The *Mycobacterium tuberculosis* SecA2 System Subverts Phagosome Maturation To Promote Growth in Macrophages

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The ability of *Mycobacterium tuberculosis* to grow in macrophages is critical to the virulence of this important pathogen. One way *M. tuberculosis* is thought to maintain a hospitable niche in macrophages is by arresting the normal process of phagosomes maturing into acidified phagolysosomes. The process of phagosome maturation arrest by *M. tuberculosis* is not fully understood, and there has remained a need to firmly establish a requirement for phagosome maturation arrest for *M. tuberculosis* growth in macrophages. Other intracellular pathogens that control the phagosomal environment use specialized protein export systems to deliver effectors of phagosome trafficking to the host cell. In *M. tuberculosis*, the accessory SecA2 system is a specialized protein export system that is required for intracellular growth in macrophages. In studying the importance of the SecA2 system in macrophages, we discovered that SecA2 is required for phagosome maturation arrest. Shortly after infection, phagosomes containing a Δ secA2 mutant of *M. tuberculosis* were more acidified and showed greater association with markers of late endosomes than phagosomes containing wild-type *M. tuberculosis*. We further showed that inhibitors of phagosome acidification rescued the intracellular growth defect of the Δ secA2 mutant, which demonstrated that the phagosome maturation arrest defect of the Δ secA2 mutant is responsible for the intracellular growth defect. This study demonstrates the importance of phagosome maturation arrest for *M. tuberculosis* growth in macrophages, and it suggests there are effectors of phagosome maturation that are exported into the host environment by the accessory SecA2 system.

Mycobacterium tuberculosis infects about one-third of the world population and, as the infectious agent of tuberculosis, causes almost 2 million deaths per year (69). With the emergence of drug-resistant strains of *M. tuberculosis*, current treatments may soon become obsolete, fueling the need for new drugs and more effective vaccines to combat the disease. A better understanding of *M. tuberculosis* pathogenesis will facilitate efforts to develop new tuberculosis control measures.

Inside the host, *M. tuberculosis* survives in a unique intracellular niche within macrophage phagosomes. Following its phagocytosis by nonactivated macrophages, *M. tuberculosis* arrests the normal process of phagosome maturation. As a result, *M. tuberculosis* resides in a phagosome that fails to acidify or fuse with late endosomes and lysosomes that supply hydrolytic enzymes and antimicrobial peptides (2, 52). This *M. tuberculosis* phagosome resembles an early recycling endosome that is accessible to transferrin and maintains a pH of ~ 6.4 (46). *M. tuberculosis*-containing phagosomes are further distinguished by diminished accumulation of vacuolar ATPase (V-ATPase), phosphatidylinositol-3-phosphate [PI(3)P], and activated Rab7, which normally accumulate on maturing phagosomes (54, 57, 62, 63, 65). While the ability of *M. tuberculosis* to block phagosome maturation is widely thought to play an important role in promoting *M. tuberculosis* intracellular growth, there are few experiments that directly address a causal relationship between phagosome maturation arrest and intracellular growth. Furthermore, the mechanism *M. tuberculosis* uses to prevent phagosomes from maturing into acidic hydrolytic compartments is not well understood but appears to be a multifactorial process involving both protein and lipid effectors (3, 8, 14, 21, 36, 46, 56, 59, 63, 67).

M. tuberculosis effectors that alter phagosome trafficking are likely molecules that are exported by the bacillus (either secreted

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

The *M. tuberculosis* SecA2 system is important for virulence (6, 33). A Δ secA2 mutant of *M. tuberculosis* is attenuated for growth in macrophages and in a mouse model of infection. A possible explanation for the function of the *M. tuberculosis* SecA2 system in pathogenesis might be to modulate host innate immune responses. Macrophages infected with the Δ secA2 mutant produce higher levels of the proinflammatory cytokines tumor necrosis

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TABLE 1 *Mycobacterium* strains used

