THE ROLE OF THE *MYCOBACTERIUM TUBERCULOSIS* SECA2 PROTEIN
EXPORT PATHWAY IN VIRULENCE

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ABSTRACT

JONATHAN TABB SULLIVAN: The Role of the *Mycobacterium tuberculosis* SecA2
Protein Export Pathway in Virulence
(Under the direction of Miriam Braunstein)

Mycobacterium tuberculosis is an intracellular bacterial pathogen that replicates in macrophages in the lung of the host. The ability to replicate in these cells of the immune system is critical to the virulence of this important pathogen. *M. tuberculosis* is thought to maintain a hospitable niche in the host through several mechanisms. *M. tuberculosis* suppresses the host innate immune response by dampening cytokine secretion and production of reactive nitrogen and oxygen species that can be toxic to bacteria. Additionally, *M. tuberculosis* manipulates macrophages by arresting the normal process of phagosome maturation into acidified and hydrolytic phagolysosomes. The process of each of these immunosuppressive functions by *M. tuberculosis* is not fully understood. Other intracellular pathogens that control the host immune response use specialized protein export systems to deliver effectors to the host cell. In *M. tuberculosis*, the accessory SecA2 system is a specialized protein export system that is required for intracellular growth in macrophages. However, we do not understand the role of SecA2 in promoting growth in macrophages. The SecA2 system has a role in dampening the host cytokine and reactive nitrogen response. However, we show that the role of SecA2 in dampening these inflammatory responses cannot explain the intracellular growth defect. In this study we discovered that SecA2 is also required for blocking phagosome

maturation. We showed that inhibitors of phagosome acidification rescued the

intracellular growth defect of the Δ*secA2* mutant, which demonstrated that the

phagosome maturation arrest defect of the Δ*secA2* mutant is responsible for the

intracellular growth defect. Our data suggests there are effectors of phagosome

maturation that are exported into the host environment by the accessory SecA2 system.

Thus, we tested a set of putative effectors of *M. tuberculosis* phagosome maturation for

SecA2 dependent export. We found that the level of one of these proteins, the secreted

acid phosphatase (SapM), was reduced in the Δ*secA2* mutant. The research presented in

this thesis establishes a role for SecA2 in promoting *M. tuberculosis* growth in

macrophages. Additionally, we demonstrate an important causal link between phagosome

maturation and arrest of *M. tuberculosis* intracellular replication.

<u>DEDICATION</u>

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List of Abbreviations

BCG – Bacille Calmette-Guerin

CF – Culture filtrate

cfu – colony forming unit

CR – Complement receptor

EEA-1 – Early endosomal antigen 1

ESX – ESAT-6 like secretion system

FACS – Fluorescently activated cell sorting

HOPS – Homotypic fusion and vacuolar sorting

IFN-γ – Gamma interferon

IL-6 – Interleukin-6

LAM – Lipoarabinomannan

ManLAM – Mannose capped lipoarabinomannan

MCD – Multi drug resistant

MR – Mannose receptor

PI(3)P - Phosphatidylinositol-3 phosphate

PI3K – Phosphatidylinositol-3 kinase

PIM – Phosphatidylinositol mannoside

RILP – Rab7 interacting lysosomal protein

RNI – Reactive nitrogen intermediates

ROS – Reactive oxygen species

T3SS – Type III secretion system

TB – Tuberculosis

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor alpha

V-ATPase – Vacuolar ATPase

WCL – Whole cell lysate

XDR – Extensively drug resistant

CHAPTER I

INTRODUCTION

Robert Koch described *Mycobacterium tuberculosis* as the bacterium that causes tuberculosis (TB) in 1882. Despite having been known as the causative agent of TB for over 100 years, *M. tuberculosis* currently infects approximately one-third of the world's population and TB is estimated to kill nearly two million people each year (107). Neither universally effective vaccines (33), nor fast-acting sterilizing drugs exist to treat TB. Additionally, *M. tuberculosis* is quickly evolving resistance mechanisms to the current drugs in use to treat infection. Taken together these factors create a need for new drugs and vaccines to combat TB. Research into the virulence mechanisms of *M. tuberculosis* will facilitate efforts to identify novel drug targets and vaccine strategies.

1.1- Challenges and limitations of current TB drugs and vaccines

M. tuberculosis has a variety of intrinsic drug resistance characteristics that make it difficult to treat with our current array of antibiotics. Antibiotics that have a cytoplasmic target in *M. tuberculosis* are impeded by a very hydrophobic cell envelope that limits drug entry, and the existence of efflux pumps that can keep cytoplasmic drug levels low by pumping drugs out of the cytoplasm (25, 43). β-lactam antibiotics that target extracellular enzymes are also inefficient due to the *M. tuberculosis* β-lactamase BlaC (47). Further, because the current TB drugs work on replicating bacteria, the drugs

in use are hindered by the unusually slow replication rate of *M. tuberculosis*, which necessitates long treatment times.

In addition to the inherent drug resistance capabilities of *M. tuberculosis*, this bacterial pathogen is quickly evolving resistance mechanisms to the current drugs used to treat TB infection. Strains that have developed drug resistance to current TB therapies are termed Multi Drug Resistant (MDR) or Extensively Drug Resistant (XDR) TB. The World Health Organization defines MDR strains as strains that are resistant to both of the first-line drugs rifampin and isoniazid. XDR strains are defined as having MDR resistance plus resistance to at least two second-line drugs.

An effective vaccine would supplant the need for drugs to treat *M. tuberculosis*. Currently, there is a live attenuated vaccine strain for TB, *M. bovis* Bacille Calmette-Guerin (BCG), that is used in most of the world. The BCG vaccine was introduced in the 1920's, but subsequent studies have shown huge discrepancies in the vaccine's efficacy, from as high as 80% protection against TB in England to 0% protection in a study conducted in India (33). Due to its questionable efficacy and the complications it presents to the diagnostic TB skin test, the BCG vaccine is not used in the United States. The current status of drugs and vaccines against TB are insufficient to deal with the emerging drug-resistant strains. Studies of the virulence of *M. tuberculosis* could lead to new drug and vaccine strategies capable of reducing the impact of drug-resistant TB.

1.2- Route of *M. tuberculosis* infection

The development of new drugs and vaccines for TB is particularly important because TB is a serious disease that causes substantial human mortality and is easily spread among

the human population. *M. tuberculosis* resides in the lungs of infected individuals. A person with active TB disease can aerosolize infectious bacteria by coughing, sneezing or even talking. When an uninfected individual inhales *M. tuberculosis*, the bacteria travel to the bronchioles and alveolar sacs within the lungs. In these spaces *M. tuberculosis* is phagocytosed by resident alveolar macrophages and contained within the macrophage in a membrane bound compartment called a phagosome (34). Macrophages generally destroy bacteria through the phagocytic pathway by creating a hydrolytic, antimicrobial, and degradative environment within the phagosome. The formation of this degradative phagosomal compartment is a multi-step process termed phagosome maturation (34). *M. tuberculosis* has the amazing ability to survive and even replicate after its uptake by macrophages. Once in macrophages, *M. tuberculosis* prevents phagosome maturation from occurring normally, which results in *M. tuberculosis* residing in a more hospitable phagosomal compartment. This early phase of bacterial replication in host phagosomes is referred to as primary infection.

It is generally believed that the ability of *M. tuberculosis* to survive and replicate during primary infection depends on the pathogen blocking the normal process of phagosome maturation of macrophages (4, 82, 102). There are, however, additional effects of *M. tuberculosis* on the host that could be critical to the success of this pathogen during primary infection. Apoptotic cell death can control replication of intracellular *M. tuberculosis* (67, 73). *M. tuberculosis* is able to reduce apoptotic cell death in favor of necrotic cell death, which promotes cell-to-cell spread of the bacteria (67). *M. tuberculosis* also dampens the inflammatory response by repressing macrophage production of cytokines. This dampening of the cytokine response affects recruitment of

immune cells that could help clear infection, potentially contributing to the ability of *M. tuberculosis* to replicate during primary infection. Because some of the cytokines whose levels are dampened by *M. tuberculosis* are able to activate macrophages to control bacillary growth, this modulation of the immune response could also be important for facilitating intracellular replication of the bacterium.

Eventually during infection, an immunocompetent host will develop a TH1-skewed adaptive immune response to *M. tuberculosis*. When the TH1 response is fully developed the growth of the bacteria is arrested and the next phase of TB infection begins. Growth is arrested by an influx of antigen specific T-cells that produce gamma interferon (IFN-γ), which activates the infected macrophages to a point where they can arrest *M. tuberculosis* replication (36). Notably, *M. tuberculosis* can persist in this state; this phase of infection is called latency. During latency *M. tuberculosis* replicates very slowly and maintains homeostasis (44). *M. tuberculosis* can emerge from latency through reactivation if the host immune system is adversely affected such that the adaptive response is dampened. Some of the more common causes of immune repression that lead to reactivation of TB disease are HIV, immunosuppressive treatments, or aging.

Reactivation of a latent *M. tuberculosis* infection leads to active bacterial replication, wasting disease and eventually death of the infected person. This phase of infection is called secondary infection.

The research in this thesis is directed at understanding the primary phase of *M. tuberculosis* infection, with the goal of helping to understand the virulence mechanisms of *M. tuberculosis* that enable replication in macrophages. In particular we studied the SecA2 protein export pathway, which was shown previously to be required for growth in

macrophages (60). We set out to understand the role of SecA2 in virulence as a way to learn more about TB pathogenesis during this important phase of infection.

Other intracellular bacterial pathogens that carve out a niche within macrophage phagosomes, such as *Salmonella* and *Legionella*, use specialized secretion systems to deliver effectors to the host cell (42, 50). Some of the effectors they deliver are involved in trafficking and control of the environment within the phagosome. *M. tuberculosis* is known to have two types of specialized secretion systems: ESX systems and the SecA2 system. One of the five ESX systems, the ESX-1 system, has been shown to have functions in promoting growth in macrophages and in the process of phagosome maturation arrest. The SecA2 system is also known to promote growth in macrophages (12, 60). Here, we further characterize the role of the SecA2 system in virulence, and find that it also has a role in phagosome maturation arrest. It has long been thought that the ability to block phagosome maturation is a keystone to *M. tuberculosis* pathogenesis (4). However, it has not been firmly established that a failure to block phagosome maturation prevents *M. tuberculosis* growth. Through the course of studying the role of the SecA2 system in promoting growth of *M. tuberculosis* in macrophages, we demonstrate in this thesis that phagosome maturation arrest is necessary for intracellular *M. tuberculosis* replication and that the failure to properly block phagosome maturation can inhibit *M. tuberculosis* growth.

1.3- Macrophages as a host defense against infection

As an intracellular pathogen, *M. tuberculosis* requires a host cell to grow *in vivo*. Upon inhalation, the first productive contact between *M. tuberculosis* and the host is

alveolar macrophages. In the lung, alveolar macrophages that reside in the bronchioles and alveolar sacs are the first line of defense of the immune system. Alveolar macrophages have the important job of detecting, consuming, degrading, and presenting antigens of bacteria and fungi to the immune system (31). Macrophages also have the capacity to clear apoptotic cells and inert particles.

Resident alveolar macrophages readily destroy foreign organisms such as bacteria; they do this through the process of phagocytosis and phagosome maturation (34, 58). Phagocytosis is the term used to describe host cell engulfment of particles greater than 0.5μm in size. Once phagocytosed, a foreign body resides in a membrane-encased compartment called a phagosome. Phagosome maturation is the subsequent process through which the phagosome is converted to an acidic, hydrolytic compartment in order to degrade the contents of the phagosomal lumen. The process of phagosome maturation is driven by sequential fusion events with endosomes and lysosomes that deliver hydrolytic enzymes and the vacuolar H^+-ATPase (V-ATPase), which drives acidification of the phagosome (35).

When containment or destruction via phagocytosis and phagosome maturation fails, macrophages can undergo apoptosis to prevent spread of intracellular bacteria. By containing the bacteria in an apoptotic body the macrophage can recruit other phagocytes, and induce phagocytosis and destruction of the apoptotic corpse. Because the bacteria are now contained in an apoptotic body the potential for them to interact with and affect the recruited phagocyte is diminished compared to direct phagocytosis of the bacteria.

1.4- Macrophage phagocytosis

Phagocytosis can lead to varied outcomes that depend on mode of entry and cargo of the initial phagosome (34). Phagocytosis begins with recognition of the particle to be consumed. This recognition is performed by surface receptors on the phagocyte (Figure 1-1). In addition to the receptors that are required for phagocytosis of bacteria, e.g. complement receptors, Fcγ receptor, mannose receptor, and scavenger receptors (34) phagocytes also sample their load with Toll-like receptors (TLRs) to determine what they are consuming (1).

Once a foreign body is recognized by receptors it is internalized through an actin dependent process. While the specific proteins vary depending on the receptor, the mechanism of phagosome formation and engulfment through each receptor is similar (Figure 1-1) and involves signaling Rho family GTPases that recruit actin nucleation factors (70, 109). Actin polymerization and branching, through the recruitment of Wiscott-Aldrich syndrome protein (WASP) and actin-related protein 2/3 (Arp2/3) then drives the creation of the phagocytic cup, which is the initial membrane extrusion of pseudopodia used to surround the target. Phagocytosis, especially of larger particles, requires extra membrane that is supplied by recycling endosomes, late endosomes and the endoplasmic reticulum; the extra membrane is required to protrude pseudopodia for surrounding the target. Myosin motors are also recruited to nascent phagosomes, and are required for the extrusion of pseudopodia and contraction of the phagocytic cup into the phagocyte (3, 23, 74). Other factors such as phosphatidylinositol (PI) kinases are recruited to the phagocytic cup, phosphorylate PI and are required for timing actin
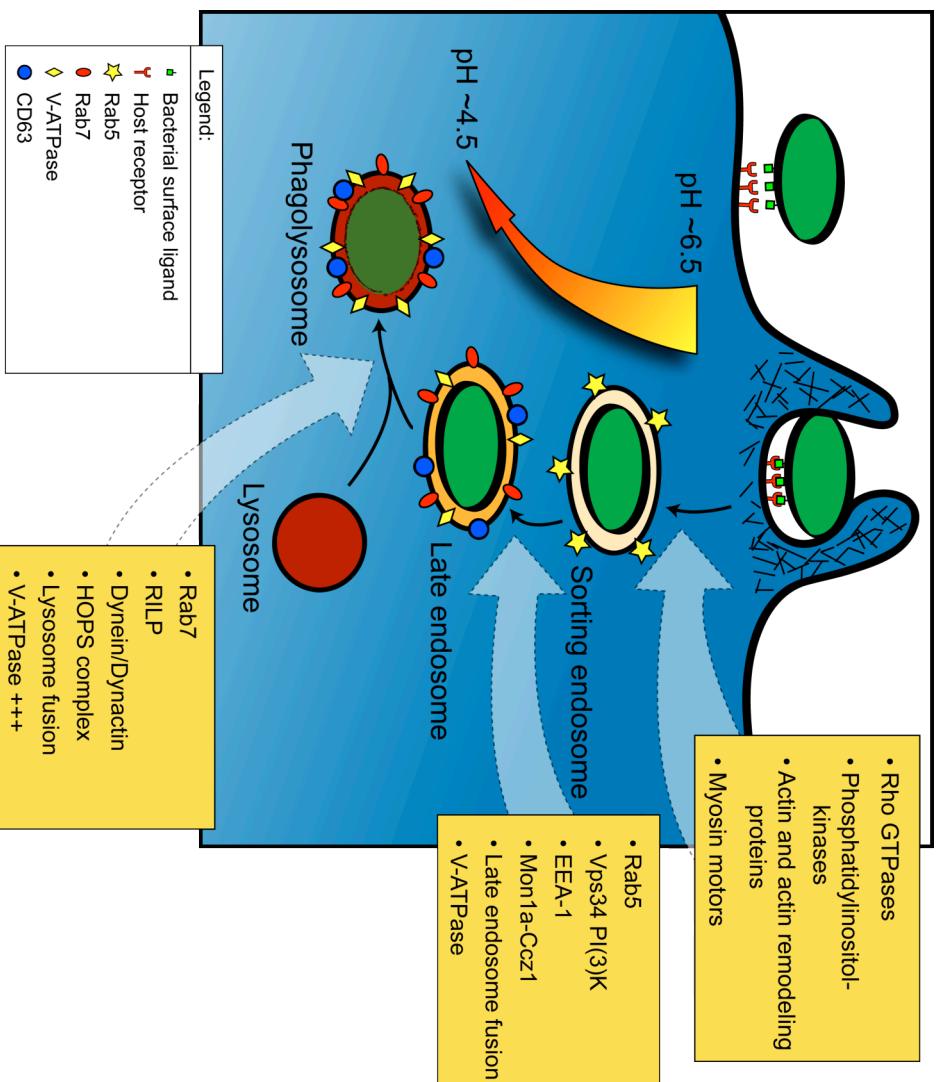
Figure 1-1. Bacteria are destroyed by macrophages through the process of phagocytosis and phagosome maturation. Briefly, phagocytic surface receptors are engaged by ligands on the bacterial surface. Engulfment is orchestrated by Rho GTPases and is an actin dependent action. After engulfment, Rab GTPases and phosphotidylinositol-3 kinase orchestrate maturation of the phagosome into a mature phagolysosome. The phagolysosome is an extremely acidic and hydrolytic compartment capable of degrading most bacteria.

Legend:
- Bacterial surface ligand
- Host receptor
- Rab5
- Rab7
- V-ATPase
- CD63

pH ~4.5

pH ~6.5

Phagolysosome

Lysosome

Late endosome

Sorting endosome

- Rab7
- RILP
- Dynein/Dynactin
- HOPS complex
- Lysosome fusion
- V-ATPase +++

- Rab5
- Vps34 PI(3)K
- EEA-1
- Mon1a-Ccz1
- Late endosome fusion
- V-ATPase

- Rho GTPases
- Phosphatidylinositol-kinases
- Actin and actin remodeling proteins
- Myosin motors

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polymerization and depolymerization (34). Coronin is a protein required for efficient

phagocytosis in the amoeba *Dictyostelium discoidium* (64). Mammalian Coronin 1A also

localizes to the nascent phagosome in phagocytic cells, but mammalian Coronin is

apparently expendable in phagocytosis, although it may play an alternate role in

phagosome maturation (52).

While the processes between phagocytosis through different receptors are

synonymous, it is likely that the final composition of the mature phagosome and/or the

kinetics of maturation depend on what route the target entered the cell (2).

1.5- Phagosome maturation

While many of the details are yet to be elucidated, there is a basic framework for

how phagosome maturation progresses. The kinetics, pathway of maturation, and final

phagosome composition depend on the cargo to be destroyed. For example, even though

both types of loads get degraded in the phagolysosome, bacterial loads lead to efficient

MHC-II peptide loading, whereas peptides from apoptotic cells do not get loaded into

MHC-II (58). The different outcomes result in inflammation and antigen presentation for

bacterial loads, whereas apoptotic "self" loads lead to anti-inflammatory phagocytosis

without antigen presentation.

After engulfment of the phagocytic load, the Rab GTPase Rab5 is quickly

recruited to the phagosome (103). This Rab5+ compartment acidifies slightly from pH 7

to a pH of about 6.1-6.5 and is referred to as a sorting endosome. The sorting endosome

is called such because it has multiple potential outcomes. The sorting endosome can enter

the recycling endosome network and be exocytosed. Alternatively, the sorting endosome

can enter the trans-golgi network where proteins are sorted for secretion, surface localization, or inclusion in endosomal or lysosomal compartments (34). The final possibility is that the sorting endosome can traffic to a mature phagolysosome. As it is the most relevant to this thesis, we will limit our discussion of these various outcomes to that of phagoslysosome maturation.

For phagolysosome maturation, active (*i.e.* GTP bound) Rab5 goes on to recruit Vps34, a phosphotydlinositol 3-kinase (PI3K) that coats the phagosomal membrane with phosphotydlinositol 3-phosphate (PI(3)P). The PI(3)P on the phagosomal membrane in turn recruits proteins containing FYVE domains, including the early endosome antigen 1 (EEA1). Antibodies against EEA1 microinjected into macrophages block phagosome maturation, suggesting a role for EEA1 in the maturation process (40). EEA1 is thought to be involved in orchestrating fusion events between the early phagosome and endocytic vesicles, but its exact role in maturation is not known (34).

An additional role for Rab5 is the recruitment of another Rab GTPase, Rab7, to the maturing phagosome. In model bead and apoptotic corpse phagosomes, Rab7 recruitment requires active Rab5 and it occurs efficiently without PI3K activity (57, 103). Thus, there appears to be a Rab5 dependent mechanism, other than promoting PI(3)P deposition, that is required for Rab7 recruitment to the phagosome. Indeed, in the maturation of phagosomes containing apoptotic corpses active Rab5 recruits active (*i.e.* GTP bound) Rab7 through the Mon1a-Ccz1 protein complex (57). However, it is not yet known whether Rab7 recruitment is through the same mechanism on bacteria-containing phagosomes.

Active Rab7 in turn interacts with Rab7 Interacting Lysosomal Protein (RILP) (17). RILP acts as a scaffold to link Rab7 to Dynamin/Dynactin, which are motor proteins that move along tubulin (54). This interaction suggests a model where activated Rab7 links the phagosome to the tubulin network in order to drive the phagosome movement toward lysosomes for fusion events to occur. Lysosomes are the terminal compartment to fuse with phagosomes during the maturation process, once fused to the phagosome the combined compartment is referred to as the phagolysosome (35). Lysosomes are vacuoles that contain high concentrations of hydrolytic enzymes and maintain a very low pH (~4.5) via a high concentration of V-ATPase in their membrane. It should be noted that while lysosomes deliver a large quantity of V-ATPase to the phagosome, V-ATPase is also delivered to the phagosome throughout the process of maturation (34). Lysosomal fusion is thought to be orchestrated by the multi-protein homotypic fusion and vacuole sorting (HOPS) complex, which assembles on the phagosomal membrane and is directly involved in activating lysosome fusion events (29).

Toll-like receptors (TLRs) may play a role in fast-tracking phagosomes for maturation (5, 10), suggesting an attractive model for how the load is recognized and directed by the phagocyte. However, there is some debate and further research will be required to tease out the function of TLRs in driving phagosome maturation (108).

1.6- *M. tuberculosis* phagosomes

For most bacteria, macrophage phagocytosis results in trafficking to highly destructive phagolysosomes where the bacterial cell is destroyed. Once degraded, bacterial antigens can be loaded into MHC and presented on the surface of the

macrophage (34). For intracellular bacterial pathogens to survive phagocytosis they need to have a mechanism to either withstand the phagolysosome environment or a way to redirect the trafficking of the phagosome to avoid the compartment altogether.

Over the years it has been shown by many different groups and readouts that in non-activated macrophages *M. tuberculosis* avoids trafficking to mature phagolysosomes (4). The different approaches used to demonstrate blockage of phagosome-lysosome fusion by *M. tuberculosis* include electron microscopy, fluorescent dextran trafficking, and staining for markers of lysosomal fusion such as lysosome associated membrane protein-1 (LAMP-1), CD-63, and lysobisphosphatidic acid (LBPA) (4, 61, 62). Further, LysoTracker and ratiometric fluorescent dyes have been used to demonstrate *M. tuberculosis* blockage of phagosome acidification (77, 93, 95). However, the mechanism employed by *M. tuberculosis* to avoid phagosome maturation remains elusive. Numerous putative *M. tuberculosis* effectors of phagosome maturation, both lipid and protein, have been identified through direct testing and mutant screens (15, 26, 41, 49, 56, 62, 77, 95, 98-100, 104). However, the mode of action for nearly all of these putative effectors remains unknown. What appears to be true from these studies is that the process is likely to be multi-factorial and complex and that *M. tuberculosis* has effectors that disrupt phagosome maturation from the beginning (phagocytosis) to the end (lysosomal fusion) of the process.

1.7- *M. tuberculosis* **phagocytosis**

Macrophages recognize *M. tuberculosis* through several receptors including the complement receptors CR1 and CR3, mannose receptor (MR), and scavenger receptors

(30). There is evidence that the initial event allowing *M. tuberculosis* to control

intracellular trafficking occurs at the step of recognition by a macrophage receptor (55).

        M. tuberculosis can be directly recognized by complement receptor CR3 on the

macrophage by a complement independent mechanism. This recognition, in the absence

of the C3b component of complement, occurs at one of two binding sites on CR3 (24).

Binding at both CR3 sites is required for activation of some antimicrobial activities of

natural-killer cells and neutrophils (101). Thus, *M. tuberculosis* may be affecting the host

cell response early on by limiting binding to one site on the CR3 receptor.

