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Mechanisms of Inhibition of Macrophage Responses to Interferonγ by *Mycobacterium tuberculosis*

by

Eleanor Zoë Kincaid

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

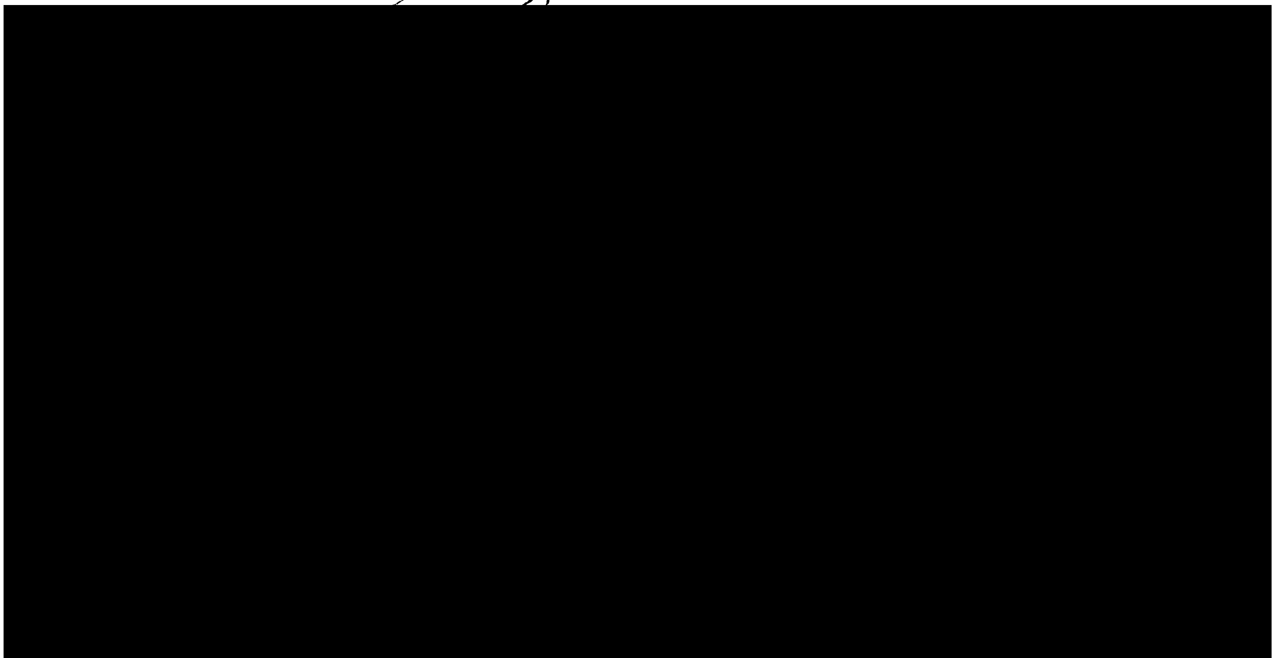
Biomedical Science

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



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First and foremost, I would like to thank my advisor, Joel Ernst. He has pushed me to become a more careful researcher, a clearer speaker and a much better writer. He is intelligent, insightful and knowledgeable on a broad range of topics. Next, I would like to thank my family for support and encouragement throughout my education but especially during my years in graduate school. The friends who have offered love, companionship, and advice are too numerous to mention, but I would like to thank Pauline Yu and Claudius Li in particular.

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I would like to thank the members of my thesis committee, Eric Verdin, Eric Brown and Anthony DeFranco for advice, guidance, suggestions, reagents and techniques. In particular, they advised me to move on from the CBP/p300 work presented in Chapter 2 and urged me to start pulling my data together into what became Chapter 3.

I am indebted to Li-Min Ting, Alejandra Solache and Vijaya Nagabhushanam, who all provided advice and hands-on training in a huge range of techniques. Niaz Banaiee was not only a skilled and insightful collaborator for the work presented in Chapter 5, but was also good company all those hours in the ABSL-3 and at the RNA

bench.

The work presented in Chapter 3 was published in the *Journal of Immunology* in 2003 and is copyright 2003 American Association of Immunologists. Under the guidance of Joel Ernst, I designed, planned and performed all of the experiments presented in that chapter. Vijaya Nagabhushanam initially validated RAW264.7 cells as a model for *M. tuberculosis* inhibition of macrophage responses to IFNγ, and made the RAW264.7 cell line stably transfected with pCIITA-LUC D1, which was used in Figure 6. I would like to thank Veronique Kiermer for outstanding advice and training on chromatin immunoprecipitation.

Chapter 4 Table 1 is a summary of work performed by several currenta nd former members of the laboratory. Claire Escaron, under the supervision of Vijaya Nagabhushanam, initially assayed inhibition of MIG, IP-10, IGTP, LRG-47, ICSBP, IRF-1 and CIITA using published primer pairs. Since that time, I have designed optimized primer pairs and retested IRF-1 and CIITA, in addition to testing NOS2, Invariant Chain, H2-IAa, H2-IEa and H2-DMa. Sarah Benson, as part of her rotation project, designed an optimized primer pair for LRG-47 and retested its inhibition. The genes are marked to indicate whose results are listed in the table. For each gene, results with optimized primer pairs was presented if available. I would like to thank Vijaya Nagabhushanam and Christian Essrich for training me in the use of SYBR Green to assay mammalian gene expression by quantitative PCR. I would also like to thank Christian Callebaut for advice on the pitfalls of using retroviral vectors for promoter studies and Tadashi Nishiya for sending me his protocols for packaging and using VSV-G pseudotyped retrovirus.

Chapter 5 was submitted to the *Journal of Immunology* as a revised manuscript on December 13, 2005. Niaz Banaiee and I contributed equally to this manuscript. Niaz, who was comentored by William Jacobs, Jr. and Joel Ernst, created the Δ*lspA* mutant strain and the Δ*lspA attB*::*lspA* complemented strain, using techniques and reagents from the Jacobs laboratory. Niaz assayed the *lspA* expression in the knock out and complemented strain and assayed differences in the mobility of three lipoproteins between wild type, mutant and complemented strains. He prepared the inoculum for all live infections, and made the *M. tuberculosis* whole cell lysates used in Figures 4 and 5. Mangala Tawde performed the Pam_3CSK_4 dose response presented in Figure 1. Jacob Bagley, under the guidance of Joel Ernst and Niaz Banaiee, performed experiments with γ-irradiated *M. tuberculosis* H37Ra that suggested that the Δ*lspA* mutant lacked TLR2 agonist activity. Under my supervision, Ulrike Buchwald optimized the conditions for secretion of IL-8 and TNFα in response to live *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv. Under Niaz's supervision, Ulrike performed the initial infection of RAW264.7 and 293-TLR2 cells with wild type, knock out and complemented *M. tuberculosis* H37Rv, and analyzed the supernatants by ELISA. For Figures 4 through 9, I performed all treatments of mammalian cells, and all subsequent analysis of cells and cell culture supernatants. These subsequent analyses included ELISA, flow cytometry and quantitative PCR.

Thanks again to friends, family, advisors and co-workers.

INVOLVEMENT OF CO-AUTHORS: THESIS ADVISOR'S STATEMENT

Eleanor Kincaid's dissertation research is represented so far in one published paper, another that is currently undergoing minor revision, and at least one additional paper on which Eleanor will be first author. Additional work on which Eleanor has made significant contributions but that are not formally part of her dissertation will provide her prominent authorship on two additional papers.

The work presented in Chapter Two represents work that was performed by Eleanor, shortly after beginning her dissertation work. Since the results excluded several potential mechanisms of *M. tuberculosis* inhibition of macrophage responses to interferon gamma, the course of her subsequent experiments was redirected to pursue alternative mechanisms. The results will be submitted for publication after her current, higher-priority work is completed and accepted.

The work presented in Chapter Three, and published in a paper in the *Journal of Immunology*, on which Eleanor is the sole first author, represents research that was led by Eleanor, although she obtained technical advice from others in the laboratory and other laboratories. In addition, Eleanor wrote the paper and modified it according to comments of the reviewers of the original manuscript. The paper demonstrates the broad range of experimental systems that Eleanor was capable of using to address questions, and has been widely cited by others.

The work in Chapter Four was generated by several members of the laboratory, but Eleanor provided the coordination and leadership of those efforts. In several cases, Eleanor initially taught the techniques to the individuals that provided the results, in some cases because Eleanor was the first in the laboratory to adopt and validate the techniques.

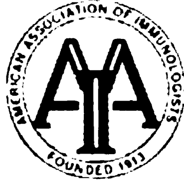
The work in Chapter Five, which is reported in a manuscript that is currently undergoing minor revision, is the result of the efforts of Niaz Banaiee, a postdoctoral fellow, and Eleanor. Eleanor's established methods and experimental systems provided the background for characterization of a mutant strain of *M. tuberculosis* that was constructed by Niaz. Eleanor performed most of the experiments characterizing cellular interactions with the *M. tuberculosis* mutant, and oversaw the remainder. In particular, she critically evaluated all of the experimental results, and the paper reflects major intellectual contributions that Eleanor provided. Eleanor also wrote most of the paper.

In addition to the results reported in her dissertation, Eleanor has initiated several projects that have been extended by other members of the laboratory. One example is a macrophage cell line that Eleanor constructed and characterized, that constitutively expresses class II transactivator (CIITA) and high levels of surface MHC class II. That cell line has been used in an ongoing project by another member of the laboratory, to determine whether inhibition of macrophage responses to interferon gamma is the sole mechanism of M. tuberculosis inhibition of class II antigen presentation in macrophages. Eleanor will be a prominent coauthor of that paper. Other contributions that Eleanor has made will continue to provide projects for present and future members of the laboratory.

The work described in this dissertation meets the standards required for the completion of the Ph.D. program in Biomedical Science.

Joel D. Ernst, M.D.

Thesis Advisor



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Abstract Eleanor Zoë Kincaid

Interferon-γ (IFNγ) is essential for the host immune response to *Mycobacterium tuberculosis*. We have found that infection of human monocyte derived macrophages, murine bone marrow derived macrophages, or human or mouse macrophage cell lines resulted in reduced cellular responses to IFNγ. This inhibition in the response to IFNγ was gene-selective and exerted at the transcriptional level. I found no defect, however, at any step in signal transducer and activator of transcription 1 activation or function. I found no decrease in signal transducer and activator of transcription 1 protein level, tyrosine or serine phosphorylation or binding to cAMP response element binding protein binding protein/p300. I also found no defect in transactivation of a synthetic γ-activated sequence construct or binding to an endogenous promoter. I observed this inhibition not only with live, virulent *M. tuberculosis*, but also with γ-irradiated or lysed *M. tuberculosis*. Using deletion constructs of the murine class II transactivator type IV promoter, I found that only a small portion of this promoter was required for sensitivity to inhibition by *M. tuberculosis*. Site-directed mutagenesis of candidate transcription factor binding sites in this region could not separate IFNγ responsiveness from sensitivity to inhibition by *M. tuberculosis*. Although purified *M. tuberculosis* 19kDa lipoprotein and synthetic lipopeptides could mimic this inhibition, we found that mature mycobacterial lipoproteins were not required in the context of whole bacteria. Toll-like receptor 2, a mammalian innate immune receptor that recognizes lipoproteins and a range of other microbial products, was required for potent inhibition. I found that *M. tuberculosis* inhibition of macrophage responses to IFNγ required extended pretreatment and new

protein synthesis. I also found that histone deacetylase inhibitors did not relieve the *M. tuberculosis* inhibition of macrophage responses to IFNγ, but instead, inhibited the response to IFNγ. Together these findings indicate that the prolonged innate immune stimulation in *M. tuberculosis* infected macrophages results in inhibition of the response to IFNγ in these macrophages. This inhibition may contribute to the failure of the immune response to clear *M. tuberculosis* infection.

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Chapter I

Introduction

Tuberculosis Infection

Mycobacterium tuberculosis is one of the most successful human pathogens – an estimated one third of the world's population is infected with *M. tuberculosis* (Dye et al. 1999; Sudre et al. 1992) with 8.8 million new cases in 2002 (Maher and Raviglione 2005). *M. tuberculosis* is also one of the leading infectious causes of death worldwide, with an estimated 1.8 million deaths in 2002 alone (Maher and Raviglione 2005). An estimated 15% of infected individuals develop active tuberculosis during their lifetime (Enarson and Rouillon 1998). Individuals who are coinfected with HIV and *M. tuberculosis*, however, have a 2-8% risk per year of developing active disease (Centers for Disease Control and Prevention 1998). As a result, *M. tuberculosis* is a leading cause of death in HIV+ individuals, causing 13% of AIDS deaths worldwide (Maher and Raviglione 2005).

The antibiotics currently available require long courses of treatment, and incomplete treatment regiments contribute to the global rise in single- and multi-drug resistant *M. tuberculosis* (Espinal and Salfinger 2005). Bacillus Calmette Guérin (BCG), a live attenuated vaccine against tuberculosis, has a variable, and at best partial, ability to prevent adult pulmonary tuberculosis (Colditz et al. 1994). Although BCG protects against the childhood form of disseminated *M. tuberculosis* (Colditz et al. 1994), immunity wanes over time, possibly due to exposure to non-pathogenic environmental mycobacteria in many parts of the world (Fine 1995).

Tuberculosis is transmitted primarily via the aerosol route. When an individual with active pulmonary tuberculosis coughs or sneezes, droplet nuclei containing *M. tuberculosis* are aerosolized. Droplet nuclei with a diameter of 1 to 5 microns can remain

suspended in the air for several hours (Wells 1934), and during this time can be inhaled by other individual. Experimental infection of mice has demonstrated that droplet nuclei in this size range can be inhaled into the alveoli (Sonkin 1951).

It is likely that the first cell to encounter the mycobacterium is the alveolar macrophage. Alveolar macrophages recognize *M. tuberculosis* via a number of cell surface molecules, including complement receptors, mannose receptors (Schlesinger 1993) and scavenger receptor class A (Zimmerli et al. 1996), resulting in phagocytosis of the mycobacterium. Once inside the macrophage, *M. tuberculosis* evades destruction by modifying the vacuole in which it resides. *M. tuberculosis* prevents both acidification of this vacuole and its fusion with components of the lysosomal pathway (Armstrong and Hart 1971; Mwandumba et al. 2004; Vergne et al. 2004). Instead of rapidly being trafficked to a lysosomal compartment, *M. tuberculosis* is retained in a compartment that resembles a recycling endosome (Clemens and Horwitz 1996; Sturgill-Koszycki et al. 1996).

Macrophages recognize *M. tuberculosis* via the innate immune receptors Toll like receptor 2 (TLR2) and TLR4 (Brightbill et al. 1999; Bulut et al. 2005; Means et al. 1999; Underhill et al. 1999). This innate immune recognition results in induction of cytokines such as TNFα and IL-12 (Brightbill et al. 1999; Underhill et al. 1999). Although *M. tuberculosis* appears to inhibit macrophage production of IL-12, dendritic cells, which are also present in the lungs, respond to *M. tuberculosis* with robust IL-12 secretion (Hickman et al. 2002). Cytokines and chemokines secreted at the site of infection direct natural killer cells, monocytes and later primed T-cells to traffic to the site. The

monocytes that are recruited to the site of infection mature into macrophages and join the developing granuloma (Saunders and Cooper 2000).

Mycobacteria enter the lymphatics and drain to the lymph nodes. *M. tuberculosis* may enter the lymphatics directly or may be carried by infected macrophages or dendritic cells. *M. tuberculosis* can establish infection in the draining lymph node, and can be disseminated to other parts of the lung (Balasubramanian et al. 1994) and, in a minority of cases, to other organs via lymphohematogenous spread (Hopewell and Jasmer 2005). Extrapulmonary tuberculosis infection accounts for approximately 15% of cases in individuals with an intact immune system (Farer et al. 1979) and up to 62% of cases in HIV infected individuals (Havlir and Barnes 1999; Small et al. 1991).

Once in the lymph node, *M. tuberculosis* antigens are presented to naive T-cells. Since dendritic cells present antigen to naive T-cells more efficiently than macrophages (Hart 1997), it is likely that dendritic cells are responsible for the education of naive T-cells in the lymph node. These dendritic cells may present antigen from apoptotic alveolar macrophages, or the dendritic cells themselves may be infected (Bodnar et al. 2001; Gonzalez-Juarrero and Orme 2001). By several weeks after initial contact, there is a robust cellular immune response to *M. tuberculosis*, including activation of CD4+, CD8+ and CD1-restricted T-cells. This immune response can be detected as a delayed type hypersensitivity reaction to subcutaneous *M. tuberculosis* purified protein derivative or the presence of antigen-specific IFNγ producing cells in the peripheral blood (Mazurek et al. 2001). Experimental infection of mice has demonstrated that this adaptive immune reaction coincides with the development of granulomas (Kobayashi and Yoshida 1996) and with control of bacterial growth (Mogues et al. 2001).

The primed T-cells traffic back to the site of infection in response to chemokines and proinflammatory signals (Saunders and Cooper 2000). The T-cells recognize infected cells and produce IFNγ. IFNγ is essential for immune control of *M. tuberculosis*, and production of IFNγ is the major contribution of effector T-cells (Flynn and Chan 2001). Mice lacking IFNγ due to gene disruption rapidly succumb to *M. tuberculosis* due to a failure to control bacterial growth (Cooper et al. 1993; Flynn et al. 1993). Humans with mutations in the IL-12/IFNγ axis of adaptive immunity are particularly susceptible to mycobacterial infections, including infections with poorly virulent mycobacteria (Dupuis et al. 2000; Jouanguy et al. 1999).

IFNγ stimulation induces macrophages to kill intracellular pathogens and to present antigens on their cell surface. IFNγ induces cultured human macrophages to kill a number of intracellular pathogens including *Toxoplasma gondii*, *Leishmania donovani, Legionella pneumophila* and *Chlamydia psittaci* (Byrd and Horwitz 1989; Murray et al. 1991; Murray et al. 1983; Nathan et al. 1983) but cannot induce these macrophages to kill *M. tuberculosis* (Douvas et al. 1985; Rook et al. 1986). Cultured murine macrophages can kill *M. tuberculosis* (Chan et al. 1992; Ehrt et al. 2001), but only if they have been primed with IFNγ before they encounter *M. tuberculosis* or *M. tuberculosis* components (Fortune et al. 2004). Reactive oxygen intermediates produced by activated macrophages were initially believed to be responsible for macrophage killing of *M. tuberculosis*, but it is now widely accepted that the primary effector of IFNγ induced macrophage antimycobacterial activity is nitric oxide production (Chan et al. 1992; MacMicking et al. 1997). Recent studies, however, have demonstrated the existence of an IFNγ-dependent,

inducible nitric oxide synthase-independent antimicrobial activity (MacMicking et al. 2003).

Despite the inability to completely clear the infection, a majority of individuals with an intact immune system hold the bacteria in check and never develop active tuberculosis (Comstock 1982). Most active disease is caused not by primary progressive disease but by reactivation at a later time (Comstock 1982). The findings that immunosuppressive therapies, as well as HIV infection, can dramatically increase the chance of progression to active disease suggest that constant immune surveillance prevents this progression.

The immune response to *M. tuberculosis* is characterized by the formation of granulomas. Granulomas start as an aggregation of infected macrophages. Some of these macrophages differentiate into epithelioid macrophages (Rich 1944). Monocytes are recruited to the site of infection, mature into macrophages and join the developing granuloma. These cells can continue to develop into epithelioid macrophages and in some cases multinucleated giant cells (Rich 1944). After the onset of cell mediated immunity, primed T cells are recruited to the granuloma (Saunders and Cooper 2000). Experimental infection of knock out mice has demonstrated that TNFα is required for proper T cell recruitment and granuloma formation (Bean et al. 1999).

The canonical tuberculosis granuloma consists of lymphocytes, activated macrophages, epithelioid macrophages and multinucleated giant cells (Stewart et al. 2003). In some granulomas, the core becomes necrotic. This necrotic tissue is solid (caseous necrosis) and contains very few bacteria. The maintenance of a stable bacterial load has been proposed to be the result of a balance between bacterial growth and

immune killing (Sever and Youmans 1957), but it is now widely believed that the bacteria are in a state of nonreplicating persistence (Munoz-Elias et al. 2005; Wayne and Sohaskey 2001). Although the actual metabolic state of these bacteria remains unclear, it has long been observed that *M. tuberculosis* in caseous granulomas are phenotypically tolerant to antibiotics that would kill *M. tuberculosis* grown in culture (Gomez and McKinney 2004; Vandiviere et al. 1956). In the majority of individuals, these caseous granulomas are well contained and over time may calcify and scar over (Grosset 2003; Rich 1944). For reasons that are not well understood, in some infected individuals, the caseous material undergoes liquefaction (Grosset 2003; Rich 1944). The softening or liquefaction of the caseum correlates with a significant increase in the number of viable bacteria in the granuloma (Long 1935). The liquefaction of the caseum can also lead to the rupture of the granuloma, resulting in cavity formation and the release of the liquefied material and mycobacteria into the airway (Long 1935). A significant number of bacteria are found in the resulting cavitary lesion (Kaplan et al. 2003), and these bacteria can be aerosolized in the form of droplet nuclei and can infect other individuals.

Work Presented in this Thesis

Persistence is a hallmark of *M. tuberculosis* infection, and the ability of *M. tuberculosis* to cause disease is linked to its ability to avoid destruction by the immune system. We have focused on the interactions of *M. tuberculosis* with naive macrophages, as a model of early events in tuberculosis infection. Macrophages routinely kill other intracellular bacteria, but become a haven for *M. tuberculosis* growth. Even after the onset of cell mediated immunity and the subsequent production of IFNγ at the site of

infection (Barnes et al. 1993; Fenhalls et al. 2002), *M. tuberculosis* can persist in macrophages.

We hypothesized that this persistence was due to inhibition by *M. tuberculosis* of the induction of antimicrobial function of infected macrophages. Indeed, pretreatment with *M. tuberculosis* or *M. tuberculosis* components inhibits IFNγ induction of microbicidal functions, as measured in reduced killing of intracellular *Toxoplasma gondii* (Ting et al. 1999) and virulent *M. tuberculosis* (Fortune et al. 2004). We and others (Fortune et al. 2004; Fulton et al. 2004; Noss et al. 2000; Noss et al. 2001; Ting et al. 1999 and Chapter 3; Kincaid and Ernst 2003) have found that *M. tuberculosis* inhibits other immunologically relevant macrophage responses to IFNγ, including MHC class II expression and antigen presentation.

Previous work in the Ernst lab found that signal transducer and activator of transduction 1 (STAT1) dimers in whole cell lysates from *M. tuberculosis* infected macrophages co-precipitated less cAMP enhancer binding protein (CREB)-binding protein (CBP)/p300 than STAT1 dimers from uninfected macrophages. This finding was interpreted as indicating that there was a defect in binding between STAT1 and CBP/p300 in *M. tuberculosis* infected cells. In Chapter 2, I present evidence that there is in fact no defect in the interaction between STAT1 and CBP/p300 in *M. tuberculosis* treated cells. I also demonstrate that histone deacetylase activity is required for macrophage responses to IFNγ.

In Chapter 3 of this thesis, I provide evidence that *M. tuberculosis* inhibits the macrophage response to IFNγ at the level of transcription of IFNγ-responsive genes. I

also demonstrate that this inhibition is gene selective, and is not the consequence of a defect in STAT1 activation or function in *M. tuberculosis* treated macrophages.

Having found a gene selective inhibition of IFNγ induced transcription in *M. tuberculosis*-infected macrophages, I investigated which characteristics of a promoter make it susceptible to inhibition by *M. tuberculosis*. Using deletion constructs of the murine class II transactivator (CIITA) type IV promoter, I determined that the only a minimal fragment of this promoter is required for both IFNγ responsiveness and sensitivity to inhibition by *M. tuberculosis*. Site directed mutagenesis of previously described transcription factor binding sites in this region of the CIITA type IV promoter could not dissociate the response to IFNγ from *M. tuberculosis* inhibition. This work is described in Chapter 4.

Following up on the finding that *M. tuberculosis* 19 kDa lipoprotein inhibits macrophage responses to IFNγ in a TLR2-dependent manner, but that *M. tuberculosis* peptidoglycan causes this inhibition in an TLR2-independent manner (Fortune et al. 2004), Chapter 5 explores the role of mycobacterial lipoproteins and host TLR2 molecules in this inhibition. We found that although mature mycobacterial lipoproteins are not required for inhibition of the macrophage response to IFNγ, TLR2 is required.

Together these findings suggest that *M. tuberculosis* takes advantage of a consequence of prolonged innate immune activation – the inhibition of IFNγ induced gene transcription – to subvert these normally antimicrobial cells and to create a niche for itself.

