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Modulation of Host Inflammatory Responses by SecA2 of *Mycobacterium marinum*

by

Brigitte Y. Watkins

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Biomedical Sciences

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GRADUATE DIVISION

of the

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by

Brigitte Y. Watkins

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Contributions of Others to the Presented Work

Chapter 2 of this dissertation will be submitted for publication to an as of yet undetermined journal. The following co-authors contributed to this work: Shilpa Joshi (Genentech, South San Francisco), Cary Austin (Genentech, South San Francisco), David Ball (Lawrence Berkeley National Laboratory), Summer Park (Genentech, South San Francisco), and Janice Kim (Genentech, South San Francisco). Eric J. Brown supervised this work.

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Mycobacterium marinum

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Brigitte Y. Watkins

Advisor: Eric J. Brown

Abstract

Mycobacterium tuberculosis, the infectious agent of tuberculosis, remains a leading cause of infectious disease worldwide due to poor control of infection, multidrug-resistant strains, and more recently, co-infection with HIV. To facilitate the study of the pathogenesis of *M. tuberculosis* and the potential discovery of new drug targets, our laboratory makes use of the *Mycobacterium marinum* model of tuberculosis infection. *M. marinum* is a very close relative of *M. tuberculosis* that causes a chronic, granulomatous disease in fish with many similarities to tuberculosis infection in humans. Of importance to the pathogenesis of Mycobacteria, are several secretion systems responsible for the export of proteins to the cell envelope and the extracellular space. SecA2 is an ATPase that provides energy for the export of a subset of proteins. This dissertation confirms that SecA2 is required for the virulence of Mycobacteria and explores the role of SecA2 in the modulation of host immune responses *in vivo*. In a zebrafish and a mouse tail model of tuberculosis infection we have shown that the SecA2 of *M. marinum* is required for the maximal induction of several pro-inflammatory cytokines including TNF-α. Additionally, SecA2 has a role in granuloma formation and/or maintenance. In vitro

studies of a Δ*secA2* deletion strain in *M. marinum* reveals that the mutant has an abnormal cell envelope by cryo-electron tomography, and increased sensitivity to SDS treatment. This work provides additional evidence supporting a role for SecA2 in modulating host immunity, and a novel role in cell wall morphology and function.

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Chapter 1

Introduction: Secretion Pathways in Mycobacteria

Abstract

Secretion systems are required by bacteria for the transport of proteins into and across membranes. The thick, waxy cell envelope of Mycobacteria presents an additional challenge to the export of proteins to the outer layers of the cell wall and to the extracellular space. *Mycobacterium tuberculosis* is a successful pathogen that has infected one third of the world's population, and kills about 1.5 million people each year. Key to its success is its ability to resist clearance by the antimicrobial mechanisms of host immunity in immunocompetent individuals. Secretion systems are important for secreting the virulence factors that mediate this survival and pathogenesis of *M. tuberculosis* within its host. This chapter of the dissertation describes the multiple secretion pathways present in Mycobacteria, and their role in virulence. Of greatest relevance to this thesis are the accessory Sec secretion systems that have been discovered in Mycobacteria and some Gram-positive bacteria.

Secretion pathways in Mycobacteria

Mycobacteria require secretion pathways to transport proteins into and across membranes to execute various tasks such as membrane and cell wall maintenance, cell division, protection from harmful environments, and pathogenesis in a host. Multiple secretion systems have been identified in Mycobacteria including the twin-arginine transport system (Tat), the recently identified ESX systems (ESX-1 to ESX-5), the general secretion pathway (Sec), and finally an accessory Sec pathway, which is the focus of this thesis.

Tat pathway

Like the Sec pathway, the Tat system is widely conserved in bacteria. The Sec system can only transport unfolded protein substrates, whereas the Tat translocase serves to transport fully folded proteins into and across the cytoplasmic membrane (1, 2, 3). Substrates of the Tat system bear a tripartite domain signal sequence similar to the Sec signal peptide: a polar N domain, a hydrophobic H domain, and basic C domain (4, 5). Between the N and H domains, lies the conserved S/T-R-R-x-F-L-K twin-arginine amino acid sequence motif (4). In non-mycobacterial species, both arginines have been shown to be essential for targeting protein substrates to the Tat machinery (6, 7, 8), with the exception of two proteins found in *Escherichia coli* and *Salmonella enterica* (9, 10). Functional Tat systems have been identified in *M. tuberculosis* and *M. smegmatis* (11, 12, 13). Mutational studies in *M. smegmatis* show that the Tat translocase proteins TatA, TatB, and TatC are required for export of β-lactamase and resistance to β-lactam

antibiotics (11, 13). Attempts to mutate the Tat proteins in *M. tuberculosis* have been unsuccessful, suggesting that the Tat pathway is essential. This presents a challenge to identifying Tat-dependent substrates in this clinically relevant species (14). Using truncated β-lactamase (′BlaC) as a reporter (11), McDonough and colleagues identified signal sequences from 13 *M. tuberculosis* proteins capable of exporting ′BlaC in a Tat-dependent manner (12), including PlcB, a phospholipase C enzyme required for virulence by *M. tuberculosis* (15, 16), and proteins with potential roles in carbohydrate and lipid metabolism, cell envelope maintenance, and nutrient import. They also demonstrated that secretion of full length PlcB required the "twin-arginine" motif (12). As has been found in other bacteria, one of the identified proteins lacked the second arginine of this motif. This finding suggests that other parts of the Tat signal peptide, or features of the mature protein itself, may assist in targeting proteins to the Tat translocase. Further studies of the Tat-dependent secreted proteins may explain why the Tat translocase is required by *M. tuberculosis* for growth, and perhaps uncover new roles in pathogenesis.

ESX Systems

Mycobacteria encode five predicted ESX secretion systems, ESX-1 to ESX-5. The first system to be identified was the ESX-1 system in *M. tuberculosis*. The ESX-1 locus, also known as the extended RD1 (region of difference 1) locus, is a cluster of genes that is deleted in all attenuated vaccine strains of *M. bovis* bacillus Calmette-Guerin (BCG), but conserved in all virulent strains of *M. tuberculosis* and *M. bovis* that were tested (17). The RD1 region is also conserved in several non-pathogenic and pathogenic mycobacteria including *M. smegmatis* and *M. marinum*. Most genes within this ESX-1

locus were found to be required for the secretion of two immunodominant antigens of *M. tuberculosis*, ESAT-6 and CFP-10 (18, 19, 20, 21, 22). Both of these proteins lack the Sec signal sequence, supporting the presence of an alternative secretion system in mycobacteria. In fact, a unique ESX-1 signal sequence was identified at the C-terminus of CFP-10 that was sufficient for secretion of a yeast ubiquitin fusion protein from *M. tuberculosis* bacilli (23). A model has been proposed for the ESX-1 system (24), but structural data is still needed to confirm the nature of the translocase and supporting members, and its location within the cell envelope.

Three regions of differences were identified in the *M. bovis* BCG strain, but early studies showed that the RD1 region is a key determinant of virulence in mycobacteria. Expression of the ESX-1 locus from *M. tuberculosis* in *M. bovis* BCG was able to enhance the immunogenicity, persistence and pathogenesis of the attenuated strain in mice (25, 26). In addition, Mycobacterial strains containing mutations and deletions of the RD1 region were found to be attenuated *in vivo,* providing further evidence that the ESX-1 locus is required for virulence of *M. tuberculosis* (18, 27, 28, 19) and *M. marinum* (20, 29). ESX-1 is reported to influence multiple aspects of pathogenesis including inhibition of pro-inflammatory responses (18), arrest of phagosome maturation (30), induction of lung necrosis (31), macrophage apoptosis (32), membrane lysis (33), cell to cell spread (19, 20), granuloma formation (34, 35), T cell recognition (36), and still controversial, phagosome escape (37). Although ESAT-6 and CFP-10 have been the primary candidates for key effector molecules of the ESX-1 system, two additional proteins (EspA and EspB) have been shown to be substrates of ESX-1 and to be required for virulence (38, 39).

The other four ESX loci (ESX-2 to ESX-5) encode ESAT-6 family members, and contain genes homologous to the founding ESX-1 gene cluster (40, 41). Of the four, the ESAT-6 homologs of ESX-3 and ESX-5, but not ESX-2 and ESX-4, have been detected in culture supernatant (42, 43). These two loci may be required for growth in vitro according to two screens for essential genes in *M. tuberculosis* (44, 45). In agreement with this data, conditional down-regulation of the ESX-3 locus has shown that this locus is required for growth and supports previous studies implicating a role for ESX-3 in zinc and iron uptake by the bacteria (46, 47, 48). The ESX-5 locus has been studied in *M. marinum* and is only found in the subclass of slow-growing mycobacteria. It has a specialized role in secreting PPE and PE_PGRS proteins that are unique to mycobacteria (49, 50). Some Gram-positive bacteria appear to have ESX loci similar to the ESX-1 (51, 52). and ESX-4 systems of mycobacteria (53). Functional systems have been identified in *Staphylococcus aureus* and *Listeria monocytogenes*. Two Esat6-like proteins, EsxA and EsxB are required for abscess formation by *S. aureus* in the murine abscess model (51). However the ESX system is not required for the pathogenesis of *L. monocytogenes in vivo* (52).

General Sec Pathway

The general secretion pathway (Sec) serves to translocate unfolded proteins into and across membranes. It is found in bacteria, archaea, and even eukaryotic cells (54), and is essential in all bacteria tested. Studies of Sec in *Escherichia coli and Bacillus subtilis* have most clearly defined the mechanics of this system. Proteins are targeted to the Sec complex machinery by a signal peptide at the N-terminus. The signal peptide

contains a positively charged N domain, a hydrophobic H domain, and a slightly polar C domain that contains the cleavage site. Gram-positive bacteria tend to have longer, more hydrophobic signal peptides than Gram-negative bacteria (55). The Sec complex is composed of SecA, the motor subunit, and SecY, SecE, and SecG, which comprise the core of the translocase channel (56). Accessory complex members that assist translocation include SecD, SecF, and YajC (57). SecA functions from the cytoplasmic side of the membrane (58), and as an ATPase, it is essential for driving proteins through the translocase core and for insertion of some inner membrane proteins (59, 60). SecA binds directly to the SecY subunit, and likely to phospholipids and anionic lipids within the membrane (61, 62, 63). To translocate a pre-protein, SecA binds the unfolded polypeptide while in an ATP bound state, transports it into the SecY pore, then releases it in its ADP bound state (64, 65). In a stepwise manner, SecA pushes 20-30 residues of the protein through the translocase per cycle of ATP binding and hydrolysis (66). Coordination of ATP hydrolysis and protein transport is orchestrated by its multiple domains including the DEAD (Asp-Glu-Ala-Asp) motor, two nucleotide-binding domains (NBD), an intramolecular regulator of ATPase 2 (IRA2), the pre-protein binding domain (PBD), and the polypeptide crosslinking domain (PPXD) (67, 68, 69, 70). Upon translocation, the signal peptide is cleaved from the pre-protein by a lipoprotein signal peptidase, allowing the protein to fold into its tertiary confirmation (71). Less is known about the folding and assembly of membrane proteins, but if a protein fails to assemble or fold correctly, the proteins in the complex are often degraded, and significant changes in the organization and lipid composition of the membrane occur (72, 73).

Accessory SecA Systems

Analysis of bacterial genome and protein databases reveals a second SecA gene, named SecA2, in some pathogenic and non-pathogenic Gram-positive bacteria and mycobacteria (See (74) for a complete list). The SecA2 of *M. tuberculosis* is 52 percent similar in amino acid sequence to the well characterized *B. subtilis* SecA. Despite their smaller size and truncated C-terminal linker, SecA2s are very similar to the conserved SecA across the entire length of the protein. Like SecA, SecA2 is an ATPase containing the DEAD motor, NBDs, IRA2, and the PBD (74). With the exception of the SecA2 in non-pathogenic *Corynebacterium glutamicum* (75), SecA2s are not essential for the viability of bacteria tested. This implies that SecA2 dependent substrates are not required for survival under standard growth conditions *in vitro*, but perhaps are important in biologically relevant environments like within host cells *in vivo*.

Some bacteria like *Streptococcus gordonii* and *Streptococcus parasanguinis* also have an accessory SecY2. *S. parasanguinis* is a primary colonizer of the tooth surface that allows the adhesion of other bacteria that cause dental caries. In this species, *secA2* and *secY2* are located within an eight-gene cluster, which includes the Fap1 protein discussed below. The genes likely make up a genomic island that was transferred horizontally from other bacteria, as suggested by the low G+C content and the presence of a putative transposase element immediately downstream of the gene cluster (76). Cell fractionation studies with polyclonal antibodies have shown that SecA2 is primarily located in the cell membrane fraction (84.3%), whereas SecA is evenly distributed between the cell membrane (49%) and the cytoplasm (51%) (77). Neither protein has been found in the culture supernatant or cell wall fractions. In an in vitro tooth model,

SecA2 was found to be important for the pathogenesis of *S. parasanguinis*. Mutation of *secA2* decreases the secretion of FimA, completely inhibits the secretion of Fap1 to the bacterial surface, and greatly reduces the amount of Fap1 present in the membrane and cytoplasm (78). FimA is an adhesin and a virulence factor that has been associated with endocarditis (79). Fap1 is a glycosylated structural protein that is required for biofilm formation, and for the assembly of the peritrichous fimbriae that mediate adhesion of the bacteria to teeth (80). To further study the role of the accessory Sec proteins in the biogenesis of Fap1, a *secY2* mutant strain was created. In this strain, a unique Fap1 precursor is expressed, and partially glycosylated Fap1 is detected in the culture supernatant and on the bacterial cell surface (76). This suggests that *secY2* is involved in glycosylation, and that partially glycosylated Fap1 is not dependent upon *secY2* for secretion. Other members of the genomic island, Gap1 and Gtf1, have also been shown to have a role in Fap1 glycosylation and biogenesis (76). Inhibition of SecA secretion with sodium azide (81) revealed that SecA does not contribute to the secretion of mature, glycosylated Fap1 (82). As noted previously, Fap1 secretion is completely inhibited in the absence of SecA2 (78, 82). To determine which features of Fap1 contribute to its targeting to SecA2 and not SecA, Fap1 variants containing different domains were expressed in *S. parasanguinis*, and tested for their dependence upon SecA2 for secretion (82). The unusually long signal peptide (68 amino acids) of Fap1 was able to target a GFP chimeric protein for secretion in the presence and absence of SecA2, but was inhibited by sodium azide, suggesting that the signal peptide alone is sufficient for secretion by the SecA-dependent pathway. A combination of three Fap1 domains were required to target a protein to the SecA2-dependent pathway: the N-terminal signal

peptide, the entire nonrepetitive region I, and part of the nonrepetitive region II. Interestingly, the two serine-rich repeat regions of Fap1 were not required for SecA2-dependent secretion, but were involved in inhibition of secretion via the SecA-dependent pathway (82). In total, studies of SecA2 in *S. parasanguinis* have suggested a role for SecA2 in pathogenesis through Fap1 and FimA secretion, have determined that the majority of SecA2 is located in the cytoplasmic membrane, and have identified protein domains involved in targeting proteins to the SecA2-dependent pathway.

Similar results have been found in the study of *S. gordonii*. *S. gordonii* is a leading cause of bacterial endocarditis (83), likely via adhesion of the bacteria to human platelets. Although SecA and SecA2 of *S. gordonii* are very similar structurally and functionally, their domains are not interchangeable, and each protein has unique biochemical properties (84), indicating that SecA2 is not redundant for SecA. Unlike *S. parasanguinis,* both SecA and SecA2-dependent secretion are inhibited by sodium azide treatment. Also unique to *S. gordonii*, both SecA2 and SecY2 are required for the secretion of a virulence factor, GspB, to the cell surface (85). Loss of GspB secretion resulted in a significant decrease in *S. gordonii* binding to platelets (85). GspB is a large cell-wall anchored protein that is heavily glycosylated in the cytoplasm prior to export (86). Like Fap1, GspB has a long signal peptide (90 amino acids), and two serine-rich repeat domains. Three glycine residues within the H region of the signal peptide were identified as being necessary for targeting GspB to the SecA2-dependent pathway (87). Replacing the glycine residues with helix-promoting residues not only decreased SecA2-dependent secretion, it also increased SecA2-independent secretion. *secA2, secY2*, and *gspB* are located in a gene cluster, within which lie three other genes (*asp1-3*) that are

required for the secretion of GspB, and are likely components of an accessory secretion system (88). *secY2-secA2* gene clusters with similar gene organization are found in *Staphylococcus aureus*, *Streptococcus agalactiae* (group B streptococcus), and *Streptococcus pneumoniae*, suggesting that these bacteria may also have an accessory secretion system for the export of a serine-rich repeat protein (85). Recent studies have confirmed that SecA2 is required for the secretion of the serine-rich adhesions SraP in *S. aureus* (89) and Srr1 in group B streptococcus (90).

