Immunoregulation of host macrophage responses by *Mycobacterium tuberculosis*

Dissertation

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By

Bin Ni

Graduate Program in Biomedical Sciences Graduate Program

The Ohio State University

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Dissertation Committee:

Professor Larry Schlesinger, M.D., Advisor

Professor Amal Amer, M.D. Ph.D.

Professor Clay Marsh, M.D.

Professor Stephanie Seveau, Ph.D.
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Abstract

One-third of the world population is infected with Mycobacterium tuberculosis (M.tb), the causative agent of tuberculosis, which leads to 1.3 million deaths annually. M.tb is a host-adapted intracellular pathogen of macrophages. A complex, evolutionally adaptive relationship exists between M.tb and the host during which the pathogen evades and subverts the immune response mounted by the macrophage. Understanding the interactions during primary infection between the macrophage and M.tb are important as they determine outcomes of infection and course of disease. In this dissertation we examined the roles of miRNAs and MAPK pathways in M.tb infection of primary human macrophages and identified two mechanisms through which M.tb can down-regulate macrophage immune responses and promote its intracellular survival.

We determined the miRNA expression profile of M.tb-infected monocyte-derived macrophages (MDM) by the NanoString nCounter miRNA Expression Assay and identified a number of miRNAs that were differentially expressed relative to the non-infected controls 24 h and 72 h after M.tb infection. Among the up-regulated macrophage miRNAs at 72 h after M.tb infection were miR-132 and miR-26a, which we show down-regulates expression of the transcriptional activator p300, a component of the IFN-γ signaling pathway that mediates transcriptional responses to IFN-γ in macrophages.
We studied effects of miR-132 and miR-26a on macrophage IFN-\(\gamma\) responsiveness using miR-132 and miR-26a specific miRNA inhibitors delivered by nucleofection to knock down miR-132 and miR-26a expression in MDMs. The miRNA knockdown MDMs demonstrated improved transcriptional, translational, and functional responses to IFN-\(\gamma\). However, we observed only limited effects of miR-132 and/or miR-26a knockdown on reducing intracellular \(M.\text{tb}\) burdens. Overall, these data suggest that during human \(M.\text{tb}\) infection there is decreased capacity for macrophages to be activated by IFN-\(\gamma\) and perform microbicidal functions.

In addition, we show that the expression of the MAP3K Tpl-2, which was previously identified as a host defense molecule against \(M.\text{tb}\) in a murine knockout model, is down-regulated during \(M.\text{tb}\) infection in human primary macrophages. Concurrently, we observed increased mRNA levels of Tpl-2, suggesting a role for miRNAs in targeting Tpl-2 mRNA or other post-translational regulatory mechanisms such as loss of stabilization by p105 in suppression of Tpl-2 protein expression. We also reported on the downstream signaling effects of \(M.\text{tb}\)-induced suppression of Tpl-2 by studying ERK and MEK activation after stimulation with toll-like receptor (TLR) ligands. \(M.\text{tb}\) Erdman lipomannan (ErdLM) stimulation of \(M.\text{tb}\)-infected macrophages showed decreased activation of ERK1/2 and MEK, congruent with decreased Tpl-2 levels after \(M.\text{tb}\) infection. However, the ERK1/2 activation did not significantly decrease in \(M.\text{tb}\)-infected MDMs after treatment with the pure TLR agonists LPS, Pam3CysSK4, and CpG ODN,
although decreased MEK phosphorylation was observed, suggesting that alternative ERK1/2 activating pathways are activated with the pure TLR ligands but not with ErdLM. These data suggest that *M.tb* has the capacity to suppress Tpl-2 and alter MAPK signaling to promote a survival advantage within the macrophage.

Rise in rates of multidrug-resistant and extensively drug-resistant tuberculosis indicate a dire need for new lines of therapies against novel targets. The results of these studies indicate that miRNAs, especially miR-132 and miR-26a, as well as MAPK pathways, including Tpl-2, may be excellent targets of host-directed therapies for TB and the knowledge gained here will benefit future studies to these ends.
Dedication

This document is dedicated to my family and friends. In particular to my mother, Ke Wang, who is the strongest person I know and who has taught me that with hard work and determination nothing is impossible. I also dedicate this document to Ross Glashan for his unwavering patience and support. This endeavor would not have been possible without the encouragement of these individuals.
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Vita

2004.................................................................Upper Arlington High School

2004-2008 ......................................................B.S. Biology, Massachusetts Institute of Technology

2008-present .....................................................Medical Scientist Training Program, The Ohio State University

2009.................................................................MD/PhD Leadership and Academic Achievement Scholarship

2011-2013 .........................................................Susan L. Huntington Dean’s Distinguished University Fellowship, The Ohio State University

2013-2014 ..........................................................Center for Microbial Interface Biology T32 training grant

2013.................................................................Keystone Symposia scholarship for Host Response in Tuberculosis meeting, Whistler, Canada

2013.................................................................12th Annual OSUWMC Research Day poster competition travel award
2013.................................Travel award to 20th Annual Midwest Microbial Pathogenesis Conference, Columbus, Ohio

2014.................................Workshop speaker, Keystone Symposia in Molecular Cell Biology of Macrophages in Human Diseases, Santa Fe, New Mexico

2014.................................Allan Yates Memorial Trainee Speaker Series, 13th Annual OSUWMC Research Day

Publications


Fields of Study

Major Field: Biomedical Sciences Graduate Program
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Abbreviations

3’UTR = 3’ untranslated region
Ago = argonaute
AP-1 = activator protein-1
BCG = *Mycobacterium bovis* BCG
BMDM = bone marrow derived macrophage
CBP = CREB-binding protein
CFP-10 = culture filtrate protein of 10 kDa
CpG ODN = CpG oligodeoxynucleotide
CR = complement receptor
DC-SIGN = dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DGCR8 = DiGeorge syndrome critical region gene
dsRNA = double-stranded RNA
DUSP = dual specificity phosphatase
ErdLM = *Mycobacterium tuberculosis* Erdman strain lipomannan
ERK = extracellular signal-regulated kinase
ESAT-6 = early secreted antigenic target of 6 kDa

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ESX-1 = ESAT-6 secretion system-1
FOXO1 = forkhead box protein O1
GAS = γ-activation sequence
HIV = human immunodeficiency virus
HSC = hematopoietic stem cell
IFN = interferon
Ig = immunoglobulin
IL = interleukin
IRAK = interleukin-1 receptor-associated kinase
JAK = janus kinase
LM = lipomannan
LPS = lipopolysaccharide
MAC = Mycobacterium avium complex
ManLAM = mannose-capped lipoarabinomannan
MAP3K = MAP kinase kinase kinase
MAPK = mitogen-activated protein kinase
MDM = monocyte-derived macrophage
MDR-TB = multidrug-resistant tuberculosis
MEK = mitogen-activated protein kinase kinase
MHC = major histocompatibility complex
MIP = macrophage inflammatory protein
miRNA = microRNA
MR = mannose receptor

*M. tb* = *Mycobacterium tuberculosis*

MyD88 = myeloid differentiation primary response gene 88

NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells

NK = natural killer

NOD = nucleotide oligomerization domain

p120RasGAP = p120 Ras GTPase activating protein

Pam₃CysSK₄ = Pam₃Cystein-Serine-(Lysine)₄

PAMP = pathogen associated molecular pattern

PBMC = peripheral blood mononuclear cell

PKA = protein kinase A

PKI = protein kinase inhibitor

PPAR = peroxisome proliferator activated receptor

PRR = pattern recognition receptor

PTPN = protein tyrosine phosphatase, non-receptor

qRT-PCR = quantitative real-time polymerase chain reaction

RD1 = region of difference 1

RISC = RNA induced silencing complex

RNAi = RNA interference

SD = standard deviation

SEM = standard error of the mean

siRNA = small interfering RNA
SOCS = suppressor of cytokine signaling
SRBC = IgG-coated sheep erythrocyts
STAT = signal transducer and activator of transcription
TAM = tumor associated macrophage
TB = tuberculosis
TCR = T cell receptor
Th1 = type 1 helper T cells
Th2 = type 2 helper T cells
TLR = toll-like receptor
TNF = tumor necrosis factor
Tpl-2 = tumor progression locus-2
TRAF = TNF receptor associated factor
TRBP = TAR RNA-binding protein
WHO = World Health Organization
XDR-TB = extensively drug-resistant tuberculosis
Chapter 1: Introduction

1.1 Tuberculosis

1.1.1 A historical perspective

The airborne infectious disease tuberculosis (TB) is an ancient disease that has plagued humans since antiquity. The earliest archaeological evidence for TB comes from mummies in Egypt that are estimated to be over 5000 years old; however, it is believed that TB is even older and originated in East Africa around the same time as the first hominids (1). Throughout the ages TB has been known by different names including phthisis in classical Greece, scrofula in the Middle Ages, and consumption in the 19th century, all of which contributed to the difficulty in connecting the signs and symptoms to identify an etiology for this disease. Some believed TB to be a result of heritable defects, while others attributed it to an infectious agent. It was not until 1882 that the bacterium Mycobacterium tuberculosis (M.tb) was identified as the etiologic agent of TB by Robert Koch in his famous presentation to the Berlin Physiological Society (2). Koch was awarded the 1905 Nobel Prize in Medicine or Physiology for this seminal discovery. Despite having identified the etiologic agent, TB remained a major killer throughout the early 1900s as there remained few effective therapies. Open air sanatoria or spas were
popular to offer TB sufferers rest and a hygienic environment to promote recovery, and also served to isolate the infected, but the first effective chemotherapy against TB, streptomycin, was not discovered until 1943 (1).

1.1.2 Current state of tuberculosis

At the time that the causative agent for TB was identified it was estimated that one of every seven people in the world was infected, today that number stands at one in three (3). According to the WHO, there were 8.6 million new cases of TB and 1.3 million deaths from the disease in 2012, making it the second leading cause of death from a single infectious agent, after HIV (4). Worldwide, approximately 2 billion people are infected by M.tb with the vast majority existing in latency. However, it is predicted that 10% of these latently infected individuals will go on to develop reactivation tuberculosis at some later point during their lifetime if they remain healthy (5). Rates of reactivation are greatly increased by immunosuppression such as during co-infection with HIV; consequently, TB is the leading cause of death in HIV patients (4). Effective chemotherapy for TB is now also under threat with rates of multi-drug resistant TB (MDR-TB), defined as having resistance to both first-line drugs isoniazid and rifampicin, on the rise globally. Even more ominous is the detection of extensively drug-resistant TB (XDR-TB), TB that is resistant to first-line as well as second-line therapeutics, in 92 nations by 2012 (4). TB primarily kills adults in the prime of life, leading to considerable loss in productivity that has dire social and economic consequences for nations with the
heaviest TB burdens; therefore, TB is a major global health emergency deserving of resources for improved control and study (6,7).