        M. tuberculosis can also be recognized by the host cell through the MR, which

recognizes the *M. tuberculosis* surface glycolipid mannose capped lipoarabinomannan

Man-LAM (87-89). When MR recognition of ManLAM-coated beads or *M. tuberculosis*

is blocked by preincubation with mannan or anti-MR antibody, phagosome maturation

kinetics are faster. Similarly, when beads or *M. tuberculosis* are shuttled through the Fcγ

receptor instead of MR by IgG opsonization, phagosome maturation kinetics are faster.

These experiments suggest that *M. tuberculosis* may selectively enter through the MR-

mediated phagocytosis in order to delay phagosome maturation. However, the

experiments testing the kinetics of phagosome maturation in these different conditions

were very short, two hours post phagocytosis for bead experiments and 30 minutes post

phagocytosis for bacteria assays, which is of questionable relevance in a long-term

chronic infection such as TB (55). This data suggests an important role for receptor

selection in the early kinetics of phagosome maturation arrest.

1.8- *M. tuberculosis* **phagosome maturation**

Once in a macrophage, the *M. tuberculosis*-containing phagosome stalls at a slightly acidic Rab5+ compartment similar to a sorting endosome. The *M. tuberculosis*-containing phagosome is slightly more acidic than a nascent phagosome at a pH of ~6.4 like a sorting endosome instead of pH 7.0, but far from the pH of a mature phagolysosome ~4.5 (82). Rab5 is an example of a protein that is recruited normally to the *M. tuberculosis*-containing phagosome but is retained, which is unusual. Not only does the *M. tuberculosis*-containing phagosome retain Rab5 (102), interestingly, it also remains accessible to surface transferrin receptor (20), indicating continued interaction with recycling endosomes that shuttle cargo between sorting endosomes and the cell membrane (35).

Another example of an abnormally retained protein is Coronin-1A, which is recruited normally to *M. tuberculosis*-containing phagosomes but is retained on the surface of a *M. tuberculosis*-containing phagosome after the phagosome is sealed (32). This is unique to *M. tuberculosis*-containing phagosomes; Coronin-1A is usually released from phagosomes after sealing.

Model phagosomes quickly build up PI(3)P on the phagosomal surface as part of the maturation process. Vps34 is the PI3K that is responsible for the build up of PI(3)P on the phagosome. Vps34 is recruited to the phagosome by active Rab5, thus localization of Rab5 at the phagosome is required for PI(3)P deposition. However, even though Rab5 is retained on *M. tuberculosis* and *M. bovis* BCG-containing phagosomes, mycobacteria clear PI(3)P from the phagosomal surface as shown by studies with BCG-containing phagosomes (99). This effect is important because PI(3)P is required for phagosome

maturation through the recruitment of EEA-1, which is thought to orchestrate phagosome

fusion events with endocytic vesicles.

A notable protein that is excluded from the *M. tuberculosis*-containing

phagosomes is Rab7 (102). The absence of Rab7 is significant in that without Rab7,

RILP recruitment and thus phagosome-lysosome fusion are blocked. By blocking

lysosomal fusion, *M. tuberculosis* resists delivery of hydrolytic activity that could be

damaging to the bacterial cell or restrict growth.

1.9- *M. tuberculosis* effectors of phagosome maturation arrest

Exactly how *M. tuberculosis* arrests phagosome maturation is unclear. What is

clear from studies of the phenomenon is that it is a complex process that seemingly

involves a wide range of protein and lipid effectors. Phagosome maturation arrest by *M.*

tuberculosis likely includes, but is probably not limited to, selecting specific phagocytic

receptors used for uptake (55), interfering with PI(3)P signaling on the phagosomal

surface (40, 98, 99), deactivating Rab GTPases or preventing their acquisition (96), and

retaining Coronin-1A on the phagosomal membrane (52).

A small subset of *M. tuberculosis* protein and lipid molecules have been directly

tested for their ability to impact phagosome maturation and signaling. Interestingly, some

of these directly tested effectors may also have essential functions in the bacteria

unrelated to their proposed moonlighting role in phagosome maturation arrest (26, 104).

Below we review the list of candidate *M. tuberculosis* molecules implicated in

phagosome maturation arrest. These candidate effectors are presented in order of the

steps in phagosome maturation in which they are proposed to act.

The following sections describe several experiments involving multiple techniques and experimental settings. One of the more common experimental approaches used is to compare model phagosomes where the phagocytic particle is a latex bead to phagosomes with latex beads coated with a *M. tuberculosis* molecule to assess the impact of that molecule on the phagosome maturation process. Other experiments use *Escherichia coli* or non-pathogenic *M. smegmatis* as the phagocytic particle, which traffic to a mature phagosome after phagocytosis. In these latter experiments, a candidate *M. tuberculosis* effector is ectopically expressed by *M. smegmatis* or the host cell to assess its potential to affect phagosome maturation. In addition, some experiments utilize *M. bovis* BCG as a surrogate for *M. tuberculosis*. Like *M. tuberculosis*, BCG is able to block phagosome maturation, but has the benefit of being non-pathogenic, so it can be used in a BSL-2 laboratory.

Mannose-capped lipoarabinomannan (ManLAM)

ManLAM is a surface localized glycolipid of *M. tuberculosis*. ManLAM is reported in two separate studies to influence phagosome maturation. Phagosomes containing ManLAM-coated beads have reduced phagosome-lysosome fusion at early time points in phagocytosis assays compared to unconjugated beads, human serum albumin-coated beads, or *M. smegmatis* LAM-coated beads (41, 55). It is possible that ManLAM affects phagosome maturation via receptor selection. The reduction in phagosome maturation depends on the mannose cap that causes recognition of the molecule by the mannose receptor on phagocytes (55). Consistent with these results, *M. tuberculosis* containing phagosomes more readily fuse with lysosomes when

phagocytosis through the MR is blocked by preincubation of macrophages with excess mannan prior to infection (55). In fact, phagosome maturation occurs more quickly when *M. tuberculosis* or ManLAM coated beads uptake is skewed to phagocytosis through receptors other than the mannose receptor, such as the Fcγ receptor via opsonization with IgG. Kang *et al.* conclude that the effect of ManLAM on phagosome maturation is caused by skewing uptake of the bacteria through the MR (55).

Phosphotidylinosotol mannoside (PIM)

PIM is another surface localized glycolipid of *M. tuberculosis*. PIM is additionally reported to be released by *M. tuberculosis* and to traffic out of the phagosome (8). Compared to uncoated beads, phagosomes containing beads coated with PIM acidify less and accumulate transferrin receptor, a hallmark of *M. tuberculosis*-containing phagosomes. However, in vitro fusion assays show that PIM does not block late endosomal fusion events but rather, stimulates fusion of early endosomes with recycling endosomes. The interaction of *M. tuberculosis*-containing phagosomes with recycling endosomes is thought to be important for delivering nutrients to the bacteria (100). The data suggests that the role of PIM is to maintain a productive interaction with recycling endosomes as opposed to preventing interactions with late endosomes.

Lipoamide dehydrogenase (LpdC)

LpdC is an essential protein in *M. tuberculosis* probably due to its predicted metabolic function as part of the pyruvate dehydrogenase complex that converts pyruvate into acetyl-CoA (85). Surprisingly, despite the need for this protein to be cytoplasmic in

order to carry out its predicted essential function and the lack of an N-terminal signal sequence for export on LpdC, this protein is reported to be secreted by *M. tuberculosis* (65).

The unexpected connection between LpdC and phagosome maturation came when LpdC was pulled down by immunoprecipitation from BCG infected macrophages with antibodies to Coronin 1A (26). As discussed earlier *M. tuberculosis*-containing phagosomes retain Coronin 1A long after it would be released from a model phagosome. Compared to wild type J774 macrophages, in J774 macrophages depleted for Coronin 1A through siRNA, BCG-containing phagosomes localize more with LAMP-1, which is a marker of late endosomes and lysosomes (52). Thus, the retention of Coronin on mycobacteria-containing phagosomes contributes to phagosome maturation arrest. In the amoeba *Dictyostelium discoideum*, Coronin is known for its function in actin remodeling for phagocytosis and cell motility; however, the function of Coronin on *M. tuberculosis* phagosomes in macrophages appears to be independent of actin remodeling. Instead, it is reported that Coronin retention on the *M. tuberculosis* phagosome activates calcineurin, which in turn creates a calcium flux that interferes with proper phagosome maturation (53).

LpdC is shown to bind Coronin 1A in a cholesterol dependent manner, and to maintain Coronin 1A at the phagosomal membrane. While the *lpdC* gene is essential and cannot be deleted, when the *M. tuberculosis lpdC* gene is introduced into *M. smegmatis* it imparts *M. smegmatis* with the ability to retain Coronin-1 on the phagosome. However, the study did not determine if *M. tuberculosis* LpdC imparted the ability to arrest phagosome maturation. This recombinant *M. smegmatis* does show increased

intracellular survival in a short macrophage infection (26). These data suggest that the

predicted metabolic enzyme LpdC is moonlighting as a phagosome maturation arrest

effector in *M. tuberculosis*.

Secreted acid-phosphatase (SapM)

SapM is a secreted *M. tuberculosis* acid-phosphatase with activity over a wide

range of pH (5.5 to 8.0) (84). SapM contains a standard N-terminal Sec signal peptide,

indicating it is likely secreted through the general secretion (Sec) machinery (84).

SapM has phosphatase activity on PI(3)P *in vitro* (99). As mentioned above,

PI(3)P is an important signaling molecule on the surface of the phagosome and it is

required for efficient phagosome maturation. Indeed, in experiments where SapM activity

was inhibited in BCG infected macrophages, PI(3)P was acquired more quickly on the

phagosomal membrane than in untreated cells, suggesting that SapM does have a role in

preventing or slowing phagosome maturation (99). However, this experiment used

molybdate to inhibit SapM, which is a broad inhibitor of phosphatases and likely has

pleiotropic effects. Further study is still required to clarify the importance of SapM in

phagosome maturation arrest.

Nucleoside diphosphate kinase (NdkA)

NdkA is like LpdC in being another *M. tuberculosis* protein that is predicted to

have a metabolic function in the bacterial cytosol, in this case phosphorylating nucleoside

diphosphates (NDPs) to create nucleoside triphosphates (NTPs). The experiments of Sun

et al. argue for an additional function of NdkA in blocking phagosome maturation (96). It

is worthwhile to point out, however, that standard Ndk activity has not been demonstrated with NdkA. Instead, Ndk activity has been demonstrated for the *M. tuberculosis* adenylate kinase (Adk) protein. The *M. tuberculosis* NdkA protein has been shown to have phosphatase activity against GTP producing GDP (96). Rab5 and Rab7 are important host proteins for phagosome maturation and both require GTP for activity. When bound to GDP, Rab5 and Rab7 are inactive. *M. tuberculosis* NdkA has been shown to act on GTP that is bound to Rab5 or Rab7, suggesting it could act directly on the GTP-bound forms *in vivo* (96).

Despite lacking a signal sequence NdkA is reported to be secreted by *M. tuberculosis* and BCG grown in culture. Compared to wild type, a BCG strain depleted of NdkA by an antisense knock-down method is enriched in mature phagosomes four hours post-infection, indicating that NdkA plays a role in preventing phagosome maturation. The same BCG knockdown strain has decreased survival in macrophages compared to wild type indicating an important role for NdkA for the bacteria during intracellular life (96).

Protein kinase G (PknG)

PknG is a protein kinase that has been shown to phosphorylate serine residues on a peptide substrate *in vitro* (59). Based on results from testing a *M. tuberculosis pknG* mutant in a mouse model, PknG is clearly important for *M. tuberculosis* infection (22). However, the specific function of PknG is controversial as a result of two contrasting studies.   One study reports PknG to function in blocking phagosome maturation through an unknown process (104). PknG is found to be secreted at low levels (22), and even

detected in the cytoplasm of BCG infected host cells (104). The secreted nature of PknG is consistent with the protein being an effector secreted into the host cytoplasm. Additionally, *M. smegmatis* engineered to express *M. tuberculosis* PknG acquires the ability to block phagosome-lysosome fusion, as evidenced by decreased LAMP-1 localization to phagosomes containing the PknG expressing recombinant strain compared to wild type *M. smegmatis*. Additionally, compared to BCG expressing wild type PknG, BCG carrying a kinase dead allele of PknG was found more readily in LAMP-1 positive phagosomes, indicating an important role for PknG and its kinase activity in blocking phagosome maturation (104). In contrast to these above data, a second study, which used a *pknG* mutant of *M. tuberculosis* suggests a completely different role for PknG during infection. *M. tuberculosis pknG* mutants are slow growing and levels of glutamine and glutamate build up in the mutant, suggesting a role for PknG in amino acid metabolism (22).

Protein tyrosine phosphatase (PtpA)

PtpA is a tyrosine phosphatase of *M. tuberculosis* that is proposed to act on host phosphorylation signaling cascades. Separate studies report roles for *M. tuberculosis* PtpA in either phagocytosis or phagosome maturation arrest. One study found that ectopic expression of PtpA in Raw264.7 cells repressed phagocytosis of *M. tuberculosis* and zymosan. Zymosan is yeast cell wall particles often used as model phagocytic targets (18).

In a second study of PtpA, the phosphatase was shown to dephosphorylate the vacuolar protein sorting-associated protein 33B (VPS33B). VPS33B is a component of

the multi-protein homotypic fusion and vacuole sorting (HOPS) complex. The HOPS complex assembles on the phagosomal membrane and is directly involved in activating lysosome fusion events (29). In an *in vitro* vesicle fusion assay, addition of active PtpA and VPS33B together, but not individually, reduced fusion events (6), suggesting that PtpA activity on VPS33B is important for blocking vesicle fusion.

In a third study of PtpA, a connection between PtpA and V-ATPase was identified. Lysosomes maintain a high concentration of V-ATPase in their membranes. When expressed ectopically by the host cell PtpA binds to the H subunit of V-ATPase and prevents lysosome fusion with *E. coli*-containing phagosomes. This block in phagosome-lysosome fusion requires both binding to V-ATPase and the phosphatase activity of PtpA (106). PtpA appears to be involved in blocking the final step of phagosome maturation, phagosome-lysosome fusion, and thus protects the bacteria from destruction by the hydrolytic mature phagolysosome. Interestingly, PtpA appears to affect phagosome maturation and survival in human cell lines, but not in mice, suggesting a host specific effect of PtpA (6, 45, 106).

Other candidate effectors of *M. tuberculosis* phagosome maturation arrest

Four independent transposon mutant screens of *M. tuberculosis* and *M. bovis* BCG have produced lists of genes that when mutated result in failure to prevent phagosome maturation. However, there is strikingly little overlap between the genes identified in the various phagosome maturation mutant screens (15, 62, 77, 95). The differences between these screens remain to be resolved but they may lie in the different mycobacterial strains, cell lines, or readouts of phagosome maturation used.

From two of the screens for effectors of phagosome maturation, components of the ESX-1 specialized secretion system have been shown to be required for phagosome maturation arrest (14, 62). ESX-1 has long been known to be required for virulence, but how ESX-1 is involved in virulence remains unknown. Phagosome maturation arrest may be part of the ESX-1 system's role in virulence. Lipid metabolic genes are another category of commonly identified genes in these screens, possibly due to the role of LAM, PIM and potentially other lipids in phagosomal trafficking. Interestingly, three of the four screens picked up genes directly involved in the molybdopterin biosynthetic pathway. Molybdopterin is an enzyme cofactor that is used by bacteria in carbon, nitrogen, and sulfur metabolism. *M. tuberculosis* and *M. bovis* have an expanded set of molybdopterin biosynthetic genes compared to *M. smegmatis* suggesting potential importance in virulence (105). It is possible that there is an effector of phagosome maturation that uses molybdopterin as a cofactor. In addition to a role in phagosome maturation arrest, molybdopterin biosynthetic genes have elsewhere been implicated in virulence in diverse animal models such as: monkeys, mice, and human cell lines (16, 28, 83).

1.10- *M. tuberculosis* modulation of other innate immune responses

***M. tuberculosis* inhibition of host cell apoptosis**

Another mechanism *M. tuberculosis* employs to promote replication in macrophages is to avoid host-protective apoptosis. This effect skews host cell death toward necrosis, which has the effect of promoting cell-to-cell spread of *M. tuberculosis*.
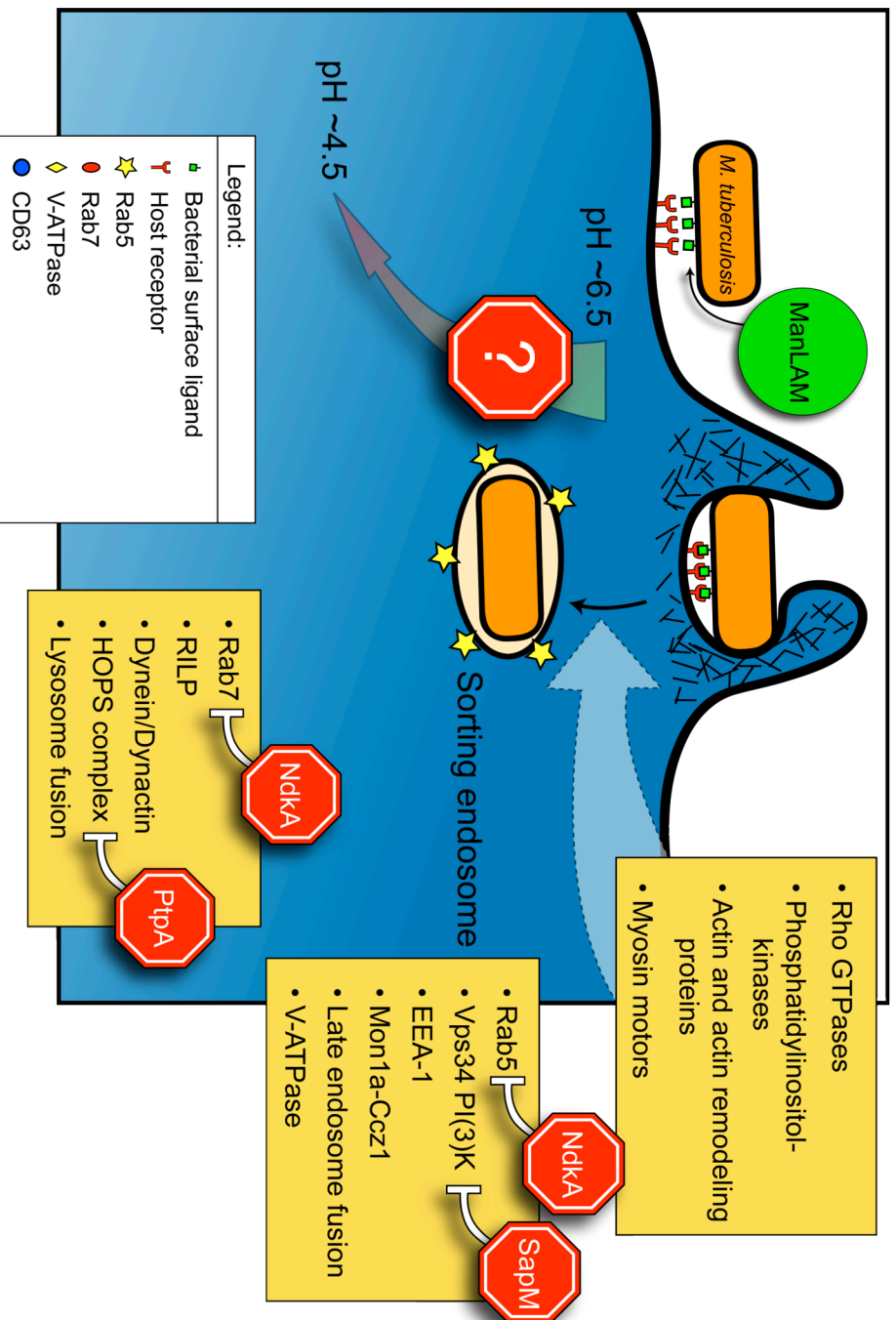
Figure 1-2. *M. tuberculosis* arrests normal phagosome maturation. Through a process that has yet to be fully elucidated *M. tuberculosis* halts maturation at a very early stage. Several bacterial components have predicted roles in blocking phagosome maturation. The *M. tuberculosis* effectors of phagosome maturation include both protein and lipid-based molecules.

Further, apoptosis is shown to be a mechanism macrophages can use to kill or contain mycobacteria (27, 39, 67). These studies demonstrate a correlation between apoptosis and reduced intracellular replication or even killing of mycobacteria. In addition to the innate role of apoptosis in clearing bacterial infection, increased apoptosis can help establish a more robust adaptive immune response by antigen presentation via apoptotic corpses leading to better control of *M. tuberculosis* (48).

There are a few proposed mechanisms for how *M. tuberculosis* limits host cell apoptosis. One mechanism involves blocking apoptosis caused by the extrinsic pathway. The extrinsic pathway refers to apoptosis caused by a signal external to the dying cell. TNF-α is a potent signal for initiating the extrinsic pathway. One study shows that *M. tuberculosis* infected macrophages increase secretion of TNF-α receptor2 (TNFR2), as a result of increased IL-10 production, which titrates up active TNF-α and prevents apoptosis induction through the extrinsic pathway (7). A second way *M. tuberculosis* inhibits apoptosis is through repression of reactive oxygen species (ROS). ROS are potent regulators of apoptosis through both the extrinsic and intrinsic pathways (92). One study shows that the *M. tuberculosis* NADPH dehydrogenase NuoG prevents TNF-α mediated (extrinsic) apoptosis by reducing the ROS response to infection (66).

M. tuberculosis inhibition of host cell inflammatory response

Another hallmark of *M. tuberculosis* infection is the dampening of the innate immune response. Macrophages have many receptors such as Toll-like receptors that detect pathogens and signal the cell to respond accordingly. In general, responding to a

bacterial pathogen means a robust cytokine response that signals there is danger, causes

macrophage activation and coordination of the adaptive immune response.

Macrophages infected with *M. tuberculosis* do mount an innate immune response

(9). However, that immune response is dampened by live *M. tuberculosis*, and some

purified components of the bacteria on their own have the ability to dampen the response

(38, 68, 71, 72, 75). This effect is important because some of the cytokines influenced by

M. tuberculosis, specifically TNF-α, IL-6 and RNI, have the ability to control *M.

tuberculosis* intracellular replication and activate macrophages. Thus, this dampening of

cytokine responses could be important to limiting macrophage activation and control of

M. tuberculosis. Additionally, because the adaptive immune response is coordinated by

signals from the innate response, dampening of the innate response can limit the scale

and effectiveness of the adaptive response so as to make long-term persistence of *M.

tuberculosis* possible (9).

1.11- Protein export systems and their role in virulence

To facilitate their interactions with the host, bacterial effectors are most

commonly proteins or lipids that are exported to the bacterial cell surface or released into

the host environment. In order for proteins to move from their site of synthesis in the

cytoplasm to the cell wall and beyond, protein export systems are required.

Conserved protein export

One protein export system that is found in all bacteria, is the general Sec protein export

pathway. This system is used for the bulk of protein export in bacteria and is essential to

bacterial life. Preproteins, which contain a N-terminal Sec signal peptide, are exported across the cytoplasmic membrane in an unfolded state by the Sec system. Once exported, the signal peptide is cleaved and the protein folds into its mature form.

A central component of the Sec system is the SecYEG integral membrane translocon, which serves as the pore through which exported proteins travel across the cytoplasmic membrane. In addition to the YEG pore, the SecA ATPase is essential for targeting cytoplasmic preproteins to the translocon, and then supplying the energy to push the protein through the pore (reviewed in (69, 76)).

Specialized protein export

The general Sec pathway is important for exporting a wide range of proteins, including virulence factors. However, bacterial pathogens often additionally employ specialized secretion systems for the purpose of delivering virulence factors into a host cell. Some specialized secretion systems offer mechanisms to deliver proteins directly to the host cell cytoplasm making them ideal for effector delivery. Good examples of these types of specialized secretion systems are the Type III secretion systems (T3SS) found in many pathogens including *Salmonella*, *Pseudomonas*, *Shigella*, and *Yersinia* (42); and the Type IV secretion system used by *Legionella* (19). Notably, some T3SS and T4SS systems are used to affect phagocytosis and phagosome trafficking (42, 50).