SecA2 has also been studied in pathogenic *Listeria monocytogenes*. Nineteen proteins have been identified as being SecA2-dependent for their secretion, only 7 of which have signal peptides (91, 92). Several studies have shown that SecA2 has a role in the smooth to rough phenotypic transition that has been linked to the ability to colonize diverse environments (109, 110, 111). In these studies, SecA2 was required for secretion of normal levels of the p60, MurA, and NamA, CwhA autolysins. So it appears that autolysins mediate the smooth to rough phenotypic variation, and SecA2 is required for the secretion of those autolysins. SecA2 is also required for the secretion of the *Listeria* adhesin protein (LAP), which promotes adhesion of the bacteria to intestinal epithelial cells *in vitro,* and manganese-SOD (MnSOD), which protects the bacteria from reactive oxygen and nitrogen species *in vitro* (93, 92). In vivo studies of the *ΔsecA2* mutant have shown that SecA2 is required for persistence and growth of *L. monocytogenes* in mice (91). Mutations in *p60* and *namA* were unable to reproduce this large defect, suggesting that other SecA2-dependent proteins mediate virulence of the bacteria *in vivo* (91). Analysis of the host immune response to *ΔsecA2* has shown that SecA2 is required to confer secondary protective responses to wild-type *L. monocytogenes.* Although *ΔsecA2*

induces a CD8+ T cell response equivalent to that of wild-type bacteria, and is able to invade the host cell cytosol, and spreads to the same percentage of neutrophils, dendritic cells, and macrophages in the spleen of infected mice, mice infected with *ΔsecA2* are not protected against a secondary challenge with wild-type bacteria (94). Therefore, in *L. monocytogenes,* SecA2 is required for virulence by the bacteria, but is also required for secondary protective immunity by the host *in vivo*.

SecA2 in Mycobacteria

Most relevant to this thesis, are the studies of SecA2 in Mycobacteria. Unlike *S. gordonii and S. parasanguinis*, Mycobacteria do not have a SecY2, and no proteins have been identified that likely make up an accessory translocase. However, while attempting to identify and characterize SecA in *M. smegmatis,* Braunstein and colleagues made the first identification of a SecA homologue present in all *Mycobacterium* species, SecA2 (95). In this study, failed attempts to delete *secA*, except in the presence of a plasmid copy of s*ecA* from *M. smegmatis* or BCG, confirmed that it is essential in mycobacteria, consistent with SecA in all other bacteria tested. To test whether SecA2 is redundant for SecA if expressed constitutively, *secA2* driven by the constitutive *hsp60* promoter was expressed while trying to make a Δ*secA* strain via allelic-exchange. Production of a Δ*secA* strain was still not achieved, indicating that *secA2* is not functionally redundant for *secA*. Mutation of *secA2* in *M. smegmatis* causes the bacteria to produce smaller colonies when grown on rich agar (95). Since this defect was rescued by complementation with *secA2,* the defect was clearly caused by the secA2 mutation. No growth defect was observed on minimal agar plates, nor in rich liquid medium. The Δ*secA2* mutant was also

found to be more sensitive than wild-type *M. smegmatis* to sodium azide, an established SecA inhibitor (81). Based on this observation, the authors suggest that SecA2 may assist SecA with secretion via the general Sec pathway (95). In a recent study, a conditional SecA mutant was created in *M. smegmatis* to test whether SecA and SecA2 are dependent upon one another for secretion of select proteins (96). Depletion of 95% of SecA did not affect the global expression of proteins as indicated by the presence of control proteins like cystolic GroEL. The authors did not state what percentage of proteins failed to be secreted upon depletion of SecA, but they showed evidence that a known SecA-dependent protein, MspA, was not exported to the cell wall fraction. Consistent with their hypothesis, the SecA2-dependent lipoprotein *Msmeg1712* (described below) failed to be localized to the cell wall fraction, and accumulated in the cytosolic fraction upon depletion of SecA (96). Based on this finding, it appears that SecA and SecA2 may both be required for the secretion of a subset of proteins. This gives further support to the model that SecA2 may facilitate the export of some proteins through the general Sec system.

Biochemical analysis of SecA2 in *M. tuberculosis* has confirmed that it is indeed an ATPase (97, 96). SecA2 is able to bind and hydrolyze ATP. Substitution of the conserved lysine within the nucleotide binding site (Walker A motif) with an arginine or alanine prevented ATP-binding *in vitro*(97). Additionally, expression of the *secA2(K115R)* mutant in the *ΔsecA2* strain failed to complement its growth defect in macrophages. A similar mutant created in *M. smegmatis, secA2(K129R),* also fails to bind ATP, and fails to export the two SecA2-dependent lipoproteins described earlier, indicating that ATP binding is required for secretion of SecA2-dependent proteins (96).

When expressed in the Δ*secA2* strain, *secA2(K129R)* fails to complement the rich agar growth defect and azide hypersensitivity of the mutant. In fact, *secA2(K129R)* appears to be dominant negative. When expressed in wild-type *M. smegmatis*, the bacteria exhibit the same defects as the Δ*secA2* mutant, even though wild-type secA2 is present (96). Thus, it is possible that SecA2(K129R) is able to interact with the same proteins as wild-type SecA2. However since SecA2(K129R) is not a functional SecA2, the protein complexes formed are also nonfunctional. Localization of SecA in *M. smegmatis* by subcellular fractionation and immunoblotting, is consistent with the localization of this protein in *S. parasanguinis.* SecA was found to be evenly distributed between the cell envelope and soluble fractions, and the absence of SecA2 had no effect on this distribution (96). Unlike the SecA2 of *S. parasanguinis,* which is located primarily in the membrane fraction, the majority of SecA2 in *M. smegmatis* is found in the soluble cytosolic fraction. Interestingly, the *secA2(K129R)* mutant is primarily associated with the cell envelope fraction. The authors suggest that the *secA2(K129R)* mutant may be trapped in a complex at the membrane, while wild-type SecA2 interacts transiently with the membrane-embedded translocase to facilitate protein export. Since SecA2(K129R) is unable to bind ATP as described above, it is possible that this mutant protein is unable to be released from the complex until it has bound and hydrolyzed ATP, i.e. completed a cycle of pushing a protein through the translocase and releasing the protein.

Several proteins dependent upon SecA2 for secretion have been identified in Mycobacteria. 1D- and 2D-PAGE analysis of cell wall and membrane fractions of *M. smegmatis* identified two SecA2-dependent lipoproteins that may have a role in sugar catabolism: Msmeg1712 and Msmeg1704 (98). Both are predicted to have N-terminal

signal sequences. To test whether SecA2 is required for global lipoprotein export, lipoprotein extracts and three known Mycobacterial lipoproteins were analyzed by 1D-PAGE. SecA2 was not required for the export of any of the proteins tested, nor for the normal composition of the lipoprotein extracts, suggesting that SecA2 is not required for the global export of lipoproteins (98). A 2D-PAGE analysis of culture filtrate proteins from a Δ*secA2* mutant in *M. tuberculosis* revealed three SecA2-dependent proteins, none of which have a signal peptide: superoxide dismutase (*sodA*), α-crystallin/HspX (*acr*), and a protein of unknown function (*rv0390*) (99). A catalase-peroxidase (KatG), which also lacks a signal peptide, was identified as SecA2-dependent by immunoblot analysis of wild-type and Δ*secA2* mutant culture filtrates. The 2D-PAGE analysis also identified proteins whose secretion was increased in the Δ*secA2* mutant: an immunogenic protein of unknown fuction (MPT63), ribosomal protein L12 (rpIL), cold shock protein (cspA), and another isoform of α-crystallin/HspX (*acr*). Different isoforms of *acr* have been observed in other studies of *M. tuberculosis* proteins (112, 113), however it is unclear how SecA2 influences their localization and function. Since SecA functions as a dimer (100), and SecA2 may assist SecA in secretion (95), the authors of this last described study suggest that SecA2 may also function as a dimer and have 2 separate roles. A SecA2 homodimer may serve to export proteins lacking a Sec signal sequence, while a SecA2-SecA heterodimer assists SecA in export.

Further characterization of the Δ*secA2* strain created in *M. tuberculosis* has shown that it has no growth defects in liquid media (99), but has a smooth colony phenotype when grown on agar containing Tween 80 (96) and grows less well in macrophages (101). *In vivo* studies have demonstrated a role for SecA2 in the virulence of *M.*

tuberculosis. Wild-type C57BL/6 mice, and B and T-cell deficient SCID mice infected with the Δ*secA2* mutant lived longer than mice infected with wild-type bacteria (99). CFU analysis of several organs from the infected mice showed that the Δ*secA2* mutant is attenuated for growth during the first twenty-days of infection. Thereafter, growth stops and bacterial numbers remain constant, and the difference in CFUs between the strains remains relatively constant (99). These data suggest that SecA2 promotes growth and survival of *M. tuberculosis in vivo* before adaptive immunity is established. The role of SecA2 in protection from innate immunity was tested *in vitro*. The Δ*secA2* mutant is attenuated for growth in unactivated murine bone marrow-derived macrophages (BMDMs) (101). Both wild-type and the mutant are equally inhibited for growth in IFN-γ activated BMDMs. Attenuation in unactivated BMDMs, but not in activated BMDMs, is consistent with the early defect for growth found *in vivo*, suggesting that SecA2 is required for protection against innate immunity. Since SecA2 is required for the secretion of two antioxidant enzymes, SodA and KatG, the authors of this study hypothesized that SecA2 may be required by *M. tuberculosis* for protection against the macrophage oxidative burst. To test this, BMDMs isolated from phox$^{-/-}$ mice that lacked various components of the macrophage oxidase complex were infected with the Δ*secA2* mutant and wild-type *M. tuberculosis.* The Δ*secA2* mutant was still attenuated for growth, indicating that defective secretion of SodA and KatG does not explain the growth defect of the mutant, and SecA2 likely has a role in other mechanisms that promote bacterial growth and survival in macrophages. Hence, the role of SecA2 in the activation of macrophage inflammatory responses to *M. tuberculosis* infection was evaluated. At 24 hours post-infection, *ΔsecA2* infected BMDMs secreted more TNF-α and IL-6, than wild-

type infected macrophages (101). No difference was found in the production of reactive oxygen intermediates (ROI) at 0, 4, and 24 hours post-infection. However, ΔsecA2 induced more reactive nitrogen intermediates (RNI) in both unactivated and activated BMDMs at 48, 72, and 96 hours post-infection. These findings show that SecA2 modulates the host immune response *in vitro*. In addition, *ΔsecA2* induced greater expression of MHC class II (I-Ab and HLA-DRA) in IFN-γ activate murine BMDMs and human monocytes (THP-1 cells) as measured by qRT-PCR (101). This last finding suggests a role for SecA2 in inhibiting adaptive immunity, which is contrary to their *in vivo* data of an early defect in growth, before the onset of adaptive immunity (99). Although the majority of the data supports a role for SecA2 in modulating the innate immune response, additional studies are necessary to confirm this, and to clarify or exclude a role in modulating the adaptive immune response.

One published study has suggested increased apoptosis due to increased ROI as an alternative explanation for the decreased virulence of *ΔsecA2 in vivo* (102). A few studies have linked pro-apoptotic *Mycobacterium* strains to decreased levels of SodA (103, 104). In addition, multiple studies in different systems have linked high levels of superoxide anions with induction of apoptosis (For review see 105). Hinchey and colleagues were able to show that the *ΔsecA2* mutant in *M. tuberculosis* induced more apoptosis, and more caspase activity than wild-type bacteria of infected THP-1 cells *in vitro* (102). Additionally, the pro-apoptotic phenotype could be reversed by expression of a SecA-dependent SodA construct in the *ΔsecA2* mutant that restored secretion of SodA to the culture filtrate. The authors next suggest that increased apoptosis of infected cells may lead to increased antigen presentation via MHC class I molecules to T cells, as has

been suggested by other studies (106, 107). To test this *in vivo*, mice were infected with strains of *M. tuberculosis* expressing the H-2K^b-binding sequence of OVA. OVA-responsive CD8$^+$ T-cells isolated from the spleen were then quantified by IFN-γ ELISPOT at 7 days post-infection. The *ΔsecA2*-OVA strain induced significantly more OVA-responsive CD8$^+$ T-cells than wild-type-OVA bacteria. However, no difference was found in the response of splenocytes to purified protein derivative (PPD), nor to peptide 25 (the MHC class II-presented epitope of Ag85B). The authors speculate that the response to PPD and peptide 25 is dominated by CD4$^+$ T-cells, so enhanced T-cell priming by *ΔsecA2* is limited to CD8$^+$ T-cells. Using an *in vivo* CFSE based cytolytic activity assay, *ΔsecA2*-OVA infected mice exhibited significantly more target-specific killing. Additionally, *ΔsecA2*-OVA was able to induce significantly more proliferation of transferred OVA/H-2K^b-reactive T cells *in vivo*, that could be reversed with expression of the SodA construct described earlier. Contrary to the mouse study of *ΔsecA2* that suggested no role for SecA2 in adaptive immunity (99), this study suggests that an absence of SecA2 enhances the adaptive immune response, specifically priming of CD8$^+$ T-cells. Further studies are necessary to determine if this increased priming has any affect on a primary infection with *M. tuberculosis*, or if it is only of benefit to the host in the context of a low dose vaccination, followed by a higher dose secondary infection. The ability of *ΔsecA2* to induce a strong CD8$^+$ T-cell response, and its attenuated virulence *in vivo* led the authors of this study to test its effectiveness as a vaccine strain. Immunization of mice with *ΔsecA2* caused a robust increase in memory T-cell populations, and yielded better protection from secondary challenge with wild-type *M. tuberculosis* than BCG in mice, as evidenced by increased survival, less inflammation, and slightly less bacterial

burden. Similar results were obtained with immunization of guinea pigs (102). In a recent study, a *ΔlysA ΔsecA2* double mutant of *M. tuberculosis* was shown to induce significantly stronger antigen specific $CD8^+$ T-cell responses than *ΔlysA ΔpanCD* and *ΔRD1 ΔpanCD* strains in neonatal mice (108). These two studies suggest that the *ΔsecA2* mutant, in combination with other genetic mutations, may be a promising vaccine platform for *M. tuberculosis.*

Mycobacterium marinum model of tuberculosis

Our laboratory makes use of the *M. marinum* model of tuberculosis to study the pathogensis of Mycobacteria. Some of the advantages of working with *M. marinum* instead of *M. tuberculosis* are that it is not a human pathogen so is less dangerous to work with in the laboratory, and it grows about 5 times faster (114). Additionally, *M. marinum* is the closest genetic relative to the tuberculosis complex. Importantly, many of the virulence factors found in *M. tuberculosis* are present in *M. marinum* (114). One major difference between the two strains is that *M. marinum* is a natural pathogen of fish and frogs, not humans. Fortunately the immune systems of zebrafish and humans are quite similar, and the innate and adaptive branches are conserved (115, 116). Therefore, not surprisingly, *M. marinum* causes a granulomatous disease similar to that caused by *M. tuberculosis* in humans (114). For the reasons described above, the results of our studies with *M. marinum* are likely to contribute to understanding the pathogenesis of *M. tuberculosis*.

Summary

Mycobacteria contain several specialized secretion systems in addition to the general Sec pathway, which are important for the pathogenesis of the bacteria. Preliminary studies of SecA2 in *M. smegmatis* suggest that SecA2 does not have its own secretion system and may facilitate secretion via the general Sec pathway. Of the SecA2-dependent proteins discovered in Mycobacteria, no common feature has been identified to help predict what type of proteins require SecA2 for their secretion. More studies are necessary to determine if and under what conditions expression of SecA2 is regulated, and to clarify which substrates require SecA2 and why. Despite its accessory role in secretion, it is clear that SecA2 is required for the virulence of Mycobacteria *in vivo*. One study of the *ΔsecA2* mutant *in vivo* has suggested that SecA2 promotes growth and survival of *M. tuberculosis* before the onset of adaptive immunity (99). However, as described previously, one study has shown that SecA2 may have a role in modulating the $CD8^+$ T-cell response to *M. tuberculosis* that influences secondary immunity, but its role in primary immunity was not addressed (102). Therefore, the role of SecA2 in modulating host immunity to primary infection is unclear. This thesis aims to investigate the role of SecA2 in modulating host immune responses during primary infection, as they relate to the requirement of SecA2 for the virulence of Mycobacteria. Specifically, we will test the requirement of SecA2 for virulence by analyzing the growth and pathogenesis of an *M. marinum ΔsecA2* mutant *in vivo.* We will investigate the role of SecA2 in modulating host immunity by histopathological analysis of the inflammation, measurement of cytokine induction, and testing the adaptive immune response from the draining lymph nodes *ex vivo*. These studies will test the hypothesis that SecA2 is required for virulence

during the innate immune response of the host, and influences pro-inflammatory cytokine induction *in vivo*.

Chapter 2

SecA2 of *M. Marinum* modulates Host Inflammatory Responses

Abstract

In addition to the essential SecA, all Mycobacteria and some Gram-positive bacteria possess the SecA homologue, SecA2. SecA2 is an ATPase that facilitates the translocation of a subset of proteins in bacteria. Multiple studies in several different bacteria, including Mycobacteria, have shown that SecA2 has an important role in virulence. To investigate the role of SecA2 in modulating host immune responses *in vivo*, we made use of two *Mycobacterium marinum* models of tuberculosis infection: the established zebrafish model, and the recently described mouse model. *M. marinum* is a very close relative of *Mycobacterium tuberculosis* that causes a chronic, granulomatous disease in fish with many similarities to *M. tuberculosis* infection in humans. Here we show that a Δ*secA2* mutant of *M. marinum* is attenuated for virulence in both models tested. In zebrafish, SecA2 was required for granuloma formation, for maximal induction of TNF-α and IL-1β, and for the growth and survival of *M. marinum in vivo*. In the mouse model, SecA2 was required for increased granuloma formation, most prominently after the onset of adaptive immunity, and for maximal induction of TNF-α and IFN-γ. Infection of Rag2 knockout mice revealed that adaptive immunity is required for control of *M. marinum.* Notably, the Δ*secA2* mutant was no longer attenuated compared to wild-type strains during infection of Rag2 knockout mice, suggesting that SecA2 may not be required for disease in the absence of adaptive immunity. Analysis of *M. marinum* bacilli by cryo-electron tomomography revealed unique protrusions from the cell envelope of the Δ*secA2* mutant. Additionally, the mutant is sensitive to SDS treatment, suggesting a role for SecA2 in normal cell wall function. These results have provided evidence for a

role of SecA2 in modulating granuloma formation and TNF-α induction *in vivo*, and cell wall morphology and function *in vitro*.

