in macrophages [Fig. 20A]. This result indicates that the full length protein in its native form is required for the full spectrum of virulence. The mutant did grow slightly better than when the N- or C-terminus is expressed singularly, however this difference is not very significant. The N-terminus does appear to play a direct role in virulence, but full virulence requires expression of the full length protein and secretion of ESAT-6. It is possible that EspB translocates through the ESAT-6 formed pore and a tight association is required for targeting.

3.2.2 The role of the EspB cleavage site in secretion and virulence

Because the full length protein is required for virulence complementation of *esp*B::tn, I wanted to see if cleavage of EspB is required for virulence. Our lab discovered that cleavage of EspB occurs between Leu346 and Ser347 [Fig. 18]. Three mutants of EspB at the cleavage site were created and expressed in *esp*B::tn; in the first mutant both the L346 and S347 amino acids (*esp*B::tn+pEspB:ΔL346-S347) were removed, for the second only L346 (*esp*B::tn+pEspB:ΔL346) was removed, and for the third

only S347 (*esp*B::tn+pEspB:ΔS347) was removed. I found that EspB is expressed and secreted in all three mutants [Fig. 19, lanes 4-6]. Both the full length protein and the N-terminal fragment are detected in the culture filtrate, which would indicate incomplete cleavage. The ratio of cleaved to uncleaved, however is the same as in the *esp*B::tn+EspB complement strain [Fig. 19, lane 3]; therefore, this incomplete cleavage is more likely a result of protein overexpression. Ohol et al, 2010 ran EspB culture filtrate on a high resolution gel and detected three separate N-terminal fragments; two of which are a result of cleavage by MycP1, the ESX-1 serine protease that is required for complex assembly. They used mass spectroscopy and identified one of the cleavage sites at position Ala392, while our result identified a cleavage site at position L346. That leaves still another site for cleavage by some other, unknown protease; possibly one of the MycP1 homologs from another ESX system. Also, we have not yet confirmed that our mutants are defective for cleavage at the L346-S347 site; therefore it is possible that cleavage is still occurring there.

The level of ESAT-6 in the culture filtrate is increased in all three of these cleavage mutants compared to WT and the complement strain; which is what Ohol et al., 2010 found in *Mtb* when they created a protease defective MycP1 mutant that is no longer able to cleave EspB at two of its cleavage sites. They detected an increase in the culture filtrate levels of several ESX-1 substrates, and suggest that MycP1 cleavage of EspB might be important for moderating the degree of ESX-1 secretion. It is also possible that the inhibition of EspB cleavage prevents the dissociation of ESAT-6 with EspB and consequently increases stability of ESAT-6 in the culture supernatant. Our lab has previously demonstrated that several ESX-1 secreted proteins, including

ESAT-6 are not stable in the culture filtrate (data not shown). Nonetheless, my results show that the residues of the L346 cleavage site alone are important for the modulation of ESAT-6 protein secretion in the culture filtrate.

The ΔL346-S347 mutant shows a defect in intracellular growth that is the same as *esp*B::tn [Fig. 20B]. This mutant is also defective at virulence in zebrafish [Fig. 21]. Therefore, the L346-S347 residues are absolutely required for the virulence function of EspB, presumably due to their role in the cleavage of EspB by MycP1. This result is not surprising because the cleaved N-terminal fragment could be important for virulence and the prevention of cleavage may block its virulence function. It is also possible that cleavage releases ESAT-6 to exert its virulence function and preventing cleavage results in reduced virulence. My data are consistent with the finding by Ohol et al., 2010 that cleavage of EspB increases the level of ESX-1 substrates in the secretion milieu. Therefore, it is also possible that an excess amount of ESAT-6 secretion increases the antigenic targets that are sensed by the host immune system and the bacteria are unable to remain safely undetected.

3.2.3 The role of the EspB WXG motif in secretion and virulence

ESAT-6 and CFP-10 are founding members of a superfamily of proteins characterized by their small size of ~100 amino acids and the presence of a highly conserved three residue, tryptophan-variable-glycine (WXG), motif. The *Mtb* genome alone has 22 of these WXG100 proteins, many of which are associated with the five duplicated ESX secretion systems. In an attempt to determine the function of ESAT-6, Palen in 2002 searched for homologes in other bacterial species, using very

senisitive parameters. He found several dozen homologues within the Actinobacteria, but also related proteins in low G+C Gram-positive species. Though the homology was deemed significant, the actual sequence similarity was relatively low, save the conservation of a WXG motif and a small size of around 100 amino acids. These proteins also showed a tendency to cluster with other ESAT-6-like proteins in the genome, have extensive coiled-coil domains, and none had a signal peptide. Pallen noticed that some, such as the *Bacillus subtilis* YukE protein, were located near FtsK/SpoIIIE ATPases in the genome. Another group discovered that WXG100 proteins were secreted in *Staphylococcus aureus* (Burts, et al., 2005).

The ESX-1 WXG100 proteins, ESAT-6 and CFP-10, have a WXG motif in the hairpin of their helix-turn-helix. The indole functional group of the tryptophan residue is free to associate with other proteins, since it is not involved in the tight 1:1 association of these two proteins [Fig. 5; section 1.6.2]. The purpose of this motif being so highly conserved is unknown; it could be structural, or important for secretion, or it could play a role in virulence. Since this superfamily of proteins is present in both pathogenic and non-pathogenic species, the WXG motif cannot be solely attributed to playing a role in virulence; though one group did show that mutating the tryptophan in the WXG motif of ESAT-6 did not disrupt secretion, but did cause attenuation (Brodin et al., 2005).

Our lab noticed that the WXG motif is present in other, larger ESX-1 secreted substrates besides ESAT-6 and CFP-10. They cannot be included in the WXG100 superfamily because they are well over 100 amino acids in length. In larger proteins the presence of a three residue motif may not seem quite as significant, but the fact

that it is so highly conserved increases the significance. The ESX-1 secreted protein, Mh3864 has a WXG motif at position W56-G58, EspA has a WXG motif at position W55-G57, and EspB has a WXG motif at position W176-G178 [Fig. 18]. The WXG motif is highly conserved across mycobacterial species for all three of these proteins. As shown in Figure 23, there are several mycobacterial genomes that have an EspB homolog and even the most distantly related of them have a highly conserved WXG motif. The motif is present in the middle of the N-terminus at relatively the same position. What's more, *Mtb* and *Mm* both have several EspB paralogs in their genome, presumably associated with the five different ESX secretion systems. *Mm* has six EspB paralogs and all show conservation of the WXG motif at relatively the same position. Because this motif is so highly conserved in EspB it seems likely that it is involved in secretion or protein function. I wondered if mutating the WXG motif in EspB would result in an attenuation phenotype similar to what was observed with ESAT-6.