M. tuberculosis **specialized protein export**

The *M. tuberculosis* genome is known to encode two types of specialized secretion systems: the ESX systems and the SecA2 system (21). In *M. tuberculosis*, there

are five homologous ESX secretion systems (ESX1-5). ESX-1 is important for virulence. The ESX-1 system is deleted from the genome of *M. bovis* BCG, which accounts for some of the attenuation of virulence of the BCG vaccine strain (78). In *M. tuberculosis*, ESX-1 protein export is required for virulence in macrophages and mice (37, 46, 94). Further, the ESX-1 system is shown to be important for blocking phagosome maturation (15, 62), and dampening of the inflammatory response to *M. tuberculosis* (63, 94). However, at this point, neither the mechanism through which ESX-1 performs these virulence functions, nor the responsible secreted proteins are known. There are four other ESX systems in *M. tuberculosis*. ESX2-5 are homologous to the ESX-1 system, but are predicted to be expendable in virulence (80, 86).

The other known specialized secretion system in *M. tuberculosis* is the SecA2 accessory Sec pathway (11). Mycobacteria and some Gram-positive bacteria are unique in that they have two copies of *secA* (81). The two gene products are termed SecA1 and SecA2. Each SecA has unique functions. In mycobacteria SecA1 performs the bulk of protein export for the cell as part of the general Sec pathway described above; thus, SecA1 is essential. In contrast, SecA2 is required for the export of a small subset of proteins, and is dispensable for *in vitro* growth (11).

Central to this thesis is the fact that the SecA2 protein export pathway is required for full virulence of *M. tuberculosis* (12, 60). A *M. tuberculosis* deletion mutant of the *secA2* gene termed Δ*secA2* is defective for growth in macrophages and in mice (12, 60). The thrust of this thesis is to understand the cause of the virulence defect in the Δ*secA2* mutant and elucidate the role of the SecA2 protein export pathway in *M. tuberculosis*

virulence. To this end, we followed up on phenotypes of the Δ*secA2* mutant in macrophages that could possibly account for the role of the SecA2 system in virulence.

The Δ*secA2* mutant induces an increased inflammatory response in infected macrophages compared to wild type *M. tuberculosis*. In response to infection with the Δ*secA2* mutant, macrophages secrete more TNF-α, IL-6, and reactive nitrogen intermediates (RNI) (60). These three host molecules are stimulated by *M. tuberculosis* through the TLR-2/MyD88 system (13, 51, 79, 90, 91, 97). Furthermore, TNF-α, IL-6, and RNI are all established as being essential for controlling *M. tuberculosis* replication in the host. Thus, in Chapter Two we explored the possibility that the role of the SecA2 system in virulence is to limit protective cytokine responses. Our results indicated that in the context of macrophages and mice, neither MyD88 responses nor TNF-α can account for the Δ*secA2* mutant intracellular growth defect.

Second, the Δ*secA2* mutant of *M. tuberculosis* induces more apoptosis than wild type *M. tuberculosis* in infected macrophages (48). As mentioned above, *M. tuberculosis* inhibition of apoptosis could promote intracellular replication of the bacillus. Therefore, in Chapter Three we examined the role of the increased apoptosis observed with the Δ*secA2* mutant to determine if it accounts for the Δ*secA2* mutant intracellular growth phenotype. Our results indicate that an effect on apoptosis is also insufficient to explain the intracellular replication phenotype of the Δ*secA2* mutant. Also in Chapter Three we tested a role for SecA2 in phagosome maturation arrest and demonstrate for the first time that the SecA2 system is required for *M. tuberculosis* blocking phagosome maturation.

The Δ*secA2* mutant resides in phagosomes that are more acidic than phagosomes containing wild-type *M. tuberculosis*. Additionally, the Δ*secA2* mutant-containing phagosome show signs of fusion with lysosomes, which wild type *M. tuberculosis* avoids. Finally, by chemically blocking the acidification of the Δ*secA2* mutant-containing phagosome we were able to effectively rescue its intracellular growth phenotype. This data indicates that the SecA2 export pathway promotes *M. tuberculosis* replication in macrophages by blocking phagosome maturation.

The data presented in Chapter Three suggests that SecA2 is involved in secreting one or more effectors of phagosome maturation. Therefore, in Chapter Four we tested a set of candidate effectors of phagosome maturation arrest for evidence of secretion by the SecA2 system. We found that SecA2 has an effect on the levels of SapM, a putative effector of PI(3)P on the surface of phagosomes. This intriguing result opens up new questions for future research. Can the effect of the SecA2 system on SapM explain the role of the SecA2 system in macrophages? How does SecA2 influence SapM levels?

This thesis provides a better understanding of how SecA2 promotes *M. tuberculosis* growth in macrophages. Additionally, it demonstrates the importance of phagosome maturation arrest for *M. tuberculosis* virulence. It is likely that *M. tuberculosis* secretes multiple effectors of phagosome maturation. The phagosome maturation phenotype of the Δ*secA2* mutant we show in this thesis indicates there is either a single critical effector or multiple effectors that depend on the SecA2 protein export pathway. Future studies will continue to work towards identifying the SecA2-dependent effectors of phagosome maturation arrest, achieving two goals: understanding

the role of the SecA2 system in virulence and identifying substrates of the SecA2 protein export pathway.

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CHAPTER II

EXAMINING THE ROLE OF THE INCREASED INFLAMMATORY RESPONSE TO
THE Δ*SECA2* MUTANT OF *MYCOBACTERIUM TUBERCULOSIS* [1]

2.1- Introduction

Resident alveolar macrophages are often the first immune cells encountered by

bacteria that get inhaled into the lung. These macrophages are well equipped to consume

and digest most bacteria that land in the alveolar spaces of the lung.   However, during

pulmonary infection, *Mycobacterium tuberculosis* replicates within these macrophages in

the lung. In the case of *M. tuberculosis* infection, it is thought that the bacteria can

survive and replicate inside macrophages because primary macrophage innate immune

responses are blocked or dampened by the pathogen (10).

Macrophages have many receptors such as Toll-like receptors (TLRs) that detect

pathogens and signal the cell to respond accordingly. In general, responding to a bacterial

pathogen means a robust cytokine response that signals there is danger, causes

macrophage activation and coordination of the adaptive immune response. While

macrophages infected with *M. tuberculosis* do mount an innate immune response (2), the

degree of the response is dampened by live *M. tuberculosis* and even some purified

components of the bacteria (8, 17-20). The effect of *M. tuberculosis* on the immune

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response is likely to be important because some of the regulated immunomodulatory molecules, specifically TNF-α, IL-6 and reactive nitrogen intermediates (RNI) can control *M. tuberculosis* intracellular replication and induce killing of bacteria in macrophages (4, 6). Additionally, dampening of the innate response can affect the adaptive response so as to make long-term persistence possible (2).

TNF-α, IL-6 and RNI are all shown to contribute to the production of a successful host response to *M. tuberculosis* infection in a low dose aerosol mouse model of infection (7, 15, 16). In mice defective for TNF-α, IL-6 or RNI *M. tuberculosis* reaches a higher bacterial burden in the lung and the mice die from infection in 40 days as compared to ~200 days in wild-type C57BL/6 mice (7, 15, 16). The host adapter molecule MyD88 is required for TLR signaling responses to *M. tuberculosis* that include TNF-α, IL-6, and RNI responses (reviewed in (11, 12, 21)). Like the mice deficient for TNF-α, IL-6 or RNI, in mice lacking MyD88 *M. tuberculosis* replication during the primary phase of infection is unchecked (7, 15, 16, 22) and mice succumb to the infection within 40 days of exposure to a low dose (100-200 cfu) of bacteria in the lung.

The SecA2 accessory protein export pathway is important for virulence in *M. tuberculosis* (3, 13). A Δ*secA2* mutant of *M. tuberculosis*, which carries an in-frame unmarked deletion of the *secA2* gene, is defective for growth in cultured primary bone marrow derived macrophages and during the acute phase of mouse infection (3, 13). During the acute phase of infection, *M. tuberculosis* replicates in macrophages, which links the macrophage growth defect and the *in vivo* growth defect. In order to understand the role of the SecA2 export pathway in *M. tuberculosis* in macrophages, we previously analyzed macrophage responses to infection with the Δ*secA2* mutant. Bone marrow-

derived macrophages infected with the Δ*secA2* mutant, compared to the wild type H37Rv

strain of *M. tuberculosis*, produce higher levels of TNF-α, IL-6 and RNI indicating a role

for the SecA2 pathway in dampening the immune response (13). Because all these

immunomodulatory molecules upregulated in Δ*secA2* mutant infected macrophages are

controlled by MyD88 we proposed that the increased inflammatory response elicited by

the mutant would depend on MyD88. We also hypothesized that intracellular replication

of the Δ*secA2* mutant was inhibited as a result of increased macrophage production of

inflammatory cytokines leading to more highly activated macrophages. In this chapter

we explored further the role of the increased inflammatory response to the Δ*secA2* mutant

of *M. tuberculosis*.

2.2- Results

**MyD88 is required for the inflammatory response of macrophages to infection with
the Δ*secA2* mutant.**

　　　M. tuberculosis elicits an innate immune response through TLRs 2, 4, and 9.

These TLRs signal through the adapter molecule MyD88, although TLR-4 also has a

MyD88-independent pathway. The Δ*secA2* mutant of *M. tuberculosis* elicits a more

robust macrophage response than H37Rv, as shown by increased TNF-α, IL-6 and RNI

production. Because MyD88 is required to induce all three of these immunomodulatory

molecules in responses to *M. tuberculosis*, we tested whether the more robust response to

the Δ*secA2* mutant was MyD88 dependent. Because SecA2 has previously been

demonstrated to export two lipoproteins of *M. smegmatis* (9), we also tested the role of

TLR-2 in the inflammatory response. TLR- recognizes exported lipoprotein agonists.

To determine if the increased macrophage response to the Δ*secA2* mutant is MyD88-dependent, we compared TNF-α and IL-6 levels in supernatants from bone marrow macrophages prepared from C57BL/6 or MyD88 deficient (MyD88$^{-/-}$) mice that were infected with H37Rv or the Δ*secA2* mutant. In response to infection with H37Rv or the Δ*secA2* mutant, MyD88$^{-/-}$ macrophages did not produce detectable levels of TNF-α or IL-6 (Figure 2-1A). This result indicates that MyD88 is absolutely required for the TNF-α and IL-6 response to both H37Rv and the Δ*secA2* mutant. Further, this result indicates that the more robust response elicited by the Δ*secA2* mutant depends on the MyD88 pathway.

To determine if TLR-2 is also required for this cytokine response, macrophages from TLR-2$^{-/-}$ mice were infected with H37Rv or the Δ*secA2* mutant and the level of secreted TNF-α was assayed. Although considerably reduced from what is seen with C57BL/6 macrophages, TNF-α secretion was detected from infected TLR-2$^{-/-}$ macrophages. Interestingly, 2-fold more TNF-α was produced by TLR-2$^{-/-}$ macrophages infected with the Δ*secA2* mutant in comparison to H37Rv, which is similar to the 2-fold difference in TNF-α levels induced by the same strains with C57BL/6 macrophages (Figure 2-1B). From these experiments, we conclude that TLR-2 signaling occurs in response to the Δ*secA2* mutant; however, the increased inflammatory response to the Δ*secA2* mutant is not TLR-2 dependent. While these data rule out increased TLR-2 signaling as the source of the increased inflammatory response to the Δ*secA2* mutant, they does not rule out the possibility of there being increased signaling through other TLRs.

Increased TNF-α does not explain the Δ*secA2* mutant growth defect.

Based on our past data showing increased TNF-α production by macrophages infected with the Δ*secA2* mutant, we hypothesized that the higher levels of TNF-α could be responsible for the intracellular growth defect of the Δ*secA2* mutant. TNF-α is a pro-inflammatory cytokine with effects that include activation of antimicrobial activity in macrophages and host cell apoptosis. TNF-α is shown to have a crucial role in controlling replication of *M. tuberculosis* in mice (7). Additionally, TNF-α can work synergistically with another mediator (e.g. interferon gamma) to arrest *M. tuberculosis* intracellular replication in cultured macrophages (4, 6). Thus, the higher levels of TNF-α elicited by the Δ*secA2* mutant could act to control *M. tuberculosis* replication.

To determine if the higher TNF-α levels are responsible for the intracellular growth defect of the Δ*secA2* mutant, we compared bacterial replication in macrophages from TNF-α$^{-/-}$ and C57BL/6 mice infected with H37Rv or Δ*secA2* mutant *M. tuberculosis*. If the increased level of TNF-α observed in Δ*secA2* mutant infected macrophages is the cause of the intracellular growth phenotype, removing TNF-α from macrophages should abrogate the Δ*secA2* mutant growth phenotype. These experiments demonstrated the Δ*secA2* mutant growth defect is not recovered in TNF-α$^{-/-}$ macrophages when compared to C57BL/6 macrophages (Figure 2-2A). Thus, elevated levels of TNF-α do not explain the Δ*secA2* mutant intracellular growth defect.
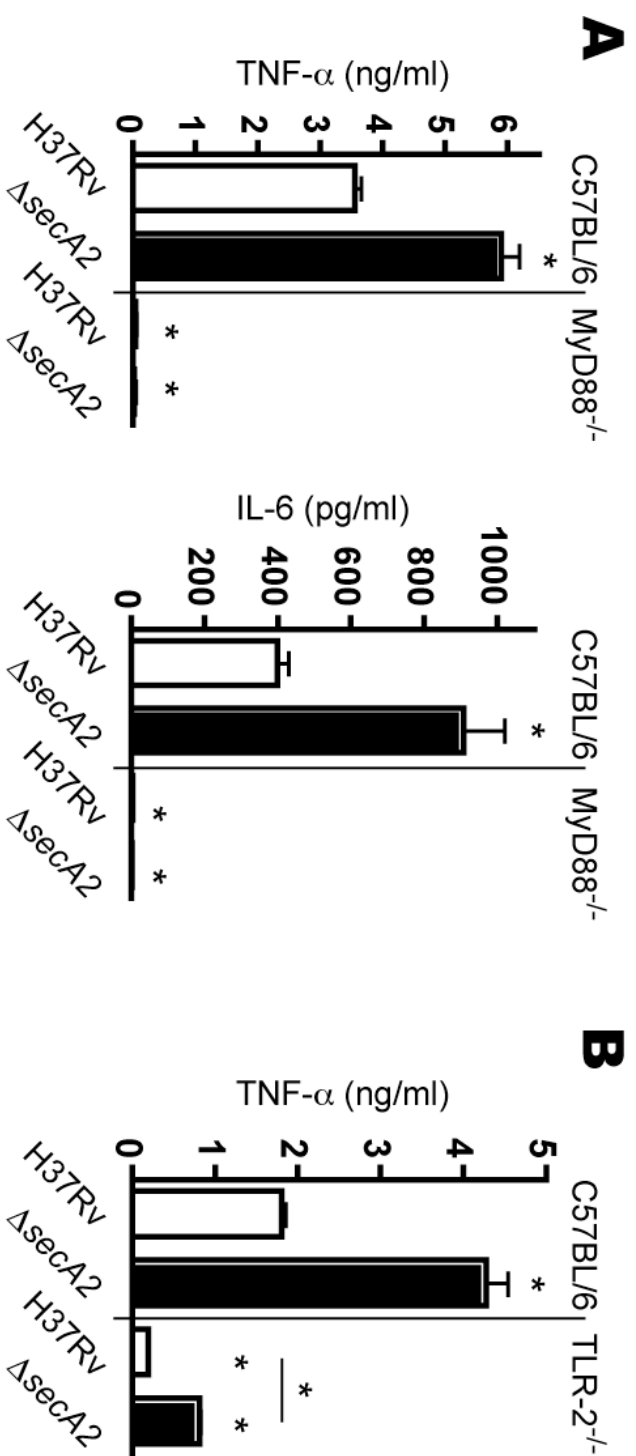
Figure 2-1. MyD88 and TLR-2 are required for TNF-α and IL-6 production in response to the ΔsecA2 mutant of M. tuberculosis. ELISA data showing TNF-α and IL-6 levels in supernatants of *M. tuberculosis* infected macrophages. Macrophages were infected for 24 hours at an MOI of 1:1 with wild type or the ΔsecA2 mutant, supernatants collected and assayed for cytokine levels. A representative experiment is shown. A. TNF-α and IL-6 levels were tested in C57BL/6 or MyD88⁻/⁻ macrophages. B. TNF-α levels were tested in C57BL/6 or TLR-2⁻/⁻ macrophages. Bars show mean of three replicate wells +SD. *p ≤ 0.05 compared to H37Rv in C57BL/6 macrophages, by Student's *t* test.

A MyD88 dependent response to the Δ*secA2* mutant does not explain the growth defect.

As another way to determine if the more robust innate response to the Δ*secA2* mutant is responsible for the mutant's attenuation, we infected macrophages from MyD88$^{-/-}$ mice. As mentioned above, our prior studies identified three immunomodulatory molecules that are increased in macrophages infected with the Δ*secA2* mutant and all are known to be under the control of MyD88. Further, we showed MyD88$^{-/-}$ macrophages fail to produce detectable levels of TNF-α and IL-6 in response to *M. tuberculosis* (Figure 2-1). However, the central role of MyD88 in TLR and IL-1β signaling makes MyD88$^{-/-}$ mice more broadly defective in innate immune response signaling. Therefore, if the Δ*secA2* mutant elicits more MyD88-dependent signaling in macrophages then there may be MyD88-dependent factors, other than TNF-α, that are responsible for the intracellular growth phenotype of the Δ*secA2* mutant. To test this possibility we infected macrophages from MyD88$^{-/-}$ mice. If the Δ*secA2* mutant intracellular growth defect is due to a more robust MyD88-dependent response to *M. tuberculosis*, then the Δ*secA2* mutant phenotype should be abrogated and the mutant should behave like H37Rv in MyD88$^{-/-}$ macrophages.

Like in the TNF-α$^{-/-}$ macrophages, the Δ*secA2* mutant remained defective for growth in MyD88$^{-/-}$ macrophages when compared to H37Rv (Figure 2-2B). These data indicate that, at least on its own, a MyD88-dependent inflammatory response to the Δ*secA2* mutant is not responsible for the intracellular growth defect.

The Δ*secA2* mutant is attenuated in mice even in the absence of TNF-α or MyD88.

The above experiments were performed with cultured macrophages in a single cell system. We tested the importance of TNF-α and MyD88-dependent immune responses to phenotypes of the Δ*secA2* mutant in a whole animal *in vivo* model of infection. In this model, mice are infected with approximately 200 cfu/lung using a whole body aerosol delivery system (Madison Aerosol Chamber). Over time, we monitored bacterial burden in the lungs of infected mice to determine rate of bacterial growth in the lung. In this model, H37Rv grows from ~200cfu/lung to ~1 x 10^7 cfu/lung during the first three weeks of infection. The initial growth phase is followed by a persistence phase in which an antigen specific TH1 response causes the lung burden to plateau and maintain homeostasis for the remainder of the infection. The Δ*secA2* mutant has a growth defect during the first three weeks and reaches a lower final burden of ~1 x 10^6 cfu/lung (3). At three weeks, post-infection the Δ*secA2* mutant still enters a persistence phase and the lung burden maintains homeostasis through the rest of the infection.

To determine if TNF-α and MyD88 are involved in the Δ*secA2* mutant's attenuation *in vivo*, we infected C57BL/6, TNF-α$^{-/-}$, or MyD88$^{-/-}$ mice with H37Rv or the Δ*secA2* mutant. If the Δ*secA2* mutant is defective for growth in mice because it fails to repress TNF-α or MyD88-dependent immune responses, then infection of mice lacking these regulatory molecules will abrogate the attenuated phenotype. Both TNF-α$^{-/-}$ and MyD88$^{-/-}$ mice are highly susceptible to *M. tuberculosis* infection (7, 22). When infected

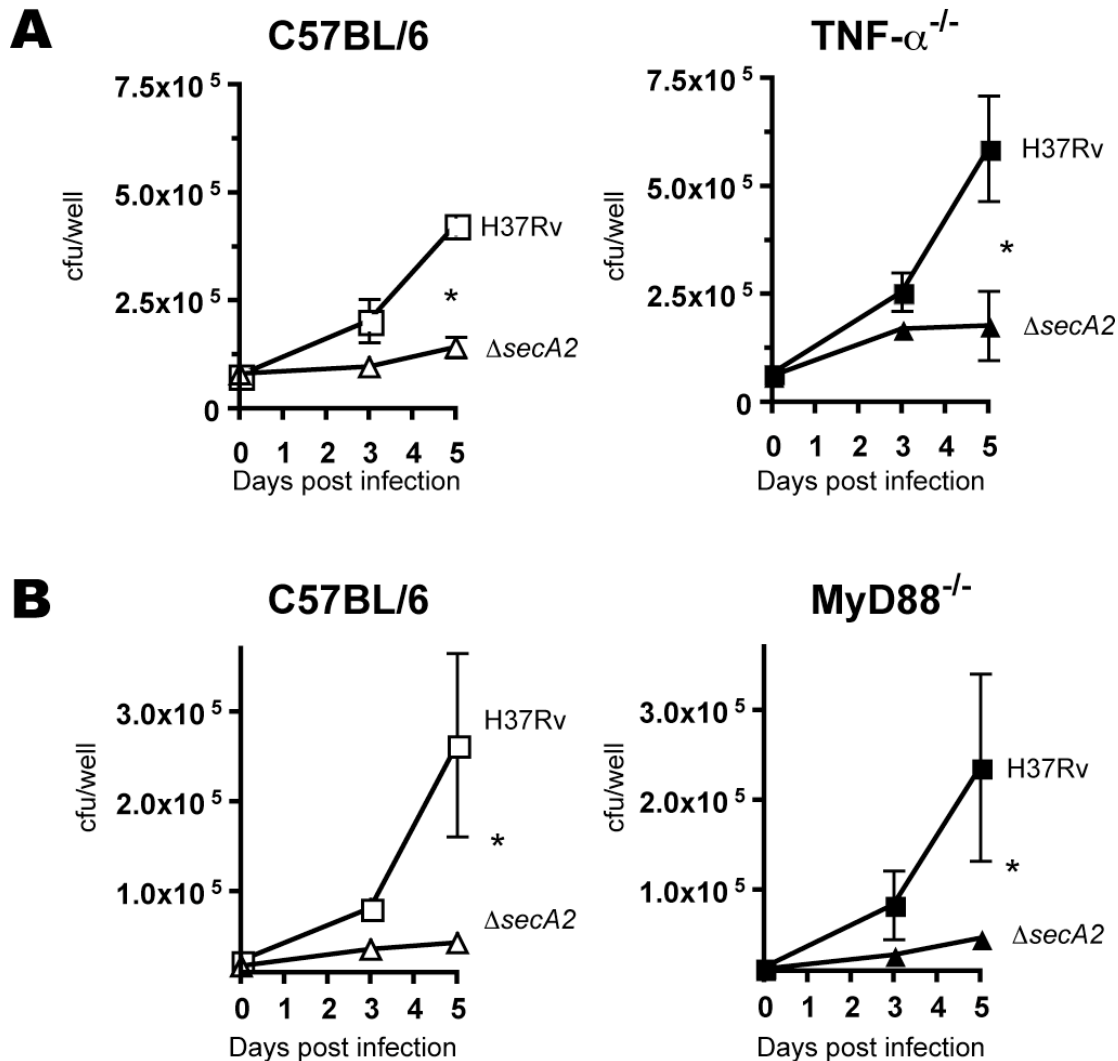**Figure 2-2. The Δ*secA2* mutant remains attenuated in macrophages lacking TNF-α or MyD88.** A. C57BL/6 or TNF-α^{-/-} macrophages infected with wild type or the Δ*secA2* mutant and monitored for intracellular replication over five days. Shown is a representative experiment of three. Points are means of triplicate wells +/- SD. *p ≤ 0.05 as determined by Student's *t* test. C. C57BL/6 or MyD88^{-/-} macrophages infected with wild type or the Δ*secA2* mutant and monitored for intracellular replication over five days. Shown is a representative experiment of three. Points are means of triplicate wells +/- SD. *p ≤ 0.05 as determined by Student's *t* test.

with *M. tuberculosis*, compared to C57BL/6, both TNF-$\alpha^{-/-}$ and the MyD88$^{-/-}$ strains of mice have 10-100 fold higher lung burdens at the three-week time point. This is due to the failure to establish TH1 control of the infection as normally occurs by the three-week timepoint, and both mouse strains succumb to infection within 40 days of exposure as opposed to the 200 day timepoint.

