The *Mycobacterium tuberculosis* Phosphate-Sensing Pst/SenX3-RegX3 System Regulates ESX-5 Secretion to Evade Host Immunity

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ABSTRACT

The *Mycobacterium tuberculosis* Type VII secretion system ESX-5, which has been implicated in virulence, is activated at the transcriptional level by the phosphate starvation responsive Pst/SenX3-RegX3 signal transduction system. Deletion of *pstA1*, which encodes a Pst phosphate transporter component, causes constitutive activation of the response regulator RegX3, hyper-secretion of ESX-5 substrates and attenuation in the mouse infection model. We hypothesized that constitutive activation of ESX-5 secretion causes attenuation of the $\Delta pstA1$ mutant. To test this, we uncoupled ESX-5 from regulation by RegX3. Using electrophoretic mobility shift assays, we defined a RegX3 binding site in the *esx-5* locus. Deletion or mutation of the RegX3 binding site reversed hyper-secretion of the ESX-5 substrate EsxN by the $\Delta pstA1$ mutant and abrogated induction of EsxN secretion in response to phosphate limitation by wild-type *M. tuberculosis*. Deletion of the *esx-5* RegX3 binding site (ΔBS) suppressed attenuation of the $\Delta pstA1$ mutant in *Irgm1*^{-/-} mice, suggesting that constitutive ESX-5 secretion limits *M. tuberculosis* evasion of host immune responses that are independent of *Irgm1*. However, the $\Delta pstA1\Delta BS$ mutant remained attenuated in both *NOS2*^{-/-} and C57BL/6 mice, suggesting that factors other than ESX-5 secretion also contribute to attenuation of the $\Delta pstA1$ mutant. In addition, a $\Delta pstA1\Delta esxN$ mutant lacking the hyper-secreted ESX-5 substrate EsxN remained attenuated in *Irgm1*^{-/-} mice, suggesting that ESX-5 substrates other than EsxN cause increased susceptibility to host immunity. Our data indicate that while *M. tuberculosis* requires ESX-5 for virulence, it tightly controls secretion of ESX-5 substrates to avoid elimination by host immune responses.

INTRODUCTION

Pathogenic bacteria often regulate the activity of specialized protein secretion systems that are required for virulence to ensure release of secreted effectors only at the appropriate stage of infection. Tight control of secretion system activity may limit recognition by the host immune system or prevent expression of complex secretion machines that restrict growth (1, 2).

Mycobacterium tuberculosis, the causative agent of tuberculosis, encodes five Type VII or ESX specialized protein secretion systems, of which ESX-1, ESX-3 and ESX-5 have been shown to promote pathogenesis (3). *M. tuberculosis* regulates activity of each of these secretion systems in response to signals encountered in the host. Iron limitation activates ESX-3 (4), which plays a role in both iron scavenging and inhibiting phagosome maturation (5, 6). ESX-1 permeabilizes the phagosomal membrane to allow bacterial access to the host cell cytoplasm (7-9). ESX-1 secretion is regulated by two signal transduction systems, PhoPR and MprAB, that respond to acidic pH and cell wall stress, respectively, signals that *M. tuberculosis* encounters in the phagosome (10-13). We recently demonstrated that *M. tuberculosis* activates ESX-5 secretion in response to inorganic phosphate (P_i) limitation (14). RegX3, a response regulator activated during P_i limitation, directly activates transcription of a subset of *esx-5* genes leading to increased production of ESX-5 secretion system core components and enhanced secretion of the EsxN and PPE41 substrates (14).

Though the precise function of ESX-5 remains unclear, it appears to influence nutrient acquisition to enable *M. tuberculosis* replication (15-17) and to promote host cell necrosis by activating the inflammasome and stimulating IL-1 β secretion (18, 19). In the related pathogen *Mycobacterium marinum*, ESX-5 secretes most proteins that belong to the mycobacteria-specific PE and PPE protein families (16, 20). The *M. tuberculosis* PE and PPE proteins are strongly immunogenic in mice; immune responses to PE and PPE antigens depend on a functional ESX-5 secretion system, suggesting that *M. tuberculosis* also secretes many PE and PPE proteins via ESX-5 (21). ESX-5 is also likely to be active during infection since T cells specific for the ESX-5 substrate EsxN have been detected in humans with latent tuberculosis (22, 23).

Activation of the RegX3 response regulator and induction of ESX-5 secretion is inhibited during growth in P_i -replete conditions by the Pst P_i uptake system (24). Deletion of *pstA1*, which encodes a Pst system trans-membrane component, causes constitutive activation of RegX3,

constitutive expression of *esx-5* genes, and hyper-secretion of ESX-5 substrates, independent of P_i availability (14). We previously demonstrated that a $\Delta pstA1$ mutant is attenuated during the chronic phase of infection in wild-type C57BL/6 mice and exhibits strongly reduced replication and virulence in two immune-deficient strains of mice, $NOS2^{-/-}$ and $Irgm1^{-/-}$, that fail to control infection with wild-type *M. tuberculosis* (24). $NOS2^{-/-}$ mice lack the interferon-gamma (IFN- γ) inducible nitric oxide synthase that generates toxic reactive nitrogen species (25). Although $NOS2^{-/-}$ mice are assumed to have a cell-intrinsic defect in their ability to control *M. tuberculosis* replication (26), they also fail to inhibit neutrophil recruitment to the lung, which creates a nutrient-rich environment that enhances *M. tuberculosis* replication (27, 28). *Irgm1* encodes an IFN- γ inducible GTPase that was originally described to restrict *M. tuberculosis* replication in a cell-intrinsic manner by mediating phagosome acidification, possibly via induction of autophagy (29, 30). However, *Irgm1* is also required for hematopoietic stem cell renewal (31); $Irgm1^{-/-}$ mice become leukopenic upon infection with intracellular pathogens, including mycobacteria (32), which also likely contributes to their profound susceptibility to infection. We previously demonstrated that attenuation of the $\Delta pstA1$ mutant in $NOS2^{-/-}$ mice was due to the constitutive activation of *RegX3*; a $\Delta pstA1\Delta regX3$ double mutant progressively replicated in the lungs and caused death of the animals (24). It remains unclear whether constitutive activation of *RegX3* similarly contributes to attenuation of the $\Delta pstA1$ mutant in either $Irgm1^{-/-}$ or C57BL/6 mice, because a $\Delta regX3$ single mutant was also attenuated in these mouse strains (24).

We hypothesized that constitutive activation of *esx-5* transcription and hyper-secretion of ESX-5 substrates driven by constitutively activated *RegX3* causes virulence attenuation of the $\Delta pstA1$ mutant. *M. tuberculosis* requires ESX-5 for replication *in vitro* (15, 33), so we were unable to construct mutants lacking ESX-5 function to test this possibility. Instead, we took a targeted approach to uncouple ESX-5 from regulation by *RegX3*. We defined the *RegX3* binding site in the *esx-5* locus and generated targeted mutations that disrupt *RegX3* binding. Mutation of the *RegX3* binding site prevented induction of *esx-5* gene expression and ESX-5 secretion

during P_i limitation by wild-type *M. tuberculosis*, and reversed the over-expression of *esx-5* genes and hyper-secretion the ESX-5 substrate EsxN by the Δ *pstA1* mutant. Deletion of the *esx-5* RegX3 binding site also suppressed attenuation of the Δ *pstA1* mutant specifically in *Irgm1*^{-/-} mice. Our results suggest hyper-secretion of ESX-5 substrates sensitizes *M. tuberculosis* to a host immune response that is independent of *Irgm1* and that *M. tuberculosis* regulates ESX-5 secretion in response to P_i availability in the host to evade this host immune response.