I created a W176A mutant and expressed it episomally in *esp*B::tn (*esp*B::tn+EspB:W176A). The secretion of EspB and ESAT-6 is much reduced in this mutant [Fig. 19, lane 7], indicating that the EspB WXG motif does play a role in ESX-1 secretion. The intracellular stability of ESAT-6 is similar to the complement strain, so it seems likely that the mutation affects secretion specifically, possibly by associating with other proteins that are important for targeting EspB to the secretion channel. This mutant, however is not defective at growth inside RAW cells [Fig. 20], nor is it attenuated in zebrafish [Fig. 21]. Therefore, it appears that the EspB WXG motif does not contribute to virulence, presumably because there is still some

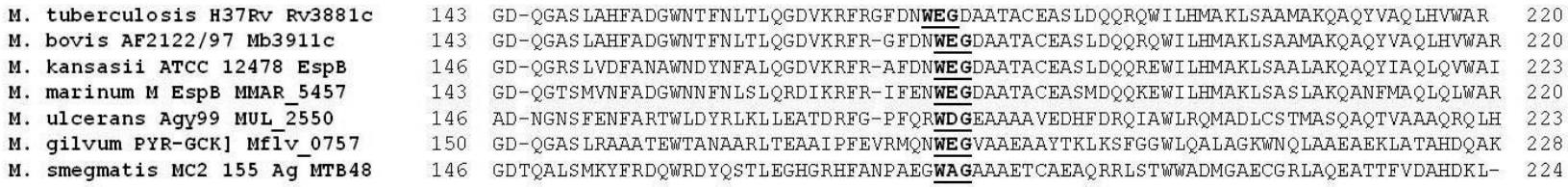

Figure 23. The WXG motif is highly conserved among EspB homologs across mycobacterial species. Partial sequence alignment of the EspB homologs in several mycobacterial species. Amino acid residues in bold indicate the conserved WXG motif. WXG is conserved even though protein identities are relatively low in species that are more distantly related to *Mtb*. The percent identities of EspB homologs compared with *Mtb* H37Rv strain Rv3881c protein are as follows: *M. bovis* AF2122/97 strain, Mb3911c protein: 98% identical. *M. kansasii* ATCC 12478 strain, EspB protein: 71% identical; *Mm* M strain, EspB MMAR_5457 protein: 68% identical; *M. ulcerans* Agy99 strain, MUL_2550 protein, 39% identical, *M. gilvum* PYR-GCK1 strain, mflv_0757 protein: 28% identical; *M. smegmatis* MC2 155 strain, Ag MTB48 protein: 27% identical.

secretion of ESAT-6 and EspB. This reduced secretion may be sufficient for full virulence function. It appears that moderate secretion of ESX-1 substrates may be sufficient for maintaining the pathogen-protection balance inside host cells. Some substrate secretion is required, but too much can be harmful.

3.2.4 EspB translocation tags

As discussed in section 1.5, it seems likely that mycobacterial proteins are translocated from the phagosome into the host cell in order to modulate their environment and ensure survival. Previous studies have indicated that ESX-1 proteins are translocated; for instance, priming of $CD8^+$ T-cells requires CFP-10 secretion (Woodsworth et al., 2008), and fractionation studies detected PknG in the host cell cytosolic fraction (Walburger et al., 2004). No study however, has yet to show direct translocation of any ESX-1 substrates.

It is my hypothesis that EspB is translocated into the host cell cytosol. This is based on the fact that EspB is absolutely required for virulence, it plays a role in the inhibition of phagolysosome fusion, and the C-terminal fragment is required for secretion of ESAT-6, while the N-terminal fragment must be present for full virulence. Translocation of secreted virulence factors has been well established in Gram-negative, type III and type IV secretion systems. Typically, a tag is used that is distinguishable once it has entered the host cell cytoplasm. In most cases, the bacteria secreting these proteins are attached to the surface of the cell, rather than inhabiting an intracellular phagosome like mycobacteria. Therefore, the design of a useful translocation tag in mycobacteria offers some unique challenges. Typical assays

require lysis of the host cell in order to analyze the tag. In the case of extracellular bacteria, all non-translocated proteins can be washed away before lysis, ensuring that tags are not activated during lysis to give a false positive result. Proteins secreted by mycobacteria into the phagosome cannot be washed away before lysis, so unless the reaction is enzymatic and can be stopped by putting the cells on ice, it may be difficult to distinguish translocated from phagosomal proteins.

Another challenge using EspB is finding the best place to insert a tag since it is cleaved upon secretion. If it is placed at the C-terminus then it will be cleaved from the N-terminus and N-terminal translocation cannot be detected. Likewise, the tag may interfere with secretion since the C-terminus is absolutely required for EspB full length protein secretion as well as secretion of ESAT-6. If the tag is placed at the N-terminus then it may also interfere with secretion since the N-terminus is known to bind to the Rv3879c protein, presumably as a chaperone to bring it to the ESX-1 channel (McLaughlin et al., 2007). Alternatively, the tag can be inserted in the middle of the protein at some position upstream of the cleavage site. The risk is that this could change important conformations that aid in secretion or translocation.

A third challenge is finding a tag that can be secreted through the ESX-1 channel. It is not yet fully known how proteins are targeted for secretion. C-terminal sequences play a role in secretion; however there is no predictable sequence that is required. It was shown that the last seven amino acids of CFP-10 can secrete the yeast ubiquitin protein (DiGiuseppe Champion et al., 2006); therefore it does appear that a secretion signal is sufficient for secretion of even unrelated proteins. That result indicates a

high likelihood that a translocation tag could be secreted; however it's not known what size of tag can be tolerated. If it is too big it may not fit through the channel.

The EspB N-terminal fragment can be secreted even without expression of the C-terminus. The full length protein is cleaved by MycP1 in the periplasmic space, so getting through the cytosolic membrane does not necessarily require the cleavage site. Taking advantage of this seemingly plausible position in which to tag the protein, I added three different tags to the C-terminal end of the N-terminal 1-346 amino acid fragment of EspB. These three tags have been used in both type III and type IV secretion systems for identification of translocated proteins and my initial goal was to see if they could be secreted in the ESX-1 system.

The first tag I tried was the adenylate cyclase (Cya) tag. It has been a very successful reporter system for detecting the translocation of many type III and type IV secreted proteins (Sory and Cornelis, 1994; Nagai et al., 2005). Adenylate cyclase is activated by calmodulin, which is only produced in eukaryotic cells. Once activated, adenylate cyclase converts ATP to cAMP and this increase in cAMP can be measured. An increase in cAMP levels would indicate that the tagged-protein has entered the cytosol. This assay requires lysis of infected macrophages in order to extract cAMP. Because it is an enzymatic reaction, it may be possible to limit further activity from proteins secreted into the phagosome by putting the cells on ice.

One potential problem with using this tag is that it is big; 398 residues. It is possible that the tag may interfere with secretion. I tagged the C-terminal end of the EspB N-terminal fragment with Cya (EspB-N1-346-Cya), expressed it in the *esp*B::tn mutant,

and checked to see if it was secreted. Unfortunately, it was not [Fig. 24A]. This could be due to the size of the tag or perhaps its location on the protein.

The second tag I used was the glycogen synthase kinase (GSK) tag. Previously, GSK was used to detect the translocation of both type III and type IV secreted proteins (Garcia et al., 2006). GSK has a serine residue that can only be phosphorylated by protein kinases in the eukaryotic cell cytosol. There are commercial antibodies available that can detect GSK and phosphospecific GSK, so infected cells are lysed and then analyzed by SDS-PAGE. This allows for the quantitative comparison between the detection of total tagged-protein versus translocated tagged-protein that has been phosphorylated in the host cytosol. This tag has the benefit of being very small, only 12-residues; so the likelihood of the tag interfering in the secretion process is greatly reduced.