We infected TNF-$\alpha^{-/-}$ or MyD88$^{-/-}$ mice with wild type or the $\Delta secA2$ mutant of *M. tuberculosis*. We then assayed bacterial burden in the lungs of mice over a three-week period. The experiments demonstrated that even in TNF-$\alpha^{-/-}$ or MyD88$^{-/-}$ animals, the $\Delta secA2$ mutant exhibited a replication defect compared to H37Rv (Figure 2-3). These data do not support a role for TNF-α or MyD88 in the $\Delta secA2$ mutant *in vivo* growth defect. These results also reinforce the results from testing the strains in macrophages.

2.3- Discussion

The $\Delta secA2$ mutant has an intracellular growth defect, and elicits an increased inflammatory response from macrophages compared to H37Rv. In this study we tested whether the increased inflammatory response to the $\Delta secA2$ mutant is the cause of the mutant's intracellular growth defect. Using macrophages derived from TNF-$\alpha^{-/-}$ and MyD88$^{-/-}$ mice and the mice themselves, we determined if elevated MyD88-dependent responses, or TNF-α in particular, account for the $\Delta secA2$ mutant intracellular and *in vivo* growth defects.
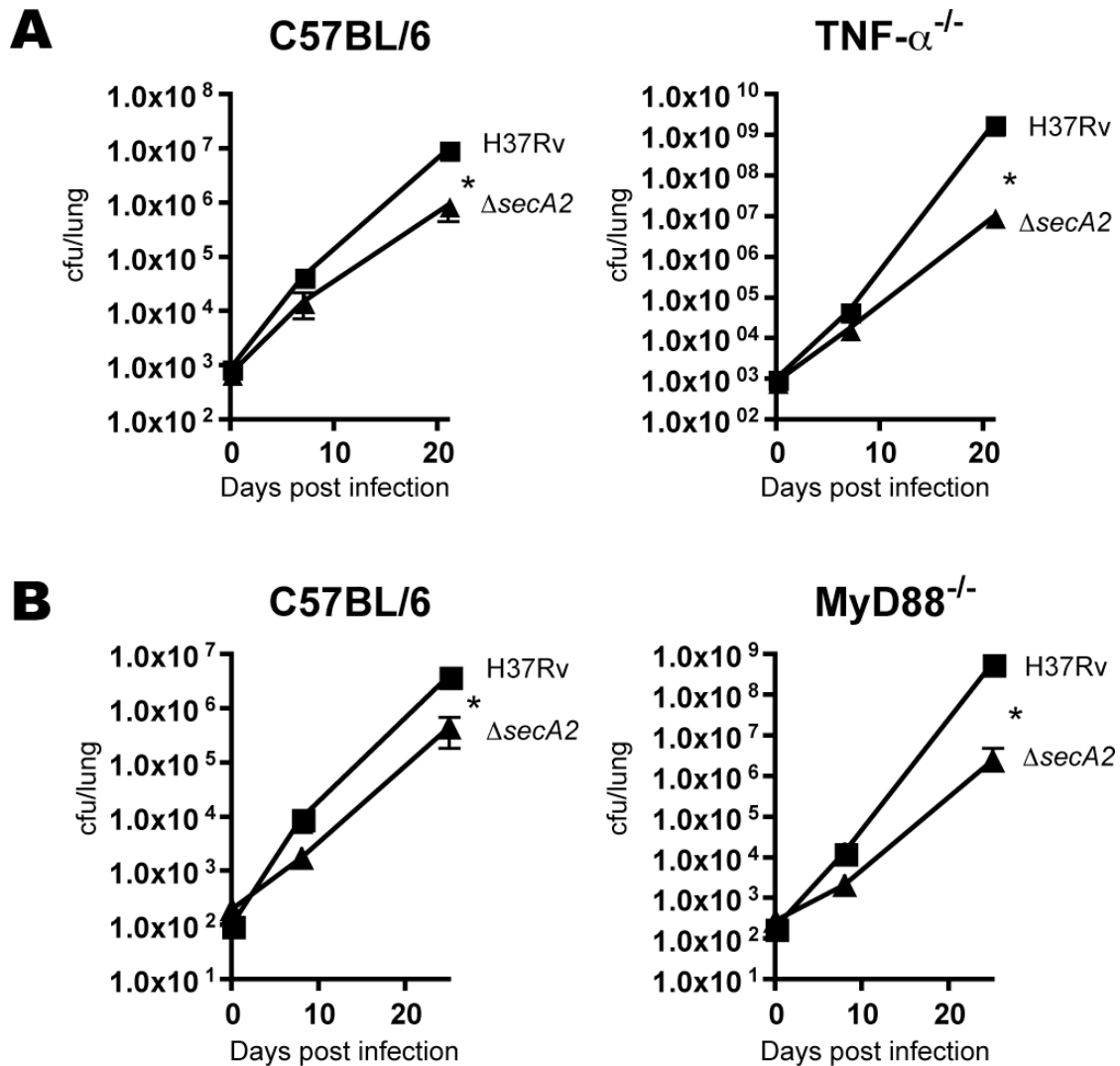
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Figure 2-3. The Δ*secA2* mutant remains attenuated in mice lacking TNF-α or MyD88. A. Lung burden of C57BL/6 or TNF-α$^{-/-}$ mice infected via aerosol with H37Rv or Δ*secA2* mutant *M. tuberculosis*. Points represent mean lung burden from four animals +/- SD. *p ≤ 0.05 compared to H37Rv by Student's *t* test. B. Lung burden of C57BL/6 or MyD88$^{-/-}$ mice infected via aerosol with H37Rv or Δ*secA2* mutant *M. tuberculosis*. Points represent mean lung burden from four animals +/- SD. *p ≤ 0. 05 compared to H37Rv by Student's *t* test.

First, we showed that the Δ*secA2* mutant did not elicit a detectable TNF-α or IL-6 response in MyD88^{-/-} macrophages. This result reinforces past reports of MyD88 being required to elicit these responses to *M. tuberculosis*. In addition, our results showed that MyD88 is essential for the Δ*secA2* mutant response. We further showed that in TLR-2^{-/-} macrophages the TNF-α response was reduced to both H37Rv and the Δ*secA2* mutant when compared to C57BL/6 macrophages, but that there was still a significant difference in TNF-α elicited by the Δ*secA2* mutant versus H37Rv. Thus, we conclude that the cause of the increased inflammatory response to the Δ*secA2* mutant is TLR-2 independent, although TLR-2 does amplify the amount of TNF-α produced in response to both H37Rv and the Δ*secA2* mutant. Recently, a *hip1* mutant of *M. tuberculosis* was reported to induce more robust MyD88-dependent responses by macrophages. However, in the case of *hip1*, it appears that the more robust immune response observed with the mutant is TLR-2 dependent, suggesting the mutant elicits increased TLR2 signaling to achieve this effect.

In assessing the significance of the increased immune responses of Δ*secA2* mutant infected macrophages, we found that the Δ*secA2* mutant has an intracellular growth defect even in macrophages derived from MyD88^{-/-} and TNF-α^{-/-} mice. These macrophage experiments revealed that TNF-α and MyD88 are not required to control the intracellular growth of the Δ*secA2* mutant. However, it is important to point out that we do not know the scope of the increased inflammatory response and there may still be important MyD88-independent factors. With this caveat in mind we can only rule out MyD88-dependent inflammation in the Δ*secA2* mutant growth defect.

In addition to the macrophage growth assays we performed mouse infections in TNF-$\alpha^{-/-}$ and MyD88$^{-/-}$ mice. The growth of the $\Delta secA2$ mutant in these mice recapitulated the macrophage results that showed that the $\Delta secA2$ mutant phenotype was not rescued in either the TNF-$\alpha^{-/-}$ of MyD88$^{-/-}$ mice. Thus, we conclude that there must be other factors besides dampening MyD88 responses that the SecA2 system is doing to promote growth in macrophages and mice.

Earlier, our laboratory had obtained preliminary data from testing the $\Delta secA2$ mutant in MyD88$^{-/-}$ and TNF-$\alpha^{-/-}$ macrophages that led to the opposite conclusions (unpublished results). In these earlier studies, the mutant phenotype of the $\Delta secA2$ mutant was not maintained but rescued in MyD88$^{-/-}$ and TNF-$\alpha^{-/-}$ macrophages (14). We believe the most likely explanation for the discrepancy in results is that different batches of L-929 cell conditioned media (LCM) were used to differentiate bone marrow cells into macrophages in the different experiments. LCM contains granulocyte monocyte-colony stimulating factor (GM-CSF) (5), an important component in stimulating hematopoietic stem cells to differentiate into macrophages. However, LCM is media recovered from cells grown in culture and it contains many secreted factors. The composition of LCM can vary batch to batch, and different batches could affect bone marrow differentiation differently. We do, in fact, see differences in the number of adherent macrophages recovered from bone marrow flushed from a femur with different batches of LCM. The batch-to-batch variability could also be influencing the activation status of macrophages such that they are more or less sensitive to inflammatory cytokines, which could have influenced our experiments. Most importantly, for the macrophage data presented in this chapter the results are entirely consistent with the results of testing the mutant in the more

relevant whole animal model of infection. In our studies presented here the absence of

TNF-α or MyD88 did not influence growth of the $\Delta secA2$ mutant in either cultured bone

marrow macrophages or murine infection. These results indicate there must be another

role for the SecA2 system, besides regulating MyD88 responses, in promoting

intracellular growth.

2.4- Materials and Methods

Bacterial strains and growth conditions

In this study we used *Mycobacterium tuberculosis* wild type strain H37Rv, and

the $\Delta secA2$ mutant (mc^{2}3112) generated in the H37Rv background (3). *M. tuberculosis*

strains were cultured in liquid Middlebrook 7H9 media or solid 7H10 supplemented with

0.05% Tween 80, 0.5% glycerol, 1× albumin dextrose saline (ADS). For plating organ

homogenates from murine infections, cyclohexamide (10μg/ml) was incorporated into

7H10 agar to inhibit fungal growth.

Animals

C57BL/6 mice acquired from Charles River Labs were used in aerosol infections

and for bone marrow-derived macrophages. MyD88^{−/−} mice on the C57BL/6 background

(1) were acquired from Dr. Shizuo Akira (WPI Immunology Frontier Research Center,

Research Institute for Microbial Diseases, Osaka University, Osaka Japan). TNF-α^{−/−} mice

on the C57BL/6 background were acquired from Dr. Jonathon D. Sedgwick (SP

Biopharma, Palo Alto, CA). TLR2^{−/−} mice on the C57BL/6 background were acquired

from Dr. Carsten Kirsching (Institute of Medical Microbiology, Immunology and Hygiene, Technical University Munich, Munich, Germany). All mice were housed in sterile caging and provided sterile food and water. All animal protocols were followed strictly as approved by the UNC Institutional Animal Care and Use Committee (IACUC).

Aerosol infection and necropsy

Aerosol infection of mice was performed using a Madison aerosol chamber. Mice were exposed to an aerosol generated from *M. tuberculosis* that was grown to log-phase and washed once and resuspended in PBS containing 0.05% Tween 80 at a concentration of 1.2×10^7 colony forming units (cfu)/ml. The mice were exposed to aerosols for 15 minutes with a 20-minute purge to clear the chamber resulting in an approximate dose of 200 cfu/lung. At various time points mice were euthanized, their lungs homogenized and plated for cfu on 7H10 agar to enumerate lung burden.

Making L-929 conditioned media (LCM)

L-929 conditioned media is made by first recovering the L-929 cells from liquid nitrogen stocks. A 2 ml aliquot of frozen L-929 cells is thawed as quickly as possible, and immediately added to 10 ml of prewarmed complete Dulbecco modified Eagle medium (DMEM; Sigma). "Complete" DMEM contains 10% fetal bovine serum (FBS; Gibco), 2mM L-glutamine, and 1× nonessential amino acids. The cells are then centrifuged at 200 × g for 5 minutes to pellet cells. The supernatant is decanted and cell pellet resuspended in 10 ml complete DMEM to wash. The L-929 cells are then centrifuged at 200 × g for 10 minutes to pellet cells and wash a second time to remove residual DMSO from the

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freezing media. After 2 washes the cells are resuspended in 4 ml prewarmed complete

DMEM and plated in one well of a 6-well tissue culture dish and incubated at 37°C and

5% CO_2. After 24 hours nonadherent cells were washed away and fresh complete DMEM

added to the well. When the cells grew to confluency, they were lifted from the well with

1× trypsin-EDTA at 37°C for ten minutes, then washed twice in warm DMEM to remove

trypsin-EDTA. The cells were split into multiple wells of a 6-well tissue culture dish and

grown again to confluency. At confluency the cells are again lifted with trypsin-EDTA as

before, and resuspended at 5×10^3 cells/ml of complete DMEM. The resuspended cells

were plated with 25 ml on 100 mm × 20 mm tissue culture treated dishes, and incubated

at 37°C and 5% CO_2. The cells were grown until just past confluency (~6-7 days), i.e. the

monolayer has a cobblestone appearance due to cells starting to round up because of

crowding. The supernatant is collected, filtered through a 0.22 μm filter, and aliquoted

for freezing at -80°C.

We tested all batches of LCM for mycoplasma contamination by PCR using the

MycoAlert test kit (Lonza). Using fluorescence activated cell sorting (FACS) we looked

at surface markers (CD11b, CD11c, MHC-I, MHC-II, and F4/80) to determine the purity

of our macrophage population *i.e.* to determine what percent of adherent cells were

macrophages. After selecting for the adherent population of cells we stained the

macrophage population with fluorescently conjugated FACS antibodies. Fluorescently

conjugated antibodies were acquired from eBioscience. FACS was performed on a CyAn

ADP LX 9 color flow cytometer (Dako) at the UNC FACS core facility. The data were

analyzed using Summit version 4.3 (Dako). We found that higher concentrations of LCM

produced higher numbers of adherent cells, so we tested each batch of LCM to determine

the best concentration that would produce 3.0×10^7 macrophages from two femurs. For all LCM batches tested, >95% of the adherent cells cultured tested positive for macrophage surface markers, which were CD11b^{high}, CD11c^{low}, MHC-I$^+$, MHC-II$^+$, and F4/80^{high}. At lower concentrations of LCM the number of adherent cells was lower, but the percentage of macrophages in the adherent population did not change. Even with these quality controls, we believe there remains batch-to-batch variation in LCM that can affect the outcome of experiments with cultured macrophages.

Macrophage infections

For bone marrow-derived macrophages, mice were euthanized by CO_2 asphyxiation and cervical dislocation. Femurs were extracted and flushed with complete DMEM. Bone marrow cells were washed and resuspended and plated in complete DMEM containing 20% L-929 cell conditioned media (LCM). After six days at 37°C, 5% CO_2, the cells were lifted off the plates using cold PBS EDTA 5mM and scraping. The cells were then washed twice and resuspended at a concentration of 1×10^6 macrophages/ml in complete DMEM containing 10% LCM. Macrophages were then seeded at 2×10^5 macrophages/well in eight-well chambered slide or chambered cover slips for microscopy experiments.

After resting 24 hours the macrophages were infected with *M. tuberculosis* culture grown to log-phase, and washed once with PBS containing 0.05% Tween 80 and diluted in warm complete DMEM. Macrophages were infected at an MOI of 1.0 or 0.2. After a four-hour incubation at 37°C for bacterial uptake, infected macrophages were washed

three times with pre-warmed complete DMEM. Macrophages were lysed at various time points and lysates were plated for cfu.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs for TNF-α and IL-6 were performed using OptEIA II ELISA (Becton Dickinson). Murine bone marrow macrophages were infected at an MOI of 1.0 with H37Rv or Δ*secA2* and incubated for four hours at 37°C and 5% CO_2, after which the extracellular bacteria were washed off with pre-warmed DMEM. The macrophages were then let incubate for 24 hours at 37°C and 5% CO_2. Then, the supernatants were collected and double filtered with 0.22 μm filters. For the ELISA, samples were loaded undiluted and at a 1:5 dilution. ELISAs were run as per the kit instructions.

Attributions

The authors of this work contributed in the following ways: Experiments were designed by JTS and MB. Experiments were performed by JTS and EY. Manuscript was written by JTS and MB. Funding was secured by MB.

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CHAPTER III

THE *MYCOBACTERIUM TUBERCULOSIS* SECA2 SYSTEM SUBVERTS PHAGOSOME MATURATION TO PROMOTE GROWTH IN MACROPHAGES[1]

3.1- <u>Introduction</u>

Mycobacterium tuberculosis infects about one-third of the world population and, as the

infectious agent of tuberculosis, causes almost two million deaths per year (69). With the

emergence of drug-resistant strains of *M. tuberculosis* current treatments may soon

become obsolete, fueling the need for new drugs and more effective vaccines to combat

the disease. A better understanding of *M. tuberculosis* pathogenesis will facilitate efforts

to develop new tuberculosis control measures.

Inside the host, *M. tuberculosis* survives in a unique intracellular niche within

macrophage phagosomes. Following its phagocytosis by non-activated macrophages, *M.*

tuberculosis arrests the normal process of phagosome maturation. As a result, *M.*

tuberculosis resides in a phagosome that fails to acidify or fuse with late endosomes and

lysosomes that supply hydrolytic enzymes and antimicrobial peptides (2, 52). This *M.*

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[1] Adapted for this dissertation from: Sullivan, J. T., Young, E. F., McCann, J. R., & Braunstein, M. (2012). The Mycobacterium tuberculosis SecA2 System Subverts Phagosome Maturation to Promote Growth in Macrophages. Infect Immun.

tuberculosis phagosome resembles an early recycling endosome that is accessible to transferrin and maintains a pH of ~6.4 (46). *M. tuberculosis* containing phagosomes are further distinguished by diminished accumulation of vacuolar ATPase (V-ATPase), phosphatidylinositol-3-phosphate [PI(3)P], and activated Rab7, which normally accumulate on maturing phagosomes (54, 57, 62, 63, 65). While the ability of *M. tuberculosis* to block phagosome maturation is widely thought to play an important role in promoting *M. tuberculosis* intracellular growth, there are few experiments that directly address a causal relationship between phagosome maturation arrest and intracellular growth. Furthermore, the mechanism *M. tuberculosis* uses to prevent phagosomes from maturing into acidic hydrolytic compartments is not well understood, but appears to be a multi-factorial process involving both protein and lipid effectors (3, 8, 14, 21, 36, 46, 56, 59, 63, 67).

   M. tuberculosis effectors that alter phagosome trafficking will likely be molecules that are exported by the bacillus (either secreted or surface localized) and positioned to interact with host cell processes. With the intracellular pathogens *Legionella* and *Salmonella*, specialized secretion systems are used to deliver effectors of phagosome trafficking (9, 13). *M. tuberculosis* has two types of specialized protein export systems: the ESX systems and accessory SecA2 system (16). The ESX-1 secretion system has previously been shown to block phagosome maturation in *M. tuberculosis* infected macrophages (8, 36). Here, we investigated the accessory SecA2 protein export system of *M. tuberculosis* and its role in virulence and phagosome maturation arrest. Mycobacteria are unusual in having two distinct SecA ATPase proteins (SecA1 and SecA2) (27, 51). SecA2 is an accessory SecA that is required for exporting a small subset of proteins out

of the cytoplasm. SecA1, as the housekeeping SecA, is essential and functions in the general Sec pathway that is used for the majority of protein export that occurs in mycobacteria.

The *M. tuberculosis* SecA2 system is important for virulence (6, 33). A Δ*secA2* mutant of *M. tuberculosis* is attenuated for growth in macrophages and in a mouse model of infection. A possible explanation for the function of the *M. tuberculosis* SecA2 system in pathogenesis might be to modulate host innate immune responses. Macrophages infected with the Δ*secA2* mutant produce higher levels of the proinflammatory cytokines TNF-α and IL-6 and increased levels of reactive nitrogen intermediates (RNI). These immunomodulatory factors have roles in controlling *M. tuberculosis* during host infection (19, 34, 38) and they are induced by *M. tuberculosis* through MyD88-dependent signaling pathways (Reviewed in (29)). Macrophages infected with the Δ*secA2* mutant also exhibit higher levels of apoptosis, which is attributed to defective SodA secretion by the Δ*secA2* mutant (25).

Here we studied the Δ*secA2* mutant to take a closer look at the role of SecA2 during *M. tuberculosis* growth in macrophages. We examined the role of apoptosis and increased MyD88-dependent inflammatory responses in controlling growth of the Δ*secA2* mutant in macrophages. While these macrophage responses did not appear to explain the role of SecA2 in promoting intracellular growth, we did discover an important role for SecA2 in blocking phagosome maturation. We go on to show that SecA2 dependent phagosome maturation arrest is required for the growth of *M. tuberculosis* in macrophages.

3.2- Results

Increased apoptosis does not account for the intracellular growth defect of the *M. tuberculosis* Δ*secA2* mutant.

The ability of *M. tuberculosis* to inhibit host cell apoptosis could be important for intracellular growth of the bacillus (41, 44). The Δ*secA2* mutant has a proapoptotic phenotype, which has been attributed to defective SodA secretion (25). Given this phenotype of the Δ*secA2* mutant, we set out to test if increased apoptosis accounts for the growth defect of the mutant in macrophages. To test this possibility we took advantage of a plasmid expressing an extra copy of SodA, termed αSodA. Unlike the endogenous *M. tuberculosis* SodA that lacks an obvious signal sequence for export, αSodA has a Sec signal sequence fused to the N-terminus of the enzyme (25). When αSodA is expressed by the Δ*secA2* mutant (Δ*secA2*-α*sodA*) it restores the ability of the Δ*secA2* mutant to release SodA activity into culture media and it reverses the proapoptotic phenotype of the mutant (25). It is worthwhile to mention that the details of αSodA release, particularly whether it is secreted by the Sec pathway, remain to be worked out. Nonetheless, because αSodA suppresses the propapoptotic phenotype of the Δ*secA2* mutant, we could test the Δ*secA2*-α*sodA* strain for growth in murine bone marrow-derived macrophages as a way to determine if altered apoptosis is responsible for the intracellular growth phenotype of the Δ*secA2* mutant. In these experiments, both the Δ*secA2* mutant and the Δ*secA2*-α*sodA* strain failed to grow in macrophages while the parental H37Rv strain and the complemented Δ*secA2* mutant strain grew approximately 10 fold over 5 days (Figure 3-1A). These results demonstrated that the Δ*secA2* mutant remains defective for intracellular growth even if the enhanced apoptosis phenotype is suppressed.

We similarly tested the role of apoptosis in the attenuated phenotype of the Δ*secA2* mutant in mice. C57BL/6 mice were infected via the aerosol route with the Δ*secA2*-α*sodA* strain and *in vivo* growth and persistence of this strain was compared to that seen with murine infection with the Δ*secA2* mutant and H37Rv. Here too, the attenuated phenotype of the *secA2* mutant was not suppressed by αSodA expression. In fact, in mice αSodA expression by the Δ*secA2* mutant actually exacerbated the *in vivo* growth defect (Figure 3-1B). Taken together, these results indicated that increased apoptosis and the defect in SodA secretion are, at least on their own, unable to explain the attenuated phenotypes of the Δ*secA2* mutant in macrophages or mice. Thus, there must exist another role for the SecA2 system in *M. tuberculosis* virulence.

The Δ*secA2* mutant resides in acidified phagosomes.