RESULTS

Defining a RegX3 binding site in the *esx-5* locus. We previously demonstrated that RegX3 directly regulates ESX-5 activity at the transcriptional level via binding to a 125 bp sequence within the *ppe27-pe19* intergenic region in the *esx-5* locus (Fig. 1A) (14). RegX3 was not included in a prior study that mapped the binding sites of most *M. tuberculosis* transcription factors (34), so a RegX3 consensus binding sequence has yet to be described. To more precisely define the *esx-5* RegX3 binding site, we conducted competitive electrophoretic mobility shift assays (EMSAs) using purified recombinant His₆-RegX3. Our previous work demonstrated that RegX3 binds within the sequence -151 to -27 bp relative to the *pe19* start codon (Probe A, Fig. 1A and 1B) (14). Binding reactions including excess unlabeled competitors comprising the 5' (-151 to -91) or 3' (-90 to -28) halves of Probe A demonstrated that RegX3 binds to the 5' region; only addition of the 5' competitor resulted in reversal of the mobility shift (Fig. 1B). These data indicate that RegX3 binds within -151 to -91 bp relative to the *pe19* start codon.

To further define the *esx-5* RegX3 binding site, we performed additional competitive EMSAs using the 61 bp -151 to -91 segment as the labeled probe (Fig. 1A and 1C, 5' Probe) and a series of unlabeled competitors that truncate the 5' Probe sequence at either the 5' or 3' end, added in excess. A complete list of competitors tested and their ability to compete with the

5' Probe for RegX3 binding is provided in Table S1. Competitors that defined the 5' and 3' ends of the RegX3 binding site are shown (Fig. 1C). Excess unlabeled Competitor 1, which truncates the 5' Probe at the 5' end, reversed the mobility shift, indicating that RegX3 binds to this competitor (Fig. 1C and 1D). However, RegX3 did not bind Competitor 2, which truncates an additional three bp at the 5' end, since the mobility shift was unperturbed (Fig. 1C and 1D), indicating that one or more base pairs removed from Competitor 2 are essential for RegX3 binding. Therefore, the 5' end of the RegX3 binding site is located near position -128 relative to the *pe19* start codon. Similarly for the 3' end, excess Competitor 3 reversed the mobility shift, indicating RegX3 can bind to this sequence, but excess Competitor 4, which eliminates an additional three bp from the 3' end, failed to alter the mobility shift (Fig. 1C and 1D). These data demonstrate that the three bp removed from Competitor 4 relative to Competitor 3 are required for RegX3 binding, and thus define the 3' end of the RegX3 binding site at -102 relative to the *pe19* start codon. Collectively, our data indicate that RegX3 binds to a 27 bp sequence located at -128 to -102 relative to the *pe19* start codon in the *esx-5* locus.

Defining essential sequence elements for RegX3 binding *in vitro*. RegX3 is a member of the OmpR/PhoB family of winged helix-turn-helix response regulators that typically bind to direct repeat DNA sequences (35). We previously identified an imperfect direct repeat separated by a 5 bp spacer in the 5' Probe sequence (DR1 and DR2, Fig. 1C) (14). Further examination revealed a third imperfect direct repeat (DR3) 5' of the first two and separated from DR1 by a 6 bp spacer (Fig. 1C). All three direct repeats are contained within the -128 to -102 region relative to the *pe19* start codon. To determine if these sequence elements are required for RegX3 binding, EMSAs were performed using competitor DNA harboring mutations in the individual direct repeats or spacer elements. For each direct repeat element, all five bp of the direct repeat were altered by transversion (Fig. 1C). We altered the spacer sequence between DR1 and DR2 by either adding or removing three base pairs (Spc+3 and Spc-3, respectively, Fig. 1C). Each mutated unlabeled competitor was tested for the ability to compete with the 5' probe for binding

to RegX3 when added in excess. The mutated DR3 competitor reversed the mobility shift, indicating RegX3 can still bind this sequence (Fig. 1E). However, the mutated DR1 or DR2 competitors both failed to reverse the mobility shift, indicating that RegX3 cannot bind these mutated sequences (Fig. 1E). These data indicate that the DR1 and DR2 sequence elements are required for RegX3 binding *in vitro*. Altering the spacing between DR1 and DR2, either by adding or removing 3 bp, abrogates RegX3 binding, since the Spc+3 and Spc-3 competitors also failed to reverse the mobility shift (Fig. 1E). This indicates that RegX3 requires a 5 bp spacer between DR1 and DR2 for *in vitro* binding. The 27 bp RegX3 binding site sequence, including DR1 and DR2, located approximately 100 bp upstream of the *pe19* start codon is consistent with RegX3 functioning as a transcriptional activator of *esx-5* genes (14).

RegX3 binding site mutations in the $\Delta pstA1$ mutant reverse *esx-5* over-expression and hyper-secretion of EsxN. We previously demonstrated that *esx-5* transcripts are over-expressed during growth in P_i -replete conditions in the $\Delta pstA1$ mutant due to constitutive activation of RegX3 (14). To determine if *esx-5* over-expression also depends upon the *esx-5* RegX3 binding site that we defined, we introduced three distinct RegX3 binding site mutations at the intergenic region 5' of *pe19* on the chromosome of the *M. tuberculosis* $\Delta pstA1$ mutant. The DR2 direct repeat mutant ($\Delta pstA1_{DR2}$) harbors the transversion mutations in DR2 identical those tested for RegX3 binding *in vitro* (Fig. 1C). The spacer mutant ($\Delta pstA1_{Spc+3}$) harbors three additional bp between DR1 and DR2, identical to the Spc+3 mutation tested for RegX3 binding *in vitro* (Fig. 1C). Finally, the binding site deletion mutant ($\Delta pstA1\Delta BS$) harbors a deletion of the complete 27 bp RegX3 binding site located at -128 to -102 bp relative to the *pe19* start codon. We tested expression of *esx-5* genes in the $\Delta pstA1_{DR2}$, $\Delta pstA1_{Spc+3}$ and $\Delta pstA1\Delta BS$ binding site mutants grown in standard P_i -rich medium (Fig. 2A). The $\Delta pstA1$ mutant exhibited significant over-expression of the *pe19* and *espG5* transcripts ($P < 0.0001$) and more than 3-fold over-expression of *eccD5* as compared to the WT control (Fig. 2A). As previously reported, over-expression of these transcripts was dependent on RegX3 since expression of each gene was

restored to the WT level in the $\Delta pstA1\Delta regX3$ mutant (Fig. 2A) (14). In both the $\Delta pstA1_{DR2}$ and $\Delta pstA1\Delta BS$ mutants, transcription of *pe19*, *espG₅*, and *eccD₅* was similarly restored to levels that were nearly the same as and not significantly different from the WT control (Fig. 2A). Both the $\Delta pstA1_{DR2}$ and $\Delta pstA1\Delta BS$ mutants also exhibited statistically significant reductions in *pe19* and *espG₅* transcription relative to the $\Delta pstA1$ parental control (Fig. 2A). The *pe19*, *espG₅*, and *eccD₅* transcripts were detected at intermediate levels in the $\Delta pstA1_{Spc+3}$ mutant that were not significantly reduced as compared to the $\Delta pstA1$ parental strain (Fig. 2A). These data demonstrate that the RegX3 binding site within the *esx-5* locus, and the DR2 sequence in particular, is required for RegX3-mediated over-expression of *esx-5* genes in the $\Delta pstA1$ mutant.

