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The *M. tuberculosis* ESX-1 secretion pathway modulates the host-pathogen interaction and is regulated through coupling of transcription and secretion

by

Sridharan Raghavan

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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[redacted]

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Abstract

The *M. tuberculosis* ESX-1 secretion pathway modulates the host-pathogen interaction and is regulated through coupling of transcription and secretion

Sridharan Raghavan

Mycobacterium tuberculosis is the causative agent of tuberculosis, a primarily pulmonary disease that is responsible for approximately one million deaths annually. *M. tuberculosis* is exquisitely adapted to survival within the eukaryotic host and is capable of persisting in a latent state for decades after infection. The ability of *M. tuberculosis* to evade and manipulate the host immune response is believed to contribute to its survival for such long periods of time after initial infection. My thesis work has focused on the bacterial mechanisms, particularly an alternate protein secretion system, underlying the modulation of macrophage cytokine production by *M. tuberculosis*.

In this work, I first describe the results of a genetic screen to identify bacterial genes required for the suppression of macrophage cytokine production. This work revealed that two major virulence determinants in *M. tuberculosis*, the ESX-1 secretion system and the pathogenic lipid phthiocerol dimycocerosate, are critical to modulation of macrophage innate immune responses. I further characterize the macrophage signaling pathways that are targeted for manipulation by the ESX-1 system and identify a small subset of macrophage genes regulated by bacterial infection that are specifically modulated by the ESX-1 pathway. Many of the genes identified in this work do not have

previously described functions in the immune system, suggesting novel avenues for study of bacterial pathogenesis and the resistance to *M. tuberculosis* infection.

In the second part of this work, I describe the characterization of a novel member of the ESX-1 protein secretion pathway, Rv3849. Through a number of complementary approaches, I demonstrate that Rv3849 is a transcriptional activator of an operon required for function of the ESX-1 pathway. Furthermore, I show that Rv3849 is itself a secreted substrate of the ESX-1 system. I propose and provide data supporting a model in which Rv3849 transcriptionally activates ESX-1 secretion in response to intracellular cues and then is exported through the ESX-1 pathway as a form of negative feedback. The identification of a regulator of ESX-1 secretion and characterization of its activity is an important step forward in understanding a critical element in the interaction between *M. tuberculosis* and the host.

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Chapter 1.

Introduction

I. Overview of *M. tuberculosis* pathogenesis

Mycobacterium tuberculosis is the causative agent of tuberculosis, a major global disease burden and proximal cause of over one million deaths each year. Furthermore, nearly one-third of the world's population is latently infected with the bacteria with the potential for development of active disease later in life. The treatments for tuberculosis are limited to active disease; no antibiotics exist to target latent bacteria. Also of note, the frontline antibiotics against *M. tuberculosis* were developed decades ago, and drug-resistant bacteria have emerged over the last several years ((CDC), 2007). Of particular concern are *M. tuberculosis* strains that are extrensively drug resistant, designated XDR *M. tuberculosis*, one of which was at the root of an outbreak in South Africa in 2005 in which nearly every infected individual succumbed to tuberculosis (Gandhi et al., 2006). The worldwide infection burden of *M. tuberculosis* coupled with the developing drug resistance of disease-causing bacteria make understanding *M. tuberculosis* pathogenesis, specifically details of the interactions between the host and pathogen, important from a global health perspective.

The most common route of entry of *M. tuberculosis* is through the respiratory route. Once inside the lung, the bacterium is thought to be phagocytosed by alveolar macrophages, an encounter that initiates the host immune response as well as triggers the intracellular life cycle of *M. tuberculosis*. A number of host immune molecules and cells have been shown to be critical to control of *M. tuberculosis* growth in mice, including tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), and CD4+ T-cells (Caruso et al., 1999; Flynn and Bloom, 1996; Flynn et al., 1993; Flynn et al., 1995a). Inside murine macrophages, the antimicrobial function of inducible nitric oxide synthase

(iNOS) is critical to control of bacterial growth (Chan et al., 1995). Another cytokine, interleukin-12 (IL-12), produced by macrophages and dendritic cells, is important in initiating the Type I immune response, characterized by IFN-γ and CD4+ T-cells, that is critical to control of *M. tuberculosis*. IL-12 is, therefore, is an important molecule in directing the differentiation of the immune response to one that is optimal for combating intracellular bacteria (Altare et al., 1998b; de Jong et al., 1998; Flynn et al., 1995b).

Less is known about how exactly the host immune response recognizes *M. tuberculosis* and initiates a response. Toll-like receptors (TLRs) are critical to recognition of various pathogen-associated molecules (Kopp and Medzhitov, 2003), and in the case of *M. tuberculosis* multiple TLRs, specifically TLR2, TLR4, and TLR9, participate in activating the macrophage response to infection (Bafica et al., 2005; Means et al., 1999). A number of other receptors have been implicated in recognition of *M. tuberculosis*, including the mannose receptor as well as the opsonin-recognizing Fc receptor and complement receptor 3 (Aderem and Underhill, 1999; Ernst, 1998; Hickman et al., 2002). Furthermore, the intracytoplasmic recognition receptor NOD2 has been implicated in synergizing with TLR2 for maximal activation of macrophages (Ferwerda et al., 2005). The mechanism underlying *M. tuberculosis* recognition by macrophages is complex and the signaling events downstream of bacterial recognition that lead to an innate immune response remain unclear.

One can also examine the host-pathogen interaction from the perspective of *M. tuberculosis*. Upon entering the macrophage, the bacteria undergo a broad transcriptional reprogramming, activating genes hypothesized to be important for survival in the nutrient-poor phagosome and for resistance to the inhospitable phagosomal environment

(Schnappinger et al., 2003; Schnappinger et al., 2006). In addition, the bacteria evade or manipulate various aspects of the host immune response. For one, *M. tuberculosis* halts the maturation of the phagosome at a point where it resembles an early endosome, preventing the development of the vacuole into a more harmful phagolysosome (Russell, 2003). Furthermore, *M. tuberculosis* suppresses the production of inflammatory cytokines such as IL-12 and mutes the signaling response to IFN-γ. The bacterial manipulation of specific elements of the host response suggests that the targeted aspects of the immune response are of particular importance for control of *M. tuberculosis* growth. The mechanisms by which *M. tuberculosis* manipulates host immune responses is poorly understood, and my thesis work was aimed at clarifying the host-pathogen interactions that underlie bacterial suppression of inflammatory cytokine production.

II. Alternative secretion systems and their role in bacterial pathogenesis

Many bacteria utilize alternate protein secretion systems to manipulate the host and engineer a survival niche. The effects on the host of secreted effectors can vary from suppressing immune pathway signaling to inducing phagocytosis of non-phagocytic cells to preventing phagocytosis (Mota et al., 2005; Navarro et al., 2005; Schlumberger and Hardt, 2006). The unifying theme of the diverse applications of alternative protein secretion pathways is the creation of a growth niche within the host. Another common feature of alternative protein secretion systems is that they are often associated with pathogenicity islands, genomic content that is found in pathogens but absent in closely-related non-pathogens (Galán and Wolf-Watz, 2006; Winstanley and Hart, 2001).

The most well characterized alternative secretion system is the Type III secretion system of gram-negative bacteria. The Type III system is remarkable for the diversity of effector proteins that have been adapted for secretion through its pore (Lee and Schneewind, 1999; Mota et al., 2005; Navarro et al., 2005; Schlumberger and Hardt, 2005; Schlumberger and Hardt, 2006). As described above, Type III effectors target disparate eukaryotic cell processes to achieve a wide variety of outcomes. In fact, the variety of cellular targets correlates to the diversity of lifestyles adopted by the bacteria that express the Type III pathway. Extracellular, intracellular vacuolar, and intracellular cytosolic bacteria all express Type III systems with differential targets (Mota and Cornelis, 2005). Furthermore, Type III systems are adapted to mediate interactions between infecting bacteria and different cell types - macrophages, epithelial cells, and fibroblasts, for example.

M. tuberculosis does not contain a Type III secretion system, but in recent years work from our lab and others has identified an alternative secretion system of vital importance to bacterial growth *in vivo* (Guinn et al., 2004; Hsu et al., 2003; Stanley et al., 2003). This multi-protein complex, named the ESX-1 secretion system for its role in export of the small protein ESAT-6, is required by *M. tuberculosis* for growth early during infection of mice. The core machinery of the ESX-1 system is conserved and functional in non-pathogenic mycobacteria (Converse and Cox, 2005). However, the ESX-1 system is lost in the avirulent vaccine strain *M. bovis BCG* (Cole et al., 1998; Pym et al., 2003), revealing some similarity to the positive selection for Type III secretion systems in pathogenic gram-negative bacteria.

Unlike the in depth characterization of Type III secretion systems, including structural analysis of the export complex, the understanding of ESX-1 secretion is still in its infancy. The core complex is encoded at one genomic locus, termed Region of Difference 1 (RD-1), and includes a putative pore as well as accessory components believed to be involved in substrate recognition and force generation (Converse and Cox, 2005; Guinn et al., 2004; Hsu et al., 2003; Stanley et al., 2003). In the last 5 years, during my thesis work, two significant findings have added to our understanding of the ESX-1 system. First, an additional genomic locus, with some homology to but unlinked from RD-1, encoding at least three genes required for ESX-1 function was identified (Fortune et al., 2005; MacGurn et al., 2005). Interestingly, two of the proteins encoded at this locus are also substrates of the ESX-1 system. This finding was significant for two reasons: it demonstrated that the entire secretion system is not confined to the RD-1 genomic region, and it expanded the repertoire of ESX-1 substrates. Secondly, a signal sequence present at the C-terminus of one ESX-1 substrate was found to be essential for interactions between that substrate and core components of the secretory machine (Champion et al., 2006). Though the specific sequence found to be necessary and sufficient for ESX-1-mediated export on CFP-10 is not found elsewhere in the *M. tuberculosis* proteome, the identification of a C-terminal sequence enhances our understanding of substrate recognition and delivery by the secretory pathway. In my thesis work, I have identified two additional members of the ESX-1 pathway and characterized in detail the role of one of these novel members of the secretion system.

III. Introduction to thesis work

At the time that I initiated my thesis work, we were just beginning to understand the ESX-1 system and its importance to *M. tuberculosis* pathogenesis. Little was known about the detailed interactions between the bacteria and macrophages beyond the initial characterization of various phenomena, some of which are described earlier. I chose to study the mechanism by which *M. tuberculosis* suppresses the production of inflammatory cytokines, specifically IL-12. To address this question, I decided to perform a genetic screen to identify *M. tuberculosis* genes that are required to modulate macrophage cytokine production. Surprisingly, this work caused a convergence between two areas of interest for our lab: detailed understanding of *M. tuberculosis*-macrophage interactions and ESX-1 secretion.

Though ESX-1 secretion has a clear role early in infection of a eukaryotic host, little had been known about its function in *in vivo* growth. One aspect of my work presented here involves functional characterization of the role of ESX-1 in interactions between *M. tuberculosis* and host macrophages. Through the genetic screen mentioned above, I identified ESX-1 secretion as a modulator of macrophage innate immune responses. This finding emerged from work to elucidate the mechanism by which *M. tuberculosis* suppresses macrophage cytokine production. In addition to assigning a role for ESX-1 secretion in early interactions between the host and microbe, I identified two novel members of the ESX-1 pathway.

Later in the thesis, I describe the characterization of a novel ESX-1 pathway member, Rv3849. Like all ESX-1 pathway components, Rv3849 is required by *M. tuberculosis* for growth early during the course of infection in mice. One distinguishing feature of Rv3849 is that it is encoded at an independent genomic locus from the core

components of the ESX-1 pathway. Interestingly, I found that Rv3849 is a transcriptional activator of the ESX-1 system, leading us to rename the protein EspR (*E*SX-1 *s*ecreted *p*rotein *R*egulator). In addition, Rv3849 is subject to an interesting form of negative feedback; it is secreted by the ESX-1 pathway to diminish its intracellular transcriptional activity. Since little was known about the regulation of ESX-1 pathway function, the identification of a regulator of this virulence determinant is a significant advance for *M. tuberculosis* pathogenesis.

Chapter 2.

Modulation of macrophage innate immune responses by *M. tuberculosis* requires novel ESX-1 protein secretion components and the lipid PDIM

Abstract

Mycobacterium tuberculosis has evolved multiple virulence mechanisms to subvert immune responses in order to create a habitable environment in a mammalian host. In particular, *M. tuberculosis* inhibits innate macrophage responses, including the production of interleukin-12 (IL-12). We performed a genetic screen to identify bacterial mutants that fail to suppress host IL-12 and found that the ESX-1 protein secretion system and the phthiocerol dimycocerosate (PDIM) synthesis pathway were required for modulating IL-12 secretion. The screen also identified two novel members of the ESX-1 secretion system, Rv3849 and PE2, both of which are required for IL-12 suppression. The elevated IL-12 production induced by mutant *M. tuberculosis* is dependent on signaling through the host receptor TLR2 and the adaptor molecule MyD88. Finally, global gene expression analysis revealed that, in addition to IL-12, the ESX-1 pathway modulates a specific subset of TLR2-activated genes.

Introduction

Modulating immune responses in order to enhance virulence is a common strategy employed by a wide array of pathogens (Munter et al., 2006). *Mycobacterium tuberculosis* is an intracellular bacterium that avoids clearance by the host immune system and persists to cause disease many years after exposure. Among the virulence strategies employed by *M. tuberculosis* is the active suppression of innate immunity, including inhibiting the production of the cytokine interleukin-12 (IL-12) by macrophages (Giacomini et al., 2001; Nau et al., 2002). Suppression of IL-12 production is shared by diverse pathogens including *M. tuberculosis*, *Histoplasma capsulatam*, and

Leishmania major (Marth and Kelsall, 1997; Reiner et al., 1994). Research into the pathogenicity of these microbes has revealed host and pathogen molecules involved in cytokine suppression and a number of models have been proposed; however, a clear mechanism of IL-12 inhibition has yet to emerge (Giacomini et al., 2001; Marth and Kelsall, 1997; Nigou et al., 2001; Pathak et al., 2005; Stanley et al., 2003). In the case of *M. tuberculosis*, IL-12 suppression is known to occur at the level of transcription and appears to be an active process as live *M. tuberculosis* can block the induction of IL-12 by *E. coli* (Nau et al., 2002).

IL-12, a heterodimer of p35 and p40 subunits, acts on T cells to promote the release of interferon-gamma (IFN-γ) and induce polarization towards a Type I, or T_H1, immune response. Humans deficient in IL-12 or the IL-12 receptor are highly susceptible to mycobacterial infection (Altare et al., 1998a; Altare et al., 1998c; de Jong et al., 1998), and knockout mice lacking IL-12 p40 are unable to control *M. tuberculosis* infection (Cooper et al., 2002; Holscher et al., 2001). Administration of exogenous IL-12 enhances resistance to *M. tuberculosis* infection in BALB/c mice, which mount only a weak T_H1 response (Flynn et al., 1995b). Thus IL-12 levels are key for establishing and maintaining the sensitive balance between immune modulation by the pathogen and immune activation by the host (Feng et al., 2005).

Analysis of gene expression changes in macrophages stimulated with purified TLR ligands or infected with various bacterial pathogens has revealed a stereotypical inflammatory response that includes IL-12 (Jenner and Young, 2005; Kopp and Medzhitov, 2003; Nau et al., 2002). Comparative analysis of the gene expression response of macrophages to infection with a variety of bacteria revealed that though *M.*

tuberculosis activates the majority of the stereotypical response, it can decrease the induction of specific genes, including IL-12 p40 (Nau et al., 2002). Activation of the broad transcriptional response and IL-12 induction that does occur following *M. tuberculosis* infection requires signaling through TLR2 and TLR9, and mice lacking both TLR2 and TLR9 or the adaptor molecule MyD88 exhibit increased susceptibility to *M. tuberculosis* infection compared to wild-type mice (Bafica et al., 2005; Scanga et al., 2004; Shi et al., 2005). In addition to TLR signaling, the type I interferon receptor also plays an important role in inducing molecules critical to *M. tuberculosis* control (Shi et al., 2005). Analysis of gene expression changes following infection of wild-type and mutant macrophages lacking TLR2, TLR4, TLR9, MyD88, or type I interferon receptor has begun to reveal the contributions of individual signaling molecules to the stereotypical transcriptional response to *M. tuberculosis* (Ehrt et al., 2001; Shi et al., 2005; Shi et al., 2003).

Since *M. tuberculosis* specifically inhibits a subset of genes, within the stereotypical response, bacterial factors responsible for this modulation of the immune response are likely important for virulence. Genetic screens have identified bacterial genes required for survival in mice and macrophages, some of which are important for controlling immune responses. The ESX-1 secretion system, a Sec-independent alternate protein secretion pathway in *M. tuberculosis*, is one such immuno-modulatory pathway in *M. tuberculosis* that is required for virulence (Fortune et al., 2005; Guinn et al., 2004; Hsu et al., 2003; MacGurn et al., 2005; Stanley et al., 2003). Several components of the ESX-1 system have been identified, including three proteins containing putative ATPase domains (Rv3870, Rv3871, and Rv3876) and a multiple transmembrane domain-

containing protein (Rv3877) that may serve as a channel. The primary substrates of this alternate secretion pathway are the immunodominant antigens ESAT-6 and CFP-10, though an additional substrate has recently been identified (Fortune et al., 2005; MacGurn et al., 2005). In addition to exhibiting decreased virulence, mutants in ESX-1 secretion system components or in the substrate ESAT-6 elicit elevated levels of IL-12 from macrophages. However, the mechanism by which this pathway modulates host responses is unclear.

Mycobacteria are unique from other bacteria in the complexity of the lipids in the cell wall, and it has been proposed that lipid components of the mycobacterial cell wall engage in unique interactions with immune cells (Brennan and Nikaido, 1995; Karakousis et al., 2004). Indeed, two related lipids synthesized and secreted by *M. tuberculosis*, phthiocerol dimycocerosate (PDIM) and phenolic glycolipid (PGL), are critical virulence determinants and play a role in modulating host responses to infection (Camacho et al., 1999; Cox et al., 1999; Manca et al., 2001; Reed et al., 2004; Rousseau et al., 2004). *M. tuberculosis* strains that fail to synthesize PDIM or PGL, a glycosylated version of PDIM, elicit elevated levels of the cytokines IL-6 and TNF-α from macrophages (Reed et al., 2004; Rousseau et al., 2004). Though neither PDIM nor PGL has been shown to suppress IL-12 levels, their role in modulating macrophage responses highlights the importance of unique lipids synthesized by *M. tuberculosis* in the host-pathogen interaction.

Here we have taken a forward genetic approach to identify pathogen pathways important for regulating IL-12 production in response to *M. tuberculosis* through a series of genetic screens. Surprisingly, we identified novel ESX-1 secretion components,

indicating both the importance of this system to cytokine regulation and the power of the screen to identify novel members of pathways important for bacterial survival. In addition, we found that the pathogenic lipid PDIM is critical to limiting the cytokine response to *M. tuberculosis*. We found that elevated cytokine levels induced by mutants in either of these pathways required TLR2, suggesting that wild-type *M. tuberculosis* interferes with signaling through the TLR pathway. Finally, we used global gene expression profiling to further characterize the macrophage response to *M. tuberculosis* and to identify a specific subset of TLR2-dependent responses, in addition to IL-12, modulated by the ESX-1 secretion system.