1.1.3 *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* is the causative agent of TB; it is a slow growing acid fast bacterium whose natural reservoir are humans. The route of infection for *M.tb* is almost always through the lung, as a result of inhalation of airborne droplets containing the bacterium released by infected individuals. Inhaled bacteria are eventually deposited in the lung alveolus where as few as one to five bacteria can result in infection. *M.tb* in the alveoli is engulfed by the resident alveolar macrophages, which are unable to completely kill and clear the bacteria. Instead, *M.tb* uses the macrophage as its cellular host. Primary *M.tb* infection is generally mild and self-limited, often going unnoticed. However, during this time bacillemia can occur, resulting in deposition of bacteria in other organs that serve as a nidus for extrapulmonary reactivation at a later stage (5). Most *M.tb* infections are usually contained and result in latency; even though the infection is not completely eradicated, there are no clinical signs of disease until reactivation occurs. Despite the global health importance of TB, we still have only limited therapies and therapeutic targets for *M.tb*. *M.tb* employs a variety of mechanisms to avoid immune detection and to persist intracellularly. Our failure to completely understand all of the avenues utilized by *M.tb* for persistence creates a barrier in our attempts to develop new and more effective treatment strategies.
1.2 The Lung

1.2.1 Human lung anatomy

The lungs are the respiratory organs of the body and in humans are comprised of five lobes, three on the right side of the body and two on the left, each surrounded by the pleural membranes (8). Upon inhalation air enters through the nose and mouth, traveling down the trachea before reaching the first branches of the two main bronchi and entering the roots of the lungs. From there each bronchus branches into successive generations of bronchi and eventually bronchioles before reaching the terminal bronchioles that contain the alveolar ducts. At approximately the 23rd generation of branching the alveolar ducts end in grape-like clusters of small cavities known as the alveoli where oxygen from inspired air is taken up into the meshwork of capillaries enveloping the alveolus and carbon dioxide is returned to the outside through exhalation (9). Functionally the respiratory tract can be divided into the conducting zone and the respiratory zone. The conducting zone, comprised of the nose, pharynx, larynx, trachea, bronchi, bronchioles, and terminal bronchioles, serves to warm and moisten air before it enters the respiratory zone, consisting of the respiratory bronchioles and the alveolar ducts, where gas exchange occurs.

1.2.2 Upper and lower airway responses to particulates and microorganisms

In the course of normal breathing the respiratory tract is constantly exposed to the outside environment and must have effective means for removal of inhaled particulates and
microorganisms. In general, a combination of mechanical, soluble, and cellular defenses are very successful in maintaining a clean airway. Anatomical division of the respiratory tract can be made into the upper and lower respiratory airways. The upper respiratory airway consists of the nasal cavity and sinuses, as well as the pharynx and larynx; here the mechanical means of host defenses predominate. Nasal hairs prevent initial entry by filtration while the tortuous structure of the nasopharynx acts as an additional barrier and promotes contact of particulates with mucociliary defenses (10). A blanketing layer of mucus and ciliated cells can capture and propel microorganisms by ciliary movement out to the oropharynx for removal. Finally, the sneeze and cough reflexes can clear material that have accrued and irritates in the upper respiratory airway (11). In addition to mechanical means of defense, cells of both the upper and lower respiratory airways can produce a number of anti-microbial molecules including lysozyme, complement, antibodies, defensins, cathelicidins, and collectins (11). For the most part these mechanisms are effective in preventing entry of large particles greater than 5 µm in diameter, but smaller microorganisms, such as \textit{M.tb} can bypass these defenses to the lower respiratory airway to gain entry into the alveoli (10).

\textit{1.2.3 Immunity in the alveolus}

The alveolus is the terminal structure of the bronchial tree and the location of gas exchange in the lung. Structurally, the alveoli are clusters of small open air sacs that promote gas exchange by optimizing the surface area across which gases can diffuse; a normal adult lung is estimated to have an alveolar epithelium the size of a tennis ball
Each alveolus is enmeshed in a fine capillary bed and has delicate walls that are only two cells thick and so that oxygen and carbon dioxide can freely diffuse across this barrier into and out of blood. The resident alveolar macrophages are the first line of defense against microorganisms that have evaded upper respiratory airway defense mechanisms and entered the alveolus (13). Alveolar macrophages must promptly phagocytose and destroy invaders in order to protect the gas exchange functionality of the alveolus. However, careful balance in immune responses must be maintained to prevent overt inflammatory damage to the alveoli that can result in loss of gas exchange capabilities.

1.3 Macrophages

1.3.1 Introduction

Macrophages are a phenotypically heterogenic group of cells serving diverse functions related to the different tissues in which they reside. Polarization of macrophages allows these cells to take on specific effector functions appropriate for their microenvironmental context. In the most general terms, macrophages can be classified into two effector types: classically activated/Th1 type or alternatively activated/Th2 type. However, most current literature acknowledges the fact that macrophage function actually represents a spectrum from classical to alternative activation and that the biological activities vary greatly among mammalian species and activating stimulus.
1.3.2 Classical macrophage activation

The canonical classically activated macrophage is a result of a resting macrophage being exposed to two signals. The first signal is the cytokine IFN-γ produced by the lymphocytes CD4\(^+\) T helper 1 (Th1) cells, CD8\(^+\) T cytotoxic 1 (Tc1) cells, or natural killer cells, which primes the macrophage for classical activation. The second signal the macrophage must receive for classical activation is not absolute and can be another cytokine such as TNF-α, a microbe product like LPS, or a pharmacological agent like taxol. Once activated, the classically activated macrophage can perform an array of effector functions such as increased antigen presentation, increased synthesis and release of pro-inflammatory mediators, and increased capacity for phagocytosis (14). In classically activated murine macrophages the most reliable marker is nitric oxide production for oxidative killing of pathogens; however, human macrophages do not reliably produce nitric oxide even after activation (15). Other markers of a classically activated macrophage include release of TNF-α and IL-12, which act in a feedback loop to increase Th1 differentiation and IFN-γ production. Classical activation also alters the macrophage cell surface molecule composition via increased MHC Class II presentation, increased expression of the T cell co-stimulatory molecule CD86, and decreased mannose receptor (MR) expression. In addition, a variety of chemokines including CXCL10, MIP-1\(\alpha\), and MIP-1\(\beta\) are produced for initiation of a robust inflammatory response (15). The primary biological function of classically activated macrophages is to travel to sites of
inflammation, phagocytose and degrade pathogens, and coordinate a robust immune response.

1.3.3 Alternative macrophage activation

In contrast to classically activated macrophages, alternatively activated macrophages are produced as a result of exposure to IL-4, which is produced by T helper 2 (Th2) cells. They may also be activated by IL-13, another cytokine secreted by Th2 cells that partially shares receptor complexes with IL-4 (16). Th1 and Th2 activity are mutually exclusive as a result of IL-10 inhibition of Th1 during Th2 activation. Similarly, alternatively activated macrophages do not produce nitric oxide, instead shuttling L-arginine into a pathway for use by arginase (15). However, human macrophages do not significantly activate arginase levels; therefore, arginase is not an ideal marker for human alternative macrophage activation. The alternatively activated macrophage generally mediates Th2 type immune responses characterized by eosinophilia, basophilia, and enhanced B cell class switching and antibody production (14). In antithesis to classical activation, alternatively activated macrophages are generally anti-inflammatory in character, producing high levels of the anti-inflammatory cytokine IL-10 and displaying increased MR expression on their cell surface. Alternatively activated macrophages also demonstrate poor macrophage-mediated killing of pathogens, but play an important role in parasite control. In general, alternatively activated macrophages are believed to promote resolution of inflammation and wound healing.
Alveolar macrophages are one of three cell types in the alveolus and a major resident mediator of cellular immune functions. It is estimated that there are over twelve alveolar macrophages per alveolus (13). Through irradiation studies alveolar macrophages were determined to originate from monocyte precursors recruited from peripheral blood which undergo differentiation in the lung to become relatively long-lived resident lung phagocytes (17). However, there is some evidence for local production of macrophages (18). Alveolar macrophages are uniquely adapted to functioning in the lung microenvironment where they act as sentinels against invading organisms but also serve to limit inflammation and minimize lung injury for preservation of alveolar function. Due to their functions in limiting inflammation in the alveolar space, alveolar macrophages are sometimes described as alternatively activated macrophages. However, alveolar macrophages do not precisely fit the paradigm of alternatively activated macrophages as they display increased oxidative killing of pathogens over alternatively activated macrophages and stronger mediation of inflammatory immune effects. Yet the inflammatory responses generated by alveolar macrophages are significantly less than that of classically activated macrophages. Therefore, the dichotomy of classical versus alternative macrophage activation may not be the most appropriate characterization of macrophage types, and alveolar macrophages serve as an example of a macrophage that straddles the line between both groups and are termed “immunoregulatory” by our group.
1.4 Macrophage defenses against *M.tb*

1.4.1 *M.tb* recognition receptors

Within the alveolus the first-line defenders against *M.tb* are the resident alveolar macrophages, which possess a variety of receptors for recognition and engagement of *M.tb* (19). Many of these receptors expressed by macrophages consequently regulate innate immune response pathways, such as cytokine production, after engaging *M.tb*, including the mannose receptor (MR), complement receptors (CRs), the nucleotide oligomerization domain (NOD)-like receptors, and the Toll-like receptors (TLRs) (20,21). In addition, *M.tb* can also interact with the macrophage via scavenger receptors and surfactant protein receptors, especially in the lung microenvironment (22). However, outcomes of engagement through these receptors may not be equal, as some receptors may promote pathways favorable to *M.tb*. For example, a virulent *M.tb* strain appears to preferentially bind via the MR and as a result limits phagosome-lysosome fusion (23,24). In addition, *M.tb* has been shown to alter host receptor expression and signaling in ways that promote its own survival, for instance, through increased expression of the PPARγ receptor or by limiting activation of the MAPK pathways (25,26).