Introduction

Mycobacterium tuberculosis was identified as the causative agent of tuberculosis in humans over 100 years ago, yet many of the mechanisms underlying the success of this pathogen are still elusive. One-third of the world's population is estimated to be infected with *M. tuberculosis*, but only 5-10% will develop active disease. This translates into more than 9 million new cases and over 1.7 million deaths each year (25). Therefore, it its likely that most individuals exposed to the pathogen are able to mount a protective immune response that contains and/or eliminates the infection. Identifying host and pathogen factors that mediate susceptibility versus immunity to *M. tuberculosis* are of great interest in eradicating this disease. *M. marinum* is a model species for tuberculosis infection that causes a tuberculosis-like disease in ectotherms like fish and frogs. Less commonly, it is able to cause localized disease in humans in the form of skin lesions (26), and disseminated disease in humans that are immunocompromised (27, 28). *M. marinum* is the closest genetic relative to the tuberculosis complex, and as such, shares common virulence factors with *M. tuberculosis* (29). Additionally, mice and zebrafish infected with *M. marinum* develop caseating granulomas that are characteristic of active pulmonary disease in humans (30, 31, 11). Granuloma formation, maturation, and maintenance is a complex process that is thought to benefit the host by containing the bacteria, but may also benefit the bacteria by creating a niche for growth (32, 33). Transmission of the pathogen is thought occur when the host's immune system fails to maintain these caseaous granulomas, causing them to rupture and release live bacilli into the bronchioles (34).

Several protein secretion systems are required for growth and virulence of mycobacteria (35). The general secretion system (Sec) is essential in all bacteria tested, and has been best studied in *Escherichia coli and Bacillus subtilis*. One of the members of this system, SecA, is an ATPase that drives the translocation of unfolded proteins across the cytoplasmic membrane via the SecYEG translocase (36, 37). All mycobacteria, and some Gram-positive bacteria, contain a second SecA protein, SecA2 (38). SecA2 of mycobacteria is required for the secretion of several proteins, including the antioxidant enzymes SodA and KatG (14, 1). Mutation of *secA2* in *M. tuberculosis* has revealed a role for SecA2 in the growth, survival, and virulence of the pathogen *in vivo,* before the onset of adaptive immunity (1). Studies of the Δ*secA2* mutant in murine bone marrow-derived macrophages suggest that SecA2 is required for inhibition of both the innate and adaptive immune response (2). Additional studies have shown that the mutant is able to increase priming of antigen specific T-cells *in vivo*, and increase secondary protection to *M. tuberculosis* in mice and guinea pigs vaccinated with the Δ*secA2* mutant, or the *ΔlysA ΔsecA2* double mutant (3, 39). Although SecA2 is clearly required for virulence, its role in modulating the innate versus the adaptive immune response *in vivo* is less defined. Here we utilize two models of *M. marinum* infection to investigate the virulence and growth of Δ*secA2* as it relates to host pathology, cytokine induction, and the adaptive immune response *in vivo*. Contrary to *M. tuberculosis* studies, our data shows that SecA2 may not required for growth before the onset of adaptive *in vivo*. Additionally, SecA2 appears to modulate the adaptive immune response to *M. marinum* infection, and is required for normal cell envelope morphology *in vitro*.

Results

Construction of a *M. marinum* Δ*secA2* mutant

Previous studies have shown that Δ*secA2* in *M. tuberculosis* is partially attenuated for growth and virulence in the mouse model of infection (1, 2), and has the potential to serve as a safe vaccine platform (3, 4). However, the host immune response to Δ*secA2* mutant infection *in vivo* has not been characterized completely. To investigate the role of SecA2 in modulating the host immune response, we created a kanamycin-resistant insertion mutant of *secA2* (*MMAR_2698*) in *M. marinum*. Homolgous recombination was utilized to replace the first 530 N-terminal amino acids of *secA2* with a kanamycin-resistance cassette, leaving the last 277 amino acids uninterrupted (5) (Figure 2.1A). To complement the mutant, *MMAR_2698,* driven by the mycobacterial hsp60 promoter, was stably inserted into the Δ*secA2* mutant chromosome at the *attB* site (39). The Δ*secA2* + *secA2* complement strain ($SA2^{comp}$) grown in 7H9 broth expresses 2.25 fold more *secA2* mRNA than wild-type *M. marinum*, whereas *secA2* was not detected in the Δ*secA2* mutant by quantitative RT-PCR, indicating that SecA2 protein is likely not present in the mutant, and present in the complemented strain (Figure 2.1B).

When grown in liquid media, the ΔsecA2 mutant exhibits an equivalent generation time during the exponential phase of growth as compared to wild-type and $SA2^{comp}$ (Figure 2.1C), consistent with the reported growth of the *M. tuberculosis* Δ*secA2* mutant *in vitro* (1). The ratio of the generation time of ΔsecA2 and $SA2^{comp}$ to that of wild-type averaged 0.96 ± 0.09 (range of 0.92 to 1.09) and 1.06 ± 0.1 (range of 0.96 to 1.16), respectively in 4 independent experiments.

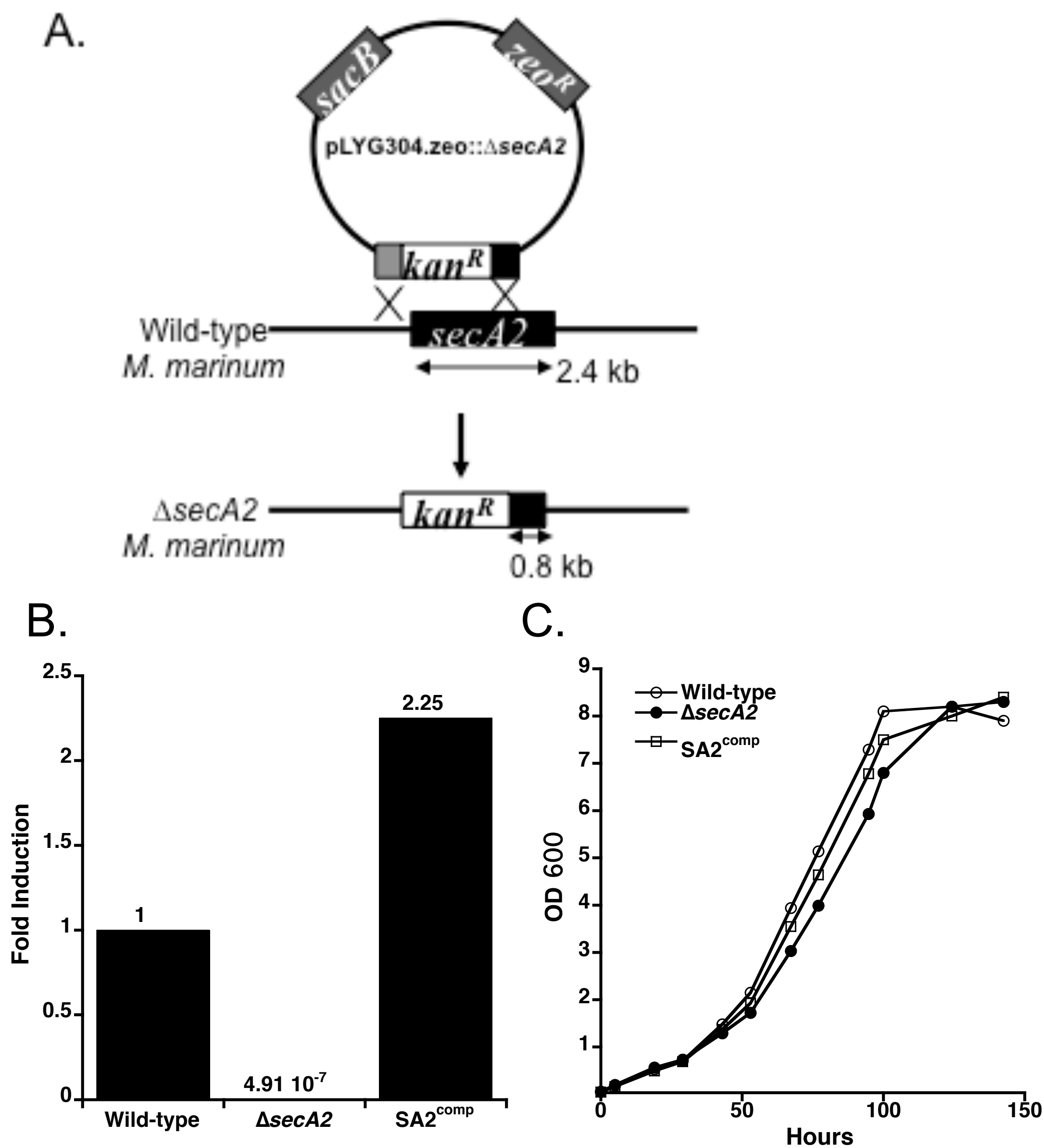


Figure 2.1 Deletion of *secA2* in *M. marinum.* (A) Schematic diagram of construction of the M. marinum Δ*secA2* mutant. Approximately 1.6 kb of the secA2 N-terminus was replaced with a kanamycin resistance cassette by homologous recombination. (B) qRT-PCR analysis of *secA2* mRNA expression in Δ*secA2* and SA2^{comp} relative to that of wild-type *M. marinum* grown in 7H9 broth to mid-log phase. (C) Growth of the M. marinum cultures in 7H9 broth. Optical density at 600 nm was measured over time.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Reference/ Source
zf β-actin	TGCTGTTTTCCCCTCCATTG	TTCTGTCCCATGCCAACCA	16
zf il-1β	TGGACTTCGCAGCACAAAATG	GTTCACTTCACGCTCTTGGATG	17
zf il-12	TCTAACTTCAGCGCAGTGGA	TGCGGTGGTGTAGTGAGTG	18
zf ifn-γ	CTTTCCAGGCAAGAGTGCAGA	TCAGCTCAAACAAAGCCTTTCGCT	8
zf tnfa	GATGGTGTCCAGGAGGAAAG	CAGAGTTGTATCCACCTGTT	18
Mm secA2	GTGGGTCAACTCGTCAAA	CTCTTCGGAGTACAGATCGA	This work
Mm sodA	ACAAGCTGCTGATCTTCCAGGTCT	TGGCGAAGTCGACCTTCACATTCT	This work

Table 2.1 Primers used in this study for quantitative RT-PCR. The primers listed above were used for quantitative RT-PCR using QuantiFast SYBR Green. Primers created in this study were designed at www.idtdna.com. Sequences for all zebrafish cytokine primers were obtained from published studies.

Δ*secA2* is required for virulence and granuloma formation in zebrafish

To assess whether SecA2 is required for virulence of *M. marinum in vivo*, we infected adult zebrafish, one of its natural hosts. Zebrafish infected with 10^4 wild-type bacilli all died between 12 and 25 days post-infection (Figure 2.2A). Fish infected with SA2^{comp} died within the same time frame, with the exception of one fish. At the time of death, the abdomens of the zebrafish were visibly hemorrhaging, a sign of inflammation. However, zebrafish infected with 10^4 Δ*secA2* bacilli never showed hemorrhaging, and survived 38 weeks until euthanization at the end of the study. SecA2 in *M. marinum* appears to be unambiguously required for virulence in this natural host.

It is possible that the Δ*secA2* mutant fails to kill the fish because it is more susceptible to clearance by the host, or is somehow able to persist without causing disease. To address these possibilities, zebrafish were infected as before, and colony forming units (CFUs) were enumerated from the whole fish at various time points post-infection (Figure 2.2B). Wild-type and SA2^{comp} showed steady growth throughout the first 11 days of infection. In contrast, while the initial growth of the Δ*secA2* mutant in vivo was no different than wildtype, the growth of the mutant peaked at 7 days post-infection, suggesting a role for SecA2 in continued growth and survival of mycobacteria in vivo. Histopathological studies of the liver and pancreas at 7 days post-infection, the timepoint at which the bacterial burdens were indistinguishable, indicated that all three strains had inflammatory foci of inflammation, but the Δ*secA2* infected fish had less granulomas and less inflammation over all (Figure 2.3). The foci of inflammation were of a lymphohistiocytic character in all strains. We were unable to identify the specific cell

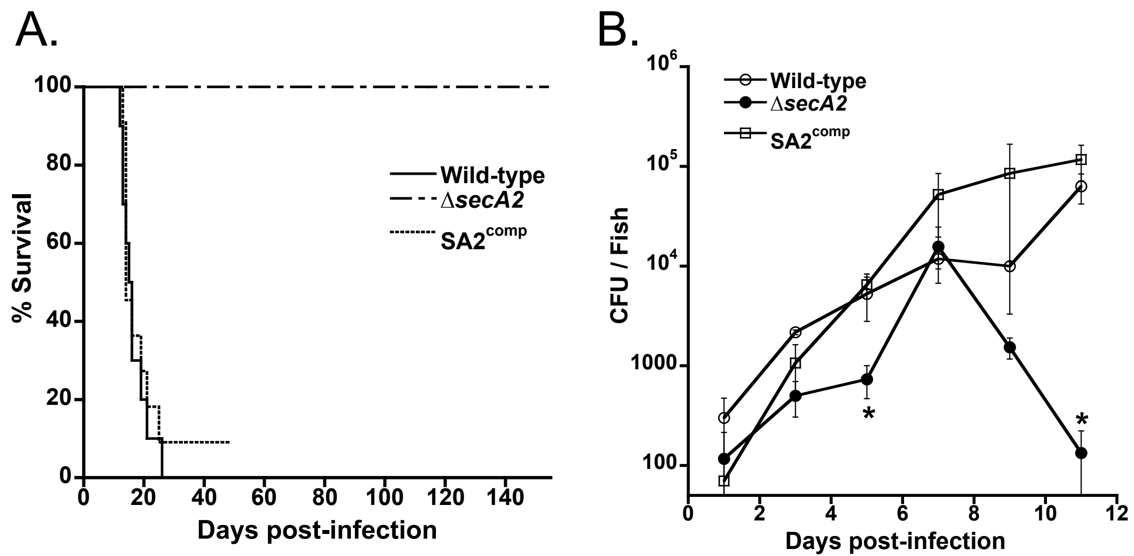
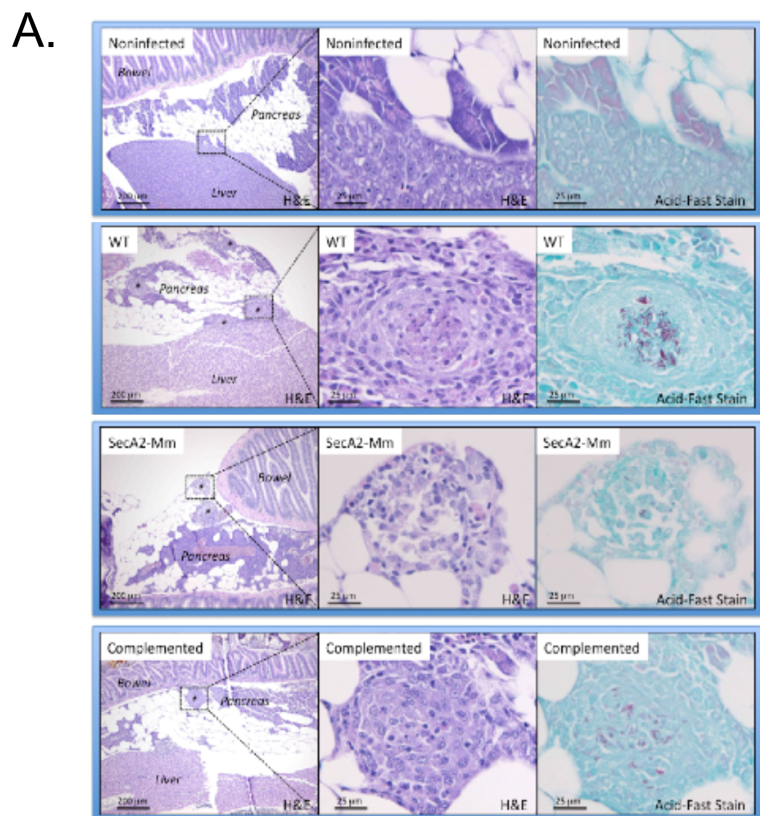


Figure 2.2 *M. marinum* ΔsecA2 is attenuated for virulence in zebrafish. Zebrafish were infected via intraperitoneal injection with 10^4 wild-type, Δ*secA2*, and SA2^{comp} *M. marinum*. (A) Survival of fish was observed over time (wild-type n=10, Δ*secA2* n=12, SA2^{comp} n=11). (B) Bacterial burden in the whole fish was quantified by enumeration of CFUs at multiple time points post-infection. The values represent the mean ± standard error of the mean of 6 fish per time point for Δ*secA2* and SA2^{comp}, and 3 fish for wild-type. Student's t-test (*P<0.03) for Δ*secA2* in reference to wild-type and SA2^{comp}.