Results

M. tuberculosis-*mediated IL-12 suppression in murine macrophages*

The dominant inhibitory effect of *M. tuberculosis* infection on IL-12 induction, including the ability to inhibit signals from strong stimulants such as LPS, was originally demonstrated in human macrophages (Giacomini et al., 2001; Nau et al., 2002). To determine if this activity was manifest in murine macrophages, we infected bone marrow derived macrophages (BMDM) from C57BL/6 mice, challenged with LPS, and measured IL-12 p40 by ELISA. As in human macrophages, infection of BMDM with *M. tuberculosis* blocked the majority of IL-12 production elicited by LPS treatment alone (Fig 1A). This dominant suppression displayed dose-dependence as infection with increasing multiplicity of infection (MOI) of *M. tuberculosis* resulted in additional suppression of LPS-induced IL-12 p40 production (Fig 1A).

Since IL-12 p40 is primarily regulated transcriptionally, we measured p40 mRNA levels by quantitative real-time PCR (qPCR) on total RNA harvested from macrophages infected with live and heat-killed *M. tuberculosis*. Although a small amount of p40 mRNA was induced by live bacteria, heat-killed *M. tuberculosis* induced IL-12 to five-fold greater levels (Fig 1B), demonstrating that *M. tuberculosis* inhibition of IL-12 secretion requires an active process that limits p40 transcriptional activation.

Screen for M. tuberculosis *mutants that fail to suppress IL-12 p40*

To elucidate the mechanism utilized by *M. tuberculosis* to suppress IL-12 production, we took a genetic approach to identify mycobacterial mutants that fail to suppress IL-12 production by macrophages. We reasoned that cytokine regulation would be important for *M. tuberculosis* survival in host tissues, so we screened a set of 80 mutants previously determined to be attenuated for growth in mice to ascertain if any of these mutants were defective for IL-12 suppression (Cox et al., 1999). For this screen, BMDM were infected at an MOI of 10, culture supernatants were harvested 24 hours post-infection, and secreted IL-12 p40 levels were measured by ELISA. The screen yielded nine mutants that induced macrophage IL-12 production at least three-fold greater than wild-type, though none to the level of heat-killed bacteria (Fig 1C). Neither *mmpl8*$^{-}$ nor *pcaA*$^{-}$, two other mutants attenuated in the lungs of mice, induced significantly more IL-12 p40 than wild-type, demonstrating that aberrant cytokine induction is not a general characteristic of all attenuated *M. tuberculosis* mutants (Fig 1C).

Of the nine genes identified in the screen as required for muted IL-12 induction, seven had known functions. Importantly, these seven genes clustered into two known biochemical pathways. First, *fadD26*, *mmpL7*, and *drrC* are genes involved in the production or secretion of the lipid PDIM (Fig 1C). Second, genes involved in the ESX-1 secretion system, *esxA*, *Rv3870*, *Rv3871*, and *Rv3877*, are required for IL-12 inhibition (Fig 1C). Although we had previously found that ESX-1 secretion was required for modulation of macrophage responses, it was unexpected to identify multiple genes involved in PDIM synthesis and secretion through this screen.

In the remaining two mutants, the disrupted genes were not previously characterized. The first mutant carries a transposon insertion thirteen nucleotides upstream of the gene *Rv3849*, which encodes a 14kD protein that is conserved throughout actinomycetes but shares little homology to other proteins encoded by *M. tuberculosis*. The second mutant is disrupted in the *PE2* gene (*Rv0152c*), encoding a 56kD member of the PE family of proteins. The role of the PE family remains unclear, though multiple functions have been ascribed to different PE proteins (Brennan and Delogu, 2002).

Rv3849 and PE2 are novel ESX-1 genes

Because most of the mutants that induced elevated IL-12 levels were defective for PDIM or ESAT-6 biogenesis, we tested whether the mutants in *Rv3849* or *PE2* were defective for synthesis or secretion of PDIM or ESAT-6. To monitor PDIM, we utilized a method that allowed us to radioactively label PDIM and identify the lipid in whole cell and surface exposed fractions. Briefly, we labeled lipids for 18 hours through the addition of ^{14}C-propionic acid to log phase cultures. Cells were then pelleted, washed, and lipids

were separated into a whole cell and cell wall associated fractions which were then separated by thin layer chromatography (TLC) (Converse et al., 2003). In wild-type cells, PDIM is secreted and thus present in both fractions, whereas in the *mmpL7* transporter mutant the lipid is present only in the cell pellet fraction (Fig 2, lanes 1, 2, 5, 6). Analysis of lipids from *PE2*$^{-}$ and *Rv3849*$^{-}$ bacteria revealed PDIM is present at levels comparable to wild-type in both surface-associated and whole cell lipid fractions indicating that neither gene is required for PDIM synthesis or secretion (Fig 2, lanes 1, 3, 4, 5, 7, 8).

We next tested whether mutants in *PE2* or *Rv3849* were defective in ESX-1 secretion by assaying cell pellets and culture supernatants for the presence of ESAT-6 and CFP-10 by immunoblotting. In wild-type cells, ESAT-6 and CFP-10 were present predominantly in the supernatant, whereas in *Rv3877* mutant bacteria they were confined to the cell pellet (Fig 3A). Like *Rv3877*$^{-}$ cells, both *PE2* and *Rv3849* mutants fail to secrete ESAT-6 and CFP-10 (Fig 3A). Introducing a single copy of the respective gene under the control of its native promoter restored ESAT-6 secretion and IL-12 suppression to the *PE2* and *Rv3849* mutants (Fig 3B). Therefore, our genetic screen identified two novel components of the ESX-1 system. The identification of these mutants along with the known ESX-1 genes in our screen indicates that this alternate protein secretion pathway is central to modulation of macrophage responses.

To further characterize the ESX-1 pathway mutants, we measured bacterial growth in the lungs of intravenously infected C57BL/6 mice. In the first three weeks following infection, wild-type *M. tuberculosis* grew approximately two logs in the lungs. In contrast, the *Rv3849* and *PE2* mutants exhibited a striking growth defect in the first 5 days post-infection (Fig 3C and D). After the initial 5 days, both mutants recovered

normal growth kinetics and achieved nearly comparable bacterial numbers to wild-type *M. tuberculosis* by three weeks post-infection (Fig 3C and D). This pattern of growth in the lungs is nearly identical to that seen with other ESX-1 secretion mutants under similar infection conditions (Stanley et al., 2003). The specific early growth defect of the ESX-1 mutants during *in vivo* infection in addition to their failure to control cytokine production by macrophages *ex vivo* suggests that the ESX-1 system is critical for manipulating macrophage activity during the early, innate immune response.

Increases in pro-inflammatory cytokine production are not a result of decreases in anti-inflammatory cytokine production

A possible mechanism for IL-12 suppression suggested by Giacomini *et al.* is the reciprocal induction of the anti-inflammatory cytokine IL-10 by *M. tuberculosis* (Giacomini et al., 2001). To address the possibility that the IL-12 suppressionless mutants simply fail to induce IL-10, we measured IL-10 protein and mRNA levels following *M. tuberculosis* infection of macrophages. IL-10 protein was undetectable by ELISA in culture supernatants of macrophages infected with wild-type, mutant, or heat-killed *M. tuberculosis* at 24 hours post-infection. IL-10 mRNA was close to the limit of detection by qPCR, and no difference was observed in transcript levels from macrophages infected with wild-type, mutant, or heat-killed bacteria (data not shown).

We also repeated an experiment performed by Giacomini *et al.* in which soluble IL-10 receptor was added to macrophages to neutralize secreted IL-10. As previously reported, the addition of soluble IL-10 receptor led to increased IL-12 levels released by macrophages infected with wild-type *M. tuberculosis* (Fig 4A). Two lines of evidence,

however, suggested that IL-10 was not solely responsible for the IL-12 p40 suppression. First, the amount of p40 elicited by wild-type *M. tuberculosis* in the presence of soluble IL-10 receptor was much lower than the amount elicited by IL-12 suppressionless mutants or heat-killed *M. tuberculosis*. Secondly, the amount of p40 elicited by mutant and heat-killed *M. tuberculosis* increased further in the presence of soluble IL-10 receptor (Fig 4A). Together, these results argue that although IL-10 induction by *M. tuberculosis* can lower IL-12 levels, the increased IL-12 levels induced by IL-12 suppressionless mutants are not due to a lack of IL-10.

Similarly, variations in virulence between clinical *M. tuberculosis* isolates are correlated with the induction of type I interferons (IFN-α and -β). Specifically, strains with greater virulence in mice induce higher levels of type I interferons, which may in turn lead to decreased IL-12 production (Manca et al., 2001). However, wild-type *M. tuberculosis* still suppressed IL-12 production in type I interferon receptor knockout macrophages, demonstrating that type I interferons are not responsible for IL-12 inhibition (data not shown).

Finally, to more generally test the possibility that wild-type *M. tuberculosis* induces macrophages to secrete a diffusible inhibitory molecule that results in diminished IL-12 p40, we transferred culture media from infected macrophages onto LPS-stimulated macrophages in an attempt to confer inhibition in *trans*. LPS-induced IL-12 p40 was not suppressed by the addition of media from *M. tuberculosis*-infected macrophages (Fig 4B). The culture medium from *M. tuberculosis*-infected macrophages also failed to suppress p40 induced by heat-killed *M. tuberculosis*. In conclusion, the low level of pro-inflammatory cytokine induction by wild-type *M. tuberculosis* does not result solely from

the production of a diffusable anti-inflammatory cytokine or other inhibitory molecule. Taken together, these results support the notion that *M. tuberculosis*-mediated inflammatory cytokine suppression is cell-autonomous.

IL-12 induction by M. tuberculosis *requires Toll signaling*

Since IL-12 is induced by TLR signaling, it is possible that wild-type *M. tuberculosis* inhibits IL-12 induction by interfering with TLR signals. To test this idea, we infected macrophages lacking the common TLR adaptor molecule MyD88 with both wild-type and mutant *M. tuberculosis* and measured IL-12 p40 transcript by qPCR. The ability of mutants from both classes (PDIM and ESX-1) to elicit elevated levels of IL-12 p40 was severely attenuated in macrophages lacking MyD88 (Fig 5A). Likewise, in the absence of TLR2, the dominant receptor activated by *M. tuberculosis*, IL-12 p40 induction by PDIM and ESX-1 secretion mutants was greatly diminished (Fig 5B). In the case of both $MyD88^{-/-}$ and $TLR2^{-/-}$ macrophages, mutant *M. tuberculosis* still elicited more IL-12 p40 than wild-type bacteria, suggesting additional minor pathways to IL-12 induction activated more strongly by mutant bacteria than wild-type (Fig 5A and B). Although TLR4 can respond to some *M. tuberculosis* ligands (Means et al., 1999), this receptor was not essential for elevated IL-12 induction by mutant *M. tuberculosis* (data not shown). These results indicate that elevated IL-12 p40 transcription in response to PDIM and ESX-1 mutant *M. tuberculosis* results predominantly from either greater activation of or a failure to interfere with MyD88-dependent signaling through TLR2.

Mutant M. tuberculosis *induces elevated levels of multiple TLR2 targets*

Modulating host immune responses is often accomplished by secreted bacterial virulence factors requiring specialized alternative secretion pathways. To identify the full complement of responses affected by the ESX-1 system, we measured the global transcriptional response of macrophages to wild-type and ESX-1 mutant *M. tuberculosis*. We utilized spotted oligonucleotide microarrays covering 88% of the open reading frames (ORFs) in the mouse genome (Mouse Exonic Evidence-based Oligonucleotide, MEEBO, arrays) (Verdugo and Medrano, 2006). Bone marrow derived macrophages were infected with wild-type and ESX-1 mutant *M. tuberculosis*, and total RNA was harvested over a 16 to 24 hour timecourse post-infection. The relative abundance of each mRNA was measured through a standard two-color competitive hybridization between amplified polyA RNA from each infection time point and a reference RNA (Eisen and Brown, 1999). To depict induction or repression over a time course, we divided the expression value at each time point by the value for the corresponding gene at time zero (uninfected macrophages). To identify the set of genes differentially induced or repressed by ESX-1 mutant *M. tuberculosis*, the relative abundance of each mRNA species following infection with mutant *M. tuberculosis* at each timepoint was divided by the relative abundance of the corresponding mRNA following infection with wild-type bacteria. Remarkably, the results of this analysis revealed that out of nearly 2000 genes that are regulated by *M. tuberculosis* infection, only 48 genes were at least three-fold more highly induced or repressed in response to ESX-1 mutant than to wild-type bacteria (Fig 6A). These 48 genes fell into a group of 12 genes expressed at a lower level in response to mutant bacteria than to wild-type bacteria (Group 1) and a group of 36 genes

expressed more highly in response to mutant bacteria than to wild-type bacteria (Group 2).

The analysis in Figure 6A does not reveal whether this subset of genes were induced or repressed following infection. To reveal these changes, we extracted the temporal expression profiles of the 48 differentially expressed genes from the original timecourse analysis. Temporal analysis of these genes revealed that all Group 1 genes are induced more highly by wild-type bacteria than by ESX-1 mutant *M. tuberculosis* following infection (Fig 6B). Interestingly, the timecourse data also reveals that Group 2 consists of 2 subsets: 14 genes that are induced more highly upon infection with mutant bacteria than with wild-type bacteria, including IL-12 p40; and 22 genes that are repressed more strongly following infection with wild-type bacteria than with mutant *M. tuberculosis* (Fig 6B).

Because elevated IL-12 p40 induction by ESX-1 mutant bacteria depends on TLR2, we measured global gene expression responses of $TLR2^{-/-}$ macrophages infected with wild-type and ESX-1 mutant *M. tuberculosis*. Interestingly, the differential expression of Group 1 genes is maintained in macrophages lacking TLR2 (Fig 6C); these genes are more highly expressed in macrophages infected with wild-type bacteria than those infected with ESX-1 mutant bacteria despite the absence of TLR2. In contrast, the differential expression of the Group 2 genes is lost in the absence of TLR2 (Fig 6C) as the relative abundance of these transcripts is comparable in $TLR2^{-/-}$ macrophages following infection with wild-type or mutant *M. tuberculosis*. This analysis identified a set of 36 genes that are aberrantly expressed in response to ESX-1 mutant *M. tuberculosis* that are TLR2-dependent.

M. tuberculosis *induces a broad gene expression response, but ESX-1 system targets a narrow subset of TLR2-dependent genes*

We next sought to determine what proportion of the overall TLR2-dependent response was represented by the 36 genes modulated by the ESX-1 system. To this end, we used MEEBO arrays to perform similar analyses to previous studies which utilized microarrays to measure macrophage responses to *M. tuberculosis* infection and to determine the contribution of TLR signaling to those responses (Ehrt et al., 2001; Shi et al., 2005; Shi et al., 2003). First, we monitored the temporal transcriptional response of wild-type macrophages to wild-type *M. tuberculosis* and found that 1963 genes are at least three-fold more highly induced or repressed following *M. tuberculosis* infection (Fig 7A). The breadth of the response exceeds those previously reported (Ehrt et al., 2001; Shi et al., 2005; Shi et al., 2003), likely due to the more extensive coverage of the MEEBO arrays used here. Additionally, we observed that the number of genes repressed by infection is comparable to the number of genes induced by infection, a finding noted previously (Ehrt et al., 2001; Shi et al., 2003).

To identify genes that require TLR2 or MyD88 for their induction or repression following *M. tuberculosis* infection, the relative abundance of each mRNA species at each timepoint following infection of mutant macrophages was divided by the relative abundance of the corresponding mRNA at the corresponding timepoint following infection of wild-type macrophages. The number of genes whose expression is at least four-fold altered in the absence of TLR2 or MyD88 (583 genes) exceeds previous findings (Fig 7B) (Shi et al., 2005; Shi et al., 2003). As in previous studies, however, this

still represents fewer than half of the responses regulated by *M. tuberculosis* infection, in agreement with the suggestion that MyD88 signaling is particularly important for priming macrophages to respond to other signals (Shi et al., 2005; Shi et al., 2003). However, we also discovered that the positive and negative amplitude of the overall response is noticeably diminished in the absence of MyD88 and TLR2 (Fig 7A). By comparing the amplitude of the expression changes of the 1963 genes regulated by infection, we found an average decrease in gene induction or repression of 30% in MyD88$^{-/-}$ macrophages and 29% in TLR2$^{-/-}$ macrophages relative to wild-type macrophages. This can be seen as a general dampening of the amplitude of the global transcriptional response (Fig 7A). Though this reduction could be considered minor for a single gene, the cumulative effect of small changes over a large number of genes could result in significant functional consequences, suggesting a more integral role for MyD88 in regulating early gene expression following exposure to *M. tuberculosis* than previously described (Shi et al., 2003).

Interestingly, the 36 TLR2-dependent genes aberrantly expressed in response to ESX-1 mutant bacteria represent only a small subset of the total TLR2-dependent response of macrophages to *M. tuberculosis*. This finding clearly demonstrates that the ESX-1 secretion system specifically modulates a subset of TLR2-dependent host responses. The failure of ESX-1 mutants to modulate early transcriptional responses in macrophages correlates with their early growth defect *in vivo*.

Discussion

Many pathogens have devised intricate interactions with the host immune system in order to create a protected survival niche. Genes required for these interactions have been well-characterized in a diverse array of bacteria, many of which rely on specialized secretion systems for interacting with the host. Far less has been discovered about the interactions between mycobacteria and their hosts. The adaptation of *M. tuberculosis* to life within the host is particularly remarkable in that it can survive within the host for decades. This may be due to the fact that *M. tuberculosis* has co-evolved with humans for tens of thousands of years (Gutierrez et al., 2005). In the work presented here, we have identified bacterial genes required for one specific host-pathogen interaction: the modulation of macrophage responses to *M. tuberculosis*. The ESX-1 secretion system and the pathogenic secreted lipid PDIM are required for limiting the macrophage response to *M. tuberculosis*. From a diverse collection of mutants attenuated *in vivo*, only mutants in these two discrete pathways were deficient in controlling cytokine levels, indicating that *M. tuberculosis* utilizes specific mechanisms to subvert innate immune responses. Modulation of the host response by secreted molecules such as ESX-1 substrates and PDIM is reminiscent of the importance of Type III and Type IV secretion systems for the pathogenicity of Gram negative bacteria.

It was surprising that two seemingly disparate pathways are each required to modulate macrophage responses to *M. tuberculosis*. Until now, no connection has been made between ESX-1 secretion and PDIM secretion. Mutants in ESX-1 secretion have previously been shown to be required to modulate macrophage responses to *M. tuberculosis* (Stanley et al., 2003). Similarly, PDIM and related lipids have been shown to play a role in regulating immune responses to *M. tuberculosis* (Reed et al., 2004;

Rousseau et al., 2004). The requirement of both pathways suggests cooperation between ESX-1 secretion and PDIM towards creating the optimal macrophage environment for *M. tuberculosis*.