Abbreviations

BCG, bacillus Calmette-Guérin; CBP, cAMP enhancer binding protein (CREB)-binding protein; CIITA, class II transactivator; STAT1, signal transducer and activator of transduction 1; TLR, Toll-like receptor;

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Chapter II

Mycobacterium tuberculosis inhibits macrophage responses to IFNγ without blocking STAT1 binding to CBP/p300 and independent of histone deacetylase activity

Abstract

Mycobacterium tuberculosis is a leading infectious cause of death worldwide. A major component of this pathogen's success is its ability to persist and progress in the face of a concerted host immune response. Interferon-gamma (IFNγ) is essential for control of *M. tuberculosis* infection, and a block of full IFNγ activation of infected macrophages may explain this immune evasion. The inhibition of macrophage responses to IFNγ is demonstrated in an inability to kill or inhibit the growth of *Toxoplasma gondii*, as well as in a defect in induction of IFNγ responsive genes. Although multiple steps in the IFNγ pathway could be blocked, no defect is seen in STAT1α protein level, serine- or tyrosine-phosphorylation or nuclear translocation. Previous work in the Ernst laboratory suggested a defect in the binding of STAT1 and the transcriptional coactivators CREB-binding protein (CBP) and p300. To determine the importance of this finding, I tested STAT1 binding to CBP/p300 using a complementary technique. I found no defect in the binding of CBP/p300 to STAT1 when the reciprocal precipitation of CBP/p300 was performed. I also investigated the importance of histone acetylation and deacetylation in the *M. tuberculosis* inhibition of macrophage responses to IFNγ. I found that, unlike many genes whose transcription is repressed by histone deacetylase activity, several IFNγ responsive genes require a histone deacetylase activity for their expression. Together, these findings suggest that *M. tuberculosis* disrupts the macrophage response to IFNγ by a mechanism distinct from inhibition of either CBP/p300 binding to STAT1 or histone acetyltransferase activity.

Introduction

Previous work in the Ernst laboratory demonstrated that *M. tuberculosis* infection of human monocyte derived macrophages inhibits responses to IFNγ (Ting et al. 1999). In these studies, *M. tuberculosis* infection did not inhibit STAT1 tyrosine or serine phosphorylation, dimerization, nuclear translocation or recognition of specific DNA sequences. Results from co-precipitation studies, however, suggested a defect in the interaction between STAT1 and the transcriptional coactivators p300 and CBP in *M. tuberculosis* infected macrophages.

CBP and p300 are closely related transcriptional coactivators required for the transcription of genes regulated by a number of transcription factors, such as STAT1, AP-1, NF-AT, NF-κB and nuclear receptors (Horvai et al. 1997; Kamei et al. 1996; Ohmori and Hamilton 2000; Sisk et al. 2000). They are required to bridge specific transcriptional transactivators with the general RNA transcription machinery. Both proteins are present in limiting quantities in the cell, and different transcription factors are thought to compete for this limited pool of CBP/p300. In this way, CBP/p300 can act as a molecular switch between opposing signals in the cell.

In addition to its role in mediating the interaction between STAT1 and the basal transcriptional machinery, CBP/p300 has histone acetyltransferase (HAT) activity. In recent years, the vital role of chromatin remodeling as an initiating event is gene transcription has been recognized. Enzymes that acetylate or deacetylate histones set the stage for further chromatin remodeling, and seem to act as a central switch between activation and repression pathways in the cell (Eberharter and Becker 2002). In addition, CBP/p300, and many other proteins initially described at HATs, can acetylate

transcription factors, components of the basal transcriptional machinery and non-histone chromatin proteins (Sterner and Berger 2000). Analogous to the post-translational modulation of protein activity by kinases and phosphatases, the balance of HATs and histone deacetylases (HDACs) sets the activation level of their protein targets (Kouzarides 2000).

I assayed the effect of HDAC inhibitors on *M. tuberculosis* inhibition of macrophage responses to IFNγ and found that inhibition of HDACs resulted in a loss of IFNγ responsive gene expression. I also tested the interaction between STAT1 and CBP/p300 using an alternate technique, and found no defect in their interaction.

Materials and Methods

Cell Culture and M. tuberculosis

Human monocytes were isolated from buffy coats (Stanford University Blood Center) by Ficoll-Paque (Amersham Pharmacia Biotech) separation followed by selection by adherence to tissue culture treated 6-well plates (Costar) for 1 h in RPMI 1640 supplemented with 2mM L-Glutamine (both from Gibco) and 1% heat inactivated human male AB serum (Sigma). Adherent cells were allowed to mature in RPMI 1640 supplemented with 2mM L-glutamine and 2.5% AB serum for 6 d. After 6 d, cell monolayers were washed and subsequently incubated with fresh culture medium supplemented with 2.5% AB serum.

THP-1 cells (American Type Culture Collection) were grown in RPMI 1640 medium supplemented with 10% heat inactivated FCS and 2 mM L-Glutamine (all from Gibco). For each experiment, cells were seeded at 1.5 x 10^5 cells per well of a 6-well plate and treated with 10 nM PMA (Sigma) in RPMI 1640/10% FCS overnight. RAW264.7 cells (ATCC) were grown in DMEM medium supplemented with 10% heat inactivated FCS and 2 mM L-Glutamine (all from Gibco).

A 50 mg/ml (dry weight) stock suspension of gamma-irradiated *M. tuberculosis* H37Rv (Colorado State University, Fort Collins, CO, NIH, NIAID Contract N01 AI-75320) was prepared by syringe shearing to disrupt clumps of bacteria. Briefly, the material was suspended at 250 mg/ml in D-PBS and passed 10 times each through 18 ga, 21 ga, 25 ga and 27 ga needles. The stock was further diluted to 50 mg/ml, aliquotted and stored at –80°C. Each aliquot was thawed and passed 10 times through a 27 ga needle immediately before use.

Coimmunoprecipitation

Monocyte-derived macrophages were incubated with 500 μg/ml gamma-irradiated bacteria and 10μg/ml Polymixin B (Sigma) in RPMI supplemented with L-glutamine and 2.5% heat inactivated human male AB serum for 4 h. After 4 hours at 37°, extracellular bacteria were removed by washing 3 times with RPMI 1640, and fresh RPMI 1640/2.5% AB serum was added. After 2 days of *M. tuberculosis* treatment, the cell culture medium was changed, and two hours later, cells were treated with 20ng/ml human rIFNγ (Genetech, $3x10^7$ U/mg) or medium alone. After 15 or 30 minutes, cells were lysed on ice in non-denaturing whole cell lysis buffer (Nandan and Reiner 1997). Briefly, cells were lysed in high salt buffer containing 400 mM KCl, then diluted to a final KCl concentration of 100 mM (final buffer composition: 0.1% Triton X-100, 10 mM HEPES pH 7.3, 2 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM NaF, 100 mM KCl, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A). Protein concentration was determined by Bradford Assay (BioRad). 250μg protein and 2μg anti-CBP (CT) polyclonal antibody (Upstate Biotechnology) were used per immunoprecipitation. This antibody binds both CBP and p300. Samples were incubated overnight at 4° with rotation. 20μl of Pierce Ultralink Immobilized Protein A/G beads were then added per reaction and samples were incubated 2 h at 4° with rotation. Samples were then washed 4 times with 100mM KCl buffer. Samples were eluted from the beads by 5 m incubation at 100°C in the presence of 1x Laemmli Sample Buffer (50mM Tris H-Cl pH 6.8, 10% glycerol, 2% SDS, 0.1% Bromophenol blue, 1% β-mercaptoethanol). Experiments with PMA differentiated THP-1 cells were performed as

described for monocyte derived macrophages, except that RPMI 1640 supplemented with 2.5% FCS and 10nM PMA was used instead of RPMI 1640 supplemented with 2.5% human AB serum.

Coimmunoprecipitated STAT1 was detected by immunoblotting, performed as previously described (Ting et al. 1999). Immunoprecipitation of CBP/p300 in all samples was confirmed by immunoblotting. Antibodies used were: monoclonal anti-phospho-tyrosine (Y701) STAT1 at 1:1000 and monoclonal anti-CBP/p300 at 1μg/ml (both from Zymed). Zymed HRP-conjugated goat anti-mouse was used at 1:2500 as a secondary antibody. Bound antibodies were detected with Amersham ECL-Plus reagent. Chemiluminescence was detected by exposure of X-ray film.

Decreased CD64 expression was confirmed by FACS or ELISA for each coimmunoprecipitation experiment. For FACS analysis, cells were scraped, washed, and stained with FITC-conjugated anti-CD64 (Ancell), counterstained with propidium iodide (1 μg/ml; Sigma), and analyzed on a Becton Dickinson FacSort (10,000 live cells per condition). For ELISA analysis, cells were lysed in ELISA lysis buffer (0.3% NP-40, 2 mM EGTA, 4 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin). Lysates were incubated in 96-well plates coated with 200 ng/well human IgG1 (Sigma), and bound CD64 was detected using HRP-conjugated anti-CD64 monoclonal Ab (clone 32.2) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma).

HDAC Inhibitor Treatments

For TSA treatment of THP-1 cells, cell were matured by overnight treatment with 10nM PMA, and then pretreated with Trichostatin A (TSA, Calbiochem) for 4 h before *M. tuberculosis* treatment. Medium was then replaced with 1.5 ml of RPMI 1640/2.5% FCS containing 500 μg/ml gamma-irradiated bacteria and TSA. After 4 hours at 37°, extracellular bacteria were removed by washing 3 times with RPMI 1640, and fresh RPMI 1640/2.5% FCS media containing 10 nM PMA and the indicated concentration of TSA was added. Forty-eight hours after addition of irradiated bacteria, the THP-1 cells were treated with 200 pg/ml human IFNγ for 16-24 hours before cells were analyzed by FACS as described above.

For HC-Toxin and TSA treated RAW264.7 cells, cells were pretreated with HDAC inhibitor for 1 hr, followed by 4 or 8h treatment with 20ng/ml murine rIFNγ (0.2-2×10^8 U/mg, BD Pharmingen.) For experiments with RAW264.7 cells and gamma-irradiated *M. tuberculosis*, cells were pretreated with HC-Toxin (Biomol International, LP) for 1 h before 8h treatment with 200 μg/ml *M. tuberculosis* in the presence of HC-Toxin. Cells were then treated with murine IFNγ for 4 h. In each case, total RNA was harvested after 4 or 8 h murine IFNγ treatment.

mRNA quantitation.

Total RNA was harvested using Qiagen RNeasy columns. DNA was removed using DNA-free (Ambion). Total RNA was quantitated using RiboGreen (Molecular Probes). One μg of each sample was reverse transcribed using the Reverse Transcription System (Promega). Reverse transcription was primed with random hexamers and oligo(dT) (0.5μg each per reaction). The cDNA equivalent of 10 ng of total RNA (50 ng for

CIITA) of each sample was analyzed by quantitative PCR using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) on an MJ Research Opticon 2. The murine CIITA primers (recognizing all forms) were forward (5'-GAAGTTCACCATTGAGCCATTTAA) and reverse (5'-CTGGGTCTGCACGAGACGAT). The murine GAPDH primers were forward (5'-TGTGTCCGTCGTGGATCTGA) and reverse (5'-CCTGCTTCACCACCTTCTTGA). Relative values were determined by comparing the threshold cycle of each sample to a standard curve consisting of serial dilutions of a positive control cDNA sample.

Results

No decrease in STAT1 bound to CBP/p300 in M. tuberculosis *infected cells.*

Previous work in the Ernst laboratory demonstrated, using oligonucleotide precipitation, that STAT1 dimers from *M. tuberculosis* infected macrophages precipitated less CBP/p300 than STAT1 dimers from uninfected macrophages (Ting et al. 1999). This result was interpreted to indicate that STAT1 binding to CBP/p300 is decreased in *M. tuberculosis* infected macrophages. In the oligonucleotide precipitations, STAT1 dimers were precipitated using a double stranded oligonucleotide containing a gamma-activated sequence (GAS). There is, however, an increased abundance of STAT1 in *M. tuberculosis* infected human macrophages (Ting et al. 1999 and Chapter 3; Kincaid and Ernst 2003), and the excess STAT1 in infected cells could confound the interpretation STAT1 co-precipitation experiments. In addition, since CBP/p300 molecules should be limiting compared to STAT1 dimers in IFNγ treated cells, the proportion of CBP/p300 molecules able to bind STAT1 is arguably more biologically relevant than the proportion of STAT1 dimers able to bind CBP/p300. To determine the effect of *M. tuberculosis* infection on the proportion of CBP/p300 molecules that can bind STAT1 in IFNγ treated macrophages, I immunoprecipitated CBP/p300 from untreated and *M. tuberculosis* treated macrophages and analyzed the eluate for coprecipitated STAT1. I found no decrease in the amount of tyrosine phosphorylated STAT1 coimmunoprecipitated in *M. tuberculosis* treated monocyte derived macrophages compared to control macrophages (Figure 1). I found similar results with the monocytic cell line THP-1 (unpublished data).

HDAC Inhibitors block IFNγ-stimulated Gene Expression

Since *M. tuberculosis* inhibits transcriptional responses to IFNγ without inhibiting STAT1 activation, I focused on steps between transcription factor activation and mRNA expression. In particular, the HAT function of CBP/p300 led me to examine the role of the balance between acetylase and deacetylase activity in macrophage responses to IFNγ. I tested the effects of pretreatment with histone deacetylase inhibitors on macrophage cell lines. I used concentrations of TSA, a potent HDAC inhibitor, from 1nM to 1μM and found that, although higher concentrations of TSA (greater than 100 nM) inhibited IFNγ-induced surface expression of CD64 in THP-1 cells, there was no concentration of TSA that relieved *M. tuberculosis* inhibition of CD64 expression (Figure 2A). This is consistent with my finding (Chapter 3; Kincaid and Ernst 2003) that *M. tuberculosis* treatment does not alter histone acetylation at the CD64 promoter.

To distinguish between the consequences of inhibition of macrophage HDAC activity, and nonspecific effect of TSA, I tested a structurally unrelated HDAC inhibitor, HC-Toxin. I found that induction of MHC class II transactivator (CIITA) expression by IFNγ was inhibited in RAW264.7 cells treated with 0.5 μM, 5 μM or 50 μM HC-Toxin or 300 nM TSA (unpublished data). In addition, I found that pretreatment with 5μM HC-Toxin did not relieve the *M. tuberculosis* inhibition of the macrophage response to IFNγ (Figure 2B).

Discussion

Previous work in the Ernst laboratory demonstrated a defect in IFNγ induction of gene expression in *M. tuberculosis* infected macrophages with no defect in STAT1 activation (Ting et al. 1999). There was, however, an apparent decrease in the association between STAT1 and the transcriptional coactivators CBP/p300. This experiment was performed by precipitating STAT1 dimers and assaying coprecipitated CBP/p300. When I performed the complementary experiment, however, by precipitating CBP/p300 molecules and assaying coprecipitated STAT1, I found no evidence of a defect in STAT1 binding to CBP/p300. Since CBP/p300, unlike STAT1 dimers, are present in cells in limiting quantities, the coimmunoprecipitation of STAT1 by CBP/p300 is more likely to represent the availability of CBP/p300 molecules for transcription of IFNγ-responsive genes than the original STAT1 coprecipitation experiment. My data indicate that CBP/p300 are not sequestered or otherwise unavailable to bind STAT1 in *M. tuberculosis* treated cells. The anti-phosphoSTAT1 antibody used in these experiments was exquisitely sensitive, and I could not exclude the possibility that my coimmunoprecipitations were contaminated with STAT1 carried over from the cell lysates. However, by this time, I had evidence (Chapter 3; Kincaid and Ernst 2003) that *M. tuberculosis* inhibition of macrophage responses to IFNγ was gene-selective. The finding that *M. tuberculosis* inhibits the expression of only a subset of IFNγ responsive genes argues against a global defect in CBP/p300 interaction with STAT1, and I focused on the mechanism of gene selective inhibition.

Since STAT1 activation, including in vitro DNA binding, appeared to be normal in *M. tuberculosis* treated cells, I investigated steps between transcription factor

activation and mRNA expression. We hypothesized a defect in the assembly and retention of the transcriptional apparatus at IFNγ responsive promoters. Since changes in the balance between histone acetylase and deacetylase activities in the cell can have a drastic impact on gene transcription, I assayed the effect of two potent and specific HDAC inhibitors, TSA and HC-Toxin. HDACs inhibitor treatment of cells causes an accumulation of acetylated protein, often resulting in activation of gene expression, in part by reversing the silencing effects of histone deacetylation (Dressel et al. 2000). If *M. tuberculosis* was inhibiting gene expression by increasing HDAC activity in the cell, HDAC inhibitors should reverse this inhibition. Instead I found that TSA or HC-Toxin treatment did not release *M. tuberculosis* inhibition of macrophage responses to IFNγ, but itself inhibited the response to IFNγ.

My results with HDAC inhibitors in macrophage cell lines are consistent with the recent finding that HDAC activity is required for the response to IFNγ in colorectal carcinoma and adenocarcinoma cell lines (Klampfer et al. 2004). In addition, two other groups (Chang et al. 2004; Sakamoto et al. 2004) found that HDAC inhibitor treatment of fibroblasts resulted in an impairment of Type I Interferon-stimulated gene expression. Together, these finding indicate that HDAC activity plays a vital role in the transcriptional response to interferons, although it is unclear whether deacetylation of histones is required or if the deacetylation activity is focused on other factors.

Since HDAC activity could be required at many different points in the response to IFNγ, I looked more specifically at the effects of *M. tuberculosis* treatment of macrophages on the endogenous CD64 promoter. As described in Chapter 3, I found no

defect in histone acetylation or STAT1 binding in response to IFNγ in *M. tuberculosis* treated cells.

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Abbreviations

CBP, cAMP enhancer binding protein (CREB)-binding protein; CIITA, MHC class II transactivator; HAT, histone acetyltransferase; HDAC, histone deacylase; TSA, Trichostatin A.

Figures

Figure 1. *M. tuberculosis* does not inhibit STAT1 binding to CBP/p300. Human monocyte-derived macrophages were treated with gamma-irradiated *M. tuberculosis* or left untreated. Two days later, cells were treated with human IFNγ for the indicated times, and CBP/p300 was immunoprecipitated from protein extracts as described in the materials and methods. Immunoprecipitated CBP/p300 (B) and coimmunoprecipitated STAT1 (phospho-Y701) (A) were detected by immunoblotting. Cells were analyzed by flow cytometry to confirm a significant reduction of surface CD64 expression.

Figure 1

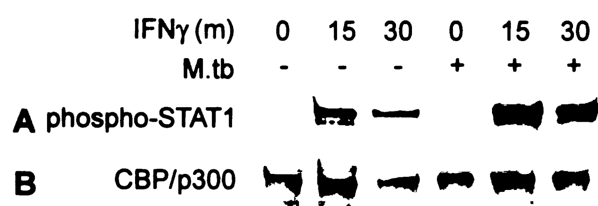
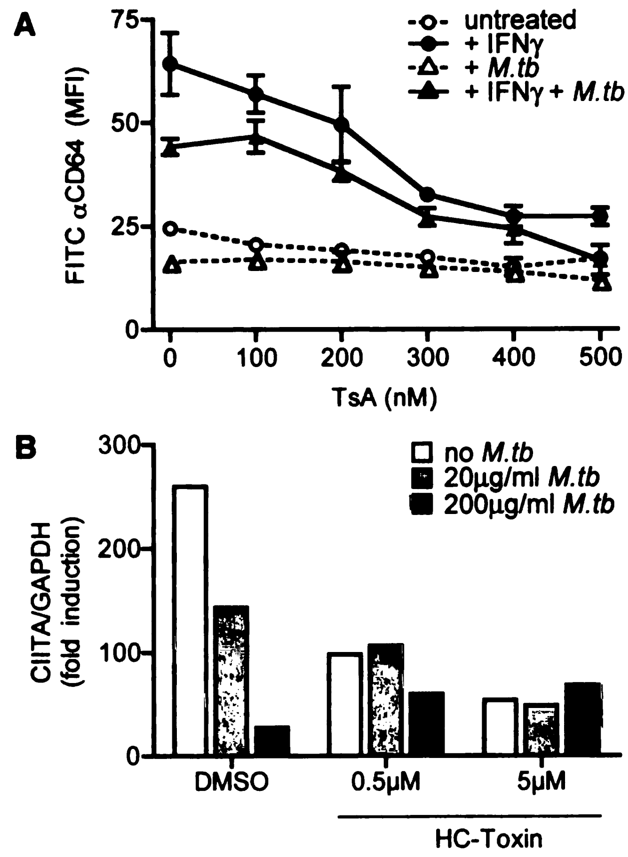


Figure 2. HDAC inhibitors inhibit macrophage response to IFNγ. (A) PMA-differentiated THP-1 cells were pretreated with TSA for 4 h, followed by 4 h *M. tuberculosis* treatment. 2 days later, cells were treated with human IFNγ overnight. Surface expression of CD64 was then analyzed by flow cytometry. Shown are untreated cells (open circle), human IFNγ-stimulated cells (closed circles), *M.tb* treated cells (open triangles) are cells pretreated with *M.tb* followed by human IFNγ treatment (closed triangles). (B) RAW264.7 cells were pretreated with HC-Toxin for 1 h before 8h treatment with the indicated dose of *M. tuberculosis* in the presence of HC-Toxin. Cells were then treated with murine IFNγ for 4 h. Total RNA was harvested and CIITA mRNA expression was assayed by quantitative real-time RT-PCR. All values were normalized to GAPDH. Results are shown as fold induction compared to uninfected sample without murine IFNγ.

Figure 2



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Chapter III

Mycobacterium tuberculosis exerts gene-selective inhibition of transcriptional responses to IFN-γ without inhibiting STAT1 function

Mycobacterium tuberculosis exerts gene-selective inhibition of transcriptional responses to IFN-γ without inhibiting STAT1 function[1]

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Running Title: Selective inhibition of response to IFN-γ by *M.tuberculosis*

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Mycobacterium tuberculosis is a highly successful human pathogen. A major component of this success is the pathogen's ability to avoid eradication by the innate and adaptive immune responses throughout the course of infection. IFN-γ, a potent activator of the microbicidal activities of macrophages, is essential for control of *M. tuberculosis* infection, but is unable to stimulate macrophages to kill *M. tuberculosis*. We have found that infection of the human monocytic cell line, THP-1, resulted in reduced cellular responses to IFN-γ, manifested as impaired induction of CD64 surface expression and transcription. This defect in transcription occurred despite normal activation of STAT1 in infected macrophages: there was no decrease in STAT1 tyrosine or serine phosphorylation, nuclear translocation, or binding of a minimal IFN-γ response sequence. Assays of STAT1 function in *M. tuberculosis*-treated cells also revealed no defect in activation of a minimal γ-activated sequence construct or STAT1 recruitment to and binding at the endogenous CD64 promoter. In addition, *M. tuberculosis* did not affect histone acetylation at the CD64 promoter. The inhibition of transcription was gene selective: while transcription of CD64 and class II Transactivator were decreased, certain other IFN-γ-responsive genes were either unaffected or were increased by *M. tuberculosis*. These results indicate that *M. tuberculosis* inhibits the response to IFN-γ by a mechanism distinct from either suppressor of cytokine signaling-1 inhibition of STAT1 phosphorylation or protein inhibitor of activated STAT interference with DNA binding, and indicate that other mechanisms of inhibition of IFN-γ responses remain to be discovered.

Tuberculosis remains a major worldwide health problem. *Mycobacterium tuberculosis* is estimated to have infected one-third of the world's population, and causes at least 3 million human deaths per year. Although most infected individuals control the infection through a cell-mediated immune response, ~10% of infected people are unable to contain the infection and progress to active disease despite an apparently effective immune response. A major challenge in the study of the host-pathogen interaction in tuberculosis is to define the mechanisms used by *M. tuberculosis* to avoid eradication by the immune response. One potential mechanism is inhibition, by *M. tuberculosis*, of effector mechanisms of the cellular immune response.

Of several immune effectors involved in control of *M. tuberculosis* infection, IFN-γ is the most thoroughly understood. Several lines of evidence support an essential role for IFN-γ in control of *M. tuberculosis* infection. IFN-γ knockout mice challenged with *M. tuberculosis* succumb rapidly, due to a failure to limit bacterial growth (1, 2). In humans, individuals with certain mutations in IFN-γ Receptor 1 (IFNGR1)[3] show a predisposition to infection with poorly virulent mycobacterial strains as well as to severe and recurrent tuberculosis (3). More generally, there is a correlation between the functional severity of mutations that disrupt IFN-γ-mediated immunity, and the extent of susceptibility to mycobacterial infection (4).

Despite the vital role of IFN-γ-mediated immunity in control of *M. tuberculosis*, the immune response is rarely, if ever, effective in clearing the infection. *M. tuberculosis* growth is controlled, but latent infection can reactivate in the future, followed by disease progression. *M. tuberculosis* infection persists in the face of significant amounts of IFN-γ present in infected individuals at sites of infection (5-7) including in the granuloma itself

(8, 9). The observation that IFN-γ is present at sites of *M. tuberculosis* infection suggests that the inability of the immune response to eradicate *M. tuberculosis* is a consequence of a limited response to, rather than defective production of, IFN-γ.

IFN-γ acts primarily through regulation of gene expression (10), and induces macrophages to kill intracellular pathogens including *Toxoplasma*, *Leishmania*, *Legionella*, and *Chlamydia* in vitro (11, 12). IFN-γ, however, cannot induce either monocyte-derived macrophages (MDMØ) or alveolar macrophages to kill *M. tuberculosis* (13, 14). This suggests that *M. tuberculosis* is either resistant to the IFN-γ-responsive microbicidal mechanisms of macrophages, or alternatively, may block macrophage responses to IFN-γ.

IFN-γ signaling is initiated when the cytokine binds as homodimers to its receptor composed of two subunits, IFNGR1 and IFNGR2, resulting in receptor dimerization. Dimerized receptors initiate activation of receptor-associated Janus kinase1 (JAK1) and JAK2 by transphosphorylation. These kinases phosphorylate the IFNGR, allowing the recruitment of STAT1; JAK1/JAK2 are also responsible for phosphorylation of STAT1 on tyrosine 701. Tyrosine-phosphorylated STAT1 homodimerizes, and these homodimers translocate to the nucleus and regulate (induce or repress) gene expression (15). In addition, IFN-γ activates other signaling pathways, such as specific mitogen-activated protein kinases (10, 16), that may augment or attenuate responses to IFN-γ in a cell-specific manner.

