HOST TRANSCRIPTIONAL REGULATION DURING A MYCOBACTERIAL INFECTION AND DEVELOPING A MECHANISM OF DRUG TARGETING THROUGH FOLATE DRUG CONJUGATES

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by

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Abstract

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*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is responsible for nearly 2 million deaths each year. The rise in HIV co-infections and the neglect of TB control programs has led to TB’s re-emergence as a serious public health concern.

An improved understanding of host immune responses following *Mycobacterium* infection can provide insights into the pathogenesis of virulent mycobacteria and identify potential targets for rational anti-TB drug design. Our work indicates that infection with isogenic strains of *M. tuberculosis* results in differential activation of various transcription factors. Previous studies have demonstrated that expression of the folate receptor (FR) is primarily restricted to tumor cells and activated macrophages. We hypothesize that the FR may also be expressed on activated, tuberculosis-infected macrophages. Addressing the biology behind FR expression in *Mycobacterium*-infected
mouse and human macrophages may lead to new mechanisms to treat drug-sensitive and drug-resistant tuberculosis.
DEDICATION

For my brother, PFC Jason D. Hoang, who makes me prouder than words can express.

Semper fi, Marine!
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>BCG</td>
<td><em>Mycobacterium bovis</em> Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CFP</td>
<td>Culture filtrate protein</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FR</td>
<td>Folate receptor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug-resistant</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced folate carrier</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>XDR</td>
<td>Extensively drug-resistant</td>
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</table>
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Marine. For this and so much more, I am fiercely proud and beyond privileged to call PFC Jason D. Hoang my younger brother.

According to the American theologian Reinhold Niebuhr, “Nothing that is worth doing can be achieved in a lifetime”, and “nothing we do, however virtuous, can be accomplished alone.” The truth of these words applies to the contents of this thesis. Its merits are the result of the contributions of those listed above; its shortcomings I unequivocally claim as my own.
CHAPTER ONE

INTRODUCTION

1.1 Background

Tuberculosis (TB) is one of the world’s oldest human afflictions and continues to be one of the leading killers among the infectious diseases, despite the widespread use of a live, attenuated vaccine and the availability of several antibiotic compounds. Examination of skeletal remains from ancient Egypt and various Neolithic sites indicates that TB was found throughout the world up to 4000 years ago. It is believed that members of the genus *Mycobacterium* were originally found in soil and evolved to live in mammals. The domestication of cattle may have allowed for the passage of a mycobacterial pathogen from domesticated livestock to humans. Genetic analyses suggest that *Mycobacterium bovis*, which causes TB-like disease in cattle, evolved at the same time as *Mycobacterium tuberculosis*, the causative agent of pulmonary tuberculosis in humans (Stead 1997).

In the fifth century B.C., Hippocrates described patients with *phthisis*, or consumption, i.e., the wasting away associated with chest pain and coughing that is frequently associated with blood in the sputum. While Hippocrates thought tuberculosis was a hereditary disease, the Greek philosopher Aristotle and the Roman physician Galen
stressed the disease’s contagious nature. Though Robert Koch would conclusively demonstrate in 1882 that TB is caused by a bacterium, environmental factors play a major part in disease transmission.

In the 16th and 17th centuries, Europe experienced rapid growths in both population and large urban centers, leading to the continent becoming the epicenter for many TB epidemics. The disease peaked in Europe in the first half of the 19th century, during which time it was estimated that a quarter of all European deaths were due to TB. It is estimated that TB accounted for one billion deaths from 1700 to 1900. In March of 1882, presenting his findings that \textit{M. tuberculosis} is the bacterium that causes tuberculosis, Robert Koch reminded his audience:

\begin{quote}
If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like. One in seven of all human beings dies from tuberculosis. If one only considers the productive middle-age groups, tuberculosis carries away one-third, and often more. (“Tuberculosis”, Nobelprize.org 2003)
\end{quote}

European immigrants brought the disease across the Atlantic, and while the mortality rate never rivaled the levels found in Europe, large urban centers like New York City and Boston had TB death rates of 6-7 per 1000 in 1800. During the second half of the 19th century, mortality rates decreased largely due to improved housing and sanitation conditions and the implementation of public health measures (Smith 2003).

1.2 Current worldwide statistics

Aided by the widespread use of the BCG vaccine, the development of antibiotics in the 1950s, and improved public health practices, TB morbidity and mortality rates steadily declined in the 20th century in developed countries. This downward trend in the
number of new TB cases ended in the mid-1980s, which was attributed to the emergence of HIV/AIDS. Underdeveloped nations are still experiencing a large TB burden (Smith 2003).

Approximately one-third of the world population is currently infected with tuberculosis. Because the immune system of immunocompetent individuals is able to contain the bacteria, only 5-10% of infected individuals will ever develop active disease in their lifetime. An untreated individual with active TB is estimated to infect an average of 10-15 new persons each year. Left untreated, TB is fatal for up to 60% of those with active disease (Kaye and Frieden 1996).

Tuberculosis is a global health concern that accounts for over 2 million deaths (one death every 15 seconds) annually, a number that is higher than it was just twenty years ago. TB incidence rates highlight the uneven worldwide distribution of TB burden. Nearly all TB deaths occur in the developing world, where poverty rates are high and where healthy living conditions and adequate medical care are in short supply. The global TB crisis is exacerbated by the limited availability of high quality antibiotics, non-compliance with recommended treatment regimens, and the emergence of drug-resistant TB strains in India and countries of South America and the former Soviet Union (Iseman 1994).

1.3 The immune response to *Mycobacterium* infection
1.3.1 Receptors and cell signaling pathways

*M. tuberculosis* is transmitted via aerosol droplets and enters alveolar passages of exposed individuals. The recognition and phagocytosis of *Mycobacterium* is mediated by
several different receptors on the host cell. Bacteria are phagocytosed by resident macrophages upon contact with macrophage mannose receptors (MR), which recognize mannose and fucose sugars on pathogenic surfaces, and complement receptors (Schlesinger 1993). The alveolar glycoprotein surfactant protein A can upregulate activity of the MR, leading to enhanced binding and ingestion of *M. tuberculosis* (Gaynor et al. 1995). Complement receptor 3 (CR3) is estimated to mediate approximately 80% of complement-opsonized phagocytosis of mycobacteria, and the receptor can also mediate non-opsonized phagocytosis (Schlesinger et al. 1990; Le Cabec et al. 2002). In addition, the membrane protein CD14, found on mature myeloid cells, can also mediate phagocytosis by acting as a receptor for components of the mycobacterial cell wall, such as peptidoglycan and lipoarabinomannan (LAM) (Dziarski 2003; Means et al. 1999). The structurally conserved membrane Toll-like receptors (TLRs) recognize various pathogen-associated molecular patterns. TLR signal transduction is mediated by the adaptor protein MyD88, leading to the recruitment of various kinases and NF-κB activation.

In individuals with a healthy immune system, acute mycobacterial infections can be controlled with various immune mediators, such as interleukin12 (IL-12), tumor necrosis factor alpha (TNF-α), nitric oxide (NO) and interferon gamma (IFN-γ). The phagocytosis and processing of mycobacterial antigens also triggers cellular immune responses. Processed mycobacterial antigens are presented by major histocompatibility complex class II (MHC-II) molecules, leading to the activation of CD4+ and CD8+ T cells (Bhatnagar 2006).
1.3.2 Unique properties of the genus *Mycobacterium*

All *Mycobacterium* species share a characteristic hydrophobic, waxy cell wall that is rich in mycolic acids. This cell wall is thicker than those found in many other bacteria and confers upon the bacteria both hardiness and a degree of resistance to many common antibiotics and chemotherapeutic reagents. The composition of the mycobacterial cell wall includes three unique insoluble macromolecules: arabinogalactan, peptidoglycan, and mycolic acid. Mycobacterial mycolic acids are distinguishable from those found in other closely related, Gram-positive bacteria (e.g., *Corynebacterium*, *Nocardia*, and *Rhodococcus*) due to their length and complexity. The outermost layer of the cell wall is rich in complex and strain-specific lipids, including LAM and 19-kDa lipoprotein (Chatterjee and Khoo 2001). Pathogenic mycobacteria, in general, contain mannosylated LAM (ManLAM), whereas non-pathogenic species, such as *M. smegmatis*, contain uncapped (AraLAM) or phosphoinositol-capped LAM (Bhatnagar 2006).

1.3.3 Mechanisms of immune system evasion

Macrophages ingest microorganisms and other foreign materials via phagocytosis. Ingested particles are housed within the phagosome, which undergoes a series of fusion and fission events with cellular vesicles that eventually lead to the formation of the phagolysosomes. In the normal phagosome maturation process, fusion of the phagosome with the lysosome creates a highly microbicidal environment that is characterized by acidic pH, degradative lysosomal enzymes, and the formation of reactive oxygen (ROI) and nitrogen (RNI) intermediates. Previous studies in mice have demonstrated that RNIs
are very effective against virulent mycobacteria (Chan et al. 1992). Furthermore, resistance to RNIs is correlated with \textit{M. tuberculosis} virulence (O’Brien et al. 1994).

Intracellular pathogens have developed ways to avoid the hostile microenvironment of the phagolysosome. Pathogenic mycobacteria inhibit phagosome-lysosome fusion, thereby preventing the acidification of the mycobacterial phagosome. Residing within an early endosome confers a selective advantage to the pathogen, as \textit{M. tuberculosis}-infected macrophages exhibit decreased expression of MHC-II proteins and decreased MHC-II presentation bacterial antigens, which results in less immunosurveillance by host CD4+ T cells (Noss et al. 2001). This selective advantage appears to be mediated during the early phase of mycobacterial entry into the macrophage by the interaction of Toll-like receptor 2 (TLR2) with the \textit{M. tuberculosis} 19-KDa lipoprotein (Thoma-Uszynski et al. 2001). Though the mechanism by which pathogenic mycobacteria prevent phagosome maturation is unknown, the normal phagosome maturation process is controlled by the successive recruitment of the Rab proteins, the small GTPases that are involved in the coordination of endosomal trafficking. Early endosomes are characterized by Rab5 expression; late endosomes are Rab7-positive. \textit{M. tuberculosis} phagosomes are positive for early endosomal markers, such as Rab5 and transferrin receptors (TfR), but they are negative for the late endosomal marker Rab7 (Via et al. 1997). It is still unclear whether this exclusion of Rab7 is directly responsible for blocking phagosome maturation. Without lysosomal interaction, the phagosome does not acidify, and the bacilli remains metabolically active within an early endosome environment (Kelley and Schorey 2003; Kelley and Schorey 2004).
1.3.4 Granuloma formation

In the lung, infected macrophages secrete chemokines that attract lymphocytes, neutrophils, and inactivated monocytes to the site of infection, though none of these cell types are particularly effective at killing the bacteria (Fenton and Vermeulen 1996; van Crevel et al. 2002). Focal lesions, or granulomas, composed of macrophage-derived giant cells and lymphocytes form at sites of infection. The infection is essentially walled off, and the spread of the mycobacteria is, in general, effectively contained. As cellular immunity develops, the bacilli-containing macrophages at the center of the granuloma are killed. This results in the formation of a caseous center that is surrounded and contained by fibroblasts, lymphocytes, and monocytes. It is postulated that *M. tuberculosis* may remain alive and dormant within the caseous tissue for decades. However, the mycobacteria are unable to multiply within this region due to the acidic pH environment, the presence of toxic fatty acids, and the low availability of oxygen. In persons with intact immune systems, the infection may be permanently arrested at this stage. The granulomas subsequently heal, leaving small fibrous and calcified lesions (Smith 2003).

1.3.5 Latency vs. active disease

An *M. tuberculosis* infection that is efficiently enclosed is referred to as latent or persistent TB. As long as the infection is contained, the infection can persist throughout the lifetime of the individual in an asymptomatic and non-transmissible state. The strength of the host’s cellular immune response determines whether the TB infection is arrested at the granuloma or whether the disease progresses to the next stage.
Active infection results if the individual fails to contain the initial infection in the lung or if a latently infected individual’s immune system should become weakened. Factors that may weaken the immune system include the use of immunosuppressive drugs, malnutrition, aging, and HIV co-infection. The breakdown of cell-mediated immunity leads to the liquefaction of the granuloma center, which serves as a rich media in which bacteria are able to undergo uncontrolled replication. The viable *M. tuberculosis* are able to escape from the granuloma and spread within the lungs (active pulmonary TB) and/or migrate in the blood and lymphatic system to other tissues (miliary or disseminated TB). At this stage, the individual becomes infectious and the initiation of antibiotic therapy is required. TB in adults is almost always pulmonary, with varying degrees of lung damage resulting from necrosis, cavitation, and bleeding of pulmonary tissues. Anoxia results from destruction of the parenchymal cells that are involved in oxygen uptake, obstruction of bronchiolar passageways by granulomatous growths, and the release of blood from the liquefied granulomas into the lung tissue. Uncontrolled *M. tuberculosis* proliferation results in extensive pulmonary damage, ultimately leading to death by suffocation from insufficient oxygen (Smith 2003).

1.4 Anti-tuberculosis drug development

1.4.1 Shortcomings of currently prescribed regimens

Treatment of latent tuberculosis infection can effectively reduce the risk of developing active TB. In low-incidence countries, treating latent TB cases with isoniazid for 6-9 months is an essential component of tuberculosis control. The decision to treat latent TB patients involves weighing the likelihood that the patient will develop active
TB against the risk of adverse effects incurred by drug therapy (Smith et al. 2011). As shown in Table 1, first-line antibiotics are associated with numerous, and sometimes severe, side effects.

Treatment of active TB requires the use of multiple antibiotics for a period of at least six months. These first-line drugs include isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB). Because mycobacterial mutations arise spontaneously and can result in drug resistance, single-drug treatment of active TB is prone to failure. The probability that an *M. tuberculosis* strain is resistant to any one of the first-line drugs ranges from $1 \times 10^7$ to $1 \times 10^{10}$. With combination therapy, the probability that a strain is resistant to all four drugs is lowered to $1 \times 10^{33}$. The standard course of treatment involves the combination of all four first-line drugs for two months, followed by a four-month course with only isoniazid and rifampin.