I tagged the C-terminal end of the EspB N-terminal fragment with GSK (EspB-N1-346-GSK), expressed it in the *esp*B::tn mutant, and checked to see if it could be secreted. I found that it was abundantly secreted [Fig. 24A], however when I probed the cell lysate and culture filtrate of this strain with the phosphospecific-anti-GSK antibody, it was detected in both fractions [Fig. 24B, lanes 1-2]. I also tagged the Antigen 85b protein which is secreted via the Sec system and it too showed phosphorylated GSK in the bacterial cell [Fig. 24B, lane 3]. This indicates that *Mm* has a protein kinase that can phosphorylate GSK. This is actually quite interesting since phosphorylation of eukaryotic GSK by AKT is involved in the inhibition of apoptosis. Perhaps *Mm* has a serine protease that can target host GSK in order to promote its survival inside the phagosome? This is an interesting question;

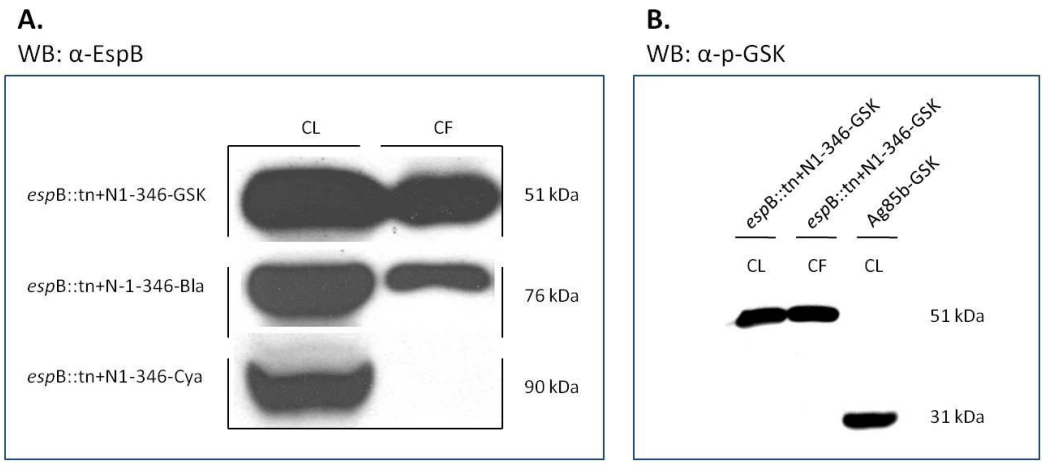

Figure 24. The potential of using various tags to determine EspB translocation. Panel A) Western blot detecting tagged-EspB expression and secretion in *Mm* cell lysate (CL) and culture filtrate (CF) grown in 7H9 broth. The EspB N-terminal 1-346 fragment was tagged with GSK, Bla, or Cya at the C-terminal end and expressed episomally in *esp*B::tn. Panel B) Western blot detecting phosphorylated GSK (p-GSK) in *Mm* CL and CF grown in 7H9 broth; *esp*B::tn with episomal expression of the EspB N-terminal 1-346 fragment with a C-terminal GSK tag, or WT *Mm* episomally expressing Ag85b tagged at the C-terminus with GSK.

unfortunately though, it means that GSK cannot be used to detect protein translocation in *Mm*.

Thirdly, I used the β-lactamase (Bla) tag. This tag has also been used to successfully detect type III and type IV translocated proteins in Gram-negative bacteria (Charpentier and Oswald, 2004; de Jong et al., 2008). With this tag, cells are viewed directly under fluorescence microscopy, which means they do not have to be lysed and so the intracellular location of *Mm* is not such an issue. Non-fluorescent CCF2/AM, when introduced to eukaryotic cells will diffuse passively across the plasma membrane. Once inside, the cellular esterases quickly convert it to the β-lactamase substrate, CCF2. This form is charged, so it is trapped inside the cell and emits green fluorescence. When Bla-tagged proteins enter the cytoplasm, the β-lactam ring of CCF2 is cleaved, resulting in a shift to the blue fluorescence spectrum. Using fluorescence microscopy, translocated proteins can be visualized directly and in living cells. They should be the only ones fluorescing blue. This assay is very sensitive; fewer than 100 Bla molecules can be detected within a cell, and fluorescence can be quantified from cells infected in a 96-well plate, using a microplate reader.

This tag is also big, though smaller than Cya. It is 263 residues. It is possible that the tag may interfere with secretion. It is, however in an unfolded state in the cytosol, so more likely to go through the channel than a globular protein tag like GFP. I fused the C-terminal end of the EspB N-terminal 1-346 amino acids fragment with Bla (EspB-N1-346-Bla), expressed it in the *esp*B::tn mutant, and checked to see if it can be secreted. Fortunately, I was able to detect the fused protein in the culture filtrate

[Fig. 24], indicating that this tag does not block secretion. The next step would be to fuse this tag to the full length protein, though the challenge there is finding a location that won't interfere with secretion.

Another potential problem of using this tag is that mycobacteria have an endogenous β-lactamase protein that makes them resistant to β-lactam antibiotics. This protein is cell wall associated so it likely never enters the cytosol; however, if it did contribute to background fluorescence then an Mm β-lactamase knock out strain would have to be made first.

Future work to determine EspB translocation could potentially prove to be very useful for vaccine development. If EspB is translocated and can transport a tag to the cytosol, it could also perhaps transport antigens that may boost protection.

3.2.5 Chemical inhibition of ESX-1 activity

EspB is an important virulence protein secreted by ESX-1, and cleavage of EspB is required for virulence and for the negative regulation of ESX-1 secretion (Ohol et al., 2010). It is now known that the protease responsible for cleaving EspB is MycP1. This protein is anchored to the cytosolic membrane and its protease domain is thought to be present in the periplasmic space between the membrane and the cell wall. This location may be accessible to drugs that can permeate the cell wall. Inhibition of MycP1 secretion would likely cause attenuation since it is required for assembly of the ESX-1 machinery. Inhibition of MycP1 protease activitiy would also likely cause attenuation due to the effects already observed with non-cleaved EspB (Ohol et al., 2010).

In our lab, we regularly use a cocktail of protease inhibitors supplied by Sigma-Aldrich®, in order to limit protein degradation of *Mm* short-term cell lysate and culture filtrate samples. When I added this cocktail to growing WT *Mm* in 7H9 liquid broth culture, I noticed that secretion of EspB, ESAT-6, and CFP-10 is ablated [Fig 25A, lane 2]. My hypothesis is that 4-(2-Aminoethyl)-benzenesulfonyl-fluoride-hydrochloride (AEBSF), the serine protease inhibitor present in the cocktail, is either inhibiting the secretion of MycP1 or the acitivity of other serine proteases that are important for the secretion of ESX-1 substrates. I tested various concentrations of AEBSF on liquid broth cultures of WT *Mm* and found that it does limit EspB, ESAT-6, and CFP-10 secretion in a dose dependent manner. At 0.9 mM, no EspB or ESAT-6 is detected, and CFP-10 secretion is much reduced [Fig. 25A, lane 5].