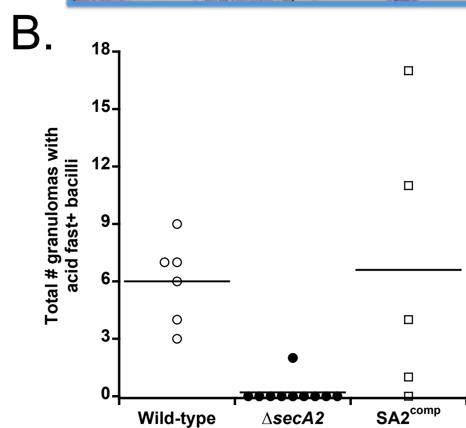


Figure 2.3 SecA2 is required for granuloma formation in zebrafish. Zebrafish were infected via intraperitoneal injection with 10^4 wild-type, Δ*secA2*, and SA2^{comp} *M. marinum*. (A) At 7 days post-infection, histopathological analysis was performed by hematoxylin and eosin (H&E) and Ziehl-Neelsen (acid fast) staining. (B) Granulomas containing Ziehl-Neelsen positive bacilli were enumerated from parasagittal sections of the liver and pancreas.

types present due to a lack of reagents available for staining immune cells in zebrafish. However, based on the organization of the inflammatory cells present, it was clear that the ΔsecA2 infected fish had fewer granulomas (Figure 2.3B). In fact, granulomas were found in only one of ten Δ*secA2* infected fish studied. Two granulomas were identified in that single fish, yielding a mean of 0.2 granulomas per fish. This is in contrast to a mean of 6.0 and 6.6 for wild-type and $SA2^{comp}$ respectively. Granuloma formation induced by $SA2^{comp}$ was more variable than that by wild-type. A possible explanation for this is that the complementing *secA2* is driven by the hsp60 promoter, not its endogenous promoter, which may cause dysregulation of SecA2 expression. Overall, it is evident that the Δ*secA2* mutant induces less inflammation in the zebrafish and is less able to incite the cells present at those sites of inflammation to organize into a granulomatous structure, even when the bacterial load of the fish is similar to that of wild-type infected fish. These data show that in addition to being required for virulence and resistance to the host immune response, SecA2 modulates host inflammation *in vivo*.

SecA2 is required for induction of pro-inflammatory cytokines IL-1ß and TNF-α *in vivo*

Reagents to study the immune response in zebrafish are fairly limited. Fortunately, many of the key cytokines and immune cells found in mammalian systems are genetically conserved in zebrafish (6, 7), allowing us to analyze the expression of various target genes in response to infection. Using quantitative RT-PCR, mRNA expression of several cytokines was quantified from infected fish at 3, 7 and 11 days post-infection (Figure 2.4, Table 2.1). At 3 days post-infection, there was no significant

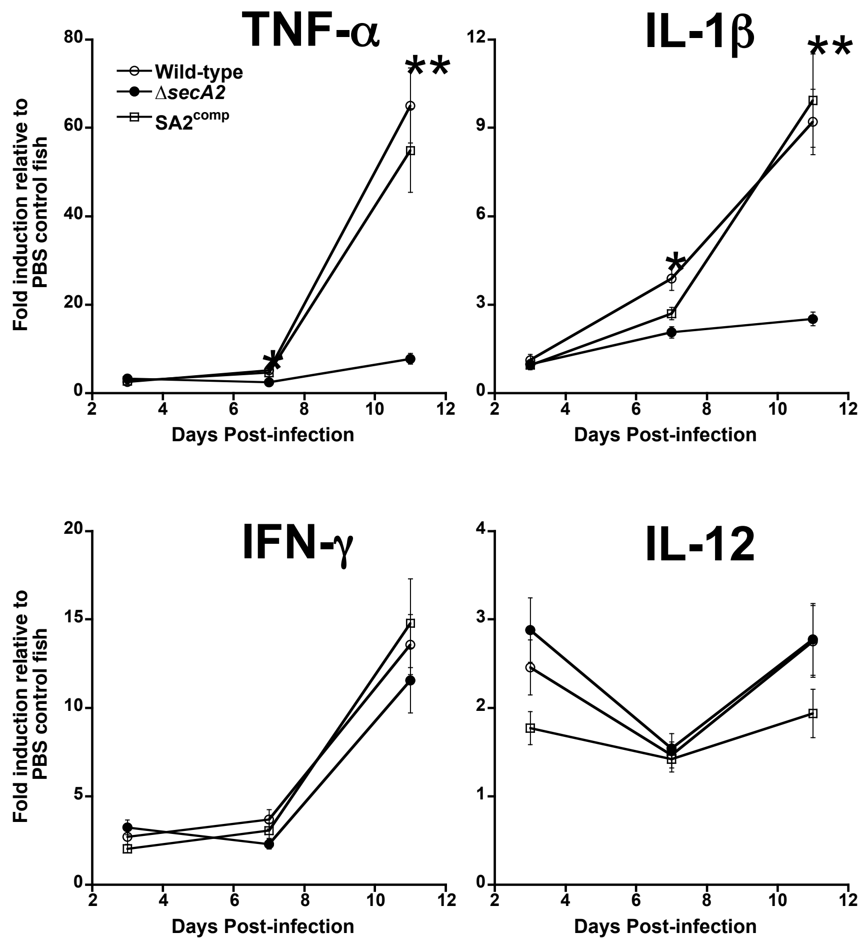


Figure 2.4 SecA2 promotes induction of IL-1ß and TNF-α in zebrafish. Zebrafish were infected via intraperitoneal injection with 10^4 wild-type, Δ*secA2*, $SA2^{comp}$ *M. marinum,* and PBS for control fish. Cytokine mRNA expression from the whole fish was measured by qRT-PCR using the gene specific primers listed in Table 1. Values represent average fold induction relative to the PBS control fish ± standard error of the mean of 8 fish per experimental group, per time point for wild-type, Δ*secA2* and $SA2^{comp}$, 6 fish for PBS control. Analysis by two-way ANOVA (treatment group x time) indicated a statistically significant difference between treatment groups over time for IL-1ß , IFN-γ, TNF-α indicating that there was significant induction of these cytokines by each *M.*

marinum strain over the PBS control (IL-1ß :$F = 8.6571$, $P<0.0001$; IFN-γ: $F=34.6181$, $P<0.0001$; TNF-α: $F = 15.1479$, $P<0.0001$). Student's t-test (*$P<0.05$, **$P<0.00001$).

induction of IL-1β relative to PBS control fish by any of the *M. marinum* strains. Wild-type and the Δ*secA2* mutant induced greater than 2- fold more IL-12, TNF-α, and IFN-γ than PBS control fish. $SA2^{comp}$ induced equivalent levels of TNF-α, and slightly less IL-12 and IFN-γ than the Δ*secA2* mutant. However, these did not differ significantly from wild-type levels. At 7 days post-infection, all three strains showed a decrease in induction of IL-12 compared to day 3 and day 11. In contrast, the infected zebrafish were able to mount a delayed IL-1β response to *M. marinum* infection on day 7 that was not present on day 3. Interestingly, the Δ*secA2* mutant induced about 2-fold less IL-1β and TNF-α than wild-type. At 7 days post-infection the bacterial load of Δ*secA2* in the zebrafish is the same as wild-type and $SA2^{comp}$ (Figure 2.2B). Therefore, the significantly reduced induction of IL-1β and TNF-α by the Δ*secA2* mutant was not caused by a decrease in bacterial load. At 11 days post-infection, wild-type and $SA2^{comp}$ induced greater than 3-fold more IL-1β than the Δ*secA2* strain. Studies of *Francisella* infection in zebrafish and mice have shown that IL-1β transcript induction in zebrafish, and active IL-1β secretion in mice requires live bacteria that are capable of escaping from the phagosome into the cytosol of host macrophages (8, 9, 10). If the requirement for live bacteria also applies to Mycobacterial infection, the low IL-1β expression in Δ*secA2* infected zebrafish may be an indication of increased bacterial clearance, as is consistent with our CFU data. This further supports a role for SecA2 in protection from the host immune response. Additionally, the wild-type and $SA2^{comp}$ induced a robust TNF-α response at 11 days post-infection that was not detected in Δ*secA2* infected fish. On the other hand, the level of IFN-γ induced by Δ*secA2* was still equivalent to that of wild-type and $SA2^{comp}$ at 11 days post-infection, even though bacterial numbers of the mutant strain were diminished.

In sum, Δ*secA2* induces less TNF-α and IL-1β than wild-type and $SA2^{comp}$ before the bacteria are cleared by its host. This difference is amplified once clearance of the bacteria from infected tissues has begun. Therefore, SecA2 has a role in modulating the host inflammatory response as indicated by its requirement for inducing TNF-α and IL-1β in zebrafish.

The Δ*secA2* mutant causes less disease in mice than wild-type *M. marinum,* yet is only minimally attenuated for growth.

A mouse model for *M. marinum* infection (11) was used to further assess the ability of SecA2 to modulate host inflammation *in vivo*. To establish a phenotype for the *M. marinum* Δ*secA2* mutant in mice, C57BL/6 mice were infected via tail vein injection with 10^7 wild-type, Δ*secA2*, or $SA2^{comp}$ bacteria. Disease caused by the bacteria was localized to cooler areas of the mouse like the tail, ears, paws, and nose, presumably because the optimal growth temperature of *M. marinum* is around 32ºC. The bacteria are able to seed in the lungs, liver, and blood, but fail to grow, and are subsequently cleared by the mice (11). The burden of visible granulomatous tail lesions in each mouse was quantified over 7 weeks by summing the length of all lesions on each tail (Figure 2.5A,B). Lesions began to appear around 9 days post-infection. In mice infected with wild-type and $SA2^{comp}$, the tail lesions continued to increase in size and number over the course of infection. For the first 3 weeks, the disease caused by the mutant was indistinguishable from the other strains. At 4 weeks, the time of peak lesion burden for the mutant, disease was already less than that for the other strains. At subsequent times, resolution of lesions was apparent for the mutant, but not for the wildtype or $SA2^{comp}$.

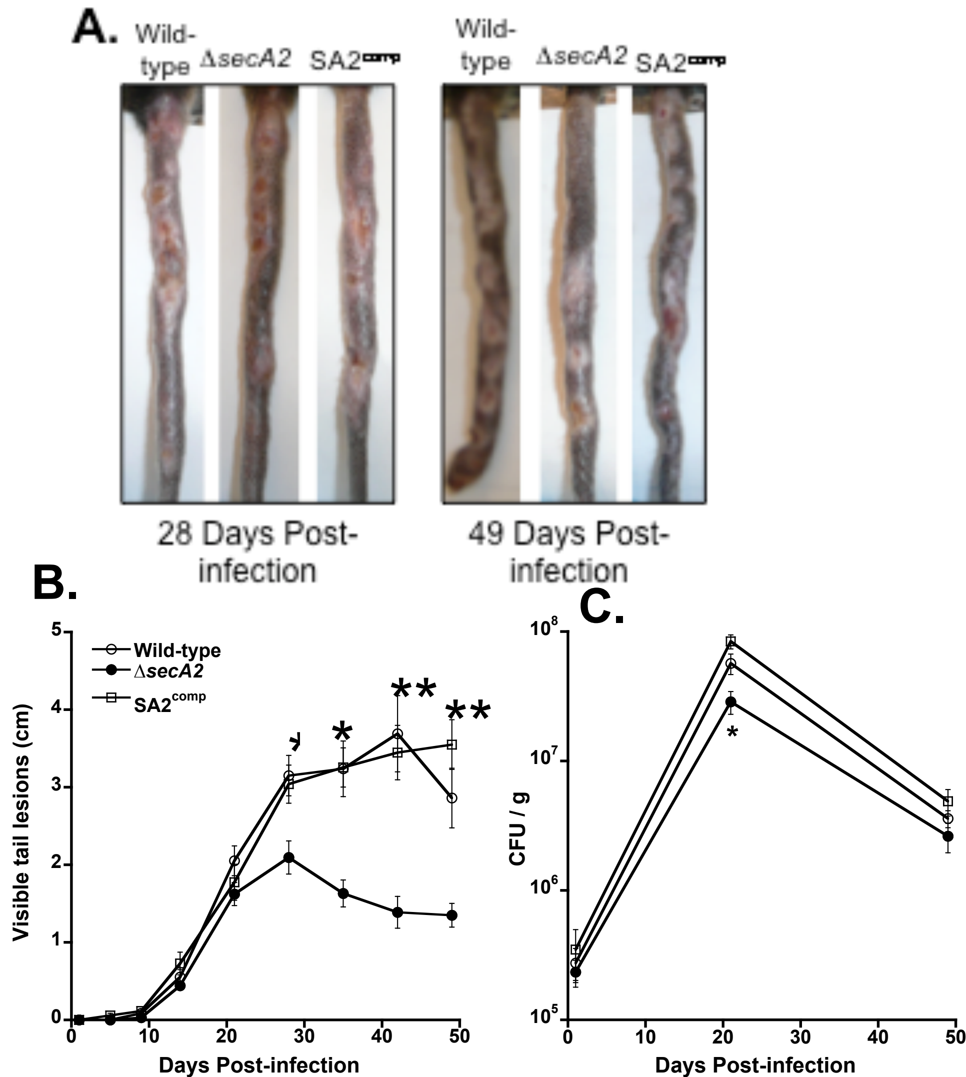


Figure 2.5 Δ*secA2* causes less disease in the mouse model of *M. marinum* infection. C57BL/6 mice were infected via tail vein injection with 10^7 wild-type, Δ*secA2*, and SA2^{comp} *M. marinum*. (A) Representative pictures of infected mouse tails at 28 and 49 days post-infection. (B) The length of each visible tail lesion was summed to give the total number of lesions on each tail at each time point. Values represent the mean ± standard error of the mean of 18 mice per experimental group. Student's t-test (*P<0.02, **P<0.0005). (C) Bacterial burden in the whole tail was quantified by enumeration of cfu's at multiple time points post-infection. Student's t-test (*P<0.03).

Hence, the Δ*secA2* mutant is able to cause disease early in infection, but is attenuated for virulence after 3 weeks. Since the onset of adaptive immunity occurs around 20 days in M. tuberculosis infected mice (12, 13), we hypothesized that the ΔsecA2 mutant is more sensitive to, and is promptly cleared by, the antimicrobial mechanisms activated by the adaptive immune response. To test this hypothesis, the growth of Δ*secA2* was measured in the mouse tail over a 7 week period by CFU analysis (Figure 2.5C). As seen in the *M. tuberculosis* mouse model, growth of *M. marinum* strains peaked at 21 days. The bacteria numbers then declined, likely due to the onset of adaptive immunity. Although the Δ*secA2* mutant bacterial load was less than wild-type at the peak of infection, and the lesions stopped growing, the Δ*secA2* bacteria were not cleared significantly more than wild-type after 3 weeks of infection. This suggests that in mice, *M. marinum* SecA2 is required for maintenance of disease after 3 weeks of infection, yet is not required for bacterial growth and survival.

The Δ*secA2* mutant causes less inflammation without altering the cellular composition of the inflammatory infiltrate in mice

It is unclear how infection of mice with the ΔsecA2 mutant leads to healing of disease without clearance of the bacteria. To further investigate this, histopathological studies of infected mouse tail lesions were performed at 2 and 7 weeks post-infection to analyze the character of inflammation in infection by the different strains, including cell types present and granuloma architecture (Figure 2.6A,B). Evaluation of the tails by H&E at 2 weeks post-infection indicated that there was equivalent inflammation induced by all three strains (Figure 2.6A). The mice had moderate to severe cellulitis, myositis,

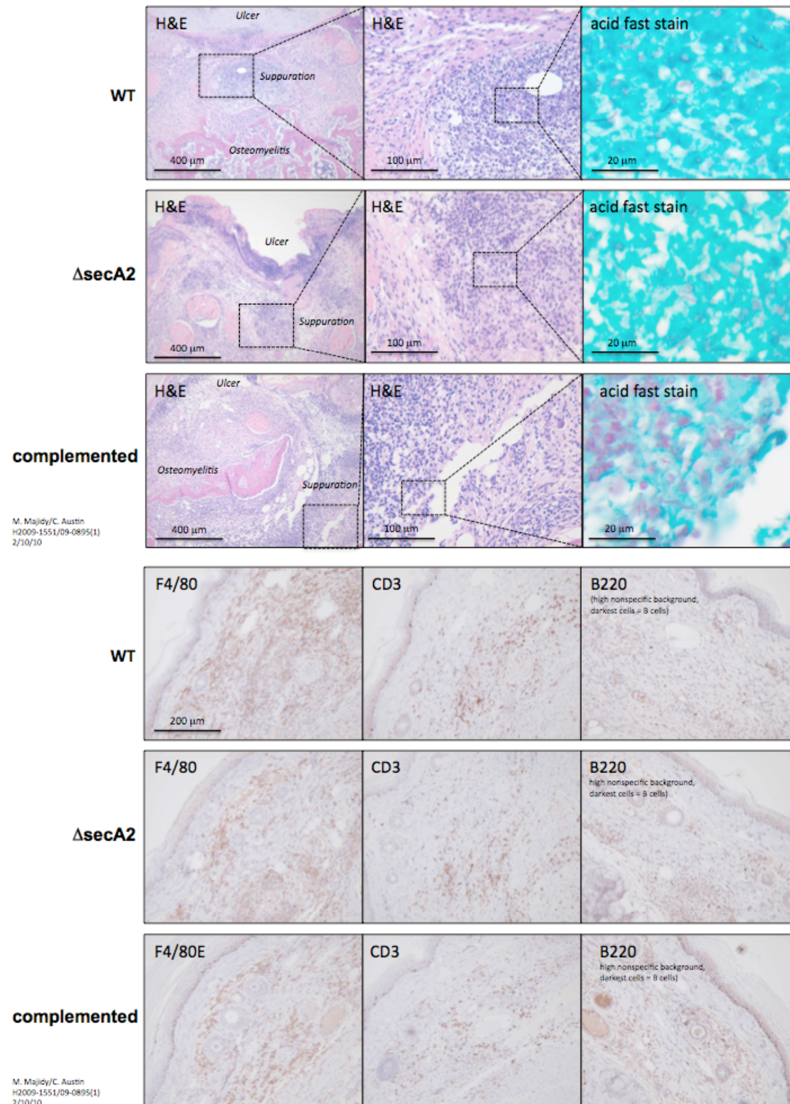


Figure 2.6 Histopathological analysis reveals similar cellular composition of inflammatory infiltrate between strains. Mice were infected via tail vein injection with 10^7 wild-type, Δ*secA2*, SA2^{comp} *M. marinum.* Cross-sections of 3 infected mouse tails per experimental group were analyzed at 7 weeks post-infection by H&E, Ziehl-Neelsen (acid fast) staining, and immunohistochemical staining for CD3 (T-cells), B220 (B-cells), and F4/80 (macrophages).

osteomyelitis, and focal ulceration of the epidermis. Immunohistochemical staining showed that the chronic inflammatory infiltrates were rich in macrophages and T-cells, with occasional B-cells. Unlike a previous study using this model (11), granulomas were not observed at this time point. Evaluation of the tails by H&E at 7 weeks post-infection indicated that the Δ*secA2* infected mice had less severe inflammation and osteomyelitis than wild-type and SA2^{comp}. However, when analyzing individual lesions, there was no difference in the character of the inflammation as observed by H&E, and immunohistochemical staining for B-cells, T-cells, and macrophages (Figure 2.6B). The inflammatory infiltrates were rich in macrophages and T-cells, with occasional multinucleated giant cells. Occasional B-cells were identified. Additionally, acid-fast positive bacilli were frequently present at sites of inflammation, however Δ*secA2* bacilli were less numerous, and less frequently observed than wild-type and SA2^{comp}, as was observed in the zebrafish analysis. Granulomas were observed, although not well formed. It is not clear when the granulomas formed, and if they were ever well formed, or if the less defined architecture is an indication of resolution of inflammation. Pathology in this model has been shown to decrease over extended periods of time despite persistence of bacterial load (11). In summary, consistent with the attenuation in tail lesion burden, histopathological analysis of the infected tails showed that Δ*secA2* infected mice had decreased inflammation at 7 weeks post-infection but the immune cell composition was the same as wild-type infected mice at 2 and 7 weeks post-infection.