Second-line drugs are used when treatment with first-line drugs is not well tolerated by the patient or if the patient is diagnosed with multi-drug resistant TB. Second-line drugs are more expensive (i.e., unavailable in many developing countries), more toxic, and weaker than first-line drugs (Raviglione and Smith 2007). Examples of second-line antibiotics include the aminoglycosides amikacin, kanamycin, and streptomycin, the closely related peptide drug capreomycin, and the fluoroquinolones (e.g., ciprofloxacin, levofloxacin, ofloxacin, and sparfloxacin) (Riska et al. 2000).

The success of combination therapy requires patients to take the drugs on schedule and to do so for the entire duration of treatment. Given the adverse effects, the involvement of multiple drugs, and the long timeline of treatment, there is a high risk of patient non-compliance to prescribed regimens. Properly implemented treatment of anti-
TB drug therapy has a success rate greater than 95% and limits the emergence of drug-resistant strains. However, as discussed in the next section, resistance to first- and second-line drugs is prevalent in many parts of the world and poses a major challenge to TB control and eradication programs.

Bacillus Calmette-Guerin (BCG) is the only currently available TB vaccine. The live, attenuated vaccine strain was produced by Albert Calmette and Camille Guerin in Paris in the 1920s by sequential passage of a virulent strain of *M. bovis*. Despite its widespread use, the BCG vaccine has not been able to lower TB incidence in recent years. While the BCG vaccine is effective in preventing miliary TB during childhood, its protective effect against pulmonary TB in adults is questionable. Though the vaccine protects against childhood manifestations of TB, the duration of the vaccine’s protective effect is short-lived, lasting only for 10-15 years. As children reach adolescence, the protective effect of the vaccine begins to decline and TB incidence begins to increase. Unfortunately, vaccine efficacy also varies with geographic location, with vaccine efficacy at its lowest levels in regions where TB prevalence is at its highest. The precise cause of this paradoxical relationship is unknown. It has been proposed that the vaccine’s protective effect is masked in areas where the population experiences high exposure to environmental bacteria. Prior contact with environmental mycobacteria may antagonize the immune response following vaccine administration (Andersen 2001).

1.4.2 Multi-drug resistant and extensively drug-resistant tuberculosis

Multi-drug resistant (MDR) TB is characterized by *M. tuberculosis* strains that are resistant to the first-line drugs isoniazid and rifampin. Extensively drug-resistant TB
XDR results when an MDR strain of *M. tuberculosis* also acquires resistance to fluoroquinolones and at least one of the following three injectable drugs: amikacin, capreomycin, and kanamycin. Inadequate treatment of TB selects for drug-resistant strains, allowing for their proliferation and eventual transmission. XDR TB patients receiving inadequate therapies can spread the infection to others, resulting in individuals who acquire a primary infection that is already extensively drug-resistant (Raviglione and Smith 2007).

Genetic analysis has indicated that the DNA sequence of *M. tuberculosis* is highly conserved, suggesting that mutations in the genome are highly correlated with drug resistance. Drug resistance is a result of sequential acquisition of gene mutations, as no plasmids conferring resistance have been identified. Mutations develop spontaneously due to the natural mutation rate of genomic DNA. Taking into consideration available statistical data on the rate of resistance to various anti-TB drugs, it is likely that every infected individual harbors bacilli that are resistant to one of the anti-TB compounds. For this reason, anti-tuberculosis therapy requires a multi-drug approach (Riska et al. 2000).

It is essential that drug-resistant TB be identified rapidly, especially in HIV co-infected patients, in order to increase the likelihood of favorable clinical outcomes and to decrease the transmission of resistant strains. Because resistance mutations accumulate sequentially, inappropriate treatment of MDR and XDR TB could result in the emergence of strains that are completely untreatable. XDR TB poses a serious threat to global public health, and its very existence exposes the weakness of tuberculosis management efforts and the shortage of the tools available for tuberculosis control. In 2011, nearly one-third of new TB cases in eastern Europe and central Asia were multi-drug resistant, and XDR
TB cases were identified in 84 countries (World Health Organization 2012). Improved understanding of the genetic mutations driving drug resistance in *M. tuberculosis* will allow for the design of pharmaceuticals that are both specific and potent in their inhibition of target genes.

1.4.3 The impact of HIV co-infection and re-emergence of TB as a global health threat

The acquired cellular immune response to *M. tuberculosis* is complex. Studies in animal models have highlighted the importance of T helper 1 (Th1) cells and IFN-γ as major effectors of macrophage activation, production of reactive oxygen intermediates, and acidification of the phagosome. Both CD4+ and CD8+ T cells accumulate in the lung during primary TB infection and assist in the containment of the infection. During chronic or latent infection, CD8+ cells mediate their effects via cytokine production and direct cytolytic activity. Human CD8+ cells are able to target and lyse infected macrophages, leading to death of both the host macrophage and the bacteria contained within. In human studies, the *in vitro* T cell response to mycobacterial antigens is predominantly mediated by CD4+ T cells (Ravn et al. 1997). CD4+ cells secrete IFN-γ, which drives macrophage activation and the killing of intracellular mycobacteria. HIV specifically infects and kills T cell populations, resulting in loss of IFN-γ production. Without stimulation from IFN-γ, the activation state of macrophages declines. Because containment of *M. tuberculosis* within the granuloma requires constant activation of macrophages, depletion of CD4+ T cells leads to reactivation of tuberculosis.

Tuberculosis is the most common opportunistic infection among HIV patients, and high HIV prevalence is a predictor of increased vulnerability to tuberculosis.
Statistics from 2011 indicate that 1.1 million, or 13%, of the 8.7 million new cases of TB were in HIV-infected individuals. An estimated 430,000 deaths, representing over 30% of the 1.4 million deaths from TB, were in persons who were HIV-positive (World Health Organization 2012).

Treatment of co-infected patients requires concomitant administration of anti-tuberculosis and antiretroviral drugs. Both therapies involve the use of multiple drugs. The risk of patient non-compliance, already a problem in anti-tuberculosis therapy, increases when therapies are combined and the pill burden multiplies. Adding to the challenges, the risks of adverse drug interactions, toxic effects, and immune reconstitution inflammatory syndrome must be taken into consideration. Questions still remain concerning the timing of antiretroviral therapy (ART) and the duration and scheduling of anti-tuberculosis drugs (Padmapriyadarsini et al. 2011). Furthermore, MDR and XDR TB have major implications for both HIV control and treatment.

1.4.4 Stagnation in the anti-tuberculosis drug pipeline

The lack of new classes of anti-tuberculosis drugs has added to the challenge of treating drug-resistant TB. Because the pipeline has stalled, new treatment regimens will be unavailable for several years. Additionally, the availability of an effective TB vaccine, which would be the most powerful tool for preventing TB and further increases in drug resistance, is not anticipated anytime in the immediate future (Raviglione and Smith 2007). Anti-TB drugs that are currently in clinical trials operate on the same chemical platforms as existing drugs and are unlikely to overcome the pitfall of drug resistance.
Figure 1.1 The discovery timeline of currently available anti-tuberculosis drugs. All first-line (red circles) and second-line (teal circles) drugs were all discovered between the 1940s and the 1970s. For three decades, little work was done on TB drug development. Dotted lines and open circles indicate that these drugs are not the first in their class. (Ginsberg 2009)

1.4.5 Enhancing antibiotic efficacy through folate-based drug targeting

Globally, *M. tuberculosis* is the leading cause of death by a single infectious agent (Andersen 2001; Riska et al. 2000). It has become increasingly clear that existing TB control and strategies have only been partially successful. Given the variable efficacy of the BCG vaccine, the spread of HIV/AIDS, the emergence of MDR and XDR TB, and socio-economic inequality in TB endemic regions, new approaches and innovations in the field of rational drug design are necessary the World Health Organization’s goal of TB eradication is to be achieved. The general consensus in the TB research and clinical communities is that new drugs are needed to 1) reduce the duration of TB treatment from the current 6-9 month regimen and 2) to combat MDR and XDR TB.
One promising approach for the development of targeted TB therapy involves the coupling of drugs or toxins to the vitamin folate. Factors that make folate an attractive ligand for use in drug targeting include its low molecular weight, lack of immunogenicity, solubility in water, and ease of conjugation chemistry (Hilgenbrink and Low 2005). Targeted folate-based applications include targeted immunotherapy with low molecular weight pharmaceuticals and conjugation with radioactive agents for non-invasive imaging.

The membrane-associated α and β isoforms of the folate receptor (FR) bind folate and folate-linked cargos with high affinity. FR-α is overexpressed in malignant tumors (Sudimack and Lee 2000). Activated macrophages, which can cause or exacerbate inflammatory and autoimmune diseases, express high levels of FR-β. Folate conjugates bind to FRs with high affinity and enter the cell via receptor-mediated endocytosis. Eventually the FR and folate conjugate dissociate from each other, with the FR recycling back to the cell surface and the folate conjugate being released into the cellular endosome (Low et al. 2007).

Limited expression of the FR is observed on healthy cells, but the receptor is overexpressed almost exclusively on pathologic cells, such as cancer cells and activated macrophages. This limited distribution allows for targeting of folate-linked drugs specifically to diseased cells, thereby minimizing damage to healthy cells and tissues. These properties of the folate receptor have been exploited to great success in the development of targeted cancer chemotherapeutics. For example, desacetylvinblastine hydrazide failed in Phase I clinical trials due to its extreme toxicity. Coupling the drug to
folate, however, allows for targeted drug delivery and better tolerance (Low and Kularatne 2009).

Because activated macrophages overexpress FR-β and because *M. tuberculosis* infection leads to macrophage activation, folate conjugation of antibiotics could have major implications for targeted anti-TB therapy. Targeting currently used drugs to infected macrophages would reduce the occurrence and severity of adverse side effects associated with systemic drug treatment. Improving drug efficacy could also potentially shorten the duration of treatment. The benefits of folate-targeted anti-TB therapeutics could lead to greater patient compliance and curtail the emergence of drug-resistant *M. tuberculosis* strains.
## TABLE 1

PROPERTIES OF FIRST-LINE ANTI-TUBERCULOSIS DRUGS

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Adverse Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (INH)</td>
<td>Formation of free radicals that inhibit mycolic acid synthesis</td>
<td>-Fever</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Cutaneous pruritis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Epigastric pain</td>
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<tr>
<td></td>
<td></td>
<td>-Clinical hepatitis</td>
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<tr>
<td></td>
<td></td>
<td>-Behavioral changes</td>
</tr>
<tr>
<td>Rifampin (RIF)</td>
<td>Inhibition of mycobacteria gene transcription by blocking DNA-dependent RNA polymerase</td>
<td>-Gastrointestinal reactions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Discoloration of body fluids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Exanthema</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Flu-like symptoms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Hepatotoxicity</td>
</tr>
<tr>
<td>Pyrazinamide (PZA)</td>
<td>Mechanism not fully understood, may inactivate enzymes required for mycolic acid biosynthesis</td>
<td>-Nausea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Hyperuricemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Dermatitis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Hepatotoxicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Kidney failure</td>
</tr>
<tr>
<td>Ethambutol (EMB)</td>
<td>Interferes with biosynthesis of arabinogalactan, interferes with enzymes involved in arabinose polymerization</td>
<td>-Retrobulbar neuritis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Nausea and vomiting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Hyperuricemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Skin rash</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Hematological symptoms</td>
</tr>
</tbody>
</table>

Adverse effects of anti-tuberculosis drugs may necessitate modification or discontinuation of treatment. (Arbex et al. 2010; Riska et al. 2000)
CHAPTER TWO

CHARACTERIZING THE IMPORTANCE OF VARIOUS TRANSCRIPTION FACTORS IN THE ACTIVATION OF THE MURINE TNF-α AND NOS2 PROMOTERS FOLLOWING IN VITRO MYCOBACTERIAL INFECTION

2.1 Introduction

The obligate intracellular pathogen *Mycobacterium tuberculosis* is the causative agent of pulmonary tuberculosis, and it is estimated that one-third of the world’s population is currently infected. Similar to other pathogenic mycobacteria species, *M. tuberculosis* prevents phagosome-lysosome fusion, thereby interfering with the macrophage’s ability to mount a proper immune response and facilitating the growth and survival of the mycobacteria. Current understanding of the mechanism(s) underlying the pathogenicity of *M. tuberculosis* is still limited. Previous studies have shown that macrophage responses differ in terms of tumor necrosis factor alpha (TNFα) and nitric oxide (NO) production following infection with either the opportunistic pathogen *Mycobacterium avium* or non-pathogenic *Mycobacterium smegmatis* (Roach and Schorey 2002). TNF-α and NO play important roles in host defense against intracellular pathogens. The production of TNF-α and NO is regulated by the transcriptional regulator protein complex nuclear factor kappa-light-chain-enhancer of activated B cells
(NF-κB) and the mitogen-activated protein kinases (MAPKs). Previous work has demonstrated that MAPK activation in mycobacteria-infected murine macrophages is inversely correlated with the pathogenicity of the bacteria in the mouse, suggesting that the ability to limit MAPK activation may serve as a mycobacterial virulence factor (Roach and Schorey 2002).

Interestingly, comparison of infections with isogenic strains of *M. tuberculosis* has indicated that strains derived from the same *Mycobacterium* species are also capable of inducing differential macrophage responses. The avirulent (H37Ra) and virulent (H37Rv) strains of *M. tuberculosis* were derived from the virulent H37 strain that was isolated from a human pulmonary tuberculosis patient. Among the reasons for the attenuation of the H37Ra strain is a single nucleotide mutation in the *phoP* gene of the PhoPR two-component system (Lee et al. 2008). Two-component systems (2CS) are highly conserved prokaryotic signal transduction modules (Ryndak et al. 2008). In bacteria, 2CS regulatory proteins function as sensory and adaptive proteins in response to a variety of environmental stimuli. For instance, the PhoP/PhoQ 2CS has been shown to control the transcription of virulence genes that are essential for the survival of intracellular pathogens, such as *Salmonella, Shigella,* and *Yersinia* species, within host cells (Perez et al. 2001).