I then wanted to see if AEBSF could limit intracellular growth of *Mm* in RAW cell macrophages, which would indicate that this inhibitor might be useful as a drug against mycobacterial infection. I first tested to make sure that adding AEBSF to uninfected RAW cells would not harm the cells. According to Sigma Aldrich ®, the maximum AEBSF concentration that can be tolerated by RAW cells is 0.25 mM; so I incubated the cells with 0.25 mM AEBSF for 6 and 24 hours, then used a resazurin cell viability assay to check for differences in growth between treated and untreated cells. After 6 hours, the cell viability was reduced by about 25%, however, by 24 hours, the cells recovered to non-treated levels [Fig. 25B].

I then pretreated *Mm* with AEBSF before adding it to RAW cells to see if intracellular growth is reduced. Cells were washed and processed in PBS to remove

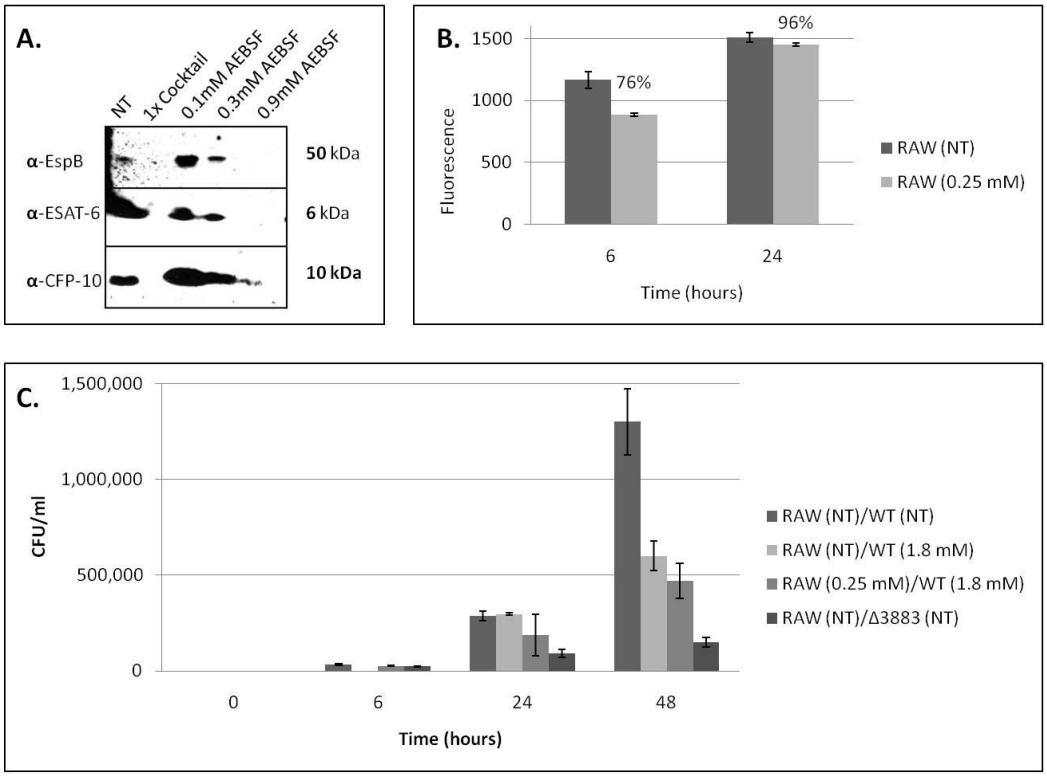

Figure 25. AEBSF can inhibit ESX-1 secretion and growth inside RAW cells. Panel A) *Mm* WT Sautons broth cultures were spun down, the pellets were washed, then resuspended in new broth to remove any previously secreted proteins. The cultures were then treated with nothing (NI), 1x concentration of the Sigma Aldrich ® protease inhibitor cocktail, 0.1 mM AEBSF, 0.3 mM AEBSF, or 0.9 mM AEBSF for two hours. The culture filtrates were then collected and analyzed via Western blot for the secretion of EspB, ESAT-6, and CFP-10. Panel B) Untreated RAW cells and cells treated with 0.25 mM AEBSF for 6 and 24 hours were analyzed for cell viability by adding alamar blue, which is an indicator dye of metabolic activity. Error bars represent the standard deviation of six wells per sample and time point. Panel C) RAW cells were left untreated (NT) or treated with 0.25 mM AEBSF and then incubated for 0, 6, 24, or 48 hours with WT *Mm* that was either untreated (NT) or pretreated for one hour with 1.8 mM AEBSF, or with untreated Δ*mh*3883 at an MOI of 2. Infections were serially diluted and plated for enumeration of CFUs. Error bars represent the standard deviation of each infection in triplicate.

all of the culture filtrate and yield a single cell suspension. I then incubated the bacteria with 1.8 mM AEBSF for one hour so that subsequent secretion would be exposed to AEBSF inhibition. Then I infected RAW cell monolayers, with pretreated bacteria both with and without 0.25 mM AEBSF added to the media. For each timepoint I lysed the cells and assayed for bacterial CFU [Fig. 25C]. I found that at 48 hours post-infection, pretreating the bacteria with AEBSF reduced the intracellular growth of *Mm* by about 50%. Adding 0.25 mM AEBSF to the infection media reduced it even further; however the difference is not very significant. The Δ*mh*3883 (*myc*P1) mutant showed an even more significant reduction of intracellular growth, which is expected because this mutant was previously shown to be completely defective in ESX-1 secretion (Ohol et al., 2010).

This very preliminary data is interesting; however I was not able to get consistent results in subsequent repeat attempts. Also, pretreating the bacteria with AEBSF may reduce intracellular growth, however adding AEBSF to the host cells themselves doesn't offer too much more protection, at least at the 0.25 mM concentration. When I added only 0.25 mM AEBSF to the cells without pretreating the bacteria there was no change in intracellular growth (data not shown). Unfortunately, higher concentrations are likely to be toxic to the RAW cells. What this very preliminary data does show however, is that the general targeting of serine proteases can affect ESX-1 secretion; therefore a drug that specifically targets MycP1 secretion may prove to be useful at treating mycobacterial infection.