1.4.2 Phagocytosis

Once taken up by the macrophage through one of the macrophage cell surface receptors microorganisms are placed in an intracellular compartment known as the phagosome. The phagosome is itself not hydrolytic, but maturation of the phagosome through a series of
molecular events culminating in fusion with lysosomes produces the acidic phagolysosome, which is able to break down ingested particles and microorganisms (27). Following phagocytosis, M.tb can limit phagosome-lysosome fusion and is able to take up residence and replicate within phagosomes in the macrophage (28,29). This is accomplished through a variety of mechanisms including preferential entry of the macrophage through the MR (24). Once in the phagosome M.tb can block the accumulation of ATPases and GTPases on the phagosome surface required for phagosome maturation through secretion of a number of M.tb virulence factors including ESAT-6, CFP10, and SecA 1/2 (30). Additionally, M.tb can also interfere with membrane trafficking and calmodulin-dependent calcium flux through a number of pathogen lipoglycans such as the mannose-capped lipoarabinomannan (ManLAM), which is a mimic of host phosphatidylinositols, and can falsely signal a phagosome maturation state (31). While fusion with late endosomes/lysosomes is limited, fusion with early endosomes is maintained, allowing M.tb to gain access to nutrients.

While the traditional view is that M.tb survives and replicates within a membrane bound compartment in the macrophage, there exist reports suggesting that M.tb can escape from the phagosome and live within the cytosol (32). These studies report observations of M.tb within the macrophage without visible host membranes by electron microscopy. However, these cytosolic M.tb bacilli were usually observed late in the course of infection and remained only a minority of bacteria. Therefore, these observations are also consistent with the concept of phagosomal damage; especially in light of the finding that
the *M.tb* secretion system ESX-1 is required for “phagosomal escape”, since ESX-1 is known to damage the phagosome (33).

### 1.4.3 Macrophage activation

“Classical” activation of macrophages by Th1 signals, especially IFN-γ, allows the macrophage to implement a genetic program designed for the destruction of microorganisms. Among the microbicial functions induced in these macrophages are production of pro-inflammatory cytokines such as IL-12 and TNF, increased phagocytosis and oxidative killing of pathogens, as well as increased antigen presentation (34). These responses are especially effective against intracellular pathogens such as *M.tb*, and consequently, IFN-γ is a critical molecule for host defenses against *M.tb*, as evidenced by the profound susceptibility to *M.tb* infection of IFN-γ deficient mice (35,36). However, *M.tb* can also avoid or prevent macrophage activation, which is normally initiated with pathogen entry, thereby reducing the macrophage’s microbe-killing abilities through mechanisms such as preventing the activation of IFN-γ-response genes (37,38). It has been previously observed that *M.tb* pre-infection in human macrophages results in reduced transcriptional responses to IFN-γ treatment, and that this was a result of interruption in transmission of the IFN-γ signal to the nucleus (39). In addition, dramatic increases in transcription of *M.tb* genes have been observed within activated macrophages as compared to resting macrophages, suggesting that *M.tb* has developed specific adaptations in response to survival within activated macrophages (40).
1.4.4 Autophagy

Autophagy is a cellular process wherein cytoplasmic components are broken down and recycled for cellular maintenance. Often induced during cellular starvation, autophagy is a means through which surplus organelles are degraded to support cellular viability by means of envelopment by a double membrane forming the autophagosome. Like the phagosome, the autophagosome eventually delivers materials to the lysosome for degradation. Autophagy is another means through which intracellular bacteria can be eliminated. The importance of this pathway in *M. tb* infection is clearly demonstrated by Atg5 deficient mice, which show a susceptibility to *M. tb* infection similar to that of IFN-γ deficient mice (33). Studies on whether induction of autophagy could overcome *M. tb* inhibition of phagosome maturation indicate that multiple inducers of autophagy (physiological, pharmacological, or immunological) could decrease intracellular bacterial burdens over a short course of induction (41). Therefore, autophagy is yet another mechanism through which macrophages can eliminate intracellular pathogens. However, there is emerging evidence that *M. tb* may be able to modulate host induction of autophagy by down-regulating autophagy molecule LC3 (42).

1.4.5 Apoptosis

If other means of controlling an intracellular pathogen fail, the macrophage has one last option. It can delete infected cells from the population in a controlled cell death process through apoptosis, thereby also killing pathogens within the cell. However, *M. tb* can suppress macrophage apoptosis, permitting the accumulation of a high intracellular
bacillary load and eventual bacterial escape from the expended macrophage through necrosis (43). Inhibition of apoptosis appears to be a characteristic of virulent M.tb, since avirulent and attenuated strains of mycobacteria are demonstrated to induce higher levels of apoptosis. Although the exact mechanism behind M.tb inhibition of apoptosis is as yet unknown, a number of pathways leading to apoptosis have been observed to be affected by M.tb infection. For example, the cytokine TNF-α is a potent inducer of apoptosis and is an important cytokine in the formation and maintenance of granulomas, which is rich in the number of apoptotic cells, but virulent M.tb has been observed to decrease TNF secretion through up-regulation of host miR-125b expression (30,44,45). In addition, it has been hypothesized that M.tb may be able to preferentially drive macrophages towards necrosis, rather than apoptosis, by inhibiting plasma membrane repair through products of its ESX-1 secretion system and by inducing inner mitochondrial membrane damage in a pathway separate from mitochondrial induced apoptosis (46). Although the macrophage is well adapted for control of invading pathogens, M.tb is uniquely able to subvert these defense mechanisms and utilizes many pathways to escape macrophage killing and establish growth.

1.5 MicroRNAs

1.5.1 Discovery

The first miRNA, lin-4, and its mRNA target, lin-14, were discovered in studies of Caenorhabditis elegans development by the Ambros and Ruvkun laboratories. Null
mutations of the two genes were observed to have opposing phenotypes, suggesting that one was a negative regulator of the other. Ambros, along with Lee and Feinbaum, carried out studies of lin-4 but could not identify a protein product for the gene; instead they observed only two small RNA transcripts that were 61 nucleotides and 22 nucleotides in length (47). At the same time Ruvkun, along with Wightman and Ha, discovered that lin-14 was regulated post-transcriptionally and that the lin-14 3’ untranslated region (3’UTR) was necessary for regulation (48). In 1993 the Ambros and Ruvkun laboratories published in independent reports the same conclusion: lin-4 regulates translation of the lin-14 mRNA by binding to its 3’UTR. These studies uncovered a novel mechanism of cellular regulation, ushering in a new era for non-coding RNAs. However, it was seven years later before a second miRNA, let-7, was discovered, also in *C. elegans*. Not only did let-7 also produce no protein yet have the ability to regulate translation of other genes, but it was conserved across species, including having detectable expression in the majority of human tissues (47). Consequently, these studies led to the discovery of a new class of regulatory molecules called miRNAs and resulted in the identification of thousands of miRNAs across species.

1.5.2 miRNA function and biogenesis

miRNAs are endogenous noncoding small RNAs that function as gene regulators by mediating translational repression or degradation of target mRNAs, which can have widespread effects on signaling pathways (49). Since their discovery, miRNAs have been implicated in a variety of biological processes such as development and patterning,
oncogenesis, apoptosis, and cellular differentiation, including immune lineage
differentiation (50). miRNAs are transcribed from intergenic or intragenic regions of the
genome by RNA Polymerase II or III as a long stem loop hairpin containing pri-miRNA;
pri-miRNAs may contain one or multiple miRNAs. Still within the nucleus pri-miRNAs
are processed by the Microprocessor complex, composed of the RNase III Drosha and its
binding partner DiGeorge syndrome critical region gene (DGCR8), into 60-80 nucleotide
pre-miRNAs (51). Pre-miRNAs are exported from the nucleus by Exportin 5 to the
cytoplasm where further processing by the RNase III Dicer and the TAR RNA-binding
protein (TRBP) occurs. Dicer activity produces a small double-stranded RNA duplex
containing the mature miRNA and an anti-sense passenger strand. Shortly after
processing, the miRNA duplex is separated and the mature miRNA strand is incorporated
into Argonaute (Ago) protein-containing complexes called the RNA-induced silencing
complex (RISC), while the passenger strand is usually degraded. Guided by imperfect
complementary base pairing of the miRNA in RISC with 3’UTR sequences of the
mRNA, mRNAs can be targeted for translational repression or degradation (52). The
exact mechanisms by which RISC functions to repress translation or cleave mRNAs
remain unknown at this time (48).

1.5.3 miRNAs and siRNAs: similarities and differences

Five years after the initial discovery by Ambros and Rukvun of the regulatory
relationship between lin-4 and lin-14 the laboratories of Fire and Mello reported that
exogenous double-stranded RNAs (dsRNA) approximately 500 base pairs in length could
silence genes in *C. elegans* in a specific fashion. The phenomenon reported by Fire and Mello was later named RNA interference (RNAi). In depth studies demonstrated that the RNAi pathway also required processing by Dicer for the formation of small dsRNAs approximately 20-25 nucleotides in length, termed small interfering RNAs (siRNA). Furthermore, a single strand of the siRNA duplex is selectively incorporated into RISC for repression of gene targets. In terms of processing and effector function siRNAs and miRNAs share many similarities. With both siRNA and miRNA processing by Dicer and incorporation into RISC is required for function. Additionally, both classes of molecules recognize target mRNAs based on Watson-Crick base pairing (53). However, three important distinctions can be made between miRNAs and siRNAs as similar but fundamentally separate classes of molecules. Firstly, miRNAs are generally considered to be endogenous gene products transcribed from the cell’s own genome through appropriate transcriptional regulatory pathways, whereas siRNAs are exogenous in source. Secondly, miRNAs are processed from single-stranded stem loop hairpin containing precursors while siRNAs originate as large true double-stranded molecules with full complementarity. Thirdly, miRNAs often bind with imperfect complementarity to mRNA targets resulting in both translational repression and mRNA degradation. However, siRNAs more commonly bind with perfect complementarity to initiate direct cleavage of target mRNAs. Despite these differences, siRNAs and miRNAs are closely related classes of molecules with similarities in biogenesis and function, and knowledge gained from studies of one class has contributed immensely to understanding of the other.
1.5.4 miRNAs in the immune system

Recent years have seen increasing recognition of a miRNA role in regulation of the immune system. Many studies have been undertaken to study expression of miRNAs in various subsets of immune cells with the finding that selective expression of certain miRNAs correspond with the differentiation of pluripotent hematopoietic stem cells (HSC) into various lineages of immune cells (54). Among the best studied miRNA roles in immune cell development are miR-181a and miR-155.