RegX3 is a global response regulator that activates and represses many genes outside of the *esx-5* locus (24). To determine if the RegX3 binding site mutations that we introduced perturbed regulation exclusively at the *esx-5* locus, we examined transcription of other genes that are over-expressed by the $\Delta pstA1$ mutant in a RegX3-dependent manner, but that are not associated with *esx-5* (24). The *udgA* and *mgtA* transcripts were over-expressed by the $\Delta pstA1$ mutant relative to both the WT and $\Delta pstA1\Delta regX3$ strains (Fig. S1). Both *udgA* and *mgtA* transcripts remained significantly over-expressed in the $\Delta pstA1_{DR2}$, $\Delta pstA1_{Spc+3}$ and $\Delta pstA1\Delta BS$ mutants (Fig. S1). These data demonstrate that mutation of the RegX3 binding site sequence within the *esx-5* locus does not globally alter RegX3 activity.

To determine if the decreased transcription of *esx-5* genes in the RegX3 binding site mutants translates to changes in stability or activity of the ESX-5 secretion system, we monitored production of ESX-5 conserved components and secretion of the ESX-5 substrates EsxN and PPE41 by the $\Delta pstA1$ RegX3 binding site mutants. We observed hyper-secretion of the ESX-5 substrates EsxN and PPE41 and over-production of the cytosolic ESX-5 chaperone EspG₅ and ESX-5 secretion machinery components EccB₅ and EccD₅ by the $\Delta pstA1$ mutant relative to the WT control (Fig. 2B). This response required RegX3 (Fig. 2B), consistent with our

prior report (14). We detected reduced amounts of the EspG₅, EccB₅ and EccD₅ proteins in all three $\Delta pstA1$ RegX3 binding site mutants as compared to the $\Delta pstA1$ mutant (Fig. 2B). EsxN hyper-secretion was reversed in both the $\Delta pstA1_{DR2}$ and $\Delta pstA1\Delta BS$ mutants, reaching levels that were undetectable, comparable to both the WT and $\Delta pstA1\Delta regX3$ mutant controls (Fig. 2B). We detected EsxN secretion by the $\Delta pstA1_{Spc+3}$ mutant but at a 6-fold reduced abundance as compared to the $\Delta pstA1$ mutant (Fig. 2B). Secretion of PPE41 was also decreased in each of the RegX3 binding site mutants relative to the $\Delta pstA1$ parental strain, but remained approximately 2-fold increased as compared to the WT control (Fig. 2B). It is possible either that RegX3 controls PPE41 secretion by a mechanism independent of its regulation of *esx-5* transcription, or that decreased secretion of EsxN frees the ESX-5 secretion apparatus to translocate other substrates including PPE41. The ModD control confirmed equivalent loading of the culture filtrate fraction; the GroEL2 control confirmed equivalent loading of the cell lysate fraction and demonstrated that cell lysis did not contaminate the culture filtrate (Fig. 2B). These results indicate that the RegX3 binding site in the *esx-5* locus is required for the over-production of ESX-5 secretion system core components and hyper-secretion of EsxN by the $\Delta pstA1$ mutant.

Mutation of the RegX3 binding site in the *esx-5* locus prevents ESX-5 induction during phosphate limitation. We previously demonstrated that P_i limitation triggers ESX-5 activity in WT *M. tuberculosis*, and that this response requires RegX3 (14). To determine if the RegX3 binding site is also required for induction of *esx-5* transcription in response to P_i limitation, we generated a strain lacking the RegX3 binding site in the WT Erdman strain background (ΔBS) and conducted qRT-PCR experiments to monitor *esx-5* gene expression. The WT, $\Delta regX3$ and ΔBS strains were grown in either P_i-free medium or P_i-replete medium as a control. In P_i-replete conditions, *esx-5* transcripts were expressed at a basal level in all of the strains (Fig. 3B). Statistically significant increases in *pe19* and *espG₅* transcription were detected for the ΔBS mutant, but the changes were less than 1.5-fold (Fig. 3B). The *pe19*, *espG₅* and *eccD₅*

transcripts were induced 7.9, 4.9, and 3.7-fold, respectively, by WT *M. tuberculosis* during growth in P_i -free medium relative to the P_i -replete control (Fig. 3A). The $\Delta regX3$ mutant failed to induce *pe19*, *espG₅* or *eccD₅* transcription in response to P_i limitation, consistent with our previous reports (Fig. 3A) (14, 36). The ΔBS mutant similarly failed to induce *pe19*, *espG₅* or *eccD₅* transcription in response to P_i limitation (Fig. 3A); the level of each transcript was significantly different from that of the WT control and not significantly different from that of the $\Delta regX3$ mutant (Fig. 3A). These data demonstrate that the RegX3 binding site in the *esx-5* locus is required for activation of *esx-5* transcription in response to P_i limitation.

We evaluated production of ESX-5 conserved components and secretion of the ESX-5 substrates EsxN and PPE41 during P_i limitation in the ΔBS mutant by Western blotting. Production of EspG₅, EccB₅ and EccD₅ was induced in WT *M. tuberculosis* during P_i limitation (Fig. 3C), as previously demonstrated (14). Increased production or stability of EspG₅, EccB₅ and EccD₅ during P_i limitation was abrogated in both the $\Delta regX3$ and ΔBS mutants (Fig. 3C). The GroEL2 control confirmed equivalent loading of cell lysate proteins (Fig. 3C). Secretion of EsxN and PPE41 was induced in the WT strain during P_i limitation, as previously reported (14) (Fig. 3C). Induction of EsxN secretion during P_i limitation was prevented by either the $\Delta regX3$ or the ΔBS mutation (Fig. 3C). The $\Delta regX3$ mutant also exhibited modestly reduced PPE41 secretion during P_i limitation, as previously reported (14) (Fig. 3C). In contrast, the ΔBS mutant induced PPE41 secretion during P_i limitation similarly to the WT control (Fig. 3C), consistent with our results demonstrating intermediate PPE41 secretion by the $\Delta pstA1\Delta BS$ mutant. The ModD control confirmed equivalent loading of the P_i -limited culture filtrate fraction (Fig. 3C); the decreased secretion of ModD during P_i limitation relative to the P_i -replete control (Fig. 3C) was consistent with our previous report (14). The GroEL2 control confirmed that cell lysis did not contaminate the culture filtrate (Fig. 3C). Collectively, these data suggest that the RegX3 binding site within the *esx-5* locus is necessary for induction of EsxN secretion during P_i limitation.

The *esx-5* RegX3 binding site deletion suppresses attenuation of the $\Delta pstA1$ mutant in *Irgm1*^{-/-} mice.