We have previously reported that *M. tuberculosis* inhibited responses to IFN-γ in human MDMØ (17). This effect was exerted at the level of mRNA expression of IFN-γ-responsive genes, despite normal activation of STAT1; we found no defect in proximal

steps in the JAK-STAT pathway. We also reported that infection of human MDMØ with *M. tuberculosis* was associated with a decrease in IFN-γ-induced association of the transcriptional coactivator(s) CBP/p300 with STAT1 in an assay wherein CBP/p300 was detected after oligonucleotide affinity precipitation of STAT1. In an initial attempt to extend those findings, we performed the complementary experiment; that is, we immunoprecipitated CBP/p300 and assayed associated STAT1. With this approach, we could not find evidence of a defect in STAT1 binding to CBP/p300. Since the amount of STAT1 binding to CBP/p300 was more likely to correlate with the function of a transcriptional complex at STAT1-dependent genes, we investigated the function of STAT1 in more detail.

To further understand the mechanism(s) whereby *M. tuberculosis* inhibits cellular responses to IFN-γ, we have used the cell line THP-1. We found that the effect of *M. tuberculosis* on IFN-γ responsive gene expression that we had found in primary human MDMØ also occurred in THP-1 cells. We also report in this work that the effect of *M. tuberculosis* is limited to a subset of IFN-γ-responsive genes, that inhibition of transcription of specific genes occurs despite recruitment of STAT1 to endogenous promoters, and does not affect histone acetylation at the promoters of IFN-γ-responsive genes.

Materials and Methods

Cell Culture, M. tuberculosis *and Flow Cytometry*

THP-1 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (Invitrogen, San Diego, CA). For each experiment, cells were seeded at 1.5 x 10^5 cells/well of a six-well plate and treated with 10 nM PMA (Sigma-Aldrich, St. Lois, MO) in RPMI 1640/10% FCS overnight.

Live *M. tuberculosis* was cultured, prepared, and quantitated as previously described (17). A 50 mg/ml (dry weight) stock suspension of γ-irradiated *M. tuberculosis* H37Rv (Colorado State University, Fort Collins, CO; National Institutes of Health, National Institutes of Allergy and Infectious Diseases, Contract N01 AI-75320) was prepared by syringe shearing to disrupt clumps of bacteria. Briefly, the material was suspended at 250 mg/ml in Dulbecco's PBS (D-PBS) and passed 10 times each through 18-, 21-, 25- and 27-gauge needles. The stock was further diluted to 50 mg/ml, aliqotted and stored at –80°C. Each aliqot was thawed and passed 10 times through a 27-gauge needle immediately before use.

Before addition of *M. tuberculosis*, PMA-containing media was removed from the adherent THP-1 cells, and was replaced with 1.5 ml of RPMI 1640/2.5% FCS containing *M. tuberculosis* at a multiplicity of infection of 12.5 live bacteria or 500 μg/ml γ-irradiated bacteria. After 4 hours at 37°C, extracellular bacteria were removed by washing three times with RPMI 1640, and fresh RPMI 1640/2.5% FCS media containing 10 nM PMA was added.

Forty-eight hours after addition of live or irradiated bacteria, the THP-1 cells were treated with IFN-γ for 16-24 hours before cells were scraped, washed, and stained for

flow cytometry. For experiments with live *M. tuberculosis*, cells were stained with PE-conjugated anti CD64 (Ancell, Bayport, MN) or isotype control before washing and fixation with 4% paraformaldehyde for 1 hour. After treatment with paraformaldehyde, cells were analyzed by flow cytometry (10,000 total events per sample). For experiments with irradiated *M. tuberculosis,* cells were stained with FITC-conjugated anti-CD64 (Ancell), counterstained with propidium iodide (1 μg/ml; Sigma-Aldrich), and analyzed by flow cytometry (10,000 live cells per condition).

Unless otherwise indicated, 20 ng/ml ($3x10^7$ U/ml) human rIFN-γ (Genentech, South San Francisco, CA) was used in all experiments. This concentration is ~5-fold above the concentration that elicited maximal CD64 surface expression after 16-htreatment of PMA-differentiated THP-1 cells.

Western blotting

To analyze phosphorylation of STAT1, after 2 days of *M. tuberculosis* treatment, the cell culture medium was changed, and 2 h later, cells were treated with IFN-γ or medium alone. After 15 or 30 min, cells were lysed on ice in radioimmunoprecipitation buffer buffer with the addition of Complete-Mini protease inhibitor cocktail (Roche, Basel, Switzerland), and phosphatase inhibitor cocktails I and II (Sigma-Aldrich). Immunoblotting was performed as previously described (17). Abs used were: NEB (Beverly, MA) monoclonal anti-phosphotyrosine (Y701) STAT1 at 1:1000, Upstate Biotechnology (Lake Placid, NY) polyclonal anti-phosphoserine (S727) STAT1 at 1μg/ml, and Zymed (San Francisco, CA) monoclonal anti-STAT1α at 1μg/ml. Annexin I (detected with rabbit polyclonal anti-Annexin I at 1:50,000) was used as a loading

control. Zymed HRP-conjugated goat anti-mouse or goat anti-rabbit was used as a secondary Ab and bound Abs were detected with Amersham (Arlington Heights, IL) ECL-Plus reagent. Chemiluminescence was detected by exposure of x-ray film.

EMSA

After 2 days of *M. tuberculosis* infection, the cell culture medium was changed, and 2 h later, cells were treated with IFN-γ (20 ng/ml) or medium alone. After 15 or 30 minutes, cells were lysed on ice in nondenaturing whole cell lysis buffer (18). Briefly, cells were lysed in high salt buffer containing 400 mM KCl, then diluted to a final KCl concentration of 100 mM (final buffer composition: 0.1% Triton X-100, 10 mM HEPES, pH 7.3, 2 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM NaF, 100 mM KCl, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A). Whole cell lysates were preincubated with specific or nonspecific competitor oligonucleotide, Ab, isotype control, or buffer alone for 10 minutes at room temperature, before the addition of 3.5 pmol ^{32}P end-labeled double stranded γ-activated sequences (GAS)-containing oligonucleotide and 20-min incubation at room temperature. Probes were end-labeled with Redivue adenosine 5'-(γ-^{32}P)-triphosphate (Amersham Pharmacia Biotech, Piscataway, NJ) using T4 polynucleotide kinase (NEB). The sequence of the GAS oligonucleotide is derived from the CD64 promoter (19) (GTATTTCCCAGAAAAGGAAC); the nonspecific competitor is a scrambled version of the CD64 promoter (TCTAAATTTAGTCCAGTAACTGCA). For supershift analysis, 1μg specific Ab (monoclonal anti-STAT1 N-term, Transduction Laboratories, Lexington, KY) or IgG1 control (PharMingen, San Diego, CA) was used. The EMSA incubation

buffer consisted of the nondenaturing whole cell lysis buffer listed above with the addition of 1mM DTT and 50μg/ml poly(dI-dC). Samples were run in a 4% acrylamide, 2.5% glycerol, 0.5x Tris-borate-EDTA gel for 3-4 h. The gel was then dried and exposed to film overnight.

Decreased CD64 expression was confirmed by ELISA for each EMSA experiment. The cells were lysed in ELISA lysis buffer (0.3% Nonidet P-40, 2 mM EGTA, 4 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin). Lysates were incubated in 96-well plates coated with 200 ng/well human IgG1 (Sigma-Aldrich), and bound CD64 was detected using HRP-conjugated anti-CD64 mAb (clone 32.2) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich).

mRNA quantitation and Actinomycin D treatment

After 2 days of infection, cells were treated with IFN-γ (20 ng/ml) for up to 12 hours before total RNA was harvested using Qiagen (Valencia, CA) RNeasy columns. A total of 1 μg of each sample was reverse transcribed using the Reverse Transcription System (Promega, Madison, WI). The cDNA equivalent of 10 ng of total RNA (100 ng for class II transactivator (CIITA)) of each sample was analyzed by quantitative PCR using SYBR Green 2x Mastermix (Applied Biosystems, Foster City, CA) on an ABI PRISM 7700 Sequence Detection System. Relative values were determined by comparing the threshold cycle of each sample to a standard curve consisting of serial dilutions of a positive control cDNA sample. The oligonucleotide primers used were: CD64 488-845 (ATGGCACCTACCATTGCTCAGG,

CCAAGCACTTGAAGCTCCAACTC), CIITA 3222-3713 (TGACCTGGGTGCCTACAAACTC, GCAAGATGTGGTTCATTCCGC), GAPDH 536-899 (TTGGTATCGTGGAAGGACTCATG, TCGCTGTTGAAGTCAGAGGAGAC), guanylate-binding protein-1 (GBP1) 716-1225 (TGAAACTTTTAACCTGCCCAGACT, GCCGCTAACTCCTTTTGAAATAGA), IFN consensus sequence-biding protein (ICSBP) 82 – 278 (AGTGGCTGATCGAGCAGATT, AGTGGCTGGTTCAGCTTTGT) indoleamine-2,3-dioxygenase (IDO) 916-1118 (GGCAAAGGTCATGGAGATGT, CTGCAGTCTCCATCACGAAA), IFN regulatory factor 1 (IRF-1) 727- 1175 (GGCCCTGACTCCAGCACTGTC, GCTACGGTGCACAGGGAATGG), monokine induced by γ (MIG) 711- 910 (CCACATCCCACTCACAACAG, AGGCCTGTAGGCTGATTCAA).

For analysis of mRNA decay rates, after 2 days of infection, cells were treated for 12 h with 20 ng/ml IFN-γ, followed by addition of fresh medium containing 5 μg/ml actinomycin D (Sigma-Aldrich). Total RNA was harvested up to 8 h later using Qiagen RNeasy columns. Samples were analyzed by slot blot. Briefly, samples were denatured in 10mM NaOH then transferred to a positively charged nylon membrane (Bio-Rad, Hercules, CA) by vacuum using a Bio-Rad Bio-Dot SF. The blot was UV irradiated to crosslink RNA to the membrane, then blocked in ExpressHyb (Clontech, Palo Alto, CA) at 68°, before addition of ^{32}P-labeled probe and incubation at 68°C for 2 hours to overnight. Probes were cloned and radiolabelled as previously described (17). Blots were washed twice at room temperature in 2x SSC/0.05% SDS and twice at 50°C in 0.1x SSC/0.1%SDS. Radioactive signals on blots were quantitated using a Storm 840

PhosphorImager (Molecular Dynamics, Sunnyvale, CA. Blots were stripped using boiling 0.5% SDS and reblocked before addition of each subsequent probe.

Transfection and Plasmids

RAW 264.7 cells were grown in DMEM medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine. One day before transfection, cells were seeded at 2×10^6 per 10-cm dish. Cells were transfected with 5 μg pGAS-luciferase reporter plasmid (Stratagene, La Jolla, CA) using TransIT transfection reagent (Mirus, Madison, WI). Eight hours later, cells were split into 12-well plates allowed to re-adhere overnight. CIITA promoter activity was monitored using stable transfectants. Cells were stably cotransfected with a construct containing –477 to +83 of the CIITA promoter IV-driving luciferase (20) and pCDNA 3.1 neo (Invitrogen). Cells were selected and maintained in 400μg/ml Geneticin (Invitrogen). Cells were treated with 632 μl 500 μg/ml γ-irradiated *M. tuberculosis* or left untreated. Twenty-four hours after addition of bacteria, fresh medium with or without 20 ng/ml (transient transfectants) or 2 ng/ml (stable transfectants) murine rIFN-γ (BD PharMingen) was added. After overnight treatment with IFN-γ, cells were washed with D-PBS and lysed using Passive Lysis Buffer (Promega). Luciferase activity was measured using Promega Luciferase Assay reagent in a TD-20/20 Luminometer (Turner Designs, Palo Alto, CA). Luciferase values were normalized to protein concentration using the BCA Protein Assay (Pierce, Rockford, IL). For analysis of surface expression of MHC class II, untransfected RAW 264.7 cells were treated with *M. tuberculosis* and IFN-γ as described for the transient transfectants. These cells were then scraped, washed, and stained for flow cytometry.

Cells were stained with PE-conjugated anti-mouse I-A/I-E (BD PharMingen), counterstained with propidium iodide (1 μg/ml; Sigma-Aldrich), and analyzed by flow cytometry (10,000 live cells per condition).

Chromatin Immunoprecipitation

THP-1 cells were seeded at 2.25 x 10^7 cells per 15-cm dish and treated with 10 nM PMA. Cells were treated with 22.5 ml per dish of γ-irradiated *M. tuberculosis* at 500 μg/ml as described above. After 2 days, 2 ng/ml human IFN-γ was added. Chromatin immunoprecipitations were performed using reagents from Upstate Biotechnology (Lake Placid, NY) chromatin immunopreciptation (ChIP) Kit. After 4 hours of IFN-γ treatment, formaldehyde was added to a final concentration of 1%, and cells were incubated at room temperature with gentle agitation for 10 min. Glycine was then added to a final concentration of 250 mM, and cells were incubated 5 min at room temperature. Cells were washed with D-PBS and lysed in SDS lysis buffer with the addition of Complete-Mini protease inhibitor cocktail (Roche), 10mM sodium butyrate, and phosphotase inhibitor cocktails I and II (Sigma-Aldrich). The lysate was sonicated in a Branson 250 Sonicator with a 1/8 inch tapered micro tip, using 14 pulses of 10 s each at 25% power output. The resulting chromatin solution was diluted (1/10 for anti-acetylated histone IPs, 1/50 for anti-STAT1 IPs) and precleared as described in the Upstate Biotechnology protocol. Precleared lysates were incubated overnight at 4°C with 5 μg of either anti-acetylated histone H3 or anti-acetylated histone H4 polyclonal Abs or 8 μg anti-STAT1 (C-terminal) polyclonal Ab (all from Upstate Biotechnology). Complexes were bound to protein A agarose and washed, and DNA was eluted and purified, all as described in the

Upstate Biotechnology Chip Kit protocol. Five percent of the resulting DNA was used in each PCR reaction. Quantitative PCR was performed in triplicate on an ABI PRISM 7700 Sequence Detection System using SYBR Green 2x Mastermix (Applied Biosystems). Oligonucleotides used were: CD64 promoter −110 to + 46 (GGGAGAGATGGGCTAACAGGTATG, TTGAAGAGGTTCTGCTGGTGGC), GAPDH promoter, −172 to +17 (AAAAGCGGGGAGAAAGTAGGGC, AACAGGAGGAGCAGAGAGCGAAGC)

Results

M. tuberculosis *inhibits responses to IFN-γ in THP-1 Cells*

We have previously shown that live and γ-irradiated *M. tuberculosis* inhibited responses to IFN-γ in human MDMØ(17). Because of the experimental limitations of human MDMØ, we characterized the human monocytic leukemia cell line THP-1 as an alternative to primary cells. We found that PMA-differentiated THP-1 cells upregulated surface expression of FcγRI (CD64) in response to IFN-γ, and we found that infection of these cells with live *M. tuberculosis* resulted in inhibition of the response to IFN-γ comparable to that seen in MDMØ (Fig. 1). In addition, we found that γ-irradiated *M. tuberculosis* also inhibited IFN-γ induction of CD64 in THP-1 cells (data not shown).

M. tuberculosis *inhibits THP-1 cell responses to IFN-γ without affecting proximal events in IFN signaling.*

As an initial step in characterizing the mechanism of *M. tuberculosis* inhibition of responses to IFN-γ in THP-1 cells, we examined STAT1 content and IFN-γ-stimulated tyrosine phosphorylation of STAT-1. There was no decrease in STAT1α protein content in THP-1 cells treated with γ-irradiated *M. tuberculosis* (Fig. 2*A*), in contrast to the decrease that has been reported for the transcription factors upstream stimulatory factor 1 and regulatory factor X 5 in cells infected with *Chlamydia trachomatis* (21). In fact, the abundance of STAT1 was increased in THP-1 cells exposed to *M. tuberculosis*, as we previously observed in primary MDMØ. Before addition of IFN-γ, there was low background STAT1 tyrosine phosphorylation in both untreated and γ-irradiated *M. tuberculosis*-treated THP-1 cells (Fig. 2*B*). Within 15 min of IFN-γ treatment, there was

robust phosphorylation of STAT1α and STAT1β on tyrosine 701 in both *M. tuberculosis*-treated and control cells. There was no decrease or delay in STAT1 tyrosine phosphorylation in response to IFN-γ in the *M. tuberculosis*-treated cells compared to that of control cells (Fig. 2*B*). Similar results were seen in PMA-differentiated THP-1 cells infected with live *M. tuberculosis*: there was no difference in the rate or extent of STAT1 tyrosine phosphorylation in response to IFN-γ (data not shown). Because the transcriptional activity of STAT1 is confined to STAT1α, and STAT1β is transcriptionally inactive (22, 23), we examined THP-1 cells to determine whether *M. tuberculosis* altered the relative amounts of STAT1α and STAT1β in THP-1 cells. We found no change in the relative abundance of STAT1α compared to STAT1β after treatment with *M. tuberculosis* (data not shown).

In addition to tyrosine phosphorylation, phosphorylation of STAT1α at serine 727 is required for full transcriptional activity (24). Unlike human MDMØ, we found a significant level of serine-phosphorylated STAT1 in PMA-treated THP-1 cells in the absence of IFN-γ stimulation. Serine 727 phosphorylation of STAT1α was further increased following addition of IFN-γ treatment and this was not diminished by prior exposure to *M. tuberculosis* (Fig. 2*C*).

Since we found that IFN-γ induced STAT1 serine and tyrosine phosphorylation were not decreased in *M. tuberculosis*-exposed cells, we conclude that defects in STAT1 phosphorylation do not account for the defects in responsiveness to IFN-γ that we observed. This also implies that steps proximal to STAT1 activation, including expression and function of IFNGR1, IFNGR2, JAK1 and JAK2 are unaffected by *M. tuberculosis* under the conditions of our experiments.

M. tuberculosis *does not affect STAT1 dimerization or DNA binding.*

Tyrosine phosphorylation is required for formation of STAT1 homodimers; these dimers are then competent to bind DNA containing a consensus γ-activated sequence (GAS). To determine whether *M. tuberculosis* interferes with dimerization and GAS binding of the STAT1 in response to IFN-γ, we used an EMSA using a 20-bp oligonucleotide probe containing the GAS and flanking sequences from the human CD64 promoter (19). Lysates from THP-1 cells that had not been treated with IFN-γ showed no detectable GAS binding activity. Lysates from uninfected cells that had been treated with IFN-γ for 15 or 30 minutes showed significant GAS binding, and there was no decrease in GAS binding in lysates from *M. tuberculosis*-infected cells at either time point. This GAS oligonucleotide binding was specific, as indicated by inhibition in the presence of a 50-fold excess of unlabelled CD64 GAS oligonucleotide, but not by a 50-fold excess of unlabeled control oligonucleotide. All shifted complexes in lysates from infected cells contained STAT1, as evidenced by a complete supershift of this band with an anti-STAT1 Ab (Fig. 3).

M. tuberculosis *inhibits IFN-γ-induced increases in CD64 mRNA in infected THP-1 cells*

Despite normal STAT1 activation in *M. tuberculosis*-infected cells, there was a significant defect in CD64 surface expression. Because this decrease in surface expression could reflect a defect in mRNA transcription, RNA degradation, protein synthesis or protein trafficking, we examined the steady state level of CD64 mRNA in live *M. tuberculosis*-infected and control cells in response to IFN-γ. We found that,

comparable to MDMØ, PMA-treated THP-1 cells expressed a low basal level of CD64 mRNA in the absence of IFN-γ. After 4 and 8 hours of IFN-γ treatment, control cells exhibited an almost 9-fold increase in CD64 mRNA. *M. tuberculosis*-infected cells expressed 34 and 41% less CD64 mRNA than uninfected cells at 4 and 8 h, respectively (Fig. 4*A*). Similar results were found in THP-1 cells treated with γ-irradiated *M. tuberculosis* (Fig. 4*B*). Because this decrease in mRNA level was of a magnitude comparable to the decrease in CD64 surface expression, we concluded that the defect imposed by *M. tuberculosis* is exerted at the level of mRNA expression.

M. tuberculosis *does not accelerate the decay of CD64 mRNA.*

The decrease in CD64 mRNA levels in *M. tuberculosis*-infected THP-1 cells could be the result of either decreased transcription or increased degradation. To distinguish between these two possibilities, we assayed the degradation rate of CD64 mRNA. We treated *M. tuberculosis*-infected THP-1 cells with IFN-γ for 12 h before changing to fresh media containing 5 μg/ml actinomycin D. RNA harvested before the addition of actinomycin D indicated that CD64 mRNA steady state level was decreased in infected compared to uninfected cells after 12 hours of IFN-γ treatment (data not shown). After addition of actinomycin D, however, the rate of decay of CD64 message in uninfected and infected cells was indistinguishable (Fig. 5). These results indicate that the decrease in CD64 (or CIITA, data not shown) message is not due to increased degradation, and therefore is likely to be due to decreased transcription.

M. tuberculosis *inhibits transcription from the IFN-γ-responsive CIITA IV promoter*

To examine the effect of *M. tuberculosis* on transcription from an IFN-γ responsive promoter, we used a promoter construct containing –477 to +83 of the murine CIITA promoter IV-driving luciferase (20). Because our efforts to achieve adequate transfection efficiencies to analyze responses in *M. tuberculosis*- and IFN-γ-treated THP-1 cells met with limited success, we used the murine macrophage-like cell line RAW 264.7 for these experiments, because *M. tuberculosis* inhibits responses to IFN-γ in these cells by a mechanism that is indistinguishable from that in THP-1 cells (V. Nagabhushanam and J.D. Ernst, unpublished observation). In RAW 264.7 cells stably transfected with the CIITA promoter IV-driving luciferase construct, luciferase expression increased ~4-fold after overnight treatment with IFN-γ. In *M. tuberculosis*-treated cells, however, there was a marked defect in the response to IFN-γ (Fig. 6). The observation that *M. tuberculosis* inhibited transcriptional activation of a reporter gene driven by a promoter fragment provides further evidence that the effect of *M. tuberculosis* is exerted at the level of transcriptional activation of IFN-γ-responsive genes.

A minimal GAS promoter is insufficient for inhibition by M. tuberculosis

To further examine IFN-γ signaling and STAT1 function in *M. tuberculosis*-treated cells, we used a minimal 4x GAS promoter driving luciferase. In the absence of IFN-γ, there was very low luciferase expression in RAW264.7 cells transfected with the GAS promoter-driving luciferase construct. After overnight treatment with IFN-γ, luciferase expression increased ~200-fold in control cells. In *M. tuberculosis*-treated cells, luciferase expression was induced to a slightly higher level in response to IFN-γ

(Fig. 7*A*). The amount of *M. tuberculosis* and the duration of treatment used in these experiments were able to inhibit IFN-γ signaling in RAW cells; untransfected cells treated in parallel showed a marked defect in IFN-γ induction of MHC class II surface expression (Fig. 7*B*). The observation that *M. tuberculosis* did not inhibit transcriptional activation from a synthetic GAS element indicates that STAT1 is functionally activated to a normal extent, and suggests that additional or alternative elements are required for *M. tuberculosis* inhibition of transcription.

M. tuberculosis *does not inhibit STAT1 binding to the endogenous CD64 promoter*

Although STAT1 is competent to bind to and drive transcription from minimal GAS sequences in *M. tuberculosis*- treated cells, neither EMSA nor transient transfection of reporter constructs necessarily reflects the STAT1-binding activity at endogenous promoters. Standard EMSA conditions may overestimate the stability of STAT1 binding (25), and transient transfections may not reflect the chromatin structure and accessibility of the native promoter. We therefore used chromatin immunoprecipitation to determine whether *M. tuberculosis* inhibits transcriptional responses to IFN-γ by altering recruitment or binding of STAT1, and/or decreasing histone acetylation at the endogenous CD64 promoter in intact THP-1 cells.

Using modified experimental conditions, which revealed a 50-60% inhibition of mRNA expression in *M. tuberculosis*-treated cells (Fig. 8*A*), we performed ChIP analysis using an anti-STAT1 Ab, to determine whether *M. tuberculosis* inhibits CD64 expression by decreasing STAT1 recruitment to or binding at the endogenous CD64 promoter. We used a saturating amount of anti-STAT1 Ab, as determined in pilot experiments. In the

absence of IFN-γ treatment, association between STAT1 and the endogenous CD64 promoter was not significantly different from the mock-immunoprecipitation control (data not shown). In contrast, the amount of CD64 promoter DNA coprecipitated with STAT1 increased ~5 fold after 30 minutes of IFN-γ treatment, indicating a rapid recruitment of STAT1 to the CD64 promoter (Fig. 8*B*). However, there was no significant difference in the amount of STAT1-associated CD64 promoter in *M. tuberculosis*- treated cells compared to control cells. By mixing lysates of unstimulated and IFN-γ-stimulated cells in varying ratios, we confirmed that the immunoprecipitation method used in these assays was sufficiently sensitive to detect a 25% decrease in STAT1 binding to the CD64 promoter (data not shown). The finding that there was no decrease in CD64 promoter coprecipitated with STAT1 indicates that there was no detectable defect in STAT1 recruitment to or stabilization at the native CD64 promoter in response to *M. tuberculosis*, despite an ~50% decrease in CD64 mRNA in the same experiment (Fig.8*A*).