In a murine model of B cell differentiation, increased expression of miR-181a in HSC resulted in an increase in the number of B cells, suggesting that miR-181a regulates B cell differentiation (55). A miR-181a role in T cell development is still inconclusive due to inconsistent data, but miR-181a has been shown to modulate T cell sensitivity by targeting a number of tyrosine phosphatases including DUSP5, DUSP6, SHP2, and PTPN22, that negatively regulate TCR signaling. Naturally occurring expression of miR-181a during early T cell development can therefore regulate positive or negative selection during thymic development (56).

miR-155, transcribed from an exon of the non-protein encoding gene bic, has been shown to regulate B and T cell maturation. miR-155 knockout mice are unable to generate a protective immune response after immunization due to decreased IgG1 antibody levels and memory B cell development; both are due to decreased expression of the transcription factor PU.1. In T cells loss of miR-155 promotes differentiation into the Th2
rather than Th1 lineage, resulting in production of Th2 cytokines IL-4, IL-5, and IL-10 along with attenuated IFN-γ release in response to IL-2 (56). In addition to its roles in adaptive immunity, miR-155 also has multiple roles in innate responses. In dendritic cells miR-155 can down-regulate expression of DC-SIGN and alter pathogen binding, while in macrophages and microglia miR-155 depresses SOCS-1 to increase inflammatory cytokine production (57-59).

A miRNA role has also been discovered in oncogenic dysregulation of immune cell development. Since miRNAs play a central role in immune cell development it is not surprising that they may also mediate oncogenesis when regulation of miRNA expression goes awry. The first report for a miRNA role in cancer came from the Croce laboratory in 2005 (60). Cimmino, et al. reported that the majority of chronic lymphocytic leukemia cells possessed either deletion of or misregulation in miR-15 and miR-16. Furthermore, miR-15 and miR-16 expression was inversely correlated with expression of Bcl2, an anti-apoptotic factor. Therefore, these two miRNAs promoted apoptosis and acted as “tumor suppressors” that when lost resulted in development of cancer (61).

In the case of miRNAs and innate immunity one of the most-studied immune roles for miRNAs is in regulation of the TLR pathways, which mediate a myriad of immune functions including, but not limited to, cytokine production, phagocytosis, apoptosis, and antigen processing and presentation (62). Many miRNAs have been observed to target the TLRs or TLR-pathway signaling molecules, suggesting that miRNAs may act as
“fine-tuners” of TLR signaling (63-65). This may be especially important in the lung microenvironment where a delicate balance must be struck between activating inflammation and preserving gas exchange. At the same time, studies have also demonstrated that miRNA expression can be regulated by TLR signaling (66,67). miRNAs such as miR-155 and miR-146 are up-regulated during TLR activation with opposite effects. In addition to its role in T and B cell development, miR-155 was induced after TLR activation by both bacterial and viral ligands and served to positively drive release of inflammatory mediators (54). On the other hand, increases in miR-146 expression were the result of only non-nucleic acid activation of TLRs and negatively regulated inflammation in a feedback mechanism (68). While we do not yet have a clear picture of the global impact miRNAs may have on innate immunity, it is evident that a complex intertwining relationship exists between miRNAs and innate immune pathways.

1.5.5 miRNAs in macrophage responses

A robustly developing area of study is in miRNA regulation of macrophage responses, both in macrophage differentiation and activation as well as in contributions to macrophage mediated pathological states. As noted earlier, macrophages encompass groups of cells with a wide array of effector abilities in response to the specific microenvironmental milieu encountered by the cells. Macrophages can be broadly generalized into two activation states with expression of characteristic gene programs: classical activation due to IFN-γ exposure and alternative activation after IL-4 stimulus. Studies of individual miRNA effects on macrophage polarization as well as systematic
searches for miRNAs involved in classical or alternative activation have been carried out. For example, Chaudhuri, et al. reported that over-expression of miR-125b promotes activation of macrophages and increased responsiveness to IFN-\(\gamma\) as a consequence of decreased IRF-4 expression (69). In contrast, Banerjee et al. observed that over-expression of miR-125a-5p promoted expression of markers of alternative activation after IL-4 induction (70). An additional function for miR-155 in macrophages appears to be modulation of the alternative activation phenotype by targeting the IL-13 receptor \(\alpha_1\), the receptor for IL-13, a pro-alternative activation phenotype cytokine (71). Several expression profiling studies using microarray or next-generation sequencing technologies have also been undertaken for unbiased screening of miRNAs in polarized macrophages. While a number of miRNAs were reported to be specifically expressed in various polarized states, there is no consensus between studies, likely as a result of variations in the polarization schemes and cell types utilized (72-74). Dysregulation of miRNAs are also under study in pathological macrophage states such as tumor associated macrophages (TAM) or foamy cells in atherosclerosis (75,76). Although as yet, no single set of miRNAs has emerged as governors of macrophage responses, these studies will have tremendous impact in our understanding of how macrophages are activated and may in the future serve as therapeutic targets for macrophage-mediated diseases.

### 1.5.6 miRNAs in immune responses to bacterial pathogens

Initial work on the miRNA role in infectious disease has focused on viral pathogens. Through these studies we have learned that both the host and the invading viruses have
the capacity to express miRNAs for their own gain. However, understanding of miRNA functions during bacterial infection has lagged behind. Only in recent years has a role for miRNAs in immune responses to bacterial infections begun to be defined (77). Among the first pieces of evidence that bacterial infections can alter miRNA expression was in a gastric epithelial infection model of *Helicobacter pylori*. Microarray analysis indicated that expression of miR-155 and miR-146 were increased during infection, later determined to be as a result of exposure to *H. pylori* virulence factors VacA and GGT (78). However, subsequent studies were not able to recapitulate these findings, but did identify involvement of additional miRNAs and their targets in *H. pylori* infection of human gastric mucosa (79,80). Intracellular pathogens such as *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* can also alter miRNA expression in immune cells during infection. Deep sequencing of *Salmonella*-infected murine macrophages indicated an increase in core immunoregulatory miRNAs miR-155 and miR-146a, but interestingly also saw decreased expression of several members of the let-7 miRNA family, which was shown to target production of IL-6 and IL-10. The opposing effect of IL-6 and IL-10 on inflammation makes decrease of let-7 family miRNAs during *Salmonella* infection a noteworthy finding, which the authors of the study attribute to promoting a balanced immune response during *Salmonella* infection (81). As in the case with *Salmonella* infection, decreases in miR-29 expression in natural killer cells (NK) after systemic *L. monocytogenes* infection also results in modulation of host immune responses to the pathogen. In this study, a model was proposed wherein down-regulation of miR-29 allows for increased secretion of IFN-γ by NK cells to promote host resistance
to intracellular pathogens such as *L. monocytogenes*, and sequestration of miR-29 by expression of a miR-29 “sponge” target resulted in lower *L. monocytogenes* burdens (38).

miRNA responses to pertinent mycobacteria species including *Mycobacterium bovis* BCG, the vaccine strain for tuberculosis, and *Mycobacterium avium hominissuis*, a member of the *M. avium* complex (MAC) have been reported by several studies. Both of these members of the *Mycobacterium* genus can be opportunistic pathogens in individuals with already altered immune systems and therefore, findings in miRNA functions should be evaluated in this context. Among the cellular functions found to be regulated by miRNAs during infection with non-*M.tb* mycobacteria are apoptosis, inflammation, and MyD88 signaling. Ghorpade et al. studied miR-155 mediated transduction of apoptosis signals by targeting PKA negative regulator PKI-α in a murine model of BCG infection and found that miR-155 knockout severely compromised BCG-induced apoptosis in macrophages (82). In contrast, *M. avium* infection resulted in increased expression of let-7e and miR-29a, which was associated with decreased expression of the pro-apoptotic caspases caspase 3 and caspase 7, respectively (83).

Another miRNA that may promote bacterial survival is expression of miR-146a in BCG-infected murine macrophages. Consistent with its previously observed anti-inflammatory effect in macrophages after LPS treatment, miR-146a was also demonstrated to down-regulate IRAK-1 and TRAF-6, two components of the TLR/NF-κB pathway that mediate the inflammatory response, thereby facilitating BCG survival (84). A separate report
from Xu, et al. indicated that miR-142-3p could also target IRAK-1 in a murine macrophage cell line after BCG infection (85).

The data surrounding BCG modulation of MyD88 signaling via miRNAs is much murkier. In three separate reports four miRNAs, miR-150, miR-31, miR-149, and miR-203, were identified as negative regulators of the TLR signaling mediator MyD88, which may have important downstream effects on mycobacterial infection, as well as interactions with other pathogens. Xu et al. observed a decrease in miR-149 expression after BCG infection of the murine macrophage cell line RAW264.7 along with corresponding increase in MyD88 levels (86). However, sonic hedgehog driven miR-31 and miR-150 expression during BCG infection of BMDM and primary human macrophages reduced MyD88 expression, leading to suppression of TLR2 responses (87). Exogenous expression of miR-203 in RAW264.7 cells also down-regulated MyD88 resulting in decreased inflammatory signaling and responses after BCG infection, but it is unclear what role miR-203 may play in the natural course of BCG infection (88). In summary, although much progress has been made in identifying a role for miRNAs in response to bacteria, there is still much work to be accomplished before we achieve full understanding of what function miRNAs are playing in immune responses to bacterial infections.
In recent years a number of studies were published examining miRNAs in virulent \textit{M.\textit{tb}} infection with hopes of producing advances in diagnostics or therapeutics (89,90). However, like studies involving other mycobacteria the results of these studies on miRNAs in \textit{M.\textit{tb}} infection remain unclear on which miRNAs and targets are true prognosticators of disease and players in pathogenesis. Several studies have been carried out comparing miRNA profiles in TB patients (active or latent) with healthy controls, yet each report generates a set of altered miRNAs containing little overlap with previous reports. One major caveat that hinders progress through these studies is the heterogeneity of sample sources. miRNA profiles of TB patients have been obtained from serum and sputum, which mostly rely on detection of extracellular sources of miRNAs; miRNA profiles obtained in this fashion may not be truly indicative of \textit{M.\textit{tb}}-induced processes (91-95). Even the studies that examined miRNA expression in human cells utilized mixed cell populations such as peripheral blood mononuclear cells, which may mask cell type-specific miRNA responses during \textit{M.\textit{tb}} infection (96,97). In addition, the majority of miRNA profiling studies in TB patients fail to follow up on targets for altered miRNAs, knowledge of which would aid in identification of the most important miRNAs for diagnostic biomarkers (98-100).