The ability of *M. tuberculosis* to interfere with phagosome maturation is another property of the bacillus proposed to be important to intracellular growth (47, 52, 66). Because secreted and surface localized proteins of *M. tuberculosis* are good candidates for being involved in phagosome maturation arrest, we tested the potential for the SecA2 export system to influence phagosome maturation. Murine bone marrow-derived macrophages were infected with H37Rv, the Δ*secA2* mutant or complemented strain and we scored the bacilli for co-localization with markers of phagosome maturation using widefield fluorescence microscopy.
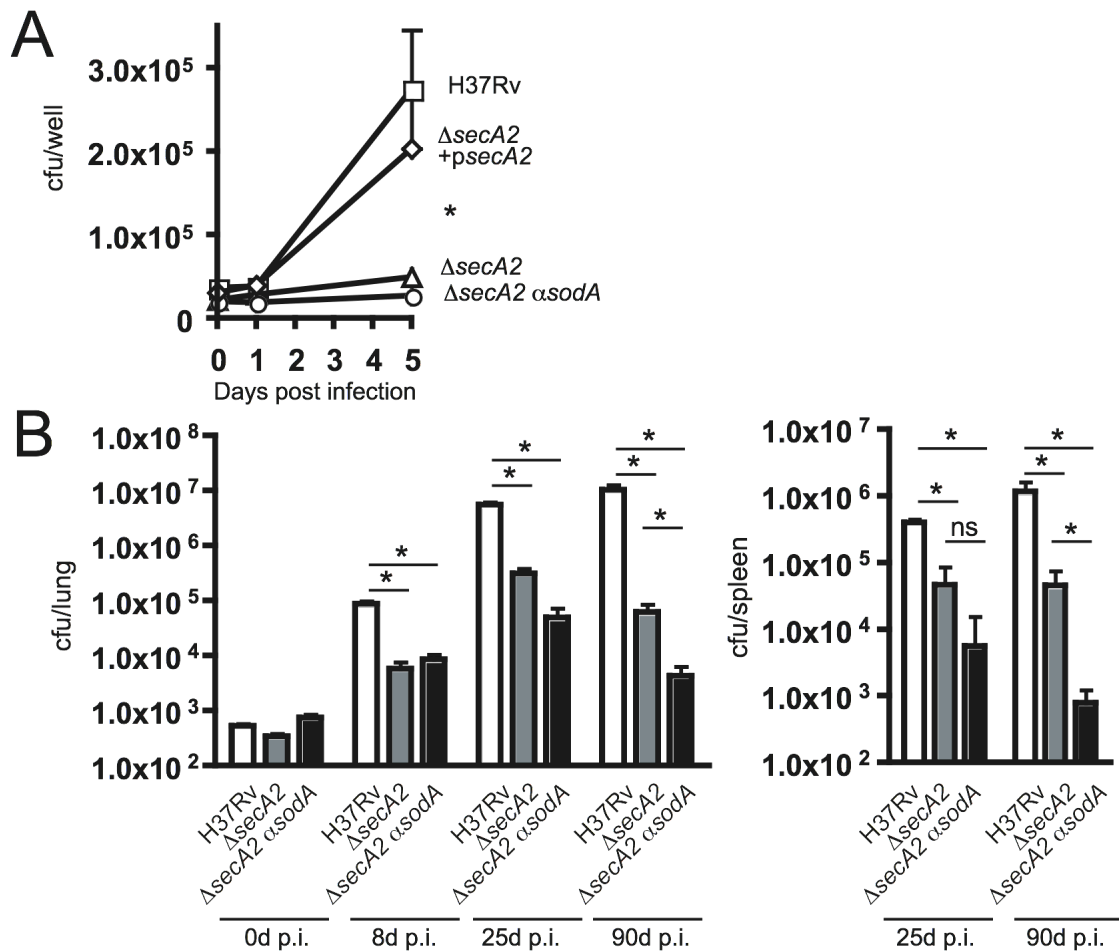
Figure 3-1. Reversal of the Δ*secA2* mutant apoptosis phenotype does not rescue growth in macrophages or mice. A. Non-activated bone marrow-derived macrophages (BMDM) were infected with H37Rv, Δ*secA2,* Δ*secA2* + *psecA2* (complemented strain), or Δ*secA2* + α-*sodA* strains of *M. tuberculosis* and intracellular replication monitored as described. The data shown is plotted in linear scale and is representative of two experiments, points represent means of triplicate wells and error bars represent SD, * $p < 0.05$ by student's *t* test. B. Lung and spleen burden in mice infected through aerosol route at an initial dose of ~200 cfu/lung with H37Rv, Δ*secA2,* or the Δ*secA2* + α-*sodA* strain. We determined cfu counts by plating lung or spleen homogenates at various time points for viable bacteria. Data shown is a single experiment, bars represent mean organ burden from four mice and error bars represent SD, * $p < 0.05$ by Student's *t* test.

To detect bacilli in macrophages we took advantage of the recently described autofluorescence of mycobacteria (45). Using scanning spectrophotometry, we first experimentally determined the optimal peak excitation and emission wavelengths (415nm and 470nm, respectively) for autofluorescence of paraformaldehyde fixed *M. tuberculosis*. These wavelengths can be detected by fluorescence microscopy using a standard CFP filter set. To validate the use of autofluorescence to localize *M. tuberculosis*, we evaluated a series of GFP expressing wild-type and mutant *M. tuberculosis* strains and compared GFP and autofluorescence signals. GFP expression is commonly used to visualize *M. tuberculosis* in macrophages (65, 70). All GFP positive cells were visible by autofluorescence (Figure 3-2); this was also true with bacilli in macrophages (data not shown).

It is well-established that in non-activated macrophages, wild-type *M. tuberculosis* is primarily found in non-acidified phagosomes (46, 52, 54, 66). To assess acidification of phagosomes containing the Δ*secA2* mutant we used LysoTracker Red (Invitrogen), which is an acidotropic dye frequently employed in studies of phagosome maturation (66, 70). By measuring co-localization of LysoTracker and *M. tuberculosis* autofluorescence signals, the parental H37Rv strain was found to largely avoid phagosome acidification, as reported previously (46, 54). In comparison, we detected the Δ*secA2* mutant in a significantly higher percentage of acidified phagosomes and this phenotype was reversed in the complemented strain (Figure 3-3A).
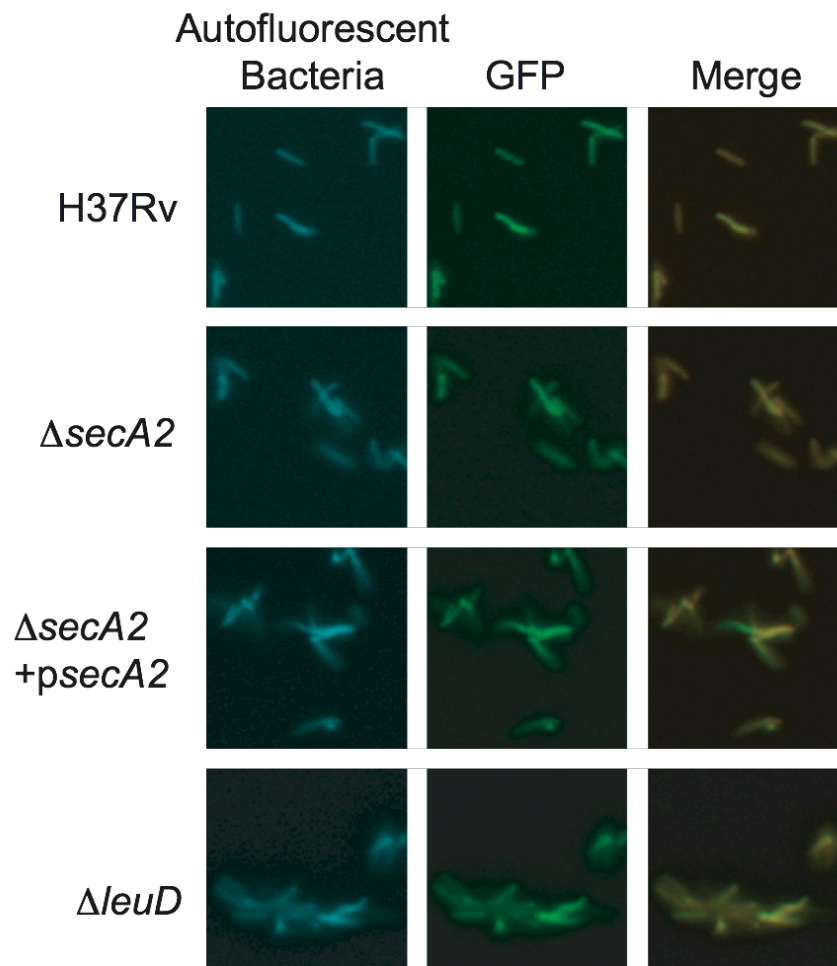
Figure 3-2. *M. tuberculosis* **autofluorescence can be used to identify bacilli by microscopy.** *M. tuberculosis* strains used in this study carrying a GFP expression plasmid were grown to mid-log phase and fixed in 4% paraformaldehyde in PBS. Fixed bacteria were loaded into the well of a chambered cover slip and visualized in the CFP and GFP channels on a widefield fluorescence microscope. Autofluorescence in the CFP channel is compared to GFP fluorescence in strains expressing GFP from a plasmid. The overlap is demonstrated in the merged images where yellow color indicates a positive correlation.

Experiments with GFP expressing versions of these strains, where GFP was used to localize the bacilli, gave the same results as obtained by scoring autofluorescence (data not shown). The Δ*secA2* mutant's association with acidified phagosomes was evident as early as one-hour post infection (Figure 3-3A).

We compared the phagosome acidification phenotype of the Δ*secA2* mutant to that of a mutant defective for ESX-1 secretion (Δ*eccD1*). As reported previously for *esx-1* mutants, the Δ*eccD1* mutant exhibited a higher association with LysoTracker positive phagosomes in comparison to H37Rv (Figure 3-3B) (8). The *esx-1* mutant phenotype was repeatedly less dramatic than the Δ*secA2* mutant phenotype. This finding that both a Δ*secA2* mutant and *esx-1* mutants are associated with increased phagosome acidification joins a list of other similarities reported for these mutants (20, 24, 28, 33, 37, 55). This raised the possibility that the SecA2 and ESX-1 systems might work together to export critical effector proteins of phagosome maturation. To test this possibility, we assayed phagosome acidification of a *M. bovis* BCG Δ*secA2* mutant. BCG lacks ESX-1 because the chromosomal locus encoding the system is deleted (49). As was the case in *M. tuberculosis*, a BCG Δ*secA2* mutant exhibited a higher association with LysoTracker positive phagosomes than the parental BCG Pasteur strain (Figure 3-3C). This result indicated that the role(s) of the SecA2 system in arresting phagosome maturation is independent of the ESX-1 system.
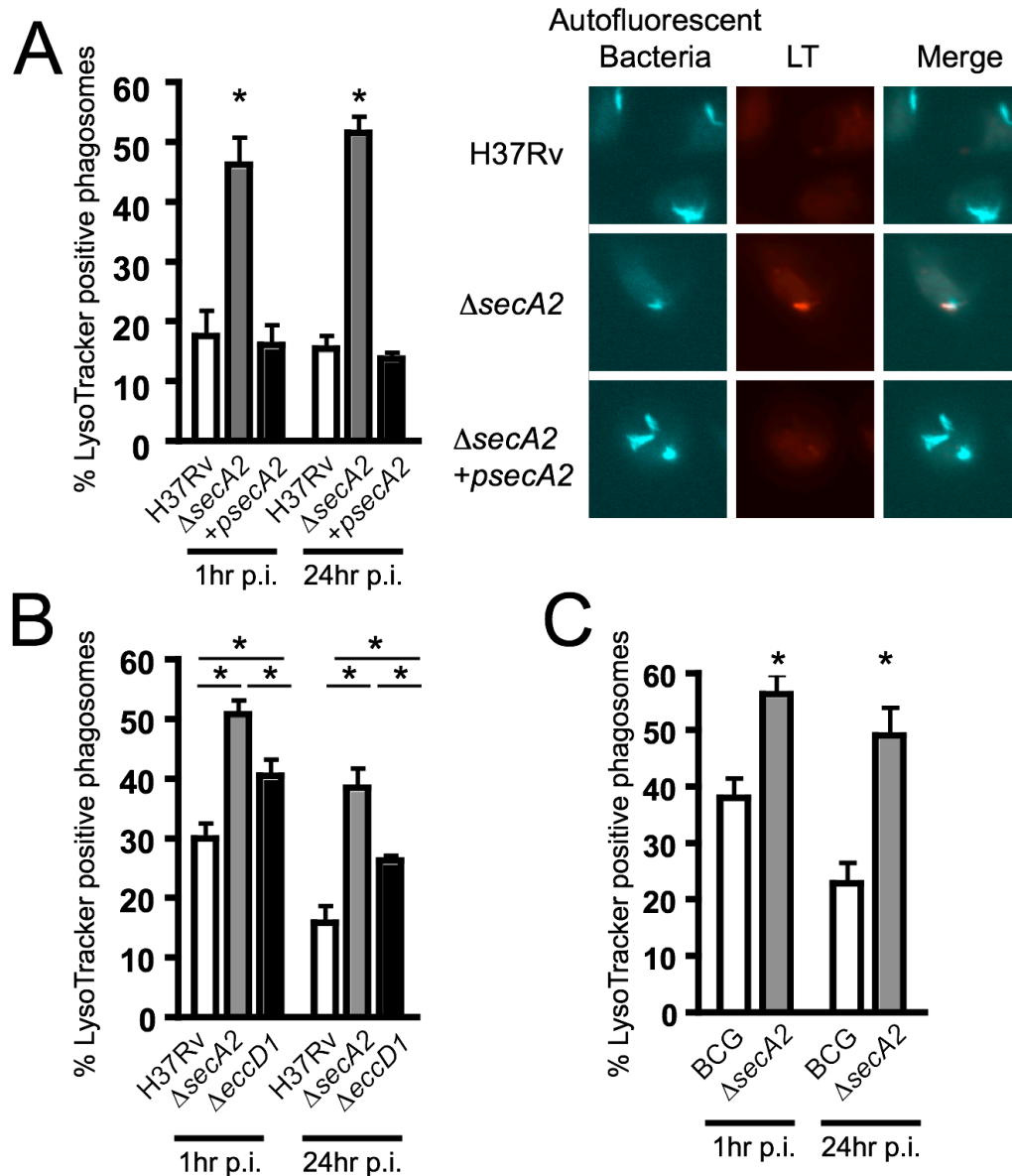
Figure 3-3. Compared to H37Rv, the Δ*secA2* mutant is enriched in LysoTracker positive phagosomes. Non-activated BMDMs were infected with A. H37Rv, the Δ*secA2* mutant or complemented strain, B. H37Rv, Δ*secA2,* or Δ*eccD1,* or C. BCG Pasteur or a Δ*secA2* mutant on the BCG Pasteur background. At indicated times the slides were stained with LysoTracker and scored for LysoTracker positive phagosomes as described above. Also shown, sample microscopy images from the experiment in panel A (LT = LysoTracker). Shown are representative experiments of at least three independent experiments. Bars represent mean percentage of bacteria-containing phagosomes that stain positive for LysoTracker; error bars represent SD of three replicate wells, each well having >100 phagosomes scored. *p≤0.05 by Student's *t* test when compared to WT.

The localization of the Δ*secA2* mutant to acidified phagosomes is not a general property of *M. tuberculosis* mutants with intracellular growth defects.

Increased association of the Δ*secA2* mutant with acidified phagosomes could result from an inability to carry out a role in blocking phagosome acidification, such as failure to secrete an inhibitor of phagosome maturation. Alternatively, the Δ*secA2* mutant could be delivered to acidified phagosomes as a secondary consequence of a failure to grow in macrophages. In considering the second possibility, we asked whether unrelated mutants that fail to replicate in macrophages are also found in acidified phagosomes. For this reason, we investigated the acidification status of phagosomes containing the *M. tuberculosis* leucine auxotroph (Δ*leuD*). The Δ*leuD* mutant is a metabolic mutant that fails to synthesize leucine and fails to grow in macrophages (26). Unlike the Δ*secA2* mutant, the Δ*leuD* mutant resembled H37Rv in its association with non-acidified phagosomes, even 72 hours post infection, indicating that this mutant maintained the ability to block phagosome acidification (Figure 3-4A). We additionally screened for phagosome acidification defects of *M. tuberculosis* transposon mutants recently identified as being defective for intracellular growth (40). Transposon mutants in *rv0199*, *mce1A*, or *mce2F* also resembled H37Rv in ability to block phagosome acidification (Figure 3-4B). These data indicated that localization to acidified phagosomes, as assessed by LysoTracker staining, is not true for all *M. tuberculosis* mutants that are defective for intracellular growth. Along with the finding that the Δ*secA2* mutant is observed in acidified phagosomes quickly (1hr) post-infection (Figure 3-3, 3-4A), these results argue for a specific role for the SecA2 system in blocking phagosome acidification.
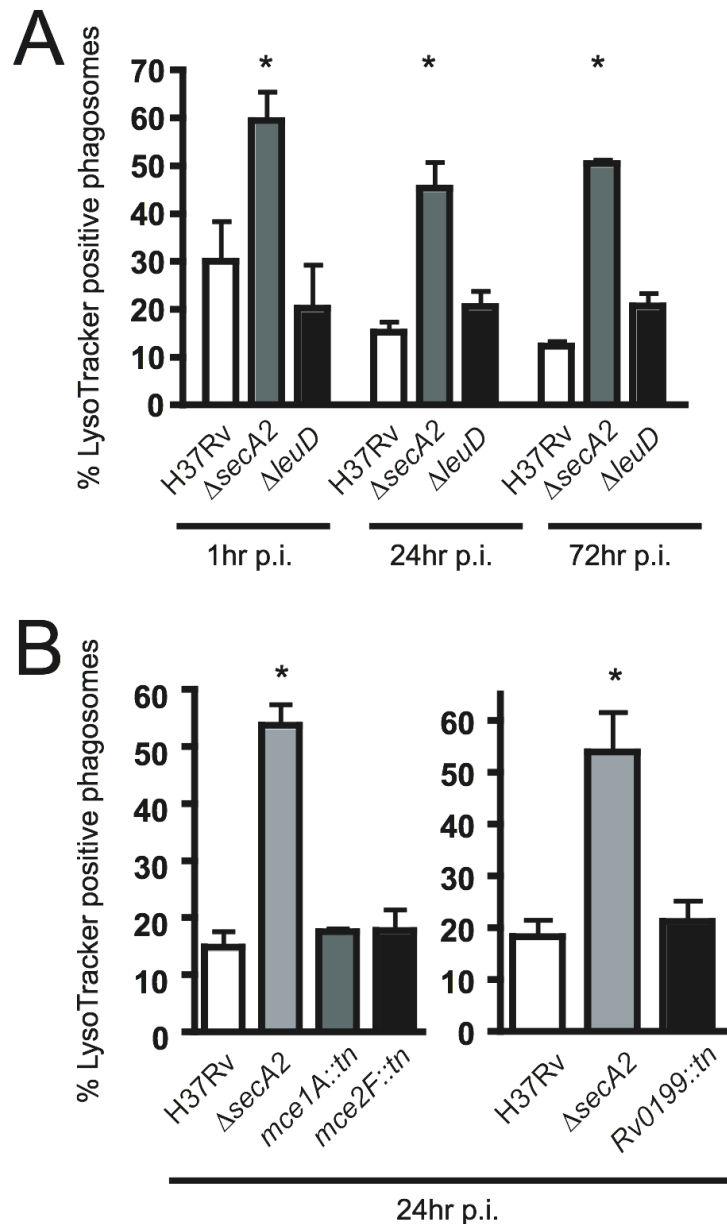
Figure 3-4. Not all *M. tuberculosis* mutants with intracellular growth defects are enriched in LysoTracker positive phagosomes. Non-activated BMDMs were infected with A. H37Rv, Δ*secA2* or Δ*leuD* B. H37Rv, Δ*secA2* , *mce1A::tn*, *mce2F::tn* or *rv0199::tn*. At indicated times the slides were stained with LysoTracker and scored for LysoTracker positive phagosomes as described above. Shown are representative experiments of at least three independent experiments. Bars represent mean percentage of bacteria-containing phagosomes that stain positive for LysoTracker; error bars represent SD of three replicate wells. *p≤0.05 by student's *t* test when compared to H37Rv.

Other markers of maturation are associated with phagosomes containing the ΔsecA2 mutant.

Vacuolar ATPase (V-ATPase) is a molecular motor that drives a proton gradient across membranes. V-ATPases are used by eukaryotic cells to acidify vacuoles. It is reported that *M. tuberculosis* phagosomes do not acidify, at least in part, because V-ATPase is either prevented from associating with or quickly degraded from the phagosome (54, 57). We wanted to determine if the higher association of the ΔsecA2 mutant with acidified phagosomes correlates with higher V-ATPase association. Macrophages infected with the ΔsecA2 mutant, H37Rv, or complemented strain were immunostained with antibodies to murine V-ATPase and co-localization was scored. We found a significantly higher percentage of phagosomes containing the ΔsecA2 mutant stained positive for V-ATPase than phagosomes containing H37Rv or the complemented strain (Figure 3-5A). The increased association with V-ATPase positive phagosomes seen with the ΔsecA2 mutant was equivalent to that seen with the ΔeccD1 mutant. Phagosome acidification is a relatively early step in phagosome maturation (17). To further characterize the ΔsecA2 mutant containing phagosome for evidence of phagosome/lysosome fusion, we immunostained infected macrophages for markers of late-endosomal/lysosomal fusion (CD63 and Rab7). Wild-type *M. tuberculosis* is reported to prevent phagosomes from maturing to a CD63 and Rab7 positive state (12, 31, 65). In comparison to phagosomes containing H37Rv or the complemented strain, a higher percentage of ΔsecA2 mutant-containing phagosomes stained positive for CD63 and Rab7. Once again, the phenotype of the ΔsecA2 mutant was similar to that of the ΔeccD1 mutant (Figure 3-5B, C).
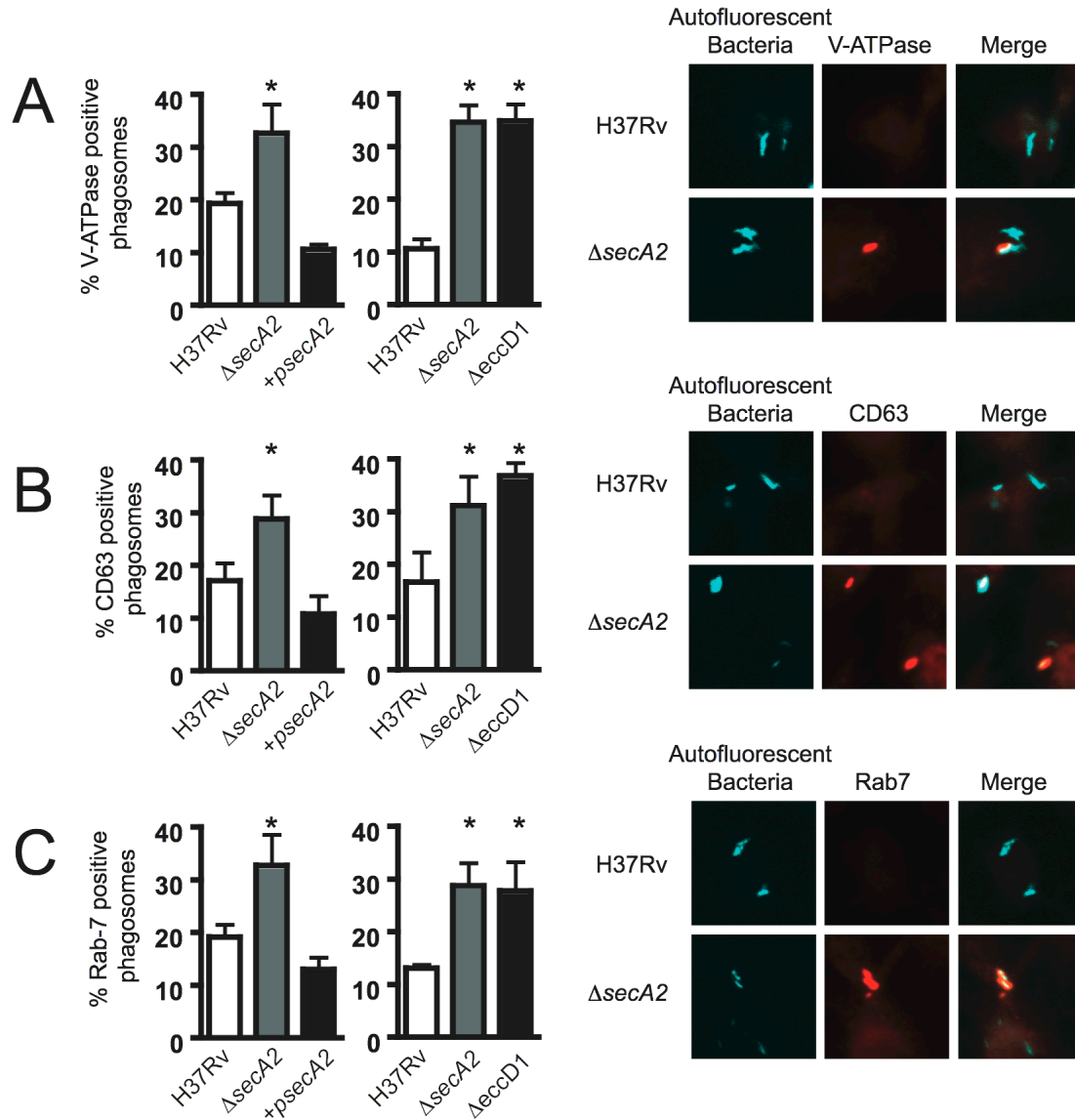
Figure 3-5. Compared to H37Rv, the Δ*secA2* mutant is enriched in phagosomes positive for late endocytic/lysosomal markers. Non-activated BMDMs were infected with H37Rv, Δ*secA2*, complemented Δ*secA2*, or the Δ*eccD1* mutant for 24 hours and immunofluorescently stained for markers of phagosome maturation as described above. A. V-ATPase, B. CD63 and C. Rab7. A representative experiment of three independent experiments is shown. Bars represent mean percentage of bacteria-containing phagosomes that stain positive for marker; error bars represent SD of three replicate wells, each well having >100 phagosomes scored. *p≤0.05 by Student's *t* test compared to H37Rv. Representative microscopy images for each set of markers are shown.