SecA2 is required for maximal induction of TNF-α and IFN-γ *in vivo*

The observations in Δ*secA2* infected mice of decreased tail lesions and less inflammation by histolopathological studies, yet no difference in bacterial numbers suggested the hypothesis that SecA2 is required specifically for modulation of the host inflammatory response. Cytokine profiles at 3 and 6 weeks post-infection were obtained from the tails of uninfected naive mice, and mice infected with Δ*secA2*, wild-type, and SA2^{comp} (Figure 2.7). At 3 weeks post-infection, there was significant and equivalent induction of IL-12p40 and IL-1ß by all strains. Interestingly, the Δ*secA2* mutant induced less IFN-γ, less TNF-α, and more IL-10 than wild-type M. marinum or SA2^{comp}, although the latter two differences reached statistical significance only when the mutant was compared to SA2^{comp}. Although not statistically significant, Δ*secA2* also induced less secretion of the inflammatory cytokine IL-17. At 6 weeks post-infection, all strains induced an increase in secretion of IL-12p40, and no difference in IL-1ß secretion. The fact that the Δ*secA2* mutant induced equivalent levels of these cytokines suggests that IL-12p40 and IL-1ß induction is not influenced by SecA2. The secretion levels of TNF-α were decreased in all *M. marinum* infected mice suggesting that this cytokine is primarily induced during early infection. IL-10 secretion was most decreased in Δ*secA2* infected mice, and was equivalent to that induced by wild-type and SA2^{comp}. IL-17 and IFN-γ induction by the mutant was unchanged from 3 to 6 weeks post-infection, whereas the levels in mice infected with wild-type and SA2^{comp} bacteria were decreased. Consistent with the zebrafish model, Δ*secA2* induces less pro-inflammatory TNF-α *in vivo* while bacteria burden is still comparable to that of wild-type infected animals. Distinctly, IFN-γ was diminished in mice but not in fish, and IL-1ß was diminished in fish but not in mice

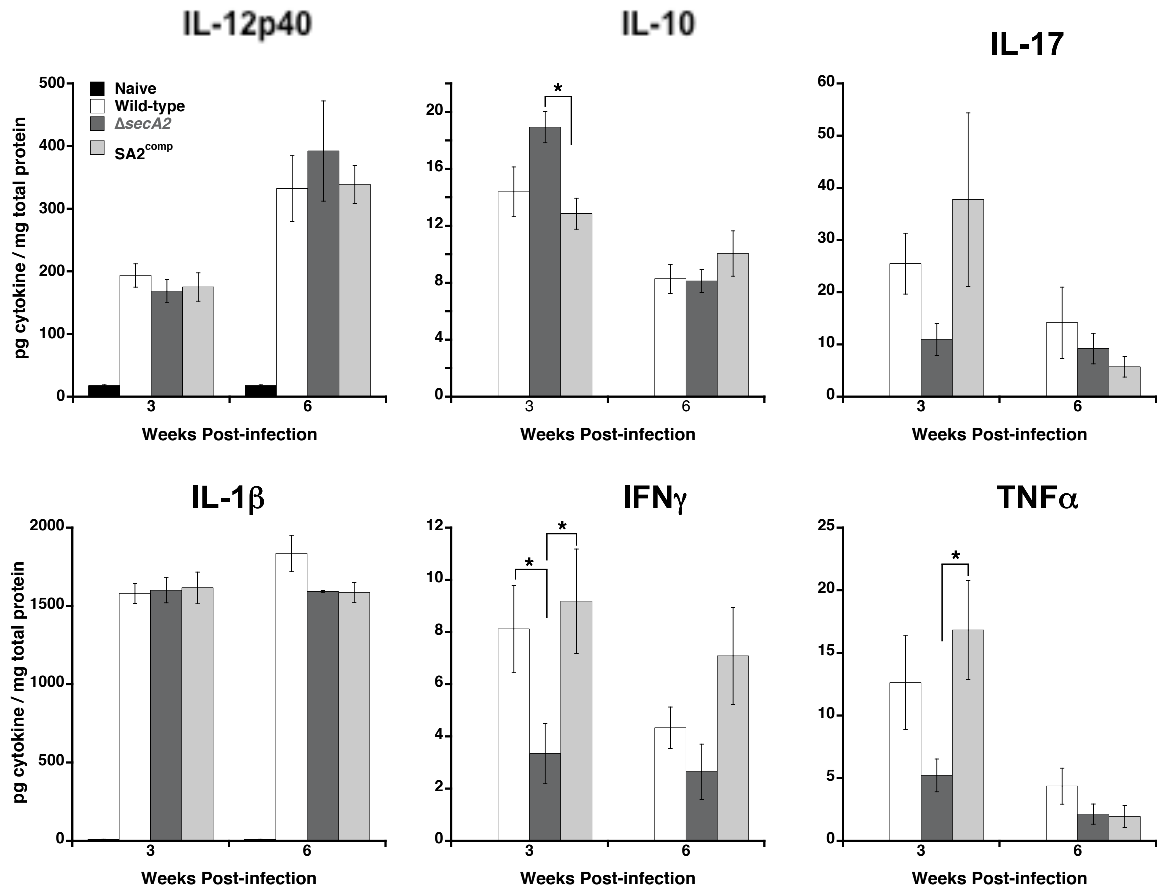


Figure 2.7 SecA2 is required for maximal induction of TNF-α and IFN-γ in mouse tails. C57BL/6 mice were infected via tail vein injection with 10^7 wild-type, Δ*secA2*, and SA2^{comp} *M. marinum*. Naive mice received no treatment. At 3 and 6 weeks post-infection, cytokine levels were quantified by Luminex from the whole tail of each experimental and control group. Values represent the average amount of cytokine detected (pg/ml), divided by the total amount of protein present in each tail suspension (mg/ml) ± standard error of the mean of 6 mice per experimental group, per time point. Analysis by two-way ANOVA indicated a statistically significant difference between treatment groups over time for all cytokines measured indicating that there was significant induction of each cytokine by *M. marinum* strains over the naive control. Student's t-test (*P<0.04).

indicating that these two models have unique cytokine responses to *M. marinum* infection.

Rag2 knockout mice are susceptible to Δ*secA2* infection

The kinetics of disease following infection by the Δ*secA2* mutant and wild-type strains in both zebrafish and mice suggested that a major role for secA2 was manifest only after the onset of adaptive immunity. To better examine only the innate immune response, Rag2 knockout mice, which lack mature B and T-cells, were infected with the *M. marinum* Δ*secA2* and $SA2^{comp}$ (Figure 2.8). Infection of these immunodeficient mice led to disease with a different macroscopic appearance than in wt mice. The lesions were less well defined, and were filled with yellow colored exudates (Figure 2.8A). Moreover, unlike infection of immunocompetent mice, the mice were made ill by the bacteria and began to die 3 weeks post-infection. There was no difference in mortality rate or in the size and extent of lesions between Δ*secA2* and $SA2^{comp}$ in Rag-deficient mice (Figure 2.8B,C). As stated previously, the optimal temperature for growth of *M. marinum* is about 32ºC*,* and therefore it fails to grow in the internal organs of wild-type mice. Diagnostic necropsy with H&E and Ziehl-Neelsen stains of the mouse tissue revealed no bacterial colonization of the lungs nor liver although some inflammation was present. The spleen showed marked extramedullary hematopoiesis. Analysis of the limbs, tail, and nasal tissue reveal cellulitis, osteomyelitis, and numerous acid fast positive bacilli associated with the severe inflammation. As expected, no granulomatous structures were found. Therefore, adaptive immunity is required for control of *M. marinum* in mice; in the absence of adaptive immunity, the Δ*secA2* strain is equally pathogenic as the wildtype.

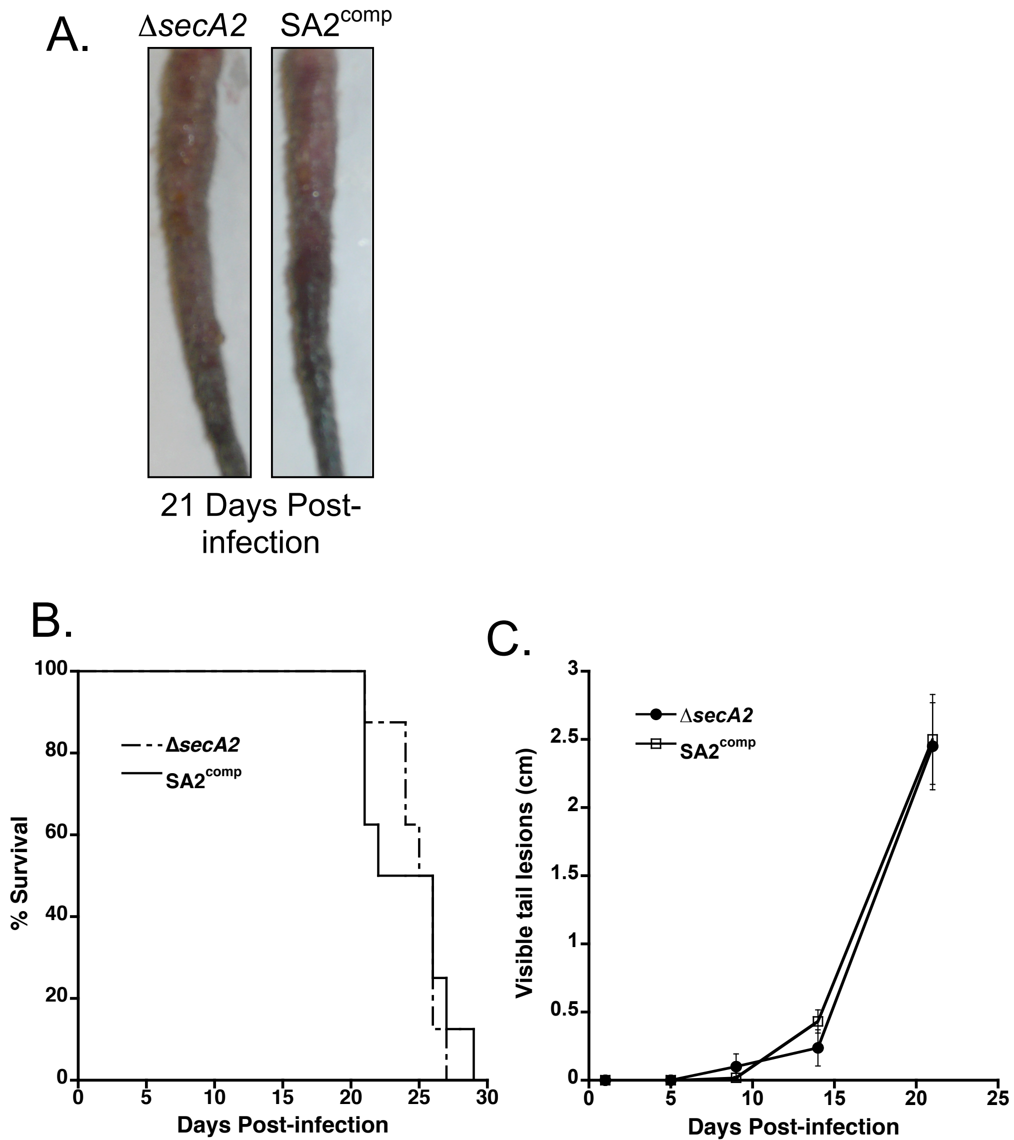


Figure 2.8 Rag2 knockout mice are susceptible to ΔsecA2 infection. Rag2 knock out mice were infected via tail vein injection with 10^7 Δ*secA2* and SA2^{comp} *M. marinum*. (A) Representative pictures of infected mouse tails at 21 days post-infection. (B) Survival of the mice was monitored over time (Δ*secA2* n=7, SA2^{comp} n=7). (C) The length of each visible tail lesion was summed to give the total number of lesions on each tail at each time point. Values represent the mean ± standard error of the mean of 7 mice per experimental group.

The lymph node immune response to *M. marinum* is independent of SecA2

Because secA2 appeared to exert its influence on disease after the onset of adaptive immunity, we characterized its role in the adaptive immune response during infection. We first assayed the population of B-cells, $CD4^+$ T-cells, and $CD8^+$ T-cells within the draining inguinal lymph nodes of infected mice by FACS at 3 and 6 weeks post-infection (Figure 2.9A). All three strains caused a striking increase in B-cells in the draining lymph nodes. However, no difference in the lymph node cell population was found between Δ*secA2* and the wildtype strain. We next tested the response of the lymph node cells to *M. marinum* antigens. The culture filtrate of mycobacteria cultures contains highly immunogenic proteins including ESAT-6, CFP-10, and the 19kDa adhesin. Concentrated culture filtrates from cultures of wild-type, Δ*secA2*, and $SA2^{comp}$ were used to stimulate lymph node cells from infected animals *ex vivo*, and cell proliferation and IFN-γ production were measured (Figure 2.9B). Lymph nodes isolated from mice infected with wild-type, Δ*secA2*, and $SA2^{comp}$ responded equally well to each strain of culture filtrate. However, since the lymph nodes are predominately populated with B-cells, it is possible that the proliferation response observed in this assay is also dominated by B-cells. To specifically assay the T-cell response, IFN-γ secreted from activated T-cells was measured by ELISA (Figure 2.9C). At 3 weeks post-infection, T-cells from mice infected with the Δ*secA2* mutant responded significantly more to *M. marinum* antigens than T-cells from wild-type infected mice. By 6 weeks post-infection, wild-type and Δ*secA2* T-cells respond equally well. However T-cells from $SA2^{comp}$ infected mice appear to respond less well, suggesting that 2-fold over expression of SecA2 may able to inhibit