There exists a number of promising studies of miRNAs in \textit{M.\textit{tb}} infection with validated targets. Some, however, were carried out exclusively in murine models or murine cell lines. Although miRNAs are conserved between species, regulation of their expression
may not be identical, and so expression patterns of these miRNAs and relationships with their target mRNAs must be validated in human model systems (101-103). Human \(M. tb\) miRNA studies have identified a number of miRNAs and their validated targets in an array of cell types from macrophages to T cells to neutrophils, indicating that diverse cell populations contribute to \(M. tb\) control and miRNAs may have different functions in different cell types. One of the first reports of functional effects for a \(M. tb\) altered miRNA came from Liu et al. detailing an increased expression of miR-144* in the peripheral blood mononuclear cells of pulmonary TB patients. Increased expression of miR-144* during \(M. tb\) infection was later localized to the T cell population and observed to inhibit IFN-\(\gamma\) and TNF-\(\alpha\) secretion, two important cytokines for \(M. tb\) control (104). Soon after, Rajaram et al. reported increased miR-125b expression with virulent \(M. tb\) infection, but not with avirulent \textit{Mycobacterium smegmatis}, an increase that resulted in destabilization of TNF mRNA and decreased TNF-\(\alpha\) secretion in human primary macrophages (45). These studies indicate that modulation of cytokine production by miRNAs may be an effective mechanism for \(M. tb\) to escape immune control. In monocytes an increase in miR-582-5p expression was observed in patients with active TB and validated as a negative regulator of FOXO1, resulting in decreased apoptosis (105). Finally, a host-protective increase in miR-223 expression was observed during miRNA expression profiling of TB patient blood and lung tissue by Dorhoi et al., which was followed by functional studies in a miR-223 knockout mouse (106). These studies indicated that miR-223 directly targets chemokines CXCL2 and CCL3 and cytokine IL-6 to regulate neutrophil chemotaxis and inflammatory function.
1.6 Summary

On respiration the human lung is exposed to a constant barrage of environmental pollutants; the alveolus is a delicate structure whose physiology must be preserved for gas exchange. Consequently, the lungs and respiratory tract contain many redundant layers of strategies for the elimination of airborne particulates and microorganisms. The airborne pathogen *M. tb* is able to evade these defenses and can gain entry to the alveolus, where it encounters the alveolar macrophage. The alveolar macrophage initiates a controlled immune defense response against *M. tb* and also possesses a variety of mechanisms for eliminating the pathogen. However, *M. tb* is able to subvert many of these macrophage defenses to establish infection within the macrophage itself. The large number of latently infected individuals and the alarming increase in the rate of MDR- and XDR-TB globally are impetuses for finding improved detection techniques and treatments for TB. Understanding the means through which *M. tb* can subvert host defenses, for instance through alteration of macrophage signaling and miRNA expression, can generate novel targets for host-directed therapies that when used in conjunction with traditional antibiotic therapies can curb the global health crisis which TB poses.

1.7 Specific Aims

Since *M. tb* is primarily an intracellular pathogen of macrophages and through co-evolution with humans is well-adapted to survival within the macrophage by
manipulating its defenses, it is essential that we study the altered macrophage responses during *M. tb* infection. Only when we understand how *M. tb* manipulates the macrophage’s defense mechanisms can we begin to develop targeted host-directed therapies to prevent establishment of infection. Emerging evidence suggests that miRNAs may be a double-edged sword in immune responses to *M. tb* infection, having the ability to both hinder and help the host; therefore, we hypothesize that miRNAs may also regulate human macrophage responses to *M. tb*. Additionally, herein we explore the role of Tpl-2 in human macrophage responses to *M. tb* as it is a recently identified host resistance factor against *M. tb* and other intracellular pathogens in murine knockout models and may be post-transcriptionally regulated. The specific aims of this dissertation are as follows:

1. Identify differentially expressed human macrophage miRNAs during virulent *M. tb* infection by miRNA expression profiling and establish a schema for finding and validating mRNA targets of altered miRNAs.

2. Demonstrate that miR-132 and miR-26a, which are up-regulated during *M. tb* infection, regulate human macrophage IFN-γ responses by targeting transcriptional co-activator p300.

3. Determine how MAPK pathway signaling molecule and host immune resistance factor Tpl-2 expression is modulated during *M. tb* infection in human macrophages and the effects of its suppression on macrophage signaling responses.
Chapter 2: Human macrophage microRNAs are significantly altered by

*Mycobacterium tuberculosis* infection

2.1 Introduction

MicroRNAs (miRNA) are endogenous noncoding small RNAs that function as gene regulators most commonly by mediating translational repression or degradation of target mRNAs, which can have widespread effects on signaling pathways (107). Since their discovery, miRNAs have been implicated in a variety of biological processes such as development and patterning, oncogenesis, apoptosis, and cellular differentiation, including immune lineage differentiation (108). Viruses are also known to be elicitors of both miRNA-mediated pro- and anti-viral mechanisms (109,110). In recent years an increasingly fruitful avenue of research is in host miRNA responses to bacterial pathogens. Infection by a number of important human bacterial pathogens including *Listeria monocytogenes, Helicobacter pylori, Francisella tularensis, Salmonella enterica, Mycobacterium avium*, and also *Mycobacterium tuberculosis (M.tb)* have been observed to alter host miRNA expression (38,45,78-81,83,111).

Previously our laboratory has identified miR-125b as a miRNA up-regulated early (3 h) during virulent *M.tb* infection of primary human monocyte-derived macrophages (MDM)
(45). In comparison, miR-125b expression was not significantly altered in MDMs over the same time period in infection with avirulent *Mycobacterium smegmatis*. However, another miRNA, miR-155, was found to be significantly up-regulated in MDMs during avirulent mycobacterial infection and remained unchanged during virulent *M. tb* infection. Further work elucidated the functional roles of these two miRNAs in human macrophages as regulators the cytokine TNF, which plays an important role in the establishment and maintenance of *M. tb* infection (112). It is based on this previous work that we hypothesize there are additional macrophage miRNAs that play a role in *M. tb* pathogenesis.

Our laboratory is interested in early *M. tb* infection processes mediated by the innate immune system, which determines the outcome of active or latent infection. Therefore, we have opted to focus on early miRNA changes in human macrophages, which are the first phagocyte immune responders against *M. tb* in the lung microenvironment and also participate in various tissues during dissemination. Equally important is the decision to examine miRNA expression changes in *M. tb* infection of human macrophages. Not only is tuberculosis a uniquely human disease with humans being the only natural reservoir of *M. tb*, but there are significant differences between human and murine immune responses to *M. tb*; therefore, it is essential to investigate miRNA responses to *M. tb* infection in a human model of *M. tb* infection (113).
We decided to study a relatively new mechanism, miRNAs, within the context of an ancient disease, tuberculosis, in order to enhance our understanding of host immune responses to *M. tb*. We identify global changes in miRNA expression after *M. tb* infection in a human primary macrophage model. By surveying global miRNA expression profiles, we are able to take an unbiased approach to identifying miRNA targets relevant in *M. tb* infection of humans. Elucidating the role host miRNAs play in *M. tb* pathogenesis has the potential to inform on new diagnostic approaches and therapeutic targets for tuberculosis.

2.2 Materials and Methods

2.2.1 Buffers and reagents

Dulbecco’s PBS without Ca$^{2+}$ and Mg$^{2+}$, RPMI medium 1640 with L-glutamine, HEPES buffer, and TRIzol were purchased from Invitrogen. 7H11 agar plates were prepared with Bacto Middlebrook 7H11 from Difco Laboratories, oleic acid-albumin-dextrose-catalase enrichment medium, and glycerol. Human serum albumin was purchased from CSL Behring.

2.2.2 Bacterial strains

Lyophilized *Mycobacterium tuberculosis* H$_{37}$R$_v$ (ATCC #25618) was obtained from the American Tissue Culture Collection, reconstituted, and used as described (114). Briefly, *M. tb* were grown for 9-14 days on Middlebrook 7H11 agar plates and scraped into a 2 mL polypropylene tube containing two sterilized 3 mm glass beads in 1 mL RPMI. The
samples were pulse vortexed 5 times and the resulting suspension settled for at least 30 minutes to remove bacterial clumps. The settled suspension was removed to a second tube for an additional 10 minutes to help further settle out any bacterial clumps. The concentration of bacteria and the degree of clumping ($\leq 10\%$) in the final suspension were determined by Petroff-Hausser chamber counting; bacteria prepared in this fashion are $\geq 90\%$ viable by CFU assay.

2.2.3 Isolation and culture of human monocyte-derived macrophages (MDM)

Blood obtained from healthy, Purified Protein Derivative negative human volunteers using a protocol approved by The Ohio State University Institutional Review Board (IRB) was processed as described (23,115). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood on a Ficoll-Paque cushion (GE Healthcare) and cultured in Teflon wells (Savillex) in RPMI 1640 medium containing 20% autologous human serum for five days at 37°C/5% CO$_2$ for differentiation of monocytes to monocyte-derived macrophages (MDM). On day 5 MDM were harvested from Teflon wells and cultured for an additional 7 days in RPMI with 20% autologous human serum for infection experiments.

2.2.4 M.tb infection of human macrophages

Twelve day old MDM were incubated with M.tb at a multiplicity of infection (MOI) of 5:1 in RHH medium (RPMI 1640 medium with 10mM HEPES and 0.4% human serum albumin) for 30 min at 37°C in 5% CO$_2$ on a platform shaker for equal dispersion of
bacteria followed by an additional 90 min incubation without shaking. MDM were washed three times with warm RPMI and repleted with RPMI containing 2% autologous human serum for the remainder of the experiment.

### 2.2.5 NanoString nCounter miRNA expression profiling

The digital multiplexed NanoString nCounter miRNA assay (NanoString Technologies) was used for miRNA expression profiling of 24 and 72 h *M.tb*-infected MDM (n = 4 individual donors). 100 ng of high quality total RNA, including small RNAs, isolated by phenol-chloroform extraction was used as input material. Small RNAs were prepared for detection by ligating a specific DNA tag (miR-tag) onto the 3’ end of each mature miRNA with the aid of a bridge oligomer according to the manufacturer’s instructions. miR-tags serve not only to normalize the wide range of miRNA melting temperatures but also aid in identification between homologous miRNA family members, enabling single base pair discrimination. Excess tags were removed by restriction digestion and the resulting material was hybridized with miRNA:tag-specific nCounter Capture and barcoded Reporter probes. Hybridization reactions were incubated at 64°C for 18h according to manufacturer’s instructions before removal of excess probes by a magnetic bead-based purification process; purified hybridized probe complexes were immobilized on a streptavidin-coated cartridge using the nCounter Prep Station. The nCounter Digital Analyzer was used to count individual fluorescent barcodes and quantify target miRNA molecules present in each sample. For each assay, a high-density scan (600 fields of
view) was performed. Data normalization and analysis was performed by the Ohio State University Bioinformatics Core Facility according to the manufacturer’s guidelines.