To determine if constitutive hyper-secretion of ESX-5 substrates contributes to attenuation of the $\Delta pstA1$ mutant we infected C57BL/6, NOS2^{-/-} and *Irgm1*^{-/-} mice via the aerosol route with ~100 CFU of WT, $\Delta pstA1$, or $\Delta pstA1\Delta BS$ *M. tuberculosis* strains. All *Irgm1*^{-/-} mice succumbed to infection with WT Erdman by 4 weeks post-infection, and bacterial loads reached over 10⁹ in the lungs (Fig. 4A). *Irgm1*^{-/-} mice controlled replication of the $\Delta pstA1$ mutant after 2 weeks post infection (Fig. 4A), consistent with previous results (24). In contrast, the $\Delta pstA1\Delta BS$ mutant replicated progressively in the lungs of *Irgm1*^{-/-} mice. At 4 weeks post-infection, mean bacterial CFU in the lungs of *Irgm1*^{-/-} mice infected with the $\Delta pstA1\Delta BS$ mutant were increased 40-fold as compared to mice infected with the $\Delta pstA1$ mutant, though this difference did not achieve statistical significance (Fig. 4A). However, by 6 weeks post-infection, the $\Delta pstA1\Delta BS$ mutant reached nearly the same final bacterial burden in the lungs of *Irgm1*^{-/-} mice as the WT control (Fig. 4A). At 6 weeks, the bacterial burden of the $\Delta pstA1\Delta BS$ mutant in the lungs was over 1000-fold higher than the $\Delta pstA1$ mutant, and this difference was statistically significant (Fig. 4A). In these experiments, several of the *Irgm1*^{-/-} mice infected with the $\Delta pstA1\Delta BS$ mutant were moribund at the 6 week time point, while our previous experiments demonstrated that *Irgm1*^{-/-} mice infected with the $\Delta pstA1$ mutant all survive for at least 14 weeks (24). These data suggest that attenuation of the $\Delta pstA1$ mutant in *Irgm1*^{-/-} mice is due, at least in part, to constitutive activity of the ESX-5 secretion system and increased secretion of one or more ESX-5 substrates. These data further suggest that hyper-secretion of ESX-5 substrates sensitizes *M. tuberculosis* to a host immune response that is independent of *Irgm1*.

To determine if the modest attenuation of the $\Delta pstA1\Delta BS$ mutant relative to the WT control might be due to the ΔBS mutation, we performed similar aerosol infection experiments with the ΔBS mutant. The ΔBS mutant exhibited a modest but statistically significant decrease in lung bacterial burden at the 4 week time point compared to the WT control, but all mice were

moribund by the 5 week time point and were euthanized (Fig. 4A). These data suggest that the partially attenuated phenotype of the $\Delta pstA1\Delta BS$ mutant in *Irgm1*^{-/-} mice may be due to an inability to induce ESX-5 secretion in response to P_i limitation.

In *NOS2*^{-/-} mice, in contrast, the ΔBS mutation had no statistically significant effect on the ability of either the $\Delta pstA1$ mutant or WT bacteria to replicate in the lungs (Fig. 4B). These data suggest that other factors besides increased ESX-5 secretion contribute to the attenuation of the $\Delta pstA1$ mutant in *NOS2*^{-/-} mice.

Similarly, in C57BL/6 mice, deletion of the *esx-5* RegX3 binding site sequence failed to suppress the attenuated phenotype of the $\Delta pstA1$ mutant during the chronic phase of infection (Fig. 4C). There were no statistically significant differences in lung bacterial burden between the $\Delta pstA1$ and $\Delta pstA1\Delta BS$ mutants at any time point (Fig. 4C). In addition, at each time point the CFUs in the lungs of C57BL/6 mice infected with either the $\Delta pstA1$ mutant or the $\Delta pstA1\Delta BS$ mutant were significantly different from the WT control (Fig. 4C). To determine if the ΔBS mutation causes attenuation, we infected C57BL/6 mice by the aerosol route with the ΔBS mutant. We observed modest but statistically significant decreases in lung bacterial burden at the 2 week and 4 week time points in ΔBS -infected mice relative to mice infected with the WT control (Fig. 4C). However, by 12 weeks post-infection, CFU in the lungs of both WT- and ΔBS -infected mice were similar (Fig. 4C). Taken together, these data suggest that other factors besides increased ESX-5 secretion contribute to attenuation of the $\Delta pstA1$ mutant during the chronic phase of infection in C57BL/6 mice and that regulation of ESX-5 secretion in response to P_i limitation enhances acute phase replication of *M. tuberculosis* in the lungs.

EsxN hyper-secretion does not cause attenuation of the $\Delta pstA1$ mutant.

To investigate whether attenuation of the $\Delta pstA1$ mutant is due to inappropriate hyper-secretion of the ESX-5 substrate EsxN specifically, we deleted *esxN* in both WT and $\Delta pstA1$ mutant backgrounds. The $\Delta esxN$ deletion was verified by PCR (data not shown) and qRT-PCR; the *esxN* transcript was not detected in either $\Delta esxN$ mutant (Fig. 5A). Deletion of *esxN* did not

alter abundance of the downstream *espG₅* transcript in either the WT or Δ *pstA1* mutant background (Fig. 5A), suggesting that the Δ *esxN* deletion is not polar. To verify that EsxN is not produced or secreted by the Δ *esxN* and Δ *pstA1 Δ *esxN* mutants, we performed Western blots. While secreted EsxN was not detected in either the WT or Δ *esxN* strains, a protein or proteins that reacted with our anti-EsxN anti-serum was still detected in the secreted fraction of the Δ *pstA1 Δ *esxN* mutant, though at a decreased level compared to the Δ *pstA1* parental control (Fig. 5B). These data suggest that while EsxN itself is hyper-secreted by the Δ *pstA1* mutant, our anti-EsxN anti-serum also detects one or more of the four EsxN paralogs encoded outside the *esx-5* locus that each exhibit >92.5% amino acid sequence identity with EsxN (37). Similar cross-reactivity of anti-EsxN anti-serum was previously described (17). Our data further suggest that secretion of one or more of these EsxN paralogs is also increased in the Δ *pstA1* mutant.**

We performed additional Western blots to verify that the Δ *esxN* deletion did not alter production of ESX-5 core components. Because co-dependent secretion of substrates has been observed for the ESX-1 secretion system (38-40), we also examined if deletion of *esxN* altered PPE41 secretion. Deletion of *esxN* in WT *M. tuberculosis* did not change production of the ESX-5 proteins EspG₅ or EccB₅, but did cause an increase in PPE41 secretion (Fig. 5B). It is possible that in the absence of EsxN, other ESX-5 substrates like PPE41 are more efficiently secreted. Both EspG₅ and EccB₅ were also produced at similar levels in the Δ *pstA1 Δ *esxN* double mutant compared to the Δ *pstA1* mutant (Fig. 5B). The Δ *pstA1 Δ *esxN* mutant hyper-secreted PPE41, like the Δ *pstA1* mutant (Fig. 5B). The ModD and GroEL2 controls demonstrate equivalent loading of culture filtrate and cell lysate fractions, respectively (Fig. 5B). Overall, our data suggest that EsxN is not required for production or stability of the ESX-5 components EccB₅ or EspG₅ and that EsxN and PPE41 are secreted independently.**

To investigate if deletion of *esxN* could reverse attenuation of Δ *pstA1* mutant, like the Δ *BS* mutation, we infected Irgm1^{-/-}, NOS2^{-/-} and C57BL/6 mice via the aerosol route with ~100 CFU the Δ *pstA1 Δ *esxN* mutant. In contrast to the Δ *pstA1 Δ *BS* mutant that replicated**

progressively in the lungs of *Irgm1*^{-/-} mice (Fig. 4A), replication of the Δ *pstA1* Δ *esxN* mutant was well controlled in *Irgm1*^{-/-} mice (Fig. 5C). There was no significant difference in CFU recovered from the lungs of Δ *pstA1*- and Δ *pstA1* Δ *esxN*- infected *Irgm1*^{-/-} mice at any time point (Fig. 5C). The Δ *pstA1* Δ *esxN* mutant remained attenuated in *NOS2*^{-/-} mice and during the chronic phase of infection in C57BL/6 mice (Fig. 5D and 5E), similar to the Δ *pstA1* Δ *BS* mutant. Bacterial burdens in the lungs were not significantly different in either *NOS2*^{-/-} mice or C57BL/6 mice infected with the Δ *pstA1* mutant or the Δ *pstA1* Δ *esxN* mutant at any time point (Figs. 5D and 5E). These data suggest that ESX-5 secreted factors other than EsxN contribute to the attenuation of the Δ *pstA1* mutant in *Irgm1*^{-/-} mice.