Strain	Description	Reference or source
<i>M. tuberculosis</i>		
H37Rv	Wild type	Trudeau Institute
mc ² 3112	H37Rv Δ secA2	6
MBTB74	H37Rv Δ secA2 attB::secA2	This study
MBTB109	H37Rv Δ secA2 attB::asodA	25, this study
mc ² 3032	H37Rv Δ leuD	26
MBTB3	H37Rv Δ eccD1	This study
PM638	H37Rv Δ blaC	18
MBTB204	PM638; mce1A::tn	40
MBTB156	PM638; mce2F::tn	40
MBTB183	PM638; rv0199::tn	40
MBTB157	H37Rv; gfp	This study
MBTB158	mc ² 3112; gfp	This study
MBTB159	MBTB74; gfp	This study
MBTB390	mc ² 3032; gfp	This study
<i>M. bovis</i> BCG		
BCG Pasteur	Wild type	Staten Serum Institut
MB544	BCG Pasteur Δ secA2	This study

factor alpha (TNF- α) and interleukin 6 (IL-6) and increased levels of reactive nitrogen intermediates (RNI). These immunomodulatory factors have roles in controlling *M. tuberculosis* during host infection (19, 34, 38), and they are induced by *M. tuberculosis* through MyD88-dependent signaling pathways (reviewed in reference 29). Macrophages infected with the Δ secA2 mutant also exhibit higher levels of apoptosis, which is attributed to defective SodA secretion by the Δ secA2 mutant (25).

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

MATERIALS AND METHODS

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

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

ing the deletion and positioned between the two flanking sequences. PacI-digested pKO-3877 was then ligated to PacI-cut phAE159 and *in vitro* packaged (Stratagene) into lambda phage particles that were recovered by transduction in *Escherichia coli*. The resulting recombinant mycobacteriophage was then used to transduce H37Rv at the nonpermissive temperature of 39°C for 4 h. The transduced cells were pelleted, resuspended in 7H9 medium with 0.1% Tween 80, and plated on 7H10 agar with hygromycin. Hygromycin-resistant colonies obtained at 3 weeks were screened for allelic exchange by Southern blotting to confirm deletion of *eccD1*.

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

Antibodies and reagents. Antibodies to mammalian markers (CD63, Rab7, and V-ATPase B1/B2) and fluorophore-conjugated secondary antibodies were acquired from Santa Cruz Biotechnology. The α -SodA construct (pMV3 α -sod) was a kind gift from William Jacobs, Jr. (Albert Einstein College of Medicine, Bronx, NY) (25). Bafilomycin A1 (Sigma) and concanamycin A (Santa Cruz) were stocked at 1,000 $\times$ in dimethyl sulfoxide (DMSO).

Animals. C57BL/6 mice acquired from Charles River Laboratories were used in aerosol infections and for bone marrow-derived macrophages (BMDM). MyD88^{-/-} mice on the C57BL/6 background (1) were acquired from Shizuo Akira (WPI Immunology Frontier Research Center, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). All mice were housed in sterile cages and provided sterile food and water. All animal protocols were followed strictly as approved by the UNC Institutional Animal Care and Use Committee (IACUC).

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

Macrophage infections. Bone marrow-derived macrophages were made as follows. Mice were euthanized by CO₂ asphyxiation and cervical dislocation. The femurs were removed, and bone marrow was flushed out with supplemented Dulbecco modified Eagle medium (DMEM) (Sigma). The DMEM was supplemented with 10% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, and 1 $\times$ nonessential amino acids (complete DMEM). The bone marrow cells were washed once, resuspended, and plated in complete DMEM containing 20% L-929 cell-conditioned medium (LCM). After 6 days at 37°C, 5% CO₂, the cells were lifted off the plates using cold PBS-5 mM EDTA and scraping. The cells were then washed twice and resuspended at a concentration of 1×10^6 macrophages/ml in complete DMEM containing 10% LCM. The macrophages were then seeded at 2×10^5 /well in eight-well chambered slides or chambered coverslips for microscopy experiments.

After resting 24 to 48 h, the macrophages were infected with *M. tuberculosis* culture grown to log phase, washed once with PBS containing 0.05% Tween 80, and diluted in warm complete DMEM. The macrophages were infected at a multiplicity of infection (MOI) of 1.0 for microscopy or 0.2 for intracellular-growth assays. After a 4-h incubation at 37°C for bacterial uptake, the infected macrophages were washed three times with prewarmed complete DMEM. The zero-hour time point of these experiments represents the time after the washes were complete. For kinetic growth assays, the macrophages were lysed at various time points, and the lysates were plated for CFU. For microscopy, coverslips were taken at various time points and fixed for at least 1 h in 4% paraformaldehyde (PFA) in PBS, pH 7.4. For experiments using bafilomycin A1 (Sigma) or concanamycin A (Santa Cruz), the inhibitors or equivalent

vehicle control was added to the macrophages 30 min prior to infection. The inhibitors were maintained throughout the 5-day infection.