One simple model for the dual requirement of ESX-1 secretion and PDIM is that both secreted PDIM and ESX-1 substrates are required for signaling inhibition in macrophages. This could occur through the cooperative action of PDIM and secreted *M. tuberculosis* proteins on one or more inflammatory signaling molecules. It is also possible that PDIM and ESX-1 secretion are required for proper formation of the *M. tuberculosis* cell wall. Defects in cell wall production may alter the interaction between *M. tuberculosis* and macrophage receptors, such as TLR2. The microarray results presented here contradict the latter model. Our data indicate that only a subset of TLR2-dependent genes is more highly induced by ESX-1 mutant bacteria than wild-type *M. tuberculosis*. In contrast, inappropriate signaling through TLR2 would most likely appear as uniform upregulation of all TLR-dependent genes. Reed *et al.* have shown that purified PGL, a lipid related to PDIM, can suppress macrophage immune responses (Reed et al., 2004). This suggests a possible role for pathogenic mycobacterial lipids outside the context of the cell wall, and PDIM may act similarly to modulate macrophage responses. It has previously been shown that mycobacterial lipids gain access to disparate membrane compartments in macrophages (Beatty et al., 2000), so it is possible that PDIM is able to influence events within macrophages once secreted by *M. tuberculosis*. It is intriguing to speculate that both proteins secreted by the ESX-1 system and PDIM gain access to macrophage signaling molecules and act independently to disrupt signaling.

We have identified two novel members of the ESX-1 alternative protein secretion system. Until now, genes in the ESX-1 secretion pathway have either been closely linked in one genomic locus or homologous to genes within that locus (Fortune et al., 2005; Guinn et al., 2004; Hsu et al., 2003; MacGurn et al., 2005; Stanley et al., 2003). The two novel ESX-1 secretion pathway proteins, PE2 and Rv3849, are unlinked and non-homologous to previously described ESX-1 components, making their identification through this screen even more notable. The *PE2* gene encodes a 56kD member of the PE family of proteins. Though PE2 lacks a signal sequence, other PE proteins have been localized outside the bacterial cell membrane (Delogu et al., 2004). The previously described Rv3877 protein transports ESAT6 and CFP10 out of the cytosol (Guinn et al., 2004; Hsu et al., 2003; Stanley et al., 2003), but it has been unclear whether an ESX-1 component is required for transit across the mycobacterial cell wall. It is possible that PE2 performs this function.

The ESX-1 and PDIM mutants identified in our work are the first *M. tuberculosis* mutants described with altered TLR-dependent responses in macrophages. Inappropriate activation of TLR responses by the ESX-1 mutants correlates with their defective growth early after infection of mice. Importantly, only a subset of TLR2-dependent responses is altered by ESX-1 mutant *M. tuberculosis*, revealing a defined group of macrophage genes that are specifically modulated by *M. tuberculosis*. Though the mechanism is unclear, Ogawa *et al.* demonstrated that certain nuclear receptors can activate or repress subsets TLR-regulated genes (Ogawa et al., 2005). Given our observation that *M. tuberculosis* suppresses a subset of TLR2 responses, it is interesting to propose a model in which *M.*

tuberculosis activates a specific nuclear receptor pathway or another anti-inflammatory pathway in order to modulate specific macrophage responses to infection.

Other host molecules have recently been described for their role in dampening TLR-dependent responses in macrophages, including IRAK-M and IkappaBNS (Kobayashi et al., 2002; Kuwata et al., 2006). In particular, Pathak *et al.* demonstrated that IRAK-M expression is induced by *M. tuberculosis* infection and required for suppression of LPS-induced IL-12 p40 (Pathak et al., 2005). In our microarray experiments, we found that IRAK-M and IkappaBNS were induced by both wild-type and ESX-1 mutant *M. tuberculosis*, making them unlikely players in the differential induction of TLR2 targets by mutant bacteria (Figure 8). IRAK-M and IkappaBNS induction by *M. tuberculosis* is TLR2-dependent, however, suggesting a potential role for these two host proteins in the *M. tuberculosis*-mediated, TLR2-dependent gene repression observed in the microarray experiments reported here and in previous studies (Ehrt et al., 2001; Shi et al., 2003).

Our gene expression analysis has expanded on the work of others and has provided an abundance of new information about the global transcriptional changes in macrophage biology in response to *M. tuberculosis*. The greater transcript coverage of the MEEBO arrays yielded a more comprehensive dataset of transcriptional responses to infection with *M. tuberculosis*, including numerous regulated genes whose role in response to infection is currently unknown. In other respects, our results resemble previous global transcript analyses of *M. tuberculosis*-infected macrophages (Ehrt et al., 2001; Shi et al., 2005; Shi et al., 2003). Two particularly interesting observations are the large number of genes repressed by *M. tuberculosis* infection and the numerous genes

affected by *M. tuberculosis* infection independently of MyD88 (Ehrt et al., 2001; Shi et al., 2003). The great majority of studies of macrophage responses to pathogens focus on the genes upregulated following infection; however, down-regulated genes may play an equally important role to the survival of the host cell or the pathogen. As demonstrated in previous studies, MyD88 and TLR2 significantly influence only a minority of *M. tuberculosis*-regulated genes, suggesting that additional, undescribed antimicrobial pathways are activated during *M. tuberculosis* infection (Ehrt et al., 2001; Shi et al., 2005; Shi et al., 2003). Interestingly, we observed a global muting of the macrophage transcriptional response to *M. tuberculosis* in the absence of MyD88 that has not been previously described. This finding suggests an additive or synergistic role for Toll signaling in the early gene expression response of macrophages to *M. tuberculosis* infection.

As increasingly comprehensive arrays are used for global gene expression analyses, the number of genes regulated by infection greatly exceeds our understanding of immune responses to bacteria. Comparative analyses of macrophage responses to wild-type bacteria and avirulent mutants as described here help identify specific genes involved in control of *M. tuberculosis* infection. The 48 genes differentially induced or repressed by wild-type bacteria compared to ESX-1 mutants define a set of macrophage responses modulated by *M. tuberculosis*. This group of genes is likely of particular importance either to control of infection or survival of the bacteria within the host, and represent promising targets for further study of the macrophage-*M. tuberculosis* interaction.

Materials and Methods

Bacterial Strains and Plasmids

M. tuberculosis Erdman cultivation and generation of bacterial mutants were as previously described (Cox et al., 1999). For infections, bacteria were grown in Middlebrook 7H9 medium supplemented with oleic acid-albumin-dextrose-catalase (OADC), 0.5% glycerol, and 0.05% Tween-80, and for culture supernatant preparation bacteria were grown in Sauton's medium (MacGurn et al., 2005). Bacteria were heat-killed by boiling for 20 minutes. The *Rv3849*$^{-}$ strain carries a transposon insertion 13 nucleotides upstream of the initiation codon and is named STM11D6; the *PE2*$^{-}$ strain carries a transposon insertion 78 nucleotides into the open reading frame and is named STM8B7. Complementation plasmids were generated by PCR and cloning *Rv3849* (pSR211) and *PE2* (pSR214) from *M. tubeculosis* Erdman along with 200 nucleotides of upstream promoter sequence into pMV306.Kan.

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). TLR2$^{-/-}$ and MyD88$^{-/-}$ mice were generous gifts of Dr. Ralph Isberg (Tufts University Medical School, Boston, MA) and Dr. Dan Portnoy (University of California, Berkeley, Berkeley, CA), respectively. Both knockout mouse strains were at least 10 generations back-crossed to C57BL/6. All mice were housed and treated humanely as described in an animal care protocol approved by the UCSF Institutional Animal Care and Use Committee.

Protein Preparation and Analysis

Concentrated culture supernatants were collected as previously described (Stanley et al., 2003). *M. tuberculosis* strains were grown to mid-log phase in Middlebrook 7H9 media supplemented with 0.05% Tween-80, diluted back to OD600=0.05 in Sauton's media supplemented with 0.05% Tween-80, and grown to mid-log phase. Cells were washed, diluted to OD600=0.05 in Sauton's media supplemented with 0.005% Tween-80 and grown for 5 days in roller bottles. Culture supernatants were harvested by centrifugation, filter sterilized, and concentrated. Cell lysates and culture supernatants were separated by SDS-PAGE, and specific proteins were visualized by immunoblotting with antibodies against ESAT-6 (Hyb 76-8), CFP-10 (K8493), or GroEL (HAT5).

Lipid Preparation and Analysis

M. tuberculosis lipid preparation and thin-layer chromatography (TLC) analysis was performed as previously described (Bligh and Dyer, 1959; Converse et al., 2003; Jain and Cox, 2005). Briefly, *M. tuberculosis* lipids were labeled by adding ^{14}C-propionate to actively growing cultures and incubating at 37 ° C for 16 hours. Surface-exposed lipids were extracted by sonicating gently in hexanes and separated from cell-associated lipids by centrifugation. Lipids from both fractions were extracted as previously described (Bligh and Dyer, 1959; Converse et al., 2003; Jain and Cox, 2005) and separated on 10cm x 10cm HPTLC plates (Alltech Associates, Deerfield, IL, USA) using hexanes:ether (9:1).

Macrophage Infection

Macrophages were derived from bone marrow progenitors by culturing with macrophage-colony stimulating factor (M-CSF), as previously described(MacGurn et al., 2005), and frozen at -80 ° C in media plus 20% fetal calf serum and 10% DMSO. Frozen cells were plated one day prior to infection. *M. tuberculosis* cultures were grown in Middlebrook 7H9 supplemented with oleic acid–albumin–dextrose-catalase (OADC), glycerol, and 0.05% Tween-80 to mid-log phase. Inocula were washed in PBS and diluted into DMEM supplemented with 10% Horse Serum. Bacterial inocula were added to macrophages at an MOI of 10. Following phagocytosis for 2 to 4 h, macrophages were washed with PBS and incubated in fresh media at 37 ° C. For cytokine protein concentration measurements, macrophage supernatants were collected and filtered at specified timepoints and assayed for IL-12 p40 concentrations by ELISA (BD Biosciences, Palo Alto, CA, USA). For quantitative real-time PCR measurements and microarray analysis, macrophage total RNA was prepared with the RNeasy Midi Kit (Qiagen Inc, Valencia, CA, USA).

Quantitative Real-Time PCR

Three micrograms of macrophage total RNA was reverse transcribed using oligo-deoxythymidine (oligo-dT) priming, and PCR was performed on first strand cDNA using gene-specific primers for IL-12 p40, or β-actin, using SYBR Green (Sigma-Aldrich, St. Louis, MO, USA) for quantification. Primer sequences were: β-actin-F: 5'-aggtgtgatggtgggaatgg; β-actin-R: 5'-gcctcgtcacccacatagga; p40-F: 5'-ggaagcacggcagcagaata ; p40-R: 5'-aacttgagggagaagtaggaatgg (Overbergh et al., 1999). Relative quantities of specific transcripts were determined from a standard curve

generated for each transcript using a reference cDNA and normalized to actin transcript levels.

Mouse Infection

M. tuberculosis cultures were grown to mid-log phase, washed and resuspended in PBS-Tween. Mice were infected intravenously through the tail-vein with 1e6 colony-forming-units. Lungs, spleens, and livers were harvested, homogenized, and plated for bacterial colonies at specified time points as previously described (Converse et al., 2003; Cox et al., 1999; Stanley et al., 2003).

Microarray Analysis

One microgram of total macrophage RNA was amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion Inc, Austin, TX, USA). A reference RNA was created by pooling equal amounts of all aRNAs generated from an experiment. Four micrograms of amplified RNA (aRNA) was labeled with Cy5 and hybridized competitively with reference aRNA labeled with Cy3 (GE Healthcare, Piscataway, NJ, USA) (Eisen and Brown, 1999). Spotted arrays were generated using the Mouse Exonic Evidence-Based Oligonucleotide (MEEBO) oligonucleotide set (Illumina, San Diego, CA, USA). Hybridizations were done at 63 ° C for 20-36 hours, and arrays were scanned on a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). Features on the array were identified using SpotReader software (Niles Scientific, Portola Valley, CA, USA) and included in further analysis only if they met quality control criteria. Relative expression levels were calculated as the ratio of the median Cy5 intensity to the median

Cy3 intensity for each feature on the array. Total Cy5 and Cy3 levels were normalized for each array in NOMAD (http://ucsf-nomad.sourceforge.net/) prior to expression analysis. Data from unflagged features was filtered, and average-centered hierarchical clustering was performed in the Cluster program (Eisen et al., 1998). Filtered and clustered data were visualized using the Java TreeView program (Eisen and Brown, 1999; Eisen et al., 1998). For timecourse analysis, prior to filtering and clustering, relative expression of each transcript at each timepoint was divided by the relative expression of that transcript in uninfected macrophages. For comparative analysis of responses of different macrophage strains or to different bacterial stimuli, the relative expression level of each transcript in one experimental condition was divided by the relative expression level of that transcript in another experimental condition.

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Figure 2.1. Live *M. tuberculosis* inhibits IL-12 production. (A) IL-12 p40 was measured by ELISA from BMDM culture supernatants 4, 12, and 24 h after infection with *M. tuberculosis* and/or stimulation with LPS. Means of duplicate measurements and standard deviations are shown. **(B)** IL-12 p40 mRNA was measured by qPCR and normalized to actin mRNA levels over a 24 h timecourse after infection with live or heat-killed *M. tuberculosis*. Means of triplicate measurements and standard deviations are shown. **(C)** IL-12 p40 protein was measured by ELISA in BMDM culture supernatants 24 hours after infection with wild-type *M. tuberculosis*, heat-killed bacteria, and nine mutants that fail to suppress IL-12 production from macrophages. *MmpL8*$^{-}$ and *pcaA*$^{-}$ are attenuated mutants that can inhibit IL-12 production. Shown are means of two replicate measurements and standard deviations. One representative experiment of at least three is shown in all panels.

Figure 1

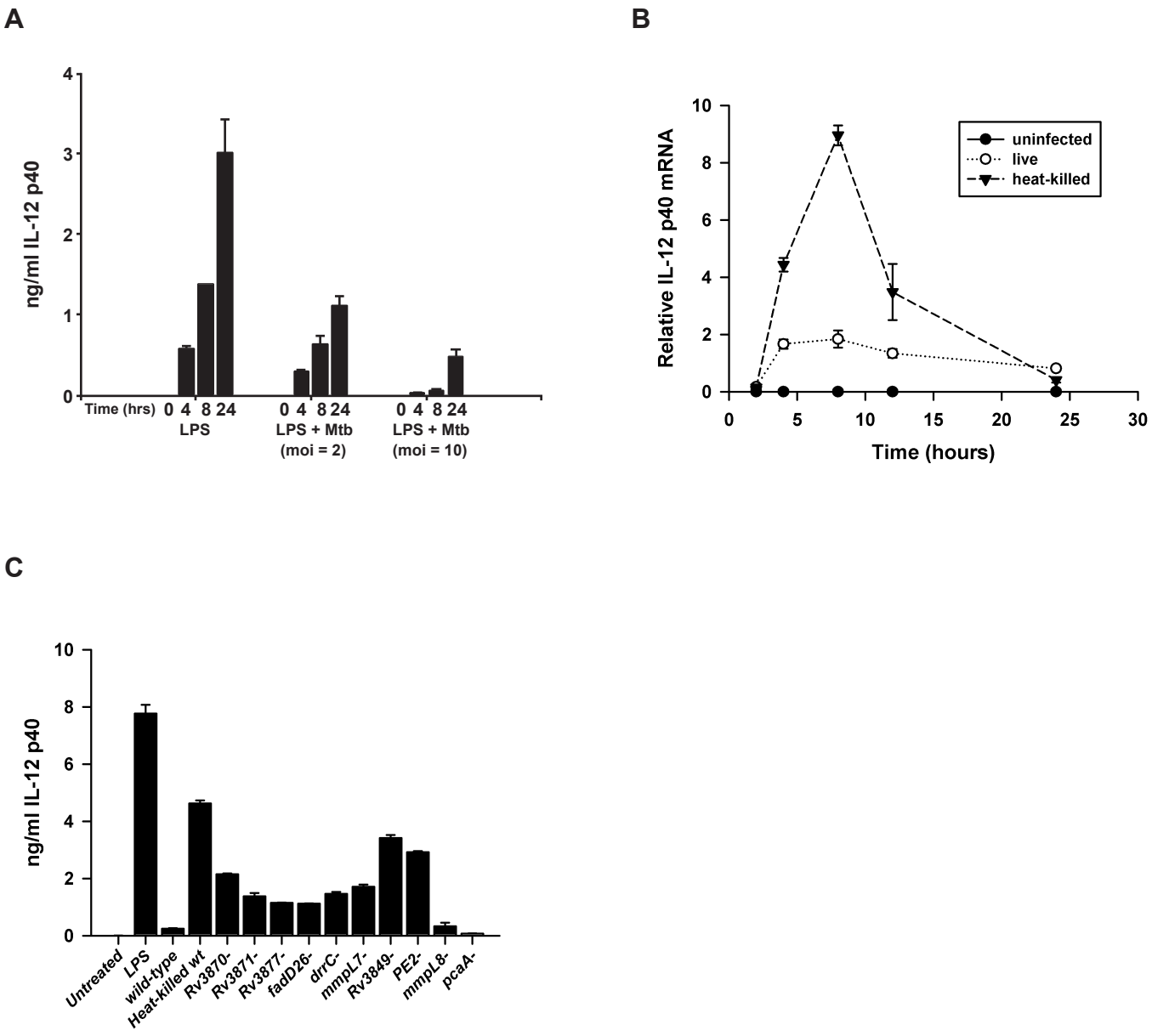

A
ng/ml IL-12 p40
Time (hrs) 0 4 8 24
LPS
0 4 8 24
LPS + Mtb
(moi = 2)
0 4 8 24
LPS + Mtb
(moi = 10)
B
Relative IL-12 p40 mRNA
uninfected
live
heat-killed
Time (hours)
C
ng/ml IL-12 p40
Untreated
LPS
wild-type
Heat-killed wt
Rv3870-
Rv3871-
Rv3877-
fadD26-
drrC-
mmpL7-
Rv3849-
PE2-
mmpL8-
pcaA-

Figure 2.2. *Rv3849*⁻ and *PE2*⁻ mutants are proficient in PDIM synthesis and secretion. Surface-exposed and cell pellet lipids were labeled with ^{14}C-propionic acid and extracted from log phase cultures. PDIM separates into two spots (methoxy and keto forms) when separated on TLC (31, 45). *MmpL7*⁻ mutant cells make but fail to secrete PDIM.