DISCUSSION

We previously demonstrated that the virulence-associated ESX-5 secretion system is regulated at the transcriptional level by the Pst/SenX3-RegX3 system that stimulates ESX-5 secretion in response to P_i limitation. By precisely defining the RegX3 binding site in the *esx-5* locus and creating targeted mutations that specifically disrupt RegX3-mediated regulation of ESX-5 secretion, we show here that regulation of ESX-5 secretion contributes to *M. tuberculosis* pathogenesis. Our data suggest that the Δ *pstA1* mutant is attenuated in *Irgm1*^{-/-} mice due to hyper-secretion of ESX-5 substrates caused by constitutive activation of RegX3. Our data further suggest that this attenuation is caused by ESX-5 substrates other than EsxN. Our results are consistent with a recent report demonstrating that secretion of the PE_PGRS subfamily of PE proteins, which are likely ESX-5 substrates, is associated with reduced *M. tuberculosis* virulence in Balb/c mice (20, 41). We conclude that *M. tuberculosis* requires precise regulation of ESX-5 secretion during infection for pathogenesis and that ESX-5 substrates other than EsxN play a direct role in the interaction with the host.

While our previous work demonstrated that RegX3 directly controls ESX-5 secretion at the transcriptional level and defined a region 5' of the *pe19* gene to which RegX3 binds (14), the precise binding site remained unknown. Here we identified a RegX3 binding site sequence at -

128 to -102 bp relative to the *pe19* start codon that consists of three imperfect direct repeats. We further demonstrated that the two 3' direct repeats and 5 bp spacer with the sequence 5'-GGTGCcaactGGTGA-3' are necessary for RegX3 binding *in vitro* and transcriptional regulation of *esx-5* genes *in vivo*. In this respect, RegX3 acts similarly to the *Escherichia coli* PhoB response regulator that also responds to P_i limitation by binding to direct repeat sequences (*pho* boxes) in the promoters of regulated genes (42). The RegX3 binding site sequence in the *esx-5* locus is upstream of a transcriptional start site that was mapped at -38 relative to the *pe19* start codon (43), consistent with RegX3 acting as a transcriptional activator of *esx-5* genes. By creating mutations in or deleting this RegX3 binding site sequence on the *M. tuberculosis* chromosome, we demonstrate that regulation of ESX-5 secretion by RegX3 in response to P_i availability requires this sequence.

Attenuation of the Δ *pstA1* mutant specifically in *Irgm1*^{-/-} mice was almost completely suppressed by deletion of the RegX3 binding site in the *esx-5* locus, suggesting that the Δ *pstA1* mutant is attenuated in these mice due to constitutive ESX-5 secretion. These data also suggest that the Δ *pstA1* mutant is sensitive to some host factor other than *Irgm1* due to constitutive ESX-5 secretion. *Irgm1* and NOS2 act independently to control *M. tuberculosis* replication (29), so it is possible that constitutive ESX-5 secretion causes increased susceptibility of the Δ *pstA1* mutant to NOS2-generated nitrosative stress. Alternatively, the Δ *pstA1* mutant may fail to induce the generalized leukopenia that is typically observed in infected *Irgm1*^{-/-} mice (32), leading to improved control of the infection. In *Irgm1*^{-/-} mice, IFN- γ produced in response to infection causes the lymphatic collapse by stimulating autophagic death of effector T cells (31). Effector T cells may more efficiently recognize and control replication of the Δ *pstA1* mutant due to its constitutive secretion of antigenic ESX-5 substrates, so that T cell containment of infection occurs despite reduced T cell abundance. The Δ *pstA1* mutant could also interfere with IFN- γ production or signaling due to constitutive ESX-5 secretion. Manipulation of cytokine responses is a plausible explanation considering that ESX-5 has previously been implicated in activating

the inflammasome and triggering IL-1 β production by infected cells (18, 19). Finally, the susceptibility of *Irgm1*^{-/-} mice to infection with intracellular pathogens can be reversed by deletion of a second IFN- γ regulated GTPase *Irgm3* (44). In *Irgm1*^{-/-} cells, mislocalization of effector immunity-related GTPases (IRGs) causes damage to lysosomes, but in cells lacking *Irgm1* and *Irgm3* the effector IRGs localize to lipid droplets and damage to lysosomes is prevented (45). It is possible that an ESX-5 secreted protein or proteins interferes with the function of either *Irgm3* or the effector GTPases to prevent lysosomal damage and enable *Irgm1*^{-/-} mice to contain replication of the Δ *pstA1* mutant. We intend to explore these ideas in our future studies.

While constitutive ESX-5 secretion attenuates the Δ *pstA1* mutant in *Irgm1*^{-/-} mice, the ESX-5 substrates responsible for this phenotype remain to be determined. Our data suggest that attenuation is not caused by hyper-secretion of EsxN since the Δ *pstA1* Δ *esxN* mutant remained attenuated in *Irgm1*^{-/-} mice. It is possible that one or more of the EsxN paralogs (EsxI, EsxL, EsxO, or EsxV) plays some role in this process. We could still detect secretion of one or more of these proteins from the Δ *pstA1* Δ *esxN* mutant using our EsxN anti-serum. However, secretion of all EsxN paralogs was undetectable in the Δ *pstA1* Δ *BS* mutant, suggesting that decreased production of ESX-5 core components reduces secretion of all EsxN paralogs. Our future plans include deleting genes encoding each of the EsxN paralogs individually and in combination to determine whether these proteins collectively influence pathogenesis.

Alternatively, PE and/or PPE proteins secreted via ESX-5 may play a role in attenuation of the Δ *pstA1* mutant. PE and PPE proteins are strongly immunogenic in mice in a manner dependent on secretion via ESX-5 (21). In addition, some PE and PPE proteins can directly manipulate the functions of host cells (46-48) and, as discussed above, secretion of the PE_PGRS subfamily in particular has previously been associated with reduced virulence (41). We are currently working to define the *M. tuberculosis* ESX-5 secretome using strains we developed that conditionally express the ESX-5 core component EccD₅ (33) and will explore the potential of these secreted

substrates to influence pathogenesis.

Although our data indicate that ESX-5 hyper-secretion causes attenuation of the $\Delta pstA1$ in *Irgm1*^{-/-} mice, aberrant ESX-5 secretion does not contribute substantially to the chronic phase persistence defect of the $\Delta pstA1$ mutant in C57BL/6 mice. We recently described that the $\Delta pstA1$ mutant also exhibits increased release of membrane vesicles (MV) derived from the inner membrane that contain immune-modulatory lipoproteins and lipoglycans (33, 49). Importantly, increased MV release by the $\Delta pstA1$ mutant was independent of ESX-5 secretion system activity (33). We speculate that aberrant MV production could also contribute to attenuation of the $\Delta pstA1$ mutant. The MV-associated lipoprotein LpqH (also known as the 19kDa lipoprotein) is a potent TLR2 ligand and signaling through this pathway causes pleiotropic effects on the innate immune system that include promoting the production of the pro-inflammatory cytokines IL-1 β , IL-12p40, and TNF α (50), reducing surface MHC class II expression on macrophages (50-52), and inducing host cell apoptosis and nitric oxide-independent antimicrobial activity (49, 53, 54). Additionally, LpqH contributes to CD4⁺ T cell activation (55). It is unclear from these studies if increased release of LpqH would be beneficial or detrimental to bacterial survival and pathogenesis. We are actively exploring the mechanism of enhanced MV release by the $\Delta pstA1$ mutant to determine the importance of regulated MV production in *M. tuberculosis* pathogenesis.