Macrophage staining and microscopy. To stain with LysoTracker, medium was replaced with prewarmed DMEM plus 100 nM LysoTracker Red DND99 (Invitrogen) and returned to 37°C, 5% CO₂ for 1 h. For immunofluorescence, the medium was aspirated from the wells at the endpoint of infection, and the coverslips were submerged in 4% PFA for at least 1 h. To stain for immunofluorescence, fixed macrophages in chambered coverslips were submerged in PBS to remove residual PFA. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature, washed in PBS, and blocked in PBS plus 10% serum from the same source as the secondary antibody. Primary antibodies were used at a 1:100 dilution in PBS plus 3% serum and incubated overnight at 4°C. After extensive washing in PBS, secondary antibodies conjugated to fluorophores were used at 1:100 in PBS plus 3% serum and incubated at room temperature for 1 h. After secondary antibodies were washed away, Fluoromount-G (Southern Biotech) was added to each well. As an important control, we showed that normal rabbit IgG and secondary antibody alone did not stain *M. tuberculosis*-containing phagosomes.

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

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

RESULTS

Increased apoptosis does not account for the intracellular-growth defect of the *M. tuberculosis* $\Delta secA2$ mutant. The ability of *M. tuberculosis* to inhibit host cell apoptosis could be important for intracellular growth of the bacillus (41, 44). The $\Delta secA2$ mutant has a proapoptotic phenotype, which has been attributed to defective SodA secretion (25). Given this phenotype of the $\Delta secA2$ mutant, we set out to test if increased apoptosis accounts for the growth defect of the mutant in macrophages. To test this possibility, we took advantage of a plasmid expressing an extra copy of SodA, termed α -SodA. Unlike the endogenous *M. tuberculosis* SodA, which lacks an obvious signal sequence for export, α -SodA has a Sec signal sequence fused to the N terminus of the enzyme (25). When α -SodA is expressed by the $\Delta secA2$ mutant ($\Delta secA2$ - α -sodA) it restores the ability of the $\Delta secA2$ mutant to release SodA activity into the culture medium and it reverses the proapoptotic phenotype of the mutant (25). It is worthwhile to mention that the details of α -SodA release, particularly whether it is secreted by the Sec pathway, remain to be worked out. Nonetheless, because α -SodA suppresses the propapoptotic phenotype of the $\Delta secA2$ mutant, we could test the $\Delta secA2$ - α -sodA strain for growth in murine bone marrow-derived macrophages as a way to determine if altered apoptosis is responsible for the intracellular growth phenotype of the $\Delta secA2$ mutant. In these experiments, both the $\Delta secA2$ mutant and the $\Delta secA2$ - α -sodA strain failed to grow in macrophages while the parental H37Rv strain and the complemented $\Delta secA2$ mutant strain grew approximately 10-fold over 5 days (Fig. 1A). These results demonstrated that the $\Delta secA2$ mutant remains defective for intracellular growth even if the enhanced-apoptosis phenotype is suppressed.

We similarly tested the role of apoptosis in the attenuated phe-

FIG 1 Reversal of the $\Delta secA2$ mutant apoptosis phenotype does not rescue growth in macrophages or mice. (A) Nonactivated BMDM were infected with the H37Rv, $\Delta secA2$, $\Delta secA2$ plus *psecA2* (complemented strain), or $\Delta secA2$ plus α -sodA strain of *M. tuberculosis*, and intracellular replication was monitored as described in the text. The data shown are plotted on a linear scale and are representative of two experiments; the points represent means of triplicate wells, and the error bars represent standard deviations (SD). *, $P < 0.05$ by Student's *t* test. (B) Lung and spleen burdens in mice infected through the aerosol route at an initial dose of ~ 200 CFU/lung with H37Rv or the $\Delta secA2$ or $\Delta secA2$ -plus- α -sodA strain. We determined CFU counts by plating lung or spleen homogenates at various time points for viable bacteria. The data shown are from a single experiment; the bars represent the mean organ burdens from four mice, and the error bars represent SD. *, $P < 0.05$ by Student's *t* test. d, day(s); p.i., postinfection.