Enzymes that acetylate or deacetylate histones (histone acetyltransferasesa and histone deacetylases, respectively), in conjunction with other chromatin-remodeling factors, play a vital role in gene regulation. Histone acetylation, due to its impact on chromatin organization, can act as a central switch between permissive and repressive chromatin structure (26). To determine whether *M. tuberculosis* inhibits IFN-γ-dependent gene transcription by decreasing the acetylation of histones, we examined the extent of acetylation of histones H3 and H4 at the CD64 promoter. We found that the amount of CD64 promoter DNA coprecipitated with acetylated histone H3 increased ~3-fold after 30 minutes of IFN-γ treatment, and that a similar amount of coprecipitated

CD64 promoter was detected in *M. tuberculosis*-treated cells compared to untreated cells (Fig. 8*C*). Likewise, there was no detectable difference in acetylated histone H4 at the CD64 promoter in *M. tuberculosis*-treated THP-1 cells (Fig. 8*D*).

The inhibitory effect of M. tuberculosis *is restricted to a subset of IFN-γ-responsive genes.*

To determine whether *M. tuberculosis* inhibits transcription of all IFN-γ-responsive genes, we extended our analysis of RNA expression to include additional genes, using real time RT-PCR. As previously noted, CD64 mRNA levels were decreased in *M. tuberculosis*-infected cells after 4 and 8 hours of IFN-γ treatment. CIITA, the transcriptional coactivator required for MHC class II expression, was induced ~100-fold after 4 and 8 h of IFN-γ treatment. This induction was ~40% less in *M. tuberculosis*-infected cells (Fig. 9*A*).

In contrast to CD64 and CIITA, whose expression was inhibited in *M. tuberculosis*-infected cells, several IFN-γ-responsive genes were slightly or substantially increased in the same experiment. In infected cells, there was a slight increase in the IFN-γ induction of GBP-1 (Fig. 9*B*), an IFN-γ responsive GTPase. IRF-1 (Fig. 9*C*), a transcription factor required for the transcription of a subset of IFN-γ-responsive genes, also showed a slight increase in its IFN-γ induction. In contrast, *M. tuberculosis* infection caused an 3- to 5-fold increase in IFN-γ induction of RNA encoding indoleamine-2,3-dioxygenase (IDO, Fig. 9*D*).

In a longer time course in *M. tuberculosis*-treated cells, RNA expression of the transcription factor ICSBP (Fig. 9*E*) and the chemoattractant MIG (Fig. 9*F*) were not

decreased in *M. tuberculosis*-treated cells at any time point, despite a decrease in CD64 mRNA at all time points in the same experiment (Fig. 4*B*). These results show that the effect of *M. tuberculosis* does not extend to all IFN-γ-responsive genes, and also confirm that the inhibition of IFN-γ responses is not the result of a global depression of transcription in these cells.

Discussion

To extend our previous work on *M. tuberculosis* inhibition of IFN-γ signaling, we found that PMA-treated THP-1 cells exhibited a comparable effect by *M. tuberculosis*, and provided greater experimental flexibility and reproducibility. This indicates that the mediators of *M. tuberculosis*-induced inhibition of responses to IFN-γ are not restricted to primary human macrophages. In addition, in every assay in which live infection and treatment with γ-irradiated *M. tuberculosis* were compared, the results were consistent between the two systems.

Several distinct mechanisms of inhibition of IFN-γ signaling have been described. *Mycobacterium avium* (27) and *Leishmania donovani* (28) have both been shown to inhibit IFN-γ signaling by downregulating IFNGR expression at the cell surface. In contrast, our finding of normal phosphorylation of STAT1 in *M. tuberculosis*-infected cells indicates that proximal signaling steps are not inhibited. Normal phosphorylation of STAT1 in response to IFN-γ also indicates that suppressor of cytokine signaling-1, which regulates IFN-γ signaling by blocking JAK1/2 phosphorylation of STAT1, is not responsible for the defect.

Another physiological inhibitor of IFN-γ signaling, protein inhibitor of activated STAT, acts by blocking DNA binding by activated STAT1 (29). Because our EMSA, supershift and ChIP experiments indicated that GAS binding was normal in *M. tuberculosis*-infected THP-1 cells, inhibition of DNA binding of STAT1 is not likely to be involved in *M. tuberculosis* inhibition of IFN-γ signaling.

Decreased expression of certain IFN-γ-responsive genes is not the result of a general transcription block, because expression of luciferase under the control of a

minimal GAS promoter was unaffected by *M. tuberculosis* treatment of macrophages and IFN-γ-responsive genes such as IRF-1, GBP-1 and IDO were expressed to a normal or increased extent. Our observation that CIITA, which is required for MHC class II expression, is among the genes whose induction was markedly inhibited by *M. tuberculosis*, is consistent with the finding that *M. tuberculosis* is capable of inhibiting MHC class II mRNA expression in IFN-γ-treated murine macrophages (30, 31). This also emphasizes a specific means whereby *M. tuberculosis* inhibition of IFN-γ responses could limit the recognition of infected macrophages, since IFN-γ may be unique in its ability to induce MHC class II on macrophages.

The finding that actinomycin D treatment did not reveal any change in the degradation rate of CD64 or CIITA mRNA in *M. tuberculosis*-infected cells, together with the defect in luciferase expression driven by a portion of the CIITA IV promoter in *M. tuberculosis*-treated cells supports the conclusion that *M. tuberculosis* exerts its effect at the level of transcription. This transcriptional defect, however, was not the result of decreased STAT1 access or binding to the CD64 promoter, because chromatin immunoprecipitation experiments showed no decrease in STAT1 bound to the endogenous CD64 promoter in *M. tuberculosis*-treated THP-1 cells. Consistent with STAT1 binding to the promoter, histone acetylation, a marker for remodelled chromatin and transcriptionally accessible promoters, was normal in *M. tuberculosis*-treated cells. Despite a defect in certain IFN-γ-responsive genes in *M. tuberculosis*-infected cells, we have found no defect in STAT1 function at any step from protein level and phosphorylation to binding of the native CD64 promoter and recruitment and/or activation of histone acetyltransferases. Because a subset of IFN-γ-responsive genes

were inhibited by *M. tuberculosis*, we expect that specific elements and regulatory mechanisms distinguish genes that are sensitive to inhibition from those that are resistant to inhibition. Our observations, together with those of others (17, 30-38), that virulent mycobacteria and mycobacterial components can inhibit cellular responses to IFN-γ suggests that this is one general mechanism whereby the bacteria can survive in humans and experimental animals that develop a cellular immune response to Ags produced by the bacteria. In addition, these observations may help to explain recent findings that IFN-γ responses do not correlate with assays of mycobacterial growth inhibition after bacillus Calmette-Guérin (BCG) vaccination in humans (39). In addition, in a study comparing BCG vaccination of rhesus and cynomolgus monkeys, two highly related macaque species, found that there was no correlation between purified protein derivative-induced production of IFN-γ by PBMCs and protection conferred by BCG vaccination (40). Together, these observations suggest that efforts to develop new vaccines for tuberculosis should not solely rely on T lymphocyte production of IFN-γ as the in vitro correlate of potential efficacy of candidate vaccines. Moreover, further understanding of the underlying mechanisms whereby *M. tuberculosis* inhibits cellular responses to IFN-γ may allow means for circumventing these mechanisms, and achieve more effective containment or eradication of the bacteria by a cellular immune response.

Acknowledgements

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Figure 1. *M. tuberculosis* inhibits induction of CD64 surface expression in THP-1 cells. THP-1 cells were infected with live *M. tuberculosis* or left uninfected. Two days later, cells were treated overnight with IFN-γ. Cells were stained with PE-labeled anti-CD64, before fixation with 4% paraformaldehyde. Ten thousand cells for each condition were examined by flow cytometry. *A*, Fluorescence intensity vs. cell number and, *B*, mean CD64 fluorescence (mean ± SD for triplicate samples). Representative of seven independent experiments.

Figure 1

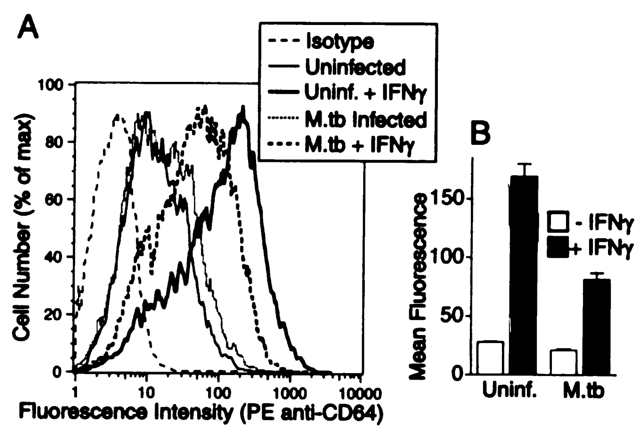


Figure 2. *M. tuberculosis* does not inhibit IFN-γ-stimulated STAT1 tyrosine phosphorylation . THP-1 cells were treated with γ-irradiated *M. tuberculosis* or left untreated. Two days later, cells were treated with IFN-γ for the indicated times, and protein extracts were examined by immunoblotting. One microgram of protein was loaded per lane. Blots were incubated with Ab specific for STAT1α(*A*), tyrosine (Y701)-phosphorylated STAT1 (*B*), or serine (S727)-phosphorylated STAT1 (*C*). Equivalent loading was confirmed using anti-annexin I. Bound Ab was visualized by ECL. Cells were analyzed by flow cytometry to confirm a significant reduction of surface CD64 expression in each experiment. Representative of four (*A,B*) and two (*C*) independent experiments.

Figure 2

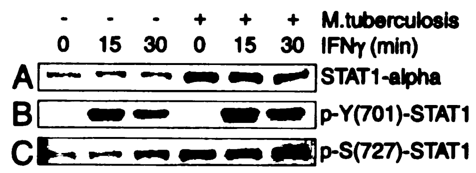


Figure 3. *M. tuberculosis* does not affect STAT1 dimerization or GAS binding in THP-1 cells. THP-1 cells were infected with live *M. tuberculosis* or left uninfected. Two days later, cells were treated with IFN-γ for the indicated times. Lysates were incubated with 3.5 pmol ^{32}P end-labeled GAS-containing oligonucleotide. Protein extracts are shown from untreated THP-1 cells (*A*), THP-1 cells treated with IFN-γ for 30 minutes (*B*), uninfected cells with no IFN-γ (*C*), uninfected cells after 15 min IFN-γ (*D*), uninfected cells after 30 min IFN-γ (*E*), *M. tuberculosis*-infected cells with no IFN-γ (*F*), infected cells after 15 min IFN-γ (*G*), infected cells after 30 min IFN-γ (*H*). Lysates from infected cells treated with IFN-γ for 15 min were preincubated with nonspecific competitor (*I*), specific competitor (*J*), 1 μg IgG1 isotype control (*K*), or 1 μg anti-STAT1 N-term Ab (*L*). Decreased CD64 expression was confirmed by ELISA. Results are representative of three independent experiments.

Figure 3

controls
\- +

\- - - + + + + + + + M.tuberculosis
0 15 30 0 15 30 15 15 15 15 IFNγ (min)
NS S IgG1 Ab

A B C D E F G H I J K L

Figure 4. *M. tuberculosis* inhibits IFN-γ-induced increases in CD64 mRNA in infected THP-1 cells. THP-1 cells were infected with live *M. tuberculosis* (*A*)or treated with γ-irradiated *M. tuberculosis* (*B*). Two days later, cells were treated with IFN-γ. Total RNA was harvested after 0 (no treatment) or at the indicated times. mRNA was assayed by quantitative real-time RT-PCR; all values were normalized to GAPDH. Results are shown as fold induction compared to uninfected sample without IFN-γ. Results are representative of seven (A) and four (B) independent experiments.

Figure 4

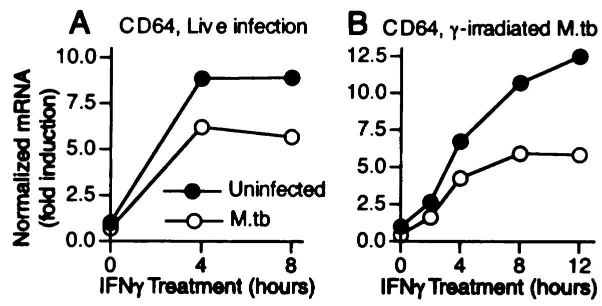


Figure 5. *M. tuberculosis* does not accelerate degradation of CD64 mRNA. THP-1 cells were infected with live *M. tuberculosis* or left uninfected. Two days later, cells were treated with IFN-γ for 12 h, followed by addition of 5 μg/ml actinomycin D. Total RNA was harvested at the indicated times. CD64 expression was assayed by slot blot, and signals on blots were quantitated using a Storm PhosphorImager. CD64 values were normalized for GAPDH hybridization for each sample. CD64 mRNA at time 0 (before the addition of actinomycin D) was plotted as 100%. Results are representative of four independent experiments.

Figure 5

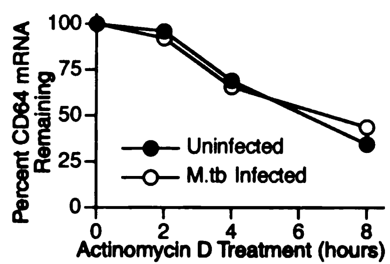


Figure 6. *M. tuberculosis* inhibits luciferase expression from IFN-γ-responsive CIITA promoter construct. Cells stably transfected with pCIITA-luciferase were treated with γ-irradiated *M. tuberculosis* or left untreated. Twenty-four hours later, cells were treated with murine IFN-γ overnight or left untreated. Luciferase values were normalized to protein concentration in each sample. Results are expressed as fold induction compared to uninfected sample without IFN-γ. Results are representative of two independent experiments.

Figure 6

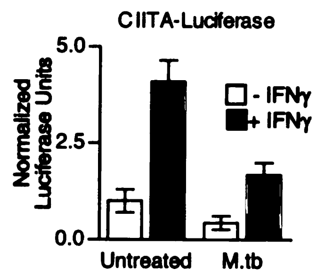


Figure 7. *M. tuberculosis* does not inhibit transcription from a minimal GAS promoter in RAW 264.7 cells. Cells were transfected with 5 μg pGAS-luciferase. Cells were treated with γ-irradiated *M. tuberculosis* or left untreated. Twenty-four hours later, cells were treated with murine IFN-γ overnight or left untreated. *A*, Luciferase values were normalized to protein concentration in each sample. Results are expressed as fold induction compared to uninfected sample without IFN-γ. Untransfected cells were stained with PE-conjugated anti-mouse I-A/I-E and 10,000 cells for each condition were examined by flow cytometry. *B*, Mean I-A/I-E fluorescence (mean ± SD for triplicate samples). Results are representative of five (*A*) and two (*B*) independent experiments.

Figure 7

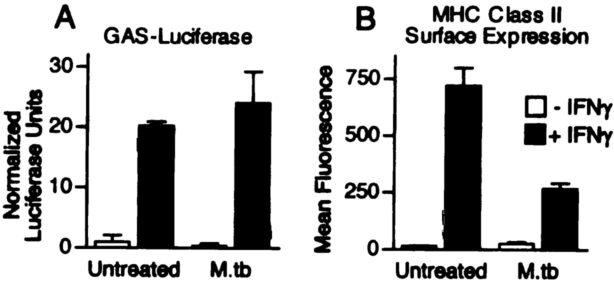


Figure 8. *M. tuberculosis* does not inhibit STAT1 binding to the endogenous CD64 promoter. THP-1 cells were treated with γ-irradiated *M. tuberculosis* or left untreated. Two days later, cells were treated with IFN-γ for 30 min or left untreated. *A*, *M. tuberculosis* inhibited induction of CD64 mRNA by ~50% in macrophages treated with this concentration of IFN-γ. Chromatin solution was prepared, as described in *Materials and Methods*. The resulting chromatin solution was immunoprecipitated using anti-STAT1 (C-terminus) (*B*); anti-acetylated histone H3 (*C*); anti-acetylated histone H4 (*D*). DNA enriched by immunoprecipitation was assayed by real-time PCR. All PCR was performed in triplicate. Values in the anti-STAT1 ChIP represent triplicate immunoprecipitations, normalized to the input chromatin solution. Values in anti-acetylated Histone H3 and anti-acetylated Histone H4 ChIPs represent a single immunoprecipitation, in which CD64 promoter values for each immunoprecipitation were normalized to values from the GAPDH promoter as a loading control. Data shown are from an experiment performed with 2ng/ml IFN-γ. Qualitatively similar results were observed with a higher concentration (20ng/ml) of IFN-γ.

Figure 8

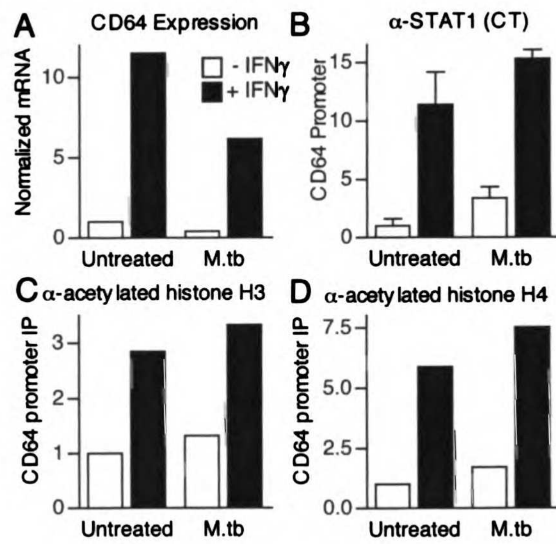
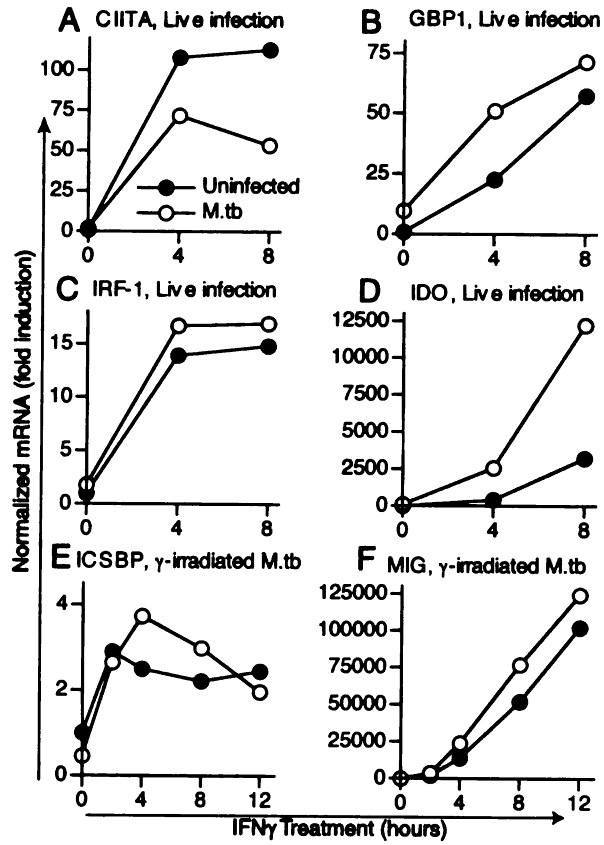


Figure 9. The inhibitory effect of *M. tuberculosis* is limited to a subset of IFN-γ-responsive genes. THP-1 cells were infected with live *M. tuberculosis* or treated with γ-irradiated *M. tuberculosis*, as indicated. Two days later, cells were treated with IFN-γ or left untreated. Total RNA was harvested after 0 (no treatment) or at the indicated times. mRNA was assayed by quantitative real-time RT-PCR with primers specific for: *A*, CIITA; *B*, IRF-1; *C*, GBP-1; *D*, IDO; *E*, ICSBP; or *F*, MIG. All values were normalized to GAPDH. Results are shown as fold induction compared to uninfected sample without IFN-γ. Decreased CD64 expression was confirmed by quantitative real-time RT-PCR. Each gene was assayed in three independent experiments.

Figure 9



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Footnotes

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[3] Abbreviations used in this paper: INFGR, IFN-γ receptor; BCG, bacillus Calmette-Guérin; ChIP, chromatin immunoprecipitation; CIITA, class II transactivator; D-PBS, Dulbecco's PBS; GAS, γ-activated sequence; GBP-1, guanylate-binding protein-1; ICSBP, IFN consensus sequence-binding protein; IDO, indoleamine-2,3-dioxygenase; IRF-1, IFN regulatory factor 1; JAK, Janus kinase; MDMØ, monocyte-derived macrophage; MIG, monokine induced by γ.

Chapter IV

Characterization of murine CIITA type IV promoter region required for inhibition of IFNγ-responsiveness by *Mycobacterium tuberculosis*

Abstract

IFNγ is a potent activator of the microbicidal activities of macrophages, and IFNγ-mediated immunity is essential for control of *Mycobacterium tuberculosis* infection. The immune response, however, is rarely, if ever, effective in clearing the infection; *M. tuberculosis* persists in the face of significant amounts of IFNγ present at sites of infection. One mechanism by which *M. tuberculosis* limits the response to IFNγ is inhibition of transcriptional responses to IFNγ. We found that, in the murine cell line RAW 264.7, expression of class II transactivator and class II transactivator-dependent genes were profoundly inhibited and that this inhibition of transcriptional responses was reflected in a comparable inhibition of MHC class II surface expression. Based on these observations, I assayed the effects of *M. tuberculosis* on transcription from a fragment of the class II transactivator type IV promoter. I found that *M. tuberculosis* inhibited luciferase expression in response to IFNγ from both the full-length promoter fragment (1.4 kb) and from a 256-bp fragment. This minimal fragment has been shown to contain three transcription factor binding sites: γ-activated sequence, E-box and IFN regulatory factor response element. Site-directed mutagenesis of each of these sites could not dissociate the response to IFNγ from the inhibitory effects of *M. tuberculosis*.

Introduction

CD4+ T cells play an important role in the control of *M. tuberculosis*. Mice lacking CD4+ cells due to genetic knock out or antibody depletion succumb rapidly to *M. tuberculosis* infection (Caruso et al. 1999; Muller et al. 1987; Scanga et al. 2000). Humans with reduced CD4+ cells due to HIV infection have accelerated progression to active tuberculosis (Centers for Disease Control and Prevention 1998).

CD4+ T cells recognize peptide in the context of MHC class II molecules on the surface of antigen presenting cells, including macrophages. Surface expression of antigen loaded MHC class II molecules requires coordinated expression of several accessory proteins in addition to the MHC class II molecules themselves; these accessory molecules include invariant chain (Ii) and H2-M. The expression of MHC class II-α and -β subunits, Ii, and H2-M is controlled by the class II transactivator (CIITA). Three isoforms of CIITA, each with their own promoter, result in cell-type specific regulation of CIITA expression (LeibundGut-Landmann et al. 2004). In macrophages, the expression of CIITA types I and IV is induced by IFNγ (Pai et al. 2002).

We have previously found that *M. tuberculosis* infection of macrophages inhibits the IFNγ induction of CIITA expression in human and murine cells (Fortune et al. 2004 and Chapter3; Kincaid and Ernst 2003). The inhibition of CIITA mRNA expression was reflected in a comparable inhibition of MHC class II surface expression (Nagabhushanam et al. 2003 and Chapter 3; Kincaid and Ernst 2003). We found inhibition of CIITA and another IFNγ-responsive gene, CD64, despite normal activation of signal transducer and activator of transcription 1 (STAT1), including STAT1 tyrosine or serine phosphorylation and DNA binding (Ting et al. 1999 and Chapter 3; Kincaid and Ernst

2003). I also found that STAT1 in *M. tuberculosis* treated cells could act as a transactivator for a synthetic γ-activated sequence (GAS) driving luciferase and could bind stably to a native IFNγ-responsive promoter in intact macrophages (Chapter 3; Kincaid and Ernst 2003). These findings, in conjunction with the finding that not all IFNγ responsive gene transcription is inhibited by *M. tuberculosis*, suggest that some feature of IFNγ responsive promoters renders them susceptible to inhibition by *M. tuberculosis*. I found that the 265-bp fragment of the CIITA type IV promoter, which contains the GAS, E-box and IFN regulatory factor response element (IRF-E) is sufficient for inhibition of IFNγ responsiveness by *M. tuberculosis*. Site-directed mutagenesis of the IRF-E resulted in loss of IFNγ-responsiveness while constructs in which the GAS or E-box had been mutated showed decreased IFNγ-inducibility but retained sensitivity to inhibition by *M. tuberculosis*.

Materials and Methods

Cells, M. tuberculosis, *and* Pam_3CSK_4.

RAW264.7 (American Type Culture Collection, Manassas, VA) were grown in DMEM medium supplemented with 10% heat inactivated FCS and 2 mM L-Glutamine (all from Gibco). γ-irradiated *M. tuberculosis* H37Rv (Colorado State University, Fort Collins, CO, NIH, NIAID Contract N01 AI-75320) was prepared as previously described (Chapter 3; Kincaid and Ernst 2003). (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys 4-OH, 3HCl (Pam_3CSK_4, Calbiochem) was dissolved in water at 1mg/ml, aliquotted and stored at -70°C.

mRNA quantitation

Cells were treated with 200µg/ml γ-irradiated *M. tuberculosis* as previously described (Chapter 3; Kincaid and Ernst 2003) or left untreated. After 24 hours of treatment, cells were treated with 20ng/ml rmurine rIFNγ ($0.2\text{-}2x10^8$ U/ml, BD Pharmingen) for 4, 8 or 24 hours as indicated before total RNA was harvested using Qiagen RNeasy columns. Any contaminating DNA was removed using DNA-free (Ambion). DNAse-treated total RNA was quantitated using Ribogreen (Molecular Probes). A total of 1 µg of each sample was reverse transcribed using the Reverse Transcription System (Promega). Reverse transcription was primed with random hexamers and oligo(dT) (0.5µg each per reaction). The cDNA equivalent of 5-100 ng of total RNA of each sample was analyzed by quantitative PCR using SYBR Green 2 Mastermix (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection System or SYBR Green Jumpstart Taq ReadyMix (Sigma) on a DNA Engine Opticon2 Fluorescence Detection System (MJ Research).