While constitutive activation of ESX-5 secretion contributes to attenuation of the $\Delta pstA1$ mutant, regulation of ESX-5 secretion by RegX3 appears to play only a minor role in *M. tuberculosis* pathogenesis. *regX3* mutants are attenuated during chronic infection of C57BL/6 mice (24, 56), but the ΔBS mutant that we constructed, which fails to induce transcription of *esx-5* genes or secretion of ESX-5 substrates in response to P_i limitation *in vitro*, was only modestly attenuated during the acute phase of infection and persisted normally in the chronic phase of infection. Our data suggest that other regulatory targets of RegX3 besides ESX-5 influence *M. tuberculosis* persistence and that other regulators may contribute more substantially to

controlling ESX-5 activity during infection. Indeed several transcription factors have been reported to bind within the *esx-5* locus and induce transcription of *esx-5* genes (34, 57). It is possible that one or more of these regulators plays an important role in controlling ESX-5 secretion during infection, which we plan to investigate in our future studies.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. tuberculosis* Erdman and the derivative Δ *pstA1*, Δ *regX3*, and Δ *pstA1* Δ *regX3* mutant strains were previously described (24). Construction of strains harboring mutations in the *esx-5* RegX3 binding site sequence is described below. Bacterial cultures were grown at 37°C with aeration in Middlebrook 7H9 liquid medium (Difco) supplemented with albumin-dextrose-saline (ADS), 0.5% glycerol and 0.1% Tween-80 or on Middlebrook 7H10 agar medium (Difco) supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC, BD Biosciences) and 0.5% glycerol, unless otherwise noted. Sauton's medium (3.67 mM KH₂PO₄, 2 mM MgSO₄·7H₂O, 9.5 mM citric acid, 0.19 mM ammonium iron (III) citrate, 26.64 mM L-asparagine, 6% glycerol, 0.01% ZnSO₄, pH 7.4) or P_i-limited Sauton's medium (Sauton's containing 2.5 μM KH₂PO₄, buffered with 50 mM MOPS, pH 7.4) were used to grow cultures for protein isolation. P_i-free 7H9 medium was prepared as previously described (24). Frozen stocks were prepared by growing liquid cultures to mid-exponential phase (OD₆₀₀ 0.8-1.0) in complete 7H9 medium, then adding glycerol to 15% final concentration, and storing 1 ml aliquots at -80°C.

Cloning. Constructs for deleting *esxN* or introducing mutations in the *esx-5* locus RegX3 binding site were generated in the pJG1100 allelic exchange vector, which contains the *aph* (kanamycin resistance), *hyg* (hygromycin resistance), and *sacB* (sucrose sensitivity) markers (58). Genomic regions ~800 bp 5' and 3' of the sequence to be mutated were PCR amplified from the *M. tuberculosis* Erdman genome using the primers in Table S2. Forward primers to amplify the 5' region were designed with a PaeI restriction site; reverse primers to amplify the 3'

region were designed with an *Ascl* restriction site. For deletion of *esxN*, the reverse primer to amplify the 5' regions and the forward primer to amplify the 3' region were designed with *AvrII* restriction sites in-frame with the start and stop codons, respectively. Resulting PCR products were cloned in pCR2.1 (Invitrogen) and sequenced. The 5' and 3' regions were removed from pCR2.1 by restriction with *PacI*/*AvrII* and *AvrII*/*Ascl*, respectively, and ligated with pJG1100 digested with *PacI*/*Ascl* to generate the in-frame Δ *esxN* deletion construct. For the *esx-5* RegX3 binding site mutations, the forward and reverse primers for amplifying the 3' and 5' regions of homology, respectively, contained the mutation to be introduced and were designed with overlapping sequence at the 5' ends to allow PCR products to be joined by overlap extension PCR (59) before cloning in pCR2.1. Sequence-confirmed binding site mutation constructs were removed from pCR2.1 by restriction with *PacI*/*Ascl* and ligated to similarly digested pJG1100.

Strain construction. *M. tuberculosis* strains harboring the Δ *esxN* deletion or *esx-5* RegX3 binding site mutations were generated by two-step allelic exchange, as previously described (24). Integration of the pJG1100 construct at the correct location was confirmed by colony PCR on heat-inactivated cell lysates using the primer pairs for detection of the 5' and 3' homologous recombination (Tables S2 & S3). Clones with the plasmid integrated were grown without antibiotics, diluted and plated on 7H10 containing 2% sucrose for counter-selection of the pJG1100 vector. Sucrose resistant isolates were screened by colony PCR on heat-inactivated cell lysates using primers for the detection of the deletion or mutation (Tables S2 & S3). The *esx-5* RegX3 binding site mutations were verified by Sanger sequencing of the resulting PCR products.

Purification of His₆-RegX3. Recombinant His₆-RegX3 was expressed and purified from *Escherichia coli* BL21 (DE3) containing pET28b+::regX3 by affinity chromatography using Ni-NTA agarose (Qiagen) as previously described (14).

Electrophoretic mobility shift assays. Double-stranded DNA probes were PCR amplified using *M. tuberculosis* Erdman genomic DNA as template and appropriate primers (Table S4).

Probes were labeled with the DIG Gel Shift Kit, 2nd Generation (Roche), following the manufacturer's protocols. Binding reactions with 0.5 ng of DIG-labeled probe, binding buffer (Roche), poly[d(I-C)], poly L-lysine, and 0.5 µg purified His₆-RegX3 in 20 µl total volume were incubated at room temperature for 15 min. Binding reactions including a 400-fold excess of unlabeled competitor (200 ng) were incubated for 15 min at room temperature prior to adding the DIG-labeled probe, then incubated an additional 15 min. DNA-protein complexes were resolved by electrophoresis on 5% native polyacrylamide gels, transferred and UV-crosslinked to nylon membranes (Roche). Membranes were washed with wash buffer (DIG wash and block buffer set, Roche), blocked for 30 min in blocking solution (Roche) and incubated with anti-DIG-AP antibodies (Roche) at a 1:10,000 dilution for 30 min at room temperature. Labeled probes were detected using CDP-Star ready-to-use substrate (Roche). Membranes were exposed to film (Blue lite autorad film, Genemate) and developed using a film processor (Konica, SRX-101A).

Quantitative RT-PCR. To measure gene expression in P_i-rich conditions, bacteria were grown in complete Middlebrook 7H9 medium to mid-exponential phase (OD₆₀₀ 0.4-0.6). To test induction of gene expression during P_i starvation, cultures were grown in 7H9 to mid-exponential phase (OD₆₀₀ 0.4-0.6), washed twice and resuspended at OD₆₀₀ 0.2 in P_i-free 7H9, and then grown at 37°C with aeration for 24 hr. Bacteria were collected by centrifugation (3700 x g, 10 min, 4°C). Total RNA was extracted using TRIzol (Invitrogen, CA) with 0.1% polyacryl carrier (Molecular Research Center, Inc) by bead beating with 0.1 mm zirconia beads (BioSpec Products). Equivalent amounts of total RNA were treated with Turbo DNase (Invitrogen) and converted to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with random hexamer primers and the following parameters: 10 min at 25°C (annealing of primers), 60 min at 50°C (elongation), and 5 min at 85°C (heat inactivation of reverse transcriptase). cDNA was stored at -20°C.