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

The $\Delta secA2$ mutant resides in acidified phagosomes. The ability of *M. tuberculosis* to interfere with phagosome maturation is another property of the bacillus proposed to be important for intracellular growth (47, 52, 66). Because secreted and surface-localized proteins of *M. tuberculosis* are good candidates for being involved in phagosome maturation arrest, we tested the potential for the SecA2 export system to influence phagosome maturation. Murine bone marrow-derived macrophages were infected with H37Rv, the $\Delta secA2$ mutant, or a complemented strain, and we scored the bacilli for colocalization with markers of phagosome maturation using wide-field fluorescence microscopy.

To detect bacilli in macrophages, we took advantage of the recently described autofluorescence of mycobacteria (45). Using scanning spectrophotometry, we first experimentally deter-

FIG 2 *M. tuberculosis* autofluorescence can be used to identify bacilli by microscopy. The *M. tuberculosis* strains used in this study carrying a GFP expression plasmid were grown to mid-log phase and fixed in 4% paraformaldehyde in PBS. The fixed bacteria were loaded into the well of a chambered coverslip and visualized in the CFP and GFP channels on a wide-field fluorescence microscope. Autofluorescence in the CFP channel is compared to GFP fluorescence in strains expressing GFP from a plasmid. The overlap is demonstrated in the merged images, where yellow indicates a positive correlation.

mined the optimal peak excitation and emission wavelengths (415 nm and 470 nm, respectively) for autofluorescence of paraformaldehyde-fixed *M. tuberculosis*. These wavelengths can be detected by fluorescence microscopy using a standard CFP filter set. To validate the use of autofluorescence to localize *M. tuberculosis*, we evaluated a series of green fluorescent protein (GFP)-expressing wild-type (WT) and mutant *M. tuberculosis* strains and compared GFP and autofluorescence signals. GFP expression is commonly used to visualize *M. tuberculosis* in macrophages (65, 70). All GFP-positive cells were visible by autofluorescence (Fig. 2); this was also true with bacilli in macrophages (data not shown).

It is well established that in nonactivated macrophages, wild-type *M. tuberculosis* is primarily found in nonacidified phagosomes (46, 52, 54, 66). To assess the acidification of phagosomes containing the Δ secA2 mutant, we used LysoTracker Red (Invitrogen), which is an acidotropic dye frequently employed in studies of phagosome maturation (66, 70). By measuring the colocalization of LysoTracker and *M. tuberculosis* autofluorescence signals, the parental H37Rv strain was found to largely avoid phagosome acidification, as reported previously (46, 54). In comparison, we detected the Δ secA2 mutant in a significantly higher percentage of acidified phagosomes, and this phenotype was reversed in the complemented strain (Fig. 3A). Experiments with GFP-expressing versions of these strains, where GFP was used to localize the bacilli, gave the same results as obtained by scoring autofluorescence (data not shown). The Δ secA2 mutant's associ-

FIG 3 Compared to H37Rv, the Δ secA2 mutant is enriched in LysoTracker-positive phagosomes. Nonactivated BMDM were infected with H37Rv, the Δ secA2, mutant or the complemented strain (A); H37Rv, Δ secA2, or Δ eccD1 (B); or BCG Pasteur or a Δ secA2 mutant on the BCG Pasteur background (C). At the indicated times, the slides were stained with LysoTracker and scored for LysoTracker-positive phagosomes, as described in the text. Also shown in panel A are representative images from at least three independent experiments (LT, LysoTracker). The bars represent mean percentages of bacterium-containing phagosomes that stain positive for LysoTracker; The error bars represent SD of three replicate wells, with each well having >100 phagosomes scored. *, $P \leq 0.05$ by Student's *t* test compared to the WT.

ation with acidified phagosomes was evident as early as 1 h postinfection (Fig. 3A).