Previous work has demonstrated a link between PhoP function and the secretion of ESAT-6 and CFP-10. These secreted mycobacterial proteins are immunodominant antigens in a majority of TB cases (Brodin et al. 2004), though it is unclear what effect the secretion of these proteins has on the ability of *M. tuberculosis* to cause human disease or on disease severity (Ryndak et al. 2008). In the pathogenic mycobacterial
species *M. marinum*, ESAT-6 and CFP-10 are necessary for intra-macrophage growth, as well as for the inhibition of phagosome maturation (Xu et al. 2007).

Because of the emergence of multi-drug resistant *M. tuberculosis* strains and the variable efficacy of the currently used BCG vaccine, there is a need for new anti-tubercular therapies. The transcriptional regulator PhoP is a potential target candidate, as it plays a major role in *M. tuberculosis* pathogenicity and its inactivation leads to severely impaired growth in animal models (Gonzalo-Asensio et al. 2008; Ryndak et al. 2008). In preclinical tests, *M. tuberculosis phoP* mutants have shown promise as safe and effective live vaccine candidates. Though the *phoP* mutant demonstrates a similar antigenic capacity as the BCG vaccine, its ability to persist in the host may confer a higher level of protective immunity against tuberculosis in mice (Gonzalo-Asensio et al. 2008; Martin et al. 2006)

As the inactivation of a transcriptional regulator protein has been implicated in the attenuation of the H37Ra strain, we were interested in evaluating how infections with isogenic strains of *M. tuberculosis* differ in their ability to activate the various transcription factors that are recruited to the TNF-α promoter. Significantly higher levels of TNF-α production have been observed in H37Ra-infected macrophages as compared to H37Rv-infected macrophages (Yadav and Schorey 2006). TNF-α is an essential cytokine that is involved in the containment of mycobacterial infections. Transcriptional activation of TNF-α is a highly regulated process that requires the recruitment of several nuclear transcription factors and co-activator proteins. The TNF-α promoter contains binding sites for numerous transcription factors, including Ets/Elk, Egr, and ATF-2 (Fig. 2.1). The region between the start site to 200 nucleotides upstream is highly conserved
between mice and humans (Kuprash et al. 1999). Within this region are four Ets/Elk sites, which are located at -76, -84, -117, and -180. The Egr binding site is located between the -117 and -180 Ets/Elk sites. The ATF-2 site is located within the CRE (cyclic adenosine monophosphate (cAMP) response element) site, which has been shown to be essential for TNF-α promoter activation in response to several stimuli in a variety of cell types (Roach et al. 2005). Because TNF-α promoter activity involves specific and coordinated interactions between transcription factors and co-activator proteins (Falvo et al. 2000), mutation of binding sites within the TNF-α promoter are hypothesized to likely disrupt promoter activity.

Following infection with intracellular pathogens, macrophages become activated and produce large quantities of nitric oxide, a potent host defense molecule capable of killing *Mycobacterium*. The production of nitric oxide is dependent upon the activation of the inducible nitric oxide synthase (iNOS), also known as nitric oxide synthase 2 (NOS2) (Chan et al. 2001). The NOS2 promoter (NOS2 -1700) contains numerous transcription factor binding sites that cluster in two regions: Region I encompasses -300 to +10, and Region II is located between -1100 and -800. There are two NF-κB binding sites within the NOS2 promoter, one in each region. The Region I NF-κB binding site is located at -80; the Region II binding site is located at -971 (Chan et al. 2001; Lee and Schorey 2005). The NF-κB transcription factor family is an evolutionarily conserved group that functions in response to infection or stress. Previous studies have shown that the transcriptional regulator NF-κB is essential for induction of the NOS2 promoter and that the association of NF-κB with other transcription factors leads to maximal activation of gene expression (Lee and Schorey 2005). Therefore, we were interested in examining
whether NF-κB is differentially activated by *M. smegmatis* and *M. tuberculosis* infections. Mutational analysis demonstrated the functional importance of the NF-κB binding site for NOS2 promoter activity.

Differential macrophages responses are observed following infection with different members of the genus *Mycobacterium*. These differential responses may be due to differences in macrophage immune responses at the transcriptional level. Macrophages were transfected with reporter vectors for various transcription factors and infected with either virulent *M. tuberculosis* H37Rv, attenuated *M. tuberculosis* H37Ra, or the non-pathogenic soil microbe *M. smegmatis*. Differential responses were observed for both the TNF-α and NOS2 promoters, as well as for the various transcription factors under investigation, indicating that pathogenic *Mycobacterium* are able to modulate the host immune response at the transcriptional level.

2.2 Materials and Methods

2.2.1 Cell culture and stable transfection

The murine macrophage cell line RAW 264.7 was grown in macrophage culture media composed of Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 20 mM HEPES, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS) (all supplements from HyClone). Selection media was supplemented with the antibiotic HyQ G418 Sulfate (HyClone) at 800 μg/mL. Maintenance media contained 400 μg/mL of G418 Sulfate.

Reporter vectors containing specific promoters or multiple repeats of a transcription factor binding element were previously cloned into a Basic-pGL3-luc vector.
and transferred into competent DH5α cells (Geister 2008). To generate stable cell lines in RAW 264.7 cells, glycerol stocks of transformed cells containing the plasmid of interest were streaked onto LB-Amp plates, and a single colony was selected after overnight incubation at 37°C. The single colony was inoculated into 60 mL of LB-Amp and grown overnight at 37°C with constant agitation. Plasmids were isolated using Promega’s PureYield Plasmid Midiprep System. DNA concentration, 260/230 and 260/280 ratios were determined using a Nanodrop2000. DNA from all midipreps was diluted to 0.4 μg/μL to standardize the transfection process. RAW 264.7 cells were plated at 3 x 10⁵ cells per well in 6 well plates. Transfections were performed using FuGene6 (Roche) in accordance with the manufacturer’s protocol. FuGene6 was mixed with DNA prior to the addition of DNA. Plasmid DNA was added in varying amounts for FuGene6: DNA ratios of 3:1, 3:2, and 6:1. The mixture (100 μL total volume) was added to the cells in a drop-wise fashion and with gentle swirling for even distribution. Cells were incubated for 24 hours in a 37°C/5% CO₂ incubator before the media was replaced with fresh macrophage culture media. 48 to 72 hours later, the cells were rinsed with PBS and dislodged following incubation with PBS/10 mM EDTA. Cells were centrifuged and resuspended in 20 mL of selection media and transferred to large tissue culture dishes (150 x 20 mm). After approximately 3 weeks, individual colonies were isolated and transferred to 6 well plates containing selection media. Once confluent, the clones were plated at 3 x 10⁵ and treated with E. coli-derived lipopolysaccharide (LPS) at 200 ng/mL for 2 hours. Transcription factor activity was measured by luciferase assay, with untreated and LPS-treated non-transfected RAW cells both serving as negative controls. The clone(s) which exhibited the greatest differential in luciferase intensity as
compared to controls were selected for further use. The selected clones were considered stable and were grown in maintenance media for subsequent experiments.

2.2.2 Mycobacterium strains and growth conditions

M. smegmatis strain MC²155 and the M. tuberculosis strains H37Ra and H37Rv were grown on Middlebrooks 7H10 agar plates supplemented with oleic acid, albumin, D-glucose, and NaCl (OADC) and Tween-20. After 72 hours, a single colony of M. smegmatis was used to inoculate Middlebrooks 7H9 broth supplemented with OADC and Tween-20 and grown with vigorous shaking at 37°C for 3 to 10 days. The culture was centrifuged and the pellet was resuspended in 7H9/OADC with 15% glycerol, aliquoted, and stored at -80°C.

Single colonies of the M. tuberculosis strains were selected for inoculation after 3 weeks of growth on agar plates. The M. tuberculosis strains were grown in 7H9/OADC at 37°C with constant agitation. At log phase, approximately 3 weeks post-inoculation, the cultures were centrifuged and resuspended in 7H9 containing 40% glycerol and 0.05% Tween-80. The aliquots were frozen at -20°C.

2.2.3 Complement opsonization and in vitro infection

Uptake assays were performed to determine the volume of mycobacteria needed to obtain a macrophage infection ratio of approximately 80%. RAW 264.7 cells were seeded on glass coverslips (Fisher Scientific) and allowed to adhere overnight. Mycobacteria stocks were thawed and centrifuged. The pellet was then resuspended in macrophages culture media containing 10% normal horse serum (GIBCO) as a source of
complement proteins. The bacteria were incubated in a 37°C water bath for at least 2 hours before infection. Bacteria were added to the RAW cells in varying doses. Each dose was performed in triplicate. After 4 hours, macrophages were fixed with 2% paraformaldehyde (PFA), rinsed with PBS, and stained with either acridine orange (Sigma-Aldrich) or TB Auramine M (BD Bioscience) for *M. smegmatis* and *M. tuberculosis*, respectively. Slides were visualized using fluorescent microscopy. Percent phagocytosis was determined by dividing the number of infected cells by the total number of cells counted. At least ten random fields were counted per coverslip and at least 200 total cells were counted per treatment condition.

2.2.4 Luciferase assays

Luciferase assays were performed according to the manufacturer’s protocol (Promega Luciferase Assay System E1500). Briefly, cells were lysed in 1X passive lysis buffer and vortexed prior to plating in opaque multiwall plates. An LMax II 384 microplate luminometer (Molecular Devices) was used to measure light intensity following the addition of 100 μL of Luciferase Assay Reagent to each well.

2.2.5 Statistical analysis

Data were analyzed using the paired Student’s *t* test and one-way analysis of variance. Statistical analysis was performed for technical replicates as well as between experimental data sets. Differences were considered statistically significant for *p* values < 0.05.
2.3 Results

2.3.1 Differential TNF-α promoter activity following mycobacterial infections

A TNF-α promoter construct was stably transfected into RAW 264.7 cells. Following infection with either *M. smegmatis* or isogenic strains of *M. tuberculosis*, the macrophages were lysed and TNF-α promoter activity was measured by luciferase assay. Mycobacterial infection led to increased TNF-α promoter activity for all strains tested. However, cells infected with non-pathogenic *M. smegmatis* exhibited the highest level of TNF-α promoter activity. Infection with isogenic strains of *M. tuberculosis* demonstrated that the strains differentially activate the TNF-α promoter, with *M. tuberculosis* H37Ra infection resulting in elevated, though not statistically significant, TNF-α promoter activity as compared to *M. tuberculosis* H37Rv infection (Fig. 2.2).

2.3.2 *Mycobacterium* infection results in differential NF-κB activity

Macrophages stably transfected with the NF-κB reporter vector were infected with mycobacteria for 4 hours. NF-κB activity, measured in luciferase units, was drastically elevated following mycobacterial infection. Similar to the trend seen with RAW cells transfected with the TNF-α promoter construct, *M. smegmatis* infection led to the highest levels of NF-κB activity. *M. tuberculosis* H37Rv infection led to the lowest levels of NF-κB activity, and infection with *M. tuberculosis* H37Ra produced an intermediate level of NF-κB activity (Fig 2.3).
2.3.3 Ets/Elk is differentially activated between *M. smegmatis* and *M. tuberculosis*-infected macrophages

Macrophages from a stable cell line expressing the Ets/Elk reporter vector were infected with mycobacteria, and Ets/Elk activation was evaluated by measuring Ets/Elk-driven luciferase activity at 4 hours post-infection. As seen in Figure 2.4, Ets/Elk is differentially activated following infection with different species of *Mycobacterium*. Infection with non-pathogenic *M. smegmatis* led to the highest levels of Ets/Elk activation. In contrast, infection with the pathogenic H37Rv strain of *M. tuberculosis* led to the lowest levels of Ets/Elk activation. This diminished Ets/Elk activation would infer low levels of TNF-α production, which would be advantageous to the pathogen and detrimental to the host. Infections with isogenic strains of *M. tuberculosis* did not result in statistically significant differences in the activation of Ets/Elk.

There are four Ets/Elk binding sites within the TNF-α promoter. Previous work by Geister (2008) demonstrated that mutation of the -84, -117, or -180 binding sites leads to limited decreases in TNF-α promoter activity following mycobacterial infection. However, the TNF-α promoter activity remains above levels observed in uninfected macrophages for all three mutants. Additionally, the pattern of TNF-α promoter activity (*M. smegmatis* > H37Ra > H37Rv) which had been previously observed is maintained across all three mutants (unpublished data). Site-directed mutagenesis of the -76 site, on the other hand, resulted in knockdown of TNF-α promoter activity to levels below those seen in uninfected cells transfected with the wild-type TNF-α promoter construct. Even infection with LPS, a very potent stimulator of TNF-α production, showed less than a two-fold increase in TNF-α promoter activity compared to untreated cells (Fig. 2.5).
2.3.4  Egr activity is higher following infection with pathogenic mycobacteria

Macrophages stably transfected with the Egr reporter vector were infected with mycobacteria or LPS. While infection and LPS treatment lead to elevated Egr activity, the fold changes were not as pronounced as those observed with the Ets/Elk or the NF-κB reporter vectors. LPS-treated macrophages had the greatest Egr activity. In contrast to the previously mentioned transcription factors, Egr activity is elevated following *M. tuberculosis* infection compared to *M. smegmatis* infection. Though infection with *M. tuberculosis* H37Rv resulted in the highest level of Egr activity for the three *Mycobacterium* species tested, differences in Egr activity were not statistically significant between the isogenic *M. tuberculosis* strains. However, differences in Egr activity were statistically significant between *M. tuberculosis* H37Rv and *M. smegmatis* infections (Fig. 2.6), which suggests that virulent infections may promote Egr activity. In comparison to other transcription factors of the TNF-α promoter, increases in Egr activity are modest following all treatments.