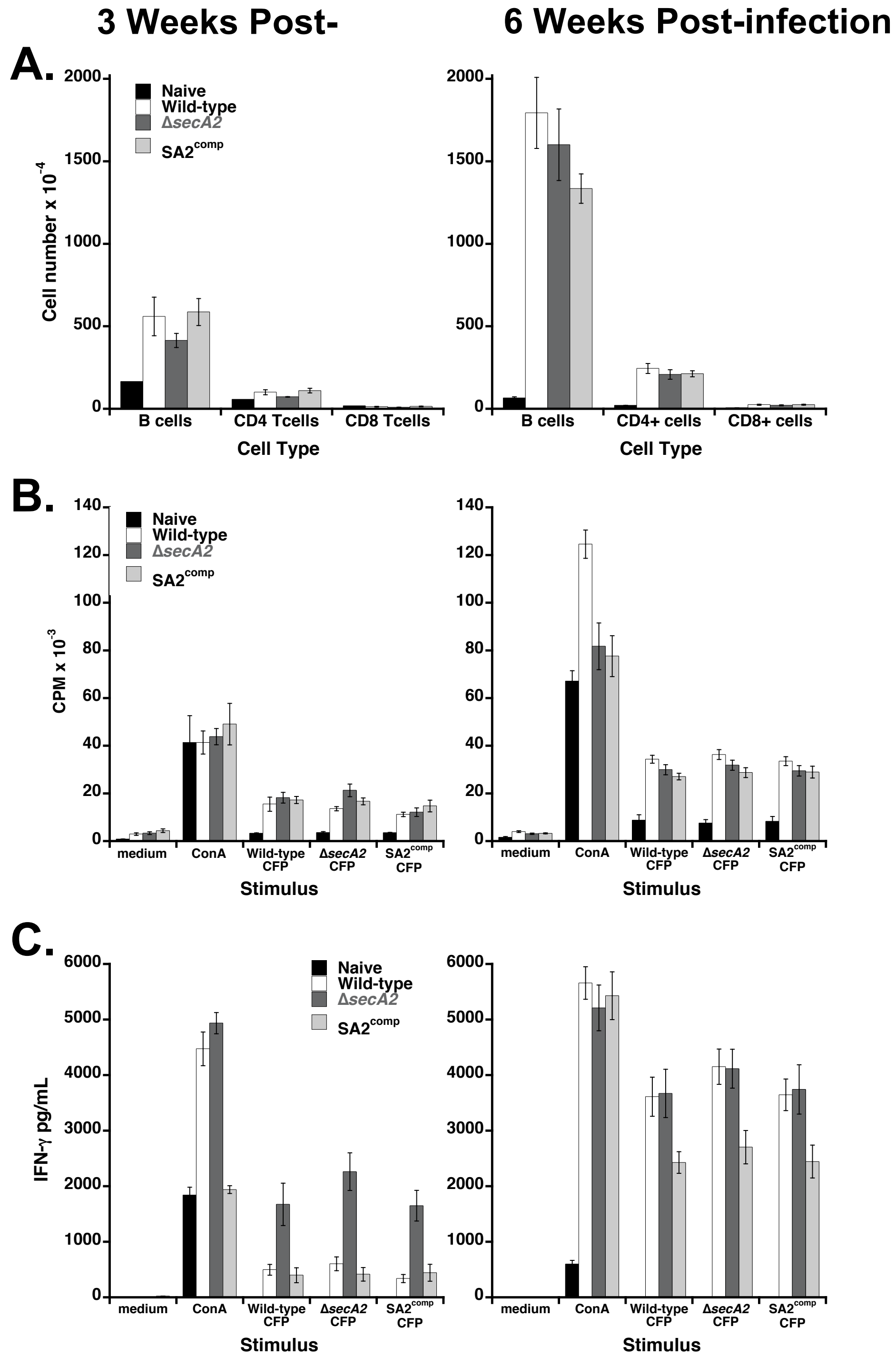


Figure 2.9 The lymph node response to *M. marinum* culture filtrate antigens *ex vivo.*

Bilateral, tail-draining inguinal lymph nodes were excised from C57BL/6 mice infected

via tail vein injection with 10^7 wild-type, Δ*secA2*, and SA2^{comp} *M. marinum,* and from uninfected naive mice at 3 and 6 weeks post-infection. (A) Lymph node cells were stained with anti-CD4-APC (1:400), anti-CD8-FITC (1:200), anti-B220-PE (1:200), and analyzed by FACS. Values represent the mean number of cells ± standard error of the mean of 6 mice per experimental group for the 6 week time point, and 3 mice for the 3 week time point. (B-C) 10^6 lymph node cells/mL were incubated for 48 hours with 10 μg/mL of *M. marinum* antigens purified from the culture filtrate of each strain grown in Sauton's medium. (B) Cells were then pulsed with [3H]thymidine to measure proliferation. Values represent the mean counts per minute of [3H]thymidine detected 18 hours post-pulse ± standard deviation of 6 mice per experimental group for the 6 week time point, and 3 mice for the 3 week time point. (C) 48 hours post-stimulation, T-cell responses were analyzed by measurement of IFN-γ present in the supernatant of lymph node cell cultures by ELISA. Values represent mean IFN-γ concentration ± standard error of the mean of 6 mice per experimental group for the 6 week time point, and 3 mice for the 3 week time point.

IFN-γ secretion by T-cells *ex vivo*. These data indicate that Δ*secA2* stimulates a normal adaptive immune response within the draining lymph nodes, but may induce it more rapidly.

The Δ*secA2* mutant is not cleared by activated macrophages

Although the zebrafish and mouse tail models of *M. marinum* infection both revealed a role for SecA2 in modulating host inflammation, the requirement of SecA2 for survival of *M. marinum in vivo* differed. The mutant was able to persist as well as wild-type bacteria in the mouse, before and after the onset of adaptive immunity. We therefore hypothesized that the Δ*secA2* strain would grow as well as the wild-type strain in macrophages *in vitro*. To test this hypothesis, we infected murine bone marrow derived macrophages (BMDM) with the three strains of *M. marinum* and assayed intracellular bacterial growth by CFU analysis (Figure 2.10A). Consistent with early growth in the mouse model, Δ*secA2* grew as well as wild-type. To mimic the *in vivo* macrophage state after the onset of adaptive immunity, the experiment was repeated in BMDMs activated with IFNγ and LPS (Figure 2.10B). In agreement with our hypothesis, the macrophages were able to inhibit growth of the Δ*secA2* mutant, but were not able to clear the mutant bacteria. To investigate cytokine induction by the Δ*secA2* mutant in macrophages, the level of TNF-α was measured from the supernatant of infected macrophages 24 hours post-infection (Figure 2.10C). Consistent with the 3 day zebrafish data, no difference was found between strains.

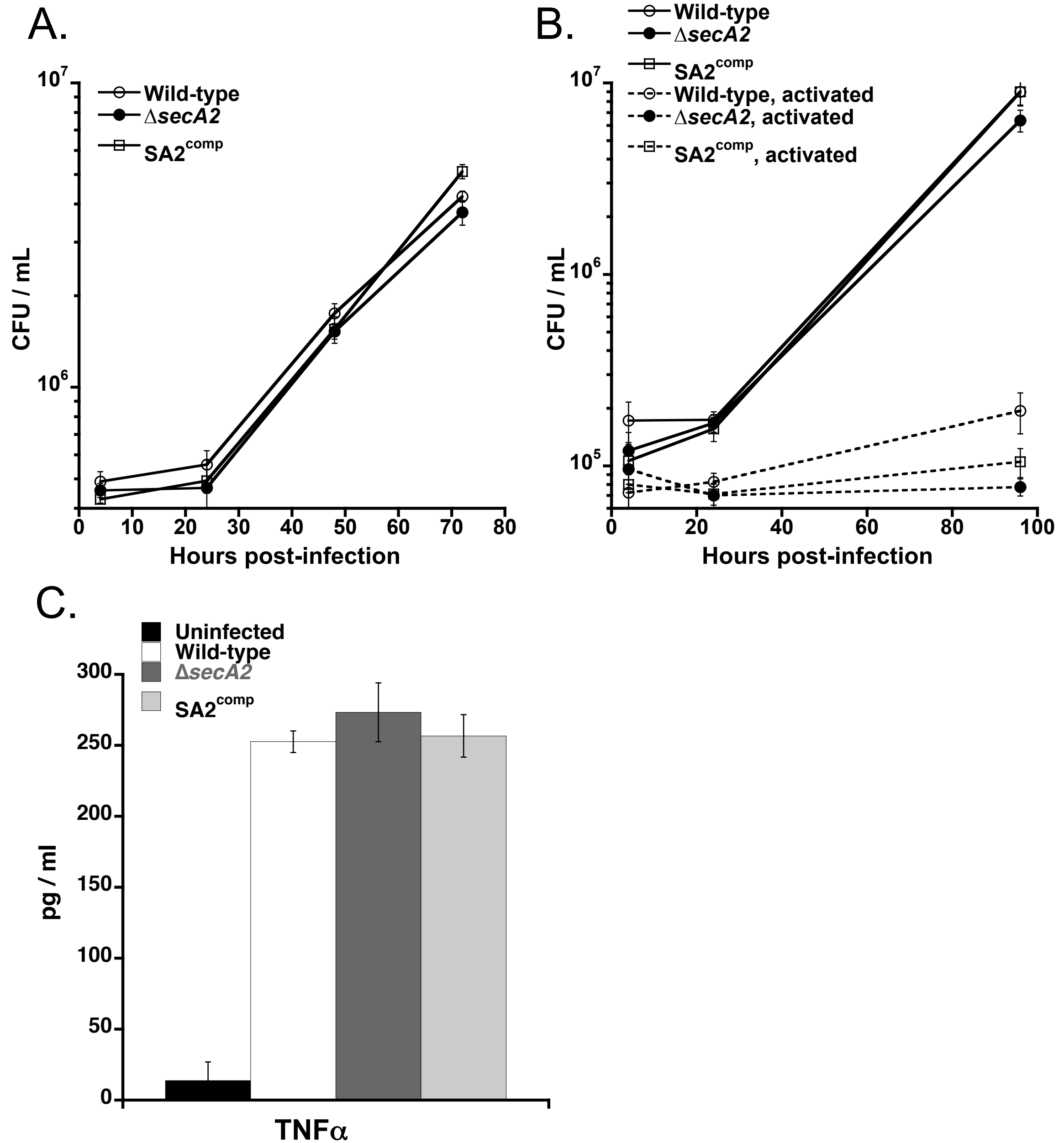
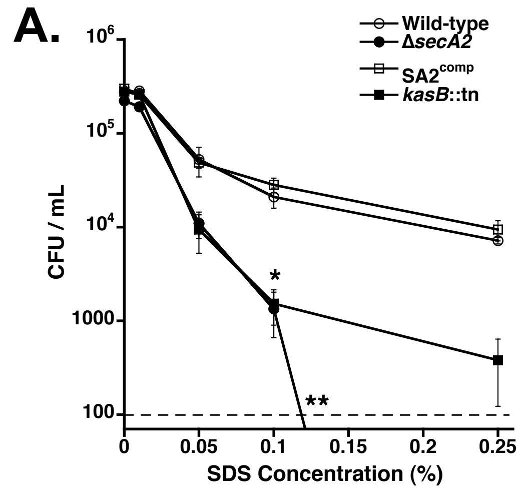


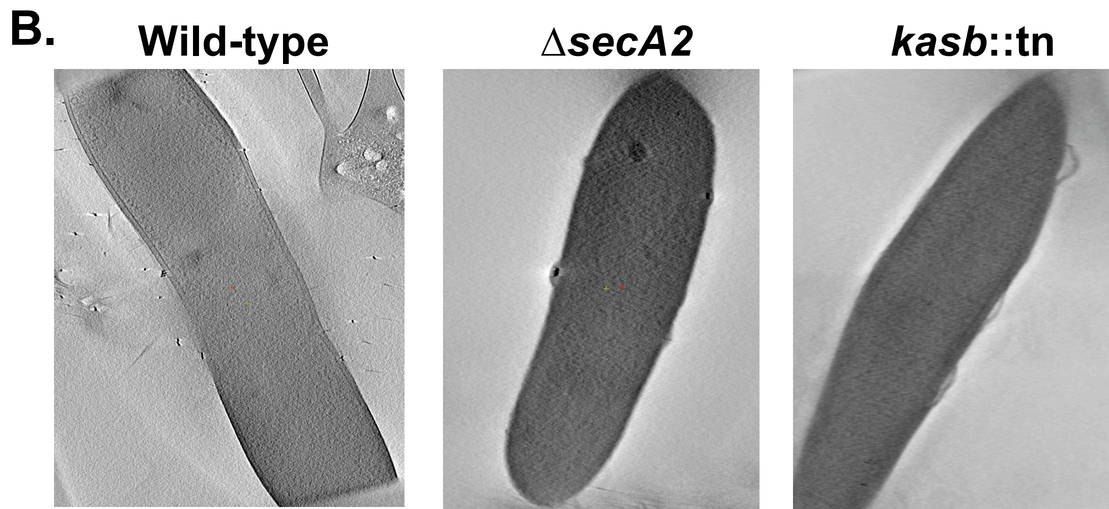
Figure 2.10 Δ*secA2* is not attenuated for growth nor TNF-α induction in macrophages. Bone marrow –derived macrophages (BMDMs) isolated from C57BL/6 mice were infected with wild-type, Δ*secA2*, and SA2^{comp} *M. marinum.* Intracellular bacterial growth was determined by enumeration of cfu's from macrophage lysates at various times post-infection. (A) BMDMs were infected at a multiplicity of infection

(MOI) of 3. (B) BMDMs were infected at a MOI of 1. For activation, macrophages were incubated with IFNγ (30ng/mL) and LPS (1ng/mL) for 24h before infection. (C) The concentration of TNF-α in the supernatants of infected BMDMs was measured by ELISA 24 hours post-infection.

The Δ*secA2* mutant has an abnormal cell envelope *in vitro*

Two lipoproteins have been identified in *M. smegmatis* that are dependent upon SecA2 for secretion to the cell envelope (14). We hypothesized that the SecA2 of *M. marinum* may also be required for localization of proteins to the cell envelope. Upon analysis of *M. marinum* cell envelope fractions by 1D SDS-PAGE, we were unable to identify any proteins dependent upon SecA2 for localization to either the cell wall or the cell membrane (Figure 2.11A). To further investigate a role for SecA2 in cell envelope structure, we visualized whole bacilli by cryo-electron tomography. Unlike traditional electron microscopy, this technique preserves cellular architecture, including the cell envelope, and allows for 3D reconstruction of the intact bacterial cell (Milne 2009). Analysis of the Δ*secA2* mutant revealed large protrusions from the cell envelope that were not present in wild-type, and differed from the ribbon-like loops observed in a kasb::tn mutant that has a defect in mycolic acid synthesis (Figure 2.11B). 25% of the Δ*secA2* bacilli analyzed had this defect. The contents of these protrusions is unknown, but this phenotype suggests a role for SecA2 in maintenance of cell wall composition. To test the integrity of the cell wall, the Δ*secA2* mutant was subjected to several stresses *in vitro*. Compared to wild-type and SA2^{comp}, Δ*secA2* was equally sensitive to hydrogen peroxide, rifampin and isoniazid (data not shown). The mutant did show increased sensitivity to the detergent sodium dodecyl sulfate (SDS) (Figure 2.11C). A low concentration of 0.25% SDS was able to kill the Δ*secA2* mutant without any effect on wild-type growth. Thus, SecA2 is required for normal morphology and integrity of the cell envelope.





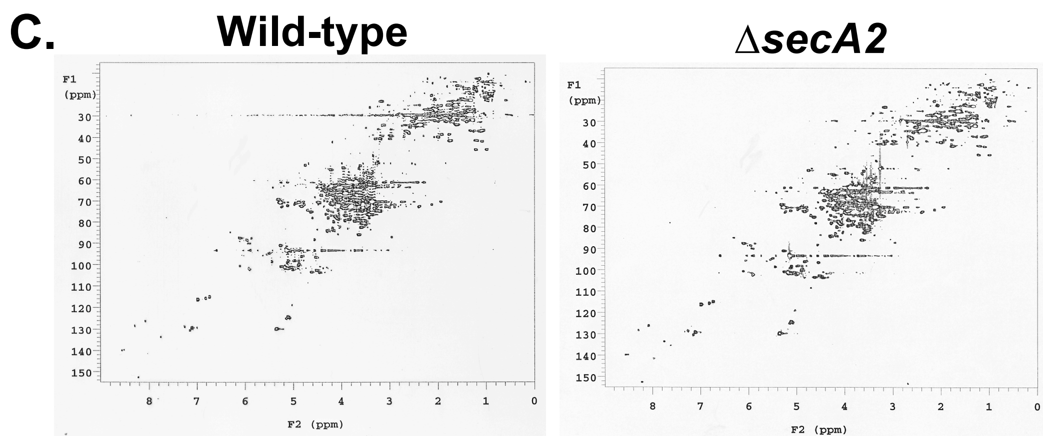


Figure 2.11 Δ*secA2* is SDS sensitive and has abnormal cell envelope morphology *in vitro*. (A) Wild-type, Δ*secA2,* SA2^{comp}, and *kasb*:tn *M. marinum* strains were incubated

with various concentrations sodium dodecyl sulfate (SDS) (0.01, 0.05, 0.1, and 0.25% v/v) in 7H9 broth for 20 h at 30ºC. Sensitivity was determined by enumerating cfu's. (B) Cell envelope morphology of wild-type, Δ*secA2, kasb*:tn *M. marinum* strains grown in 7H9 broth was analyzed by cryo-electron tomography. Representative images are shown. (C) Crude lipid extracts isolated from wild-type and Δ*secA2 M. marinum* grown in 7H9 broth were analyzed by 2D ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) NMR. Axes: F1 = δ^{13}C , F2 = δ^{1}H.

To see if the cell wall defects observed in the Δ*secA2* mutant were due to abnormal lipid composition, we analyzed the lipid content of the mutant and wild-type *M. marinum* by 2D ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) NMR. This technique allows for rapid qualitative and quantitative analysis of lipid populations (15). Crude lipid extracts were obtained from bacteria grown in 7H9 broth supplemented with ^{13}C. Analysis of the extracts did not reveal any differences in aromatics, glycolipids, and aliphatic chains of lipid molecules (Figure 2.11D). However, significant overlap of signals was observed. Therefore, further analysis of the bacterial lipids by multiple methods is necessary to determine whether a defect in lipid composition is present.