We compared the phagosome acidification phenotype of the Δ secA2 mutant to that of a mutant defective for ESX-1 secretion (Δ eccD1). As reported previously for *esx-1* mutants, the Δ eccD1 mutant exhibited greater association with LysoTracker-positive phagosomes than H37Rv (Fig. 3B) (8). The *esx-1* mutant phenotype was repeatedly less dramatic than the Δ secA2 mutant phenotype. This finding that both a Δ secA2 mutant and *esx-1* mutants are associated with increased phagosome acidification joins a list of other similarities reported for these mutants (20, 24, 28, 33, 37, 55). This raised the possibility that the SecA2 and ESX-1 systems might work together to export critical effector proteins of phagosome maturation. To test this possibility, we assayed the phagosome acidification of an *M. bovis* BCG Δ secA2 mutant. BCG lacks ESX-1 because the chromosomal locus encoding the system is deleted (49). As was the case in *M. tuberculosis*, a BCG Δ secA2 mutant exhibited greater association with LysoTracker-positive phagosomes than the parental BCG Pasteur strain (Fig. 3C). This result indicated that the role(s) of the SecA2 system in arresting phagosome maturation is independent of the ESX-1 system.

FIG 4 Not all *M. tuberculosis* mutants with intracellular growth defects are enriched in LysoTracker-positive phagosomes. Nonactivated BMDM were infected with the H37Rv, $\Delta secA2$, or $\Delta leuD$ (A) or H37Rv, $\Delta secA2$, *mce1A::tn*, *mce2F::tn*, or *rv0199::tn* (B) strain. At the indicated times, the slides were stained with LysoTracker and scored for LysoTracker-positive phagosomes as described in the text. Shown are representative data from at least three independent experiments. The bars represent mean percentages of bacterium-containing phagosomes that stain positive for LysoTracker; the error bars represent SD of three replicate wells. *, $P \leq 0.05$ by Student's *t* test compared to H37Rv.

The localization of the $\Delta secA2$ mutant to acidified phagosomes is not a general property of *M. tuberculosis* mutants with intracellular-growth defects. Increased association of the $\Delta secA2$ mutant with acidified phagosomes could result from an inability to carry out a role in blocking phagosome acidification, such as failure to secrete an inhibitor of phagosome maturation. Alternatively, the $\Delta secA2$ mutant could be delivered to acidified phagosomes as a secondary consequence of failure to grow in macrophages. In considering the second possibility, we asked whether unrelated mutants that fail to replicate in macrophages are also found in acidified phagosomes. For this reason, we investigated the acidification status of phagosomes containing the *M. tuberculosis* leucine auxotroph ($\Delta leuD$). The $\Delta leuD$ mutant is a metabolic mutant that fails to synthesize leucine and fails to grow in macrophages (26). Unlike the $\Delta secA2$ mutant, the $\Delta leuD$ mutant resembled H37Rv in its association with nonacidified phagosomes, even 72 h postinfection, indicating that the mutant maintained the ability to block phagosome acidification (Fig. 4A). We additionally screened for phagosome acidification defects of *M. tuberculosis* transposon mutants recently identified as being defective for intracellular growth (40). Transposon mutants in *rv0199*, *mce1A*, or *mce2F* also resembled H37Rv in the ability to block phagosome acidification (Fig. 4B). These data indicated that localization to acidified phagosomes, as assessed by LysoTracker staining, is not

the case for all *M. tuberculosis* mutants that are defective for intracellular growth. Along with the finding that the $\Delta secA2$ mutant is observed in acidified phagosomes quickly (1 h) postinfection (Fig. 3, 4A), these results argue for a specific role for the SecA2 system in blocking phagosome acidification.

Other markers of maturation are associated with phagosomes containing the $\Delta secA2$ mutant. V-ATPase is a molecular motor that drives a proton gradient across membranes. V-ATPases are used by eukaryotic cells to acidify vacuoles. It has been reported that *M. tuberculosis* phagosomes do not acidify, at least in part, because V-ATPase is either prevented from associating with or quickly degraded from the phagosome (54, 57). We wanted to determine if the greater association of the $\Delta secA2$ mutant with acidified phagosomes correlates with greater V-ATPase association. Macrophages infected with the $\Delta secA2$ mutant, H37Rv, or the complemented strain were immunostained with antibodies to murine V-ATPase, and colocalization was scored. We found that a significantly higher percentage of phagosomes containing the $\Delta secA2$ mutant stained positive for V-ATPase than phagosomes containing H37Rv or the complemented strain (Fig. 5A). The increased association with V-ATPase-positive phagosomes seen with the $\Delta secA2$ mutant was equivalent to that seen with the $\Delta eccD1$ mutant.