Gene-specific primers are listed in Table 2. Relative values were determined by comparing the threshold cycle of each sample with a standard curve consisting of serial dilutions of a positive control cDNA sample. Percent inhibition was calculated by subtracting the IFNγ induced induction of gene expression in *M. tuberculosis* treated cells from the induction in control cells, and dividing the difference by the induction in control cells.

Western blotting

To analyze phosphorylation of STAT1, RAW264.7 cells were treated with γ-irradiated M. *tuberculosis* for 1, 8, 16 or 24 h or were left untreated. Cells were then treated with IFNγ or medium alone for 30 minutes. Cells were lysed on ice in RIPA buffer with the addition of Complete-Mini protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktails I and II (Sigma). Immunoblotting was performed as previously described (Ting et al. 1999). Antibodies used were: Zymed monoclonal anti-phospho-tyrosine (Y701) STAT1 at 1:1000 and Zymed monoclonal anti-STAT1α at 1μg/ml. Annexin I (detected with rabbit polyclonal anti-Annexin I at 1:50,000) was used as a loading control. Zymed HRP-conjugated goat anti-mouse or goat anti-rabbit was used as a secondary antibody and bound antibodies were detected with Amersham ECL-Plus reagent. Chemiluminescence was detected by exposure of X-ray film.

Promoter constructs for transfection and retroviral transduction

The plasmids containing -1404 to +83 of the murine CIITA type IV promoter cloned into pGL3-basic (pCIITA-LUC full length) and two derivatives containing truncated

fragments of the CIITA type IV promoter (pCIITA-LUC D1 and pCIITA-LUC D2) were provided by Dr. Etty Benveniste (University of Alabama School of Medicine)(O'Keefe et al. 2001). To create the pQ-CIITA-LUC plasmid: The ClaI/ClaI fragment of pCIITA-LUC full length (containing -1404 to +83 of the murine CIITA type IV promoter, the luciferase open reading frame, and the simian virus 40 (SV40) polyadenylation sequence) was cloned into pQCXIN (BD Clontech) grown in SSC110 and digested with ClaI. ClaI digestion of pQCXIN grown in a *dam-* strain yielded a fragment containing the hybrid 5' long terminal repeat (containing the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter), the Ψ+ packaging signal, the ampicillin resistance gene, the Col E1 origin of replication, the 3' Moloney murine leukemia virus (MoMuLV) long terminal repeat with a deletion in the U3 region, the SV40 promoter and the SV40 origin of replication, but lacking the cytomegalovirus immediate-early (CMV IE) promoter, the multicloning site, the internal ribosomal entry site and the neomycin resistance gene. The polyadenylation sequence derived from pCIITA-LUC full length was removed using ClaI/XbaI. The ends were digested with mung bean nuclease (New England BioLabs) and blunt-end ligated. The CIITA-LUC D3 construct was created by amplifying 256 bp of the mCIITA-IV promoter and the multicloning site from full-length pQ-CIITA-LUC using Platinum Pfx (Invitrogen) and cloning this fragment into the TA-cloning vector pCR2.1 (Invitrogen). The BamHI/NotI fragment (containing -173 to + 83 of murine CIITA type IV promoter) was cloned into BamHI/NotI digested pQ-CIITA-LUC. To create the pQ-CIITA-LUC mut GAS, mut E-box and mut IRF-E plasmids, full-length pQ-CIITA-LUC was subjected to site directed mutagenesis using the Quickchange II system (Stratagene). Oligonucleotides are listed in Table 3.

Transduction with vesicular stomatitis virus membrane glycoprotein (VSV-G) pseudotyped retrovirus.

VSV-G pseudotyped virus containing pQ-CIITA-LUC full length or one of the derivative plasmids was prepared by cotransfecting each plasmid with pVSV-G into GP2-293 cells (both from BD Clontech). Retroviral supernatants were harvested 48 hours later and were stored at –75°. RAW 264.7 cells were infected with VSV-G pseudotyped retrovirus by centrifugation for 1 hour at 1675rcf followed by a 6 h incubation at 37°/5% CO2, all in the presence of 10μg/ml Polybrene (Sigma). Retroviral supernatants were removed and cells were allowed to recover overnight before further treatment.

Luciferase Assay

Cells were lysed using Passive Lysis Buffer (Promega) and luciferase was quantitated using Luciferase Assay Reagent (Promega) in a TD 20/20 Luminometer (Turner Designs). Luciferase values were normalized to protein concentration in each sample. Protein concentrations were determined using BCA Protein Assay (Pierce). For each promoter construct, all values were normalized to the value of the untreated control sample in each experiment.

Results

Gene-selective inhibition of RAW264.7 response to IFNγ without inhibition of STAT1 activation.

Like human monocyte derived macrophages and THP-1 cells (Ting et al. 1999; and Chapter 3; Kincaid and Ernst 2003), the murine macrophage cell line RAW264.7 is susceptible to *M. tuberculosis* inhibition of responses to IFNγ (Fortune et al. 2004). To determine if this inhibition of gene expression required inhibition of STAT1 activation, I assayed STAT1 tyrosine phosphorylation in RAW264.7 cells treated with *M. tuberculosis* for 1, 4, 16 and 24 h. I found that, comparable to results with human macrophages, the inhibition of RAW264.7 cell responses to IFNγ is not the result of inhibition of STAT1 phosphorylation (Figure 1).

To examine the extent of inhibition of IFNγ-responsive genes in RAW cells, we compared the inhibition of several genes with important immune functions. We found that *M. tuberculosis* inhibition of IFNγ responses was gene-selective (Table 1). While the induction of inducible nitric oxide synthase (NOS2) was not inhibited, and the induction a several other genes was partially inhibited, CIITA and genes whose expression is dependent on CIITA were profoundly inhibited

Construction of the CIITA-luciferase retroviral construct

Based on the observation that expression of CIITA and CIITA-dependent genes is significantly more inhibited than transcription of other IFNγ-responsive genes, I assayed the effects of *M. tuberculosis* on transcription from a fragment of the CIITA type IV promoter (O'Keefe et al. 2001). The full length promoter construct contains the

previously described murine CIITA type IV promoter which contains nuclear factor GMa (NF-GMa), GAS, E-box and IRF-E transcription factor binding sites as well as two activator protein 1 (AP-1) sites (Muhlethaler-Mottet et al. 1997). The full length construct also contains approximately 1 kb of sequence of unknown function, which is downstream of the CIITA type III exon 1 but upstream of the known CIITA type IV promoter. A construct containing a truncation of the full length promoter (D1) contains only the previously described CIITA type IV promoter driving luciferase (Figure 2a).

To determine if the *M. tuberculosis* inhibition of endogenous IFNγ responsive promoters could be reproduced with a promoter construct driving luciferase, I used a cell line stably transfected with CIITA-LUC D1. I found that these cell expressed luciferase in response to IFNγ treatment, and that there was a marked defect in the response to IFNγ in *M. tuberculosis* treated cells (Chapter 3; Kincaid and Ernst 2003). In addition, the fragment of the CIITA type IV promoter from –231 to +83 was found to be sufficient for sensitivity to inhibition by γ-irradiated *M. tuberculosis*, native 19kDa lipoprotein and a synthetic lipopeptide based on the 19kDa lipoprotein (Fortune et al. 2004). The –231 to +83 fragment, however, still contains most of the previously described transcription factor binding sites in the CIITA type IV promoter including one predicted AP-1 site and the inhibitory NF-GMa site (see figure 2a).

Due to concerns that clone-to-clone differences in transcription between individual stable transfectants would confound analysis of deletion constructs, I used VSV-G pseudotyped retrovirus to deliver deletion constructs to RAW cells. I cloned fragments of the murine CIITA type IV promoter driving luciferase into the pQCXIN self-inactivating retroviral vector from Clontech (Figure 2b). I used a self-inactivating

vector to prevent promoter interference and to provide a more accurate representation of transcription from the CIITA type IV promoter. For the same reason, I removed the CMV IE promoter from this construct. I also removed the internal ribosomal entry site and neomycin resistance gene from this construct, to ensure that the viral transcript would not be too large to be efficiently packaged. Lastly, I removed the polyadenylation sequence following the luciferase gene to allow the U3 region to be properly transcribed. Since the GAS, E-box and IRF-E transcription factor binding sites had previously been shown to be essential for full IFNγ responsiveness of this construct (O'Keefe et al. 2001), I created a minimal construct with only the 256-bp fragment that contained these three transcription factor binding sites (Figure 2a).

M. tuberculosis *and synthetic analog of 19kDa lipoprotein inhibit luciferase expression from full length and 256-bp fragment of CIITA promoter.*

Comparing the full length and minimal constructs, I found that *M. tuberculosis* inhibited luciferase expression in response to IFNγ from both the full length promoter fragment and from the minimal promoter fragment (Fig. 3). This finding indicates that the distal 1.2 kb containing two AP-1 sites and the NF-GMa site was not required for *M. tuberculosis* inhibition of the CIITA type IV promoter. NF-GMa has been shown to have negative regulatory effect on the CIITA type IV promoter (Muhlethaler-Mottet et al. 1997), and although the presence of this site was not required for *M. tuberculosis* inhibition of the response to IFNγ, removal of this site and the upstream 1.2 kb did result in significantly higher basal and induced luciferase expression from this construct (unpublished data). I found similar results when I treated RAW264.7 cells with

Pam_3CSK_4, a synthetic triacylated lipopeptide instead of γ-irradiated *M. tuberculosis* (Fig 3).

Site directed mutagenesis of candidate transcription factor binding sites could not dissociate the response to IFNγ from the inhibitory effects of M. tuberculosis.

To investigate the role of the transcription factor binding sites present in this minimal fragment of the CIITA type IV promoter, I used site directed mutagenesis to disrupt the GAS, E-box and IRF-E individually. I performed site-directed mutagenesis in the context of the full length CIITA type IV promoter, since this construct had a consistently higher fold induction by IFNγ (Fig. 3). I mutated nucleotides that had previously been shown to be required for function of these transcription factor binding sites (O'Keefe et al. 2001)(see Fig. 2C). Primers used for this mutagenesis are listed in Table 3. I found that mutation of the IRF-E resulted in a loss of responsiveness to IFNγ, while mutation of the GAS or E-box resulted in diminished responsiveness to IFNγ and continued susceptibility to inhibition (Fig. 4). I also found that the IRF-E was required for response to IFNγ in the context of the minimal 256-bp fragment of the CIITA type IV promoter (unpublished data).

Discussion

We have previously described inhibition of the response to IFNγ in human and murine macrophages infected with *M. tuberculosis* (Fortune et al. 2004; Ting et al. 1999 and Chapter 3; Kincaid and Ernst 2003). Like human macrophages, mouse macrophages are sensitive to *M. tuberculosis* inhibition of responses to IFNγ, and, although the range of affected genes differs, a similar pattern is seen in both cases: *M. tuberculosis* causes a gene-selective inhibition of IFNγ dependent mRNA induction without inhibiting STAT1 activation.

Although transient transfection of RAW264.7 cells gave a robust signal when I assayed IFNγ induction of luciferase activity downstream of six synthetic GAS sequences (Chapter 3; Kincaid and Ernst 2003), I had inconsistent results when I assayed a large fragment of the CIITA type IV promoter driving luciferase using transient transfection. By using stable transfectants, I was able to overcome these technical problems and I found that a fragment of the CIITA type IV promoter could reproduce the inhibition seen at the endogenous CIITA promoter in *M. tuberculosis* treated cells. Although the difference between transient and stable transfection may simply reflect the percentage of cells carrying the plasmid, it is possible that the promoter construct must be integrated into the genome in order to fully mimic the endogenous promoter. Using the stable transfectants, we found that the region upstream of the previously identified CIITA type IV promoter was not required for *M. tuberculosis* inhibition of IFNγ induction of luciferase expression from this promoter (Fortune et al. 2004 and Chapter 3; Kincaid and Ernst 2003).

Due to concerns that clone-to-clone differences in stable transfectants might mask subtle differences between the activity of different promoter constructs, I used VSV-G pseudotyped retroviral transduction to compare these constructs. Promoter constructs introduced into cells by retroviral transduction should also integrate into the genome, but increased transduction efficiency allowed me to use bulk transduced cells, thereby averaging out differences in integration site. I found that, of the previously identified transcription factor binding sites in the CIITA type IV promoter, only the region containing the GAS, E-box and IRF-E was required for both induction by IFNγ and inhibition by *M. tuberculosis*. Site-directed mutagenesis of each of these three sites, however, could not dissociate the response to IFNγ from the inhibitory effects of *M. tuberculosis*. In particular, I found that mutation of the IRF-E abrogated all IFNγ responsiveness of this promoter construct. A previous report (O'Keefe et al. 2001) found that mutation of the IRF-E did not result in a total loss of responsiveness to IFNγ, but this difference may reflect a difference between transient transfection and retroviral transduction.

The finding that I could not dissociate the response to IFNγ from the inhibitory effects of *M. tuberculosis* suggests either that there is an as yet unidentified transcription factor binding site in this region, or that *M. tuberculosis* may inhibit CIITA induction by interfering with the binding of STAT1, upstream stimulatory factor 1 (USF1) or IFN regulatory factor 1 (IRF1) to its promoter. This possibility could be tested using electrophoretic mobility shift to detect any changes in IFNγ inducible binding to a minimal fragment of the CIITA type IV promoter in *M. tuberculosis* treated cells. In addition to assaying in vitro binding, chromatin immunoprecipitation would allow us to

determine if the binding and stabilization of these transcription factors at the endogenous promoter is inhibited in *M. tuberculosis* treated cells.

Of the three transcription factor binding sites in the minimal CIITA type IV promoter construct, the absolute requirement for the IRF-E, combined with the partial reduction of IRF-1 expression in *M. tuberculosis* treated cells makes the IRF-1 transcription factor an attractive candidate. Since IRF-1 has been shown to be required for full IFNγ induction of CIITA in vivo (Hobart et al. 1997), a relatively small change in IRF-1 expression could play a significant role in the inhibition of CIITA expression. However, the induction of NOS2, another IFNγ responsive gene whose induction is dependent on IRF1 (Kamijo et al. 1994), is not inhibited in *M. tuberculosis* treated RAW 264.7 cells (Table 1) or bone marrow derived macrophages treated with *M. tuberculosis* components (Fortune et al. 2004). An alternate possibility is that there is a change in IRF-2 expression in *M. tuberculosis* treated cells. Although IRF-2 contains a repression domain and represses IRF-1-induced transcriptional activation in many settings (Taniguchi et al. 2001) there is evidence that IRF-2 overexpression in macrophages actually increases both basal and IFNγ-induced transcription from the CIITA type IV promoter (Giroux et al. 2003). It has been reported that IRF-1 and IRF-2 co-occupy the IRF-E in the CIITA type IV promoter, and that the presence of IRF-2 stabilizes IRF-1 binding, resulting in increased transactivation (Xi and Blanck 2003). The role of IRF-2 (either positive or negative) in any decrease in IRF-1 binding to the CIITA type IV promoter would need to be evaluated.

If STAT1, USF-1 and IRF-1 are found to bind properly to the endogenous CIITA type IV promoter in *M. tuberculosis* treated cells, it would suggest that *M. tuberculosis* is

inducing a negative regulatory factor that binds at the CIITA type IV promoter and prevents recruitment or activation of the basal transcriptional machinery. Recruitment and phosphorylation of RNA polymerase II at the CIITA type IV promoter will be examined, as a marker of the function of the transcription complex.

The profound inhibition of IFNγ induction of CIITA and MHC class II expression may play an important role in *M. tuberculosis* evasion of the host adaptive immune response. In mice and humans infected with *M. tuberculosis*, there is a robust CD4+ T cell response, with significant numbers of antigen specific effector T cell in the peripheral blood (Lalvani et al. 2001; Pathan et al. 2001). Dendritic cells, which are able to present antigen to naïve T cells but which do not require IFNγ for MHC class II surface expression, may be responsible for the education of these cells. Although effector T cells are able to traffic to the site of infection and produce IFNγ (Barnes et al. 1993; Fenhalls et al. 2002), the immune response is ultimately unable to clear *M. tuberculosis* infection. Our findings (Chapter 5), along with those of others (Hmama et al. 1998; Noss et al. 2000), demonstrating a defect in MHC class II expression in macrophages infected with live *M. tuberculosis*, suggest that defective recognition of *M. tuberculosis* infected macrophages may play an important role in *M. tuberculosis* immune evasion.

Acknowledgements

Sarah Benson and Claire Escaron contributed to the table of genes inhibited by *M. tuberculosis* treatment. Vijaya Nagabhushanam initially validated RAW264.7 cells as a model for *M. tuberculosis* inhibition of macrophage responses to IFNγ. Vijaya Nagabhushanam and Christian Essrich provided advice and discussion in the use of SYBR Green to assay mammalian gene expression by quantitative PCR.

Abbreviations

AP-1, activator protein 1; CIITA, class II transactivator; CMV, cytomegalovirus; CMV IE, cytomegalovirus immediate-early; GAS, γ-activated sequence; ICSBP, IFNg-induced GTP binding protein; Ii, invariant chain; IP-10, IFNγ-induced protein 10; IRF, IFN regulatory factor; IRF-E, IFN regulatory factor response element; NF-GMa, nuclear factor GMa; MIG, monokine induced by γ; MoMuLV, Moloney murine leukemia virus; MSV, mouse sarcoma virus; NOS2, inducible nitric oxide synthase; Pam_3CSK_4, (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys 4-OH, 3HCl; STAT, signal transducer and activator of transcription 1; SV40, simian virus 40; USF-1; upstream stimulatory factor 1; VSV-G, vesicular stomatitis virus membrane glycoprotein.

Table 1. Expression of CIITA and CIITA-dependent genes is more profoundly inhibited than other IFNγ responsive genes.

Gene	% Inhibition	IFNγ (h)	Function
inducible nitric oxide synthase (NOS2)	-3%	8	production of NO and related RNIs
monokine induced by γ (MIG)[1]	26%	4	chemoattractant
IFNγ-induced protein 10 (IP-10)[1]	32%	4	chemoattractant
IFNγ-induced GTP binding protein (IGTP)[1]	34%	4	host resistance to intracellular pathogens
large G-protein-related protein of 47 kDa (LRG-47)[2]	40%	4	host resistance to intracellular pathogens
IFN consensus sequence binding protein (ICSBP)[1]	52%	4	transcription factor
IFN regulatory factor 1 (IRF-1)	52%	4	transcription factor
invariant chain (Ii)	88%	24	MHC class II trafficking and peptide loading
class II transactivator (CIITA)	93%	8	transcriptional coactivator
H2-IAα	95%	24	MHC class II molecule
H2-IEα	97%	24	MHC class II molecule
H2-DM	98%	8	MHC class II peptide loading

[1] Experiments performed by Claire Escaron. [2] Experiments performed by Sarah Benson

Table 2. Oligonucleotide primers used for Quantitative PCR

Gene	Position*	Forward	Reverse
CIITA	2511-2586†	GAAGTTCACCATTGAGCCATTTAA	CTGGGTCTGCACGAGACGAT
GAPDH	711-787	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
GAPDH§	438-909	CAATGCATCCTGCACCACCAA	GTCATTGAGAGCAATGCCAGC
H2-DM	111-186	TGACCCACAGAACCACACATTC	GGTCTCCGAGAGCCCTATGTT
I-Ad α	592-667	GAGGAGCCGGTTCTGAAACA	CACACACCACAGTTTCTGTCAGC
ICSBP	438-627	GATCAAGGAACCTTCTGTGG	GAAGCTGATGACCATCTGGG
I-Ed α	498-574	CGATCACCTCTTCCGCAAAT	GATCCACCTCACAGTCATAGAAATCA
IGTP	74-553	TCAAGCGCCTCATCAGCCCGTGGT	TGGCCAGCTTCACATGATTTGAG
iNOS	200-549	ACAAGCTGCATGTGACATCG	GGCAAAGATGAGCTCATCCA
Ii	139-214	CAGGCCACCACTGCTTACTTC	GCAGGTTCTGGGAGGTGATG
IP-10	162-592	CCTATCCTGCCCACGTGTTG	CGCACCTCCACATAGCTTACA
IRF-1	643-710	GTGTCACCCATGCCTTCCA	TCTTCGGCTATCTTCCCTTCCT
LRG-47	254-321	CTGGCAATGGCATGTCATCT	AGCCGAGGCATCTTCATCAT
MIG	1-381	ATGAAGTCCGCTGTTCTTTTCC	TTATGTAGTCTTCCTTGAACGAC

* Position relative to start of coding sequence

† Position relative to start of the coding sequence of CIITA type IV. These primers also amplify CIITA type I and type III.

§ Longer GAPDH amplicon used in experiments testing ICSBP, IGTP, IP-10 and MIG

Table 3. Primers used for mutation of the CIITA promoter*

Site	Position†	Forward§
GAS	-182 to -137	CTTGGGTTGCATGTGGCAGC**CGA**T**CG**GAAAGCACGTGGTGGATATC
E-box	-171 to -126	TGTGGCAGCTTCTGAGAAAG**GCG**GT**A**GTGGATATCACTTTTCAGGG
IRF-E	-87 to -42	GCTGAAGGTGTAGACAGA**C**AGTG**CTC**G**A**GGGAAAAGCGCCACAGAT

* Mutated nucleotides are indicated in bold.

† Position of mutation primers relative to start of coding sequence

§ The reverse complement of the forward primer was used as the reverse primer in each case.

Figures

Figure 1. *M. tuberculosis* treatment does not inhibit STAT1 activation in RAW264.7 cells. RAW264.7 cells were treated with γ-irradiated *M. tuberculosis* for the indicated time or left untreated, followed by 30 minute IFNγ treatment. Protein extracts were examined by immunoblotting. One microgram of protein was loaded per lane. Blots were incubated with antibody specific for tyrosine (Y701) phosphorylated STAT1 (A) or STAT1α (B). Equivalent loading was confirmed using anti-annexin I. Bound Ab was visualized by enhanced chemiluminescence. Images shown are representative of 2 independent experiments.

Figure 1

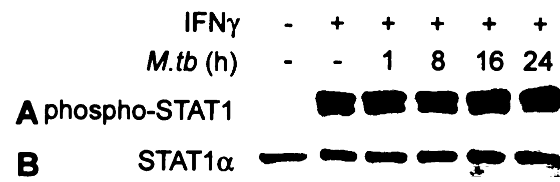


Figure 2. Promoter constructs used in this study. A, Deletion mutants of murine CIITA type IV promoter. B, Retroviral promoter constructs. These constructs consist of the CMV/MSV hybrid 5' long terminal repeat (containing the cytomegalovirus type I enhancer and the mouse sarcoma virus promoter), the Ψ+ packaging signal, a fragment of the murine CIITA type IV promoter, the luciferase open reading frame, the 3' MoMuLV long terminal repeat with a deletion in the U3 region, the SV40 promoter, the SV40 origin of replication, the Col E1 origin of replication and the ampicillin resistance selectable marker. C, Site directed mutants of the retroviral constructs.

Figure 2

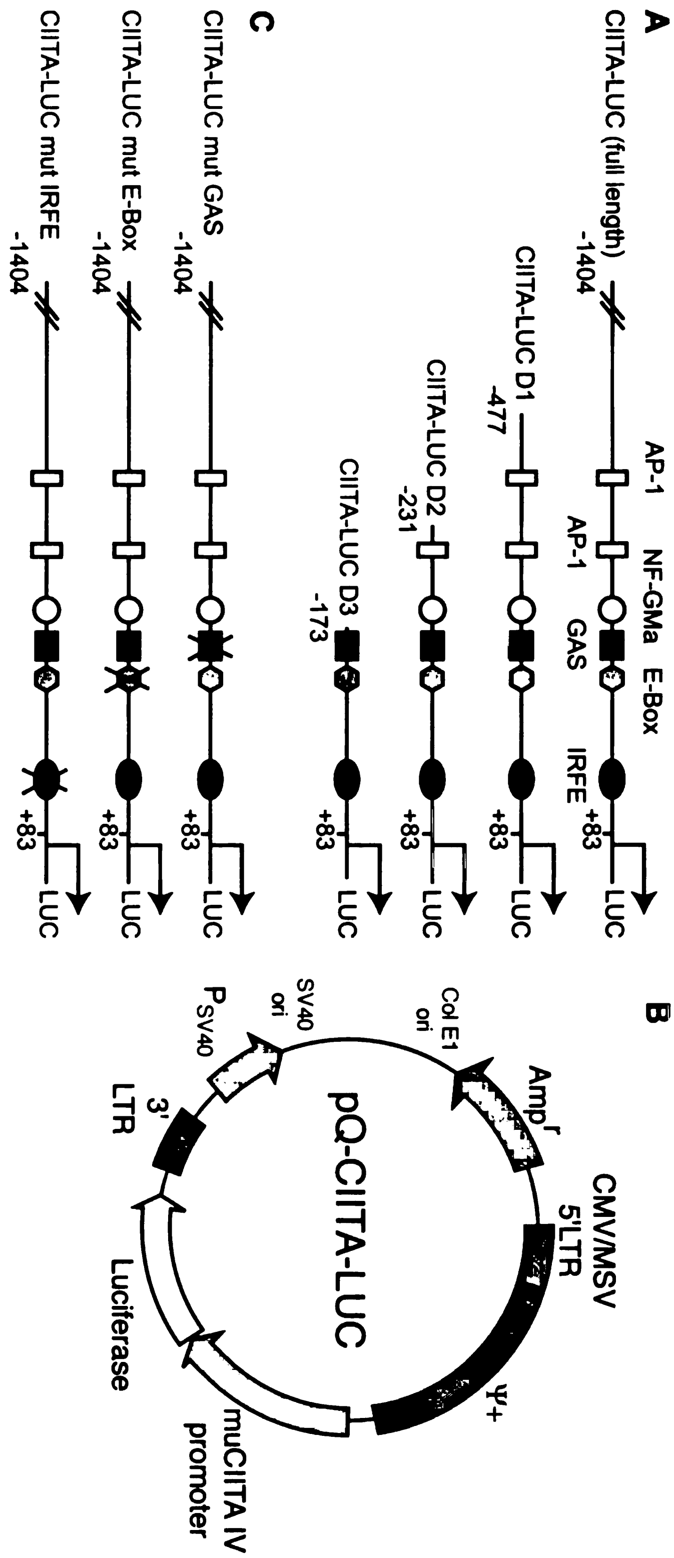


Figure 3. *M. tuberculosis* and Pam_3CSK_4 inhibit luciferase expression from full length and 256-bp fragment of CIITA type IV promoter. RAW264.7 cells were infected with VSV-G pseudotyped virus containing pQ-CIITA-LUC (A) or pQ-CIITA-LUC D3 (B) as described in *Materials and Methods*. Cells were treated with either 200μg/ml γ-irradiated *M. tuberculosis* or Pam_3CSK_4 for 8 hours or left untreated. Cells were then treated with 20ng/ml IFNγ for 12 hours or left untreated. Luciferase values were normalized to protein concentration in each sample.