Figure 2

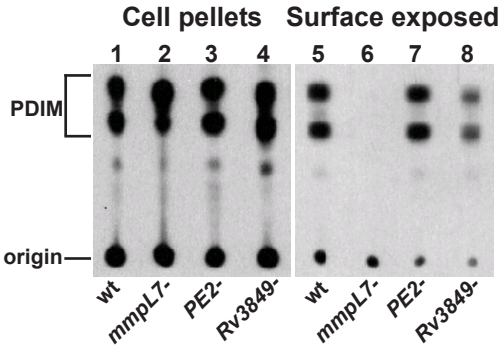
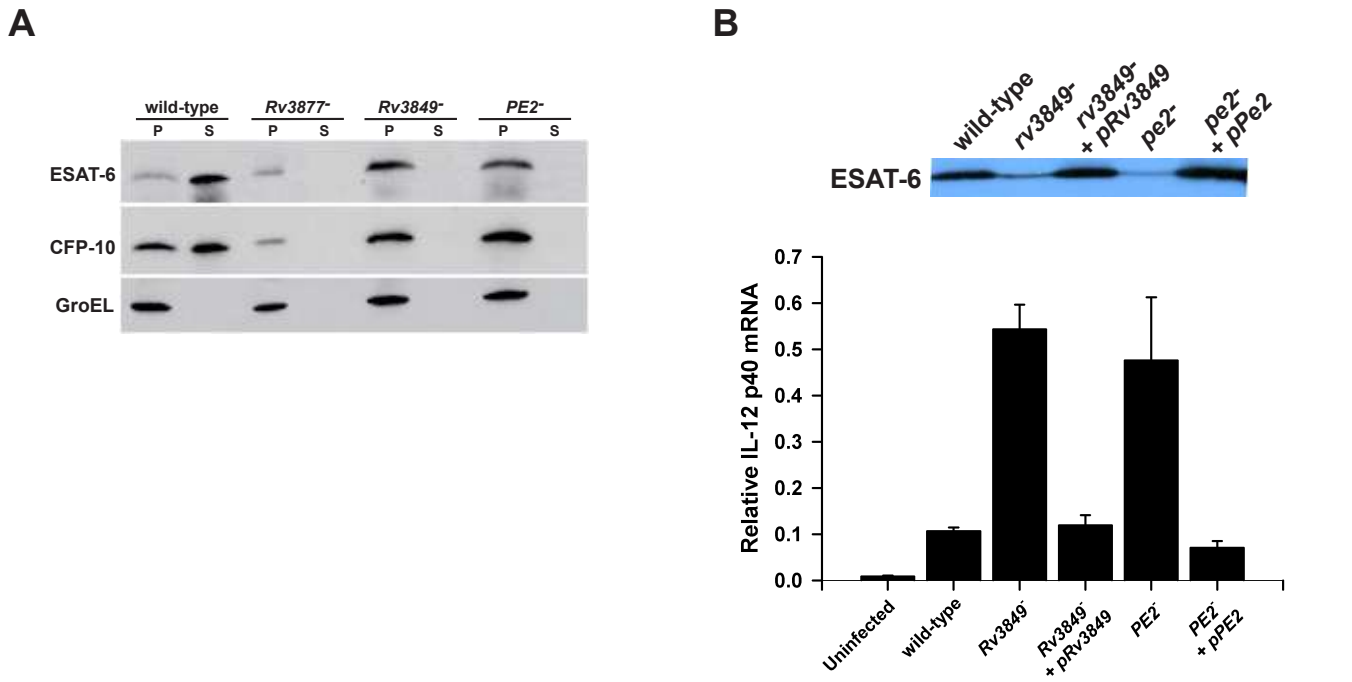
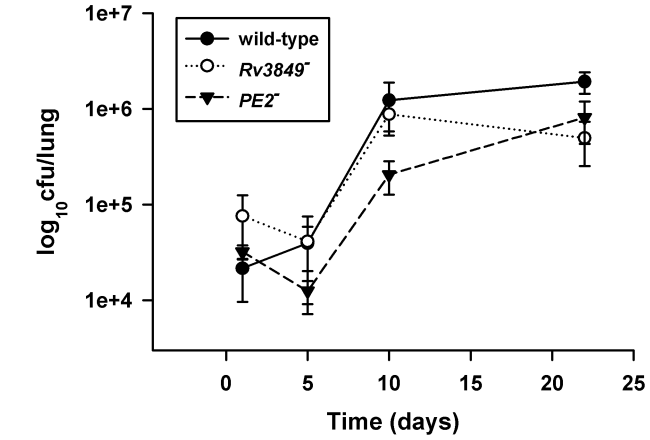

Cell pellets
Surface exposed
1
2
3
4
5
6
7
8
PDIM
origin
wt
mmpL7-
PE2-
Rv3849-
wt
mmpL7-
PE2-
Rv3849-

Figure 2.3. Rv3849 and PE2 are novel members of the ESX-1 secretion pathway. (A) ESAT-6, CFP-10, and GroEL (lysis control) were detected by immunoblotting of total bacterial cell pellets (P) and culture supernatants (S) of wild-type, *Rv3877*$^{-}$, *Rv3849*$^{-}$, and *PE2*$^{-}$ bacteria. **(B)** Expression of a single copy of *Rv3849* and *PE2* in *Rv3849*$^{-}$ and *PE2*$^{-}$ mutant bacteria, respectively, restored ESAT-6 secretion and suppression of IL-12 p40 transcription. Top: ESAT-6 was detected by immunoblotting culture supernatants. Bottom: IL-12 p40 mRNA was measured by qPCR as in Fig 1B at 8 h post-infection. Shown are means and standard deviations of triplicate measurements; one representative experiment of two is shown. **(C)** Growth of bacteria in the lungs of C57BL/6 mice following intravenous infection with 1*10^6 colony-forming units (CFUs) of wild-type and mutant *M. tuberculosis*. **(D)** Lung CFUs from (C) were normalized to initial dose at 24 hours post-infection. For each bacterial strain, means and standard deviations are shown from five mice at each timepoint.

Figure 3


A
wild-type
Rv3877-
Rv3849-
PE2-
P
S
ESAT-6
CFP-10
GroEL
B
wild-type
rv3849-
rv3849- + pRv3849
pe2-
pe2- + pPe2
ESAT-6
Relative IL-12 p40 mRNA
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Uninfected
wild-type
Rv3849-
Rv3849- + pRv3849
PE2-
PE2- + pPE2


C
wild-type
Rv3849-
PE2-
log10 cfu/lung
1e+4
1e+5
1e+6
1e+7
0
5
10
15
20
25
Time (days)

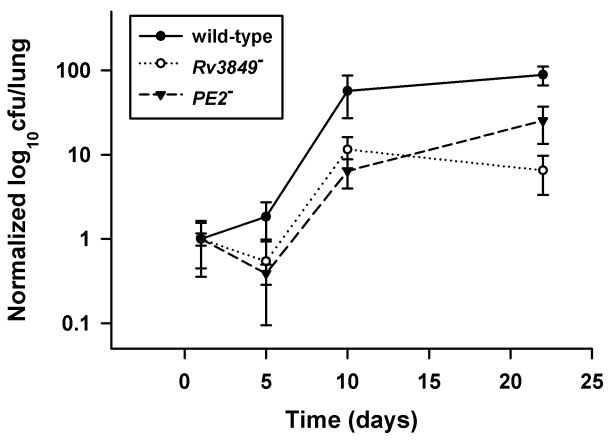

D
wild-type
Rv3849-
PE2-
Normalized log10 cfu/lung
0.1
1
10
100
0
5
10
15
20
25
Time (days)

Figure 2.4. Production of anti-inflammatory cytokines do not account for IL-12 suppression or IL-12 suppressionless phenotype. (A) IL-12 p40 protein was measured by ELISA as in Fig 1C. IL-10 was neutralized by pretreating BMDM with soluble IL-10 receptor where indicated. Means and standard deviations of duplicate measurements from one representative experiment of two. **(B)** IL-12 p40 was measured by ELISA as in Fig 1A in BMDM culture supernatants 24 hours after the indicated treatment (black bars), and 24 hours after exposing cells to spent culture supernatant from LPS-stimulated or infected cells and restimulating with LPS (grey bars). Supernatants from cells treated as indicated were filtered and applied to naïve macrophages which were then stimulated with LPS, and IL-12 p40 was measured in the supernatants after 24 hours (grey bars). Shown are means and standard deviations of one representative experiment of two.

Figure 4

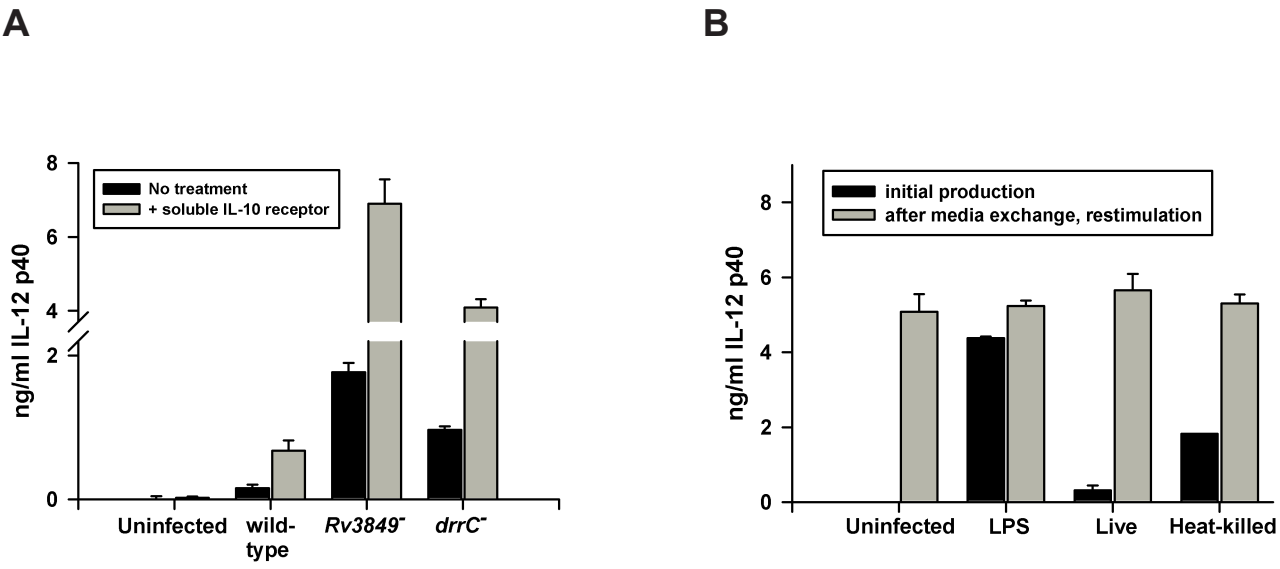

A
B
No treatment
+ soluble IL-10 receptor
ng/ml IL-12 p40
8
6
4
2
0
Uninfected
wild-type
Rv3849-
drrC-
initial production
after media exchange, restimulation
Uninfected
LPS
Live
Heat-killed

Figure 2.5. Elevated IL-12 induction by ESX-1 and PDIM mutants requires MyD88 and TLR2. IL-12 p40 mRNA was measured as in Fig 1B by qPCR 8 h following infection of wild-type C57BL/6, MyD88$^{-/-}$ **(A)**, and TLR2$^{-/-}$ **(B)** BMDMs with wild-type, *Rv3849*$^{-}$, and *drrC*$^{-}$ bacteria. Shown are averages of triplicate measurements and standard deviations for one representative experiment of at least two.

Figure 5

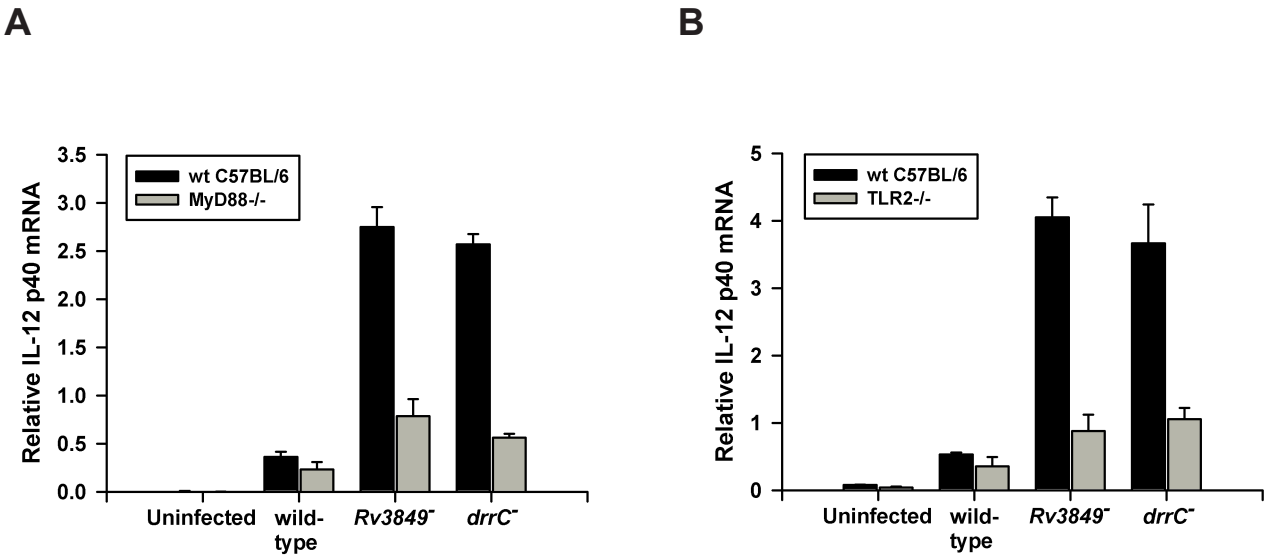

A
B
wt C57BL/6
MyD88-/-
TLR2-/-
Relative IL-12 p40 mRNA
Uninfected
wild-type
Rv3849-
drrC-

Figure 2.6. ESX-1 mutant *M. tuberculosis* causes inappropriate regulation of 48 genes following infection. **(A)** Transcriptional response of each monitored gene to ESX-1 mutant bacteria was divided by response to wild-type bacteria at each timepoint, and genes differentially regulated at least three-fold in each of three experiments were filtered and displayed. Yellow represents genes more highly expressed in response to mutant bacteria than to wild-type bacteria, and blue represents genes more highly expressed in response to wild-type bacteria than to mutant. Grey represents points for which data of sufficiently high quality were not obtained. **(B)** Expression of the 48 differentially regulated genes following infection with wild-type and ESX-1 mutant bacteria was monitored over a 16 h timecourse. Yellow represents upregulation, and blue represents downregulation. **(C)** Analysis similar to (A) in which transcriptional response of each of 48 differentially regulated genes following mutant infection is divided by response following wild-type infection in wild-type (left side) and $TLR2^{-/-}$ (right side) macrophages.

Figure 6

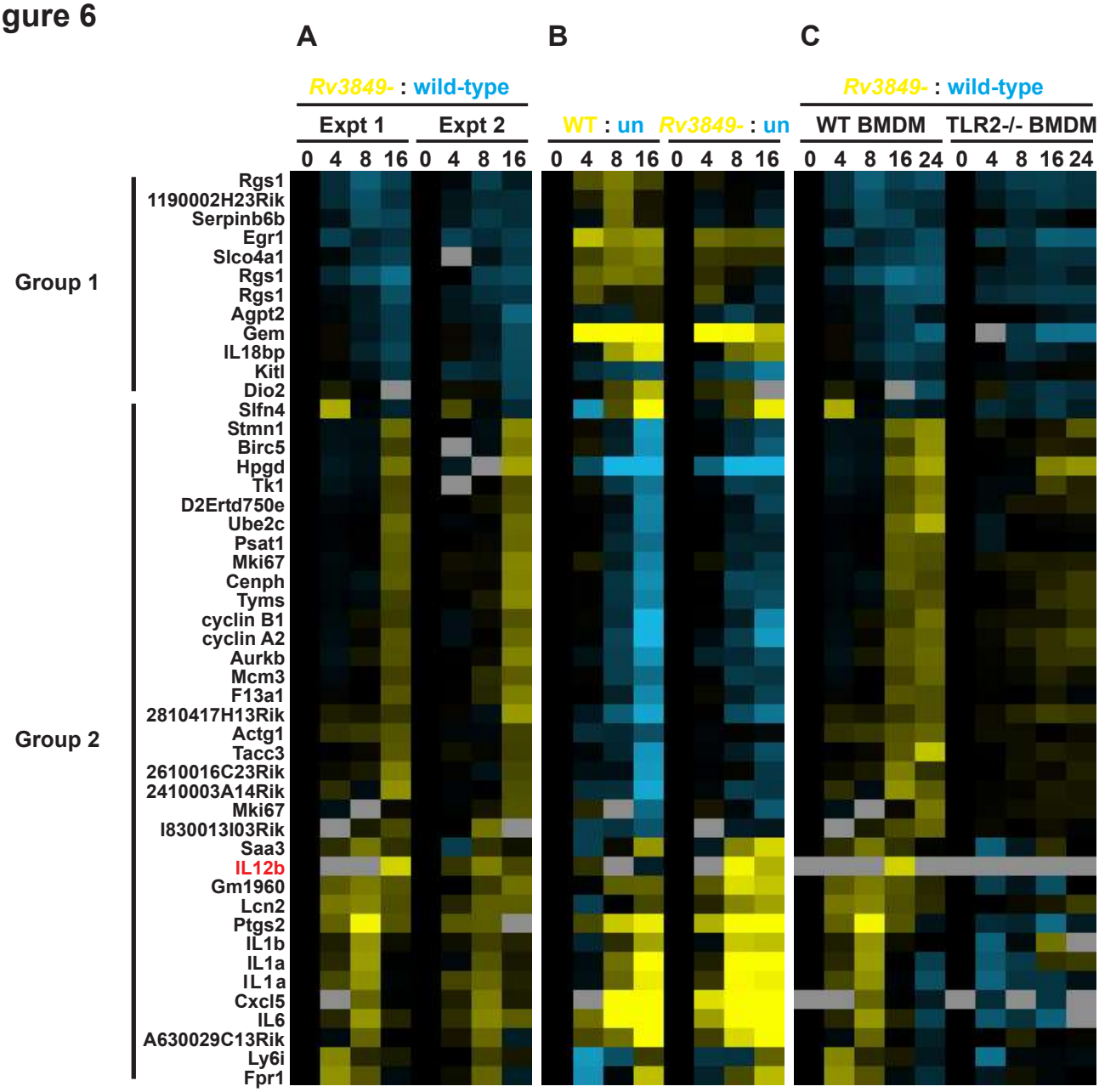

A
B
C
Rv3849- : wild-type
Expt 1
Expt 2
WT : un
Rv3849- : un
Rv3849- : wild-type
WT BMDM
TLR2-/- BMDM
0 4 8 16 0 4 8 16
0 4 8 16 0 4 8 16
0 4 8 16 24 0 4 8 16 24
Group 1
Group 2
Rgs1
1190002H23Rik
Serpinb6b
Egr1
Slco4a1
Rgs1
Rgs1
Agpt2
Gem
IL18bp
Kitl
Dio2
Slfn4
Stmn1
Birc5
Hpgd
Tk1
D2Ertd750e
Ube2c
Psat1
Mki67
Cenph
Tyms
cyclin B1
cyclin A2
Aurkb
Mcm3
F13a1
2810417H13Rik
Actg1
Tacc3
2610016C23Rik
2410003A14Rik
Mki67
I830013I03Rik
Saa3
IL12b
Gm1960
Lcn2
Ptgs2
IL1b
IL1a
IL1a
Cxcl5
IL6
A630029C13Rik
Ly6i
Fpr1

Figure 2.7. Global gene expression analysis of wild-type, TLR2$^{-/-}$, and MyD88$^{-/-}$ infected with wild-type *M. tuberculosis*. **(A)** Gene expression response to *M. tuberculosis* infection was monitored over a 24 h timecourse. Genes induced or repressed at least three-fold in at least one experiment following infection with *M. tuberculosis* are shown. Yellow represents upregulation, and blue represents downregulation. **(B)** Transcriptional response of each monitored gene following infection of TLR2$^{-/-}$ or MyD88$^{-/-}$ BMDMs was divided by response from wild-type macrophages. Genes differentially induced or repressed at least four-fold in at least one experiment are shown. Yellow represents genes more highly expressed in mutant macrophages following *M. tuberculosis* infection than in wild-type macrophages; blue represents genes expressed more highly in wild-type macrophages than in mutant macrophages.