3.3 Materials and Methods

3.3.1 Generation of EspB mutants

The transposon mutant, *esp*B::tn was previously complemented using the PLYG206-zeo episomal plasmid (Xu et al., 2007). To make the domain mutants, inverse PCR was done on the complemented gene. The following primer pairs were used: ΔL346-S347: 5' – GTTGGACGCCGCCTCGCGACCC – 3' and 5' – AAGGGCCTCGGCGTCAAACCGATGTC – 3'; ΔL346: 5' – GTTGGACGCCGCCTCGCGACCC – 3' and 5' – TCCAAGGGCCTCGGCGTCAAACCGATGTC – 3'; ΔS347: 5' – CAGGTTGGACGCCGCCTCGCGACCC – 3' and 5' – AAGGGCCTCGGCGTCAAACCGATGTC – 3'; W176A: 5' – CGCGTTCTCGAAGATCCGGAACCGCTTGATG – 3' and 5' – GAGGGTGACGCCGCTACCGCCTG – 3'; N1-346: 5' – CAGGTTGGACGCCGCCTCGCG – 3' and 5' – TAGTCCGTCAGGACAGTCGTCAGGACAGTAAGG – 3': C347-454: 5' – CATGTCGGATCGTCCTCCTTAGTGCTCCATG – 3' and 5' – TCCAAGGGCCTCGGCGTCAAACCG – 3'. PCR products were ligated, transformed into competent *E. coli* DH5α cells, and plated onto LB plates with Zeocin selection. Plasmids were purified from positive clones and confirmed by sequencing. Correct plasmids were transformed into the *M. marinum esp*B::tn mutant by electroporation. Mutants were chosen from 7H10 plates with Zeocin selection. For expression of N1-346 and C347-454 separately on the same plasmid, the N1-346

plasmid was cut at the multiple cloning site with EcoRI and BamHI. The primer pairs (5' – GAATTCCTACTTGTTGTCCTGACGGCGGCGG – 3' and 5' – GGATCCCTCCAAGGGCCTCGGCGTCAAACCG – 3') were used to amplify C347-454 and add the restriction sites. The cut plasmid and insert were ligated and then transformed into competent *E. coli* DH5α cells as with the initial mutants.

3.3.2 Preparation of short term culture filtrate and cell lysate.

Mycobacterium marinum M strain WT and mutants were cultured as previously described (Gao et al., 2003b; 2006). Short term culture filtrate and cell lysate were prepared as previously described (Xu et al., 2007).

3.3.3 Western blotting.

Proteins were separated on a 4%-20% gradient SDS-PAGE gel, and then transferred to a 0.45μm PVDF membrane. Membrane was blocked with 1% Bovine Serum Albumin (BSA) for one hour, then primary antibody was added either at room temperature for two hours or overnight at 4°C. Subsequently, the membrane was washed and then incubated with HRP-conjugated secondary antibody (BioRad) for one hour. SuperSignal® West Pico Chemiluminescent substrate (Thermo Scientific) was added to detect HRP and then developed onto X-Ray film (Thermo Scientific). Primary antibodies were used at the following dilutions: anti-ESAT-6 (Mab HYB 076-08 – Santa Cruz Biotechnology) at 1:300; anti-CFP-10 (Colorado State University) at 1:5000; anti-Rv3881c (Michael Lodes, Corixa Corporation, Seattle) at 1: 6000; anti-Fap (Eric Brown, University of California, San Francisco) at 1: 2500;

anti-GroEL (Colorado State University) at 1:100; anti-p-GSK3β (Cell Signaling) at 1:1000.

3.3.4 RAW cell maintenance and infection

RAW cells were cultured as previously described (Gao et al., 2003b). Infection of RAW cells and enumeration of intracellular CFU were as previously described (Gao et al., 2003b). Briefly, *Mycobacterium marinum* strains were added as a single cell suspension to RAW cells at a multiplicity of infection of 2 and incubated at 32°C with 5% CO_2 for two hours. To ensure the removal of extracellular bacteria, cells were washed three times with PBS then media was added for one hour containing 100 µg/ml of streptomycin. Cells were washed again and then incubation continued for each time point in DMEM growth media containing 4 µg/ml of streptomycin. At 0, 24, 48, 72, and 96 hours post infection, the cells were lysed using a 0.1% Triton X-100 solution in sterile water. Cell lysates were diluted and spotted onto 7H10 plates for enumeration of bacterial CFU.

3.3.5 Zebrafish infection

Zebrafish were obtained from Dr. Shaojun Du's lab at the University of Maryland, Biotechnology Institute in Baltimore, MD. They were maintained as previously described (Lee et al., 2003). Fish were anesthetized in 0.04% tricaine (ethyl 3-aminobenzoate methanesulphonate) (Sigma Aldrich) for 3-5 minutes then injected into the peritoneum with 5 µl of PBS containing $2x10^4$ bacteria. Eight fish were infected per strain and the experiment was maintained for 10 weeks. The same volume of PBS without bacteria was injected into control fish.

3.3.6 *In vitro* growth rates of EspB mutants.

Mm WT, *esp*B::tn, and EspB mutants were diluted in DMEM growth medium to an initial concentration of $1x10^6$ cells/ml and allowed to grow at 32°C with 5% CO_2. For each time point, the cultures were serially diluted in 1X PBS in triplicate and plated onto 7H10 agar plates for enumeration of CFU.

3.3.7 Generation of translocation tagged EspB proteins.

To create the EspB-N1-346-Cya tagged construct, amino acids 2-399 of the *B. pertussis*, *cya* gene was amplified from a plasmid containing the gene. The SpeI restriction site was added to the reverse primer, R-Cya: 5'-ACACCCGACGTCAACCAGCAATCGCATCAGGCTGGTTACGC-3' and F-Cya: 5'-CAGCACTAGTCTAGCGTTCCACTGCGCCCAGCGACG-3'. The plasmid containing the N-terminal 1-346 amino acid fragment of EspB was also amplified with a complementary Cya sequence added to the primer: F-EspB-N: 5'-CATGACTAGTCTGGAGGAAACCCCCAAGGTGGCG-3' and R-EspB-N: 5'-GCCTGATGCGATTGCTGGTTGACGTCGGGTGTGCCGCCCG-3'. PCR products were run on an agarose gel and gel purified using the Wizard SV Gel and PCR Clean-UP system from Promega. Then the two fragments were fused together via PCR using the reverse Cya primer and the forward EspB-N primer. Fusion products were run on an agarose gel and gel purified. The product and the plasmid were cut with the NotI and SpeI restriction enzymes and then the vector and insert were purified from agarose gel and ligated together.

To create the EspB-N1-346-GSK tagged construct, the GSK sequence and an SpeI restriction site was added to the forward primer and then the EspB-N1-346 fragment was amplified from the plasmid: EspBNGSK-F: 5'-GCTGACTAGTCTAACTTTCAGCGAAACTAGTAGTGCGAGGGCGACCACTCATGTTGACGTCGGGTGTGCCGCCCG-5'. R-EspB-N was used again as the reverse primer. The product was purified from an agarose gel and then the vector and insert were digested with SpeI and NotI. The pieces were gel purified and ligated together.

To create the EspB-N1-346-Bla tagged construct the *bla* gene, minus the SecA secretion signal, was amplified from the pUC19-bla plasmid. The SpeI restriction site was added to the reverse primer, EspBNbla-R: 5'-ACACCCGACGTCAACCACCCAGAAACGCTGGTGAAAGTAAAAGATGC-3' and EspBNbla-F: 5'-CAGCACTAGTCTACCAATGCTTAATCAGTGAGGCACCTATCTCAGC-3'. The plasmid containing the N-terminal 1-346 amino acid fragment of EspB was also amplified with a complementary Bla sequence added to the forward primer: EspBNBla-F: 5'-CAGCGTTTCTGGGTGGTTGACGTCGGGTGTGCCGCCCG-3' and R-EspB-N was used again as the reverse primer. PCR products were run on an agarose gel and gel purified using the Wizard SV Gel and PCR Clean-UP system from Promega. Then the two fragments were fused together via PCR using the reverse Bla primer and the forward EspB-N primer. Fusion products were run on an agarose gel and gel purified. The product and the plasmid was cut with the NotI and

SpeI restriction enzymes and then the vector and insert were purified from agarose gel and ligated together.