2.3.5  ATF-2 activity is significantly reduced in *M. tuberculosis* H37Rv-infected cells

Treatment with LPS, a potent inducer of TNF-α production, elicited the highest levels of ATF-2 activity. The transcription factor ATF-2 exhibited higher activity levels in macrophages that had been infected with avirulent *Mycobacterium* species (Fig. 2.7). Infection with *M. tuberculosis* H37Ra generated levels of ATF-2 activity that were similar to the levels observed in *M. smegmatis*-infected macrophages. Macrophages infected with virulent *M. tuberculosis* H37Rv exhibited significantly lower levels of
ATF-2 activity as compared to macrophages infected with either *M. smegmatis* or *M. tuberculosis* H37Ra. These data demonstrate that infection with isogenic strains of *M. tuberculosis* results in differential activation of ATF-2. The ability of the *M. tuberculosis* H37Rv strain to suppress ATF-2 activation may illustrate a potential mechanism of virulence.

2.3.6 Deletion of Region II results in loss of NOS2 promoter activity

The NOS2 promoter contains two NF-κB binding sites, located at -80 and -971 (Figure 2.8A). As depicted in Figure 2.8B, promoter constructs used to measure NOS2 promoter activity in RAW 264.7 cells included the full length or wild type promoter (NOS2 -1700), a construct containing a mutation at the -971 NF-κB binding site (NOS2 -971 mt), and a truncated construct lacking Region II (NOS2 -390). As illustrated in Figure 2.9, mutation of the -971 binding site led to only a slight decrease in the amount of NOS2 promoter activity following mycobacteria infection. This difference was not significant compared to promoter activity levels observed in cells transfected with the full length NOS2 promoter. NOS2 promoter activity was elevated following infection with the three strains, but activity levels were comparable between infections. This trend applied to macrophages transfected with either the NOS2 -1700 or NOS2 -971 mt constructs. In contrast, knocking out Region II eliminated NOS2 promoter activity, demonstrating the essential nature of Region II for maximal induction of NOS2 promoter activity in the context of mycobacterial infections.
2.4 Discussion

Isogenic strains of *M. tuberculosis* elicit differential responses in macrophages at the transcriptional level. These differences may contribute to the differential disease outcomes observed in mice following infection with either the H37Ra or H37Rv strain. The ability of the H37Rv strain to suppress the activity of various transcription factors, resulting in decreases in TNF-α and NOS2 promoter activities, may contribute to the strain’s virulence and provide insights into the mechanisms underlying the ability of *M. tuberculosis* to modulate host immune response to the advantage of the pathogen.

Macrophage production of TNF-α and NO following infections with pathogenic and nonpathogenic mycobacteria is regulated at the transcriptional level. These differential responses can be attributed to the increased activation of the Ets/Elk and NF-κB transcription factors following an *M. smegmatis* compared to an *M. tuberculosis* infection.

From these studies, we observed that transcription factors within the TNF-α promoter are differentially activated following mycobacterial infection. Of the 4 Ets/Elk binding sites, the -76 site appears to be essential for maximal induction of TNF-α promoter activity. Previous work had indicated that mutation of the other Ets/Elk sites led to a decrease in TNF-α promoter activity following infection, but the data with the mutated -76 Ets/Elk suggests that this is the most critical Ets/Elk site in the TNF promoter. The differential pattern of activation observed for the transcription factors NF-κB and Ets/Elk was also observed for the TNF-α promoter.

The differential pattern of activation observed for the transcription factors Egr and ATF-2, however, did not follow the same trend. In the case of Egr, infection with *M.*
*tuberculosis* H37Rv led to significantly higher Egr activity than infection with *M. smegmatis*. While this might suggest that the activation of Egr would be detrimental to pathogenic mycobacteria, the increase in transcription factor activity observed for Egr did not lead to an increase in the promoter activity of TNF-α.

The transcription factor ATF-2 exhibited the lowest activity following infection with *M. tuberculosi*sis H37Rv, similar to the trends seen with NF-κB and Ets/Elk. Infection with the H37Ra strain and *M. smegmatis* led to similar levels of ATF-2 activity. However, differential levels of ATF-2 activity were observed following infection with isogenic *M. tuberculosi*sis strains. This downgrade in ATF-2 activity may indicate a potential mechanism of virulence for the H37Rv strain. As ATF-2 is only one of three transcription factors that bind within the CRE region, it would be interesting to study the activities of CREB and c-jun in the context of a mycobacterial infection. Previous work in our lab demonstrated that macrophages transfected with a TNF-α promoter containing a CRE mutation had decreased TNF-α promoter activity compared to cells containing the wild-type promoter. Infection with *M. smegmatis, M. tuberculosi*sis H37Ra, and *M. tuberculosi*sis H37Rv resulted in similar levels of TNF-α promoter activity in cells containing the CRE mutant promoter construct (Geister 2008).

Studies with TNF-α and NOS2 promoters have demonstrated the essential nature of the -76 Ets/Elk site and Region II, respectively. The mutation of the -971 NF-κB site in Region II led to an insignificant decrease in NOS2 activity following mycobacterial infection, suggesting that the presence of one intact NF-κB site may be sufficient for the induction of NF-κB activity. Elimination of Region II, as demonstrated by the truncated promoter construct, results in drastic knockdown of NOS2 activity. This suggests that
the other transcription factor binding sites located Region II are involved in enhancing NOS2 promoter activity. Future studies involving the NOS2 promoter may involve mutating the Region I NF-κB site and the generation of a truncated NOS2 promoter that lacks Region I. The generation of these two promoter constructs would elucidate whether there is any redundancy between the two NF-κB sites. It would also shed light on whether interactions between particular transcription factors are responsible for the increased induction of NOS2 activity following infectious challenge. In a similar vein, it would be interesting to examine how the two SP1 binding sites within the TNF-α promoter interact with one another to drive TNF-α activity. In particular, it would be interesting to determine whether the mutation or absence of one of the two SP1 sites leads to the elimination of TNF-α activity or if there is any redundancy between the two binding sites.
Figure 2.1  The TNF-α promoter of *Mus musculus* contains binding sites for several transcription factors. Four Ets/Elk sites are located within this region. The binding site for transcription factor Egr is situated between the -180 and -117 Ets/Elk sites. The binding site for ATF-2 is located within the CRE site.
Figure 2.2  RAW 264.7 cells infected with H37Rv show decreased production of TNF-α and a corresponding decrease in promoter activity compared to cells infected with H37Ra or M. smegmatis. RAW 264.7 cells, transfected with -1200 TNF-α-pGL3-luc were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results are representative of 2 experiments. a, p < 0.05 compared to RC; b, p < 0.05 compared to M. smegmatis; RC, uninfected RAW 264.7 cells.
Figure 2.3  RAW 264.7 cells stably transfected with the NF-κB reporter vector were infected with mycobacteria for 4 hours, after which cell lysates were assayed for luciferase activity. Results are an average of 4 experiments.  a, p < 0.01 compared to RC; b, p < 0.05 compared to *M. smegmatis*. 
Figure 2.4  RAW 264.7 cells stably transfected with the Ets/Elk reporter vector were infected with mycobacteria. Four hours post-infection, cell lysates were assayed for luciferase activity. Results are an average of 4 experiments. a, p < 0.01 compared to RC; b, p < 0.01 compared to H37Ra; c, p < 0.01 compared to M. smegmatis.
Figure 2.5 Defining the importance of the -76 Ets/Elk binding site within the TNF-α promoter for promoter activity following mycobacterial infection. RAW 264.7 cells stably transfected with either wild-type (TNF) or -76 mutant TNF-α-pGL3-luc were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results are an average of 4 experiments. a, p < 0.05 compared to the wild-type promoter (-1200 TNF-α-pGL3-luc).
Figure 2.6  Infection with pathogenic *M. tuberculosis* H37Rv leads to greater Egr activity than infection with non-pathogenic *M. smegmatis*. RAW 264.7 cells transfected with the Egr reporter vector were infected with H37Ra, H37Rv, *M. smegmatis*, or lipopolysaccharide (LPS). Four hours after infection, Egr activity was measured by luciferase assay. Results are an average of 3 experiments. a, p < 0.05 compared to RC; b, p < 0.05 compared to H37Ra; c, p < 0.05 compared to *M. smegmatis*; d, p < 0.05 compared to LPS.
Figure 2.7 Higher levels of ATF-2 activity are observed following infection with non-pathogenic mycobacteria. RAW 264.7 cells transfected with the ATF-2 reporter vector were infected with H37Ra, H37Rv, *M. smegmatis*, or LPS. Four hours after infection, ATF-2 activity was measured by luciferase assay. Results are an average of 3 experiments. a, p < 0.01 compared to RC; b, p < 0.05 compared to H37Ra; c, p < 0.05 compared to *M. smegmatis*; d, p < 0.05 compared to LPS.
Figure 2.8  Topography of the murine NOS2 promoter (A) and the various NOS2 promoter constructs (B) (Lee and Schorey 2005). The NOS2 promoter contains numerous nuclear transcription factor binding sites that are arranged in two distinct clusters: Region I (-300 to +10) and Region II (-1100 to -800). Promoter constructs used to measure NOS2 activity following mycobacterial infection included the WT NOS2 promoter (NOS -1700), the truncated 390 bp promoter lacking Region II (NOS2 -390), and the NOS2 -971 mt construct, which contains a GGG to CTC mutation at the -971 NF-κB site.
Figure 2.9  Region II of the NOS2 promoter is essential for the maximal induction of NOS2 promoter activity. RAW 264.7 cells transfected with wild-type promoter, -971 NF-κB mt-pGL3-luc, or -390 NOS2-pGL3-luc were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results are an average of 3 experiments. a, p < 0.01 compared to the wild-type promoter (-1700 NOS2-pGL3-luc).
3.1 Introduction

*M. tuberculosis* is an intracellular pathogen that subverts the normal phagocytic process by blocking phagosome-lysosome fusion. By residing within infected macrophages, the bacterium is sequestered within the host cell, making the targeting of drugs to the pathogen a difficult task. There are currently no existing mechanisms to effectively target drugs for maximal exposure to the bacteria. The effectiveness of existing anti-TB drugs, as well as the development of future drugs, would benefit from mechanisms that target them specifically to infected macrophages.

Folate, or folic acid, is utilized in the biosynthesis of nucleotide bases and is therefore required for DNA replication in actively dividing cells (Hilgenbrink and Low 2005). In non-pathologic cells, folate uptake is carried out using a proton coupled folate transporter or the reduced folate carrier (RFC), which is present on virtually all cells in the body. Though the RFC displays micromolar affinity for folate, it is selective in facilitating the transport of folate in its reduced forms. The folate receptor (FR), on the other hand, is anchored to the cellular membrane by the glycosylphosphatidylinositol
(GPI) anchor and binds folate with nanomolar affinity. Whereas the RFC is selective in its transport of folate, the FR is able to transport many types of folate-conjugated cargo (Figure 3.1). Folate-conjugated compounds bind to FRs at the cell surface and are transported into the cell via receptor-mediated endocytosis (Antony 1992). The FR exhibits limited tissue distribution, being selectively expressed on pathologic cells, namely tumor cells and activated macrophages. These properties make the FR an attractive candidate in selective drug targeting. Because folate-conjugates are not considered substrates and because the RFC is selective in its transport, folate-conjugates are only taken up by cells expressing a functional FR. Folate conjugation can be utilized to lower the risk of undesirable side effects, which are a common consequence associated with non-targeted drug therapy. With the addition of a folate linker, non-specific drugs can be selectively delivered to pathologic cells without causing harm to normal, FR-lacking tissues.

The alpha isoform of the folate receptor (FR-α) is overexpressed on 40% of human cancers (Low and Kularatne 2009). It is often used as a diagnostic marker of various cancerous cell types, such as ovarian cancer and brain tumors, making it a potential anti-cancer target (Sabharanjak and Mayor 2004). Of note, metastatic cancers express significantly more FRs than localized cancers. In fact, FR expression can be used to characterize the grade and histological stage of tumors (Toffoli et al. 1997). The staining intensity of FR expression in the primary tumor mass also correlates strongly with poor prognosis for recovery (Hartmann et al. 2007). Expression of the FR on tumor cells may enable them to compete more aggressively for folate, which is normally found in low concentrations in human serum and extracellular fluids (Salazar and Ratnam...
2007). Folate-targeted drugs for cancer therapy are currently in Phase II human clinical trials where they have been shown to affect only cancerous cells with very little to no toxicity to nearby healthy cells.

The beta isoform of the folate receptor (FR-β) is expressed on activated macrophages. Macrophages are an important component of the innate immune system and play an essential role in controlling most types of infections. Macrophages become activated upon exposure to pathogenic components or immune factors, such as cytokines. Unregulated, chronic activation can contribute to the development of autoimmune and inflammatory diseases. Activated macrophages release chemokines, cytokines, digestive enzymes, and reactive oxygen species that alert the body to the presence of a pathogen and recruit immune cells to the site of infection. However, if the release of these secreted factors is not regulated, they can cause damage to normal tissues and contribute to pathology. For example, rheumatoid arthritis (RA) is an autoimmune disease characterized by the accumulation of activated macrophages in the synovial fluid, leading to inflammation in the joints. Macrophages isolated from the synovial fluid of RA patients are activated and positive for folate receptor expression (Xia et al. 2009). Studies looking at the use of folate-conjugated therapeutic agents to target and eliminate the disease-causing macrophages are ongoing.