Figure 7

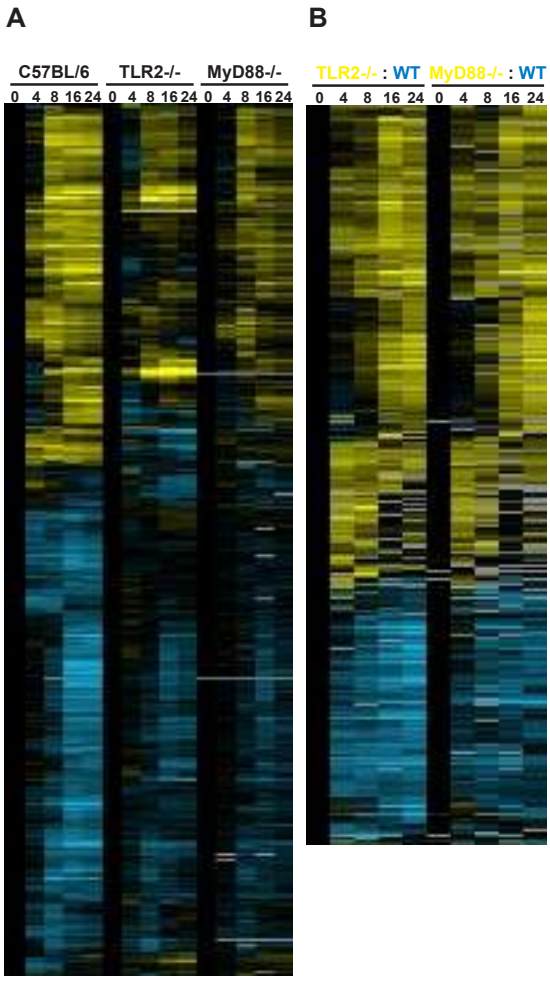
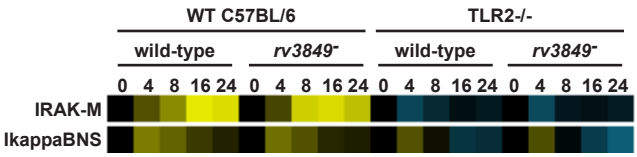

A
B
C57BL/6
TLR2-/-
MyD88-/-
0 4 8 16 24 0 4 8 16 24 0 4 8 16 24
TLR2-/- : WT
MyD88-/- : WT
0 4 8 16 24 0 4 8 16 24

Figure 2.8. IRAK-M and IkappaBNS are induced by infection with wild-type and ESX-1 mutant *M. tuberculosis* and require TLR2. Results for IRAK-M and IkappaBNS extracted from twenty-four hour timecourse of gene expression response to wild-type and *Rv3849*$^{-}$ bacteria. Yellow represents up-regulation and blue represents down-regulation.

Figure 8.


WT C57BL/6
TLR2-/-
wild-type
rv3849-
wild-type
rv3849-
0 4 8 16 24 0 4 8 16 24 0 4 8 16 24 0 4 8 16 24
IRAK-M
IkappaBNS

Chapter 3.

Rv3849 is a secreted regulator of M. tuberculosis ESX-1 secretion

Abstract

The ESX-1 pathway is a Sec-independent secretion system that is critical for *Mycobacterium tuberculosis* virulence. Through a genetic screen, we have identified Rv3849 as a novel member of the ESX-1 pathway. Although Rv3849 lacks primary sequence homology to proteins of known function, bioinformatic analysis of its predicted secondary structure suggested that Rv3849 might be a helix-turn-helix DNA binding protein. Comparative gene expression analysis of *rv3849*- bacteria and wild-type *M. tuberculosis* revealed that Rv3849 is a transcriptional activator of ESX-1 pathway components. Through genetic and biochemical studies, we show that Rv3849 binds to the promoter and activates transcription of the *Rv3616c-Rv3612c* operon that encodes two proteins required for ESX-1 secretion. Unexpectedly, we have also found that Rv3849 itself is a secreted substrate of the ESX-1 system. Taken together, our findings suggest that transcription factor secretion is a novel mechanism of feedback regulation employed by the *M. tuberculosis* ESX-1 secretion pathway.

Introduction

Many pathogenic microbes have adapted to eukaryotic hosts by evolving mechanisms to manipulate host anti-microbial processes. Alternate protein secretion systems targeting various host responses to infection are a common virulence strategy of many bacterial pathogens. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is particularly adept at evading and suppressing host immune responses, and recent studies have revealed the importance of the ESX-1 protein secretion pathway in

the interaction between the tubercle bacillus and its primary host cell, the macrophage (Stanley et al., 2007; Stanley et al., 2003). The ESX-1 system is a Sec-independent protein translocation pathway that transports proteins from inside the bacteria across the cell envelope. Two distinct genomic loci have been implicated in ESX-1 secretion, encoding both structural components of the secretory apparatus as well as secreted substrates (Fortune et al., 2005; Guinn et al., 2004; Hsu et al., 2003; MacGurn et al., 2005; Stanley et al., 2003), but little is known about how this system is regulated.

The core machinery as well as the major substrates, ESAT-6 and CFP-10, of the ESX-1 pathway are encoded at one genomic locus known as Region of Difference 1 (RD1) (Cole et al., 1998; Guinn et al., 2004; Hsu et al., 2003; Stanley et al., 2003). In a simple model for the secretory route of the principal ESX-1 substrates, the substrate pair is recognized through an interaction between Rv3871 and a signal sequence on CFP-10 (Champion et al., 2006). The substrates are then translocated across the cell membrane, and presumably through the cell envelope, through the action of Rv3870 and Rv3871 via a pore formed by Rv3877 (Stanley et al., 2003). The presence of ATPase domains on both Rv3870 and Rv3871 suggests a role for both of these proteins in satisfying the energetic requirement of secretion. In addition to these three core components, a number of other proteins encoded at RD1 are required for ESX-1 secretion (Converse and Cox, 2005).

The idea that the ESX-1 system and substrates are completely contained within the RD-1 locus has been contradicted by the more recent identification of ESX-1 substrates at an additional genomic locus (Fortune et al., 2005; MacGurn et al., 2005). The discovery of additional substrates also introduced the surprising phenomenon of

mutually-dependent secretion in which the secretion of each substrate is required for the secretion of all other substrates (Fortune et al., 2005). Mutually-dependent secretion is particularly intriguing in that it suggests that substrates exert control over the secretion activity of the entire pathway.

The phenomenon of mutually-dependent secretion has complicated the genetic dissection of effector functions of single ESX-1 substrates. To date, the specific functions of the known ESX-1 substrates remain unknown. As a whole, ESX-1 secretion has been clearly implicated in immune modulation, especially early following infection in macrophages (Stanley et al., 2007; Stanley et al., 2003; Volkman et al., 2004). It has been suggested that ESAT-6 and CFP-10 compromise host cell membrane integrity by forming pores in artificial lipid bilayers (Hsu et al., 2003). However, this role for the primary ESX-1 substrates remains controversial. The ESX-1 pathway has also been implicated in communication between the bacteria-containing vacuole and the macrophage cytoplasm (Stanley et al., 2007). The ESX-1 system is required for *M. tuberculosis* induction of interferon-β via cytosolic pathways, suggesting a role for secreted effectors of this alternative secretion pathway in interactions with cytosolic signaling molecules (Stanley et al., 2007).

In this work, we have identified a novel mutually-dependent secretion substrate of the ESX-1 system, EspR, expanding the repertoire of ESX-1 substrates and identifying another extra-RD1 locus required for ESX-1 function. Interestingly, EspR is a putative helix-loop-helix-containing protein that regulates ESX-1 activity via transcriptional activation of *Rv3616c-14c*. Furthermore, EspR activity is negatively regulated by

secretion through the ESX-1 system, suggesting a novel mechanism of feedback control of a transcription factor.

Results

Rv3849 is an ESX-1 secretion substrate and is required for the ESX-1 pathway

We have identified a transposon mutant in the *M. tuberculosis* gene *Rv3849* that fails to secrete ESAT-6. Immunoblotting for ESAT-6 in bacterial pellets (P) and culture supernatants (S) from wild-type and *rv3849*- bacteria revealed that Rv3849 is required for ESAT-6 secretion (Fig. 1). The *rv3849*- strain retains low level expression of Rv3849 (Fig. 1) because the transposon insertion is 13 nucleotides upstream of the *Rv3849* initiation codon. Because of the residual Rv3849 expression, the ESAT-6 secretion defect in *rv3849*- bacteria is not as complete as in a mutant in the putative pore, *Rv3877* (Fig. 1). Restoration of a single copy of *Rv3849* to *rv3849*- bacteria completely restored ESAT-6 secretion (Fig. 1). Rv3849 is required only for ESX-1 secretion as Mpt64, a substrate of the conventional Sec pathway, is secreted at wild-type levels by *rv3849*- bacteria (data not shown). Because Rv3849 is a small protein like most of the substrates of the ESX-1 system, we tested whether Rv3849 is itself secreted by the ESX-1 pathway. Indeed, immunoblotting for Rv3849 revealed it in both cell pellets as well as culture supernatants of wild-type *M. tuberculosis* but not in the supernatants of ESX-1 mutant bacteria (*rv3877*-) (Fig. 1). Rv3849, therefore, is another example of an ESX-1 substrate that is itself required for a functioning secretion system, like ESAT-6, CFP-10, EspA, and EspC (Fortune et al., 2005; Guinn et al., 2004; Hsu et al., 2003; MacGurn et al., 2005; Stanley et al., 2003).

Rv3849 shares structural homology with a B. subtilis transcription factor

Simple BLAST and protein motif searches failed to identify any putative functional domains of Rv3849. As an alternate method, we performed a secondary structure-based homology search using the Phyre algorithm (http://www.sbg.bio.ic.ac.uk/phyre/). Surprisingly, secondary structure-based homology search using the Phyre algorithm predicted strong secondary structure homology to the *B. subtilis* transcription factor SinR. Based on this finding, we modeled the three-dimensional structure of Rv3849 using the crystal structure of SinR as a template (Lewis et al., 1998). The results of this modeling revealed considerable similarity between the two proteins throughout their lengths, especially at the N- and C-termini (Fig. 2A). The striking degree of similarity is revealed most clearly when the modeled structure of Rv3849 is overlaid on the structure of SinR (Fig. 2B). In fact, the N-termini are nearly identical between Thr4 and Glu82 of Rv3849 which spans four alpha-helices, including helices 2, 3 and the intervening sequence which form a helix-turn-helix DNA-binding motif in SinR (Fig. 2B). Thus, although Rv3849 is an ESX-1 secreted protein, it bears a striking resemblance to a DNA binding protein.

rv3849- bacteria exhibit diminished expression of an essential ESX-1 locus

Given the potentially high degree of structural homology between Rv3849 and SinR, we hypothesized that Rv3849 is a transcriptional regulator and sought to identify bacterial genes that it regulates. Total RNA was isolated from wild-type and *rv3849-* bacteria, reverse transcribed, fluorescently labeled, and subjected to competitive

hybridization on an *M. tuberculosis* expression microarray. Of the approximately 4000 genes represented on the microarray, 11 genes exhibit reproducibly altered expression levels in *rv3849*- bacteria that are complemented by expression of a single copy of *Rv3849* (Fig. 3A). Only two operons were significantly down-regulated in the *rv3849*- mutant, the *Rv3616c-Rv3612c* operon, encoding the ESX-1 substrates EspA and EspC, and *Rv0888*, encoding a protein of unknown function. Like Rv3849, EspA and EspC are not only substrates of the ESX-1 pathway, but are also required for ESX-1 secretion. In addition to these operons, a number of genes were overexpressed in the *rv3849*- mutant (Fig. 3A), including a putative operon containing the genes MT2035, Rv1982c, and nrdF1. Quantitative RT-PCR (qPCR) on RNA harvested from wild-type, *rv3849*- mutant, and complemented *M. tuberculosis* to measure nrdF1 and Rv3615c provided additional support for the microarray data (Fig. 3B, 3C). Although Rv3849 regulates expression of a number of different genes, the decreased expression of *Rv3616c-Rv3612c* in *rv3849*- bacteria provided a possible link between Rv3849 activity and ESX-1 function. Given the regulatory function of Rv3849 in ESX-1 secretion, we have renamed this protein EspR (*E*SX-1 *s*ecreted *p*rotein *R*egulator).

EspR is a transcriptional activator that binds directly to the Rv3616c promoter

To determine if EspR directly regulates *Rv3616c-Rv3612c*, we performed electrophoretic mobility shift assays (EMSA) to assay binding of recombinantly purified MBP-EspR fusion protein to a DNA probe spanning 710 base pairs upstream of the *Rv3616c* initiation codon (Fig. 4A). The MBP-EspR fusion protein bound to the DNA

probe and retarded its mobility in a non-denaturing polyacrylamide gel, while purified His-MBP did not (Fig 4B).

To identify the DNA region bound by EspR, we made a series of EMSA probes by making approximately 100 base pair truncations from the 5' end of the *Rv3616c* promoter (Fig 4A). Purified His-MBP-EspR bound to probes that included at least 527 base pairs upstream of *Rv3616c* (Fig 4B). The 420 base pair probe was weakly bound by the fusion protein, but was dramatically reduced compared to longer probes (Fig 4B), and no binding was detectable with a shorter probe (data not shown). This analysis places the EspR binding site in the distal 300 base pairs of the original 710 base pair *Rv3616c* promoter probe, suggesting that expression of the *Rv3616c-Rv3612c* operon requires a promoter that is considerably larger than what is typical in bacteria, including mycobacteria.

Taken together, our experiments suggest that EspR is an activator of the *Rv3616c-Rv3612c* operon. To test this directly, we fused the longest promoter fragment used for the EMSA experiments to drive the expression of a *LacZ* reporter gene (Fig. 4C). A single copy of the resultant reporter construct was integrated into the genome of *Mycobacterium smegmatis*, a non-pathogenic relative of *M. tuberculosis* that lacks the *Rv3616c-Rv3612c* locus. We monitored *LacZ* expression on plates containing X-gal in the reporter strain following expression of EspR from an episomal plasmid. *LacZ* expression was weak in the absence of EspR (Fig 4D), but induced when driven by the 710 bp *Rv3616c* promoter in the presence of EspR (Fig 4D). In correlation with the *LacZ* plate assay, measurement of ß-galactosidase activity in liquid cultures revealed induction of *LacZ* in the reporter strain in the presence of EspR (Fig 4E). Together, the binding

studies and reporter studies demonstrate that EspR binds directly to the promoter of *Rv3616c* and activates transcription of the operon.

EspR mediated activation of Rv3616c-Rv3612c is required for ESX-1 secretion

As described earlier, a remarkable feature of the ESX-1 pathway is the co-dependence of secretory substrates, including EspR. Unlike the *espr-* mutant, mutation of any of the other substrates does not affect expression of the others. Therefore, it appears that the coupling of secreted factors can be mediated both transcriptionally and post-translationally. It is conceivable that secretion of EspR, in addition to its transcriptional activation of the *Rv3616c-Rv3612c* operon, may be an obligate step in ESX-1 secretion. To test the nature of EspR's role in ESX-1 function, we have made N- and C- terminal mutations in EspR to probe the coupling between EspR transcriptional activity and secretion. Furthermore, to determine if EspR plays a required role in ESX-1 secretion in addition to its transcriptional activity, we tested for functional ESX-1 secretion in *espR-* bacteria that express the *Rv3616c-Rv3612c* operon under a heterologous promoter.

First, to definitively demonstrate that the DNA-binding activity of EspR is required for ESX-1 secretion, we made point mutations in several conserved residues in the N-terminal DNA binding region of EspR. As shown in Figure 5A, there is a high degree of conservation in the N-terminus among EspR homologues in other mycobacteria as well as in more distantly related actinomycetes. We mutated highly conserved residues in this region with the aim of disrupting DNA binding activity; charged residues were mutated to alanine and hydrophobic residues were mutated to glutamate. To determine if DNA-binding activity was lost in the point mutants, we purified each from *E.coli* as an

MBP fusion protein, as previously described with the wild-type EspR. All of the mutant proteins either failed to bind DNA or exhibited drastically reduced DNA-binding activity compared to wild-type MBP-EspR (Fig. 5B).

Next we expressed the point mutated EspR variants in *rv3849- M. tuberculosis* to determine if they were sufficient to restore ESX-1 secretion. Cell pellets and culture supernatants from wild-type, *rv3849-* mutant, and *rv3849-* mutant complemented with either wild-type or mutated EspR were immunoblotted for ESAT-6. ESAT-6 is secreted in wild-type bacteria as well as *rv3849-* mutant bacteria complemented with wild-type Rv3849; however, none of the DNA-binding deficient point mutants restored functional ESX-1 secretion to *rv3849-* mutant *M. tuberculosis* (Fig. 5C). We confirmed that the failure to restore ESX-1 secretion is correlated with diminished expression of the regulated *Rv3616c-Rv3612c* locus by comparing the gene expression profiles of wild-type, *rv3849-*, and *rv3849-* mutant bacteria expressing one of two DNA-binding deficient EspR mutants (Rv3849R8A and Rv3849R21A). Point mutants that fail to bind DNA do not restore wild-type expression of EspR transcriptional targets to *rv3849-* bacteria (Fig. 5D). These data demonstrate that DNA binding and, presumably, transcriptional activation of *Rv3616c-Rv3612c* is a required function of EspR in ESX-1 secretion.

C-terminus of EspR plays a role in transcriptional activation but not DNA binding

We have so far demonstrated that EspR transcriptionally regulates ESX-1 secretion by activating expression of the *Rv3616c-Rv3612c* locus. We next attempted to uncouple EspR's transcriptional activity from its secretion by generating EspR mutants that retain the ability to activate transcription but fail to be secreted. Previous work has

identified a signal sequence at the C-terminus of CFP-10 that is necessary and sufficient for export through the ESX-1 system (Champion et al., 2006). We therefore hypothesized that an export signal may reside in the C-terminus of EspR and made a series of EspR truncation mutants by incrementally deleting five amino acids from the C-terminus of EspR (Fig. 5E), expecting to disrupt EspR secretion without affecting transcriptional activity.

We tested the ability of the C-terminal truncation mutants to restore ESX-1 secretion to *rv3849- M. tuberculosis* by immunoblotting cell pellets and culture supernatants for ESAT-6. Unexpectedly, only the mutant with smallest deletion, *rv3849Δ5*, complemented ESX-1 secretion to wild-type levels (Fig. 5F), raising the possibility that the C-terminus of EspR plays a role in ESX-1 secretion. Interestingly, each of the purified C-terminal truncation mutants bound *Rv3616c* promoter DNA as well as wild-type EspR protein (Fig. 5B), suggesting that the failure of these mutants to function in ESX-1 secretion was not due to lack of DNA recognition. Futhermore, gene expression analysis of *M. tuberculosis* expressing C-terminal mutants revealed that only the Rv3849Δ5 protein partially complemented the transcriptional defect of *rv3849- M. tuberculosis* (Fig. 5D). Even the deletion of 10 amino acids from the C-terminus completely blocked the ability of EspR to transcribe the *Rv3616c-Rv3612c* operon at wild-type levels (Fig. 5D). This deletion analysis reveals that the C-terminus of EspR is essential for it to function as a transcription factor despite being dispensable for DNA binding.