Figure 3

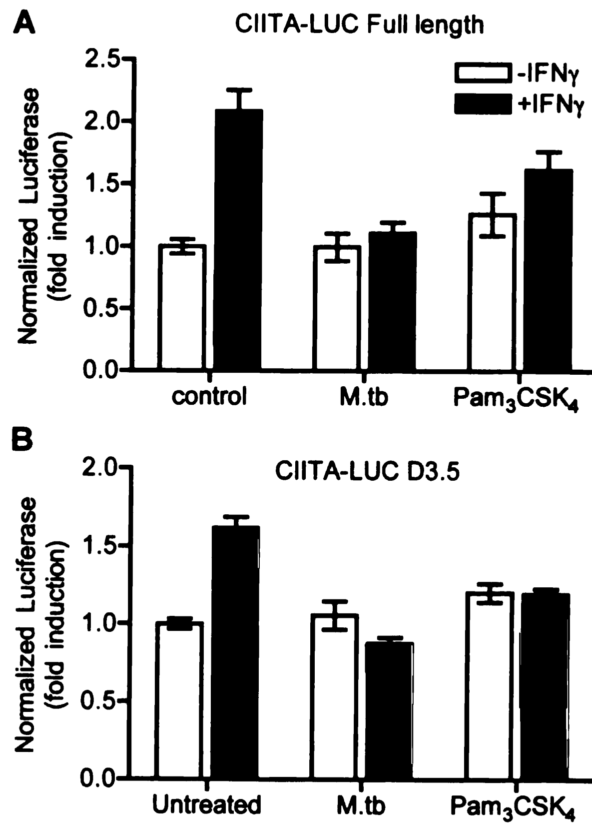
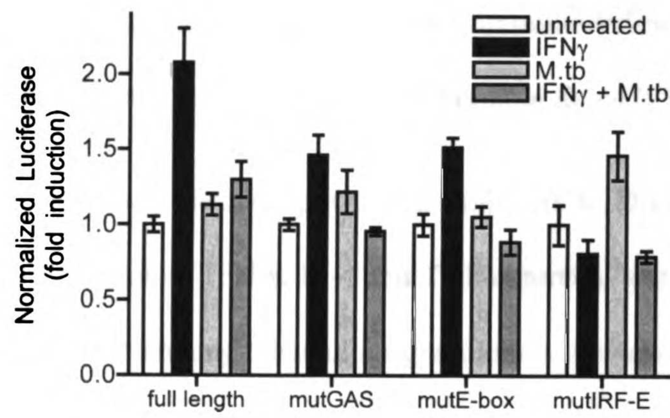


Figure 4. Mutation of GAS, IRFE and E-box does not dissociate the response to IFNγ from inhibition by *M. tuberculosis*. Experiments were performed as described for Figure 3, except that the retroviral sequence contains the full length CIITA type IV promoter or site directed mutants thereof, as indicated.

Figure 4



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Chapter V

Potent inhibition of macrophage responses to IFNγ by live virulent *Mycobacterium tuberculosis* is independent of mature mycobacterial lipoproteins but dependent on Toll-like Receptor 2[1]

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Abstract

Mycobacterium tuberculosis is a highly successful pathogen that can persist and cause disease despite an immune response. One potential mechanism for resisting elimination is by inhibiting the action of IFNγ. We have previously shown that live *M. tuberculosis* inhibits selected macrophage responses to IFNγ, and that purified *M. tuberculosis* 19 kDa lipoprotein inhibits induction of selected IFNγ-responsive genes through a Toll-like receptor 2 -dependent pathway, while peptidoglycan inhibits responses to IFNγ by a Toll-like receptor 2-independent pathway. To determine the relative contribution of lipoproteins to the inhibition of responses to IFNγ, we deleted the *M. tuberculosis* gene (*lspA*) that encodes lipoprotein signal peptidase. This revealed that *M. tuberculosis* lipoprotein processing is indispensable for stimulation of Toll-like receptor 2 reporter cells, but that the *lspA* mutant inhibits macrophage responses to IFNγ to the same extent as wild type bacteria. Macrophages lacking Toll-like receptor 2 are more resistant to inhibition by either strain of *M. tuberculosis*, suggesting that non-lipoprotein Toll-like receptor 2 agonists contribute to inhibition. Indeed, we found that phosphatidylinositol mannan from *M. tuberculosis* inhibits macrophage responses to IFNγ. *M. tuberculosis* inhibition of responses to IFNγ requires new protein synthesis, indicating that a late effect of innate immune stimulation is the inhibition of responses to IFNγ. These results establish that *M. tuberculosis* possesses multiple mechanisms of inhibiting responses to IFNγ.

Introduction

Mycobacterium tuberculosis is a highly successful pathogen that can infect, persist, and cause progressive disease in humans and experimental animals with apparently normal immune responses. This implies that *M. tuberculosis* has evolved mechanisms to avoid elimination by normal mechanisms of immunity. Individuals that are infected with *M. tuberculosis* develop apparently appropriate cellular immune responses with priming, expansion, differentiation, and trafficking of antigen specific $CD4^+$ and $CD8^+$ T-cells (Fenhalls et al. 2002; Lalvani et al. 1998; Lalvani et al. 2001; Randhawa 1990; Schwander et al. 1998) resulting in IFNγ (Barnes et al. 1993) and TNFα production at the site of infection (Fenhalls et al. 2002). The inability to clear *M. tuberculosis* despite this immune response suggests that *M. tuberculosis* may interfere with distal effector events. Defective recognition of infected macrophages by T cells and/or defective responses of infected macrophages to effectors of adaptive immunity may contribute to the ability of *M. tuberculosis* to persist and progress. One specific mechanism that could permit *M. tuberculosis* to avoid elimination by the immune response is inhibition of macrophage responses to IFNγ, an important effector of immunity to intracellular pathogens. Indeed, while IFNγ is capable of stimulating macrophages to kill intracellular Toxoplasma, Leishmania, Legionella, and Chlamydia, it is incapable of stimulating macrophages to kill *M. tuberculosis in vitro* unless IFNγ is used to prime macrophages prior to infection (Chan et al. 1992; Douvas et al. 1985; Ehrt et al. 2001; Flesch and Kaufmann 1987; Flesch and Kaufmann 1990; Fortune et al. 2004; Murray et al. 1991; Nathan et al. 1983; Nathan et al. 1984; Rook et al. 1986). Moreover,

experiments in mice have provided evidence that virulent *M. tuberculosis* evades IFNγ-dependent mechanisms of immune control *in vivo* (Jung et al. 2002).

We and others have reported that *M. tuberculosis* inhibits human and murine macrophage responses to IFNγ (Ehrt et al. 2001; Fortune et al. 2004; Fulton et al. 2004; Kincaid and Ernst 2003; Noss et al. 2000; Noss et al. 2001; Ting et al. 1999). Moreover, we have found that *M. tuberculosis* utilizes at least two mechanisms to block responses to IFNγ. One is initiated by lipoproteins, acting through Toll-like receptor 2 (TLR2) and MyD88, while the other is initiated by mycobacterial peptidoglycan, acting in a TLR2- and MyD88-independent fashion (Fortune et al. 2004). In this report we examined whether lipoprotein-mediated inhibition is dominant in the context of intact *M. tuberculosis*. We found that disruption of the *M. tuberculosis* lipoprotein signal peptidase (*lspA*) resulted in loss of mature lipoproteins and lipoprotein TLR2 agonist activity, but had no effect on the mutant strain's ability to inhibit macrophage responses to IFNγ. The disruption of TLR2 on macrophages, however, significantly reduced the cells' sensitivity to inhibition by live *M. tuberculosis* and *M. tuberculosis* lysates.

Materials and Methods

Bacteria and TLR2 agonists. All *M. tuberculosis* strains were grown in shaking cultures to mid-log phase in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol, 10% OADC, and 0.05% Tween 80. *M. tuberculosis* cultures used for infection of macrophages were grown the same medium with low-endotoxin ADC (dextrose, catalase and cell culture tested BSA, all from Sigma) substituted for OADC. This media contained less than 1 endotoxin unit per ml. γ-irradiated *M. tuberculosis* H37Rv (Colorado State University, Fort Collins, CO, NIH, NIAID Contract N01 AI-75320) was prepared as previously described (Kincaid and Ernst 2003). Phosphatidylinositol $mannan_{1+2}$ (PIM, Colorado State University) was stored at 1mg/ml in DMSO (Sigma) at -80°. (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH, 3HCl (Pam_3CSK_4, Calbiochem) and (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-(R)-Cys-(S)-Ser-(S)-Lys_4 x 3 CF3COOH (Pam_2CSK_4, InvivoGen) were stored at 1mg/ml in endotoxin-tested water (Gibco) at -80.

Mice. C57BL/6, $IL6^{-/-}$ and $TLR2^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. $TLR2^{-/-}$ mice from The Jackson Laboratory have been backcrossed onto the C57BL/6 background for 9 generations. All work with animals was approved by the NYU School of Medicine Institutional Animal Care and Use Committee.

Cell lines. RAW264.7 and HEK-293 cells (American Type Culture Collection, Manassas, VA) were grown in DMEM with 10% heat inactivated FCS and 2 mM L-

Glutamine (all from Gibco). HEK 293-TLR2 cells were provided by Dr. Douglas Golenbock (University of Massachusetts), and were grown in the same medium with 500 μg/ml Geneticin (Gibco). Cells were allowed to adhere overnight before *M. tuberculosis* treatment or infection. L929 cells (ATCC) were maintained in DMEM with 10% FCS, 2 mM L-glutamine, 100 μM nonessential amino acids, and 55 μM β-mercaptoethanol (Gibco). L cell-conditioned medium was collected from confluent L929 cultures and stored at -20°C.

lspA *knockout and complement.* A *lspA* knockout mutant was created with the conditionally replicating mycobacteriophage as described (Bardarov et al. 2002). The upper (886 bp) and lower (1125 bp) allelic exchange substrates (AES) were PCR amplified (for complete list of primers see Table 1) from H37Rv genomic DNA and ligated into pCR2.1-TOPO (Invitrogen). The AES were sequenced and subcloned into pYUB854. Following transduction of H37Rv and plating on Middlebrook 7H9 agar with ADC and hygromycin (50 μg/ml), 6 colonies were picked and screened for the absence of *lspA* transcript by real-time RT-PCR. A PCR fragment encoding the entire *lspA* ORF and the preceeding 470 bp was ligated into pMV306 (Stover et al. 1991) for complementation of the *lspA* mutant. Transformed bacteria were plated on Middlebrook 7H9 agar supplemented with ADC and 25 μg/ml of kanamycin and 4 colonies were picked and analyzed for complementation by real-time RT-PCR.

Western Blotting. Protein extraction was performed according to a published protocol (Hatfull and Jacobs Jr 2000). Briefly, 2 ml of bacterial culture at mid-log phase was

sedimented and washed once with 2 ml of PBS and resuspended in 300 μl of extraction buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.6% SDS, 10 mM NaPO4, and Roche Complete Protease Inhibitor Cocktail) and added to 0.2 ml of 0.1 mm zirconia/silica beads. The tube was vortexed for 5 min and subsequently sedimented for 2 min at 12600 x *g*. The supernatant was removed and the protein concentration determined with the BCA protein assay kit (Pierce). 0.2-1.5 μg was separated on a 10% SDS-PAGE gel and immunoblotting was performed as previously described (Kincaid and Ernst 2003). Polyclonal anti-MPT83 was provided by Dr. Harald Gotten Wiker (Gades Institutt, Norway) and incubated overnight at 4°C at a dilution of 1:2000.

Infection of Cell Monolayers. Bacteria from mid-log cultures (A_{580} ~ 0.5) were sedimented, resuspended in cell culture media, vortexed for 3 minutes in O-ring tubes containing 2 5mm glass beads (Fisher) and passed through a 5 μm sterile filter (Millipore). Bacteria were enumerated in a Petroff-Hausser chamber and also serially diluted and plated on Middlebrook 7H10 and 7H9/ADC agar plates.

Bacterial lysates for cell stimulation. Mid-log cultures of *M. tuberculosis* grown in low endotoxin media were sedimented, washed once with PBS, and resuspended in 1 ml of extraction buffer or PBS with or without protease inhibitors. Bacteria were disrupted in O-ring tubes containing 0.5 ml of 0.1 mm zirconia/silica beads and mechanically disrupted with three 1 min pulses at maximum speed in a BioSpec Products BeadBeater™ with 3 min intervals on ice. The lysates were sedimented for 1 min at 12600 x *g* and the supernatants were transferred and the efficacy of disruption was

assayed as soluble protein concentration (BCA protein assay kit; Pierce) and enumeration of viable bacteria before and after bead beating. Equivalent amounts of protein (~0.8 μg per 1×10^6 cfu) were released from all three strains upon bead beating, indicating a comparable efficiency of lysis. Furthermore, a 10^4 fold difference was seen in the number of viable bacteria before and after beadbeating of H37Rv (unpublished observations).

Isolation and culture of bone marrow-derived macrophages (BMDM). Bone marrow cells were isolated as previously described (Nagabhushanam et al. 2003), then erythrocytes were lysed using ACK lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 100 μM EDTA, pH 7.4, all from Sigma). Cells were plated in 150 x 25-mm bacterial grade petri dishes (Falcon) at 3-4 x 10^6/plate, in DMEM with 10% FCS, 20% L929-cell conditioned medium, 1 mM sodium pyruvate, 2 mM L-glutamine and 100 U/ml Penicillin/100 μg/ml streptomycin sulfate (Gibco). The cells were incubated at 37°C in 5% CO_2 for 3 days, after which the medium was replaced. Adherent cells were harvested between days 6 and 10; the cells were incubated in ice cold Dulbecco's PBS (DPBS) containing 5 mM EDTA for 20 minutes, detached from the plates by vigorous pipetting, then washed and replated at 5 x 10^5/well in 12-well tissue culture plates in DMEM supplemented with 10% FCS, 10% L929-cell conditioned medium, 1 mM sodium pyruvate and 2 mM L-glutamine.

Flow Cytometry. Following infection with live *M. tuberculosis* or treatment with PIM or Pam_3CSK_4, RAW264.7 cells were treated with 20 ng/ml recombinant murine IFNγ (400-

4000 U/ml, BD Pharmingen). Cells were rinsed with DPBS and incubated for 10 min in DPBS containing 1 mM EDTA. Cells were then scraped, washed, and stained with PE conjugated anti-mouse I-A/I-E (BD Pharmingen). For experiments with live *M. tuberculosis*, cells were fixed overnight with 1% paraformaldehyde (Sigma). Cells were analyzed on a FACSCalibur (Becton Dickinson, 10,000 total events gated by forward and side scatter).

mRNA quantitation. For experiments with Pam_3CSK_4, total RNA was harvested using Qiagen RNeasy columns. For *M. tuberculosis* cultures and cells infected with live *M. tuberculosis* or treated with *M. tuberculosis* whole cell lysates, total RNA was harvested using Trizol Reagent (Invitrogen). DNA was removed using DNA-free (Ambion). Total RNA was quantitated using RiboGreen (Molecular Probes). Ten ng of bacterial or one μg of mammalian RNA was reverse transcribed using the Reverse Transcription System (Promega). For mammalian RNA, reverse transcription was primed with random hexamers and oligo(dT). For bacterial RNA, reverse transcription was primed with gene-specific primers (listed in Table 1). The cDNA equivalent of 0.2 ng of total bacterial RNA or 10 ng of total mammalian RNA (50 ng for CIITA) was analyzed by quantitative PCR using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) on an MJ Research Opticon 2 (for primers, see Table 1). For quantitation, the relative values were determined by comparing the threshold cycle of each sample to a standard curve consisting of serial dilutions of a positive control cDNA sample.

ELISA. Cell supernatants for ELISA were harvested after the indicated time, and were stored at –80°. ELISAs were performed with optimized antibody sets specific for human IL-8 (BD Pharmingen) or murine TNFα (eBiosciences), used according to the manufacturers' directions. Samples were diluted 1:10, 1:100 or 1:1000 to allow detection within the range of each assay. Results for experiments with live or lysed *M. tuberculosis* H37Rv were quantitated using a SpectraMax 340PC^{384} spectrophotometer, all other results were quantitated using a EL_x800_{UV} spectrophotometer (Bio-Tek Instruments, Inc).

Inhibition of protein synthesis. Cells were pretreated with cycloheximide (Calbiochem) or solvent control (0.02% ethanol, Sigma) for 1 hour before addition of *M. tuberculosis* whole cell lysates. After 8 hours, media was removed and stored at –80° for ELISA and cytotoxicity assays. Cells were treated with IFNγ or left untreated for 4 hours and RNA was harvested as described above.

Results

Pam_3CSK_4, a synthetic lipoprotein unrelated to the M. tuberculosis *19 kDa lipoprotein, is a potent inhibitor of macrophage responses to IFNγ*

We and others (Fortune et al. 2004; Noss et al. 2001) have found that the *M. tuberculosis* 19 kDa lipoprotein inhibits macrophage responses to IFNγ and that this inhibition is dependent on TLR2 and MyD88. Although considerable attention has been focused on the properties of the *M. tuberculosis* 19 kDa lipoprotein, it is becoming increasingly clear that this lipoprotein is not the sole mediator of *M. tuberculosis* inhibition of macrophages. Not only is the 19 kDa lipoprotein not required for *M. bovis* bacille Calmette-Guerin (BCG)[4] inhibition of IFNγ-dependent antigen processing in infected macrophages, at least one other *M. tuberculosis* lipoprotein is capable of causing this inhibition (Gehring et al. 2004).

We have also previously found that in addition to native, full-length 19 kDa lipoprotein, a synthetic triacylated hexapeptide containing the N-terminal 6 amino acid residues of the 19 kDa lipoprotein can also inhibit macrophage responses to IFNγ (Fortune et al. 2004). To determine if treatment with an unrelated lipopeptide could reproduce this inhibition, we examined Pam_3CSK_4, a triacylated hexapeptide that has no peptide sequence identity with any *M. tuberculosis* open reading frame, as determined by BLASTP search (Altschul et al. 1997) of all non-redundant GenBank coding sequences of *M. tuberculosis*. Treatment of RAW264.7 cells with Pam_3CSK_4 profoundly inhibited IFNγ induction of MHC class II surface expression; as little as 1 nM Pam_3CSK_4 caused greater than 90% inhibition of IFNγ induction of surface MHC class II (Fig. 1). IFNγ induction of class II transactivator (CIITA) mRNA was also potently inhibited in

Pam_3CSK_4 treated cells (unpublished observations) and this inhibition was likely responsible for the defect in MHC class II surface expression. These findings are consistent with previous work (Fortune et al. 2004; Noss et al. 2001) showing the inhibitory effects of higher (micromolar) concentrations of synthetic bacterial lipopeptides on responses to IFNγ.

Construction and characterization of lspA *mutant*

The finding that a synthetic lipoprotein unrelated to the 19 kDa lipoprotein is a potent inhibitor of IFNγ suggests that other *M. tuberculosis* lipoproteins could contribute to inhibition of macrophages via TLR2. Bioinformatic analysis of the *M. tuberculosis* genome revealed 99 putative lipoproteins, accounting for approximately 2.5% of its proteome (Sutcliffe and Harrington 2004). Given the number and variety of lipoproteins and the evidence that the TLR2 agonist activity is attributable to the triacylated amino terminus rather than other protein domains, it is unlikely that deletion of any single lipoprotein would render *M. tuberculosis* unable to inhibit macrophage responses to IFNγ. To evaluate the global role of mature lipoproteins in the modulation of macrophage responses to IFNγ, we chose to disrupt the *lspA* gene which encodes prolipoprotein signal peptidase II, the enzyme that cleaves the signal sequence from diacylated prolipoproteins at a site directly preceding the lipidated cysteine residue. The exposure of the primary amine group on the newly exposed N-terminal cysteine then allows for the final acylation and creation of the mature triacylated lipoproteins capable of serving as TLR2 agonists (Fig 2A).

Genomic search of the annotated *M. tuberculosis* H37Rv genome sequence (Cole et al. 1998) revealed a single copy of a putative *lspA* (Fig 2A). A deletion mutant of *lspA* was created by homologous recombination using a specialized phage transduction system (Figure 2B) (Bardarov et al. 2002). The *lspA* mutant did not express detectable *lspA* mRNA and was unable to process MPT83, a well-characterized lipoprotein of *M. tuberculosis* (Figure 3). Complementation of the mutant with an integrating plasmid carrying *lspA* under the control of its native promoter restored prolipoprotein signal peptidase activity. These results confirm the presence of a single functional copy of *lspA* gene in H37Rv whose function is to cleave the signal peptide from prolipoproteins. Growth kinetics of the *lspA* mutant in rich broth medium (7H9 supplemented with OADC and glycerol) revealed a mild growth defect during the late-log phase (slopes, wild type 0.36 OD/24h; mutant 0.27) which corrected with complementation (unpublished observations). The *lspA* mutant also reached stationary phase at a slightly lower bacterial density as compared to wild-type (A_{580}, wild type 2.0; mutant 1.8). On 7H9 solid agar, the mutant formed raised microcolonies with smoother borders compared to wild type, compatible with alteration of the cell surface.

Prolipoprotein processing is required for M. tuberculosis *activation of HEK293 cells via TLR2.*

To characterize the role of prolipoprotein processing in generating *M. tuberculosis* TLR2 agonist activity, we examined the ability of wild-type *M. tuberculosis* H37Rv, Δ*lspA*, and the Δ*lspAattB*::*lspA* complemented strain to stimulate HEK293 cells stably transfected to constitutively express human TLR2 (293-TLR2 cells). These cells

produce IL-8 in response to IL-1β or lipopeptide TLR2 agonists (Fig. 8A and unpublished observations).

While wild type H37Rv induced 293-TLR2 cells to produce IL-8 in a dose-dependent manner, the response to Δ*lspA* bacteria was reduced by greater than 95% compared to that of wild-type H37Rv and the complemented strain (Fig. 4A). HEK293 cells that did not express TLR2 did not produce IL-8 upon treatment with any of the strains of *M. tuberculosis* tested (unpublished observations).

Since HEK293 cells are not professional phagocytes and therefore, may only detect TLR2 agonists present on the surface of the bacteria, we determined if lipoprotein TLR2 agonists were present but not exposed on the surface of live Δ*lspA* bacteria. Treatment of 293-TLR2 cells with wild-type whole-bacteria lysates induced approximately 25 fold greater amounts of IL-8 than that seen with live bacteria, but lysis of the Δ*lspA* strain did not correct the defect in activation of 293-TLR2 cells observed with live bacteria: the amount of IL-8 secreted in response to lysates from Δ*lspA* bacteria was only 2% percent of that seen with lysates from wild-type bacteria (Figure 4B). This finding indicates that the defect in stimulation of 293-TLR2 cells is not simply due to sequestration or mistrafficking of TLR-2 agonists in the Δ*lspA* mutant. Furthermore, expression of transfected CD14, a coreceptor for some TLR2 agonists, had only a minor effect on IL-8 secretion in response to lysates from the Δ*lspA* strain; in transfected cells, there was a greater than 90% decrease in the IL-8 secreted in response to the *lspA* mutant compared to the wild type strain (unpublished observations).

Macrophage activation by lipoprotein and nonlipoprotein components in M. tuberculosis.