Together, these results indicated that phagosomes containing the Δ*secA2* mutant have a higher association with V-ATPase, which could account for the observed higher percent acidification detected with LysoTracker. The Δ*secA2* mutant-containing phagosomes also have a higher association with markers indicative of late endosomal/lysosomal fusion indicating a defect in later stages of phagosome maturation arrest as well (Figure 3-5B, C).

MyD88 signaling is not responsible for the altered phagosome maturation or intracellular growth phenotypes of the Δ*secA2* mutant.

We previously reported that during macrophage infection the Δ*secA2* mutant induces higher levels of TNF-α, IL-6, and RNI in comparison to infection with H37Rv (33). All three of these immunomodulatory molecules are induced by *M. tuberculosis* through TLR and MyD88 pathways (7, 32, 50). Because TLR and MyD88 signaling are implicated in driving phagosome maturation events (4, 71), we considered the possibility that increased signaling through these pathways was responsible for the trafficking defects of the Δ*secA2* mutant. To address the significance of MyD88-dependent responses to Δ*secA2* mutant phenotypes in macrophages, we tested the Δ*secA2* mutant and H37Rv in parallel infections of primary MyD88 deficient macrophages (MyD88^{-/-}) and C57BL/6 macrophages. As reported by others, MyD88^{-/-} macrophages showed a significant decrease in level of secreted TNF-α and IL-6 in response to *M. tuberculosis* infection (22, 60). The TNF-α and IL-6 levels from H37Rv or Δ*secA2* mutant infected MyD88^{-/-} macrophages were the same and equivalent to the levels produced by uninfected macrophages (data not shown).

As in C57BL/6 macrophages, Δ*secA2* mutant-containing phagosomes in MyD88^{-/-} macrophages stained positive more than H37Rv-containing phagosomes with LysoTracker, V-ATPase, CD63, and Rab7. The difference between the Δ*secA2* mutant and H37Rv phagosomes in MyD88^{-/-} macrophages was equivalent to that seen in C57BL/6 macrophages (Figure 3-6A). We also tested the Δ*secA2* mutant for growth in MyD88^{-/-} macrophages. In MyD88^{-/-} macrophages the Δ*secA2* mutant was as defective for intracellular growth as is seen in C57BL/6 macrophages (Figure 3-6B). Aerosol infection of MyD88^{-/-} mice with the *secA2* mutant also showed that the absence of MyD88^{-/-} had no effect on the *in vivo* growth defect of the Δ*secA2* mutant in mice at early time points (data not shown). Thus, the explanation for the intracellular trafficking and growth defects of the Δ*secA2* mutant appears to be unrelated to MyD88-signaling.

Phagosome acidification is necessary for the intracellular growth phenotype of the Δ*secA2* mutant.

There are other *M. tuberculosis* mutants reported to have defects in blocking phagosome maturation (8, 31, 36, 46, 56, 67). In the majority of cases, phagosome maturation arrest mutants are also defective for intracellular growth (for example, *esx-1* mutants). However, with the exception of a few studies (23, 30, 68), the causal relationship between localization to a more mature phagosome and inhibition of growth remains largely untested. With the goal of determining if the phagosome maturation arrest defect of the Δ*secA2* mutant is responsible for the growth defect in macrophages, we used macrolide antibiotic V-ATPase inhibitors (bafilomycin A1 and concanamycin A) (15) to block acidification of Δ*secA2* mutant containing phagosomes and asked if this treatment rescued growth of the mutant.
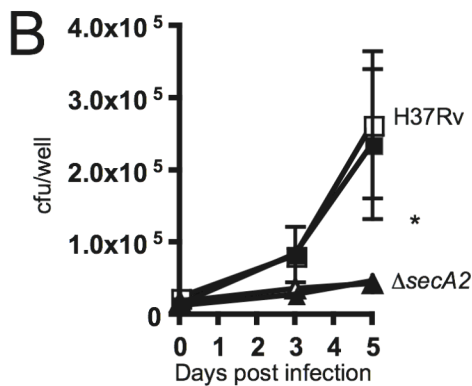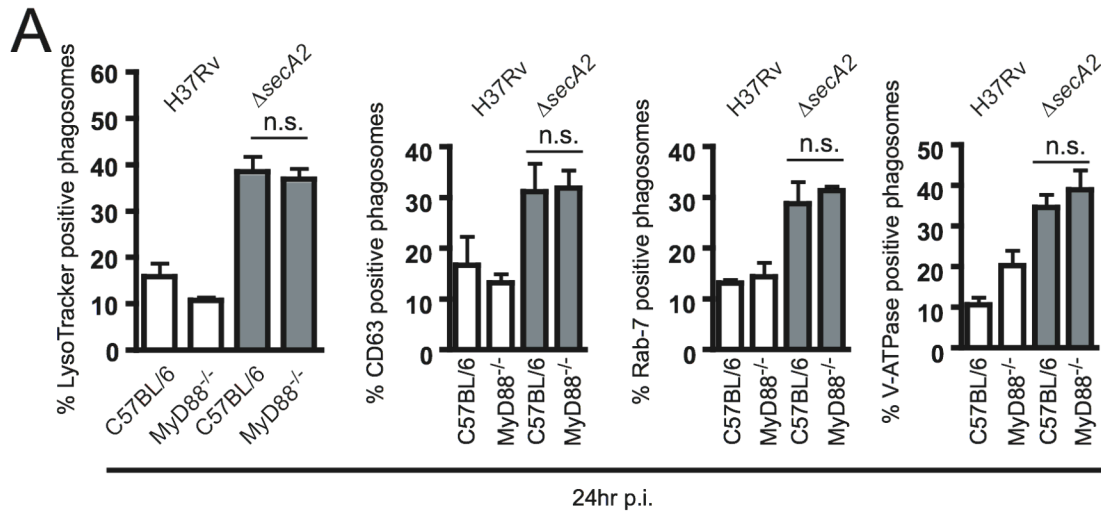
Figure 3-6. MyD88 has no effect on Δ*secA2* mutant phagosome trafficking or intracellular growth. A. Non-activated BMDMs derived from C57BL/6 or MyD88^{-/-} mice were infected with H37Rv or the Δ*secA2* mutant. At 24 hours post infection the slides were stained with LysoTracker or antibodies to CD63, Rab7 or V-ATPase and scored for marker positive bacteria-containing phagosomes as described above. Bars represent mean percentage of bacteria-containing phagosomes that stain positive for marker; error bars represent SD of three replicate wells. There are no significant differences between C57BL/6 and MyD88^{-/-} macrophages when infected with the Δ*secA2* mutant. B. Non-activated BMDMs from C57BL/6 (open symbols) or MyD88^{-/-} (closed symbols) mice were infected with H37Rv or the Δ*secA2* mutant and intracellular replication monitored as described. The data shown is plotted in linear scale and is a representative experiment of three, points are mean of three replicate wells, error bars represent SD. *p≤0.05 by student's *t* test compared to H37Rv.

To minimize pleiotropic effects of the inhibitors on macrophages, we experimentally determined a minimum concentration of each inhibitor (10nM Bafilomycin A1 and 5nM Concanamycin A) required to prevent acidification of the Δ*secA2* mutant containing phagosomes, as measured by LysoTracker co-localization at 24 hours post infection (Figure 3-7A). These concentrations had no detectable effect on macrophage viability over the course of a five-day infection (data not shown).   In contrast to untreated macrophages, the Δ*secA2* mutant and H37Rv grew equally well in macrophages treated with 10nM bafilomycin A1 (Figure 3-7B). While bafilomycin treatment increased growth of both H37Rv and the Δ*secA2* mutant in macrophages over 5 days (Figure 3-7B), the effect of bafilomycin was small and not significant for H37Rv but significantly greater for the Δ*secA2* mutant (Figure 3-7C).

To establish the specificity of this rescue, we also tested the effect of bafilomycin on intracellular growth of the Δ*leuD* mutant, which is not localized to acidified phagosomes (Figure 3-4A). Bafilomycin A1 treatment of the Δ*leuD* mutant did not rescue growth to the level of H37Rv in bafilomycin treated macrophages (Figure 3-7B, C). Experiments with 5nM concanamycin A showed the same effect of rescuing the intracellular growth defect of the Δ*secA2* mutant. Growth of the Δ*secA2* mutant was equivalent to that of H37Rv in concanamycin A treated macrophages (Figure 3-7D, E). Together, these results indicated the importance of phagosome acidification for inhibiting intracellular growth of the Δ*secA2* mutant. Our data further argues that phagosome maturation can result in inhibition of *M. tuberculosis* intracellular replication.
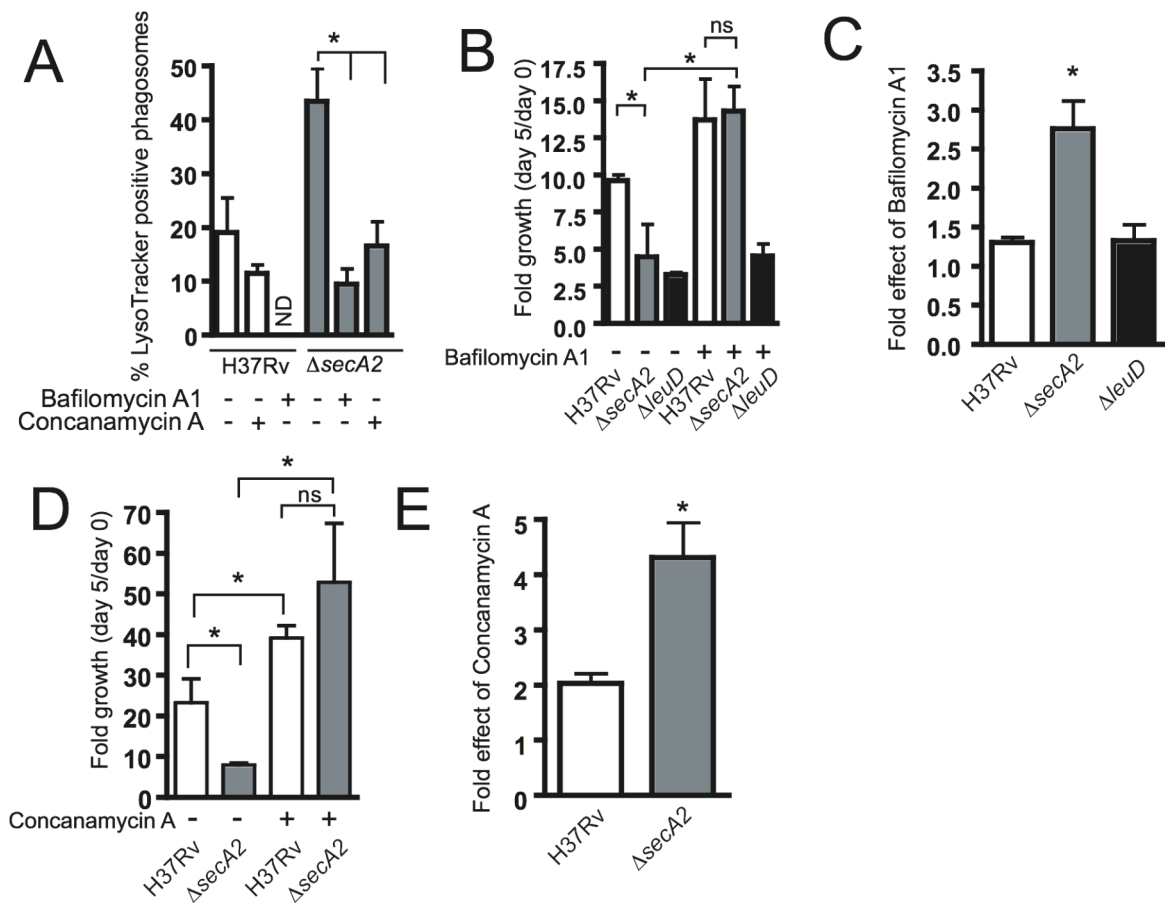
Figure 3-7. Growth of the Δ*secA2* mutant is inhibited by phagosome acidification. A. LysoTracker positive phagosomes in BMDMs infected with H37Rv or Δ*secA2* mutant and treated with bafilomycin A1 or concanamycin A. Shown are the lowest inhibitor concentrations that bring Δ*secA2* LysoTracker positive phagosomes to H37Rv levels, ND = not determined. B. Non-activated BMDMs treated with bafilomycin A1 or vehicle control (DMSO) were infected with H37Rv, Δ*secA2* or Δ*leuD* and intracellular replication monitored as described. Shown is a representative experiment of four independent experiments. Bars represent mean fold growth over five days of three replicate wells +SD. * p<0.05 by Student's *t* test. C. Data from the above experiment (7B) combined with three independent experiments to show the fold effect of bafilomycin A1 on intracellular growth compared to untreated macrophages. Bars represent mean fold effect of bafilomycin A1 in four individual experiments +SEM, *p<0.05 by Student's *t* test compared to H37Rv. D and E, as in B and C, with concanamycin A or DMSO. D. Shown is a representative experiment of three experiments. E. Bars represent mean fold effect of concanamycin in three experiments +SEM, *p<0.05 by Student's *t* test compared to H37Rv.

3.3- Discussion

The function of the accessory SecA2 export system in promoting growth in macrophages has remained elusive. Previously, we tested if the role of the SecA2 system is to protect against the oxidative or nitrosative stresses produced by macrophages during infection (33). These past studies showed that even in the absence of these reactive radical stresses the Δ*secA2* mutant remains attenuated in macrophages. Thus, the SecA2 system must have other roles in promoting *M. tuberculosis* growth in macrophages. In this study, we considered alternate explanations. We tested if the inhibition of macrophage apoptosis mediated by the SecA2 system is what promotes *M. tuberculosis* growth in macrophages. Our results from testing the Δ*secA2-αsodA* strain do not support this possibility. While expression of the *αsodA* construct in the Δ*secA2* mutant suppresses the proapoptotic phenotype, the strain remained defective for intracellular growth (Figure 3-1A). It is worthwhile to note that our results do not rule out other important roles of the apoptosis phenotype of the *M. tuberculosis* Δ*secA2* mutant as reported elsewhere, such as enabling enhanced adaptive immune responses and increased protective immunity in vaccination studies (25).

In considering other explanations for the function of the SecA2 system in macrophages we tested the Δ*secA2* mutant for the ability to arrest phagosome maturation. Our results showed the Δ*secA2* mutant to be defective in phagosome maturation arrest, as the mutant was more readily trafficked to a more mature phagosome than the parental H37Rv strain. This defect was evident quickly after infection (1hr), and not exhibited by other mutants with intracellular growth defects even at later times post-infection (72 hr).

Additionally, we showed that treating macrophages with inhibitors of the V-ATPase prevented acidification of phagosomes and rescued the intracellular growth defect of the Δ*secA2* mutant. From these results, we conclude that the accessory SecA2 export system has a specific role in blocking phagosome acidification and that the intracellular growth defect of the Δ*secA2* mutant is directly related to its defect in blocking phagosome maturation.

Correlations exist between phagosome maturation and *M. tuberculosis* growth arrest (8, 35, 46, 56), and the ability of *M. tuberculosis* to arrest phagosome maturation is generally assumed to be critical for pathogenesis and for growth in macrophages. Yet, there are a few examples of mutants that are able to grow even though they reside in more mature phagosomes (8, 36, 46), which raises the possibility that phagosome maturation arrest is not essential for *M. tuberculosis* intracellular growth. Our results showing both bafilomycin A1 and concanamycin A rescue the Δ*secA2* mutant intracellular growth defect are significant in demonstrating the ability of V-ATPase mediated phagosome acidification to control *M. tuberculosis* growth. This argues that the avoidance of phagosome acidification by virulent *M. tuberculosis* is necessary for intracellular growth. In addition to rescuing growth of the Δ*secA2* mutant, bafilomycin and concanamycin treatment also modestly increased the replication efficiency of H37Rv. This effect on H37Rv is most likely due to the inhibitors further decreasing the percentage of H37Rv associated with acidified phagosomes (Figure 3-7). A similar result was reported by Welin et al. with H37Rv infection of bafilomycin treated human monocyte derived macrophages (68).

How might phagosome acidification control *M. tuberculosis* infection? Although low pH can render media components toxic to *M. tuberculosis*, which complicates assessing growth at acid pH (61), *M. tuberculosis* replication is reported to be sensitive to low pH (48). Therefore, it is possible that phagosome acidification is directly responsible for the growth inhibition of the Δ*secA2* mutant in macrophages. Alternatively, the acidic environment may be activating the pH sensitive lysosomal hydrolases (52) and thereby limiting replication. A final possibility is that the acidification of the phagosome is driving downstream fusion events that produce the growth restricting environment (11, 58).

Because metabolically inactive bacteria are more readily found in acidified phagosomes (35), it has been suggested that mutants impaired in any aspect of growth or metabolic activity may be found in mature phagosomes as a secondary consequence of those defects, as opposed to a direct effect on phagosome maturation (47). Because we observed the Δ*secA2* mutant in more mature phagosomes at early times post-infection and because V-ATPase inhibitors rescued the growth defect, we think it unlikely that general growth or metabolic defects account for the Δ*secA2* mutant phenotype. Moreover, we tested the leucine auxotroph (Δ*leuD*) of *M. tuberculosis* as an example of a mutant severely compromised in metabolic activity. The Δ*leuD* mutant requires leucine supplementation to grow in liquid media and the mutant does not grow in macrophages, presumably due to a failure to acquire leucine from the intracellular environment (26). Somewhat surprisingly, the Δ*leuD* mutant behaved like H37Rv and maintained its ability to avoid phagosome acidification, even at the latest (72hr) time point. Although the precise level of metabolic activity of the Δ*leuD* mutant in macrophages is not known, this

result suggests that general growth or metabolic defects do not necessarily lead to a breakdown in phagosome maturation arrest.

What is currently known about the process of phagosome maturation arrest by *M. tuberculosis* suggests it is a complex process involving several effectors. It seems likely that *M. tuberculosis* has multiple effectors targeting a minimum of two parallel pathways that are critical to phagosome maturation: PI(3)P accumulation/signaling and Rab7 accumulation/activation (10, 47, 62). The phagosome maturation defect of the Δ*secA2* mutant may be due to a defect in the secretion of such effectors. Several *M. tuberculosis* secreted proteins (*i.e.* LpdC, NdkA, PknG, PtpA, and SapM) affect phagosome maturation or pathways thought to be critical for phagosome maturation (3, 14, 59, 63, 67). This list of possible effectors is likely incomplete, however, as mutant screens to find effectors of phagosome maturation suggest the existence of a much broader set of proteins involved in arresting phagosome maturation (8, 36, 46, 56). There are also *M. tuberculosis* surface lipids (lipoarabinomannan, phosphotidylinositol mannoside, and tetra-acylated sulfoglycolipid) (8, 21, 64) reported to affect phagosome maturation. The SecA2 system could be involved in surface lipid production or localization by exporting lipid synthesis or export machinery.

An alternate way that the SecA2 system could promote phagosome maturation arrest would be to limit macrophage responses that drive downstream phagosome maturation events. With this idea in mind we considered the possibility that the more robust MyD88-dependent responses elicited by the Δ*secA2* mutant (33) might be responsible for the altered trafficking of the Δ*secA2* mutant. Although our experiments with MyD88^{-/-} macrophages revealed this not to be the case, it only excludes altered

phagosome trafficking as a downstream event of MyD88 signaling. Macrophages can detect *M. tuberculosis* through MyD88-independent intracellular receptors such as the NLR family of receptors and the inflammasome, which could also drive phagosome maturation (39).

The microscopy experiments performed in the course of this work are the first to take advantage of the autofluorescence of mycobacteria to co-localize intracellular bacilli with phagosomal markers. Previously, either exogenous fluorescent proteins (GFP, RFP) or fluorescent dyes have been used to track mycobacteria in similar experiments (54, 56). While GFP fluorescence has the advantage over autofluorescence of being brighter and longer-lasting, it has the disadvantage of requiring the construction of strains that express exogenous genetic elements and there is the potential for GFP expression itself to influence virulence (42). The use of dyes to surface label the bacilli have a similar disadvantage in introducing an experimental variable that could possibly alter the course of infection (54).

The work presented here not only provides a better understanding of how SecA2 promotes *M. tuberculosis* growth in macrophages, but it also demonstrates the importance of phagosome maturation arrest for *M. tuberculosis*. It seems likely that *M. tuberculosis* has multiple effectors of phagosome maturation arrest. As is the case in the Type IV secretion system of *Legionella pneumophila*, this may mean there is substantial effector redundancy (43). The phagosome maturation phenotype of the Δ*secA2* mutant indicates there is a single critical effector or multiple effectors that depend on the SecA2 export pathway. Future studies will seek to identify such SecA2-dependent effectors of phagosome maturation arrest.

3.4- Materials and Methods

Bacterial strains and growth conditions

In this study we used *Mycobacterium tuberculosis* strains listed in Table 1. *M. tuberculosis* strains were cultured in liquid Middlebrook 7H9 media or solid 7H10 supplemented with 0.05% Tween 80, 0.5% glycerol, 1× albumin dextrose saline (ADS), and appropriate drugs kanamycin (20μg/ml) or hygromycin (50μg/ml). For plating organ homogenates from murine infections, cyclohexamide (10μg/ml) was incorporated into 7H10 agar to inhibit fungal growth. For experiments with the Δ*leuD* mutant all media was additionally supplemented with 50μg/ml L-leucine.

Mutant construction

The *M. tuberculosis* Δ*eccD1* null mutant was constructed by specialized transduction using an allelic exchange construct delivered by the temperature-sensitive mycobacteriophage phAE159, as described previously (5). The allelic exchange construct was constructed by amplifying flanking regions of *eccD1* (*rv3877*) by PCR from *M. tuberculosis* genomic DNA. An 833 bp upstream flanking sequence (ending at the second codon of *eccD1*) and an 821 bp downstream flanking sequence (starting at the fifth codon from the stop) were amplified and individually cloned into the pCR 2.1 cloning vector (Invitrogen). These flanking sequences were then sequentially cloned into pJSC284 to create plasmid pKO-3877, which has the hygromycin (*hyg*) cassette marking the deletion and positioned between the two flanking sequences. PacI digested pKO-3877 was then ligated to PacI cut phAE159 and *in vitro* packaged (Stratagene) into

lambda phage particles that were recovered by transduction in *E. coli*. The resulting recombinant mycobacteriophage was then used to transduce H37Rv at the non-permissive temperature of 39°C for 4 hours. Transduced cells were pelleted, resuspended in 7H9 media with 0.1% Tween 80, and plated on 7H10 agar with hygromycin. Hygromycin-resistant colonies obtained at 3 weeks were screened for allelic exchange by Southern blotting to confirm deletion of *eccD1*.

The Δ*secA2* null mutant in BCG Pasteur was constructed as previously reported for BCG Tice and H37Rv strains using a two-step allelic exchange method (6, 53).

Antibodies and reagents

Antibodies to mammalian markers (CD63, Rab7 and V-ATPase B1/B2) and fluorophore conjugated secondary antibodies were acquired from Santa Cruz Biotechnology. The α-SodA construct (pMV3α-sod) was a kind gift of Dr. William Jacobs Jr. (Albert Einstein College of Medicine, Bronx NY) (25). Bafilomycin A1 (Sigma) and concanamycin A (Santa Cruz) were stocked at 1000× in DMSO.

Animals

C57BL/6 mice acquired from Charles River Labs were used in aerosol infections and for bone marrow-derived macrophages. MyD88^{-/-} mice on the C57BL/6 background (1) were acquired from Dr. Shizuo Akira (WPI Immunology Frontier Research Center, Research Institute for Microbial Diseases, Osaka University, Osaka Japan). All mice were housed in sterile caging and provided sterile food and water. All animal protocols

were followed strictly as approved by the UNC Institutional Animal Care and Use Committee (IACUC).