Constitutive expression of Rv3616c-Rv3612c bypasses the ESX-1 requirement for EspR

To determine if EspR plays an obligate role in ESX-1 function in addition to its transcriptional activity, we expressed *Rv3616c,* Flag-tagged *Rv3615c,* and *Rv3614c* under the control of the constitutive GroEL promoter in *espR*- bacteria. If the sole function of EspR in ESX-1 secretion is to activate the *Rv3616c-Rv3612c* operon, ESX-1 secretion will be rescued in this *M. tuberculosis* strain despite the lack of EspR. Assaying ESX-1 secretion by immunoblotting for ESAT-6 in bacterial cell pellets and culture supernatants revealed that heterologous expression of *Rv3616c-Rv3614c* rescues ESX-1 secretion in *espR*- bacteria (Fig. 5G). Assaying for Mpt64 in cell pellets and supernatants showed that constitutive expression of *Rv3616c-Rv3614c* does not affect Sec-dependent secretion (data not shown). This experiment demonstrates that EspR lacks an essential post-translational role in ESX-1 secretion, distinguishing it from other mutually-dependent ESX-1 substrates.

EspR transcriptional activity is regulated by secretion and regulates ESX-1 secretion in vivo

Since the secretion of EspR is not required for ESX-1 function, we sought to test the role of EspR secretion in regulating its function. We propose that EspR secretion is a form of negative feedback that regulates EspR transcriptional activity. To test this model, we sought to impair EspR secretion, predicting that such a perturbation would result in increased intracellular EspR and a resultant increase and decrease in the expression of its positive and negative transcriptional targets, respectively. We tagged the N-terminus of EspR with the FLAG epitope, and, serendipitously, this modification caused EspR secretion to be impaired (Fig. 6A). Quantitation of EspR secretion by normalizing to

Mpt64 reveals that the FL-EspR protein secretion is over 8-fold reduced (Fig. 6A). We next compared the gene expression profiles of FL-EspR expressing bacteria to wild-type *M. tuberculosis*, *rv3849*- mutants, and *rv3849*- mutants complemented with untagged EspR. Interestingly, the transcriptional profile of EspR-regulated genes is inverted in FL-EspR-expressing bacteria as compared to *rv3849*- *M. tuberculosis* (Fig. 6B). The association of diminished secretion of EspR associated with increased expression of EspR-activated targets and decreased expression of EspR-repressed transcripts seen here (Fig. 6B) suggests that secretion of EspR is a mechanism to downregulate its intracellular activity.

We next sought to determine if EspR plays a role in regulating ESX-1 secretion in macrophages. To this end, we infected macrophages with wild-type and *rv3849*- bacteria and harvested bacterial RNA at 2 hours and 4 hours post-infection. We measured *Rv3615c* expression by qPCR in RNA harvested from intracellular bacteria and bacteria grown in liquid culture. *Rv3615c* expression is highly induced by 2 hours post-infection in wild-type bacteria (Fig. 6C and 6D). In *rv3849*- bacteria, however, *Rv3615c* induction is reduced compared to wild-type *M. tuberculosis* (Fig. 6C and 6D). Taken together, these results indicate that ESX-1 secretion is activated *in vivo* and requires EspR for complete induction.

Discussion

In this work we have discovered a simple mechanism by which the *M. tuberculosis* ESX-1 secretion system is regulated. We propose a model in which ESX-1 secretion cycles between OFF and ON states driven by the EspR-regulated expression of

Rv3616c-Rv3612c (Fig. 7). The ESX-1-OFF state is characterized by low expression of *Rv3616c-Rv3612c*. In response to environmental cues, including signals detected inside the macrophage, EspR activates expression of *Rv3616c-Rv3612c*, inducing ESX-1 secretion. Once the bacteria achieve an adequate level of secretion, EspR is itself exported through the ESX-1 pathway, diminishing the intracellular concentration and transcriptional activity of EspR. In support for this model, we have shown that DNA-binding activity of EspR is required for its function as an activator of ESX-1 function. Furthermore, we have demonstrated that impairing EspR secretion potentiates its transcriptional activity, suggesting that its activity is negatively regulated by ESX-1 secretion. Finally, we have shown that *Rv3616c-Rv3612c*, an operon critical for ESX-1 function, is induced in macrophages and depends on EspR for its activation.

Coupling between transcription and secretion has been described in at least two contexts previously. First, in *Salmonella typhimurium* an anti-sigma factor, FlgM, is retained within the cell, inhibiting flagellin transcription driven by σ-28, until a functional hook-basal body complex forms (Hughes et al., 1993). Upon hook-basal body assembly, FlgM is secreted from the cell, enabling σ-28-driven flagellin transcription. In this context, the coupling of secretion to transcription plays a timing role to insure ordered assembly of the flagellum. In a second example of transcription-secretion coupling, secretion of an anti-anti-repressor, ExsE, is an early event in initiating Type III secretion in *Pseudomonas aeruginosa* (Rietsch et al., 2005; Urbanowski et al., 2005). In this case, export of ExsE is an inducing event in the transition between two stable states – inactive and active secretion. In these examples, regulators of DNA-binding proteins are secreted whereas the DNA-binding regulator itself is secreted by ESX-1. Furthermore,

from the work presented here, it is clear that EspR activates the *Rv3616c-Rv3612c* operon early following infection of macrophages, and suggests that the ESX-1 system is induced by intracellular cues.

The direct secretion of a DNA-binding activator as a means of negative feedback seemingly has both advantages and disadvantages. By omitting additional regulatory proteins, the logic of the feedback system is direct and simplified. However, with fewer regulatory elements in the feedback system, robustness and tolerance to genetic mutation are sacrificed (Baker et al., 2006). This potential detriment to the system suggests the possibility of other points of regulation that enhance the robustness of ESX-1 regulation. Likewise, it is interesting that only a single operon of ESX-1-related genes is regulated by EspR. Greater understanding of ESX-1 regulation in response to intracellular signals may provide insight into why the *Rv3616c-Rv3612c* operon is specifically regulated and not other ESX-1 components. Clearly, finer studies of the dynamics of EspR regulation and specificity will allow for greater understanding of this novel form of feedback regulation and its implications for the ESX-1 system.

The detailed mechanism of EspR secretion through the ESX-1 pathway remains to be elucidated. EspR lacks a C-terminal sequence with similarity to the signal sequence identified in CFP-10 (Champion et al., 2006). Two explanations can account for the absence of a signal sequence on EspR. First, it is possible that the ESX-1 system recognizes multiple signal sequences, with an unidentified signal present on EspR. Alternatively, EspR may be secreted as a heterodimer coupled with a signal sequence-carrying protein. The prototypical substrates of the ESX-1 pathway, ESAT-6 and CFP-10, are secreted as a dimer with only CFP-10 carrying a signal sequence. Similarly,

another substrate of the ESX-1 pathway, one of the currently identified or possibly an unknown substrate, may be responsible for escorting EspR through the secretory machine. Given the role of EspR secretion in regulating its intracellular activity, clarifying the detailed mechanism of EspR secretion is critical to understanding the regulation of ESX-1 secretion *in vivo*.

For the first time we have elucidated the molecular details underlying a case of mutually-dependent secretion of ESX-1 substrates in *M. tuberculosis*. We have shown here that the mutual-dependence of EspR and ESAT-6 secretion is due to EspR's critical role as an activator of ESX-1 secretion. We have further demonstrated that the ESX-1 requirement for EspR is unlikely to involve a structural role for EspR in the secretory machine as heterologous expression of *Rv3616c-Rv3614c* makes EspR dispensable for ESX-1 secretion. The nature of mutually-dependent secretion involving other substrates remains to be elucidated. However, since transcriptional coupling between other co-dependent substrates has not been observed, post-translational mechanisms distinct from EspR's transcriptional control of the ESX-1 system are likely to explain the other instances of mutual dependence.

In addition to the *Rv3616c-Rv3612c* operon, there are a total of 12 genes that appear to be regulated by EspR. Though they do not appear to play a role in ESX-1 secretion, they may play important roles in virulence or parasitization of macrophages. In particular, it will be interesting to determine if Rv0888, the additional target of EspR-mediated activation and a predicted substrate of the TAT protein translocation system, is important for early adaptation to or manipulation of macrophages. Likewise, it will be interesting to determine if EspR-repressed targets are turned off early *in vivo*, and

possibly activated at later timepoints following infection. Furthermore, it remains a possibility that a subset of the EspR-regulated genes is not directly targeted by EspR. If so, EspR may be a part of a wider regulatory network with a critical role in virulence.

We provide the first evidence that the ESX-1 pathway is regulated during infection. In fact, the *Rv3616c-Rv3612c* operon is activated early following macrophage infection, which correlates with published reports that the *Rv3616c-Rv3612c* operon is activated by low pH (Fisher et al., 2002). The early activation of ESX-1 secretion components in macrophages is consistent with the requirement for the ESX-1 pathway early during infection of mice. Mutants in the ESX-1 pathway, including *espR*- bacteria, exhibit a characteristic early growth defect in mice, suggesting a vital role for secretion in the early stages of infection (Stanley et al., 2003). Furthermore, ESX-1 secretion is critical for modulation of macrophage responses to *M. tuberculosis*, consistent with its activation early upon macrophage infection. On the other hand, the primary substrates of the ESX-1 system, ESAT-6 and CFP-10, are highly immunogenic, so it is intriguing to speculate that it would be advantageous to *M. tuberculosis* bacteria to regulate their secretion. *M. tuberculosis* may balance the costs and benefits of ESX-1 activity by inducing secretion early upon infection, through EspR, and then turning it off as the infection progresses, through EspR secretion and possibly other mechanisms.

In conclusion, the secretion of a DNA-binding transcription factor represents a novel mechanism of transcription factor regulation. The secretion of EspR through the ESX-1 pathway, a target of its positive regulation, is a simple form of negative feedback that may allow for fine control, timing, or conditional regulation of ESX-1 substrate export. It is intriguing that a novel regulatory scheme has evolved in the context of a

critical virulence determinant of a pathogen, and it will be interesting to discover the extent of this form of direct coupling between transcription and secretion in diverse phylogeny.

Materials and Methods

Bacterial strains and plasmids

Wild-type *M. tuberculosis* is the Erdman strain, and all genetically manipulated bacteria are in this background. The *rv3849*- mutant carries a Tn5 transposon insertion 13 nucleotides upstream of the Rv3849 intiation codon. The full coding sequence of *Rv3849* plus 200 nucleotides upstream were cloned into the integrating plasmid pMV306-Kan to generate the p*Rv3849* (p*EspR*, pSR211) complementation construct. N-, and C- terminal Rv3849 mutants were generated by site-directed mutagenesis in p*Rv3849* and transformed into *M. tuberculosis*. Flag-EspR was generated by fusion PCR insertion of a 3X-Flag epitope tag upstream of EspR in p*Rv3849*. GroEL promoter-driven *Rv3616c-Rv3614c* (pSR403) was generated by fusion PCR insertion of GroEL promoter upstream of *Rv3616c* in pJAM69 (MacGurn and Cox unpublished data).

Protein purification

The coding sequence of wild-type and mutated Rv3849 was cloned by ligation-independent cloning into pLIC-HMK (a gift of Dr. James Berger, University of California, Berkeley). Plasmids were transformed into *E. coli* BL21/DE3/pLysS, and protein expression was induced for 4 hours with 1mM IPTG. His-tagged MBP-EspR fusion protein was purified on a Ni-NTA agarose column (Qiagen).

Electrophoretic mobility shift assay

Radio-labeled probe was generated by treating PCR amplified *Rv3616c* promoter sequences with T4 polynucleotide kinase in the presence of ^{32}P-γ-ATP, followed by purification over a Sephadex G-50 spin column. EMSA was performed by incubating purified protein with approximately 10^5 cpm of probe for 30 minutes at room temperature prior to electrophoresis on a non-denaturing 6% polyacrylamide gel.

Microarrays

Total *M. tuberculosis* RNA was generated by bead-beating bacterial pellet in Trizol reagent (Invitrogen), followed by chloroform extraction, isopropanol precipitation, DNAse treatment, and cleanup over an RNEasy mini column (Qiagen). 3-5 ug of total RNA was reverse transcribed in the presence of amino-allyl UTP and Cy3- or Cy5-labeled. Competitive hybridizations between Cy5-labeled experimental cDNA and Cy3-labeled reference cDNA were performed for 24 hours at 63° C. The Rv3849 regulon was defined as genes that exhibited at least 2-fold dependence on Rv3849 in two out of three experiments.

Quantitative PCR

One to 3 ug of total *M. tuberculosis* mRNA was reverse transcribed and used as template for PCR in the presence of SYBR Green. Oligonucleotides for amplification of *Rv3615c* were: oSR603 5'gagcgtctcggtgtactg3' and oSR604 5'cgtgtcgttgaactgtgagc3'. Oligonucleotides for amplification of *nrdF1* were: oSR601 5'caacctgggataccagcctg3' and

oSR 602 5'cattacgtatgagcttcc3'. Oligonucleotides for amplification of *16S rRNA* were: oAL63 5'atgctacaatcgccggtaca3' and oAL64 5'gcgttgctgatctgcgatta3'. For each sample, expression of *Rv3615c* or *nrdF1* was normalized to expression of *16S rRNA*.

For qPCR analysis of RNA isolated from *M. tuberculosis* inside macrophages, bacterial RNA was isolated by first lysing macrophages in guanidine isothiocyanate buffer. Intact bacteria were then pelleted and washed in PBS + 0.05% Tween-80, followed by bead-beating in Trizol, chloroform extraction, and cleanup over an RNEasy mini column. Total bacterial RNA was then amplified using a MessageAmp II Bacterial Amplification Kit (Ambion), and *Rv3615c* expression was measured in amplified RNA as above.

LacZ reporter assays

Rv3616c promoter fragments were PCR cloned upstream of *LacZ* in the integrating plasmid pMV306-Hyg-LacZ, and reporter constructs were transformed into *M. smegmatis*. *M. tuberculosis espR* was cloned downstream of the inducible acetamidase promoter (Triccas et al., 1998) in pHR100-Kan, and reporter strains were transformed with this expression construct. Due to sufficient leakiness of the acetamidase promoter, inducer was not used to overexpress *espR*. Doubly-transformed *M. smegmatis* clones were grown in liquid culture and then spotted onto plates containing X-gal to measure *LacZ* expression.

Structural modeling

Structural homology search was performed using the Phyre server (http://www.sbg.bio.ic.ac.uk/phyre/). Rv3849 was then modeled in the program Modeler (http://salilab.org/modeller/) using the crystal structure of SinR as a template (PDB: 1B0N). The Rv3849 model was viewed and manipulated using the program Chimera (http://www.cgl.ucsf.edu/chimera/).

Figure 3.1. Rv3849 is secreted by the ESX-1 system and is required for ESX-1 function. Rv3849 and ESAT-6 were detected in cell pellets (P) and culture supernatants (S) from wild-type, *rv3849*-, *rv3849*- complemented, and *rv3877*- bacteria.

Figure 1.

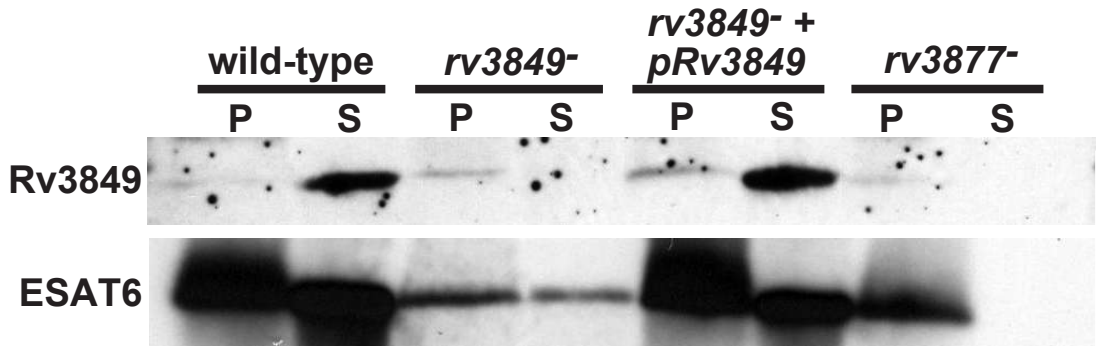

wild-type
rv3849-
rv3849- + pRv3849
rv3877-
P
S
P
S
P
S
P
S
Rv3849
ESAT6

Figure 3.2. Structural analysis of Rv3849 reveals strong predicted similarity to SinR. (A) Rv3849 primary sequence was structurally modeled using the crystal structure of SinR as a template. The structure of SinR (violet) and the predicted structure of Rv3849 (silver). **(B)** Overlay of the N-termini of SinR and Rv3849 reveals close overlap between the two structures. Arginine 8 of Rv3849, used in mutagenic analyses, is shown.

Figure 2.



Figure 3.3. Rv3849 is a transcriptional regulator that activates the *Rv3616c-Rv3612c* operon. (A) Gene expression values in *rv3849*- and complemented *rv3849*- bacteria were divided by expression values in wild-type bacteria in three experiments. Genes with at least two-fold dependence on Rv3849 in two out of three experiments are shown. Black represents no expression change, yellow represents increased expression, blue represents decreased expression, grey represents points for which data of sufficiently high quality was not acquired. Lines to the right of the cluster diagram represent likely operons. **(B)** Quantitative PCR measurement of expression of *Rv3615c* in wild-type, *rv3849*-, and complemented *rv3849*- bacteria. *Rv3615c* expression in each strain was normalized to *16S rRNA* expression. Shown are averages and standard deviations of triplicate measurements in one representative experiment of two. **(C)** Quantitative PCR measurement of *nrdF1* expression in wild-type, *rv3849*-, and complemented *rv3849*- bacteria. As above, *nrdF1* expression was normalized to *16S rRNA* expression in each sample. Shown are averages and standard deviations of triplicate measurements in one representative experiment of two.

Figure 3.