Ligation products were transformed into competent *E. coli* DH5α cells as with the EspB mutants described in section 3.3.1

3.3.8 AEBSF inhibition of *Mm* WT ESX-1 secretion.

Mm WT was grown in 30 ml Sautons liquid broth culture until confluent. The cultures were spun down at 3800 rpm for 10 minutes, and the pellets were washed and then resuspended in fresh Sautons broth to remove any previously secreted proteins. The cultures were then treated with 1x concentration of the Sigma Aldrich ® protease inhibitor cocktail, 0.1 mM AEBSF, 0.3 mM AEBSF, 0.9 mM AEBSF or left untreated for two hours. The cultures were spun again and the culture filtrate was prepared as previously described (Xu et al., 2007).

3.3.9 AEBSF affect on RAW cell viability.

A monolayer of RAW cells were seeded in a 96-well plate and incubated with 0.25 mM AEBSF or without for six or 24 hours at 37°C with 5% CO_2. Then 10% alamar blue (Serotec) was added to each well and incubated for an additional four hours. Fluorescence was read at an excitation of 560 nm and emission of 590 nm to determine metabolic activity.

3.3.10 Affect of AEBSF on *Mm* intracellular growth in RAW cells.

RAW cells were seeded into 96 well plates at a concentration of $2.5x10^5$ cells/ml and incubated at 37°C with 5% CO_2 for two days. Bacterial cultures were processed in 1x

PBS to get a single cell suspension then either treated with 1.8 mM AEBSF for one hour at 32°C, or left untreated. RAW cells were either treated with 0.25 mM AEBSF for 30 minutes or left untreated. Bacteria were added to RAW cells at an MOI of 2 and incubated at 32°C with 5% CO_2 for two hours. Cells were washed twice with 1x PBS to remove extracellular bacteria and then fresh infection media was added with 100 μg/ml streptomycin to kill remaining extracellular bacteria. Cells were incubated for 1 hour and then washed again. Fresh media with 4 μg/ml streptomycin was added and then the cells were incubated for the various time points. At each time point the cells were lysed with 0.1% Triton X-100, then serially diluted and plated onto 7H10 plates for enumeration of CFU.

3.4 Discussion

In this study, I showed that the EspB full-length protein is required for virulence. Since expression of the C-terminal fragment is sufficient to secrete some ESAT-6, the N-terminal fragment is somewhat dispensable for the codependent secretion. The C-terminal fragment alone however, is not able to complement the intracellular growth defect or attenuation in zebrafish of the *esp*B::tn mutant. Therefore, the N-terminus is required for virulence, independent of its role in aiding ESAT-6 secretion. I showed that the N-terminal fragment is able to secrete independent of ESX-1. How it gets out is not known. It has been demonstrated that the full length EspB protein absolutely requires a functional ESX-1 system for secretion (McLaughlin et al., 2007); therefore, it seems likely that the C-terminus confers specificity for EspB to secrete only via ESX-1. This may be an important part of the N-terminal virulence function since I showed that the N-terminal fragment alone is not able to complement intracellular

growth defects or attenuation in zebrafish of the *esp*B::tn mutant. The N-terminal fragment requires codependent secretion of ESX-1 substrates in order to carry out its virulence function. In fact, when I expressed the N- and C-terminus separately on the same plasmid, there was not a significant increase in intracellular growth inside macrophages. Therefore, expression of the full length protein is required for virulence. Because *esp*B::tn was previously shown to play a more profound role in the inhibition of phagolysosome fusion than the ΔCFP-10/ESAT-6 mutant (Xu et al., 2007), it is my hypothesis that the N-terminal fragment of EspB is translocated through the ESAT-6 formed pore in order to directly modulate vesicle trafficking. It is possible that the tight association of ESAT-6 and the full-length EspB protein is required for this targeting. I created several EspB N-terminal fragment fusions with potential translocation tags that have previously been used to identify translocated type III and type IV secreted proteins in Gram-negative bacteria. Two of the tags, Cya and GSK, cannot be used in the ESX-1 system; Cya is too large, and GSK can be phosphorylated by a mycobacterial kinase. The Bla tag has some potential for future studies, since I was able to show that it can be secreted.

This study also shows that the potential cleavage site of EspB at L346 is important for intracellular growth in macrophages and virulence in zebrafish. It supports the conclusion made by Ohol et al., 2010 that cleavage of EspB is required for regulating ESX-1 secretion. In my cleavage mutants I detected a significant increase in ESAT-6 secretion. ESAT-6 is an important virulence protein that is required for survival of mycobacteria inside the host phagosome, however too much secretion of ESAT-6 leads to attenuation. I agree with the hypothesis of Ohol et al., 2010 that

mycobacterial survival inside the phagosome is dependent on the ability of the bacteria to create a delicate balance between the secretion of virulence factors that modulate the phagosome environment, and being able to evade detection from the host immune system. The modulation of the phagosome environment is dependent on the secretion of ESX-1 substrates, however if too many of these strong antigenic proteins are secreted, then the host is able to activate their defenses to a level sufficient for clearing the infection. This idea is supported by the secretion profile I observed from the EspB WXG mutant. In this mutant, ESAT-6 and EspB secretion is greatly reduced, yet there is no defect in intracellular growth inside macrophages or virulence in zebrafish. Therefore, the virulence function of ESAT-6 and EspB can be accomplished without having an excess of secreted protein. Some is required, but it must be regulated.

The WXG motif is one of the factors that define the WXG100 superfamily of ESAT-6-like proteins. It is highly conserved among species, though no function has been attributed to it. Even though EspB has a WXG motif, it is not a member of the WXG100 superfamily because of its large size. Still, the motif is highly conserved in EspB homologs across mycobacterial species, indicating that it may be important for secretion or virulence. One study by Brodin et al., 2005 showed that mutating the ESAT-6 WXG motif does not lead to a secretion defect, but does cause attenuation. I found that the EspB WXG motif is involved in the codependent secretion of EspB and ESAT-6. In this mutant, secretion is much reduced for both proteins, yet the mutant is as virulent as the *esp*B::tn+EspB complement strain. Non-pathogenic species of Gram-positive bacteria have WXG100 proteins in their genome, so the

WXG motif cannot be strictly attributed to virulence. Being so highly conserved indicates that the role of WXG is structural. Perhaps for some proteins, like EspB the structural role aids in secretion, while in others, like ESAT-6, it enables the protein to carry out its virulence function. It is also possible that the ESAT-6 WXG mutation affects the secretion of other important ESX-1 substrates, and the attenuation observed by Brodin et al., 2005 is due to this secretion codependence.

Because the ESX-1 secretion system is so important for mycobacterial virulence, its constituents could prove to be excellent drug targets; either the components that make up the secretion machinery, or the virulence effectors that are secreted from it. I was able to demonstrate the potential of using the inhibitor, AEBSF to target serine proteases that are important for ESX-1 secretion. Adding AEBSF to wild type *Mm* liquid culture leads to a reduction in ESX-1 secretion and pretreatment of WT *Mm* with AEBSF leads to a reduction in intracellular growth inside RAW cells. It is possible that this drug is able to target the secretion of MycP1 which is a serine protease known to be involved in the secretion of EspB. I got inconsistent results when I tried to replicate this experiment, but future work may prove very promising.