Phagosome acidification is a relatively early step in phagosome maturation (17). To further characterize the $\Delta secA2$ mutant-containing phagosome for evidence of phagosome/lysosome fusion, we immunostained infected macrophages for markers of late-endosomal/lysosomal fusion (CD63 and Rab7). Wild-type *M. tuberculosis* is reported to prevent phagosomes from maturing to a CD63- and Rab7-positive state (12, 31, 65). In comparison to phagosomes containing H37Rv or the complemented strain, a higher percentage of $\Delta secA2$ mutant-containing phagosomes stained positive for CD63 and Rab7. Once again, the phenotype of the $\Delta secA2$ mutant was similar to that of the $\Delta eccD1$ mutant (Fig. 5B and C). Together, these results indicated that phagosomes containing the $\Delta secA2$ mutant have a greater association with V-ATPase, which could account for the observed higher percent acidification detected with LysoTracker. The $\Delta secA2$ mutant-containing phagosomes also have a greater association with markers indicative of late endosomal/lysosomal fusion, indicating a defect in later stages of phagosome maturation arrest, as well (Fig. 5B and C).

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

FIG 5 Compared to H37Rv, the $\Delta secA2$ mutant is enriched in phagosomes positive for late endocytic/lysosomal markers. Nonactivated BMDM were infected with H37Rv or the $\Delta secA2$, complemented $\Delta secA2$, or $\Delta eccD1$ mutant for 24 h and immunofluorescently stained for markers of phagosome maturation as described in the text. (A) V-ATPase. (B) CD63. (C) Rab7. A representative of three independent experiments is shown. The bars represent mean percentages of bacterium-containing phagosomes that stain positive for marker; the error bars represent SD of three replicate wells, with each well having >100 phagosomes scored. *, $P \leq 0.05$ by Student's t test compared to H37Rv. Representative microscopy images for each set of markers are shown.

infected MyD88^{-/-} macrophages were the same and equivalent to the levels produced by uninfected macrophages (data not shown).

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

Phagosome acidification is necessary for the intracellular-growth phenotype of the $\Delta secA2$ mutant. There are other *M.*

tuberculosis mutants reported to have defects in blocking phagosome maturation (8, 31, 36, 46, 56, 67). In the majority of cases, phagosome maturation arrest mutants are also defective for intracellular growth (for example, *esx-1* mutants). However, with the exception of a few studies (23, 30, 68), the causal relationship between localization to a more mature phagosome and inhibition of growth remains largely untested. With the goal of determining if the phagosome maturation arrest defect of the $\Delta secA2$ mutant is responsible for the growth defect in macrophages, we used macrolide antibiotic V-ATPase inhibitors (bafilomycin A1 and concanamycin A) (15) to block acidification of $\Delta secA2$ mutant-containing phagosomes and asked if this treatment rescued growth of the mutant. To minimize pleiotropic effects of the inhibitors on macrophages, we experimentally determined the minimum concentration of each inhibitor (10 nM bafilomycin A1 and 5 nM concanamycin A) required to prevent acidification of the $\Delta secA2$ mutant-containing phagosomes, as measured by LysoTracker colocalization at 24 h postinfection (Fig. 7A). These concentrations had no detectable effect on macrophage viability over

FIG 6 MyD88 has no effect on $\Delta secA2$ mutant phagosome trafficking or intracellular growth. (A) Nonactivated BMDM derived from C57BL/6 or MyD88^{-/-} mice were infected with H37Rv or the $\Delta secA2$ mutant. At 24 h postinfection, the slides were stained with LysoTracker or antibodies to CD63, Rab7, or V-ATPase and scored for marker-positive bacterium-containing phagosomes as described in the text. The bars represent mean percentages of bacterium-containing phagosomes that stain positive for marker; the error bars represent SD of three replicate wells. n.s., not significant. There are no significant differences between C57BL/6 and MyD88^{-/-} macrophages infected with the $\Delta secA2$ mutant. (B) Nonactivated BMDM from C57BL/6 (open symbols) or MyD88^{-/-} (closed symbols) mice were infected with H37Rv or the $\Delta secA2$ mutant, and intracellular replication was monitored as described. The data shown are plotted on a linear scale and are from a representative experiment out of three; the points are means of three replicate wells, and the error bars represent SD. *, $P \leq 0.05$ by student's t test compared to H37Rv.