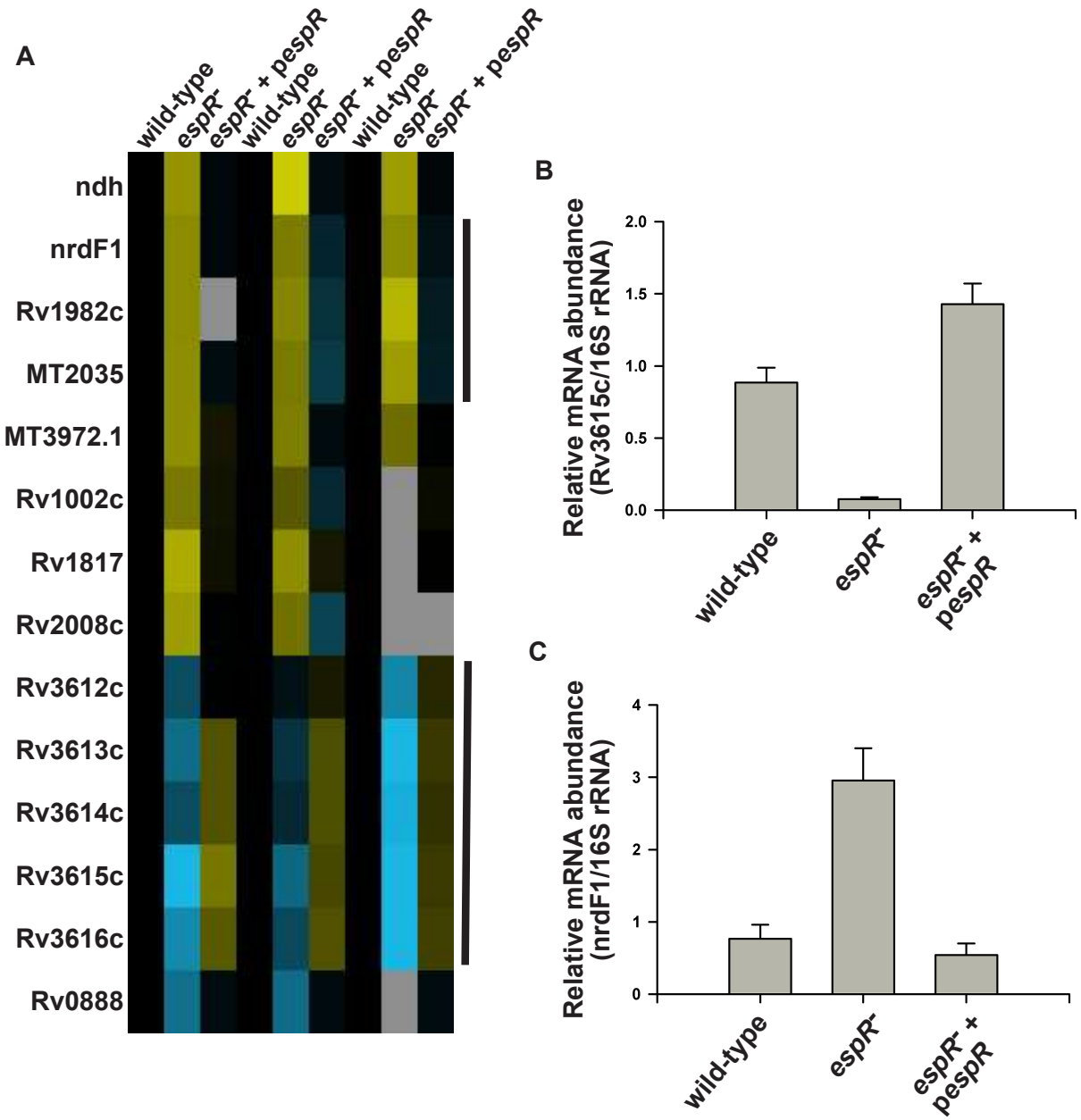

A
wild-type
espR-
espR- + pespR
wild-type
espR-
espR- + pespR
wild-type
espR-
espR- + pespR
ndh
nrdF1
Rv1982c
MT2035
MT3972.1
Rv1002c
Rv1817
Rv2008c
Rv3612c
Rv3613c
Rv3614c
Rv3615c
Rv3616c
Rv0888
B
Relative mRNA abundance (Rv3615c/16S rRNA)
2.0
1.5
1.0
0.5
0.0
wild-type
espR-
espR- + pespR
C
Relative mRNA abundance (nrdF1/16S rRNA)
4
3
2
1
0
wild-type
espR-
espR- + pespR

Figure 3.4. EspR binds to the promoter of and activates expression of the *Rv3616c-Rv3612c* operon. (A) Schematic representation of promoter fragments used as probes in electrophoretic mobility shift assays (EMSA). **(B)** EMSA was performed by incubating radiolabeled promoter fragments with purified MBP-EspR fusion protein or MBP alone for 30 minutes at room temperature, followed by separation of protein-DNA complexes on a non-denaturing 6% polyacrylamide gel and visualization of probe mobility retardation by autoradiography. **(C)** Schematic of *Rv3616c* promoter fusion to *LacZ* used to report promoter activity in *M. smegmatis*. **(D)** Patch of *M. smegmatis* expressing *Rv3616cpr-LacZ* fusion alone (top) or *Rv3616cpr-LacZ* fusion plus *M. tuberculosis* EspR (bottom) onto plates containing X-gal. **(E)** ß-galactosidase activity was measured in liquid cultures by using the colorimetric substrate ONPG in cultures of *M. smegmatis* expressing *Rv3616cpr-LacZ* fusion alone or *Rv3616cpr-LacZ* fusion plus *M. tuberculosis* EspR.

Figure 4.

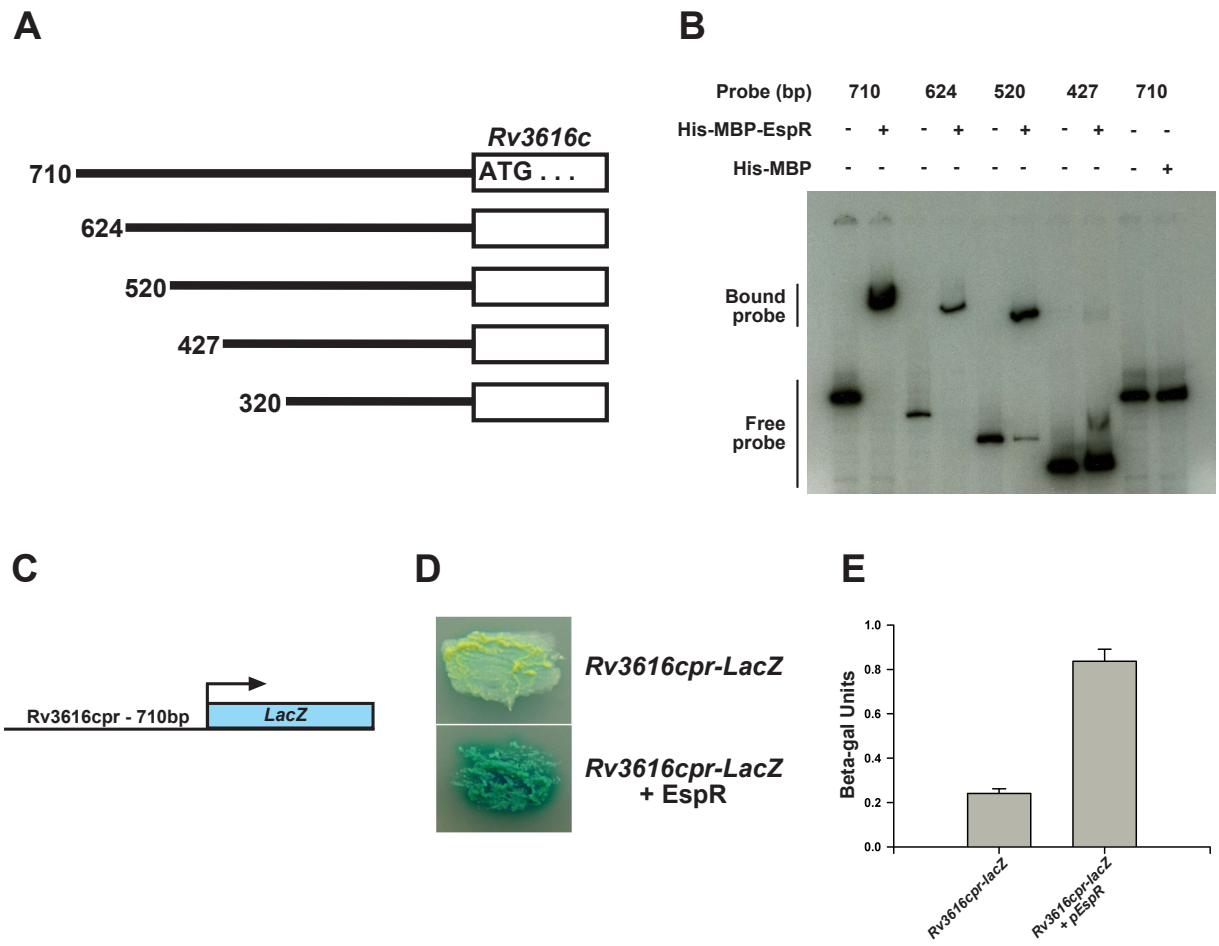

A
Rv3616c
ATG . . .
710
624
520
427
320
B
Probe (bp) 710 624 520 427 710
His-MBP-EspR - + - + - + - + - -
His-MBP - - - - - - - - - +
Bound probe
Free probe
C
Rv3616cpr - 710bp
LacZ
D
Rv3616cpr-LacZ
Rv3616cpr-LacZ + EspR
E
Beta-gal Units
1.0
0.8
0.6
0.4
0.2
0.0
Rv3616cpr-lacZ
Rv3616cpr-lacZ + pEspR

Figure 3.5. DNA binding and transcriptional activity, but not any extracellular function, of EspR are required for ESX-1 function. (A) Alignment of N-terminal primary sequence of close homologues of EspR in other mycobacteria and actinomycetes. Highly conserved residues targeted in mutagenic analysis are highlighted in red. **(B)** EMSA performed as described in Figure 4, using N-terminal point-mutated EspR and C-terminal EspR truncations and 710bp *Rv3616c* promoter fragment. N-terminal point mutations, but not C-terminal truncations, severely reduce DNA binding of EspR. **(C)** ESAT-6 was immunoblotted from cell pellets (P) and culture supernatants (S) of wild-type (Erdman) bacteria and *espR*- bacteria expressing N-terminal point-mutated EspR. **(D)** As described in Figure 3, expression level of each gene in *espR*- bacteria and *espR*- expressing wild-type or mutated EspR was divided by expression level in wild-type bacteria. Shown are the genes of the EspR regulon defined in Figure 3 for which usable data was acquired in the mutant analysis. As earlier, black represents no change, yellow represents increased expression, and blue represents decreased expression. **(E)** Alignment showing extent of C-terminal truncation series of EspR. **(F)** ESAT-6 immunoblotted in cell pellets (P) and supernatants (S) of wild-type bacteria, *rv3849*- bacteria, and *rv3849*- bacteria expressing C-terminal truncation mutants of EspR. **(G)** *Rv3616c-Rv3614c* were expressed in *rv3849*- bacteria under the control of the constitutive *GroEL* promoter. ESAT-6 was immunoblotted in cell pellets (P) and culture supernatants (S) of wild-type bacteria, *espR*- bacteria, complemented *espR*- bacteria, and *espR*- bacteria constitutively expressing *Rv3616c-Rv3614c*.

Figure 5.

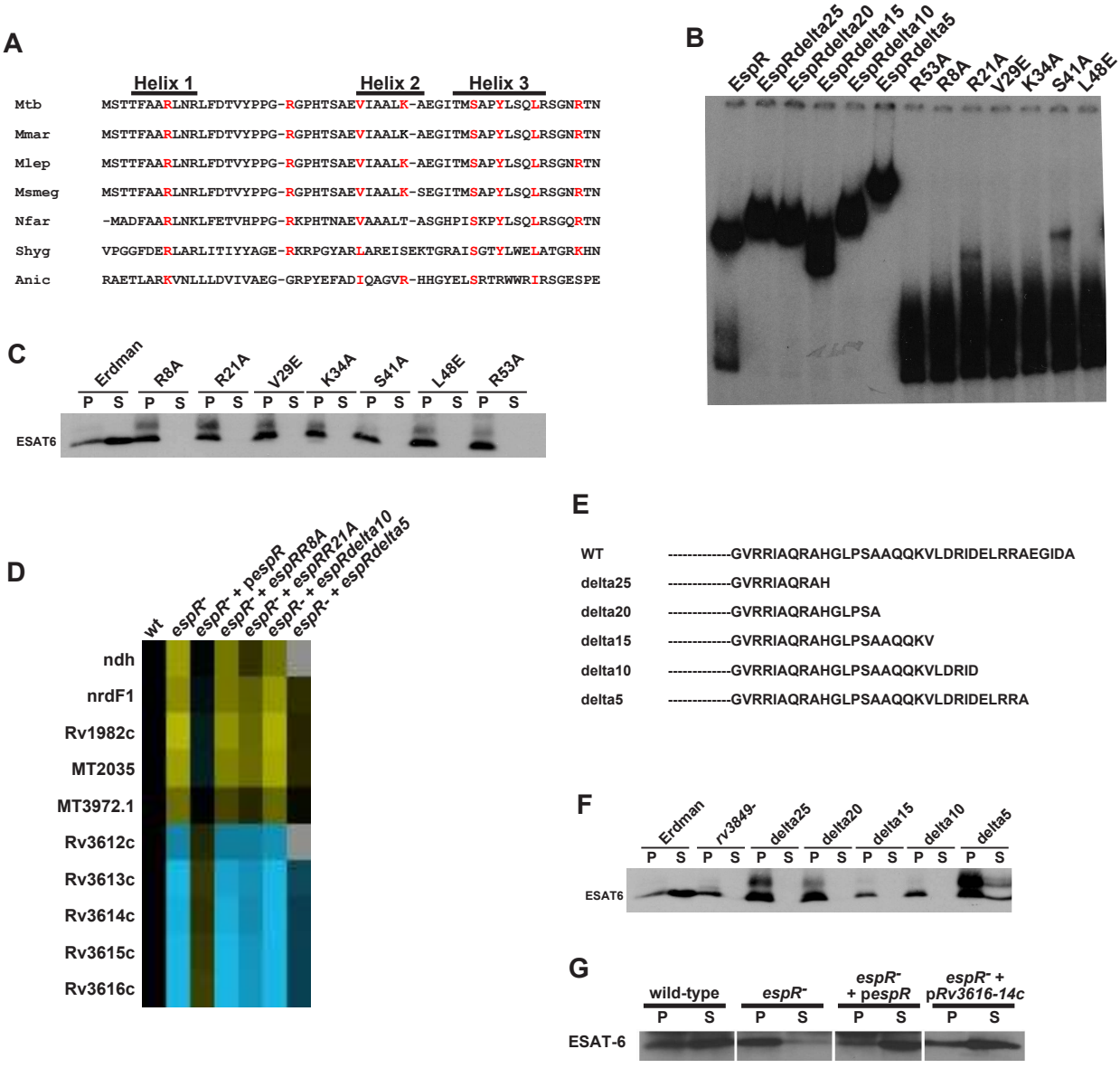

A
Helix 1
Helix 2
Helix 3
Mtb MSTTFAARLNRLFDTVYPPG-RGPHTSAEVIAALK-AEGITMSAPYLSQLRSGNRTN
Mmar MSTTFAARLNRLFDTVYPPG-RGPHTSAEVIAALK-AEGITMSAPYLSQLRSGNRTN
Mlep MSTTFAARLNRLFDTVYPPG-RGPHTSAEVIAALK-AEGITMSAPYLSQLRSGNRTN
Msmeg MSTTFAARLNRLFDTVYPPG-RGPHTSAEVIAALK-SEGITMSAPYLSQLRSGNRTN
Nfar -MADFAARLNKLFETVHPPG-RKPHTNAEVAAALT-ASGHPISKPYLSQLRSGQRTN
Shyg VPGGFDERLARLITIYYAGE-RKRPGYARLAREISEKTGRAISGTYLWELATGRKHN
Anic RAETLARKVNLLLDVIVAEG-GRPYEFADIQAGVR-HHGYELSRTRWWRIRSGESPE
B
EspR
EspRdelta25
EspRdelta20
EspRdelta15
EspRdelta10
EspRdelta5
R53A
R8A
R21A
V29E
K34A
S41A
L48E
C
Erdman
R8A
R21A
V29E
K34A
S41A
L48E
R53A
P S P S P S P S P S P S P S P S
ESAT6
D
wt
espR-
espR- + pespR
espR- + espRR8A
espR- + espRR21A
espR- + espRdelta10
espR- + espRdelta5
ndh
nrdF1
Rv1982c
MT2035
MT3972.1
Rv3612c
Rv3613c
Rv3614c
Rv3615c
Rv3616c
E
WT ----------GVRRIAQRAHGLPSAAQQKVLDRIDELRRAEGIDA
delta25 ----------GVRRIAQRAH
delta20 ----------GVRRIAQRAHGLPSA
delta15 ----------GVRRIAQRAHGLPSAAQQKV
delta10 ----------GVRRIAQRAHGLPSAAQQKVLDRID
delta5 ----------GVRRIAQRAHGLPSAAQQKVLDRIDELRRA
F
Erdman
rv3849-
delta25
delta20
delta15
delta10
delta5
P S P S P S P S P S P S P S
ESAT6
G
wild-type
espR-
espR- + pespR
espR- + pRv3616-14c
P S P S P S P S
ESAT-6

Figure 3.6. EspR transcriptional activity is regulated by secretion and is required for induction of *Rv3615c* inside macrophages. (A) EspR and Mpt64 were immunoblotted in cell pellets (P) and culture supernatants (S) of wild-type bacteria and *espR*- bacteria expressing N-terminally Flag-tagged EspR. EspR secretion was quantitated by densitometry and normalized to Mpt64 secretion using ImageJ software. **(B)** Microarray analysis, as described in Figure 3, comparing expression of EspR regulon genes in wild-type bacteria, *espR*- bacteria, complemented *espR*- bacteria, and *espR*- bacteria expressing Flag-tagged EspR. As before black represents no change in expression, yellow represents increased expression, blue represents decreased expression. Shown are EspR regulon genes for which usable data was acquired. **(C)** Expression of *Rv3615c* in wild-type and *espR*- bacteria in liquid culture (0h) and 2h and 4h following infection of bone marrow-derived macrophages was measured by quantitative PCR and normalized to expression of *16S rRNA*. **(D)** Expression of *Rv3615c* in wild-type and *espR*- bacteria 2h and 4h after infection of bone marrow-derived macrophages was normalized to expression of each strain in liquid culture (0h).

Figure 6.