Chapter 4: Significance of this work

This dissertation focuses on the importance of ESX-1 as a specialized secretion system and its mechanism of virulence inside host cells. In *Mtb* ESX-1 has been shown to be required for virulence in mice and growth in macrophages. It is also required for the inhibition of phagolysosome fusion, as well as the modulation of host immune responses which result in reduced expression of IL-12 and TNF-α (Stanley et al., 2003; Hsu et al., 2003; Guinn et al., 2004; MacGurn and Cox, 2007). In *Mm* ESX-1 has been shown to be required for virulence in zebrafish, cytolysis, escape from the phagosome, and granuloma formation (Gao et al., 2004; Tan et al., 2006; Volkman et al., 2004). Several of the individual substrates secreted via ESX-1 are also required for virulence and survival inside host cells. The functional mechanism of these substrates however, is difficult to discern given the codependent nature of their secretion. ESAT-6, CFP-10, and EspB for instance are all codependent for secretion. Therefore, the mutant phenotype observed by one could be due to reduced secretion of another.

In Chapter 2, I demonstrated using purified ESAT-6 protein that it could form pores in red blood cells and murine macrophage membranes. Identifying ESAT-6 as a pore forming toxin is a big step toward understanding the function of this protein. However, several questions still remain. ESAT-6 is involved in several important ESX-1 survival strategies; such as inhibition of phagolysosome fusion, macrophage cytokine signaling, and inhibition of TLR-2 signaling (Stanley et al., 2003; Hsu et al.,

2003; Pathak et al., 2007). Are these all direct effects of the ESAT-6 protein or are they indirectly attributed due to its pore forming function?

ESAT-6 has been shown to directly associate with both CFP-10 and EspB. The association it has with CFP-10 exhibits high affinity both inside the cytosol and also in the culture filtrate after secretion (Renshaw et al., 2002; de Jonge et al., 2007); however, the pore forming ability of ESAT-6 is not dependent on CFP-10 (Smith et al., 2008) and it has been shown that ESAT-6 and CFP-10 dissociate under acidic conditions (de Jonge et al., 2007). It seems likely that the association of ESAT-6 and CFP-10 is dependent on environmental factors that are sensed by the complex upon secretion. The acidic nature of the phagosome may cause the dissociation of ESAT-6 and CFP-10 so that ESAT-6 can insert into the host membrane.

Eventually, ESAT-6 pore formation may allow *Mm* to escape the phagosome and spread from cell-to-cell (Smith et al., 2008). This may be a feature that is *Mm* specific since the predominant thought is that *Mtb* does not escape (Clemens et al., 2002; Russel et al., 2001). Escape may be an adaptation that evolved in *Mm* given the diverse natural hosts and environments that it resides in. Even if *Mtb* doesn't escape the phagosome, it has been shown to have cytolytic properties, and the *Mtb* ESAT-6 protein can complement the *Mm* ΔESAT-6 mutant, indicating a conservation of function (Gao et al., 2004). It has also been clearly demonstrated that *Mm* is able to inhibit phagolysosome fusion and induce granuloma formation, similar to *Mtb*. Given that these two mycobacterial species have very similar persistence mechanisms, it seems likely that the pore forming function of ESAT-6 would somehow function for the benefit of *Mtb* as well.

ESX-1 secretion has been shown to be regulated at several levels. EspR as discussed in section 1.6.3, has been shown to bind the promoter of the Rv3614c-Rv3616c operon and activate transcription (Raghavan et al., 2008). This operon encodes the EspA protein that is secreted via ESX-1 and is required for virulence (MacGurn et al., 2005). EspA is codependent with ESAT-6 for secretion, which is codependent with CFP-10 and EspB; therefore, EspR secretion negatively regulates the whole ESX-1 system by reducing the transcription of *esp*A.

Cleavage of the EspB protein by MycP1 is also involved in the negative regulation of ESX-1 secretion. Recently, Ohol et al., 2010 showed that a protease deficient MycP1 protein is unable to cleave EspB, resulting in an increase in ESX-1 substrate secretion. My data in Chapter 3 supports their results; I showed that mutating the cleavage site of EspB so that it cannot be cleaved by MycP1, leads to an increase in ESAT-6 secretion. This increase in ESAT-6 secretion results in a defect of growth inside macrophages and attenuation in zebrafish. I also showed that the highly conserved WXG motif present in the N-terminal fragment of EspB is involved in the secretion of ESX-1 substrates. This mutant showed reduced secretion of both ESAT-6 and EspB. Interestingly, this reduced secretion does not cause a defect in intracellular growth inside macrophages and does not affect virulence in zebrafish. Together this data indicates that cleavage of EspB is required for regulating the amount of ESX-1 substrates that are secreted.

The regulatory controls observed thus far in the ESX-1 system seem to be important for making sure that the right amount of virulence effectors are secreted. Secretion of ESX-1 substrates is required in order for mycobacteria to create an amenable

environment inside the phagosome and survive host defense mechanisms. However, if there is too much secretion of ESX-1 substrates, it's likely that the potent antigens activate too many host defenses and the bacteria are no longer able to persist. Like the story of "The Three Bears", mycobacteria must secrete their effectors to create an infection-protection balance that is "just right".

It is possible that *Mm* is regulated differently than *Mtb*, and when environmentally triggered, it upregulates ESAT-6 secretion in order to disturb phagosome membrane integrity, allowing the bacteria to escape to the cytosol. It has been shown that *Mm* and *Mtb* do not have the exact same ESX-1 secretion profile. The EspF protein, for example is expressed much more abundantly in *Mm* than it is in *Mtb* (DiGiuseppe Champion et al., 2009). During the persistence stage of both *Mm* and *Mtb*, it is my hypothesis that ESX-1 is able to translocate the tightly regulated virulence effectors through the ESAT-6 formed-pore and modulate the host environment. This would explain how ESAT-6 is able to aid *Mm* escape, but also explains how the pore forming ability of ESAT-6 contributes to the other phenotypes observed in both *Mm* and *Mtb* that allow for intracellular survival. If pores induced by ESAT-6 allow for the translocation of other ESX-1 virulence effectors, then the question arises, which ones?

EspB seems like a likely candidate since it associates with ESAT-6, and is required for virulence and the inhibition of phagolysosome fusion (Xu et al., 2007; McLaughlin et al., 2007). It has been shown that the C-terminus is important for the secretion of ESAT-6 however, no function has yet been attributed to the N-terminus. Cleavage of EspB into a 50 kDa N-terminal fragment and an 11 kDa C-terminal

fragment are important to negatively regulate ESX-1 secretion. Interestingly, I showed that when the N terminus is expressed without the C-terminus it is still secreted independent of ESX-1, though ESAT-6 is not secreted. Apparently, the full-length protein is necessary for targeting of the EspB protein to the ESX-1 channel. Since the C-terminus is involved in secretion, then what is the purpose of the N-terminus? Is its purpose solely to aid secretion of other substrates, or does it play a more direct role in virulence?