Aerosol infection and necropsy

Aerosol infection of mice was performed using a Madison aerosol chamber. Briefly, mice were exposed to a whole body aerosol generated from *M. tuberculosis* that was grown to log-phase and washed once and resuspended in PBS containing 0.05% Tween 80 at a concentration of 1.2×10^7 colony forming units (cfu)/ml. The mice were exposed to aerosols for 15 minutes with a 20-minute purge to clear the chamber resulting in an approximate dose of 200 cfu/lung. At various time points mice were euthanized, their lungs and spleens homogenized and plated for cfu on 7H10 agar.

Macrophage infections

Bone marrow-derived macrophages were made as follows. Mice were euthanized by CO_2 asphyxiation and cervical dislocation. Femurs were removed and bone marrow flushed out with supplemented Dulbecco modified Eagle medium (DMEM; Sigma). DMEM was supplemented with 10% fetal bovine serum (FBS; Gibco), 2mM L-glutamine, and 1× nonessential amino acids (complete DMEM). Bone marrow cells were washed once and resuspended and plated in complete DMEM containing 20% L-929 cell conditioned media (LCM). After six days at 37°C, 5% CO_2, the cells were lifted off the plates using cold PBS EDTA 5mM and scraping. The cells were then washed twice and resuspended at a concentration of 1×10^6 macrophages/ml in complete DMEM

containing 10% LCM. Macrophages were then seeded at 2×10^5 macrophages/well in eight-well chambered slide or chambered cover slips for microscopy experiments.

After resting 24-48 hours the macrophages were infected with *M. tuberculosis* culture grown to log-phase, and washed once with PBS containing 0.05% Tween 80 and diluted in warm complete DMEM. Macrophages were infected at an MOI of 1.0 for microscopy or 0.2 for intracellular growth assays. After a four-hour incubation at 37°C for bacterial uptake, infected macrophages were washed three times with pre-warmed complete DMEM. The zero hour time point of these experiments represents the time after the washes were complete. For kinetic growth assays macrophages were lysed at various time points and lysates were plated for cfu. For microscopy, cover slips were taken at various time points and fixed for at least one hour in 4% paraformaldehyde (PFA) in PBS pH 7.4. For experiments using bafilomycin A1 (Sigma) or concanamycin A (Santa Cruz) the inhibitors or equivalent vehicle control were added to the macrophages 30 minutes prior to infection. Inhibitors were maintained throughout the five-day infection.

Macrophage staining and microscopy

To stain with LysoTracker, media was replaced with prewarmed DMEM +100nM LysoTracker Red DND99 (Invitrogen) and returned to 37°C 5% CO_2 for one hour. For immunofluorescence, media was aspirated from the wells at the endpoint of infection and cover slips were submerged in 4% PFA for at least one hour. To stain for immunofluorescence, fixed macrophages in chambered cover slips were submerged in PBS to remove residual PFA. Cells were then permeabilized with 0.1% Triton-X 100 in PBS for 5 minutes at room temperature, washed in PBS and blocked in PBS + 10%

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serum from the same source as the secondary antibody. Primary antibodies were used at a 1:100 dilution in PBS + 3% serum and incubated overnight at 4°C. After extensive washing in PBS, secondary antibodies conjugated to fluorophores were used at 1:100 in PBS + 3% serum and incubated at room temperature for one hour. After secondary antibodies were washed away, Fluormount-G (Southern Biotech) was added to each well. As an important control, we showed that normal rabbit IgG and secondary antibody alone did not stain *M. tuberculosis* containing phagosomes.

Widefield fluorescence microscopy was performed using an Olympus IX-81 controlled by the Volocity software package. All images were taken using a 40× oil-immersion objective. A minimum of five fields per well was captured and bacteria were scored for phagosomal markers, amounting to a minimum of 100 bacteria-containing phagosomes scored per well. For each experimental group three replicate wells were scored per experiment.

Mycobacterial autofluorescence was visualized using a CFP filter cube (Semroc) with an excitation band of 426-450nm and emission band of 467-600nm. A 3% transmission, neutral density filter was used and minimal time was spent focusing in the CFP channel to protect the autofluorescence from photobleaching.

Table 3-1. Mycobacteria strains used

Strain	Description	Reference
M. tuberculosis		
H37Rv	Wild-type	Trudeau Institute
mc^{2}3112	H37Rv Δ*secA2*	(6)
MBTB74	H37Rv Δ*secA2*, *attB*::*secA2*	This study
MBTB109	H37Rv Δ*secA2*, *attB*::α*sodA*	(25), This study
mc^{2}3032	H37Rv Δ*leuD*	(26)
MBTB3	H37Rv Δ*eccD1*	This study
PM638	H37Rv Δ*blaC*	(18)

MBTB204	PM638, *mce1A::tn*	(40)
MBTB156	PM638, *mce2F::tn*	(40)
MBTB183	PM638, *rv0199::tn*	(40)
MBTB157	H37Rv, *gfp*	This study
MBTB158	mc^{2}3112, *gfp*	This study
MBTB159	MBTB74, *gfp*	This study
MBTB390	mc^{2}3032, *gfp*	This study
M. bovis BCG		
BCG Pasteur	Wild-type	Staten Serum Institut
MB544	BCG Pasteur Δ*secA2*	This Study

3.5- Acknowledgments

Attributions

The authors of this work contributed in the following ways: Experiments were designed by JTS, JRM, and MB. Experiments were performed by JTS, JRM, and EY. Manuscript was written by JTS and MB. Funding was secured by MB.

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CHAPTER IV

TESTING A ROLE FOR THE ACCESSORY SECA2 SYSTEM IN EXPORTING *MYCOBACTERIUM TUBERCULOSIS* EFFECTORS OF PHAGOSOME MATURATION[1]

4.1- Introduction

      Mycobacterium tuberculosis, the causative agent of tuberculosis disease,

primarily resides in macrophages during infection of a host (21). After being engulfed by

macrophages through receptor-mediated phagocytosis (12), *M. tuberculosis* has the

remarkable ability to survive and grow in the normally hostile environment of

macrophages. *M. tuberculosis* manipulates phagosome trafficking to prevent acidification

and phagosome maturation (19, 20). By preventing the process of phagosome maturation

it is thought that *M. tuberculosis* creates an intracellular niche amenable to bacterial

replication.

      While it is a well-established fact that *M. tuberculosis* blocks phagosome

maturation in macrophages (1, 9, 20, 24), the process by which *M. tuberculosis* achieves

this is not yet clear. Through multiple genetic screens and direct tests of potential

effectors there is an extensive list of genes, proteins and lipids that could play some role

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in blocking phagosome maturation(2, 6, 11, 13, 15, 17, 18, 23, 26-28). Interestingly, there is minimal overlap of the factors identified by these various approaches. The list of potential *M. tuberculosis* molecules involved in phagosome maturation arrest suggests a complex process that probably involves multiple molecules working in tandem. As presented in chapter 3, the accessory SecA2 protein export system of *M. tuberculosis* is required for phagosome maturation arrest during macrophage infection (25). However, the role of the SecA2 system in blocking phagosome maturation remains to be defined. Because SecA2 is a cytoplasmic protein required for the export of a specific subset of proteins (4, 14), we hypothesize that one or more effectors of phagosome maturation are proteins exported by the SecA2 system.

In this chapter, we assay a set of potential effectors of phagosome maturation arrest (2, 7, 11, 26-28) for evidence of export by the SecA2 pathway. More specifically, we tested proteins reported to have roles in blocking phagosome maturation (LpdC, NdkA, PtpA and SapM) for SecA2 dependent export. As reviewed in chapter 1, these putative effectors of phagosome maturation arrest are implicated in diverse roles in blocking the process. LpdC is proposed to block phagosome maturation by retaining Coronin 1A at the phagosomeal membrane (11). NdkA, is proposed to interfere with GTP association with Rab GTPases on the phagosomal membrane, which are required for phagosome maturation (26). PtpA is shown to interfere with phagosome-lysosome fusion, via association with the V-ATPase and dephosphorylation of the Vps33B component of the HOPS complex (29). SapM is proposed to interfere with PI(3)P signaling by dephosphorylating PI(3)P at the phagosomal membrane (27).

4.2- Results

LpdC and NdkA are SecA2 independent.

To determine if specific *M. tuberculosis* proteins reported to effect phagosome maturation are exported in a SecA2 dependent manner, we used western blot analysis to compare the amount of proteins secreted into culture media (culture filtrates – CF) by the Δ*secA2* mutant and H37Rv strains of *M. tuberculosis*. Concentrated short-term culture filtrates were generated in the following manner. Individual strains were grown to an OD_{600} of ~1 in 7H9 Middlebrook media. The cultures were then washed and transferred to Sauton media in triplicate samples at an OD_{600} of 0.3. The 50 ml Sauton cultures were incubated in roller-bottles for 24 hours at 37°C then the cells were pelleted and the supernatant collected, filtered to remove cells and concentrated to generate the culture filtrate (CF) fraction. The cell pellets from the same cultures were fixed overnight in 10% formalin and later used to generate the whole-cell lysate (WCL) fractions. Using antibodies raised against an LpdC peptide or full-length *M. tuberculosis* NdkA we detected both LpdC and NdkA as secreted into culture filtrates by *M. tuberculosis*. As a lysis control, CF samples were shown to be devoid of the cytoplasmic SecA1 protein by western blot analysis (data not shown). However, for both LpdC and NdkA comparable protein levels were observed in culture filtrates from H37Rv and the Δ*secA2* mutant (Figure 4-1). Thus, the accessory SecA2 system does not appear to be involved in the export of these proteins.

With the same culture filtrates we also probed for the presence of PtpA using an anti-PtpA antibody. Even though in the whole cell lysate the PtpA antisera bound several proteins, a band of the predicted size of PtpA and the same size as purified PtpA protein

was visible in the whole cell lysate. However, in the culture filtrate we were unable to detect any protein of the expected size of PtpA (Figure 4-1). Our inability to detect PtpA in the culture filtrate precludes us from drawing any conclusion about the possibility of PtpA export being SecA2-dependent.

SapM protein levels are SecA2-dependent.

We also evaluated the secretion of SapM, which is a phosphatase proposed to be an effector of phagosome maturation. SapM is shown to cleave the phosphate, preferentially off of phosphotydlinositol 3-phosphate (PI(3)P) (22, 27). Because PI(3)P is an important signaling molecule on the surface of the phagosome, that is required for phagosome maturation, SapM is proposed to influence phagosome maturation by reducing PI(3)P levels on the phagosome (27).

Western blot analysis with SapM antibody recognized a single band of the predicted size (30 kD) in both the whole cell lysate and the culture filtrate fractions of *M. tuberculosis*. The level of SapM protein was reproducibly less in the culture filtrates of the Δ*secA2* mutant compared to H37Rv, and, the level of SapM protein was also reduced in the cell-associated fraction (WCL) of the Δ*secA2* mutant. Importantly, the SapM phenotype was complemented in *trans* by adding back a copy of *secA2* on a plasmid (Figure 4-2A). By looking at cell fractions on a Western blot, we found that the cell-associated SapM was almost entirely in the cytoplasm as opposed to the cell wall or membrane (Data not shown).
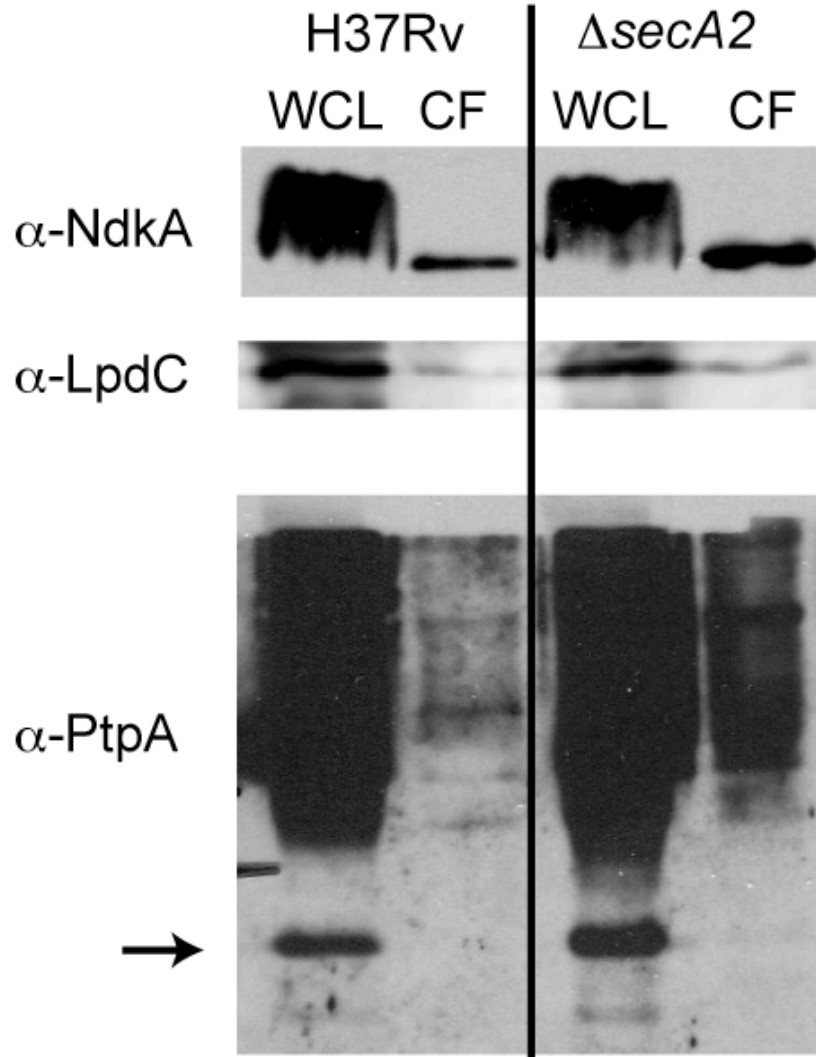
Figure 4-1. Secretion of NdkA and LpdC is independent of SecA2.
Western blot analysis from whole cell lysates and concentrated culture
filtrates prepared from the wild type H37Rv and Δ*secA2* mutant strains
of *M. tuberculosis*. NdkA and LpdC are detected in both wild type and
Δ*secA2* mutant culture filtrates at comparable levels. PtpA was not
detected in culture filtrates. A band that ran at the size of a PtpA
positive control was present in the whole-cell lysate, but absent in the
culture-filtrate fraction. WCL lanes were loaded with equal protein, 1-5
µg protein depending on available material, CF lanes were loaded for
cellular equivalents between strains.

The reduced cytoplasmic SapM levels where we found lower levels of SapM were detected in independent samples prepared from the ΔsecA2 mutant grown in different culture conditions, including media that was designed to mimic the environment of the phagosome (figure 4-2B). The difference in WCL associated SapM was quantitated and found statistically significant by densitometry of Western blots (Figure 4-2B).

Using a previously reported phosphatase assay designed to filter out background phosphatase activity in order to quantify SapM activity of *M. tuberculosis* derived samples, we assessed SapM activity in culture filtrates (22). To filter out background phosphatase activity, the assay uses tartrate in all reaction buffers. Tartrate is an inhibitor of some phosphatases, but SapM activity is unaffected by tartrate (22). Using this assay, we measured phosphatase activity in culture filtrates of the ΔsecA2 mutant compared to H37Rv and the complemented strain. We loaded equal total CF protein to each assay as determined by BCA assay. We found significantly less phosphatase activity in the supernatant of the ΔsecA2 mutant compared to H37Rv and the complemented strain (Figure4-2C). The phosphatase activity we assayed in these reactions was tartrate resistant and molybdate sensitive (data not shown), which is characteristic of SapM (27). The results from this phosphatase assay are consistent with our western blots. However, it should be noted that the assay used here may not be exclusive for SapM. The phosphatase assay we used could detect any other tartrate resistant molybdate sensitive phosphatases that may be present in *M. tuberculosis* culture filtrates.

Taken together, our results show an effect of SecA2 on SapM protein levels and a reduction in phosphatase activity that is consistent with less secreted SapM. In the

presence of a secretion defect one might expect to see the levels in the whole cell lysate be unaffected or accumulate. However, because SapM levels were reduced in the whole cell lysate as well as in culture filtrates it is not immediately clear if this difference reflects a role for SecA2 in SapM secretion.

To further characterize the relationship between lower SapM levels and the Δ*secA2* mutation, we tested if *sapM* transcript levels were also reduced in the Δ*secA2* mutant of *M. tuberculosis*. Using quantitative RT-PCR we measured the transcript levels of *sapM* relative to the *rpoB* gene, and found levels of *sapM* transcript to be equal between H37Rv and the Δ*secA2* mutant (Figure 4-2D). The *rpoB* gene encodes the Beta subunit of RNA polymerase, which works as an internal control for total RNA because it should have constant transcript levels between strains. This data rules out the possibility that lower levels of SapM protein were the result of altered *sapM* transcription in the Δ*secA2* mutant.

4.3- Discussion

In this chapter, we began the process of determining which effectors of phagosome maturation arrest are SecA2-dependent. In these initial experiments we obtained antibodies from other investigators to test putative effectors of phagosome maturation arrest for evidence of export by the accessory SecA2 system.
We compared culture filtrates from H37Rv and the Δ*secA2* mutant for LpdC and NdkA localization. The antibodies we used reacted with a single protein band of the proper size on the western blots. In these experiments, the levels of LpdC and NdkA were comparable in cultures filtrates of H37Rv and the Δ*secA2* mutant. Thus, SecA2 appears

to have no role in the export of LpdC or NdkA. We also attempted to monitor PtpA secretion by the two strains. While the antibody did identify PtpA protein in the whole cell lysate, we could not detect PtpA in culture filtrates of either H37Rv or the Δ*secA2* mutant. This negative result of testing for PtpA secretion prevents us from drawing any conclusions about the potential for the SecA2 system to be exporting this effector. Even though PtpA is demonstrated to be secreted by *M. tuberculosis* growing in host cells it is likely that the proteins is not highly expressed by in vitro grown *M. tuberculosis* (2). In order to monitor the secretion of PtpA we will need to test *M. tuberculosis* strains engineered to express the protein from a constitutive promoter.

When we tested for SapM secretion, we found that that the SecA2 system affects the levels of SapM protein in both the culture filtrate and whole cell lysate. This reduced SapM phenotype was reversed when the Δ*secA2* mutant was complemented in *trans*, which demonstrates this phenotype is attributable to the lack of SecA2. Furthermore, phosphatase assays designed to monitor SapM activity in culture filtrates were consistent with the western blotting results.

The relationship between SecA2 and SapM remains to be clarified. Quantitative RT-PCR data argues against the possibility of SecA2 indirectly affecting transcription of the *sapM* gene. Like the two known SecA2 substrates in *M. smegmatis*, *sapM* encodes a N-terminal Sec signal peptide. One possibility is that SecA2 is required for SapM export and the resulting defect in SapM export that occurs in the Δ*secA2* mutant results in degradation of the non-exported protein.
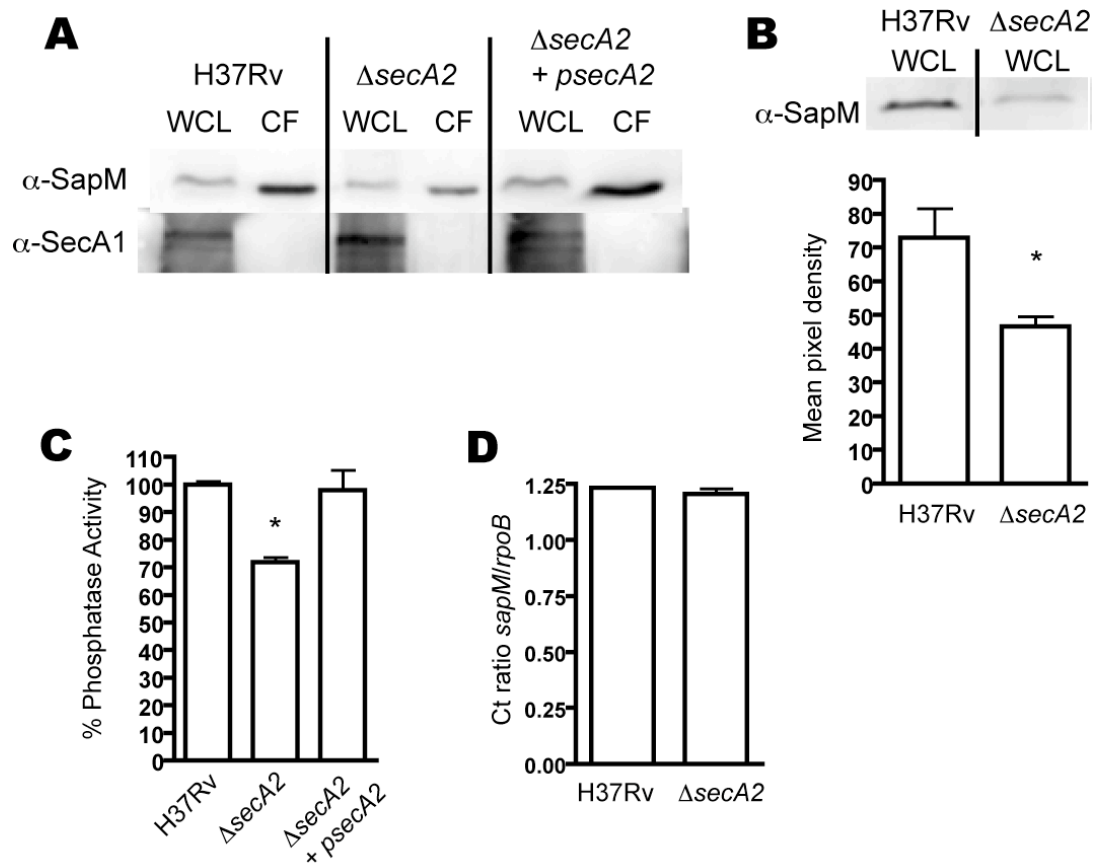
Figure 4-2. SapM levels are reduced in the Δ*secA2* mutant. A. Western blot for SapM, shown is a representative of triplicate whole cell lysate samples from H37Rv, the Δ*secA2* mutant, and complemented strain that were grown in 7H9 and Sauton media. B. Whole cell lysates from bacteria cultured in low pH 7H9 with propianate, quantitated by densitometry. Bars represent mean of independent triplicate samples +SD, *p≤0.05 by Student's *t* test. C. Phosphatase assay of concentrated supernatants, data is normalized to H37Rv as 100% activity. Strains were grown in 7H9 and Sauton media. Bars represent mean of independent triplicate samples +SD. *p≤0.05 by Student's *t* test. D. Real-time PCR data comparing transcript levels from H37Rv to the Δ*secA2* mutant. Strains were grown in 7H9 media. Bars represent mean of triplicate samples +SD.

There is precedent for non-exported proteins in the cytoplasm being degraded when their cognate export system is absent (8, 10). Thus, it is possible that the reduced level of SapM in the Δ*secA2* mutant is due to an export defect resulting in less protein secreted into culture media and degradation of the non-exported protein that accumulates in the cytoplasm. Another possibility is that the impact of SecA2 on SapM protein may be independent of a role in export and more simply be to stabilize the protein in the cytoplasm. In this case in the Δ*secA2* mutant there would be less protein in the cytoplasm available for secretion. To determine if SecA2 is involved in the process of SapM secretion we plan to employ more sensitive pulse-chase protocols that have previously been used to study protein secretion (8).

While our data shows the Δ*secA2* mutant to secrete less SapM, we also do not yet know if the reduction in SapM alone can account for the Δ*secA2* mutant defect in arresting phagosome maturation. While we do see a difference in SapM levels between H37Rv and the Δ*secA2* mutant, the difference is a modest one. One approach we plan to take to address this question is to overexpress SapM in the Δ*secA2* mutant and see if this rescues the phagosome maturation phenotype observed in the absence of the SecA2 system. If SapM overexpression can overcome the difference in phagosome maturation and intracellular growth between H37Rv and the Δ*secA2* mutant, it would argue that the defect in SapM secretion is the cause of the Δ*secA2* mutant growth defect. However, the *M. tuberculosis* process of blocking phagosome maturation is likely to be complex with potentially redundant effectors. Therefore, if SapM overexpression does not rescue the Δ*secA2* mutant growth defect, it could be because multiple effectors of phagosome

maturation, including SapM, are exported by the SecA2 system. In the mean time, continuing efforts in our laboratory are underway to identify additional SecA2 dependent exported proteins that may be effectors of phagosome maturation.