The limited expression of the folate receptor to primarily two cell types (e.g., tumor cells and activated macrophages) is of particular interest in the study of tuberculosis. As was previously mentioned, *M. tuberculosis* is phagocytosed by and resides within macrophages. If *M. tuberculosis*-infected macrophages express the FR, developing new drugs that utilize mechanisms to target infected macrophages could have
numerous benefits, including: 1) the potential to lower current drug dosages, leading to the lower possibility of adverse side effects, 2) the potential to shorten the duration of current drug treatment schedules, and 3) the possibility to use anti-tuberculosis drugs that had previously been shelved because they demonstrated host toxicity at high doses.

Building on the previous success of folate-coupled drugs for the targeted treatment of various cancers and inflammatory diseases, we hypothesize that targeting the FR on activated macrophages is an effective approach to gain access to pathogens that reside within these cells, and therefore presents a novel approach for the treatment of tuberculosis. It is predicted that macrophages containing *M. tuberculosis* will be activated to express the FR and that drugs with anti-TB activity will demonstrate increased efficacy when coupled to folate.

3.2 Materials and Methods

3.2.1 Cell culture and reagents

Folate receptor expression was examined using various compounds. The rabbit polyclonal anti-FR antibody (Santa Cruz Biotech.) and the goat anti-rabbit FITC secondary (Jackson Immuno.) were used in the microscopy and flow cytometry studies. The Folate-Oregon Green conjugate and the m909 monoclonal antibody were generously provided by Dr. Philip S. Low. HeLa cells were generously provided by Dr. Crislyn D’Souza-Schorey. Goat anti-human IgG-FITC (Santa Cruz Biotech.) was used as the secondary antibody in microscopy studies using the monoclonal m909 anti-FR-β antibody.
3.2.2 Isolation and cultivation of bone marrow-derived macrophages

Primary monocytes were isolated from the femurs of 6-8 week old female C57BL/6 mice and grown in macrophage culture media composed of Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 20 mM HEPES, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (all supplements from HyClone). The macrophage media was further supplemented with 1X Penicillin-Streptomycin solution (Cellgro) and 20% L-cell supernatant (LCS) as a source of macrophage colony stimulating factor (M-CSF). After one week, the adherent macrophages were frozen in the presence of 10% endotoxin-tested dimethyl sulfoxide (DMSO; Sigma). Thawed macrophages were cultured in non-tissue culture plastic 100 mm Petri dishes. Prior to infection, bone marrow-derived macrophages (BMDM) were plated at the needed concentration and allowed to adhere overnight. Infections were performed using macrophage culture media. After 4 hours, cells were rinsed with 1X PBS, and cells were incubated with fresh macrophage media for the duration of the experimental time point.

3.2.3 Culture and infection of human THP-1 cells

THP-1 cells were grown in complete RPMI media containing 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 1X Penicillin-Streptomycin. Cells were differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (PMA) for 48 hours. The PMA was removed, fresh media was added, and the cells remained at resting state for an additional 24 hours (at most) prior to Mycobacterium infection. THP-1 cells were rinsed with binding media (Dulbecco’s Phosphate Buffered Saline (Ca++ and Mg++) and 5.5 mM D-glucose) and
incubated in binding media containing human serum for 15 minutes in order to allow the cells to acclimatize. Human serum-conditioned mycobacteria were added to the macrophages. After 4 hours, the cells were rinsed with binding media and incubated with complete RPMI media until the designated time point.

3.2.4 *M. bovis* BCG growth conditions and fluorescent labeling

Single colonies of the *M. bovis* BCG Paris strain (ATCC) were selected for inoculation after 3 weeks of growth on Middlebrooks 7H10 agar plates. The cultures were grown in 7H9/OADC at 37°C with constant agitation. At log phase, approximately 3 weeks post-inoculation, the cultures were centrifuged and resuspended in 7H9/OADC with 15% glycerol, aliquoted, and stored at -80°C.

Fresh cultures of BCG were grown in order to prepare rhodamine-labeled stocks. Briefly, the culture was pelleted and washed with Hanks Balanced Salt Solution (HBSS; Cellgro)/1% bovine serum albumin (BSA; Sigma). Following the wash, the bacterial pellet was resuspended in boric acid buffer. Rhodamine powder was dissolved in DMSO at 1.5 mg/mL and added to the resuspended bacteria. The BCG was incubated with the dye solution for 2 hours in a 37°C water bath. The bacteria were pelleted and washed 3 times with HBSS/1% BSA. The final resuspension was performed with Middlebrooks 7H9 supplemented with 10% OADC and 15% glycerol. The rhodamine-labeled BCG was aliquoted and frozen at -80°C.

In microscopy studies involving non-rhodamine-labeled *M. bovis* BCG, the mycobacteria were localized by staining with an anti-LAM primary antibody (ATCC). A
Texas Red-conjugated goat anti-mouse IgG (Jackson Immuno.) was used as the secondary antibody for LAM.

3.2.5 Immunofluorescence microscopy studies

Cells were plated on flame-sterilized glass coverslips in 24 well plates 16-24 hours prior to infection. At the indicated time points, cells were fixed with 2% PFA (Fisher) in PBS for 1 hour at 37°C. Fixed cells were incubated with blocking solution (0.1 M NH₄Cl, 0.2% gelatin, 0.05% Triton X-100 in 1X PBS) and rinsed with IF wash buffer (0.2% gelatin, 0.1% sodium azide in 1X PBS). Antibodies were prepared in incubation media (IF wash buffer supplemented with goat serum). Coverslips were mounted onto glass microscope slides with mounting medium (buffered glycerol-propylgallate) and sealed. Slides were imaged using a Zeiss Apotome fluorescent microscope.

When indicated, cells were co-incubated with the fluorescently-tagged secondary antibody and the nuclear stain DAPI. In microscopy studies involving membrane-associated protein expression, Triton X-100 was excluded from the blocking solution. Wheat germ agglutinin (WGA)-Alexa Fluor 647 conjugate (Invitrogen) was used to label the plasma membrane.

3.2.6 Flow cytometric analysis

Samples were prepared at 1 x 10⁶ cells per well in polystyrene, round bottom, 96 well plates (Greiner Bio-One). Washes were performed with FACS wash buffer (1% BSA in filtered 1X PBS). Wells were blocked with FACS Buffer (1% BSA + 10%
normal mouse serum in filtered 1X PBS) and all antibody dilutions were prepared in FACS Buffer. Samples were thoroughly resuspended in a final volume of 200 μL of FACS buffer and analyzed using a Beckman Coulter FC-500 flow cytometer immediately following staining. In all cases, an isotype control antibody (Santa Cruz Biotech.) for the polyclonal anti-FR antibody and staining with secondary antibody alone were included. Cytomtics CXP Analysis software was used for data acquisition and analysis. Each experimental group was analyzed in triplicate, and representative data from at least three experiments are shown.

3.2.7 Preparation of bacterial lysates

1 mL of *M. bovis* BCG glycerol stock was thawed and the bacterial pellet was resuspended in 0.5 mL of 1X PBS. 1X Phenylmethylsulfonyl fluoride (PMSF) was added and the mixture was transferred to a bead-beating tube. 200 μL of beads were added and the tube was placed in a Mini-Beadbeater (BioSpec Products) for 2 x 30 second cycles at maximum shaking speed. Following centrifugation at 13,000 rpm for 5 minutes, the supernatant was isolated for protein concentration using the Pierce BCA Protein Assay Kit microplate procedure (Thermo Scientific).

3.2.8 Western blotting

Macrophages were lysed by treating the cells for 10 minutes with lysis buffer (50 mM Tris (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, 10% glycerol, and mammalian protease inhibitor cocktail (1:100 dilution; Sigma)). Protein concentration of whole cell lysates was determined using the
Pierce BCA Protein Assay Kit microplate procedure (Thermo Scientific). Equal concentrations of samples were loaded on a 10% SDS-PAGE gel, electrophoresed, and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked overnight at 4°C in 5% milk solution and probed for FR expression with the rabbit polyclonal anti-FR antibody, followed by incubation with goat anti-rabbit HRP (Pierce). Protein expression was measured by chemiluminescence using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

3.2.9 In vivo infection procedures

6- to 8-week old mice were intraperitoneally injected with sterile saline, 4% thioglycolate (provided by the Bohlson lab), or 10⁷ CFUs of rhodamine-labeled M. bovis BCG resuspended in 1X PBS/0.05% Tween-80. All injections were prepared in a final volume of 1 mL per mouse. At the designated times, macrophages were isolated by peritoneal lavage with ice-cold HBSS. Following centrifugation, the pellet was resuspended in ACK buffer to eliminate erythrocytes. Prior to analysis by flow cytometry, peritoneal cells were assessed for viability, counted, and plated in complete RPMI media overnight in non-tissue culture dishes. The following day, only the adherent cells (i.e., macrophages) were collected for subsequent analysis.

For in vivo quantitative real-time PCR (qRT-PCR) experiments, systemic infection was carried out in 6- to 8-week old mice by retro-orbital injection with either 50 μL of 1X PBS or 10⁷ CFUs of BCG in 50 μL of 1X PBS/0.05% Tween-80. At 2 and 5 days post-injection, spleens and lungs were excised, submerged in RNALater RNA Stabilization Reagent (Qiagen), and stored at 4°C.
3.2.10 Expression of FR-β by quantitative real time PCR

Total RNA was purified from animal tissues using the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen). RNA quantification and sample purity were determined using a NanoDrop2000. Reverse transcription and cDNA synthesis were performed using qScript cDNA SuperMix (Quanta Biosciences), and an equal concentration of each sample was loaded for amplification by PCR with GoTaq Green Master Mix (Promega). PCR products were loaded were loaded and run on a 2% agarose gel containing ethidium bromide, and visualized under UV light. For analysis of FR-β and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression, PCR reactions were set up utilizing PerfeCTa SYBR Green SuperMix, Low ROX (Quanta Biosciences) and detected with an ABI Prism 7900HT real-time PCR system (Applied Biosystems by Life Technologies). Custom primers for murine GAPDH and the beta isoform of the folate receptor were ordered from Invitrogen (Table 2).

Assay efficiency was validated by amplifying a dilution series for each sample in real-time and examining the resulting slope of the regression line. PCR efficiencies for all reactions ranged between 90 and 110%. Relative mRNA content was quantified using the comparative C_t (ddC_t) method.

3.3 Results
3.3.1 *In vitro* Mycobacterium infection leads to increased anti-FR binding in bone marrow-derived macrophages

Primary macrophages from C57BL/6 mice were infected with either the non-pathogenic saprophytic *M. smegmatis* or with the attenuated vaccine strain, *M. bovis*
BCG. Uninfected cells were run in parallel as a negative control. After 48 hours, the macrophages were stained with the polyclonal anti-folate receptor antibody, followed by staining with a FITC-conjugated secondary antibody. Whereas uninfected cells exhibited little to no green fluorescent signal, infected cells displayed bright, punctuate FITC labeling (data not shown). This suggested that the expression of the FR can be induced following mycobacteria infection. Subsequent studies utilized *Mycobacterium bovis* BCG as a model for tuberculosis infection. Though *M. smegmatis* is commonly used in laboratory settings, it is a non-pathogenic strain with little relevance to human health. *M. bovis* BCG is a close relative of *M. tuberculosis* and has frequently been used *in vitro* and *in vivo* to evaluate the efficacy of anti-TB drugs.

3.3.2 Anti-FR binding was strongly correlated with *M. bovis* BCG infection

In order to determine whether FR expression is a result of direct mycobacteria infection, it was necessary to visualize the mycobacteria within the macrophage. To achieve this, *M. bovis* BCG was labeled with rhodamine, a red fluorescent dye. Macrophages were then either uninfected or infected with the rhodamine-labeled *M. bovis* BCG. After 48 hours, the cells were stained for the FR and visualized using fluorescence microscopy. We could not detect FR expression in uninfected cells (Figure 3.2a). Interestingly, FR staining was observed almost exclusively on cells that were also positive for infection (Figure 3.2b), suggesting that FR expression is confined to directly infected macrophages. A similar observation was made using flow cytometry (Figure 3.3). In order to obtain a large number of cells for unbiased quantitation, flow cytometric
analysis utilized RAW 264.7 cells that were either uninfected or infected with rhodamine-labeled *M. bovis* BCG.

3.3.3 Infection with pathogenic *M. tuberculosis* resulted in anti-FR staining on infected macrophages

Preliminary studies were carried out using the pathogenic H37Rv strain of *M. tuberculosis*. Rhodamine-labeled H37Rv was used to infect bone marrow-derived macrophages for observation via IF microscopy (Figure 3.4). For this study, the bacteria were added at a concentration where approximately 70% of cells were infected. Similar to the data from studies using *M. bovis* BCG, cells that were positive for H37Rv infection were also positive for folate receptor staining, and FR was not observed on nearby cells that had not phagocytosed the mycobacteria.

3.3.4 Anti-folate receptor staining is also observed in human macrophages following *Mycobacterium* infection

Having demonstrated a strong correlation between *Mycobacterium* infection and folate receptor staining in murine macrophages, we next tested whether this correlation could also be observed in human macrophages. THP-1 cells, a human macrophage cell line, were infected at a 3:1 *M. tuberculosis* H37Rv-to-cell ratio for 72 hours in tissue culture flasks. The cells were transferred from the flasks, seeded at a concentration of 1 x 10^5 cells per glass coverslip, fixed with 2% PFA, and stained for infection and FR expression. The bacteria were labeled using an antibody against LAM, a glycolipid virulence factor associated with *M. tuberculosis*, followed by a Texas Red-conjugated
secondary antibody. Folate receptor expression was visualized using the polyclonal anti-FR antibody and a FITC-conjugated secondary antibody. As seen in Figure 3.5, uninfected THP-1 cells exhibited no anti-FR staining. Compared to the previously described microscopy studies, which were performed with primary macrophages from C57BL/6 mice, the THP-1 cells were largely negative for both infection and FR expression. These findings were not unexpected, as the infection was performed at a lower MOI, and the phagocytic ability of THP-1 cells is lower than that of BMDMs. However, similar to observations in the murine macrophages, *Mycobacterium* infection was associated with FR staining in human macrophages. In the infected THP-1 population, only 1% of the cells were negative for FR staining. A larger bystander effect, defined by the FR+, LAM- sub-population, was observed in human macrophages following infection with *M. tuberculosis* H37Rv.