the course of a 5-day infection (data not shown). In contrast to untreated macrophages, the $\Delta secA2$ mutant and H37Rv grew equally well in macrophages treated with 10 nM bafilomycin A1 (Fig. 7B). While bafilomycin treatment increased growth of both H37Rv and the $\Delta secA2$ mutant in macrophages over 5 days (Fig. 7B), the effect of bafilomycin was small and not significant for H37Rv and significantly greater for the $\Delta secA2$ mutant (Fig. 7C). To establish the specificity of this rescue, we also tested the effect of bafilomycin on intracellular growth of the $\Delta leuD$ mutant, which is not localized to acidified phagosomes (Fig. 4A). Bafilomycin A1 treatment of the $\Delta leuD$ mutant did not rescue growth to the level of H37Rv in bafilomycin-treated macrophages (Fig. 7B and C). Experiments with 5 nM concanamycin A showed the same effect of rescuing the intracellular-growth defect of the $\Delta secA2$ mutant. The growth of the $\Delta secA2$ mutant was equivalent to that of H37Rv in concanamycin A-treated macrophages (Fig. 7D and E). Together, these results indicated the importance of phagosome acidification for inhibiting intracellular growth of the $\Delta secA2$ mutant. Our data further argue that phagosome maturation can result in inhibition of *M. tuberculosis* intracellular replication.

DISCUSSION

The function of the accessory SecA2 export system in promoting growth in macrophages has remained elusive. Previously, we tested if the role of the SecA2 system is to protect against the oxidative or nitrosative stresses produced by macrophages during infection (33). These past studies showed that even in the absence of these reactive radical stresses, the $\Delta secA2$ mutant remains at-

tenuated in macrophages. Thus, the SecA2 system must have other roles in promoting *M. tuberculosis* growth in macrophages. In this study, we considered alternate explanations. We tested if the inhibition of macrophage apoptosis mediated by the SecA2 system is what promotes *M. tuberculosis* growth in macrophages. Our results from testing the $\Delta secA2$ - α -sodA strain do not support this possibility. While expression of the α -sodA construct in the $\Delta secA2$ mutant suppresses the proapoptotic phenotype, the strain remained defective for intracellular growth (Fig. 1A). It is worthwhile to note that our results do not rule out other important roles of the apoptosis phenotype of the *M. tuberculosis* $\Delta secA2$ mutant, as reported elsewhere, such as enabling enhanced adaptive immune responses and increased protective immunity in vaccination studies (25).

In considering other explanations for the function of the SecA2 system in macrophages, we tested the $\Delta secA2$ mutant for the ability to arrest phagosome maturation. Our results showed the $\Delta secA2$ mutant to be defective in phagosome maturation arrest, as the mutant was more readily trafficked to a more mature phagosome than the parental H37Rv strain. This defect was evident soon after infection (1 h) and was not exhibited by other mutants with intracellular-growth defects even at later times postinfection (72 h). Additionally, we showed that treating macrophages with inhibitors of the V-ATPase prevented acidification of phagosomes and rescued the intracellular-growth defect of the $\Delta secA2$ mutant. From these results, we conclude that the accessory SecA2 export system has a specific role in blocking phagosome acidification and

FIG 7 Growth of the $\Delta secA2$ mutant is inhibited by phagosome acidification. (A) LysoTracker-positive phagosomes in BMDM infected with H37Rv or the $\Delta secA2$ mutant and treated with bafilomycin A1 or concanamycin A. Shown are the lowest inhibitor concentrations that bring $\Delta secA2$ LysoTracker-positive phagosomes to H37Rv levels. ND, not determined. (B) Nonactivated BMDM treated with bafilomycin A1 or vehicle control (DMSO) were infected with H37Rv or the $\Delta secA2$ or $\Delta leuD$ mutant, and intracellular replication was monitored as described. Shown is a representative of four independent experiments. The bars represent the mean fold growth over 5 days of three replicate wells plus SD. *, $P < 0.05$ by Student's t test. (C) Data from the experiment described in panel B combined with three independent experiments to show the fold effect of bafilomycin A1 on intracellular growth compared to untreated macrophages. The bars represent the mean fold effect of bafilomycin A1 in four individual experiments plus the standard errors of the mean (SEM). *, $P < 0.05$ by Student's t test compared to H37Rv. (D and E) As in panels B and C, with concanamycin A or DMSO. (D) Shown is a representative of three experiments. (E) The bars represent the mean fold effect of concanamycin in three experiments plus SEM. *, $P < 0.05$ by Student's t test compared to H37Rv.

that the intracellular-growth defect of the $\Delta secA2$ mutant is directly related to its defect in blocking phagosome maturation.