2.2.6 NanoString data analysis

Specific background correction factors were applied to certain miRNAs according to manufacturer’s directions to account for non-hybridization dependent interactions of some bridge oligomers, miR-tags, or capture and reporter probes. Technical normalization of the code counts was performed using spiked in mRNA positive controls according to manufacturer’s instructions, and background was determined by the included negative controls. Each sample was then normalized to the geometric mean of the top 50 most highly expressed miRNAs. miRNAs with normalized counts $< 50$ (average background count) in all groups were removed and Student’s $t$-test was used to calculate the statistical significances of pair-wise comparisons of the remaining miRNAs with a cutoff of $P$-value $< 0.05$ considered significant. Calculations were performed using the $R$ statistical computing environment.
Figure 2.1: Overview of NanoString microRNA detection workflow.
In order to detect and quantify mature miRNAs (1) in samples each mature miRNA is complementarily paired with a sequence-specific bridge oligonucleotide (2) that facilitates the ligation of a specific DNA tag onto the 3’ end of each mature miRNA (3). Bridges and excess tags are removed via an enzymatic step (4) and the resulting tagged miRNAs are hybridized with miRNA:tag-specific nCounter capture probes containing a biotin label and barcoded reporter probes (5). The resulting miRNA-probe complexes are purified and immobilized on a streptavidin-coated cartridge via the biotin label on the capture probe using the nCounter Prep Station (6). The nCounter Digital Analyzer is used to count individual fluorescent barcodes and quantify target miRNA molecules present in each sample.
2.2.7 MicroRNA expression studies by qRT-PCR

Non-infected or *M.tb*-infected MDM in triplicate wells were lysed in TRIzol reagent and total RNA (including small RNA) was isolated according to manufacturer’s instructions. RNA quantity and quality were evaluated by NanoDrop 2000 spectrophotometer. miR-132, miR-26a, miR-221, miR-128, let-7i, and RNU44-specific cDNA were generated from RNA using the Taqman MicroRNA Reverse Transcription kit using specific reverse-transcription primers (Applied Biosystems). Expression of miR-132 and miR-26a was determined by qRT-PCR using Taqman MicroRNA Assays with RNU44 as housekeeping control (Applied Biosystems). Negative controls in all qRT-PCR included no reverse transcriptase and no template (cDNA) groups in the reactions. Triplicate samples were analyzed in duplicate wells in each experiment.

2.2.8 Transfection of human macrophages

PBMC were transfected with 30 nM of *mirVana* miR-132 mimic or with *mirVana* mimic negative control #1 (Applied Biosystems) by the Amaxa Nucleofector (Lonza Group) as described (20,116). Briefly, 5 day old PBMCs (1 × 10^7) were resuspended in 100 μl nucleofector solution, followed by the addition of siRNA or control, incubated at room temperature for 5 min, and nucleofected according to the manufacturer’s instructions.

2.2.9 Western blotting

MDM monolayers were washed once in PBS and lysed in TN-1 lysis buffer (50 mM Tris, 10 mM EDTA, 10 mM Na_4PO_7, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM
Na$_3$VO$_4$, 10 µg/mL each aprotinin and leupeptin) at 4ºC for 10 min and centrifuged at 10,000 x g for 10 min at 4ºC to remove cell debris (117). Protein concentrations of the cleared lysates were measured by the BCA-protein assay kit (Pierce). Protein-matched cell lysates were separated by SDS-PAGE and analyzed by Western blot using the p120RasGAP and β-actin antibodies (Santa Cruz). The protein band intensities were measured using the online ImageJ software provided by the NIH. Background intensity was subtracted from each sample and then normalized to the β-actin loading control.

2.2.10 Statistical analysis

The magnitude of the response from each independent experiment varied among the donors; however, in general the pattern of experimental results was the same from donor to donor. To account for this variability, we normalized the data to an internal control in each experiment when possible. A ratio of experimental results to control was obtained, and the mean ratio was then tested for a significant difference from one using $t$ statistics. An unpaired one-tailed Student’s $t$-test was used to analyze differences between groups. $P$-value < 0.05 was considered significant.

2.3 Results

2.3.1 Identification of macrophage miRNAs altered during *M.tb* infection

To identify the human macrophage miRNAs whose expression is altered during *M.tb* infection we performed global miRNA expression profiling in primary human monocyte-
derived macrophages after infection with the virulent standardized strain *M. tb* H₃₇R₇ by the Nanostring nCounter miRNA Expression Assay. miRNAs expressed by MDMs at 24 h (Fig. 2.2A) and 72 h (Fig. 2.2B) after *M.tb* infection were compared with miRNAs expressed by non-infected control MDMs. The times of 24 h and 72 h after infection were chosen to reflect the phases of entry and acclimatization to the intracellular environment (24 h) and initial rounds of intracellular replication (72 h). To identify statistically significant candidate miRNAs regulated by *M.tb* infection we first generated a subset of miRNAs expressed in MDMs (≥ 50 copies detected in at least one group and consistent in all donors tested) after data normalization and then utilized a cutoff of *P* < 0.05 when compared to the non-infected control.

Figure 2.2: Expressed macrophage miRNAs 24 and 72h after *M.tb* infection. Scatter plots show normalized counts for miRNAs expressed in MDMs (A) 24 h after

(continued on next page)
Cumulative results from four individual donors indicated that at 24 h three miRNAs, miR-490-3p, let-7i, and miR-29a, were significantly up-regulated, while three additional miRNAs, miR-361-5p, miR-23b, and miR-221 were significantly down-regulated relative to the non-infected control (Fig. 2.3A). At 72 h after infection we identified ten significantly up-regulated miRNAs: miR-132, miR-146b-5p, miR-30e, let-7i, miR-490-3p, miR-29c, miR-26a, miR-21, let-7b, and miR-29a. In addition, 21 miRNAs were significantly down-regulated at 72 h after infection relative to the non-infected control: miR-25, miR-23b, miR-331-3p, miR-423-3p, miR-548f, miR-340, miR-24, miR-107, miR-93, miR-324-5p, miR-188-5p, miR-130b, miR-410, miR-361-5p, miR-197, miR-27a, miR-128, miR-345, miR-379, miR-133a, and miR-221 (Fig. 2.3B). Of note, all six of the significantly altered miRNAs at 24 h post-infection were also significantly altered with the same patterns at 72 h post-infection (Fig. 2.3C). Therefore, we focused our efforts on further characterization of miRNA functions on the regulated miRNAs at 72 h post-M.tb infection.
Figure 2.3: Significantly altered human macrophage miRNAs after *M.tb* infection. (A) At 24 h after infection 6 differentially expressed miRNAs were identified, while at (B) 72 hours after infection 31 miRNAs were differentially expressed. (C) Venn diagram comparing MDM miRNAs altered by *M.tb* infection identified at 24 hours and at 72 hours after *M.tb* infection. All differentially expressed miRNAs identified at 24 hours were also observed at 72 hours. Data shown are cumulative from four donors (consistent all in all donors). Significantly altered miRNAs were determined using a cutoff of P<0.05 when compared to the non-infected control.
2.3.2 Validation of miRNA expression patterns by qRT-PCR

In order to validate the miRNA expression profiling results we used real-time quantitative RT-PCR (qRT-PCR) to assay expression of several up-regulated and down-regulated miRNAs for comparison to expression patterns as determined by NanoString, including miR-132 (the most up-regulated miRNA at 72 h post-infection) (Fig. 2.4A and 2.4B), miR-26a (Fig. 2.4C and 2.4D), let-7i (Fig. 2.4E and 2.4F), miR-221 (the most down-regulated miRNA at 72 h post-infection) (Fig. 2.4G and 2.4H) and miR-128 (Fig. 2.4I and 2.4J). When compared to the expression profiles as established by NanoString, the qRT-PCR validation results demonstrated great congruence between the expression patterns determined using these two techniques for these miRNAs. Additionally, qRT-PCR results confirmed that expression levels of a miRNA not determined to be significantly altered at either 24 h or 72 h after M.tb infection by NanoString was also unchanged relative to the non-infected control by qRT-PCR (data not shown). Although we cannot conclude that the patterns of alteration for these miRNAs are specific responses to M.tb, our results confirm that miR-132, miR-26a, let-7i, miR-221, and miR-128 are distinctly regulated in human macrophages after M.tb infection.
Figure 2.4: Validation of macrophage miRNA expression patterns during *M.tb* infection.

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(Fig. 2.4 continued)
Expression patterns of selected miRNAs of interest were validated by qRT-PCR. Comparison of the most up-regulated miRNA miR-132 expression in *M. tb*-infected MDMs as measured by (A) NanoString and (B) qRT-PCR, miR-26a expression as measured by (C) NanoString and (D) qRT-PCR, let-7i expression as measured by (E) NanoString and (F) qRT-PCR, miR-128 expression as measured by (G) NanoString and (H) qRT-PCR, and miR-221, the most down-regulated miRNA at 72 h after *M. tb* infection, as determined by (I) Nanostring and (J) qRT-PCR. NanoString data are normalized cumulative results from 4 individual donors (mean ± SEM). The qRT-PCR data were performed in triplicate, normalized to RNU44 endogenous control and plotted as a percentage of the non-infected control expression (black bars); shown are cumulative results from three independent experiments (mean ± SEM) except for miR-128 (n=1, mean ± SD.) *P<0.05, **P<0.01, ***P<0.001.

2.3.3 Identification of putative targets for miRNAs altered during *M. tb* infection

In order to identify the mRNA targets of the altered miRNAs identified during *M. tb* infection we utilized an approach combining in silico prediction and surveys of published literature to increase the odds of determining true targets in human macrophages. Although miRNA target prediction algorithms are perhaps the most common route for finding mRNA targets for a given set of miRNAs these databases often return large numbers of predicted targets with no consideration for biological relevance. In general, target prediction databases employ algorithms based on 3’UTR-complementarity for prediction (118). However, miRNA target selection is a complicated and not fully understood process, consequently, in silico target prediction generally yields a large number of targets with few true miRNA-mRNA relationships that may vary greatly by the cell type under study. Thus, in conjunction with predicted mRNA targets for the differentially expressed miRNAs during *M. tb* infection we cross-matched macrophage proteins with known functions in *M. tb* pathogenesis with predicted mRNA targets, as
well as assessing the literature for published targets of these miRNAs, which has the added benefit of being able to identify proven targets that are not predicted by any of the existing algorithms.