I showed that the C-terminal fragment is sufficient to secrete ESAT-6 however, this did not restore virulence. Likewise, the N-terminus is able to secrete independent of ESX-1, yet expressing only this fragment does not restore virulence either. This indicates that the N-terminus does play a role in virulence beyond secretion, yet the secretion of ESAT-6 and possibly other ESX-1 substrates is required in order for EspB to carry out this function. Determining the intracellular localization of the EspB protein would be a great breakthrough in understanding its contribution to virulence. My preliminary work in this effort demonstrated that the Bla tag could potentially be used in future studies to determine if EspB is translocated into the host cell cytosol. This tag has been used previously to identify translocated proteins of type III and type IV secretion systems, and I showed that it can be secreted in *Mm* when fused to the N-terminal fragment of EspB.

My research has contributed to a new model of ESX-1 secretion [Fig. 26]. In this model, ESAT-6 associates with the other secreted substrates in the cytosol, including EspB. Upon secretion across the cytosolic membrane, EspB is cleaved by MycP1 at the L346 cleavage site; this cleavage modulates subsequent secretion through the

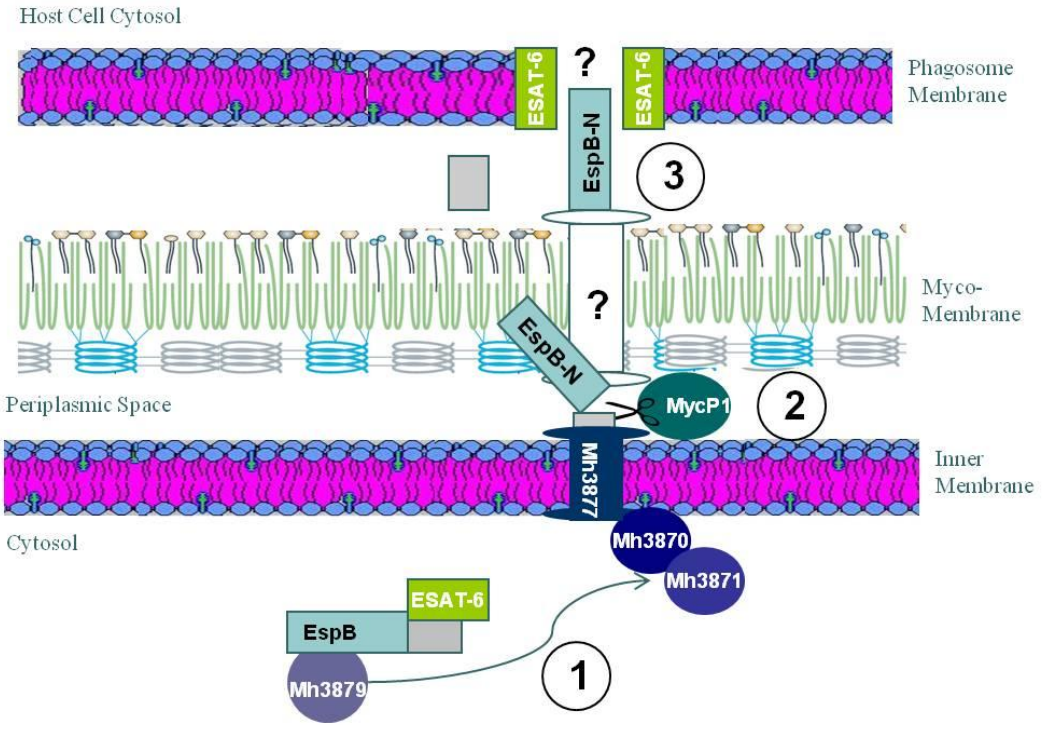

Figure 26. A new model of EspB secretion. (1) EspB is known to associate with Mh3879c and ESAT-6 in the cytosol. ESAT-6 in turn associates with other proteins that may all be targeted to the ESX-1 channel as a complex. The EspB C-terminus is required for the codependent secretion of ESAT-6, and the WXG motif in the N-terminus also seems to play a role, perhaps by interacting with other proteins in the secretion complex or the ESX-1 machinery. The EspB N-terminal fragment seems to play a direct role in virulence, though expression of the full length protein is required for full function. This is perhaps because a close association with ESAT-6 is necessary for this function. Mh3879c associates with the Mh3871 channel ATPase and may act as a chaperone to target EspB and associated proteins to be secreted. (2) Once EspB is secreted across the inner membrane channel it is cleaved by MycP1. Cleavage of EspB at the L346-S347 cleavage site is required for virulence and may act to negatively regulate secretion of other ESX-1 substrates. (3) I demonstrated that ESAT-6 is able to form pores in macrophage membranes. The purpose of this pore forming ability is not known. Because of the tight association of full-length EspB and ESAT-6 that is required for virulence, it seems plausible that ESAT-6 forms pores in the phagosome membrane and the EspB N-terminal fragment is translocated into the host cell in order to carry out its virulence function.

channel. The N-terminus then goes on to carry out its virulence function with the help of ESAT-6. It is my hypothesis that EspB translocates through the ESAT-6 formed pore into the host cell cytoplasm and its targeting enables the bacteria to persist inside a more amenable phagosome environment.

Understanding the function of ESX-1 secreted substrates has great future implications. Because the substrates are codependent for secretion, drugs that target individual substrates can lead to major disruptions in ESX-1 secretion which is required for mycobacterial virulence. Since I've shown that ESAT-6 is a pore forming toxin, it may be possible to identify drugs that interfere with its ability to insert into the membrane. It is my hypothesis that EspB is translocated through the ESAT-6 formed pore where it likely tarets a host protein(s). If future experiments confirm this hypothesis, then EspB could potentially be a very promising drug target. Its location in the cytosol would make it more accessible to drugs than proteins inside the phagosome. Future efforts to identify the host protein(s) that EspB associates with may present an assay to screen for drugs that are able to interfere with EspB function. Drugs targeting the secretion of EspB could also be used as targets for drug therapy. MycP1 could prove extremely promising given its location in the cell wall. A drug that interferes with the ability of MycP1 to associate with the secretion machinery could lead to an attenuation phenotype similar to what is observed in the *myc*P1 mutant. My preliminary data using the general serine protease inhibitor, AEBSF showed that this kind of targeting could interfere with ESX-1 secretion. Our lab has been working to develop a new vaccine against *Mtb* using *Mm*. *Mm* is an opportunistic human pathogen but we've discovered that mutation of the *iip*A gene

that is involved in cell wall assembly causes further attenuation (Gao et al., 2006). Since, *Mm* has a functional ESX-1 secretion system it secretes potent T-cell antigens, including ESAT-6 and EspB that may illicit a stronger immune response than BCG, offering better protection against *Mtb* infection. If EspB is found to be translocated into the host cell cytosol it would be able to prime $CD8^+$ T-cells. This could potentially be useful as a means to boost vaccine protection if the protein were tagged with other strong antigenic peptides.

One-third of the world's population is infected with *Mtb* and 2-3 million people die from the disease each year (World Health, 2009). This staggering statistic really underlines the importance of finding better ways to prevent and treat this devastating disease.

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