M. tuberculosis has been reported to activate macrophages through TLR2 and TLR4 (Means et al. 1999), and through one or more MyD88-independent mechanisms (Shi et al. 2003). To determine the relative contributions of lipoprotein and nonlipoprotein proinflammatory agonist activities of *M. tuberculosis*, we infected RAW264.7 cells with live wild-type H37Rv, Δ*lspA*, and the Δ*lspAattB::lspA* complement. At 8 h post infection, the *lspA* mutant stimulated RAW264.7 cells to secrete 20-30% less TNFα than RAW264.7 cells infected with wild-type bacteria at the same multiplicity of infection (MOI) (Fig. 5A). To compare TNFα secretion in response to wild type, Δ*lspA* and complemented strains at different times post-treatment, we treated RAW264.7 cells with whole cell lysates of the three strains for 4, 8, 12 and 24 h. After 8 and 12 h of treatment, significantly less TNFα was secreted by RAW264.7 cells treated with whole cell lysates from the ΔlspA strain than from the wild type or complemented strain (Fig. 5B). This difference persisted at the 24 h time point, but was no longer statistically significant. The partial response of RAW264.7 cells to the *lspA* mutant indicates that lipoproteins are responsible for a portion of the proinflammatory stimulation of macrophages in response to infection with live *M. tuberculosis*. There remains, however, significant lipoprotein independent stimulation of macrophage TNFα secretion in response to *M. tuberculosis*.

Lipoprotein-independent inhibition of the macrophage response to IFNγ.

To investigate the relative contribution of lipoproteins to *M. tuberculosis* inhibition of macrophage responses to IFNγ, we infected RAW264.7 cells with wild-type H37Rv, Δ*lspA*, and the Δ*lspAattB::lspA* complement. This revealed no difference in the

ability of the three strains to inhibit the induction of MHC class II surface expression in response to IFNγ at a wide range of MOI (Fig. 6). Similarly, we found no difference between wild-type H37Rv and Δ*lspA* in the inhibition of IFNγ induction of CIITA mRNA in infected RAW264.7 cells (unpublished observations). These results suggest that, although lipoproteins contribute to inhibition of macrophage responses to IFNγ, they are dispensable for the inhibition of macrophage responses to IFNγ by live *M. tuberculosis*.

Macrophages lacking TLR2 are more resistant to M. tuberculosis *inhibition than wild-type macrophages.*

Having found that expression of mature lipoproteins was not required for *M. tuberculosis* inhibition of macrophage responses to IFNγ, we tested the contribution of TLR2 to the inhibitory effects of wild-type *M. tuberculosis* and the Δ*lspA* mutant. If all of TLR2 agonist activity of Δ*lspA* mutant was lost, as suggested by the 293-TLR2 cells, then we would expect to see no difference in *M. tuberculosis* inhibition of $TLR2^{+/+}$ and $TLR2^{-/-}$ macrophages by the Δ*lspA* mutant and wild-type strains. Infection with either strain of *M. tuberculosis* resulted in inhibition of IFNγ induction of CIITA mRNA in $TLR2^{-/-}$ macrophages, but a significantly higher MOI was required for $TLR2^{-/-}$ compared to $TLR2^{+/+}$ macrophages (Fig. 7A). To a test a broader range of concentrations of *M. tuberculosis*, we also assayed the inhibition of IFNγ responsiveness of wild-type and $TLR2^{-/-}$ macrophages treated with *M. tuberculosis* whole cell lysates (high inocula of live *M. tuberculosis* result in death of macrophages). We found a significant difference in dose-response to *M. tuberculosis* between the two types of macrophages (Fig. 7B). In

contrast, there was relatively little difference in the inhibition caused by the wild-type and Δ*lspA* mutant strains of *M. tuberculosis*. These results confirm that while *M. tuberculosis* lipoprotein-TLR2 interactions contribute to inhibition of macrophage responses to IFNγ, they are not essential, and indicate that additional TLR2-dependent and TLR2-independent mechanisms of inhibition exist.

PIM, a nonlipoprotein TLR2 agonist, inhibits macrophage response to IFNγ

To examine the difference in sensitivity to TLR2 agonists between murine macrophages and 293-TLR2 cells, we compared the response of RAW264.7 and 293-TLR2 cells to lipopeptides and phosphatidyl inositol $mannan_{1+2}$ (PIM), a non-lipoprotein TLR2 agonist from *M. tuberculosis* (Jones et al. 2001a; Jones et al. 2001b). 293-TLR2 cells responded to stimulation by 2nM Pam_3CSK_4, a triacylated lipopeptide which signals via TLR2/TLR1 heterodimers (Takeuchi et al. 2002), with secretion of IL-8, while 10, 1, or 0.1 μg/ml PIM (10 μg/ml PIM is ~5.6μM) induced <1% as much IL-8 as Pam_3CSK_4(Fig. 8A). 293-TLR2 cells also secreted significant amounts of IL-8 in response to nanomolar concentrations of Pam_2CSK_4, the diacylated analog on Pam_3CSK_4, and macrophage-activating lipopeptide-2, a diacylated lipopeptide which signals via TLR2/TLR6 heterodimers (Takeuchi et al. 2001) (unpublished observations). RAW264.7 cells, by contrast, secreted similar amount of TNFα in response to 2nM Pam_2CSK_4, 2nM Pam_3CSK_4 and 5μg/ml PIM (Fig. 8B). In addition, treatment of RAW264.7 cells with as little as 0.5μg/ml PIM inhibited subsequent IFNγ induction of MHC class II surface expression (Fig. 8C). These results indicate that at least one nonlipoprotein TLR2 agonist which is not detected by 293-TLR2 cells is able to inhibit

macrophage responses to IFNγ, and suggest that PIM and related mycobacterial cell wall glycolipids may be responsible for the effects of the ΔlspA strain on murine macrophages.

Inhibition of macrophage response to IFNγ requires new protein synthesis.

Much of the work on TLR2 and other innate pattern recognition receptors has focused on their proinflammatory activities, and has examined acute effects of innate immune activation. Since our experiments reveal an inhibitory effect of innate immune activation on responses to IFNγ, we investigated the mechanism by which the observed inhibition occurs. We found that 8 hours of pretreatment with *M. tuberculosis* was required for maximal inhibition of responses to IFNγ (Fig. 9A). The need for extended pretreatment suggested that inhibition of the response to IFNγ requires new protein synthesis. Accordingly, cycloheximide (CHX) inhibition of macrophage protein synthesis prevented the ability of either wild-type *M. tuberculosis* or the Δ*lspA* mutant strain to inhibit IFNγ induction of CIITA gene expression (Fig. 9B). These results indicate that although *M. tuberculosis* stimulation of macrophages via innate immune receptors has an immediate proinflammatory effect, longer treatment with *M. tuberculosis* induces the expression of one or more cellular proteins which inhibit macrophage responses to IFNγ.

Discussion

In efforts to understand the mechanisms whereby *M. tuberculosis* evades elimination by an adaptive immune response, we and others have discovered that *M. tuberculosis* blocks macrophage responses to IFNγ, with consequences that include defective killing of *M. tuberculosis* (Fortune et al. 2004) and class II antigen presentation (Gehring et al. 2003; Noss et al. 2000; Noss et al. 2001; Pai et al. 2003; Ramachandra et al. 2001).

Efforts to elucidate the mechanisms used by *M. tuberculosis* to block macrophage responses to IFNγ have revealed that a purified 19 kDa bacterial lipoprotein, acting through TLR2 and MyD88, can initiate signals that disrupt IFNγ gene regulation (Fortune et al. 2004; Gehring et al. 2003; Noss et al. 2001). Moreover, we found that peptidoglycan from *M. tuberculosis* (or a component that copurifies with peptidoglycan) can act in a TLR2- and MyD88-independent manner to block responses to IFNγ (Fortune et al. 2004). While substantial attention has been focused on the ability of the 19 kDa lipoprotein to inhibit responses to IFNγ and class II antigen presentation, a 19 kDa lipoprotein-null strain of BCG was as capable as a 19 kDa lipoprotein-replete strain at inhibiting class II antigen presentation by IFNγ-stimulated macrophages (Gehring et al. 2004). Moreover, other lipoproteins and nonlipoprotein components of *M. tuberculosis* can exhibit the same effects when examined as isolated components (Fortune et al. 2004; Gehring et al. 2004). Therefore, we have used genetic modification of *M. tuberculosis* to determine the relative roles of lipoproteins and nonlipoprotein components in inhibition of macrophage responses to IFNγ, in the context of the whole bacteria.

We reasoned that since acylation of a small synthetic lipopeptide was essential for inhibition of macrophage responses to IFNγ (Fortune et al. 2004), disruption of prolipoprotein processing would provide valuable information on the relative role of *M. tuberculosis* lipoproteins for inhibition of responses to IFNγ. Therefore, we disrupted bacterial lipoprotein processing by deleting the gene (*lspA*) that encodes the lipoprotein signal peptidase of *M. tuberculosis*. We found that the Δ*lspA* mutant strain lacked ≥95% of the TLR2 agonist activity of its parental strain, as assayed by a reporter cell line. Since we found that whole-cell lysates of the Δ*lspA* mutant were also unable to stimulate 293-TLR2 cells, it does not appear that the lack of lipoprotein processing results only in sequestration of lipoproteins or other TLR2 agonists. We also found that the Δ*lspA* mutant strain induced less TNFα secretion from murine macrophages than the wild type or complemented strains. This decreased induction of TNFα was most apparent after 8 and 12 h of *M. tuberculosis* treatment. Extrapolation of the curves from the time course of treatment suggests that greater than 48 h of treatment would be required for the Δ*lspA* mutant to reach the level of TNFα induction of the wild type and complemented strains.

We then examined the relative importance and/or potency of lipoproteins in the inhibition of macrophage responses to IFNγ in the context of whole, live *M. tuberculosis*. The Δ*lspA* strain exhibited no distinguishable difference in the potency or extent of inhibiting IFNγ induction of CIITA mRNA or surface MHC class II protein in macrophages, which indicates that lipoproteins are redundant in the context of inhibition by whole *M. tuberculosis*.

In order to examine the contribution of TLR2-independent pathways in the inhibition of macrophage responses by whole *M. tuberculosis*, we compared the *M.*

tuberculosis inhibition of response to IFNγ in macrophages from $TLR2^{+/+}$ and $TLR2^{-/-}$ mice. We found that macrophages lacking TLR2 were significantly less sensitive to inhibition by *M. tuberculosis* than wild-type macrophages. This difference in sensitivity was seen using both wild-type *M. tuberculosis* and the Δ*lspA* mutant strain. This finding was surprising because it suggests that the 293-TLR2 cells do not respond well to non-lipoprotein TLR2 agonists in the Δ*lspA* mutant.

We compared the responses of macrophages and 293-TLR2 cells to PIM, a glycolipid purified from the cell wall of *M. tuberculosis.* PIM has been shown to activate CHO cells expressing human TLR2 (Jones et al. 2001b), and PIM activation of murine macrophages requires TLR2 (Jones et al. 2001a). We found, however, that PIM did not activate 293-TLR2 cells, even when the cells were transfected when human CD36. Concentrations of PIM that potently induced TNFα secretion from macrophages did not induce significant IL-8 secretion from 293-TLR2 cells. In addition, concentrations of PIM unable to stimulate 293-TLR2 cells potently inhibited macrophage responses to IFNγ. These results suggest that, in the absence of mature lipoproteins, non-lipoprotein TLR2 agonists from *M. tuberculosis* are able to inhibit macrophage responses to IFNγ. In addition to PIM, lipomannan from *M. tuberculosis* has been reported to act as a nonlipoprotein TLR2 agonist (Dao et al. 2004; Quesniaux et al. 2004). Peptidoglycans from gram-positive bacteria (Lien et al. 1999) and BCG (Uehori et al. 2003) have also been shown to exhibit TLR2 agonist activity, although the interpretation of these results is controversial (Travassos et al. 2004). It is likely that, although the 293-TLR2 cells respond to both diacylated and triacylated lipopeptides, they lack one or more coreceptors required to sense nonlipoprotein TLR2 agonists.

Recent work (Sander et al. 2004) has demonstrated that disruption of lspA in *M. tuberculosis* results in a loss of virulence in wild-type BALB/c and CBA/J mice. This attenuation may indicated a specific role for lipoproteins in virulence or it may simply reflect the requirement for one or more of the 99 putative lipoproteins processed by LspA for optimal *M. tuberculosis* viability in mouse macrophages and tissues. That work did not examine the TLR2 agonist activity of the mutant strain, nor did it examine the ability of lspA-null *M. tuberculosis* to activate macrophages.

The preponderance of attention to TLR2 and other pattern-recognition receptors has been concentrated on activating signals that result in cytokine secretion and cellular phenotypic changes such as dendritic cell maturation. The finding that a later effect of TLR2 stimulation includes inhibition of IFNγ signaling, through induction of one or more proteins, extends the spectrum of activities attributable to innate immune receptors. While our results indicate that inhibition of IFNγ signaling is relieved by inhibition of protein synthesis, we have not yet identified the essential protein(s) that mediates inhibition of responses to IFNγ. One obvious candidate was suppressor of cytokine signaling-1 (SOCS1), which can be induced by *M. tuberculosis* (Ehrt et al. 2001) and which inhibits cellular responses to IFNγ by blocking the interaction of JAK1 and JAK2 with STAT1 (Chen et al. 2000). Our previous finding that *M. tuberculosis* inhibits responses to IFNγ without inhibiting activation (Ting et al. 1999) or function (Kincaid and Ernst 2003) of STAT1 provides strong evidence against a role for SOCS1 in this context. Moreover, recent experiments (Pai et al. 2003) directly excluded a role for SOCS1 by finding that 19 kDa lipoprotein inhibited IFNγ-dependent MHC class II antigen presentation in macrophages from SOCS1-deficient mice. In addition, we have

previously reported that *M. tuberculosis*-induced IL-6 can provide 'bystander' inhibition of IFNγ induction of MHC class II (Nagabhushanam et al. 2003). To determine whether induction of IL-6 was sufficient for *M. tuberculosis* inhibition of macrophage responses to IFNγ, we tested the effects of *M. tuberculosis* treatment on IL-6$^{-/-}$ BMDM. Consistent with our previous observations, both C57BL/6 and IL6$^{-/-}$ BMDM treated with *M. tuberculosis* had a defective response to IFNγ (unpublished observations). These results indicate that although *M. tuberculosis* stimulation of macrophages via innate immune receptors has an immediate proinflammatory effect, a late effect of innate immune receptor stimulation includes induction of one or more proteins which act to inhibit macrophage transcriptional responses to IFNγ. *M. tuberculosis*, by virtue of its ability to survive in nonactivated macrophages, appears to have evolved mechanisms to take advantage of this late effect to enhance its survival in macrophages exposed to an important mediator of adaptive cellular immunity.

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Disclosures

The authors have no financial conflicts of interest.

Table 1. Primer sets used in this study.

Target	Forward	Reverse
Allelic Exchange Substrate		
Upper	<u>CTTAAG</u>ATCGCTCCCGTCTCGACAGT[1]	<u>TCTAGA</u>GCGGATCAGCCGATCCTGTT
Lower	<u>AAGCTT</u>ACACCGTAGGTCGGCGCAAA	<u>ACTAGT</u>GCCGTGCTCAACTGGAAAGG
Complement		
lspA	<u>TCTAGA</u>GCAAGCGCGTCGCGCAGATT	<u>AAGCTT</u>AACCGCACCTGCAGCAAGGC
Quantitative Reverse Transcription-PCR		
lspA reverse transcription		CGACCTACGGTGTCGAAGTC
lspA	CCTGGACTCTGGTGCGTAAT	ACCGACAAGAAATCGACGAC
murine CIITA (all forms)	GAAGTTCACCATTGAGCCATTTAA	CTGGGTCTGCACGAGACGAT
murine GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA

[1]Restriction endonuclease sites are underlined.

Figures

Figure 1. Synthetic triacylated hexapeptide inhibits macrophage response to IFNγ. RAW264.7 cells were pretreated with media alone ("0") or Pam_3CSK_4 at the concentrations indicated. 24 h later, cells were treated with IFNγ for 24 hours. Cells were stained with PE conjugated anti-mouse I-A/I-E, and analyzed by flow cytometry (10,000 total events were counted). Values shown are mean fluorescent intensity (mean ± SD of triplicate assays). Concordant results were obtained in two other experiments.

Figure 1

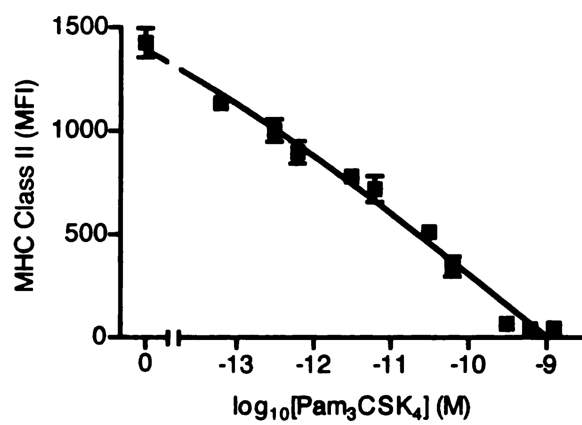


Figure 2. Disruption of *M. tuberculosis lspA* by homologous recombination. (A) Bacterial lipoprotein processing (Sutcliffe and Harrington 2004). Nascent preprolipoproteins are translocated across the bacterial membrane where prolipoprotein diacylglycerol transferase (the product of *lgt*) catalyzes the addition of diacylglycerol to the sulfhydryl group of a cysteine residue near the amino terminus and preceded by residues characteristic of a "lipobox." The signal peptide on diacylglycerol-modified prolipoprotein is cleaved on the amino-terminal side of the modified cysteine (by prelipoprotein signal peptidase II, the product of *lspA*.) The cleaved lipoprotein containing diacylglycerol can act as an agonist for TLR2/TLR6 heterodimers. Further addition of a fatty acid to the newly exposed free amine on the modified cysteine (by lipoprotein N-acyl transferase, the product of *lnt*) results in a triacylated lipoprotein that can act as an agonist for TLR2/TLR1 heterodimers. (B) Genomic map of *lspA* and flanking genes in *M. tuberculosis* H37Rv. (C) Allelic exchange substrate used to make a Δ*lspA* knockout mutant with the mycobacteriophage system. The shaded box with *hyg* cassette represents the region deleted from *lspA*. (D) Genomic fragment PCR amplified to create Δ*lspAattB*::*lspA* complement.

Figure 2

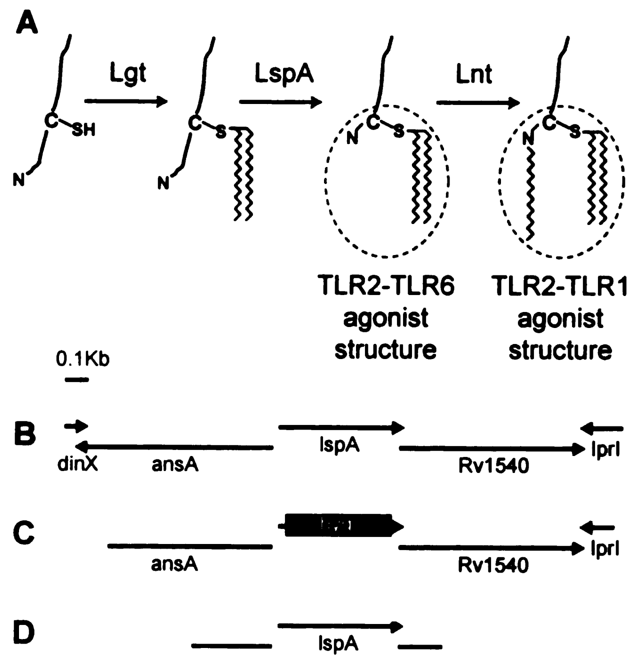


Figure 3. Phenotypic confirmation of *lspA* mutant by real-time RT-PCR, and western blot. (A) Real-time RT-PCR results showing the expression of *lspA* transcript in *M. tuberculosis* H37Rv, Δ*lspA* (*lspA* mutant), and Δ*lspAattB*::*lspA* (*lspA* complement). (B) Western immunoblot of bacterial extracts with anti-MPT83 antibody showing the migration of MPT83 from H37Rv, Δ*lspA*, and complement (*attB*::*lspA*), as indicated. The blot was loaded with 0.2 μg of total protein from the wild-type (H37Rv) and complemented mutant (*attB*::*lspA*) extract and 1.5 μg of total protein from the *lspA* mutant (*ΔlspA*), to demonstrate the mobility difference of the lspA mutant in the face of a lower abundance of Mpt83 in the mutant. Although MPT83 is present in lower abundance in the *lspA* mutant, the other two lipoproteins (19 and 38 kDa lipoproteins) we analyzed are present in similar quantities in wild-type, *lspA* mutant, and complemented *lspA* mutant (unpublished observations). Data shown is representative of 3 independent extractions.

Figure 3

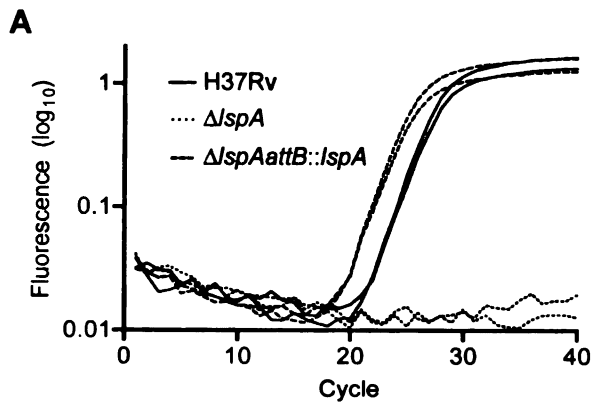


Figure 4. Prolipoprotein processing is required for *M. tuberculosis* stimulation of HEK293 cells expressing TLR2. (A) 293-TLR2 cells were infected with wild type *H37Rv* (filled squares), Δ*lspA* (open triangles) or Δ*lspAattB*::*lspA* complement (filled triangles) at a range of multiplicities of infection, as indicated. Cell supernatants were harvested after 24 hours and secretion of IL-8 into the medium was quantitated by ELISA (mean of duplicate assays). Background level of IL-8 secreted into media of untreated cells (70.9 pg/ml) was subtracted from all values. Results are representative of 4 independent experiments. Because small changes in MOI led to significant changes in IL-8 secretion, all MOIs were confirmed by quantitative culture. (B) 293-TLR2 cells were treated with whole cell lysates from wild type H37Rv, Δ*lspA* or Δ*lspAattB*::*lspA* complement at a range of dilutions, as indicated. Protein concentrations refer to soluble protein in whole cell lysates. Supernatants were analyzed as described for (A). Background level of IL-8 secreted into media of untreated cells (45.2 pg/ml) was subtracted from all values. Data is mean ± SD of triplicate assays. Results are representative of 4 independent experiments.

Figure 4

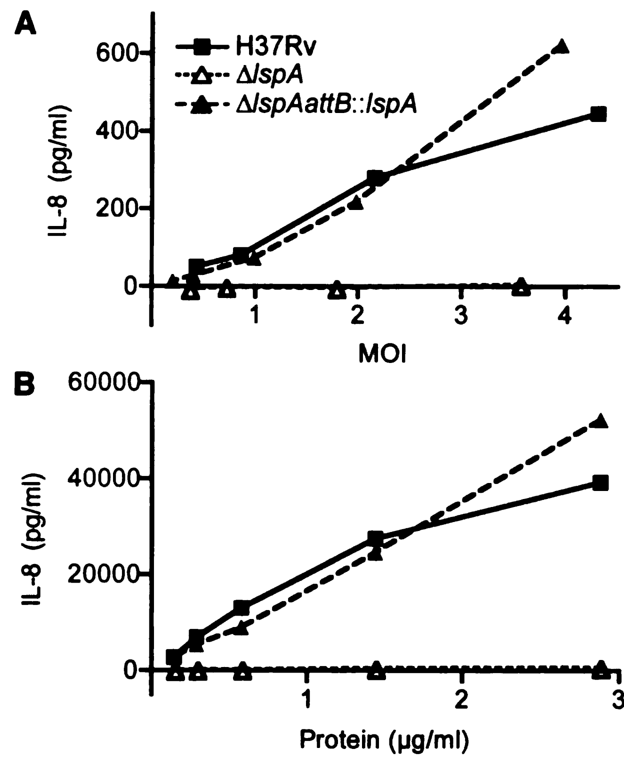


Figure 5. Induction of macrophage TNFα secretion by lipoprotein and non-lipoprotein components of intact *M. tuberculosis*. (A) RAW264.7 cells were infected with wild type H37Rv (filled squares), *ΔlspA* (open triangles) or *ΔlspAattB::lspA* complement (filled triangles) at a range of multiplicities of infection, as indicated. Cell supernatants were harvested after 8 h. (B) RAW264.7 cells were treated with 0.3μg/ml *M. tuberculosis* whole cell lysates from the indicated strains. (Protein concentrations refer to soluble protein in whole cell lysates.) Cell supernatants were harvested at the indicated times. Secretion of TNFα into the medium was quantitated by ELISA (mean ± SD of triplicate assays). Results are representative of 4 (panel A) and 2 (panel B) independent experiments.