Figure 2.5: p120RasGAP is a putative target of M.tb up-regulated miR-132 in human macrophages. p120RasGAP protein levels were assessed by Western blot of protein-matched whole cell lysates of M.tb-infected MDMs (A). Reduced p120RasGAP expression was observed at 72 h after M.tb infection, correlating with an increase in miR-132 expression. Shown is representative blot of n = 2 with cumulative quantification by densitometry in right panel, (continued on next page)
(Fig. 2.5 continued)

*P<0.05. In addition, manipulation of MDM miR-132 levels by transfection of miR-132 inhibitors (B) or mimics (C) resulted in corresponding increase or decrease of p120RasGAP protein levels, respectively (n = 1). Blots were re-probed with actin as a loading control; densitometry analysis of the immunoblot results appears in right panels.

As miR-132 was the most up-regulated miRNA at 72 h after *M. tb* infection, assigning function for this miRNA in human macrophages was of special interest. We queried a number of target prediction databases including DIANAmT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22, and Targetscan along with a literature search of known miR-132 targets. Our search returned two targets of interest for miR-132: p300 (discussed in Chapter 3) and p120 Ras GTPase-activating protein (p120RasGAP). p120RasGAP has been confirmed as a target of miR-132 in endothelial cells, smooth muscle, and axons where it is responsible for a wide array of processes varying from vascularization, axon extension, to angiotension II responses (119-122). Western blot analysis of *M. tb* infected MDM whole cell lysates demonstrates that p120RasGAP protein expression decreases at 72 h after infection, correlating with the increase in miR-132 expression as determined by NanoString (Fig 2.5A). Additionally, to demonstrate that miR-132 may regulate p120RasGAP protein levels in human macrophages, we transiently knocked-down or over-expressed miR-132 in human MDMs by transfection of a miR-132 inhibitor or a miR-132 mimic, respectively, and assessed p120RasGAP levels over time by Western blot. With knockdown of miR-132 increased p120RasGAP protein expression was observed at 48 h and 72 h after transfection relative to the negative control group (Fig. 2.5B), while over-expression of miR-132 reduced
protein levels of p120RasGAP at 48 h and 72 h after transfection compared to the corresponding negative control in MDMs (Fig. 2.5C).

2.4 Discussion

Recent years have witnessed a spate of investigation on the dysregulation and roles of miRNAs during bacterial infections (77), including *M. tb* (89). However, with some exceptions (38,45,104,106,123-125), the majority of these studies have failed to identify downstream effects on the host immune response for the miRNAs detected with altered expression. A major contributing factor to the lack of concrete targets is the use of mixed cell populations, serum, or sputum for the identification of differentially expressed miRNAs during *M. tb* infection. *M. tb* is an ancient organism that has co-evolved with its human host; therefore, it is uniquely adapted for survival within its intracellular host: the macrophage (126). To date, little data exist on how miRNAs alter human macrophage immune responses during infection, which are the first phagocyte immune responders to *M. tb* in the lung microenvironment (100). This study emphasizes the effects of primary *M. tb* infection, which determines the outcome of active or latent infection, on macrophage miRNAs using a pure primary human macrophage model of tuberculosis. We have identified 31 miRNAs differentially expressed in primary human macrophages during infection with virulent *M. tb* and demonstrated that a subset of these significantly altered miRNAs can regulate protein levels in human macrophages.
By studying miRNA changes in a pure population of human macrophages during *M. tb* infection we are able to limit our focus on effects of miRNAs on targets in the context of macrophage functions, which can have broad implications for both *M. tb* and other macrophage-mediated diseases. The subset of miRNAs identified in this study are not necessarily all specific to *M. tb* infection and determining the cellular targets for the broader response miRNAs can lead to insights into intrinsic macrophage functions.

Further expression profiling will need to be conducted in order to differentiate between the miRNAs altered in a specific *M. tb* response and generic macrophage responses to stimuli. Comparison of the current profile of virulent *M. tb* altered miRNAs against the expression profile of miRNAs altered in MDMs during infection with the vaccine strain *M. bovis* BCG can not only identify virulence specific miRNAs (the non-overlapping subset of miRNAs between the two groups), but also aid in determination of generic macrophage response miRNAs (the over-lapping subset of miRNAs). Further comparison of miRNA expression profiles against MDMs treated with various stimuli (cytokines, TLR agonists, etc.) has the potential to identify a “core” subset of miRNAs that regulate canonical human macrophage functions.

Although the magnitude of change in expression observed for miRNAs during *M. tb* infection is relatively small compared to the magnitude of changes commonly observed for mRNAs, the fold-changes seen in this study are on par with similar studies of miRNA expression during *M. tb* infection (96,98,104). Due to the mechanism of action of miRNAs, small changes in miRNA levels can cascade to large effects downstream. A
single miRNA molecule incorporated into the RNA-induced silencing complex (RISC) can result in the degradation of many copies of mRNA, leading to a significant reduction in protein levels. The effect of miRNAs can be further amplified by the reduced activity of the targeted proteins (for instance, by targeting a kinase that results in reduced phosphorylation of multiple downstream targets). Therefore, as miRNAs are high level regulators of cellular functions, small shifts in miRNA levels during infection can result in dramatic changes in macrophage responses to \textit{M.tb}.

Identifying mRNA targets for the miRNAs altered during \textit{M.tb} infection is currently an inexact science. Much of current target identification relies on predictive algorithms based on miRNA base-pairing to 3’ untranslated regions (UTR) of targeted mRNAs with an emphasis on matches in the miRNA seed sequence. However, base-pairing between miRNAs and their targets is often imperfect and this can determine whether the resulting effect is mRNA degradation or translational repression; this lack of exact base-pair matching between miRNAs and 3’ UTRs presents an obstacle to accurate target prediction. Additionally, some studies now suggest that miRNAs can exert effects by binding outside of 3’ UTRs, which further complicates prediction (127,128). Due to the limitations of \textit{in silico} miRNA target identification, many are now turning to pull-down assays in which RISC components or specific miRNAs are labeled and pulled-down along with associated mRNAs. While more likely to produce a smaller set of potential targets with greater biological relevance, this technique also requires stringent validation to prove a true direct interaction between miRNA and mRNA. Based on currently
existing target prediction methods and the existing body of literature, we have identified p120RasGAP as a putative target of miR-132 in human macrophages. We have shown correlative changes in p120RasGAP protein levels with miR-132 knockdown and over-expression in MDMs, as well as a decrease in p120RasGAP at 72 h after *M. tb* infection that correlates with miR-132 levels during infection. However, further confirmation of the miR-132/p120RasGAP relationship is needed through an increased number of experiments with multiple donors for miR-132 knockdown and over-expression. Direct interaction between miR-132 and the p120RasGAP 3’ UTR should also be demonstrated via a luciferase quenching assay, as well as assays on downstream biological function. As we gain further knowledge on the mechanism of miRNA function target prediction will become more exact, but until then biological validation of predicted relationships within cells of interest is required before miRNA targeting of a mRNA can be concluded.
Chapter 3: Mycobacterium tuberculosis decreases human macrophage IFN-γ responsiveness through miR-132 and miR-26a

3.1 Introduction

Macrophages are among the frontline defenders against invading organisms (129). However, they can also act as cellular hosts for intracellular pathogens, including Mycobacterium tuberculosis (M.tb), the causative agent of tuberculosis (130). The lymphocyte secreted cytokine IFN-γ is the predominant activator of macrophage microbicidal activity, turning on a transcriptional program for the killing of intracellular pathogens through JAK-STAT signaling downstream of the type II IFN receptor (34). Phosphorylation of STAT1 by JAK1 and JAK2 and formation of homodimers upon type II IFN receptor engagement results in translocation to the nucleus where the STAT1 homodimers engage with transcriptional co-activators, including the co-activator p300, to initiate transcription of IFN-γ induced genes (131). p300 interacts with a variety of transcription factors and has been identified as an important transcriptional mediator of immune defenses, including IFN-γ response genes, against many intracellular pathogens (39,109,132-134).
IFN-γ activity is essential for control of *M. tb* in murine models (35,36); however, the effects of IFN-γ are not as clear in humans. While lack of IFN-γ signaling predisposes individuals to infections with intracellular pathogens, including several mycobacteria species (135-138), high levels of IFN-γ have been detected in the pleural fluid of TB patients (139) and IFN-γ therapy for tuberculosis in humans has not demonstrated the same striking results as in murine models (140). These observations can be accounted for by the discovery that *M. tb* blocks IFN-γ signaling and subsequent activation of human macrophage functions by interrupting the p300 association with STAT1 homodimers within the nucleus, causing reduced binding at the γ-activation sequences (GAS) of IFN-γ response genes (39).

MicroRNAs (miRNA) are endogenous noncoding small RNAs that function as gene regulators, most commonly by mediating translational repression or degradation of target mRNAs, which can have widespread effects on downstream pathways (50). Manipulation of host miRNA expression may be another mechanism by which *M. tb* is able to subvert immune detection and persist intracellularly within macrophages. Recent discoveries from our laboratory demonstrate that virulent *M. tb* can modify the human macrophage immune response by modulating miR-125b expression, which in turn decreases levels of TNF-α, another major cytokine for *M. tb* control (45). In this study we sought to identify additional miRNAs whose expression is altered during *M. tb* infection of primary human macrophages and elucidate the functions of some of these miRNAs. We provide evidence that *M. tb* induces host miRNAs miR-132 and miR-26a. Induction of these miRNAs
reduces p300 thereby diminishing transcription of IFN-γ induced genes and macrophage responsiveness to this key lymphokine.

3.2 Materials and Methods

3.2.1 Buffers and reagents
Dulbecco’s PBS without Ca$^{2+}$ and Mg$^{2+}$, RPMI medium 1640 with L-glutamine, HEPES buffer, and TRIzol were purchased from Invitrogen. 7H11 agar plates were prepared with Bacto Middlebrook 7H11 from Difco Laboratories, oleic acid-albumin-dextrose-catalase enrichment medium, and glycerol. Human serum albumin was purchased from CSL Behring and recombinant human IFN-γ was obtained from R&D Systems.