Quantitative PCR primers to amplify internal regions of the genes of interest (*pe19*, *esxN*, *espG₅*, *eccD₅*, *udgA*, *mgtA*, and *sigA*) were designed with similar annealing temperatures (58-60°C) using either Primer Express software (Applied Biosystems) or ProbeFinder Assay Design software (Roche) and are listed in Table S5. Quantitative RT-PCR reactions were prepared using 2x SYBR Green master mix (Roche), 2.5 µM each primer and 1 µl cDNA and run on a LightCycler 480 (Roche) using the following cycle parameters: 95°C for 10 min; 45 cycles of 95°C for 10s, 60°C for 20s, and 72°C for 20s with data collected once per cycle during the extension phase; and one cycle of 95°C for 5s, 65°C for 1m, 97°C with a ramp rate of 0.11 °C/s for generation of melting curves. Cycle threshold values (C_p , Roche nomenclature) were converted to copy numbers using standard curves for each gene generated using genomic DNA. Gene copy numbers were normalized to *sigA*.

Antisera production. Rabbit polyclonal antisera against EccD₅, EsxN and PPE41 were previously described (33, 60). Synthetic antigenic peptides (EccB₅ 489-506, EHDTLPMDMTPAELVVPK; EspG₅ 283-300, KTVLDTLPYGEWKTHSRV) that were identified with Antigen Profiler and conjugated to keyhole limpet hemocyanin (KLH) were used with TiterMax Gold adjuvant (Sigma) to raise polyclonal antisera against EccB₅ and EspG₅ in rabbits (Pierce Custom Antibodies, Thermo Scientific).

Protein preparation for immunoblots. *M. tuberculosis* cultures were grown at 37°C with aeration in Sauton's medium or P_i-limited (2.5 µM P_i) Sauton's medium for five days as previously described (14) prior to protein isolation. Bacteria were collected by centrifugation (4700 x *g*, 15 min, 4°C). Culture supernatants were filter sterilized as previously described (14) and Complete EDTA-free protease inhibitor tablets (Roche) were added. Supernatants were concentrated roughly 25-fold by centrifugation (2400 x *g*, 4°C) using VivaSpin 5 kDa molecular weight cut-off spin columns (Sartorius). Whole cell lysates were prepared by bead beating with 0.1 mm zirconia beads (BioSpec Products) in PBS containing Complete EDTA-free protease inhibitors (Roche) and lysates were clarified by centrifugation as previously described (14). Cell

lysates were passaged through a Nanosep MF column with a 0.22 μ m filter (Pall Life Sciences) by centrifugation (14000 x *g*, 3 min, 4°C) to remove any remaining intact cells. Total protein concentration in each sample was quantified using the Pierce BCA Protein Concentration Assay kit (Thermo Scientific). Proteins were stored at 4°C for immediate use, or at -80°C with glycerol at 15% final concentration.

Western blotting. Culture filtrate or whole cell lysate proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Mini-PROTEAN TGX Any kD gels (Bio-Rad) and transferred to nitrocellulose membranes (Whatman) by electrophoresis. Proteins were detected by Western blotting as previously described (14) using primary anti-sera at the following dilutions: rabbit α -EsxN 1:1000; rabbit α -EspG₅ 1:1000; rabbit α -EccB₅ 1:1000; rabbit α -EccD₅ 1:1000; rabbit α -PPE41 1:1000; rabbit α -ModD 1:25000; mouse α -GroEL2 1:1,000. Appropriate secondary antibodies (either goat-anti-rabbit or rabbit-anti-mouse conjugated to HRP, Sigma) and SuperSignal West Pico substrate (Thermo Scientific) were used to detect reactive bands. Blots were imaged on an Odyssey Fc Imaging System (LI-Cor) and protein abundance was analyzed using ImageStudio software (LI-Cor).

Mouse infections. Female C57BL/6J and NOS2^{-/-} mice 6-8 weeks of age were purchased from Jackson Laboratories. Irgm1^{-/-} mice were bred under specific-pathogen-free conditions at the University of Minnesota Research Animal Resources. Mice were infected with ~100 CFU using an Inhalation Exposure System (GlasCol) as previously described (36). Infected mice were euthanized with CO₂ overdose. Bacterial CFU were enumerated by plating serial dilutions of lung homogenates on complete Middlebrook 7H10 agar containing 100 μ g/ml cyclohexamide and counting CFU after 3-4 weeks of incubation at 37°C. All animal protocols were reviewed and approved by University of Minnesota Institutional Animal Care and Use committee and were conducted in accordance with recommendations in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (61).

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FIGURE LEGENDS

Figure 1. Competitive EMSAs define a RegX3 binding site 5' of *pe19* in the *esx-5* locus.

(A) Schematic depicting the location of EMSA probes and competitors in the *esx-5* locus.

Positions of the 5' and 3' ends of probe and competitor sequences relative to the *pe19*

translational start site are indicated. (C) Sequences of the 5' Probe, truncated competitors that

defined the 5' and 3' ends of the RegX3 binding site, and mutated competitors that defined

sequence elements required for RegX3 binding. Direct repeats (red text) and the 5' and 3' ends

of each competitor relative to the *pe19* translational start site are indicated. Mutated sequences

are highlighted by underlines and green (DR3), purple (DR1), blue (DR2) or gray (spacer) text.

(B, D, and E) EMSA analysis of binding between purified His₆-RegX3 (0.5 µg), DIG-labeled

probe (0.5 ng), and unlabeled competitors (200 ng), as indicated. Results are representative of

two independent experiments.

Figure 2. Mutation of the *esx-5* RegX3 binding site suppresses over-expression of *esx-5*

genes and hyper-secretion of EsxN by the Δ *pstA1* mutant. (A) Transcript abundance of *pe19*, *espG₅* and *eccD₅* relative to *sigA* were determined by quantitative RT-PCR for the indicated strains grown to mid-logarithmic phase in 7H9 complete medium. Results are the means $\pm$ standard deviations of three independent experiments. ** $P < 0.01$, **** $P < 0.0001$. (B) The indicated strains were grown in Sauton's medium without Tween-80. Cell lysates (10 μ g) and culture filtrates (5 μ g) were separated and analyzed by Western blotting to detect the indicated proteins. Results shown are from a single experiment and are representative of two independent experiments.

Figure 3. Deletion of the *esx-5* RegX3 binding site prevents activation of ESX-5 secretion

in response to P_i limitation. (A & B) Transcript abundance of *pe19*, *espG₅* and *eccD₅* relative to *sigA* were determined by quantitative RT-PCR for the indicated strains grown to mid-logarithmic phase in P_i -free 7H9 medium (A) or P_i -replete 7H9 complete medium (B). Results are the means $\pm$ standard deviations of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, **** $P < 0.0001$. (C) The indicated strains were grown in Sauton's medium without Tween-80 (WT + P_i) or in P_i -limiting (2.5 μ M P_i) Sauton's medium without Tween-80. Cell lysates (10 μ g) and culture filtrates (5 μ g) were separated and analyzed by Western blotting to detect the indicated proteins. Results shown are from a single experiment and are representative of two independent experiments.