Figure 5

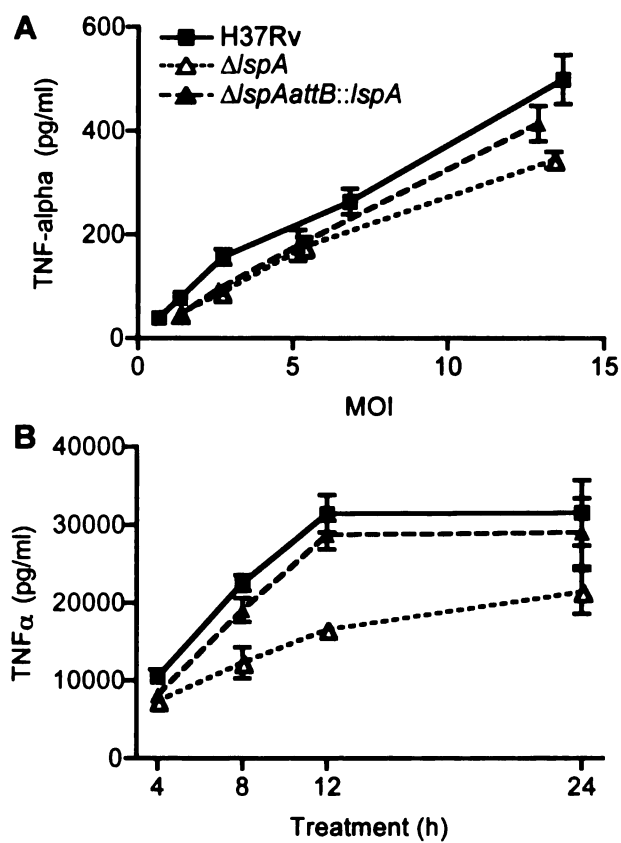


Figure 6. Lipoprotein-independent inhibition of the macrophage response to IFNγ. RAW264.7 cells were infected with wild type H37Rv (filled squares), *ΔlspA* (open triangles) or Δ*lspAattB*::*lspA* complement (filled triangles) at a range of multiplicities of infection, as indicated. After 8 h infection, cells were treated with IFNγ for 16-24 hours. Cells were stained with PE-conjugated anti-mouse I-A/I-E and analyzed by flow cytometry (10,000 events, gated by forward and side scatter, were counted). Values shown are mean fluorescent intensity (mean ± SD of triplicate assays). Results are representative of 4 independent experiments.

Figure 6

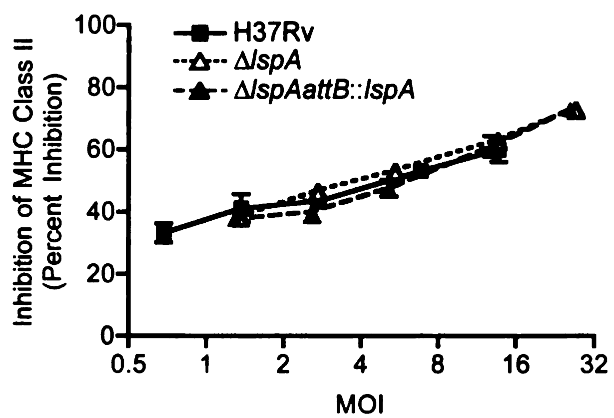


Figure 7. TLR2$^{-/-}$ macrophages are less sensitive than wild-type macrophages to *M. tuberculosis* inhibition of response to IFNγ. (A) BMDM from C57BL/6 (filled circles) or TLR2$^{-/-}$ (open circles) mice were infected with wild-type H37Rv (solid lines) or the Δ*lspA* mutant strain (dashed lines) at the indicated MOI. Infection was allowed to proceed for 24 h. (B) BMDM from C57BL/6 (filled circles) or TLR2$^{-/-}$ (open circles) mice were treated with whole cell lysates from wild-type H37Rv (solid lines) or the Δ*lspA* mutant (dashed lines), at the indicated concentrations, for 24 h. In both (A) and (B), total RNA was harvested after 4 h IFNγ treatment. CIITA expression was assayed by quantitative real-time RT-PCR with primers recognizing all forms of murine CIITA. All values were normalized to GAPDH. Results are shown as fold induction compared to uninfected sample without IFNγ. Results are representative of 3 (panel A) or 2 (panel B) independent experiments.

Figure 7

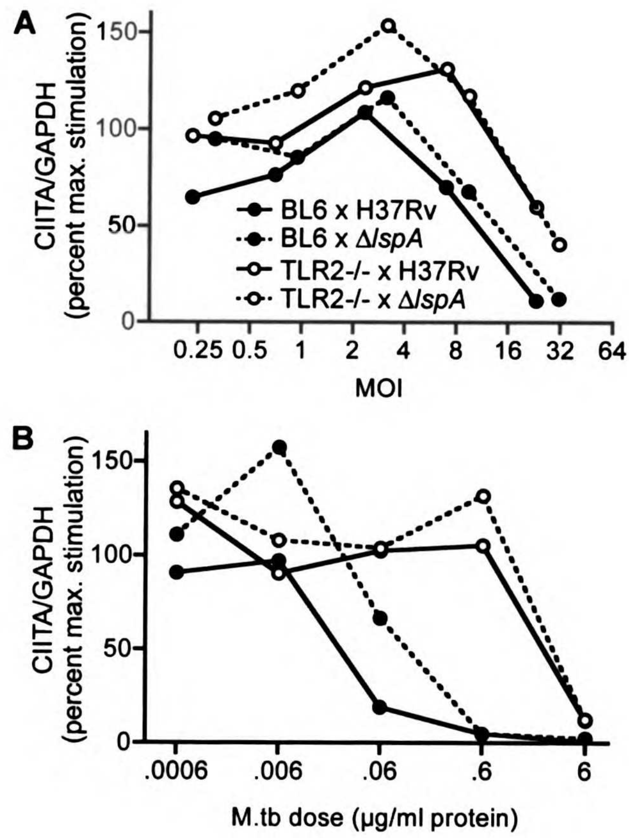


Figure 8. *M. tuberculosis* PIM inhibits macrophage response to IFNγ. (A) 293-TLR2 cells were left untreated (UT) or were treated with 2nM Pam_3CSK_4 (P3C) or the indicated doses of PIM for 24h. Cell culture supernatants were analyzed by ELISA for human IL-8. Background level of IL-8 secreted into media of untreated cells (47.2 pg/ml) was subtracted from all values. Values shown are mean of triplicate assays. Overexpression of human CD36 in these cells did not significantly increase the induction of IL-8 secretion in response to these concentrations of PIM (unpublished observation). (B) RAW264.7 cells were left untreated or were treated with 2nM Pam_2CSK_4, 2nM Pam_3CSK_4 or the indicated doses of PIM for 8h. Cell culture supernatants were analyzed by ELISA for murine TNFα. Solvent control (DMSO) did not induce IL-8 or TNFα above background levels (unpublished observation). (C) RAW264.7 cells were treated with the indicated doses of PIM for 24h, followed by 18-24h treatment with IFNγ. Cells were stained with PE conjugated anti-mouse I-A/I-E, and analyzed by flow cytometry (10,000 total events were counted). Values shown are mean fluorescent intensity (mean ± SD of triplicate assays). Solvent control (DMSO) did not inhibit IFNγ-induced MHC Class II expression (unpublished observation). Values shown are mean ± SD of triplicate assays. Results are representative of 3 (panel A), 4 (panel B) and 3 (panel C) independent experiments.

Figure 8

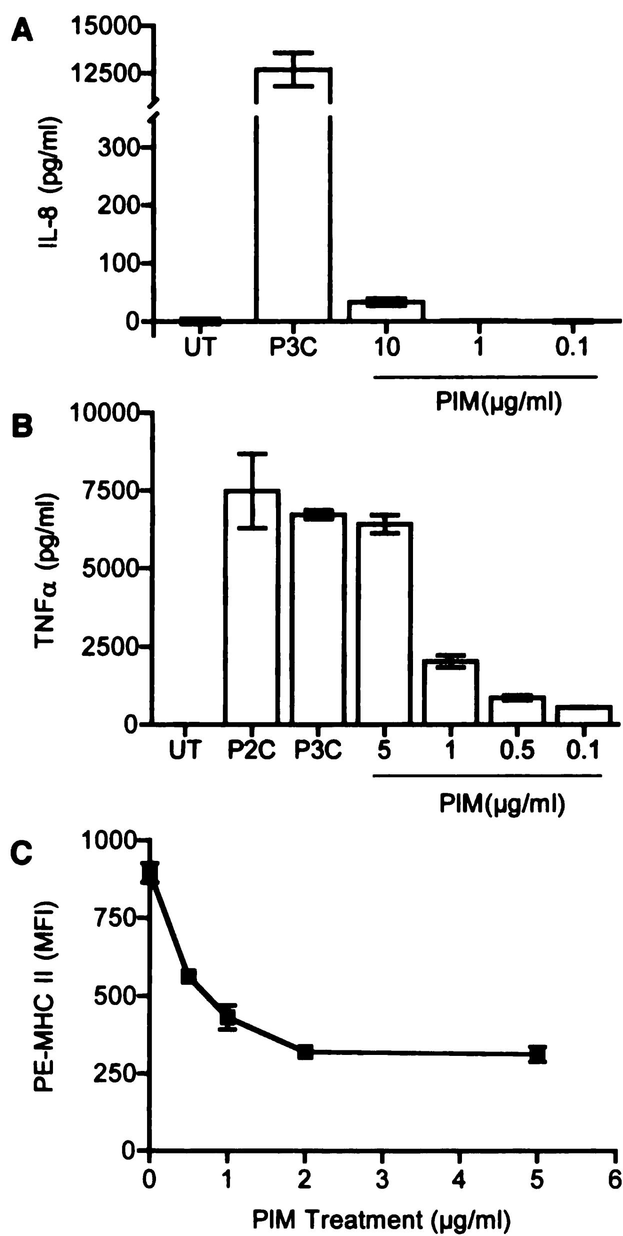
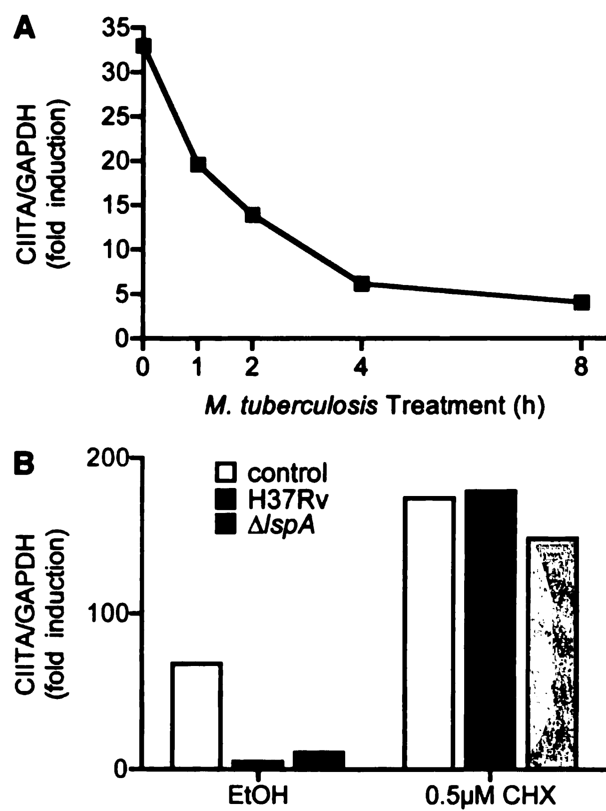


Figure 9. Inhibition of macrophage response to IFNγ requires new protein synthesis. (A) RAW264.7 cells were treated with γ-irradiated *M. tuberculosis* for the indicated times or left untreated ("0"). After *M. tuberculosis* treatment, cells were treated with IFNγ for 8 h. Inhibition of CIITA expression was not significantly different at 8 and 24 h (unpublished observations). (B) C57BL/6 BMDM were pretreated with solvent control (0.02% ethanol) or 500 nM CHX for 1 hour before 8 hours of treatment with 3 μg/ml *M. tuberculosis* whole cell lysate from wild-type (filled bars), Δ*lspA* (striped bars) or mock treatment (open bars) in the presence of cycloheximide or ethanol. Cells were then treated with IFNγ for 4 hours. In both (A) and (B), total RNA was harvested after IFNγ treatment. CIITA expression was assayed by quantitative real-time RT-PCR with primers recognizing all forms of murine CIITA. All values were normalized to GAPDH. Results are shown as fold induction compared to uninfected sample without IFNγ. The concentration of CHX used inhibited TNFα production (as a measure of protein synthesis) by *M. tuberculosis* treated BMDM by approximately 90%, respectively, while minimizing cell death. Results are representative of 3 (panel A) or 2 (panel B) independent experiments.

Figure 9



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Footnotes

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[4] Abbreviations used in this paper: 293-TLR2, HEK293 cells expressing human TLR2; AES, allelic exchange substrate; BCG, *M. Bovis* bacille Calmette-Guerin; BMDM, bone marrow derived macrophages; CHX, cycloheximide; DPBS, Dulbecco's PBS; lspA, lipoprotein signal peptidase; MOI, multiplicity of infection; Pam_2CSK_4, (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-(R)-Cys-(S)-Ser-(S)-Lys_4 x 3 CF3COOH; Pam_3CSK_4, (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH, 3HCl; PIM, *M. tuberculosis* phosphatidyl inositol $mannan_{1+2}$; SOCS1, suppressor of cytokine signaling.

Chapter VI

Conclusions

Previous work in the Ernst laboratory found inhibition of cellular responses to IFNγ in human monocyte-derived macrophages infected with live, virulent *M. tuberculosis* (Ting et al. 1999). Inhibition of activation of the antimicrobial function in *M. tuberculosis* infected macrophages was demonstrated by the failure of these cells to kill intracellular *Toxoplasma gondii*. The inhibition of IFNγ induction of CD64 expression was evident at the level of mRNA expression and surface expression of the protein. The inhibition of the macrophage response to IFNγ was not due to an inhibition of proximal steps in IFNγ signaling; there was no defect in signal transducer and activator of transduction 1 (STAT1) protein level, tyrosine or serine phosphorylation, DNA binding or nuclear translocation. There was, however, an apparent decrease in the association between STAT1 and the transcriptional coactivators cAMP enhancer binding protein (CREB)-binding protein (CBP)/p300.

In Chapter 2, I revisit the question of whether *M. tuberculosis* infection of macrophages interferes with the interaction between STAT1 and the transcriptional coactivators CBP and p300. The original experiments demonstrated a decrease in the capacity of STAT1 dimers from *M. tuberculosis* infected cells to bind CBP/p300. Using the complementary technique of assaying the capacity of CBP/p300 molecules to bind STAT1, I found no defect in CBP/p300 binding to STAT1 in *M. tuberculosis* treated cells. Since CBP/p300 are limiting in cells, the proportion of CBP/p300 molecules able to bind STAT1 molecules is arguably more important than the proportion of STAT1 molecules able to bind CBP/p300. These results, combined with the finding that the *M. tuberculosis* inhibition of macrophage responses to IFNγ is gene-selective (described in Chapters 3 and 4), suggest that there is no global defect in the interaction between

STAT1 and the transcriptional coactivators CBP and p300 in *M. tuberculosis* infected cells. I also used histone deacetylase (HDAC) inhibitors to examine the role of the balance between histone acetylase and deacetylase activities in *M. tuberculosis* treated cells. I found that treatment with HDAC inhibitors did not block *M. tuberculosis* inhibition of macrophage responses to IFNγ, but instead the HDAC inhibitors themselves inhibited the response to IFNγ. My work, along with the work of others (Chang et al. 2004; Klampfer et al. 2004; Sakamoto et al. 2004), indicates that HDAC activity is required for gene expression in response to interferons. My findings suggest that *M. tuberculosis* does not inhibit macrophage responses to IFNγ by increasing HDAC activity in macrophages.

I found that, like human monocyte-derived macrophages, THP-1 cells, a human monocytic cell line, are susceptible to *M. tuberculosis* inhibition of IFNγ responsiveness. As previously described with monocyte-derived macrophages (Ting et al. 1999), *M. tuberculosis* inhibition of THP-1 cell responses to IFNγ occurs without an inhibition of STAT1 phosphorylation or in vitro binding of double-stranded oligonucleotides that contain a γ-activated sequence (GAS). Using THP-1 cells, which provided greater flexibility and reproducibility than primary human cells, I was able to extend these findings and to demonstrate that *M. tuberculosis* inhibits macrophage responses to IFNγ without inhibiting STAT1 transactivation of a synthetic GAS sequence or STAT1 binding to the endogenous CD64 promoter. I found that this inhibition was gene-selective and was exerted at the level of transcription. This work is presented in Chapter 3.

Our consistent finding that IFNγ-induced STAT1 activation is not inhibited by *M. tuberculosis* (Ting et al. 1999 and Chapter 3; Kincaid and Ernst 2003) contrasts with the inhibition of IFNγ signaling in cells infected with *Mycobacterium avium* (Hussain et al. 1999), *Leishmania donovani* (Ray et al. 2000) or Adenovirus type 5 (Joseph and Look 2001), in which IFNγ receptor expression is impaired. Although *M. tuberculosis* infection of macrophages induces expression of suppressor of cytokine signaling 1 (SOCS1)(Ehrt et al. 2001), an inhibitor of IFNγ signaling that blocks the interaction between STAT1 and the kinases Janus kinase 1 (JAK1) and JAK2 (Chen et al. 2000), our finding that IFNγ induced STAT1 phosphorylation is not decreased in these cells indicates that SOCS1 is not contributing to the inhibition of downstream responses to IFNγ. In addition, the finding that both in vitro GAS binding and STAT1 binding to an endogenous promoter are not decreased in *M. tuberculosis* infected cells indicate that protein inhibitor of activated STAT 1, a cellular inhibitor of IFNγ signaling that block STAT1 DNA binding (Liu et al. 1998), is not responsible for the decreased response to IFNγ.

Macrophages infected with *M. tuberculosis* have a defect in MHC class II antigen presentation (Fulton et al. 2004; Noss et al. 2000). Since there is a defect in phagolysosomal fusion in *M. tuberculosis* infected macrophages (Armstrong and Hart 1971; Clemens and Horwitz 1995) it has been proposed that the defect in antigen processing and presentation is due to sequestration of *M. tuberculosis* away from intracellular MHC class II molecules (Hmama et al. 1998; Ramachandra et al. 2001) thereby preventing the expression of mature MHC class II molecules on the cell surface and/or preventing presentation of *M. tuberculosis* antigens. In addition, as part of the

block in phagolysosomal fusion, *M. tuberculosis* prevents the acidification of its phagosome, resulting in an environment too basic for optimal antigen processing and loading.

Expression of high levels of MHC class II on the surface of macrophages, however, requires IFN-γ treatment. We and others have shown that *M. tuberculosis* can inhibit macrophage responses to IFNγ, including the induction of CIITA and MHC class II expression (Fortune et al. 2004; Noss et al. 2000 and Chapter 3; Kincaid and Ernst 2003). This shortage of MHC class II molecules and accessory proteins could explain part, if not all, of the defect in antigen presentation in infected cells. These finding suggest that the *M. tuberculosis* inhibition of responses to IFNγ not only interferes with killing of *M. tuberculosis*, but also prevents recognition of *M. tuberculosis* antigens on infected macrophages.

The finding that *M. tuberculosis* inhibition of IFNγ induced transcription is gene-selective suggests that the affected promoters contain elements that render them sensitive to this inhibition. In Chapter 4, I investigate which regions of an *M. tuberculosis* inhibited promoter are required for this inhibition. We found that, of the IFNγ-responsive genes whose expression was inhibited in murine macrophages, CIITA and genes whose transcription was dependent on CIITA were the most profoundly inhibited. The IFNγ responsiveness and the sensitivity to inhibition of the endogenous promoter could be reproduced using a fragment of the CIITA type IV promoter driving luciferase. Using deletion mutants of this promoter construct, I found that a 256-base pair fragment of the CIITA type IV promoter was sufficient for induction by IFNγ and inhibition by *M. tuberculosis*. This minimal fragment of the CIITA type IV promoter has been shown to

contain three transcription factor binding sites: GAS, E-box and IFN regulatory factor response element (Muhlethaler-Mottet et al. 1997). Site-directed mutagenesis of these three sites, however, could not separate induction by IFNγ from inhibition by *M. tuberculosis*.

We have found that *M. tuberculosis* infection inhibited the response to IFNγ in human and murine primary cells and cell lines. The murine macrophage cell line RAW264.7 provided a more transfectable cell system, and murine bone marrow macrophages allowed us to take advantage of cells from genetically modified mice. Although a different range of genes was inhibited in murine compared to human cells, there were basic similarities between the cells from the two species. I found that the response to IFNγ was inhibited without inhibition of STAT1-activation, and that an extended period of pretreatment or infection was required to establish this inhibited state before IFNγ treatment. There were some difference between human and mouse macrophages; we found that certain genes that were not inhibited in human macrophages, such as IFN regulatory factor 1 and monokine induced by γ, were inhibited in murine macrophages. In addition, induction of CIITA was much more profoundly inhibited in murine compared to human cells. These findings may indicate that mouse cells are susceptible to additional mechanisms of inhibition compared to human cells.

Infection with live *M. tuberculosis* was not required for inhibition of macrophage responses to IFNγ; treatment with γ-irradiated or lysed *M. tuberculosis* could reproduce this inhibition. In every assay in which live infection and treatment with γ-irradiated *M. tuberculosis* were compared, the results were consistent between the two systems. These

findings indicate that preformed components of *M. tuberculosis* are sufficient for this inhibition, and suggest a role for innate immune sensing of *M. tuberculosis*.

Live *M. tuberculosis* has been found to have both Toll-like receptor 2 (TLR2) and TLR4 agonist activity (Means et al. 1999). Several *M. tuberculosis* TLR2 agonists have been identified, including the 19kDa lipoprotein (Brightbill et al. 1999), phosphatidylinositol mannan (Jones et al. 2001), and lipomannan (Dao et al. 2004; Quesniaux et al. 2004), and recently *M. tuberculosis* heat shock proteins 65 and 70 (Bulut et al. 2005) have been found to have TLR4 agonist activity. The observation that the same components that result in the inhibition of IFNγ signaling (Ting et al. 1999) also activate nuclear factor κB via TLR signaling (Means et al. 1999) led us to investigate the role of *M. tuberculosis* TLR agonists in the inhibition of macrophage responses to IFNγ.

Like infection with live *M. tuberculosis*, treatment with the 19kDa lipoprotein of *M. tuberculosis* inhibits macrophage responses to IFNγ (Fortune et al. 2004; Fulton et al. 2004; Gehring et al. 2003). The 19kDa lipoprotein is just one of an estimated 99 lipoproteins in the *M. tuberculosis* genome (Sutcliffe and Harrington 2004) and at least one other mycobacterial lipoprotein has been found to have the same inhibitory effect (Gehring et al. 2004). To determine the contribution of all mycobacterial lipoproteins to the inhibition of macrophage responses to IFNγ by live, virulent *M. tuberculosis*, Niaz Banaiee disrupted expression of all mature lipoproteins in *M. tuberculosis* H37Rv. and I tested the ability of this mutant to inhibit macrophage responses to IFNγ. This work is described in Chapter 5. We found that mature mycobacterial lipoproteins were not required for *M. tuberculosis* inhibition of macrophage responses to IFNγ.

The activation of macrophages via TLR2 has been found to inhibit macrophage responses to IFNγ (Fortune et al. 2004; Fulton et al. 2004; Gehring et al. 2003; Noss et al. 2001; Pai et al. 2003), but the significance of TLR2 in the context of infection with live, virulent *M. tuberculosis* was not known. In Chapter 5 we present our findings that, although live *M. tuberculosis* can inhibit macrophage responses to IFNγ in a TLR2 independent manner, TLR2 was required for potent inhibition by low doses of *M. tuberculosis*. Our findings suggest that non-lipoprotein TLR2 agonists play an important role in this inhibition. I also found that extended pre-treatment with *M. tuberculosis* was required, and that new protein synthesis was required during this pretreatment.

A number of reports have attempted to clarify the role of TLRs in *M. tuberculosis* infection. Although the effect is subtle, TLR2-deficient mice challenged with *M. tuberculosis*, given long enough or a high enough dose, have a shorter time to death than wild-type mice (Drennan et al. 2004; Reiling et al. 2002; Sugawara et al. 2003). These findings suggest that TLR2 plays a dual role in the response to *M. tuberculosis* in vivo – early induction of proinflammatory responses and subsequent inhibition of IFNγ induced activation in infected cells. This inhibition may represent a host feedback mechanism to prevent inflammation-mediated damage. *M. tuberculosis*, however, is a slow-growing pathogen able to persist within macrophages, and, therefore, is ideally situated to take advantage of this inhibition of adaptive immune responses.

This negative crosstalk between innate and adaptive immune responses could have profound consequences for vaccine development. Care should be taken to ensure that vaccine adjuvants are not inhibiting adaptive immune responses. Conversely, a

therapy that could block this negative crosstalk might allow the immune response to clear *M. tuberculosis* infection.

Using cured tuberculosis infection in mice as a vaccine model, the North group found that the secondary immune response to *M. tuberculosis* is earlier but otherwise no more effective at clearing *M. tuberculosis* infection than the primary response (Jung et al. 2005). They hypothesize that the failure of bacterial clearance is due to a failure to activate the antimicrobial function of macrophages. Our work provides a model of how this failure occurs -- *M. tuberculosis* infection of unstimulated macrophages renders these cells refractory to later IFNγ stimulation. The infected macrophages, therefore, cannot perform their function in the adaptive immune response – as antimicrobial and antigen presenting cells. Although macrophages that are recruited to the site of infection may be primed with IFNγ, and therefore able to kill *M. tuberculosis*, there remains of reservoir of infected macrophages that are refractory to mediators of the adaptive immune response.

Currently the mechanism of this inhibition is not fully understood, but future work in this area should provide not only a better understanding of IFNγ activation of macrophages but also potential therapeutic targets for the next generation of anti-tuberculosis drugs.

Abbreviations

CBP, cAMP enhancer binding protein (CREB)-binding protein; CIITA, class II transactivator; GAS, γ-activated sequence; HDAC, histone deacetylase; JAK, Janus kinase; SOCS, suppressor of cytokine signaling 1; STAT1, signal transducer and activator of transduction 1; TLR, Toll-like receptor.

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