3.3.5 Folate receptor staining is not inducible by secreted inflammatory cytokines

In both the immunofluorescence and flow cytometry analyses, only a small percentage of uninfected cells were positive for staining with the FR antibody. This suggested that the presence of secreted factors from infected macrophages did not induce FR expression on uninfected macrophages. This situation is advantageous in the design of folate-targeted drug therapies, as the effects of a folate-linked drug should be limited to infected macrophages, with negligible effects on nearby resting cells. To address the bystander effect more directly, media was collected from RAW cells at 48 hours post-*M. bovis* BCG infection. This media was filtered to remove any non-phagocytosed mycobacteria. Naïve macrophages were incubated with the filtered culture supernatant.
for 48 hours, stained for expression of the folate receptor, and analyzed by flow cytometry. Results indicate that cells treated with conditioned media did not display increased levels of anti-FR staining compared to untreated macrophages (Figure 3.6).

3.3.6 Macrophages treated with culture filtrate protein from *M. tuberculosis* H37Rv do not exhibit a significant increase in FR staining above basal levels

Previous findings by Xia et al. (2009) demonstrated that bacterial cell wall fragments were unable to upregulate folate receptor expression in cultured monocyte-derived macrophages. However, the authors did not include any bacteria from the genus *Mycobacterium* in their study. Though the authors were able to demonstrate that *in vivo* administration of various inflammatory stimuli was capable of inducing a population of FR-expressing peritoneal macrophages, they were unable to identify the specific molecular stimulus required for folate receptor expression. To test whether mycobacterial components were capable of upregulating folate receptor expression *in vitro*, RAW 264.7 cells were treated with varying concentrations of *M. tuberculosis* H37Rv-derived culture filtrate protein (CFP). Flow cytometric analysis of RAW 264.7 cells treated with CFP for 48 hours suggests that *M. tuberculosis* cell wall components induce minimal folate receptor expression in macrophages *in vitro*, as only a slight induction of anti-FR staining was observed (Figure 3.7). This experimental finding suggested that folate receptor expression is a consequence of direct infection with live, intact *Mycobacterium*. 
3.3.7 Comparable levels of anti-FR staining are observed in wild-type and knockout macrophages at 48 hours post-infection

To begin to define the pathways that induce the apparent FR expression following mycobacterial infection, macrophages from wild-type and various knockout mice were infected with *M. bovis* BCG and FR protein levels were measured by Western blot. The knockout macrophage cell lines selected for inclusion in the study were ones lacking in either receptors that recognize pathogen-associated molecular patterns (PAMPs) or in adaptor proteins known to be involved in pro-inflammatory signaling pathways. Toll-like receptor (TLR) 2 is a pattern recognition receptor that mediates the production of cytokines necessary for effective immunity (Lien et al. 1999). The mannose receptor (MR) recognizes mannose sugars, a moiety that is found on the cell surface of many pathogenic microbes. Myeloid differentiation factor 88 (MyD88) is an adaptor protein that activates the transcription factor NFκB in TLR-initiated signaling pathways, leading to a robust antimicrobial response. Previous studies have demonstrated that MyD88-/-mice have profound defects in both resistance and immune responses to *Mycobacterium avium* and higher bacterial burdens than their wild-type counterparts (Feng et al. 2003). Initial Western blot analysis (Figure 3.8) indicated that the knockout macrophages showed similar anti-FR binding as the wild-type macrophages at 48 hours post-*M. bovis* BCG infection. These results were similarly observed by immunofluorescence microscopy (Figure 3.9). Immunostaining of uninfected macrophages suggested that the knockout macrophages may express some folate receptor, whereas FR staining in the wild-type cells was almost non-existent (Figure 3.10).
3.3.8 The polyclonal anti-FR antibody displays cross-reactivity with bacterial antigens

When *M. bovis* BCG lysate was probed with the FR antibody, the resulting Western blot revealed that the antibody had cross-reacted with the bacteria. In addition to reacting with lysate from *M. bovis* BCG, the antibody also demonstrated cross-reactivity with lysates from the virulent H37Rv strain of *M. tuberculosis* and with *E. coli* (data not shown). All three bacterial lysates were also probed with the HRP-conjugated secondary antibody alone. The absence of any bands in these Western blots ruled out non-specific cross-reactivity from the secondary antibody. This indicated that the antibody was reacting with the mycobacteria and was likely not reacting with a eukaryotic FR epitope(s) in a reliable fashion. While the antibody may indeed recognize host folate receptor, this recognition is not exclusive in terms of specificity. From the Western blots, we were able to demonstrate that the polyclonal anti-folate receptor antibody, in addition to its intended target, also binds to previously untested prokaryotic protein targets. While this unexpected lack of specificity may be attributable to the polyclonal nature of the antibody, the more likely explanation is that the rabbits were exposed to bacterial contaminants during the antibody purification process.

3.3.9 The absence of FR expression in *M. bovis* BCG-infected macrophages was confirmed using other folate-targeting compounds

To evaluate anti-FR staining at the cell surface, cells were infected and then stained with the anti-FR antibody under non-permeabilizing conditions. Under these conditions, the antibody lacks access to the cytoplasm, where the phagocytosed bacteria reside. While the FR does cycle between the cytoplasm and the cell surface, at steady
state the ratio is approximately 50:50. The bacteria, on the other hand, should all be localized within the cell, as any unphagocytosed bacteria would have been removed during the media exchange at four hours post-infection or killed by the subsequent addition of antibiotic-containing media. The murine macrophages were immunostained using the commercially available FR antibody and an anti-rabbit FITC-conjugated secondary antibody. We observed very little to no green fluorescent signal in unpermeabilized macrophages following infection with M. bovis BCG (Figure 3.11).

Additionally, the use of a folate-conjugate also confirmed the absence of folate receptor on M. bovis BCG-infected macrophages (Figure 3.12). At 48 hours post-infection, the infected macrophages were washed with sterile saline and incubated with folate conjugated to the fluorophore Oregon Green for 30 minutes. HeLa cells, a cervical cancer cell line, were used as a positive control for binding of the folate conjugate. Unbound folate conjugate was removed with a sterile saline wash and the unpermeabilized cells were fixed for visualization. The lack of fluorescent signal indicates that either a) expression of the folate receptor is not induced by Mycobacterium infection, or b) FR is made but exists in a non-functional form and is therefore unable to endocytose the Oregon Green-conjugated folate.

We further examined FR expression in the context of a Mycobacterium infection by immunostaining macrophages with m909, a monoclonal antibody that selectively binds human FR-β, the isoform associated with cells of myeloid origin (Feng et al. 2011). At 48 hours post-infection, THP-1 cells were immunostained with the m909 antibody, followed by addition of a FITC-conjugated anti-human secondary antibody. It should be noted that THP-1 cells are less phagocytic than primary macrophages, and higher
mycobacterial burdens may be required before any FR expression can be observed. Our microscopy studies demonstrated that the THP-1 cells did phagocytose the bacterial targets; however, in comparison to their uninfected counterparts, the infected cells expressed no observable differences in FR-β staining (Figure 3.13). This preliminary experiment did not include a positive control for anti-m909 reactivity, as one was not readily available. For future studies with the m909 antibody, a positive control cell population would need to be of human origin and specifically express the beta isoform of the folate receptor. Examples include activated macrophages from the synovia of RA patients, tumor-associated macrophages, and human cancer cells of myelomonocytic origin, such as KG-1 myeloid leukemia cells.

3.3.10 Evaluation of FR expression in *M. bovis* BCG-infected peritoneal macrophages

Previous work had indicated that expression of the FR could not be induced *in vitro*. For example, treating murine macrophages with a combination of inflammatory cytokines, thereby mimicking the cytokine storm seen in the synovia of RA patients, did not lead to any significant elevation in FR expression above basal levels. Similarly, lipopolysaccharide, an endotoxin derived from *E. coli*, failed to induce FR expression in C57/BL6 bone marrow-derived macrophages when administered *in vitro* (Xia et al. 2009). However, LPS had been previously demonstrated to induce FR expression in peritoneal macrophages following intra-peritoneal (i.p.) injection, leading us to hypothesize that perhaps *Mycobacterium* would also only induce FR expression following *in vivo* infection.
Peritoneal lavage was collected at 3, 7, and 10 days post-infection and the adherent macrophage populations were analyzed for anti-FR binding by flow cytometry. In this *in vivo* study, thioglycolate-treated mice were intended to serve as the positive control group, as Xia et al. (2009) had previously demonstrated that nearly one-third of the macrophage population that was recruited to the peritoneum at 3 days post-thioglycolate treatment was positive for FR expression.

The results of this preliminary *in vivo* experiment were inconclusive. Though thioglycolate-induced peritonitis led to the highest percentage of FR-binding peritoneal macrophages, the population was not as substantial as the population obtained in the Xia et al. (2009) study. Additionally, the elevation in the FR-binding population was minimal compared to the negative control group. In contrast to the results of our *in vitro* studies, a high percentage of peritoneal macrophages from PBS-treated mice reacted with the anti-FR antibody. While it is unclear whether elevated basal levels of FR binding are an unintended consequence inherent to *in vivo* studies or an indication of experimental error (e.g., compromised sterility of the saline solution, internal trauma from i.p. injection, insufficient washes, etc.), the high level of background limits our assessment of the effect(s), if any, of *Mycobacterium* infection on macrophage FR expression in a mouse model. The BCG-positive, FR-positive population remained low throughout the course of the experiment, as did the total population of BCG-infected cells (Figure 3.14). The infection-positive population was measured based on the rhodamine signal from the labeled bacteria. The low infection percentages may suggest inefficient delivery of the mycobacteria into the peritoneal cavity, but it may also be an indication of the instability of the rhodamine signal in an *in vivo* setting. The use of *M. bovis* BCG that has been
transformed to express its own fluorescent signal may allow for better visualization of the infected cell population and may allow for long-term studies.

Due to the unanticipated high level of FR-binding in our negative control group and very low infection-positive population numbers, we cannot conclude whether the decreasing trend in the BCG-positive, FR-positive sub-population and the total BCG-infected population over time are suggestive of the dissemination of the infection from the peritoneal cavity. It is essential to note that this was a preliminary in vivo study and that FR expression was measured in using the polyclonal anti-FR antibody before our Western blot analyses had been performed. In light of our findings that the polyclonal antibody cross-reacts with bacterial proteins, future studies measuring FR expression following in vivo Mycobacterium infection would require the use of a different FR-binding compound. Quantitation of FR expression using either an antibody that is exclusive in its recognition of the FR or a fluorescently-labeled folate conjugate will also assist in better illustrating whether Mycobacterium-infected macrophages are positive for FR expression in an in vivo mouse model.

3.3.11 Evaluation of FR-β mRNA content in splenic and pulmonary tissues following in vivo administration of M. bovis BCG

Having demonstrated the non-specificity of the FR antibody, it became necessary to utilize a different method to examine if there was any upregulation in FR-β expression following infection. Previous work in our lab by Jennifer Zupkosky had demonstrated that there was no detectable difference in FR-β mRNA expression between uninfected and M. tuberculosis-infected macrophages. This in vitro study used primary
macrophages from murine and human sources and changes in FR expression were measured using quantitative real-time PCR. Over the course of a 96 hour infection, FR-β mRNA levels were comparable between uninfected and infected populations at each time point tested.

To further test the hypothesis that mycobacterial infection might only induce FR expression in vivo, wild-type C57BL/6 mice were retro-orbitally infected with M. bovis BCG. At two days and five days post-infection, the lungs and the spleen were harvested for RNA extraction. The synthesized cDNA was then processed using quantitative real-time PCR. The housekeeping gene GAPDH was used as the internal control, and the relative mRNA content was calculated using the ddCt method. The results of this preliminary experiment were inconclusive, largely due to variability in the expression of GAPDH between the samples tested, but it would appear that there is no difference in expression of the FR-β gene between uninfected and infected mice (Table 3). Variation in the cycle threshold (Ct) values was also observed for FR-β in uninfected tissues. If the level of FR-β mRNA transcript increases as a result of pathogenic challenge, one would predict that a) the FR-β Ct values would be consistent between uninfected tissues at both 2 days and 5 days post-infection and that b) FR-β Ct values would be lower in infected tissues as compared to uninfected tissues. However, as seen in Table 3, the FR-β Ct values for uninfected spleens varied greatly between the 2 day and 5 day samples. In contrast, FR-β Ct values were consistent between the 2 day and 5 day samples. We had hypothesized that M. bovis BCG infection would result in a decrease in FR-β Ct values, indicating gene upregulation. Unexpectedly, FR-β Ct values appear to increase following mycobacterial challenge in splenic and pulmonary tissues. At present, without consistent
Ct values for the housekeeping gene, we cannot conclude what effect an in vivo *Mycobacterium* infection has on FR-β gene expression. These preliminary observations, if confirmed, would differ from previous work, in which infection with pathogenic bacteria (e.g., *Pseudomonas aeruginosa*) was shown to elicit an increase in FR expression on peritoneal macrophages (Xia et al. 2009).

This experiment will need to be repeated and certain modifications will need to be incorporated before any conclusions about the relationship between mycobacterial infection and macrophage FR-β expression can be determined. At present, it is unclear why the GAPDH Ct values varied between all tested samples, whether due to user error, compromised primer integrity, and/or poor primer binding. However, it is essential that the variations in the internal control gene be eliminated. As previously stated, this was a preliminary experiment and better uniformity in the Ct values for the housekeeping gene may be achieved with simply more technical practice.