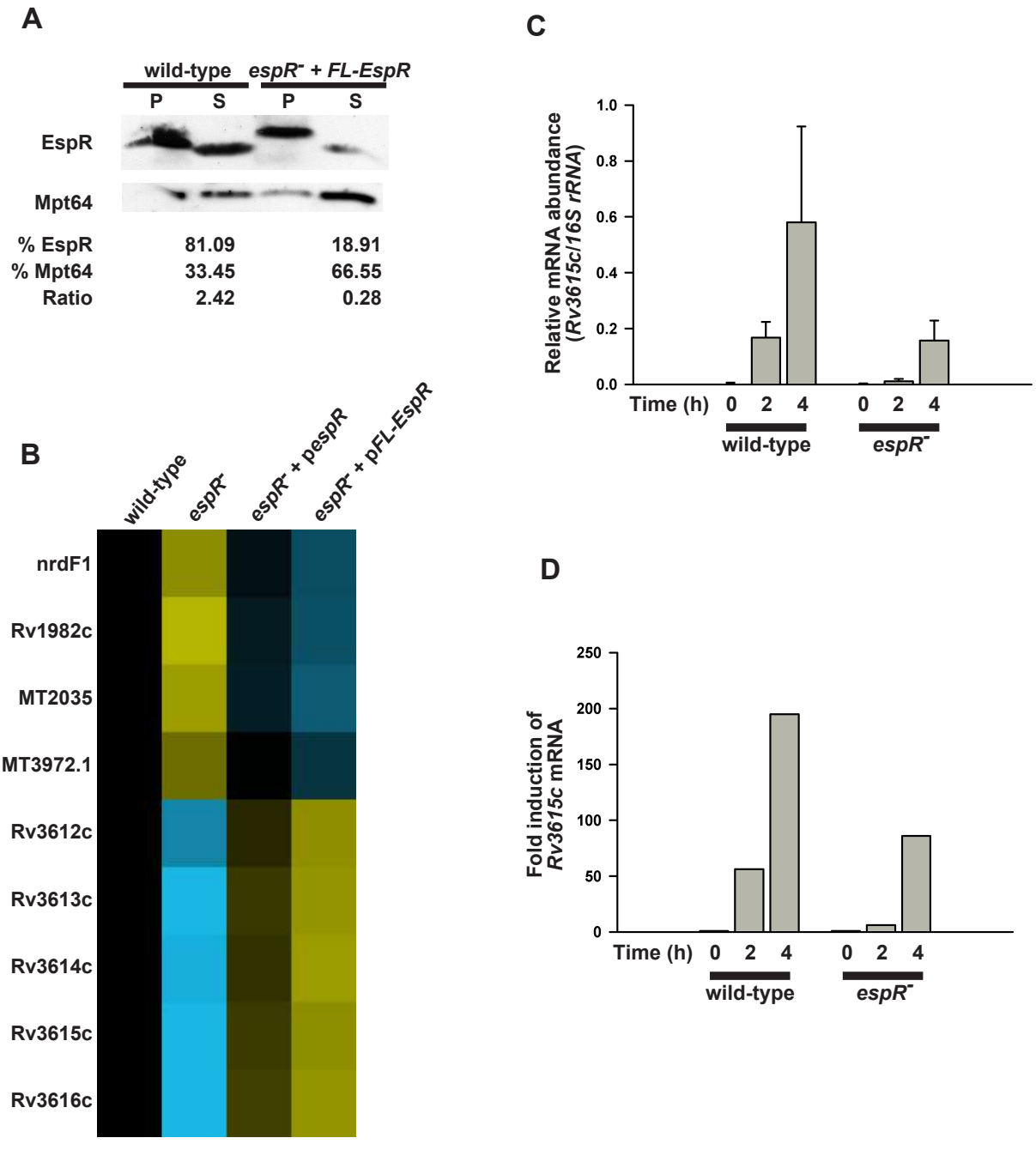
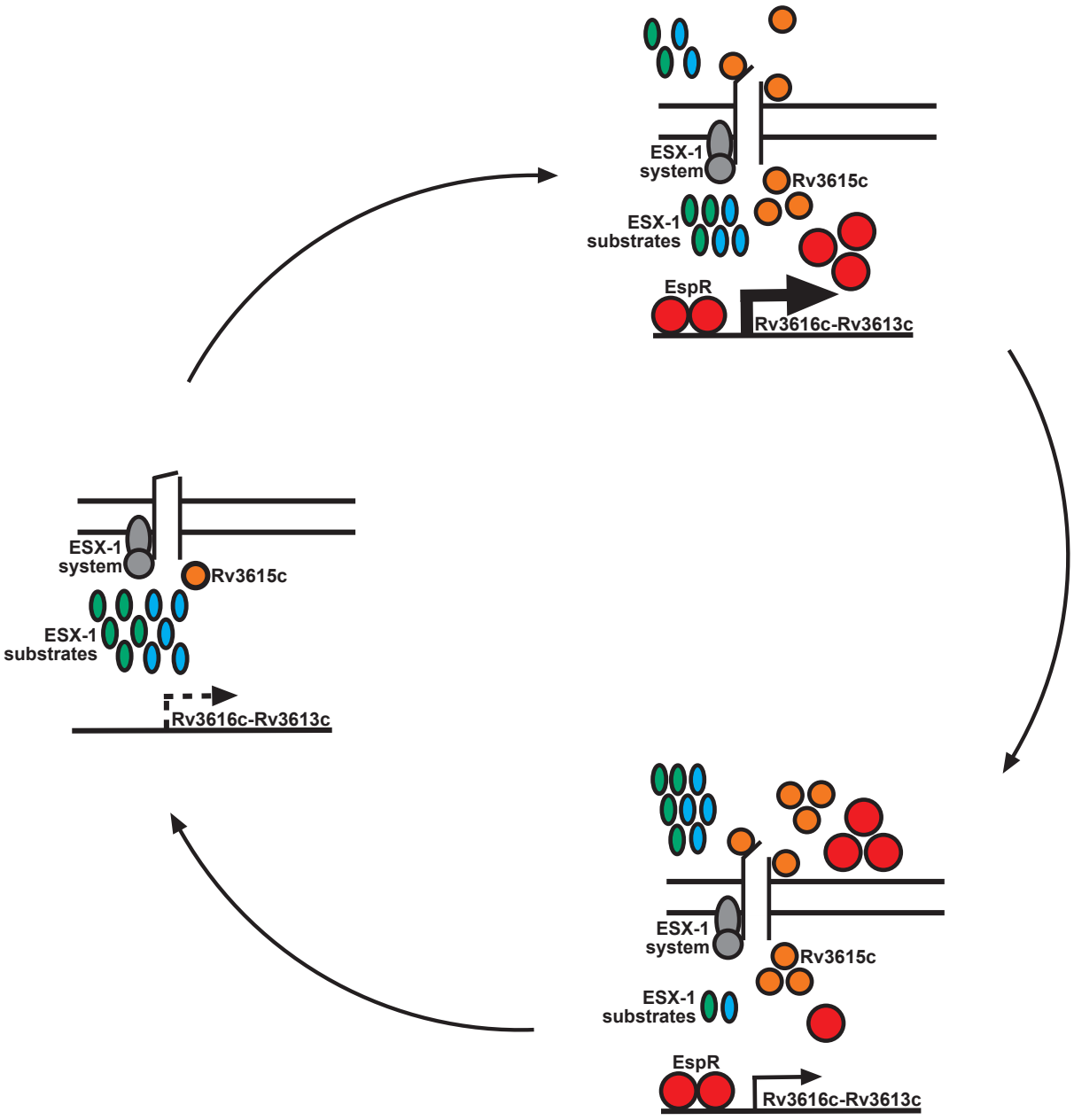

A
wild-type
espR- + FL-EspR
P
S
P
S
EspR
Mpt64
% EspR 81.09 18.91
% Mpt64 33.45 66.55
Ratio 2.42 0.28
B
wild-type
espR-
espR- + pespR
espR- + pFL-EspR
nrdF1
Rv1982c
MT2035
MT3972.1
Rv3612c
Rv3613c
Rv3614c
Rv3615c
Rv3616c
C
Relative mRNA abundance (Rv3615c/16S rRNA)
1.0
0.8
0.6
0.4
0.2
0.0
Time (h) 0 2 4 0 2 4
wild-type
espR-
D
Fold induction of Rv3615c mRNA
250
200
150
100
50
0
Time (h) 0 2 4 0 2 4
wild-type
espR-

Figure 3.7. EspR regulates activation of ESX-1 secretion and is subject to negative feedback through export via the ESX-1 pathway. ESX-1 function cycles between OFF and ON states and is activated by EspR transcriptional activity and deactivated by EspR secretion.

Figure 7


ESX-1
system
Rv3615c
ESX-1
substrates
EspR
Rv3616c-Rv3613c
ESX-1
system
Rv3615c
ESX-1
substrates
Rv3616c-Rv3613c
ESX-1
system
Rv3615c
ESX-1
substrates
EspR
Rv3616c-Rv3613c

Chapter 4.

Perspectives and Conclusions

I. New directions for *M. tuberculosis* pathogenesis

The whole-genome microarray studies presented in Chapter 2 described two findings of particular significance: first, many genes are repressed in response to *M. tuberculosis*, a poorly studied phenomenon; second, a small subset of macrophage genes is transcriptionally modulated by the ESX-1 secretion system. Though previous microarray studies have shown gene repression in response to various TLR simuli, there has been little in depth study of the repressed responses. There are two obvious explanations for the widespread repression of genes in response to infection. First, gene repression may simply represent broad reprogramming of macrophage function and be homeostatically coupled to broad gene upregulation. Second, the repressed genes may include antagonists of antimicrobial macrophage activation. Though it remains to be determined what role the repressed genes play in the macrophage response to infection, it is unlikely that these responses are purely incidental and not physiologically relevant.

The second important finding mentioned above points towards a subset of genes that may play an integral role in response to *M. tuberculosis*. Though all of the ESX-1-modulated genes may not be specifically targeted, at least some of them are likely to be regulated by the bacteria due to their importance to *M. tuberculosis* control. One group of ESX-1-regulated genes includes a number of immune molecules, IL-12 p40, IL-1β, IL-6. Most of the modulated genes, however, lack known functions in the immune system. Further study of the 48 genes targeted by the ESX-1 system may reveal critical insights into anti-mycobacterial host responses and how *M. tuberculosis* combats them.

By gaining a better understanding of both the comprehensive response to *M. tuberculosis* as well as the responses modulated by the ESX-1 secretion system, we have

identified two groups of host responses that may play important roles in control of intracellular bacteria. Understanding the role of the genes repressed by infection may provide insight into the systemic response to mycobacterial exposure. Furthermore, if this widespread repression is important for robust antimicrobial immunity, manipulation of this response in the setting of *M. tuberculosis* infection may yield interesting physiologic outcomes. On a smaller scale, the ESX-1 secretion system modulates only a set of 48 genes. This likely represents a set of genes of particular importance for *M. tuberculosis* control. Understanding their immune functions will yield new insights into antibacterial defense. In addition, manipulating the expression of these unstudied genes critical for *M. tuberculosis* may reveal novel approaches to therapeutic control of infection.

II. ESX-1 as an early virulence determinant

Previous work has shown that the ESX-1 secretion system is critical to growth in mice early in infection (Stanley et al., 2003). That work involved components of the core machinery, and in Chapter 2 here I described similar results in studies of genetic mutants in novel members of the ESX-1 pathway. A straightforward model of the early role of ESX-1 secretion ties together the early growth defect with aberrant cytokine induction of ESX-1 mutant *M. tuberculosis*. However, in a TLR2-/- mouse background, in which many of the aberrant macrophage responses are lost, the early growth defect persists. The simplest explanation for this apparent paradox is that genes whose modulation by ESX-1 is TLR2-independent are the critical determinants of early growth in mice. This set of genes includes a number of interferon-regulated genes, and work from our lab has shown that induction of Type I interferon signaling by the ESX-1 pathway in a TLR-

independent manner is an important virulence strategy for *M. tuberculosis* (Stanley et al., 2007). However, inadequate induction of Type I interferons alone does not account for the entire growth defect observed in ESX-1-deficient bacteria (Stanley, 2006). Taken together, these data suggest that multiple host-pathogen interactions dependent on ESX-1 secretion contribute to the establishment and early growth of *M. tuberculosis* in mice. To more comprehensively address the role of ESX-1 in manipulating TLR responses, growth of wild-type and ESX-1 deficient bacteria should be compared in MyD88-/- mice. This would remove the contributions of other TLR molecules to the induction or repression of ESX-1 modulated genes, enabling a more comprehensive analysis of the importance of ESX-1-mediated manipulation of TLR signaling to *M. tuberculosis* growth in mice. Finally, it is interesting to speculate that the ESX-1 pathway modulates both TLR signaling and Type I interferon signaling in a manner that yields synergistic benefits to the infecting pathogen. In the absence of either pathway alone, an appreciable phenotype may be absent or partial. Testing the growth of wild-type and ESX-1-deficient bacteria in a compound mutant in both TLR signaling as well as Type I interferon signaling taken together with growth data in singly-deficient mice will allow evaluation of potential synergy between the two pathways.

It is also possible that the early growth defect of ESX-1-deficient *M. tuberculosis* reflects systemic differences in the immune responses against wild-type versus mutant bacteria. Little work to date has explored the early immune response to *M. tuberculosis* infection on a system-wide, organismal level. Early work in the area of *M. tuberculosis* pathogenesis demonstrated the importance of a number of immune molecules and cells in control of infection (Caruso et al., 1999; Chan et al., 1995; Flynn and Bloom, 1996;

Flynn et al., 1993; Flynn et al., 1995a; Flynn et al., 1995b). However, at the time, innate immunity was poorly understood and genetic means to distinguish early and late events in the immune response did not exist. With current tools as well as an *M. tuberculosis* mutant with a growth defect that appears to be limited to the first two weeks of infection, we can begin to probe early events in the response to infection and the immunomodulatory activities evolved by the bacteria. For example, we have shown in a primary macrophage culture model that ESX-1-deficient bacteria induce elevated levels of IL-12 in a TLR-dependent manner. It remains to be shown, however, that this occurs *in vivo* and whether macrophages or other immune cells are responsible for elevated IL-12 production. Intracellular cytokine staining along with staining for specific cell surface markers of lung-associated, splenic, and blood cells over time following infection with wild-type and ESX-1-deficient bacteria will allow dynamic analysis of the systemic immune response to *M. tuberculosis* infection. Furthermore, this approach can be applied in different mouse backgrounds to expose the contributions of various signaling molecules in the systemic immune response. These types of analyses are critical to comprehensive understanding of the immune response to infection and will likely reveal insights into both the host adaptation to bacterial infection as well as the role of bacterial counter-adaptations to the host.

III. Rationale for and implications of EspR secretion

As described in Chapter 3, we have discovered a transcriptional activator of ESX-1 secretion that is regulated itself by secretion through the ESX-1 pathway. Though coupling of secretion and transcriptional regulation have been described previously, this

is the first instance in which that coupling is accomplished via secretion of a DNA-binding regulator (Hughes et al., 1993; Rietsch et al., 2005; Urbanowski et al., 2005). The direct secretion of a transcription factor as opposed to the secretion of a secondary regulator of the transcription factor, as in the previously described scenarios, is curious. As discussed in Chapter 3, the logic for this scheme may derive from the simple and direct feedback control that is afforded when the DNA-binding protein is targeted for secretion. On the other hand, additional points of regulation typically confer greater robustness and plasticity to regulatory networks (Baker et al., 2006). As a result, there may be a fitness cost associated with simplifying the ESX-1-EspR feedback loop. Dynamic studies of the activation of EspR activity in response to *in vitro* and intra-macrophage conditions and the subsequent negative feedback will yield greater clarity into the functioning of this regulatory system and its specific benefits and costs.

An alternative rationale for direct secretion of EspR is that the protein has a function outside the cell. Experiments shown in Chapter 3 refute an extracellular role for EspR in ESX-1 secretion. However, we have not ruled out an unrelated role for EspR outside the cell in another process that may also be important for virulence. There are many possible functions for EspR outside the bacteria, but I will describe only two related ideas here. The first is that EspR is able to re-enter the bacterial cell after being exported as a means of resetting the ESX-1 regulatory system. This possibility is more easily conceived if secreted EspR resides in the periplasmic space and is actually not secreted completely out of the cell. Though we believe that our assay for protein secretion is fairly selective for truly secreted proteins and not cell-wall associated or periplasmic proteins (Converse, 2005), it is a formal possibility that EspR resides in the

periplasm and is detected in the culture supernatants due to non-specific release of periplasmic contents during culturing or harvesting protein. Alternatively, it may be that most EspR is secreted completely out of the cell, but that a small fraction is retained in the periplasm. It is also possible, however, that the bacteria have developed a means of binding and taking up EspR that has been released into the culture supernatant. Regardless of the precise location of secreted EspR, it would be very interesting if it were, indeed, taken back up by the bacteria. This would suggest a model of localization-based transcription factor regulation akin to nucleocytoplasmic shuttling of eukaryotic transcription factors.

Another possible extracellular role for EspR is as a signaling molecule. Individual members of a community of bacteria signal to one another via secreted molecules (Waters and Bassler, 2005). Though such signaling has yet to be identified in *M. tuberculosis* it is presumed to exist. There are myriad possible signaling processes in which EspR might participate, if it is in fact a secreted signaling molecule. I will describe just one such speculation here. If EspR secretion by a single bacterium is a mark of that cell activating ESX-1 secretion, the secreted EspR, if detected by neighboring bacteria, might provide a signal that activates or primes ESX-1 secretion in the neighbors. This would allow transmission of a signal between bacteria communicating that extracellular conditions linked to ESX-1 activation are imminent. Conversely, given the potent immunogenicity of ESAT-6 and CFP-10, it may be in the best interest of a community of bacteria to limit activation of ESX-1 secretion to levels that are beneficial to the pathogen without initiating too strong of an immune response. Secreted EspR might act as a negative signal to bacteria, communicating that sufficient activation of ESX-1 secretion

has already occurred in one or more bacteria in the community. Though we have yet to show an extracellular role for EspR, identifying any of a number of functions it may have outside the cell would represent a major advance in our understanding of cell-cell communication in the context of *M. tuberculosis* infection.

IV. Regulation of ESX-1 paralogs and rationale for regulating ESX-1 function

There are 11 duplications of ESAT-6 and CFP-10 in *M. tuberculosis*, including 5 duplications that include additional ESX-1 component homologues (Cole et al., 1998; Gey Van Pittius et al., 2001). Various paralogs of ESAT-6 and CFP-10 have been identified in *M. tuberculosis* culture supernatants (Champion et al., 2006); however, it remains unclear how these proteins are secreted. They do not depend on the prototypical ESX-1 system for secretion, and it is hypothesized that duplicate ESX-1-like secretion machines provide the route of export for the duplicate substrates. It remains an open question whether the secretion of these proteins is important to virulence. If, as with the ESX-1 system, paralogous secretion systems are critical to growth *in vivo* and are regulated by extracellular signals, an EspR-like regulator may be implicated. However, there are no proteins in *M. tuberculosis* that share sequence homology with EspR. This could mean that secretory activity of the other loci is not regulated, or that *M. tuberculosis* encodes functional homologues of EspR that are not identifiable by primary sequence. As more is elucidated about the function and regulation of the duplicate ESX loci, it will be interesting to determine if each has a corresponding transcriptional regulator sensitive to a specific signal.

Though we have shown in Chapter 3 that the *Rv3616c-Rv3612c* operon is upregulated following entry of *M. tuberculosis* into macrophages, we have not demonstrated that this induction occurs similarly in mice. Furthermore, we have not provided evidence that ESX-1 secretion is turned off at some point during the course of macrophage or mouse infection. From the bacterial perspective, activating ESX-1 and then turning it off seems a good strategy. The ESX-1 system seems to be primarily important for modulation of early immune responses, and the primary substrates, ESAT-6 and CFP-10, are potent antigens. Given the immunogenicity of ESAT-6 and CFP-10, turning off their secretion later in infection seems optimal for *M. tuberculosis*. It will be important to gain understanding of the inducing as well as the inactivating signals sensed by the bacteria inside the host. Furthermore, we have generated a strain in which ESX-1 secretion is no longer transcriptionally regulated, providing a reagent to test the importance of transcriptional control of ESX-1 expression *in vivo.* It is intriguing to speculate that dysregulation and hyperproduction of virulence factors – ESAT-6 and CFP-10 – might be detrimental to the bacteria *in vivo* either during early growth or possibly during persistence by supporting a more vigorous and sustained immune response against the infecting pathogen.

Demonstrating the criticality of regulated expression of ESX-1 secretion to bacterial survival in the host would be a major advance in the field of *M. tuberculosis* pathogenesis. Little is known about the dynamic behavior of individual bacteria or bacterial communities inside the host. Assaying the expression patterns of a critical virulence determinant with a seemingly specific role in the early stages of infection would not only grant clarity on the workings and importance of the ESX-1 system, but

also yield insight into the adaptations between bacteria and host over the course of infection. Finally, coupling the dynamic study of the bacteria *in vivo* with dynamic study of the host response to infection would be the ultimate analysis of the host-pathogen interaction.

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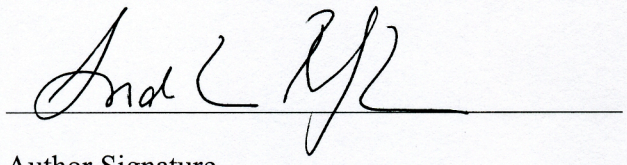
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