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

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

Because metabolically inactive bacteria are more readily found in acidified phagosomes (35), it has been suggested that mutants impaired in any aspect of growth or metabolic activity may be found in mature phagosomes as a secondary consequence of those defects, as opposed to a direct effect on phagosome maturation (47). Because we observed the $\Delta secA2$ mutant in more mature phagosomes at early times postinfection and because V-ATPase inhibitors rescued the growth defect, we think it unlikely that general growth or metabolic defects account for the $\Delta secA2$ mutant phenotype. Moreover, we tested the leucine auxotroph ($\Delta leuD$) of *M. tuberculosis* as an example of a mutant severely compromised in metabolic activity. The $\Delta leuD$ mutant requires leucine supple-

mentation to grow in liquid medium, and the mutant does not grow in macrophages, presumably due to a failure to acquire leucine from the intracellular environment (26). Somewhat surprisingly, the ΔleuD mutant behaved like H37Rv and maintained its ability to avoid phagosome acidification, even at the latest (72-h) time point. Although the precise level of metabolic activity of the ΔleuD mutant in macrophages is not known, this result suggests that general growth or metabolic defects do not necessarily lead to a breakdown in phagosome maturation arrest.

What is currently known about the process of phagosome maturation arrest by *M. tuberculosis* suggests it is a complex process involving several effectors. It seems likely that *M. tuberculosis* has multiple effectors targeting a minimum of two parallel pathways that are critical to phagosome maturation: PI(3)P accumulation/signaling and Rab7 accumulation/activation (10, 47, 62). The phagosome maturation defect of the ΔsecA2 mutant may be due to a defect in the secretion of such effectors. Several *M. tuberculosis*-secreted proteins (i.e., LpdC, NdkA, PknG, PtpA, and SapM) affect phagosome maturation or pathways thought to be critical for phagosome maturation (3, 14, 59, 63, 67). This list of possible effectors is likely incomplete, however, as mutant screens to find effectors of phagosome maturation suggest the existence of a much broader set of proteins involved in arresting phagosome maturation (8, 36, 46, 56). There are also *M. tuberculosis* surface lipids (lipoarabinomannan, phosphatidylinositol mannoside, and tetra-acylated sulfoglycolipid) (8, 21, 64) reported to affect phagosome maturation. The SecA2 system could be involved in surface lipid production or localization by exporting lipid synthesis or export machinery.

An alternate way that the SecA2 system could promote phagosome maturation arrest would be to limit macrophage responses that drive downstream phagosome maturation events. With this idea in mind, we considered the possibility that the more robust MyD88-dependent responses elicited by the ΔsecA2 mutant (33) might be responsible for the altered trafficking of the ΔsecA2 mutant. Although our experiments with MyD88^{-/-} macrophages revealed this not to be the case, it only excludes altered phagosome trafficking as a downstream event of MyD88 signaling. Macrophages can detect *M. tuberculosis* through MyD88-independent intracellular receptors, such as the NLR family of receptors and the inflammasome, which could also drive phagosome maturation (39).

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

The work presented here not only provides a better understanding of how SecA2 promotes *M. tuberculosis* growth in macrophages, it also demonstrates the importance of phagosome maturation arrest for *M. tuberculosis*. It seems likely that *M. tuberculosis* has multiple effectors of phagosome maturation ar-

rest. As is the case in the type IV secretion system of *Legionella pneumophila*, this may mean there is substantial effector redundancy (43). The phagosome maturation phenotype of the ΔsecA2 mutant indicates there is a single critical effector or multiple effectors that depend on the SecA2 export pathway. Future studies will seek to identify such SecA2-dependent effectors of phagosome maturation arrest.

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