Figure 4. Deletion of the *esx-5* RegX3 binding site in the Δ *pstA1* mutant restores

virulence in *Irgm1*^{-/-} mice. *Irgm1*^{-/-} (A), *NOS2*^{-/-} (B) or C57BL/6J (C) mice were infected by the aerosol route with ~100 CFU of the *M. tuberculosis* WT, Δ *pstA1*, Δ *BS*, or Δ *pstA1* Δ *BS* strain. Groups of mice ($n=4$) were sacrificed at the indicated time points and bacterial CFU were enumerated by plating serial dilutions of lung homogenates. Results are the means $\pm$ standard errors of the means. Results for the Δ *pstA1* Δ *BS* mutant in *Irgm1*^{-/-} mice are from one representative experiment of two independent experiments. All other results are from a single

experiment. Data for the WT control in panel B are reproduced from (24) for comparison with the ΔBS mutant. Asterisks indicate statistically significant differences between WT and ΔBS (black) or between $\Delta pstA1$ and $\Delta pstA1\Delta BS$ (red). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, $\pm P = 0.1353$.

Figure 5. Hyper-secretion of EsxN does not cause attenuation of the $\Delta pstA1$ mutant. (A)

Abundance of the *esxN* and *espG₅* transcripts relative to *sigA* was determined by quantitative RT-PCR for the indicated strains grown to mid-logarithmic phase in 7H9 complete medium.

Results are the means $\pm$ standard deviations of three independent experiments. Colored #

indicates the transcript was not detected in the corresponding mutant. (B) The indicated strains

were grown in Sauton's medium without Tween-80. Cell lysates (10 μ g) and culture filtrates (5 μ g) were separated and analyzed by Western blotting to detect the indicated proteins. Results

shown are from a single experiment and are representative of two independent experiments. (C-

E) Aerosol infection of mice. *Irgm1*^{-/-} (C), *NOS2*^{-/-} (D) or C57BL/6J (E) mice were infected with

~100 CFU of the *M. tuberculosis* WT, $\Delta pstA1$, or $\Delta pstA1\Delta esxN$ strain. Groups of mice ($n=4$)

were sacrificed at the indicated time points and bacterial CFU were enumerated by plating serial

dilutions of lung homogenates. Results are the means $\pm$ standard errors of the means and are

from a single experiment. Data for the $\Delta pstA1\Delta esxN$ mutant in *Irgm1*^{-/-} mice are representative

of two independent experiments. Data for the WT and $\Delta pstA1$ mutant controls are reproduced

from Figure 4.

Figure 1. Competitive EMSAs define a RegX3 binding site 5' of *pe19* in the *esx-5* locus. (A) Schematic depicting the location of EMSA probes and competitors in the *esx-5* locus. Positions of the 5' and 3' ends of probe and competitor sequences relative to the *pe19* translational start site are indicated. (C) Sequences of the 5' Probe, truncated competitors that defined the 5' and 3' ends of the RegX3 binding site, and mutated competitors that defined sequence elements required for RegX3 binding. Direct repeats (red text) and the 5' and 3' ends of each competitor relative to the *pe19* translational start site are indicated. Mutated sequences are highlighted by underlines and green (DR3), purple (DR1), blue (DR2) or gray (spacer) text. (B, D, and E) EMSA analysis of binding between purified His₆-RegX3 (0.5 μg), DIG-labeled probe (0.5 ng), and unlabeled competitors (200 ng), as indicated. Results are representative of two independent experiments.

Figure 2. Mutation of the *esx-5* RegX3 binding site suppresses over-expression of *esx-5* genes and hyper-secretion of EsxN by the $\Delta pstA1$ mutant. (A) Transcript abundance of *pe19*, *espG₅* and *eccD₅* relative to *sigA* were determined by quantitative RT-PCR for the indicated strains grown to mid-logarithmic phase in 7H9 complete medium. Results are the means $\pm$ standard deviations for three independent experiments. ** $P < 0.01$, **** $P < 0.0001$. (B) The indicated strains were grown in Sauton's medium without Tween-80. Cell lysates (10 μ g) and culture filtrates (5 μ g) were separated and analyzed by Western blotting to detect the indicated proteins. Results shown are from a single experiment and are representative of two independent experiments.

Figure 3. Deletion of the *esx-5* RegX3 binding site prevents activation of ESX-5 secretion in response to P_i limitation. (A&B) Transcript abundance of *pe19*, *espG₅* and *eccD₅* relative to *sigA* were determined by quantitative RT-PCR for the indicated strains grown to mid-logarithmic phase in P_i -free 7H9 medium (A) or P_i -replete 7H9 complete medium (B). Results are the means $\pm$ standard deviations for three independent experiments. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. (C) The indicated strains were grown in Sauton's medium without Tween-80 (WT + P_i) or in P_i -limiting (2.5 μ M P_i) Sauton's medium without Tween-80. Cell lysates (10 μ g) and culture filtrates (5 μ g) were separated and analyzed by Western blotting to detect the indicated proteins. Results shown are from a single experiment and are representative of two independent experiments.

Figure 4. Deletion of the *esx-5* RegX3 binding site in the $\Delta pstA1$ mutant restores virulence in *Irgm1*^{-/-} mice. *Irgm1*^{-/-} (A), *NOS2*^{-/-} (B) or C57BL/6J (C) mice were infected by the aerosol route with ~100 CFU of the *M. tuberculosis* WT, $\Delta pstA1$, ΔBS , or $\Delta pstA1\Delta BS$ strain. Groups of mice ($n=4$) were sacrificed at the indicated time points and bacterial CFU were enumerated by plating serial dilutions of lung homogenates. Results are the means $\pm$ standard errors of the means. Results for the $\Delta pstA1\Delta BS$ mutant in *Irgm1*^{-/-} mice are from one representative experiment of two independent experiments. All other results are from a single experiment. Data for the WT control in panel B are reproduced from (24) for comparison with the ΔBS mutant. Asterisks indicate statistically significant differences between WT and ΔBS (black) or between $\Delta pstA1$ and $\Delta pstA1\Delta BS$ (red). ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, ‡ $P=0.1353$.

Figure 5. Hyper-secretion of EsxN does not cause attenuation of the $\Delta pstA1$ mutant.

(A) Abundance of the *esxN* and *espG₅* transcripts relative to *sigA* was determined by quantitative RT-PCR for the indicated strains grown to mid-logarithmic phase in 7H9 complete medium. Results are the means $\pm$ standard deviations of 3 independent experiments. Colored # indicates the transcript was not detected in the corresponding mutant. (B) The indicated strains were grown in Sauton's medium without Tween-80. Cell lysates (10 μ g) and culture filtrates (5 μ g) were separated and analyzed by Western blotting to detect the indicated proteins. Results are from a single experiment and are representative of two independent experiments. (C-E) Aerosol infection of mice. *Irgm1*^{-/-} (C), *NOS2*^{-/-} (D) or C57BL6/J (E) mice were infected with ~100 CFU of the *M. tuberculosis* WT, $\Delta pstA1$ or $\Delta pstA1\Delta esxN$ strain. Groups of mice (*n*=4) were sacrificed at the indicated time points and bacterial CFU were enumerated by plating serial dilutions of lung homogenates. Results are the means $\pm$ standard errors of the means and are from single experiment. Data for the $\Delta pstA1\Delta esxN$ mutant in *Irgm1*^{-/-} mice are representative of two independent experiments. Data for the WT and $\Delta pstA1$ mutant controls are reproduced from Figure 4.