3.4 Discussion

It has become apparent over the past decade that current strategies for TB control are ineffective and outdated. The reasons for the limited success of current TB control and treatment strategies can be attributed to the HIV/AIDS epidemic, the lack of health care systems and infrastructure in regions where TB is endemic, and the continued increases in MDR and XDR cases of TB. These factors, combined with a pipeline of new anti-TB drug development that has remained stagnant for nearly the last 20 years, highlight the need for the development of new drugs that can reduce the current 6-9
month TB drug regimen, as well as drugs that can effectively combat MDR and XDR TB.

While new anti-TB drugs have demonstrated efficacy against MDR and XDR TB in pre-clinical studies or promote the early clearance of pathogenic *M. tuberculosis*, these drugs operate on the same platform as existing drugs, meaning the issue of drug resistance developing is still not fully resolved. A major drawback for the delivery of TB drugs is the lack of existing mechanisms to target drugs in a way that ensures maximal exposure to the bacteria.

Previous studies had demonstrated that activated macrophages are highly FR-positive and that naïve macrophages could be induced to express the FR expression via infection with pathogenic microbes. Therefore, we hypothesized that *Mycobacterium* infection could also induce FR expression on macrophages. Our studies have indicated that an *in vitro* *Mycobacterium* infection does not induce an increase in macrophage FR expression. Our initial studies suggested that *Mycobacterium*-infected macrophages were also highly FR-positive and that FR expression was a result of live infection. Via Western blot analysis, we discovered that our initial findings were a consequence of cross-reactivity between the polyclonal anti-FR antibody and bacterial components. Follow-up experiments with a fluorescent folate conjugate and an anti-FR-β monoclonal antibody demonstrated that the strong correlation between BCG infection and FR expression we had previously observed via microscopy and flow cytometry were attributable to the strong affinity of the polyclonal antibody for bacterial components.

Current work in the lab is focused on the synthesis of labeled folate conjugated to polyethylene glycol (PEG). Because commercially available folate receptor antibodies
have proven unreliable in their specificity, we are interested in producing FR-targeting molecules that can be made and tested for our specific needs. Direct labeling of folic acid with a fluorophore will eliminate the need for secondary staining, and the conjugate could be used for a wide variety of applications, including fluorescence microscopy, flow cytometry, and possibly histology. Activation of the gamma carboxylic acid of the folic acid and its subsequent reaction with the primary amine group of PEG should not affect the recognition or the binding of the folate compound by the folate receptor. Also, when using the aforementioned synthesis method, receptor-mediated endocytosis of the folate compound by FR-expressing cells should not be impaired. Additionally, conjugating the labeled folate to the distal end of the PEG chain will allow the molecule to remain in circulation for longer periods of time (Kim et al. 2007), which may prove useful for any future long-term in vivo studies.

Our preliminary in vivo infection studies yielded inconclusive results. Peritoneal macrophages were harvested and collected following i.p. administration of *M. bovis* BCG. Similar to the work by Xia et al. (2009), we used thioglycolate to elicit a population of highly FR-positive macrophages and sterile saline was administered to the negative control group. However, in our preliminary study, peritoneal macrophages from PBS-treated mice exhibited high basal levels of anti-FR staining, and thioglycolate-induced peritonitis failed to significantly elevate anti-FR staining. The extremely low percentage of cells that were positive for BCG infection indicated that there was a problem with the experimental set-up, which further restricted our ability to draw any conclusions regarding changes in FR expression levels in a mouse model of *Mycobacterium* infection.
We also conducted a preliminary study to assess the expression of FR-β at the mRNA level following an *in vivo* *M. bovis* BCG infection. The results of the qRT-PCR study were also inconclusive due to the non-uniform nature of the C\textsubscript{i} values of the GAPDH internal control. Though future studies are needed to confirm the relationship between *M. bovis* BCG infection and relative FR-β mRNA content in splenic and pulmonary tissues, the C\textsubscript{i} values from our preliminary study suggest a downward trend in FR-β expression following a *Mycobacterium* infection. This unconfirmed observation would be unanticipated in the context of previous findings from other studies, which would predict a decrease in C\textsubscript{i} values corresponding to an increase in FR-β expression following infection-mediated macrophage activation.
Folate conjugates are endocytosed by pathologic cells. Folate uptake in healthy tissues is mediated by the reduced folate carrier (RFC), which binds folate with low affinity. The RFC is selective in its transport of reduced forms of folic acid. Because the RFC does not recognize folate conjugates as substrates, they are taken up only by cells that express functional folate receptors (FRs). In contrast to normal cells, the glycosylphosphatidylinositol (GPI)-anchored FR is expressed on only a few cell types, namely tumor cells and activated macrophages. Folate and folate conjugates bind the FR with high affinity and enter FR-expressing cells via receptor-mediated endocytosis.

Figure 3.1  Folate conjugates are endocytosed by pathologic cells. Folate uptake in healthy tissues is mediated by the reduced folate carrier (RFC), which binds folate with low affinity. The RFC is selective in its transport of reduced forms of folic acid. Because the RFC does not recognize folate conjugates as substrates, they are taken up only by cells that express functional folate receptors (FRs). In contrast to normal cells, the glycosylphosphatidylinositol (GPI)-anchored FR is expressed on only a few cell types, namely tumor cells and activated macrophages. Folate and folate conjugates bind the FR with high affinity and enter FR-expressing cells via receptor-mediated endocytosis.
Figure 3.2  Macrophages are positive for anti-FR staining following infection with *M. bovis* BCG. (A) BMDMs from C57BL/6 mice were seeded on glass coverslips and infected with rhodamine-labeled *M. bovis* BCG at a 5:1 bacilli-to-macrophage ratio for 48 hours. Cells were stained using an antibody against the folate receptor or an isotype control antibody and with DAPI to label nuclei. Shown are the merged images; overlapping rhodamine and FITC appear yellow in merged images. (B) Cells which contained *M. bovis* BCG, FR or both were quantified by counting ~20 fields and 200 cells. (- does not contain FR/BCG and + contains FR/BCG)
Figure 3.3  A significant percentage of *Mycobacterium*-infected RAW 264.7 cells are positive for anti-FR antibody binding. Macrophages were collected for analysis by flow cytometry at 48 hours after infection with rhodamine-labeled *M. bovis* BCG. The polyclonal anti-FR antibody and FITC-conjugated anti-rabbit secondary antibody were used to stain the FR. Experiments from each group were repeated at least three times, and representative dot plots of (A) uninfected and (C) infected macrophage samples, and quantitation of subpopulations from (B) uninfected and (D) infected macrophages are included. 100,000 cells were counted for each sample. The fluorescence gate for the FR marker was set so that less than 1% of the macrophages appeared to be positive when examined with an isotype control antibody.
Figure 3.4  Anti-FR binding is observed on *M. tuberculosis*-infected macrophages. (A) BMDMs were either uninfected or infected with rhodamine-labeled *M. tuberculosis* H37Rv at a 5:1 bacilli-to-macrophage ratio for 48 hours. Cells were stained with DAPI to label cell nuclei and with either the polyclonal FR antibody or an isotype control antibody. Shown are the merged images; overlapping rhodamine and FITC appear yellow in merged images. (B) Cell populations were quantified by counting ~20 fields and 200 cells. (− does not contain FR/TB and + contains FR/TB).
Figure 3.5  The correlation between *Mycobacterium* infection and anti-folate receptor staining is also observed in human macrophages. Human THP-1 cells were infected with *M. tuberculosis* H37Rv at a 3:1 MOI for 72 hours. Cells were transferred to glass coverslips, fixed with 2% PFA, and stained. The images are from a single, representative field. (A) The polyclonal anti-FR antibody and FITC-conjugated secondary antibody were used to detect folate receptor expression. (B) Bacilli were visualized using an anti-LAM primary antibody and a Texas Red-conjugated secondary antibody. (C) Cell nuclei were labeled with DAPI, and (D) the merged image combining all three channels is shown.
Figure 3.5 (cont.) (E) Cell populations of THP-1 cells were quantified by counting ~20 fields and 200 cells. (- does not contain FR/LAM and + contains FR/LAM).
Figure 3.6  Culture supernatant from *M. bovis* BCG-infected macrophages fails to induce FR expression in naïve cells. RAW 264.7 cells were treated with rhodamine-labeled *M. bovis* BCG at a 5:1 ratio for 48 hours. Cells were stained for FR expression and analyzed via flow cytometry (red bars). The conditioned media was filtered through a 0.22 μM filter to remove any *M. bovis* BCG and then added to naïve RAW 264.7 cells for 48 hours. These treated cells (pink bars) were then stained for FR expression and analyzed via flow cytometry. Flow cytometry was performed using a Beckman Coulter FC-500 flow cytometer.
Figure 3.7  Macrophages treated with culture filtrate protein (CFP) exhibit a minimal increase in anti-FR staining as compared to untreated cells. RAW 264.7 cells were treated with varying concentrations of CFP from *M. tuberculosis* H37Rv for 48 hours (blue bars). Infection with live mycobacteria is represented by the red bar. Cells were stained for expression of the folate receptor and analyzed via flow cytometry.
Figure 3.8  Western blot analysis for the folate receptor protein in wild-type and knockout BMDMs using a polyclonal anti-FR antibody. Uninfected wild-type and knockout BMDMs do not express detectable levels of FR. At 48 hours post-BCG infection, wild-type and knockout macrophages express comparable levels of FR. The 37 kD marker is included for reference. The blot was subsequently probed for β-tubulin as a loading control. The figure is representative of two independent experiments.
Figure 3.9  Immunostaining of wild-type and knockout primary murine macrophages following *in vitro* infection with rhodamine-labeled *M. bovis* BCG. Representative merged images at 63x magnification are shown for (A) wild-type, (B) MyD88-/-, (C) MR-/-, and (D) TLR2-/- macrophages. A polyclonal anti-FR antibody and FITC-conjugated secondary antibody were used to label FR expression. Cellular nuclei were labeled with DAPI, and plasma membranes were visualized using Alexa Fluor 647-conjugated WGA.
Figure 3.9 (cont.)  (E) Quantitation of immunofluorescence data shows similar distribution of cellular sub-populations between wild-type and knockout primary macrophages at 48 hours post-\textit{M. bovis} BCG infection. Cell populations were quantified by counting ~20 fields and 200 cells.
Figure 3.10 Immunostaining of uninfected wild-type and knockout primary murine macrophages. Representative merged images at 63x magnification are shown for (A) wild-type, (B) MyD88−/−, (C) MR−/−, and (D) TLR2−/− macrophages. A polyclonal anti-FR antibody and FITC-conjugated secondary antibody were used to label FR expression. Cellular nuclei were labeled with DAPI, and plasma membranes were visualized using Alexa Fluor 647-conjugated WGA.
Figure 3.10 (cont.)  (E) Quantitation of immunofluorescence data indicates that macrophages from uninfected knockout mice are highly FR-positive. Cell populations were quantified by counting ~20 fields and 200 cells.
Figure 3.11  The limited affinity and specificity of the polyclonal anti-FR antibody were confirmed using unpermeabilized macrophages. Following a 48 hour infection with rhodamine-labeled *M. bovis* BCG, BMDMs were stained for FR expression using the polyclonal anti-FR antibody in the absence of permeabilization with Triton X-100. Images of the (A) FITC and (B) Texas Red channels and (C) the merged channels are representative of the total infected cell population.
Figure 3.12  *M. bovis* BCG-infected BMDMs were visualized for FR expression using a Folate-Oregon Green conjugate. Cells were seeded on glass coverslips and infected with rhodamine-labeled *M. bovis* BCG for 48 hours. At 48 hrs, cells were rinsed with warm PBS and incubated with Folate-Oregon Green (100 nM) for 30 minutes at 37°C. Cells were not permeabilized. HeLa cells were used as a positive control for folate conjugate binding. The FITC channel was used to measure FR expression by Oregon Green. The merged images are a composite of the FITC and rhodamine channels.
Figure 3.13  Immunostaining of infected THP-1 cells with a monoclonal antibody against FR-β indicates that FR expression is absent. PMA-differentiated THP-1 cells were infected with rhodamine-labeled *M. bovis* BCG and stained for FR expression using the monoclonal m909 antibody and FITC-conjugated anti-human secondary antibody. WGA-Alexa Fluor 647 was used to label plasma membranes. Images of the individual (A) FITC, (B) Texas Red, (C) Alexa 647 channels and (D) the merged image of a representative field are shown.
Figure 3.14  Flow cytometric analysis of peritoneal macrophages from C57BL/6 mice following *in vivo* infection. 6- to 8-week-old mice received either PBS (blue bars), thioglycolate (red bars), or $10^7$ CFUs of rhodamine-labeled *M. bovis* BCG (green bars) by i.p. injection. Adherent cells were stained for FR expression using the polyclonal anti-FR antibody. Three mice were used for each treatment condition, and at least 30,000 cells were counted per sample. Peritoneal lavage was collected at (A) 3 and (B) 7 days post-infection.
Figure 3.14 (cont.)  (C) Flow cytometric analysis of peritoneal lavage collected at 10 days post-injection. Adherent cells were stained for FR expression using the polyclonal anti-FR antibody. Three mice were used for each treatment condition (PBS: blue bars, thioglycolate: red bars, *M. bovis* BCG: green bars). Approximately 30,000 cells were counted per sample.
**TABLE 2**

**SEQUENCES OF PRIMERS USED FOR qRT-PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse GAPDH F</td>
<td>AACGACCCCTTCATTGAC</td>
</tr>
<tr>
<td>Mouse GAPDH R</td>
<td>TCCACGACATACTCAGCAC</td>
</tr>
<tr>
<td>Mouse FR- F</td>
<td>AGCACCCCTGTCACACGTTT</td>
</tr>
<tr>
<td>Mouse FR- R</td>
<td>TCATTAGGCCAGATGTGTC</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer
TABLE 3