Materials and Methods

Bacterial strains and growth conditions. Wild-type *Mycobacterium marinum* strain M, and subsequent mutant strains used in this study were derived from a human clinical isolate (ATCC BAA-535). Construction of the transposon insertion mutant *kasB*::tn and deletion mutant Δ*RD1* have been described previously (19, 20). Δ*secA2*, a *M. marinum secA2*::Kan^R insertion mutant of gene *MMAR_2698*, was constructed using a previously published double-selection strategy (5). Flanking upstream sequence was amplified using PCR with genomic DNA template and primers SecA2U5 (5' TAATACTAGT*TGAACAGCACATTCAGTC* 3') and SecA2U3 (5' TATAGATATC*TAGGCCAGGTTTGATCGG* 3'), where genomic sequences are italicized. Likewise, downstream sequence was amplified using primers SecA2D5 (5' TAATGATATC*TGGCACCGATATCCGGTT* 3') and SecA2D3 (5' TTAATGCATACTAGT*ACAGACCCCAGATCAGAAACG* 3'). These two flanking sequences were cloned sequentially into pCR 2.1-TOPO (Invitrogen). A kanamycin-resistance gene derived from pUC4k (GE Healthcare) was then inserted between the upstream and downstream segments using EcoRV. This entire piece containing the upstream/Kan^R/downstream segment was finally cloned into pLYG304 (5). The mutant was then isolated after double selection and the mutated genomic region was PCR amplified and confirmed by DNA sequencing. Δ*secA2* was complemented with *secA2* (*MMAR_2698*) by integration of this gene and the mycobacterial hsp60 promoter, into the Δ*secA2* chromosomal attB site using the pMV306.hyg shuttle vector (21). For experiments, strains were grown to mid-log phase at 30ºC, 105 rpm in Middlebrook 7H9

broth (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% Middlebrook ADC enrichment (BBL) or at 32ºC on Middlebrook 7H10 agar (Difco) supplemented with 0.5% glycerol, and 10% Middlebrook OADC enrichment (BBL). Media was supplemented with 10% sucrose, kanamycin (30µg/mL), zeocin (50µg/mL), and hygromycin (50µg/mL) as appropriate.

Zebrafish Infections. Wild-type zebrafish AB strain were purchased from the Zebrafish International Resource Center (Eugene, OR) and maintained in static tanks under conditions and standards specified by the Institutional Animal Care and Use Committee, and outlined by Cosma (22). For infections, fish were anesthetized with 0.015% MS-222 (ethyl 3-aminobenzoate methanesulfonic acid salt) for 3-5 min, then injected i.p. with 10^4 *M. marinum* in 50µl of PBS, or 50ul of PBS alone for controls. To assay bacterial load in the whole fish, fish were euthanized in 0.05% MS-222 for 20 min, bathed in 70% ethanol, then homogenized in 0.1% Triton X-100 (1.5 mL) using disposable tissue grinders (VWR 47732-450). 250µL of each fish homogenate was decontaminated of normal flora using BBL Mycoprep reagent (BD). 10-fold serial dilutions were plated on 7H10 plates for enumeration of CFUs. Viability of the *M. marinum* strains was unaffected by incubation with 0.1% Triton X-100 (1 h) and Mycoprep reagent (25 min). For histopathological analysis, At 7 days post-infection, fish were euthanized, at 7 days post-infection, fixed in 10% formalin for 7d, then processed whole for paraffin embedded histology. Serial pairs of parasagittal sections with maximal representation of pancreatic and liver tissue were stained by hematoxylin and eosin (H&E) and Ziehl-Neelsen. Total

pancreatic foci of three or more clustered acid-fast bacteria were enumerated, as well as those clusters specifically associated with recognizable granulomas.

Quantitative RT-PCR analysis. For analysis of *secA2* expression levels in *M. marinum*, cultures were grown to mid-log phase in 7H9 broth. Cultures were incubated for 5 min with lysozyme and RNA was isolated using the Qiagen RNeasy kit (Qiagen 74104). A second DNAse treatment was performed per manufacturer instructions (New England Biolabs M0303S). Quantitative RT-PCR was performed using the QuantiFast SYBR Green PCR kit (Qiagen 204054), gene specific primers listed in Table 1, and an ABI 7500 RT-PCR system. Fold induction of Δ*secA2* and $SA2^{comp}$ were calculated relative to the wild-type bacteria, using the ΔΔCt model with SodA as the reference gene, and no template and no reverse transcriptase reactions for controls. For zebrafish cytokine analysis, whole fish were euthanized in 0.05% MS-222 for 20 min, then homogenized in 1ml TRIzol (Invitrogen) per 50mg of tissue. RNA was extracted from tissues according to product instructions, and stored at -80ºC. cDNA was synthesized using Anchored Oligo(dT)20 primer (Invitrogen 12577-011), and Superscript III Reverse Transcriptase (Invitrogen 18080-044). Quantitative RT-PCR was performed as stated above. Fold induction of experimental groups were calculated relative to the PBS control group, using the ΔΔCt model with β-actin as the reference gene, and no template and no reverse transcriptase reactions for controls.

Macrophage infections. Murine bone marrow derived macrophages (BMDM) were cultured from C57BL/6 wild-type mice and infected with *M. marinum* as described previously (21). For all experiments, $4x10^5$ BMDMs were seeded in 12-well plates overnight, then infected with an MOI of 1 or 3 as described in figure legends. For activated macrophage experiments, BMDMs were incubated with recombinant mouse IFNγ (30ng/mL) and LPS (1ng/mL) for 24h before infection, and included in media 4h post-infection. TNF-α concentration was measured by ELISA (eBioscience) 24h post-infection.

Mouse infections. 12 week old, female C57BL/6 mice (Jackson - West), and Rag2 knockout mice (B6.129S6-Rag2tm1Fwa N12, Taconic) were inoculated with 10^7 *M. marinum* in 100μl of PBS via tail vein injection. For analysis of visible tail lesions, the greatest length of individual lesions were measured and summed for each tail. For analysis of bacterial load in the tail, tails were severed at the tail base, weighed, cut into 5mm pieces, and homogenized in 3 mL 0.1% Triton X-100 in DMEM, using an AHS200 homogenizer (VWR) with saw tooth adaptors (10x105 mm, Troemner). 10-fold serial dilutions of homogenates were plated on 7H10 plates for enumeration of CFUs, and calculated as CFUs per gram of tissue. For cytokine analysis, tails were severed from the tail base, immediately placed on dry ice. While on dry ice, tails were cut into 5mm pieces, frozen in liquid nitrogen, and pulverized with a biopulverizer (Biospec Products) also chilled in liquid nitrogen. The tissue was incubated on ice for 1.5h in 1 mL of PBS supplemented with complete, EDTA-free, protease inhibitor cocktail (Roche), then centrifuged twice at 20kg, 4ºC, for 20min. Supernatants were collected for Bradford

Analysis (Biorad) to determine total protein content for normalization of each tail sample, and Luminex analysis (Biorad) to quantify amount of cytokine in each tail. For histology, tails were fixed in 10% formalin, minimally decalcified in Immunocal (Decal Chemical Corp), trimmed into five or more 3 μm cross-sections, paraffin-embedded, sectioned, and stained by H&E and Ziehl-Neelsen. Additionally, immunohistochemical staining for CD3 (T-cells), B220 (B-cells), and F4/80 (macrophages) was performed.

Murine *ex vivo* lymph node assay. Tail draining, bilateral, inguinal lymph nodes (LN) were excised and placed on ice in 5 mL RPMI-1640 supplemented with 2% fetal bovine serum (FBS) (Sigma). LN were processed into single cell suspensions using a 70 μm cell strainer (Costar). For analysis of LN cell population, cells were stained with anti-CD4-APC (1:400, BD 553051), anti-CD8-FITC (1:200, BD 553031), anti-B220-PE (1:200, BD 553090) and analyzed by FACS. For *ex vivo* stimulation, LN cells were seeded 10^6/mL in 96-well U-bottom plates (Costar) in 200 μL RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 μM β-mercaptoethanol, and 10 μg/mL *M. marinum* culture filtrate (see below). After incubation for 48h at 37ºC, 5% CO_2, 110 μL of supernatant was carefully removed from each well for IFN-γ analysis by ELISA (eBioscience). Cells were then pulsed with 1μCi [3H]thymidine in 110 μL of media per well. 18 h post-pulse, cell DNA was harvested onto Unifilter plates (Perkin Elmer), and incorporated [3H]thymidine was measured using Cell count. Culture filtrate was obtained as outlined by Andersen and Rosenkrands (24). Briefly, supernatants collected from 250 mL *M. marinum* cultures grown to early-log phase in Sauton's defined medium (Teknova), were concentrated by an ammonium sulfate cut (516g/L, 4ºC, 7h), and

overnight dialysis at 4ºC in PBS. Total protein content was measured using a BCA assay.

SDS sensitivity assay. *M. marinum* ($2.5x10^5$ mL^{-1}) grown to mid-log phase in liquid media was inoculated into 7H9 containing sodium dodecyl sulfate (SDS) (0.01, 0.05, 0.1, and 0.25% v/v), and incubated for 20 h at 30ºC, 105 rpm. CFUs were enumerated on 7H10 agar plates.

Cryo-electron tomography. *M. marinum* grown to mid-log phase in 7H9 broth was prepared for microscopy as described by Comolli (23).

Lipid profiling by 2D HSQC NMR. ^{13}C labeled lipid extracts from *M. marinum* strains were prepared as described by Mahrous (15). Briefly, bacteria were grown to an optical density (at 600 nm) of 0.6 to 0.8 in 7H9 liquid media supplemented with 0.2% U-$^{13}C_6$-glucose and 0.2% U-$^{13}C_3$-glycerol for ^{13}C labeling as described by Mahrous (15). Cells were pelleted and washed with D_20 twice. A 2:1 (v/v) mixture of $CDCl_3$ and CD_3OD was used to extract total lipids. Lipids were transferred to a 5mm NMR tube for analysis. The HSQC pulse and NMR analysis were performed as described (15).

Chapter 3

Concluding Remarks

Discussion

SecA2s of several different bacteria, including Mycobacteria, are proven to have a role in virulence. Although SecA2 dependent virulence factors and their influence on host pathology have been identified in some systems (See 1 for review), such factors and mechanisms are still being defined in Mycobacteria. One study suggests that SecA2 dependent SodA secretion may indirectly inhibit priming of host adaptive immunity, but an *in vivo* role for SodA in virulence has not been shown (2). The focus of this thesis is investigation of the role that *M. marinum* SecA2 has in modulating host immune responses. Using two distinct *in vivo* models for *M. marinum* infection, we were able to show that *M. marinum* SecA2 is required for full expression of disease, as is the case in *M. tuberculosis*. Unlike *M. tuberculosis*, this requirement is not observed during the early innate phase of the host immune response. Additionally, we have shown that SecA2 is required for maximal induction of several pro-inflammatory cytokines *in vivo*, and for normal cell envelope morphology *in vitro*.

To study the role of SecA2 in virulence and host immunity to Mycobacteria, we created a Δ*secA2* deletion strain in *M. marinum* by replacing the secA2 gene with a kan^R gene. Comparison of infection of a natural host for *M. marinum*, the zebrafish, with wild-type *M. marinum* and the Δ*secA2* strain yielded striking results. The Δ*secA2* mutant failed to kill the fish, whereas wild-type and the complemented strain ($SA2^{comp}$) caused acute inflammation and killed the fish between 12 and 25 days post-infection. At day 7 post-infection, Δ*secA2* infected fish had bacterial burdens equivalent to wild-type infected fish. However, at 9 days post-infection, the zebrafish were able to clear the

Δ*secA2* bacilli, while wild-type and SA2^{comp} bacteria continued to grow and were not eliminated by the fish. This result raises the possibility that loss of pathogenicity of the Δ*secA2* strain may coincide with the onset of adaptive immunity. A study has shown that the early growth of *M. marinum* is slowed by the antimicrobial mechanisms of innate macrophages within the first few days of infection of zebrafish embryos (3). Since Δ*secA2* bacilli were able to grow to wild-type levels after 7 days of infection, it appears that SecA2 is not required for growth during the innate phase of immunity. Adaptive immunity, like in mouse tuberculosis models, is necessary to stop the growth of *M. marinum* in adult zebrafish (4), however the kinetics of the onset of adaptive immunity is unknown. Therefore, its is possible that the onset of adaptive immunity occurs after 7 days post-infection, and SecA2 has a role in promoting growth and survival of *M. marinum* once adaptive immunity has been established.

Further studies of the host immune response at 7 days post-infection revealed that granulomas were present in fish infected by wild-type *M. marinum,* including even necrotic centers. Strikingly, only one of ten Δ*secA2* infected zebrafish showed any evidence of granuloma formation. A study of the kinetics of granuloma formation and maturation in adult zebrafish would be necessary to determine if macrophage aggregates were formed in these fish but couldn't mature to, or be maintained as organized granuloma-like structures. In addition, Δ*secA2* infected zebrafish made less TNF-α and IL-1β mRNA, yet equivalent levels of IL-12 and IFN-γ. Studies in zebrafish embryos and *rag1* mutant adult zebrafish, both of which lack the ability to mount an adaptive immune response, have shown that *M. marinum* is able to induce granuloma formation in the absence of adaptive immunity (5, 4). In fact, granuloma-like aggregates form within three

days of infection in zebrafish embryos. These aggregates are initiated by the recruitment of new macrophages by the infected macrophages (5). Once the aggregates form, accelerated mycobacterial replication and spread to neighboring cells is observed (6). This suggests that Mycobacteria are able to use granulomas as a growth niche to promote its replication and dissemination *in vivo*. The maintenance of granuloma integrity in zebrafish requires TNF signaling. Knockdown of TNF receptor 1 results in increased bacterial growth within macrophages and subsequently, macrophage death and disintegration of the granuloma (7). Consistent with the decreased presence of granulomas and decreased expression of TNF-α in Δ*secA2* infected zebrafish, SecA2 may be required for creation of a growth niche within granulomas via maximal induction of TNF-α. Since granuloma formation promotes accelerated replication of Mycobacteria, perhaps the clearance of Δ*secA2* bacteria is a reflection of an absent niche for replication in the midst of ongoing bacterial elimination initiated by the onset of adaptive immunity. The Δ*secA2* mutant, wild-type, and $SA2^{comp}$ may all be equally sensitive to killing by the adaptive immune response, but the latter two strains are able to induce granuloma formation, which leads to a niche that allows for continued growth that is absent in Δ*secA2* infection. This would suggest that SecA2 is required for promotion of growth after the onset of the adaptive immune response via its role in granuloma formation and/or maintenance. Further studies of the kinetics of granuloma formation and cytokine expression in relation to the kinetics of the death and growth of the Mycobacterial strains would be needed to determine if this hypothesis is correct. Although the mechanisms by which SecA2 in pathogenesis are undefined, it is clear that in adult zebrafish, *M. marinum* SecA2 is required for virulence, for growth and survival of the bacteria during

later stages of infection, for maximal induction of some host pro-inflammatory cytokines, and has a role in granuloma formation or maintenance.

Using a mouse model for *M.marinum* infection (8), we were able to further investigate the role of SecA2 in modulating host immunity. Mice infected with the Δ*secA2* mutant experienced less disease, yet normal bacterial load after 3 weeks of infection. The onset of adaptive immunity occurs around 20 days in *M. tuberculosis* infected mice (9, 10), suggesting that in this model SecA2 may be required for disease, but not bacterial survival, after the onset of adaptive immunity. SecA2 does not appear to be required for induction of an adaptive immune response, however the induction may have been more rapid in the Δ*secA2* infected mice. T-cells from the draining lymph nodes of Δ*secA2* infected mice responded earlier to infection *in vivo* than those from wild-type infected mice, as measured by IFN-γ secretion *ex vivo*. This result suggests a role for SecA2 in inhibiting the rapidity of the adaptive immune response. This result is similar to results obtained in *M. tuberculosis* infected mice. A study has shown that at 7 days post-infection, Δ*secA2* infected mice have significantly more antigen specific T-cells as quantified by IFN-γ ELISPOT, than the wild-type infected mice (2). Additionally, rapid immune responses to *M. tuberculosis* in mice has been associated with increased host survival, despite equivalent control of bacterial growth (11). This study involved comparison of the kinetics of adaptive immunity in a *M. tuberculosis* resistant mouse strain (B6) and a susceptible strain (C3H/HeJ), and showed that the resistant mouse strain was able to mount an adaptive immune response more quickly than the susceptible strain (11). This rapid immune response was associated with accelerated dissemination of the cellular response and an increased median survival time (220 days for B6, 65 days for

C3H/HeJ), despite equivalent bacterial growth in the lungs. Although correlational, the authors suggest that the increased mortality in the susceptible strain may be due to its slower immune response to the bacteria. In the mouse model for *M. marinum* infection, the bacteria are not able to kill the mice, so the measurement of granulomatous lesion growth is used as an indicator of disease progression in the host and of bacterial virulence. Along the lines of the study previously described, if the Δ*secA2* mutant does in fact induce a more rapid immune response, as suggested by the more rapid response of the lymph node T-cells, it is possible that this more rapid response leads to the decreased pathology observed after 3 weeks of infection despite normal bacterial load. Alternatively, the adaptive immune response may cause the Δ*secA2* mutant to be less immunogenic, leading to the decreased pathology observed in these mice. After the onset of adaptive immunity, Mycobacteria may secrete a unique set of proteins, some of which may be SecA2 dependent and immunogenic, stimulating further progression of disease. If this is the case, attenuated immunogenicity of the Δ*secA2* mutant would not have been detected in our *ex vivo* lymph node stimulation assay, due to the absence of these *in vivo* specific antigens in the culture filtrates used to stimulate the lymph node cells. In support of this hypothesis, *M. tuberculosis* has been shown to express a unique transcript profile after induction of adaptive immunity *in vivo* (12). Interestingly, in that study, the transcription of alpha-crystallin (*acr*) is increased. Acr is a heat shock protein that is dependent upon SecA2 for secretion in *M. tuberculosis* (13). This protein is thought to protect Mycobacteria from the host *in vivo,* since it is regulated by the dormancy regulon (DosR) and is upregulated under hypoxic conditions (14). Additionally, Acr has been shown to be immunogenic as evidenced by the presence of anti-Acr antibodies in about

half of the smear positive patients tested in six separate studies (15). Therefore it is possible that SecA2 may be required for the secretion of proteins that have a role in protecting the bacteria, but are incidentally immunogenic. This potential attenuation of immungenicity may account for the attenuated granulomatous lesion burden, but may also account for attenuated induction of cytokines by the Δ*secA2* mutant. Cytokine analysis revealed that SecA2 is required for maximal induction of TNF-α, IFN-γ, and possibly IL-17 at 3 weeks post-infection. TNF-α has been shown to be required for the maintenance of granuloma structure in BCG infected mice (16), as has been found in *M. marinum* zebrafish studies as stated previously. Additionally, IL-17 may be required for the maturity of granulomas in *M. tuberculosis* infected mice, specifically growth of lesion size and increased presence of granulocytes (17). In summary, SecA2 of *M. marinum* appears to be required for pathogenesis after the onset of adaptive immunity in mice. Additionally, SecA2 modulates the host immune response as indicated by decreased inflammation and decreased pro-inflammatory cytokine secretion at the site of infection in Δ*secA2* infected mice. The mechanism of this modulation is unclear, but it is possible that SecA2 dependent granuloma formation and/or maintenance via TNF-α is involved.