4.4- Materials and Methods

Bacterial strains and growth conditions

In this study we used *Mycobacterium tuberculosis* wild type strain H37Rv, and the Δ*secA2* mutant (mc^{2}3112) generated in the H37Rv background (5). *M. tuberculosis* strains were cultured in liquid Middlebrook 7H9 media supplemented with 0.05% Tween 80, 0.5% glycerol, 1× albumin dextrose saline (ADS). Sauton media used for preparation of culture filtrates contains the following in one liter of media: 4 g DL-asparagine, 2 g sodium citrate, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$-7H_2O, 50 mg ferric ammonium citrate, and 48 ml glycerol. The final media is pH adjusted to 7.4 and filter sterilized through a 0.22 μm filter. Media used to mimic phagosomal conditions was 7H9 with 0.1% glycerol, 1mM proprionic acid, 0.1% tyloxapol, 0.1M MES (buffer), 0.5% BSA, and is pH adjusted to 6.5.

Culture filtrates and phosphatase assay

For culture filtrate collection, cultures at log-phase growth in 7H9 were washed in Sauton media, and added to 50ml Sauton media in roller bottles at an OD_{600} of 0.2-0.3. Cultures were incubated at 37°C for 24 hours. Then the entire 50 ml culture was centrifuged at 4000 rpm in a Sorvall Legend RT centrifuge. The supernatants were

collected and double filtered with a 0.2μm filter. Culture Filtrate proteins were

concentrated 100 fold using 15 ml capacity 10,000 MW cut off centrifuge filters

(Centricon). Samples were centrifuged at 3,000 rpm and sample was added until the

entire 50 ml was concentrated to < 500 μl. The bacterial pellet from the culture was fixed

in 10% formalin overnight and later used to prepare whole cell lysates by bead beating in

protein extraction buffer (3, 14). SapM activity was assayed as described previously (22).

Protein concentration was assayed using a BCA assay (Pierce) and equal concentration of

CFP protein (3-5 μg depending on available material) was added to each assay. In a 96

well plate 10x buffer of 1M Tris base pH 6.8 with 20 mM sodium tartrate to inhibit

background phasphatase activity was added to each assay and 50mM p-nitrophenyl

phosphate (pNPP) samples were brought to 200 μl total volume. In samples with

molybdate, 1 mM sodium molybdate was added to the sample prior to adding CF protein.

The plate was incubated at 37°C in a TECAN automated plate reader, and the absorbance

at 405nm was measured every minute for two hours. Phosphatase activity cleaves pNPP,

which creates a color change and an increase of OD_{405} absorbance. Over the linear portion

of the kinetic assay we calculated the rate of pNPP conversion by calculating the slope of

the line generated by plotting OD_{405} in the y-axis and time in the x-axis. These slopes

were then normalized to the H37Rv rate of change, which we set to 100%.

Western blotting

Equal amounts of protein samples from whole cell lysates (1-5μg depending on

available material) or concentrated culture filtrates (cell equivalents between strains)

were run on a 12% SDS-PAGE gel, and then transferred to nitrocellulose membranes.

After transfer, the membranes were blotted with primary antibodies. Antibodies to *M. tuberculosis* proteins were kind gifts of Dr. Vojo Deretic, University of New Mexico (SapM), Dr. Zakaria Hmama, University of British Columbia (LpdC and NdkA), and Dr. Yossef Av-Gay, University of British Columbia (PknG and PtpA). Antibodies were used at the following dilutions (SapM 1:2000, LpdC 1:2,000, NdkA 1:2,000, PknG 1:1,000 and PtpA 1:10,000) Secondary antibodies were either conjugated to horseradish peroxidase or alkaline phosphatase. Blots were then developed using a horseradish peroxidase or alkaline phosphatase substrate. Blots were visualized with direct film exposure or a phosphoimager. For quantitative westerns, fluorescence was quantified using a phosphorimager and ImageQuant 5.2 (Molecular Dynamics).

Quantitative real time PCR

Quantitative real-time PCR (qRT-PCR) was run on RNA samples previously prepared for microarray analysis. Bacteria were grown in roller bottles using 7H9 with Tween-80 and ADS. mRNA was prepared by Meghan Feltcher and cleaned up using a DNA-Free RNA kit (Zymo). Prior to clean-up RNA extraction was performed as follows: Bacterial culture is lysed in chloroform/methanol 3:1 mix. An equal volume of TRIzol reagent (Invitrogen) is used to separate protein and lipids from the nucleic acids. After thorough mixing of TRIzol and *M. tuberculosis* lysate, samples were centrifuged and the aqueous layer transferred to a fresh tube. Then an equal volume of isopropanol was added to the tube to precipitate nucleic acids overnight at -20°C. The nucleic acids were then pelleted by centrifugation and the pellet washed with 70% ethanol. After washing the nucleic acids were resuspended in H_2O and treated with DNAse. Primers were designed

to *sapM* (ATCGTTGCTGGCCTCATGG and AGGGAGCCGACTTGTTACC) and to *M.*

tuberculosis rpoB (ACAGAAGCTAGTCCTAGTC and

ACCGATCAGCCACTCGAAC), which was used as a normalization control as done

previously (16). Primers were tested on *M. tuberculosis* DNA to be sure that only one

product was produced. qRT-PCR reaction was run using a SensiMix Syber and

Fluorescein One-Step kit (Bioline). mRNA template was loaded at 48 ng/20 μl reaction.

Reactions were run on an iCycler (BioRad).

Attributions

The authors of this work contributed in the following ways: Experiments were designed

by JTS and MB. Experiments were performed by JTS, MEF, and EY. Manuscript was

written by JTS and MB. Funding was secured by MB.

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CHAPTER V

DISCUSSION

Mycobacterium tuberculosis infects about one-third of the world's population and causes a substantial amount of mortality worldwide (39). Currently, the vaccine to prevent *M. tuberculosis* infection is questionable at best (11), and drug regiments are extended and difficult to administer effectively, especially in developing nations where tuberculosis is endemic. Moreover, with the emergence of strains of *M. tuberculosis* that are resistant to first-line and second-line drugs, the need for new treatments and vaccines to prevent the spread of this bacterial pathogen is urgent. A better understanding of the pathogenesis of *M. tuberculosis* should help to develop new strategies for targeting tuberculosis with drugs, or for eliciting protective immunity with improved vaccines.

The research presented in this thesis focuses on the role of secreted proteins and the SecA2 protein export pathway in *M. tuberculosis* pathogenesis. In this research we used a Δ*secA2* deletion mutant strain of *M. tuberculosis* to dissect the role of the SecA2 protein export pathway in our macrophage and mouse infection models. Going into the project we already knew that the SecA2 protein export pathway had a role in virulence from data showing that the Δ*secA2* mutant is defective for growth in macrophages and mice (2, 19). However, we did not know the cause of this intracellular growth defect.

Thus, we do not know the role SecA2 plays in virulence. The Δ*secA2* mutant does not have a growth defect *in vitro*, ruling out a general slow growth phenotype(2).

At the start of this research, there were three reported phenotypes for Δ*secA2* mutant infected macrophages that could possibly explain its intracellular growth defect. First, macrophages infected with the Δ*secA2* mutant produce increased levels of reactive nitrogen intermediates (RNI). Second, the Δ*secA2* mutant induces higher levels of proinflammatory cytokines by infected macrophages(19). Third, the Δ*secA2* mutant induces more apoptosis than wild type *M. tuberculosis* in infected macrophages(16).

Increased macrophage production of RNI does not explain the Δ*secA2* mutant intracellular defect.

Previous data from our laboratory ruled out a failure to resist either RNI or reactive oxygen species (ROS) as the sole cause of the Δ*secA2* mutant growth defect. Specifically, it was shown that the Δ*secA2* mutant remains attenuated for growth in macrophages that fail to produce RNI (NOS2$^{-/-}$) or ROS (gp91$^{phox-/-}$ or p47$^{phox-/-}$)(19). While these past results do not completely rule out a role for SecA2 in protecting against or limiting reactive radical stresses, these results do indicate the existence of another role for SecA2 in promoting intracellular growth.

The increased inflammatory response elicited by the Δ*secA2* mutant is not sufficient to explain the intracellular growth defect.

Compared to H37Rv, the *M. tuberculosis* Δ*secA2* mutant induces an increased inflammatory response in infected macrophages as shown by higher levels of TNF-α, IL-6 and RNI. TNF-α, IL-6 and RNI all play important roles in controlling *M. tuberculosis*

replication in macrophages and mice (5, 12, 13, 20, 22). Therefore, we hypothesized that the increased inflammatory response elicited by the Δ*secA2* mutant may be responsible for controlling the intracellular replication of the mutant. In Chapters two and three of this thesis we tested this possibility by eliminating components of the inflammatory response and asking if this rescued the mutant phenotype. Our results indicate that the increased inflammatory response to the Δ*secA2* mutant is not sufficient to explain the intracellular growth defect. Specifically, we worked with mice and macrophages deficient in the production of TNF-α. TNF-α has been shown to be important for activating macrophages synergistically with interferon gamma, thus increasing the antimicrobial capacity of macrophages and allowing them to contain *M. tuberculosis* replication (12). We also tested whether a broader inflammatory response to *M. tuberculosis* is important for controlling the Δ*secA2* mutant using mice and macrophages lacking the adaptor protein MyD88. MyD88 has been shown to be involved through Toll-like receptor (TLR) signaling for production of TNF-α, IL-6 and RNI in response to *M. tuberculosis* (3, 15, 18, 25, 28, 34, 35, 37). However, when the Δ*secA2* mutant was tested in the TNF-α^{-/-} or the MyD88^{-/-} mice and macrophages the mutant remained attenuated for growth. Thus, while both TNF-α and MyD88 are important for controlling *M. tuberculosis* infection, neither appears to be necessary for the Δ*secA2* mutant growth defect. Our results argue that the role of the SecA2 system in promoting intracellular growth in macrophages can not simply be to dampen the macrophage inflammatory cytokine response to *M. tuberculosis*.

The pro-apoptotic phenotype elicited by the Δ*secA2* mutant is unable to explain the intracellular growth defect.

The Δ*secA2* mutant also induces higher levels of apoptosis of infected macrophages than wild type *M. tuberculosis*(16). This difference in apoptosis is attributed to the defect in SodA secretion reported for the Δ*secA2* mutant (16). Because apoptosis has been demonstrated to control and even kill multiple mycobacterial species including *M. tuberculosis* (8, 14, 24), we were interested in testing whether increased apoptosis exhibited by Δ*secA2* mutant infected macrophages was responsible for the growth defect of the Δ*secA2* mutant. To test the role of apoptosis in the Δ*secA2* mutant phenotype, we took advantage of a plasmid expressing an extra copy of SodA, termed αSodA. When αSodA is expressed by the Δ*secA2* mutant, the apoptosis phenotype of the Δ*secA2* mutant is reversed. We tested the same Δ*secA2* mutant strain expressing αSodA for growth in macrophages and mice to assess the role of apoptosis in the Δ*secA2* mutant growth defect. As shown in chapter three, even though this construct rescues the apoptosis phenotype it does not rescue either the macrophage or the mouse growth defect of the Δ*secA2* mutant. In fact, expression of αSodA in the ΔsecA2 mutant exacerbated the virulence defect of the mutant in a mouse. We conclude from these results that the pro-apoptotic phenotype of the Δ*secA2* mutant is not responsible for the growth defect, either in mice or macrophages.

Our observation that the Δ*secA2* mutant expressing αSodA exhibited a more severe attenuated phenotype than the Δ*secA2* mutant in mice was unexpected. One possibility is that overexpression of SodA succeeds in converting superoxide to peroxide but that the catalase KatG, which has been shown to be SecA2 dependent for export, is

not able to complete the detoxification and the bacteria is left in a environment that is more toxic due to high levels of peroxide. Another possibility is that ROS levels, which are important signals for the immune response (10), are deregulated by overexpression of SodA. The deregulated levels of ROS could lead to a more robust immune response, which could be acting to suppress the growth of the bacteria.

The Δ*secA2* mutant has also been shown induce a more robust cytotoxic T-cell response than H37Rv in murine infection. This adaptive immune response to the Δ*secA2* mutant is thought to be caused by the pro-apoptotic phenotype of the mutant in infected macrophages (16). The more robust T-cell response makes the Δ*secA2* mutation an attractive addition to a live attenuated *M. tuberculosis* vaccine strain. In this thesis, we tested the possibility that the pro-apoptotic phenotype of the Δ*secA2* mutant was responsible for the intracellular growth defect. In these experiments we ruled out the pro-apoptotic phenotype being responsible, at least on its own, for the intracellular growth defect of the mutant. However, our data does not preclude the hypothesis that the Δ*secA2* mutant induces a more robust T-cell immune response via increased apoptosis. Our data does, however, separate two distinct functions for SecA2: a role in suppressing apoptosis and a role promoting intracellular growth.

Thus, the results of testing the significance of the three reported macrophage phenotypes of the Δ*secA2* mutant, (increased RNI, increased inflammatory cytokine response, and increased apoptosis) failed to uncover the specific role for SecA2 in promoting growth in macrophages and mice. These results led us to consider other possibilities to explain the function of the SecA2 system in promoting intracellular growth, such as a role in modifying the normal process of phagosome maturation.

The Δ*secA2* mutant traffics to a more mature phagosome than does wild type *M. tuberculosis*.

It has long been known that *M. tuberculosis* prevents phagosome maturation(1), and blocking phagosome maturation has been considered an integral part of *M. tuberculosis* virulence. Because exported proteins are implicated in the process of *M. tuberculosis* phagosome maturation arrest, we tested the possibility that the accessory SecA2 protein export system is important for blocking phagosome maturation. Our data in chapter three shows the Δ*secA2* mutant to be defective in the process of phagosome maturation arrest. Compared to H37Rv, the Δ*secA2* mutant resides in a phagosome that is more acidic and stains positive for surface markers of phagosome maturation V-ATPase, CD63, and Rab7. However, while these markers indicate phagosome maturation it should be pointed out that they are not unique to the fused phagolysosome. Thus, we do not know if the Δ*secA2* mutant ends up in a terminally fused phagolysosome. Other assays, such as fluorescent dextran fusion assays or electron microscopy, which we have not managed to successfully use in our system, would be required to define phagosome-lysosome fusion of Δ*secA2* mutant containing phagosomes.

It has been speculated that any *M. tuberculosis* mutant that fails to grow or is metabolically inactive in a macrophage, will eventually traffic to a more mature phagosome(21, 27). This raised the possibility that the Δ*secA2* mutant is in an acidified phagosome as a secondary consequence of a failure to grow in macrophages, as opposed to being the cause of the mutant intracellular growth defect. To test whether all intracellular growth defects of *M. tuberculosis* mutants lead to localization in a mature phagosome, we examined phagosome acidification of five other mutants with

intracellular growth defects, including the leucine auxotroph Δ*leuD* mutant (17, 23).

Despite having intracellular growth defects, four out of the five mutants tested behaved

like wild type in maintaining the ability to block phagosome maturation at 24 hours post

infection. In fact, the Δ*leuD* mutant still blocked phagosome acidification out to 72 hours

post infection. The fifth mutant tested, an *esx-1* mutant, was chosen as a positive control

for a mutant known to have a defect in blocking phagosome maturation(4, 21, 40), and

our results agreed with previous findings that the *esx-1* mutant resides in a more acidic

phagosome with surface markers of phagosome maturation in comparison to H37Rv.

Our finding that four individual *M. tuberculosis* mutant strains with defects in

intracellular growth maintained a phagosome maturation block argues against the

possibility that any intracellular growth defect results in phagosome maturation. This left

us with the other possibility that the phagosome maturation that occurs with the Δ*secA2*

mutant is due to a specific defect in exporting an effector protein involved in the process

and that the failure to block phagosome maturation is the cause of the intracellular growth

phenotype.

Increased phagosome maturation leads to the Δ*secA2* mutant growth arrest.

While blocking phagosome maturation has long been considered integral to *M.*

tuberculosis intracellular survival and growth(30), surprisingly few experiments have

directly tested whether phagosome acidification and maturation directly affect *M.*

tuberculosis intracellular replication. From previous research performed in numerous

laboratories, a strong correlation exists between mutants that fail to block phagosome

maturation and mutants that have intracellular growth defects (4, 21, 26, 36). However,

the causal nature of the relationship between failure to block phagosome maturation and intracellular growth inhibition had not been defined. In addition, there are a few reports of *M. tuberculosis* mutants that fail to block phagosome maturation but grow normally in macrophages. These exceptions raise the question about the need to block phagosome maturation to allow intracellular growth.

The Δ*secA2* mutant provided us with the opportunity to test if failure to block phagosome maturation could, in fact, be growth inhibitory. To answer the question of whether phagosome maturation controls the Δ*secA2* mutant's intracellular replication, we utilized chemical inhibitors of phagosome maturation. Specifically, bafilomycin A1 and concanamycin A are inhibitors of the vacuolar ATPase (V-ATPase) the protein pump that is responsible for acidifying the phagosome(9). Treatment of infected macrophages with these inhibitors rescued the Δ*secA2* mutant from an acidified phagosome, and rescued the Δ*secA2* mutant growth defect. The inhibitors also had a mild positive effect on intracellular replication of H37Rv, which makes sense because a fraction of wild type *M. tuberculosis* is found in acidified phagosomes. Importantly, treatment with the inhibitors did not rescue intracellular growth defect of the Δ*leuD* mutant, which remains in a neutral phagosome, indicating the effect of these inhibitors was specific for the Δ*secA2* mutant.

In addition to showing the Δ*secA2* mutant growth phenotype in macrophages is attributable to its defect in blocking phagosome maturation, these experiments make a larger statement about the importance of *M. tuberculosis* avoiding phagosome maturation. Our experiments demonstrated a causal relationship between phagosome maturation and *M. tuberculosis* growth arrest.

SecA2 dependent effectors of phagosome maturation

The Δ*secA2* mutant fails to block phagosome maturation compared to H37Rv. Because the failure to block phagosome maturation appears to be specific to the lack of the SecA2 export pathway, we hypothesize that one or more effectors of phagosome maturation depend on SecA2 for export. As a starting point to identify such effectors, we looked for SecA2 dependent export of a set of putative protein effectors of phagosome maturation. As reported in chapter four, one of the proteins tested, the secreted phosphatase SapM, was present at reduced levels in the Δ*secA2* mutant. Less SapM was detected in culture filtrates prepared from the Δ*secA2* mutant. In addition, we found less cell associated SapM, indicating that decreased SapM levels may be a result of decreased cytoplasmic stability. One possibility is that cytoplasmic stability is reduced in the Δ*secA2* mutant independent of an export defect. The other possibility, for which there is precedent, is that cytoplasmic stability is compromised as a result of an export defect (6, 7). More research will be required to elucidate the role of SecA2 in SapM export.

SapM is shown to cleave phosphates from phosphatydlinositol-3-phosphate, an important signaling molecule required for phagosome-lysosome fusion(38). Thus, SapM is potentially a SecA2 dependent effector of phagosome maturation. However, phosphatase levels were only mildly reduced in the Δ*secA2* mutant secreted fraction. Additionally, the published study linking SapM and phagosome maturation arrest did not go as far as showing a direct role of SapM in phagosome maturation arrest(38). For example, it has yet to be shown that a *sapM* mutant is defective for blocking phagosome

maturation. The current data only shows that SapM preferably dephosphorylates PI(3)P, it does not directly show that this activity leads to phagosome maturation arrest (38).

Further studies of the SecA2 exported proteome and dissection of the role of SapM in phagosome maturation are required to better understand the role of SecA2 in phagosome maturation arrest. The current list of putative effectors of phagosome maturation is long and there are probably effectors with redundant functions. Thus, there may be more than one effector exported by the SecA2 system. To identify other SecA2 dependent exported proteins it may be necessary to use large-scale proteomic methods. Previous studies have employed 2D gel electrophoresis methods to discover SecA2 dependent exported proteins, and the limitations of these studies have been reached. More sensitive quantitative mass spectrometry is an alternative approach that should be considered for discovering new SecA2 dependent exported proteins.

As an alternative approach, for identifying SecA2 dependent effectors of phagosome maturation, we have also considered a genetic screen based on TraSH analysis (29, 31-33). TraSH is a transposon based genetic method used to determine genes that are required for pathogen survival in a particular environment. Using TraSH, we would compare the genes required to replicate in a macrophage to the genes required to replicate in a macrophage treated with bafilomycin A1. Because the Δ*secA2* mutant is specifically rescued in macrophages by addition of bafilomycin A1 or concanamycin A, TraSH analysis could be used to find similarly rescuable mutants in macrophages. Mutants that can be rescued like the Δ*secA2* mutant with bafilomycin A1, would be considered candidate SecA2 dependent effectors of phagosome maturation.

Conclusion

The SecA2 system has an impact on multiple components of the innate immune response, such as cytokines, apoptosis, and here we show phagosome maturation. We have shown in this thesis that the SecA2 protein export pathway is required for blocking phagosome maturation. Furthermore, blocking phagosome maturation is required for *M. tuberculosis* intracellular replication (Figure 5-1). While a role in exporting the SapM phosphatase may prove to be the important contribution of the SecA2 system to phagosome maturation arrest, we have yet to prove this to be the case. It is certainly possible that there are additional effectors of phagosome maturation arrest that are controlled by the SecA2 system. This work shows a direct causal link between phagosome maturation and *M. tuberculosis* growth arrest reasserting phagosome maturation arrest as a critical step in virulence. Further, we have opened up a new avenue for discovering effectors of phagosome maturation by finding SecA2 exported proteins.
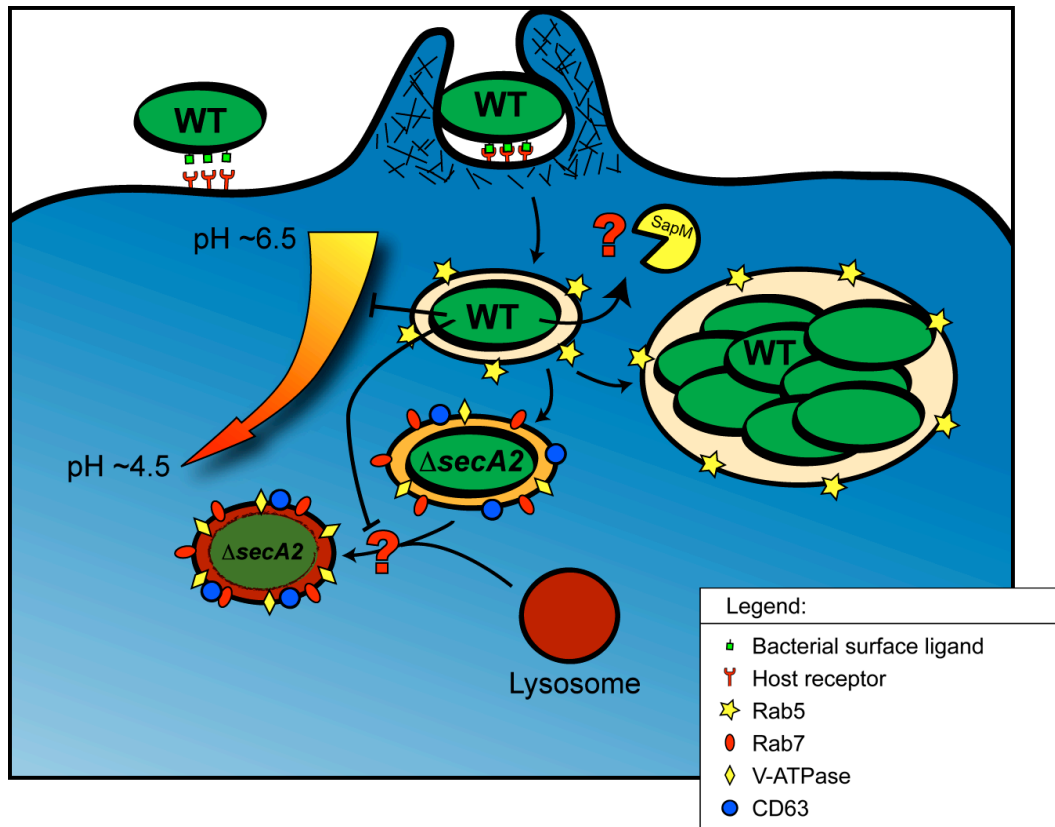
Figure 5-1. The accessory SecA2 protein export system promotes growth in macrophages by blocking phagosome maturation. In this model SecA2 is required for export of one or more effectors of phagosome maturation SapM as one potential effector and the question mark indicates other possible effectors. Without *secA2* the effectors are not properly localized to prevent phagosome maturation and the mutant containing phagosome progresses to a late-endosome or possibly a phagolysosome, and bacterial replication is arrested. It is unclear whether or not the Δ*secA2* mutant blocks phagosome-lysosome fusion.

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