RELATIVE mRNA CONTENT IN SPLENIC AND PULMONARY TISSUES FROM C57BL/6 MICE FOLLOWING IN VIVO INFECTION WITH M. BOVIS BCG

<table>
<thead>
<tr>
<th></th>
<th>GAPDH</th>
<th>FR-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>2d Uninfected Spleen</td>
<td>18.90394</td>
<td>30.73593</td>
</tr>
<tr>
<td>2d BCG-infected Spleen</td>
<td>20.82885</td>
<td>28.66864</td>
</tr>
<tr>
<td>5d Uninfected Spleen</td>
<td>18.8255</td>
<td>24.84086</td>
</tr>
<tr>
<td>5d BCG-infected Spleen</td>
<td>20.91445</td>
<td>29.53712</td>
</tr>
<tr>
<td>2d Uninfected Lung</td>
<td>21.02803</td>
<td>28.29284</td>
</tr>
<tr>
<td>2d BCG-infected Lung</td>
<td>18.90923</td>
<td>30.17145</td>
</tr>
<tr>
<td>5d Uninfected Lung</td>
<td>20.89299</td>
<td>28.25747</td>
</tr>
<tr>
<td>5d BCG-infected Lung</td>
<td>20.29884</td>
<td>28.28191</td>
</tr>
</tbody>
</table>

\[ \Delta C_t = C_t \text{ (Target)} - C_t \text{ (Normalizer)} \]
\[ \Delta C_t = C_t \text{ (Folate) - C_t (GAPDH)} \]

\[ \Delta \Delta C_t = \Delta C_t \text{ (Treatment)} - \Delta C_t \text{ (Control)} \]
\[ \Delta \Delta C_t = \Delta C_t \text{ (BCG-infected) - C_t (Uninfected)} \]

Relative mRNA content = \(2^{\Delta \Delta C_t}\)

<table>
<thead>
<tr>
<th></th>
<th>Relative mRNA content</th>
</tr>
</thead>
<tbody>
<tr>
<td>2d Spleen</td>
<td>(2^{3.9922} = 15.91373)</td>
</tr>
<tr>
<td>5d Spleen</td>
<td>(2^{-2.60731} = 0.16410)</td>
</tr>
<tr>
<td>2d Lung</td>
<td>(2^{-3.99741} = 0.06261)</td>
</tr>
<tr>
<td>5d Lung</td>
<td>(2^{-0.61859} = 0.65131)</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

SUMMARY AND FUTURE PERSPECTIVES

4.1 The complexities of *Mycobacterium* pathogenesis

In 1952, Rene Dubos cautioned that medical interventions alone would not be sufficient for the prevention or cure of tuberculosis. The widespread use of the BCG vaccine has not succeeded in lowering TB incidence in recent years. The introduction of the anti-TB drugs isoniazid, rifampin, and pyrazinamide has not resulted in the elimination of the disease. The last half of the 20th century demonstrated the failures of disease control measures, the increased susceptibility to mycobacteria infection in HIV patients, and the far-reaching consequences of increasingly drug-resistant strains of *M. tuberculosis*. Scientific advances will be needed for the development of vaccines and drugs that are more effective than the ones that are currently available. The rational development of antitubercular agents requires an understanding of how *M. tuberculosis*-host interactions allow the bacteria to circumvent host defenses, survive within the macrophage, and ultimately cause disease and mortality.

There is no simple answer to the question of what makes *M. tuberculosis* virulent. *M. tuberculosis* does not have the classical virulence factors (e.g., bacterial toxins), which are the hallmarks of other disease-causing bacterial pathogens, such as *Corynebacterium*...
diptheriae, Shigella dysenteriae, and Vibrio cholera. Though our knowledge about how M. tuberculosis causes disease remains limited, its virulence can be quantified in terms of bacterial burden and mortality. Previous experiments have demonstrated the complexity of the immune system’s response to M. tuberculosis infection. The relationship between the cytokine TNF-α and disease progression in clinical and animal models is neither simple nor consistent. For example, TNF-α is a key cytokine in the inflammatory or Th1 response and is necessary for the control of infection in a mouse model. Mice that are unable to produce or respond to TNF-α develop a diminished inflammatory granulomatous reaction following infection with live M. bovis BCG (Senaldi et al. 1996). However, excessive TNF-α production leads to early death due to severe pulmonary inflammation (Smith 2003). Other studies have indicated the involvement of factors additional to TNF-α in disease progression. For example, the reportedly hypervirulent clinical isolate M. tuberculosis CDC1551 induces TNF-α production levels that are higher than those induced by other M. tuberculosis strains. However, its virulence, as measured by bacterial burden and mortality, is not greater than the other strains (Manca et al. 1999). The converse was observed in mouse studies using M. tuberculosis H878. Interestingly, infection with M. tuberculosis H878 induced small amounts of TNF-α and other inflammatory cytokines, but higher levels of Th2 cytokines, such as IL-4 (Smith 2003). These results highlight the complex and unclear interactions between M. tuberculosis and its host. They also clearly demonstrate that an optimal balance of immunomodulators is essential. Inflammation is necessary for the control of infection, but inflammatory host responses are also responsible for extensive tissue damage and must therefore be regulated.
4.2 Modulation of macrophage immune responses by *Mycobacterium*

In the present study, we investigated the differential macrophage responses following infection with various members of the genus *Mycobacterium*. Previous work had demonstrated that the regulation of TNF-\(\alpha\) gene expression is both stimulus- and cell type-specific. Additionally, the region of the TNF-\(\alpha\) promoter containing binding sites for the transcription factors Ets/Elk, NF-\(\kappa\)B, Sp1, and the CRE site is a) responsible for the maximal induction of TNF-\(\alpha\) activity following LPS exposure and b) is highly conserved between mice and humans (Falvo et al. 2000; Lee and Schorey 2005).

We used Ets/Elk and NF-\(\kappa\)B reporter vectors in order to study the activation of these transcription factors in the context of a *Mycobacterium* infection. Our results suggest that a virulent *M. tuberculosis* infection may lead to the decreased activation of the transcription factors Ets-1 and NF-\(\kappa\)B as compared to infections with avirulent *M. tuberculosis* and non-pathogenic mycobacteria, such as *M. smegmatis*. Not only are differential transcriptional responses observed following infection with non-pathogenic versus pathogenic mycobacteria, but differential responses at the transcriptional level are also observed following infection with isogenic strains of *M. tuberculosis*. Though the H37Ra and H37Rv strains have shown very little genomic differences between them, H37Rv displays a marked advantage over H37Ra in terms of its survival and growth within animal hosts. As observed within primary murine macrophages, the doubling rate of H37Rv is over 60% faster than that of H37Ra, indicating the ability of the H37Rv strain to overcome the growth inhibitory actions of Th1-based defense mechanisms (Jung et al. 2002). In terms of bacterial viability, a higher rate of killing was seen for intracellular H37Ra as compared to H37Rv at 96 and 168 hours post-treatment with the
antimicrobial compound mercaptopyridine-N-oxide (Li et al. 2008). Differences in transcriptional responses may ultimately contribute to differences in disease outcomes. The findings of our study provide new insights into the pathogenesis of virulent *M. tuberculosis*. Our results suggest that modulation of the host immune response may facilitate the successful evasion of host defenses and enable the survival of the pathogen within the host.

4.3 The limitations of non-targeted anti-TB therapies

Currently available anti-TB drugs all operate on existing chemical platforms, which means the likelihood for drug resistance will persist. One of the major drawbacks in the physiological administration of antitubercular drugs is accessibility. Because *M. tuberculosis* is an intracellular pathogen, its residence within the infected host macrophage limits its accessibility, making it more difficult for drugs to localize to their target. Therefore, existing anti-TB drugs, as well as the development of new drugs, would benefit from mechanisms that effectively target them to the infected macrophage. These benefits include the potential to 1) lower current drug dosages, 2) minimize the likelihood of adverse drug side effects, and 3) shorten current drug treatment schedules. Together, all of these benefits may lead to improved rates of patient compliance. As seen with successful anti-cancer therapy targeting approaches, the targeting of anti-TB drugs could circumvent problems with host toxicity. Successful outcomes could lead to the re-introduction and use of effective anti-TB drugs which had been previously excluded from consideration.
Characterization of FR expression in *Mycobacterium*-infected macrophages

The relationship between *Mycobacterium* infection and FR expression in macrophages has not been previously characterized. We had hypothesized that the limited expression of the folate receptor on activated macrophages might serve as a potential mechanism whereby anti-tuberculosis drugs could be effectively targeted to infected (i.e., activated) macrophages. Though our initial *in vitro* studies suggested a correlation between live *M. bovis* BCG infection and the upregulation of FR expression, thorough testing of the polyclonal anti-FR antibody revealed the non-specific nature of the antibody and necessitated measurement of FR expression with more specific reagents. Subsequent *in vitro* studies revealed that FR expression is not upregulated in *M. bovis* BCG or *M. tuberculosis* H37Rv-infected macrophages. Though discouraging, these results were similar to those observed in previously published studies in which the molecular stimulus required for FR expression was never identified. Work by Xia et al. (2009) found that *in vivo* activation of macrophages induced the expression of a functional folate receptor. Stimuli such as live and heat-killed *P. aeruginosa*, thioglycolate, and zymosan led to the recruitment of FR-expressing macrophages when administered intra-peritoneally. However, addition of these same stimuli to macrophage cultures did not promote FR expression. These *in vitro*-stimulated macrophages were analyzed via flow cytometry and were found to express activation markers, such as CD80, CD86, and Ly6C/G. Classical activation of BMDMs using stimulants such as IFN-γ, LPS, methotrexate, immune complexes, and TNF-α led to high levels of macrophage activation markers but no detectable levels of FR expression. The authors proposed that *in vitro* differentiation of monocytes using M-CSF may select for a type of
macrophage that is different from macrophages that naturally mature \textit{in vivo}. Also relevant to our findings is the proposal that the FR may be transiently expressed on activated macrophages, suggesting that the time points selected for sampling may have failed to capture the window wherein cultured macrophages are positive for FR expression.

The results of our \textit{in vivo} experiments are presently inconclusive. Following intra-peritoneal injection of \textit{M. bovis} BCG, less than 1\% of the total cell population was infected and FR-positive. The limited number of infected cells within the peritoneal cavity makes it difficult to evaluate the FR expression in this sub-population of cells. Further studies with higher infection levels are needed in order to determine if \textit{Mycobacterium} infections induce FR expression to a similar extent as other previously established stimulants, such as thioglycolate, zymosan and pathogenic \textit{Pseudomonas}.

Our preliminary \textit{in vivo} experiments will need to be repeated. Future studies with peritoneal macrophages will require a macrophage population that is highly positive for FR expression. This population can be elicited with \textit{P. aeruginosa}, a newly prepared stock of highly immunogenic thioglycolate, or zymosan. PBS-recruited macrophages, serving as the negative control group, should express little to no FR. Aseptic technique and careful i.p. administration may reduce the high level of background observed in our pilot study. To assess the effect of \textit{Mycobacterium} infection on FR expression in peritoneal macrophages, it will be necessary to improve the delivery of the bacteria into the host. \textit{M. bovis} BCG is prone to clumping, which can be overcome by reconstituting the bacteria with a higher concentration of detergent and membrane disruption by sonication. It may also be necessary to measure infection by means other than
fluorescent labeling, either by staining the bacteria with an antibody against a component of the mycobacteria cell wall or by the use of a *Mycobacterium* strain that has been transformed to produce its own fluorescent signal. Lastly, accurate characterization of FR expression will require the use of an anti-FR compound that does not cross-react with bacterial moieties.

From a transcriptional standpoint, preliminary qRT-PCR data from *M. bovis* BCG-infected splenic and pulmonary tissues was inconclusive. For future qRT-PCR studies, it is essential that uniform GAPDH C<sub>t</sub> values be obtained in order to draw any conclusions about the relationship between *Mycobacterium* infection and FR-β gene expression. While all PCR efficiencies and 260/280 ratios were within the accepted limits in our preliminary study, future studies may require the application of more stringent limits on these values, more careful handling of RNA and reagents to minimize nucleic acid degradation, and the inclusion of more technical replicates. Most importantly, elimination of pipetting error may have the greatest effect in tightening the range of C<sub>t</sub> values for the housekeeping gene. Standardization of study design, such as the use mice that are very close in age and/or of the same gender, may reduce the variability that accompanies *in vivo* studies.

4.5 FR expression at the interface between pathogen invasion and host response

At present, it is not clearly understood what advantage upregulation of the folate receptor has to activated macrophage populations. Activated macrophages, such as *Mycobacterium*-infected macrophages, are not believed to undergo active proliferation. Though FR upregulation makes sense in the context of rapidly dividing tumor cells, the
question naturally arises as to why non-proliferating activated macrophages would upregulate the expression of a receptor for the vitamin folate, which is primarily required for DNA synthesis. There is speculation that the increase in FR expression on activated macrophages, which accumulate at sites of inflammation and in regions that experience chronic exposure to pathogens, may provide the cell with some means of preventing further pathogen damage (Xia et al. 2009). On the other hand, increased FR expression in bacteria-containing macrophages may be induced for the benefit of the pathogen. As folate is an essential nutrient for pathogen growth, increased uptake of folate by the host may confer a benefit to the intracellular pathogen. It is unclear whether the host macrophage upregulates FR expression following pathogenic challenge to the benefit of the host or whether once phagocytosed, intracellular pathogens induce an increase in FR expression and highjack host FR trafficking to the benefit of the invading pathogen.

Though the potential of the folate receptor as a mechanism for the effective targeting of antitubercular drugs remains to be determined, there are still unanswered questions regarding the underlying biology behind folate receptor expression and whether expression is modulated by mycobacteria in mouse and human macrophages. Continued in vivo studies may establish whether there is any correlation between folate receptor expression and Mycobacterium infection in mice. Successful outcomes may lead to new mechanisms to enhance the efficacy of antibiotics against tuberculosis through drug targeting.
LIST OF REFERENCES