The Δ*secA2* mutant also has a unique phenotype *in vitro*. The mutant was more sensitive than wild-type to SDS treatment, and also had unique cell wall morphology by cryo-electron tomography. Surprisingly, the mutant was more sensitive than our kasB::tn mutant that has an established cell wall permeability defect due to its defect in mycolic acid synthesis (18). These data suggest that SecA2 is required for normal cell wall morphology. Two lipoproteins have been identified in *M. smegmatis* that are dependent

upon SecA2 for secretion to the cell envelope (19). Therefore, it is possible that the SecA2 of M. marinum is also required for the secretion of cell wall proteins.

Overall, the phenotype of the Δ*secA2* mutant in the mouse tail model is consistent with the zebrafish model for *M. marinum* infection. In these models SecA2 appears to be required for virulence and modulation of the host immune response after the onset of adaptive immunity. Kurtz and colleagues have shown that SecA2 of *M. tuberculosis* inhibits the host innate and adaptive immune response *in vitro* (20). Specifically, the Δ*secA2* mutant induced greater secretion of TNF-α, IL-6, and reactive nitrogen intermediates (RNI) from murine bone marrow-derived macrophages. It also promoted increased expression of IFN-γ induced MHC class II. These data are consistent with the rapid adaptive immune response induced by Δ*secA2* of *M. marinum* in the mouse model. However our data are inconsistent with the study showing that SecA2 of *M. tuberculosis* is required for bacterial growth before the onset of adaptive immunity (13). Therefore it appears that SecA2 is required for virulence in *M. marinum* as it is in *M. tuberculosis*, however the role of SecA2 in modulating host immunity differs between the two strains. Further analysis of the host immune response during the early phase of infection would be required to determine if SecA2 of *M. marinum* is indeed required for inhibition of the host immune response upon initial infection that contributes to the increased virulence of the bacteria after the onset of adaptive immunity *in vivo*.

Future Directions

Identification of SecA2 interacting proteins

As described in Chapter 1, novel SecA2/SecY2 secretion systems have been identified in several bacterial strains. However, Mycobacteria do not have the SecY2 homologue, so a novel secretion system has not been readily identified through genomic analysis. Two studies in *M. smegmatis* have indirectly shown that SecA2 may assist SecA in secretion of proteins via the general Sec pathway (21, 22). Identifying proteins that interact with SecA2 may provide further clues as to how SecA2 facilitates the export of some Mycobacterial proteins. Such a study may not eliminate the possibility of SecA2 being a member of a novel Sec-independent secretion system, but it may support a role for SecA2 in secretion via the Sec pathway.

Investigate cell wall defect

We have shown that SecA2 is required for normal cell envelope morphology in *M. marinum*. However the cause of the defects in the Δ*secA2* mutant, and whether the SDS sensitivity is related to the cell wall protrusions is unknown. Cryo-electron tomography is still a relatively new application in regards to the study of bacterial cell wall architecture. As far as we know, the cell wall protrusions observed in the Δ*secA2* mutant have not been published elsewhere for any bacteria. Therefore, we can only speculate as to its cause and composition. Further analysis of the lipid and protein composition of the cell envelope in the Δ*secA2* mutant in comparison to wild-type bacteria, may identify specific proteins and/or lipids that are dependent upon SecA2 for

proper localization to the cell envelope. If the substrates identified have known function, or structure similar to proteins/lipids of known function, the role of SecA2 in cell envelope morphology may be clarified.

Further investigation of host immune response

The results of this thesis clearly show a role for SecA2 in virulence and modulation of the host immune response. The mechanisms involved in this modulation were not determined. As discussed previously, determining the kinetics of induction of host immunity in relation to bacterial growth and host pathology, during all phases of infection, is crucial to explaining the attenuated phenotype of the Δ*secA2* mutant. It is possible that the mutant induces a more rapid immune response that results in better control of infection and inflammation by the host. The cause of the decreased levels of pro-inflammatory cytokines observed in Δ*secA2* infected zebrafish and mice is unclear. Studies have shown that engagement of different host cell receptors by Mycobacteria, induces unique inflammatory responses (23). Determining which receptors are engaged by the Δ*secA2* mutant may help in understanding the unique cytokine profile induced by the mutant. An interesting experiment may be to add exogenous cytokine to Δ*secA2* infected zebrafish and mice at various time-points, and observe the effect on granuloma/lesion formation and bacterial growth. If granulomas were then able to form in the fish, would the Δ*secA2* bacilli be able to use this niche to grow like wild-type and eventually kill its host? Or would it still be cleared by the fish, suggesting that SecA2 is required for growth and virulence of Mycobacteria independently of granuloma formation? Similarly in the mouse model, would exogenous cytokine be able to restore

growth of the granulomatous lesions? A similar experiment, but investigating the requirement for SecA2-dependent secreted proteins, would be to inject purified culture filtrate proteins from wild-type cultures into the Δ*secA2* infected animals at various time points post-infection and observing the affect on host survival (zebrafish), the host immune response, and host pathology.

Appendix

Characterization of p60 proteins in *Mycobacterium marinum*

Abstract

NlpC/p60 proteins make up a family of cell-wall peptidases and are found in most bacterial lineages. Members of our laboratory have studied two such proteins in *Mycobacterium marinum*, iipA and iipB, which are homologous to Rv1477 and Rv1478 in *Mycobacterium tuberculosis*. Our laboratory has found that the iipA/B locus is required for Mm virulence in zebrafish, resistance to antibiotics and lysozyme *in vitro*, invasion of murine macrophages, normal cell division, and cording (a gross morphology characteristic) (1). A BLAST search of the *M. marinum* genome reveals the presence of five p60-like genes, including *iipA and iipB*. Significantly, each of these has a homologue in the *M. tuberculosis* genome. The majority of my time in graduate school was dedicated to the study of the three addition p60-like proteins, named p60-1, p60-2, and p60-3. I proposed to characterize each of these proteins in *M. marinum* through mutational analysis, to determine whether they were peptidoglycan hydrolases, and to investigate the potential role of the p60 domain in host cell invasion and intracellular survival. Unfortunately, I was unsuccessful in deleting these genes, and in making functional recombinant protein. This appendix lists the reagents that were created for these studies.

Table A.1 List of plasmids created for p60 studies

Plasmid	Description
pjsc232ΔoriM	Created a suicide plasmid (to make unmarked mutants in *M. marinum*) from pjsc232 (obtained from Jeff Cox/Scott Converse) by removing the oriM. Contains sacB, kan^R, and oriE
pMip1-U/D	pjsc232ΔoriM plus overlapping up and downstream regions of mip1 (aka iipA, Mm2284, homologous to Rv1477) to create unmarked deletion of iipA in *M. marinum*
pMip2-U/D	pjsc232ΔoriM plus overlapping up and downstream regions of mip2 (aka iipB, Mm2285, homologous to Rv1478) to create unmarked deletion of iipB in *M. marinum*
pP601-U/D	pjsc232ΔoriM plus overlapping up and downstream regions of p60-1 (Mm0043, homologous to Rv0024) to create unmarked deletion of p60-1 in *M. marinum*
pP602-U/D	pjsc232ΔoriM plus overlapping up and downstream regions of p60-2 (Mm2381, homologous to Rv1566c) to create unmarked deletion of p60-2 in *M. marinum*
pP603-U/D	pjsc232ΔoriM plus overlapping up and downstream regions of p60-3 (Mm3234, homologous to Rv2190c) to create unmarked deletion of p60-3 in *M. marinum*
pLYG304ΔoriM	Created a suicide plasmid from (to make unmarked mutants in *M. marinum*) pLYG304 by removing the oriM.
pdelta-iipA	Made knockout construct from pLYG304 consisting of a kanamycin resistance cassette bordered by the up and downstream regions of iipA to replace iipA in *M. marinum* with kanR
pdelta-iipB	Made knockout construct from pLYG304 consisting of a kanamycin resistance cassette bordered by the up and downstream regions of iipB to replace iipB in *M. marinum* with kanR
pdelta-p601	Made knockout construct from pLYG304 consisting of a kanamycin resistance cassette bordered by the up and downstream regions of p601 to replace p601 in *M. marinum* with kanR
pdelta-p602	Made knockout construct from pLYG304 consisting of a kanamycin resistance cassette bordered by the up and downstream regions of p602 to replace p602 in *M. marinum* with kanR
pdelta-p603	Made knockout construct from pLYG304 consisting of a kanamycin resistance cassette bordered by the up and downstream regions of p603 to replace p603 in *M. marinum* with kanR
piipAcomp	Created construct to complement M. marinum iipA knockout with the Mtb iipA. iipA expression driven by own promoter in pMV306 chromosomal integration plasmid. Contains attB site. Hygromycin selection.
piipBcomp	Created construct to complement M. marinum iipB knockout with the Mtb iipB. iipB expression driven by iipA promoter in pMV306 chromosomal integration plasmid. Contains attB site and V5 tag. Hygromycin selection.
pP601comp	Created construct to complement M. marinum p60-1 knockout with the Mtb p60-1. p60-1 expression driven by own promoter in pMV306 chromosomal integration plasmid. Contains attB site and V5 tag. Hygromycin selection.
pP602comp	Created construct to complement M. marinum p60-2 knockout with the Mtb p60-2. p60-2 expression driven by own promoter in pMV306 chromosomal integration plasmid. Contains attB site and V5 tag. Hygromycin selection.
pP603comp	Created construct to complement M. marinum p60-3 knockout with the Mtb p60-3. p60-3 expression driven by own promoter in pMV306 chromosomal integration plasmid. Contains attB site and V5 tag. Hygromycin selection.
piipAtet	Created construct to conditionally complement M. marinum iipA knockout with the Mtb iipA. iipA expression driven by tetracycline inducible promoter in pUV15tetORm. kanR and HygR
piipBtet	Created construct to conditionally complement M. marinum iipB knockout with the Mtb iipB. iipB expression driven by tetracycline inducible promoter in pUV15tetORm. kanR and HygR
pP601tet	Created construct to conditionally complement M. marinum p60-1 knockout with the Mtb p60-1. p60-1 expression driven by tetracycline inducible promoter in pUV15tetORm. kanR and HygR
pP602tet	Created construct to conditionally complement M. marinum p60-2 knockout with the Mtb p60-2. p60-2 expression driven by tetracycline inducible promoter in pUV15tetORm. kanR and HygR
pP603tet	Created construct to conditionally complement M. marinum p60-2 knockout with the Mtb p60-2. p60-2 expression driven by tetracycline inducible promoter in pUV15tetORm. kanR and HygR
pMip1-p60	Cloned p60 domain of Mm iipA into pSH200, an E. coli recombinant protein expression plasmid with a T7 inducible promoter

pMip2-p60	Cloned p60 domain of Mm iipB into pSH200, an E. coli recombinant protein expression plasmid with a T7 inducible promoter
piipA.smeg	Cloned Mtb iipA into pHR100 (obtained from Jeff Cox) to express recombinant iipA in *M. smegmatis*
piipB.smeg	Cloned Mtb iipB into pHR100 (obtained from Jeff Cox) to express recombinant iipB in *M. smegmatis*
piipA.yeast	Cloned Mtb iipA into pPICZαA (Invitrogen K1740-01) to express recombinant iipA in *Pichia pastoris*
piipB.yeast	Cloned Mtb iipB into pPICZαA (Invitrogen K1740-01) to express recombinant iipA in *Pichia pastoris*
piipA.pgex	Cloned a 50 amino acid peptide sequence of Mtb iipA into pGEX-KG (GST-tag plasmid) to make peptide for antibody production
piipB.pgex	Cloned a 50 amino acid peptide sequence of Mtb iipB into pGEX-KG (GST-tag plasmid) to make peptide for antibody production
pP601.pgex	Cloned a 50 amino acid peptide sequence of Mtb p60-1 into pGEX-KG (GST-tag plasmid) to make peptide for antibody production
pP602.pgex	Cloned a 50 amino acid peptide sequence of Mtb p60-2 into pGEX-KG (GST-tag plasmid) to make peptide for antibody production
pP603.pgex	Cloned a 50 amino acid peptide sequence of Mtb p60-3 into pGEX-KG (GST-tag plasmid) to make peptide for antibody production

Table A.2 List of strains created for p60 studies

Strain	Species	Description
iipA.ppicza	*Pichia pastoris*	Recombinant Mtb iipA expressed in yeast. *c-myc* and 6xHis tagged. Zeocin selection
iipB.ppicza	*Pichia pastoris*	Recombinant Mtb iipB expressed in yeast. *c-myc* and 6xHis tagged. Zeocin selection
iipA.smeg	*Mycobacterium smegmatis*	Recombinant Mtb iipA expressed in *M. smegmatis (*inducible acetamidase promoter)
iipB.smeg	*Mycobacterium smegmatis*	Recombinant Mtb iipB expressed in *M. smegmatis (*inducible acetamidase promoter)
iipApep	*Escherichia coli*	Recombinant GST-tagged Mtb iipA peptide for making antibody
iipBpep	*Escherichia coli*	Recombinant GST-tagged Mtb iipB peptide for making antibody
P601pep	*Escherichia coli*	Recombinant GST-tagged Mtb p601 peptide for making antibody
P602pep	*Escherichia coli*	Recombinant GST-tagged Mtb p602 peptide for making antibody
P603pep	*Escherichia coli*	Recombinant GST-tagged Mtb p603 peptide for making antibody
iipAko	*Mycobacterium marinum*	Knockout strain of iipA, contains some wild-type *M.marinum*
iipBko	*Mycobacterium marinum*	Knockout strain of iipB, contains some wild-type *M.marinum*
P601ko	*Mycobacterium marinum*	Knockout strain of p60-1, contains some wild-type *M.marinum*
P602ko	*Mycobacterium marinum*	Knockout strain of p60-2, contains some wild-type *M.marinum*
P603ko	*Mycobacterium marinum*	Knockout strain of p60-3, contains some wild-type *M.marinum*
iipAcomp	*Mycobacterium marinum*	Mtb iipA with V5 tag integrated into M. marinum at attB site using pMV306. Hygromycin selection. Expression driven by own promoter.
iipBcomp	*Mycobacterium marinum*	Mtb iipB with V5 tag integrated into M. marinum at attB site using pMV306. Hygromycin selection. Expression driven by iipA promoter.
P601comp	*Mycobacterium marinum*	Mtb p60-1 with V5 tag integrated into M. marinum at attB site using pMV306. Hygromycin selection. Expression driven by own promoter.
P602comp	*Mycobacterium marinum*	Mtb p60-2 with V5 tag integrated into M. marinum at attB site using pMV306. Hygromycin selection. Expression driven by own promoter.
P603comp	*Mycobacterium marinum*	Mtb p60-3 with V5 tag integrated into M. marinum at attB site using pMV306. Hygromycin selection. Expression driven by own promoter.

Table A.3 List of antibodies made for p60 studies

Antibody	Target sequence/protein	Host
5913A	*M. tuberculosis* iipA peptide	Rabbit
5913B	*M. tuberculosis* iipA peptide	Rabbit
5719A	*M. tuberculosis* iipB peptide	Rabbit
5719B	*M. tuberculosis* iipB peptide	Rabbit
5929A	*M. tuberculosis* p601 peptide	Rabbit
5929B	*M. tuberculosis* p601 peptide	Rabbit
6200A	*M. tuberculosis* p602 peptide	Rabbit
6200B	*M. tuberculosis* p602 peptide	Rabbit
5762A	*M. tuberculosis* p603 peptide	Rabbit
5762B	*M. tuberculosis* p603 peptide	Rabbit

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Chapter 1

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Chapter 2

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Appendix A

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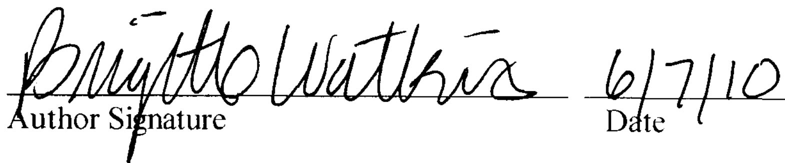
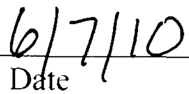

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