3.2.2 Bacterial strains
Lyophilized Mycobacterium tuberculosis H$_{37}$R$_{v}$ (ATCC #25618) was obtained from the American Tissue Culture Collection, reconstituted, and used as described (114). Briefly, M.tb were grown for 9-14 days on Middlebrook 7H11 agar plates and scraped into a 2 mL polypropylene tube containing two sterilized 3 mm glass beads in 1 mL RPMI. The samples were pulse vortexed 5 times and the resulting suspension settled for at least 30 minutes to remove bacterial clumps. The settled suspension was removed to a second tube for an additional 10 minutes to help further settle out any bacterial clumps. The concentration of bacteria and the degree of clumping (≤ 10%) in the final suspension were determined by Petroff-Hausser chamber counting; bacteria prepared in this fashion...
are $\geq 90\%$ viable by CFU assay. Alternatively, \textit{M.\textit{tb}}-Lux (H$_{37}$R$_v$ expressing the luciferase gene and substrate, generated by Dr. Abul Azad), prepared in the same way, was used for the intracellular growth assays.

\textbf{3.2.3 Isolation and culture of human monocyte-derived macrophages (MDM)}

Blood obtained from healthy, Purified Protein Derivative negative human volunteers using a protocol approved by The Ohio State University Institutional Review Board (IRB) was processed as described (23,115). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood on a Ficoll-Paque cushion (GE Healthcare) and cultured in Teflon wells (Savillex) in RPMI 1640 medium containing 20\% autologous human serum for five days at 37ºC/5\% CO$_2$ for differentiation of monocytes to monocyte-derived macrophages (MDM). On day 5 MDM were harvested from Teflon wells and used for transfection experiments.

\textbf{3.2.4 \textit{M.\textit{tb}} infection of human macrophages}

Six day old MDM were incubated with \textit{M.\textit{tb}} at a multiplicity of infection (MOI) of 5:1 in RHH medium (RPMI 1640 medium with 10mM HEPES and 0.4\% human serum albumin) for 30 min at 37ºC in 5\% CO$_2$ on a platform shaker for equal dispersion of bacteria followed by an additional 90 min incubation without shaking. MDM were washed three times with warm RPMI and repleted with RPMI containing 2\% autologous human serum for the remainder of the experiment, unless receiving further treatments.
3.2.5 Transfection of human macrophages

PBMC were transfected with 100 nM each of mirVana miR-132 and miR-26a inhibitors or mimics, along with the appropriate negative controls (Applied Biosystems) by the Amaxa Nucleofector (Lonza Group) as described (20,25) or by the Lipofectamine LTX with Plus Reagent (Life Technologies) transfection system. For the Amaxa Nucleofection system 5 day old PBMCs ($1 \times 10^7$) were resuspended in 100 μl nucleofector solution followed by the addition of siRNA or control, incubated at room temperature for 5 min, and nucleofected according to the manufacturer’s instructions. Lipofectamine LTX with Plus Reagent was used for transfection of MDMs after adherence. Briefly, the appropriate amount of Lipofectamine LTX lipid reagent in Opti-MEM medium was incubated for five minutes with the appropriate amount of siRNA and Plus Reagent in Opti-MEM medium. Adherent MDMs in tissue culture plates were washed once in warm RPMI to remove residual serum and repleted in serum-free RPMI before addition of the lipid-siRNA mixture according to manufacturer’s instructions.

3.2.6 MicroRNA and gene expression studies by qRT-PCR

MDM monolayers were treated with 40 ng/mL of recombinant human IFN-γ for varying times. M.tb-infected or IFN-γ stimulated MDM in triplicate wells were lysed in TRIZol reagent and total RNA (including small RNA) was isolated according to manufacturer’s instructions. RNA quantity and quality were evaluated by NanoDrop 2000 spectrophotometer. Total RNA (100 ng) was reversed transcribed to cDNA by SuperScript II reverse transcriptase (Invitrogen) and expression of CD64, CYBB, IRF1,
HLA-DRα, CXCL10, and CD86 were determined by qRT-PCR using Taqman Gene Expression Assays (Applied Biosystems) and normalized to β-actin as a housekeeping gene. miR-132 and miR-26a cDNA were generated from RNA using the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems). Expression of miR-132 and miR-26a was determined by qRT-PCR using Taqman MicroRNA Assays with RNU44 as housekeeping control (Applied Biosystems). Negative controls in all qRT-PCR included no reverse transcriptase and no template (cDNA) groups in the reactions. Triplicate samples were analyzed in duplicate wells in each experiment.

3.2.7 Western blotting

MDM monolayers were washed once in PBS and lysed in TN-1 lysis buffer (50 mM Tris, 10 mM EDTA, 10 mM Na₄PO₇, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na₃VO₄, 10 µg/mL each aprotinin and leupeptin) at 4°C for 10 min and centrifuged at 10,000 x g for 10 min at 4°C to remove cell debris (117). Protein concentrations of the cleared lysates were measured by the BCA-protein assay kit (Pierce). Protein-matched cell lysates were separated by SDS-PAGE and analyzed by Western blot using the following antibodies: p300 and β-actin (Santa Cruz); STAT1, JAK1, JAK2, and CBP (Cell Signaling); HLA-DR (clone L243, BD Bioscience); CD64 (clone 22.2) generously provided by Dr. Clark Anderson (The Ohio State University). The protein band intensities were measured using the online ImageJ software provided by the NIH. Background intensity was subtracted from each sample and then normalized to the β-actin loading control.
3.2.8 Confocal microscopy

Transfected MDM were adhered to glass coverslips in 24-well tissue culture plates for 2-3 hours at 37°C and washed to remove lymphocytes. MDM were treated with IFN-γ for 48 h, washed, and fixed in 2% paraformaldehyde then blocked in blocking reagent (5% BSA, 10% FBS in PBS). Coverslips were incubated with primary antibody or the appropriate isotype control for 1 h at room temperature, washed with blocking reagent, and counterstained with an Alexa Fluor conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. Nuclei were labeled with the DNA stain 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Finally, coverslips were washed and mounted on glass slides and viewed using an Olympus Fluoview confocal microscope.

3.2.9 Assessment of phagocytic function through Fcγ receptors

IgG-coated sheep erythrocytes (SRBC) were prepared as described (141) and then incubated with transfected MDMs on coverslips at a ratio of 50:1 for 1 h in RPMI at 37°C. Cells were subjected to brief hypotonic lysis for removal of extracellularly-bound SRBC and fixed in paraformaldehyde. Phagocytosis through the Fcγ receptors was confirmed by the lack of ingested SRBC by MDMs incubated with SRBC not opsonized with IgG (data not shown). Phagocytosis was measured by the total number of ingested SRBC in 100 MDMs (phagocytic index).
3.2.10 Cell enumeration of miRNA inhibitor transfected MDMs in monolayer culture

Day 5 or 6 MDMs transfected with miRNA inhibitor control, miR-132 inhibitor alone, miR-26a inhibitor alone, or combined miR-132 and miR-26a inhibitors were plated in 24 well plates, washed after 3 h, and repleted with 10% autologous serum in RPMI for 72 h. Cells were then washed once with PBS without Ca$^{2+}$ or Mg$^{2+}$ and treated with 1% Cetavlon in 0.1M citric Acid with 0.05% Naphthol blue black (Sigma-Aldrich), pH 2.2, for 15 min at room temperature. Cell lysates were then loaded on a hemacytometer and stained nuclei enumerated using phase contrast microscopy (142).

3.2.11 M.tb intracellular growth assay in MDMs

Intracellular bacterial load of M.tb in miRNA inhibitor transfected MDMs was determined by infection with M.tb-Lux, a luciferase producing virulent M.tb strain. M.tb-Lux was grown for 9-14 days on Middlebrook 7H11 agar plates and prepared as previously described for infection. At various times after initial incubation and washing infected MDMs were lysed by incubation with DNase (500 µg/mL) and 0.25% SDS. The lysis reaction was quenched by addition of 20% BSA (Sigma) and the cell lysates were collected in 2 mL polypropylene tubes containing two sterilized 3 mm glass beads. Each sample was pulse vortexed 5 times to disperse clumps and luciferase activity was measured by a single tube luminometer. Background luminescence was determined and subtracted from each sample before calculation of relative light units (RLU) per mL of cell lysate.
3.2.12 Statistical analysis

Most experiments were performed at least three independent times using three different donors and yielded similar results. The magnitude of the response from each independent experiment varied among the donors; however, the pattern of experimental results was the same from donor to donor. To account for this variability, we normalized the data to an internal control in each experiment. A ratio of experimental results to control was obtained, and the mean ratio was then tested for a significant difference from one using \( t \) statistics. In most instances an unpaired one-tailed Student’s \( t \)-test was used to analyze differences between groups. \( P \)-value < 0.05 was considered significant.

3.3 Results

3.3.1 Identification of transcriptional co-activator p300 as a target of M.tb up-regulated miR-132 and miR-26a

We selected miR-132 for further analysis since it was the most up-regulated miRNA at 72 h post-infection and its expression pattern was validated by qRT-PCR. To identify the gene targets of miR-132 we sought previously validated targets in literature as well as predicted gene targets by miRWalk, an online software for miRNA target prediction that aggregates miRNA target predictions from ten computational algorithms (DIANAmT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22, and Targetscan) (see Chapter 2 for details). Among the putative target genes of interest was transcriptional co-activator p300, previously identified as a miR-132 target in endothelial
and monocytic models of viral infection (109) and predicted by nine of ten algorithms as a target of miR-132 based on sequence. Additionally, miR-26a, another miRNA significantly up-regulated at 72 h after *M. tb* infection, was predicted to target p300 by seven of ten algorithms, although no such biological evidence yet exists in the literature. Therefore, there exists solid support for some of these differentially regulated miRNAs to have a functional role during *M. tb* infection by targeting p300, a component of an important macrophage immune response pathway.

### 3.3.2 Changes in p300 protein levels correlate with alterations in miR-132 and miR-26a expression

To establish p300 as a target of miR-132 and miR-26a in human macrophages we utilized transient knockdown or over-expression of these two miRNAs by transfection of miR-132- and miR-26a-specific inhibitors or mimics and assayed for p300 protein levels by Western blot analysis of transfected cell lysates. Confirmation of miR-132 and miR-26a knockdown and over-expression by qRT-PCR demonstrated significant decrease (Fig. 3.1A and 3.1B) or increase (Fig. 3.1C and 3.1D) in miR-132 and miR-26a expression when compared to the appropriate transfection control.
Figure 3.1: miR-132 and miR-26a knockdown and over-expression in human macrophages by transfection with miRNA inhibitors and mimics. (A, B) Knockdown or (C, D) over-expression of miR-132 and miR-26a was achieved by transfection of specific inhibitors or mimics and confirmed by qRT-PCR. Shown are representative data obtained from three independent experiments for knockdown and two independent experiments for over-expression (mean ± SD, *$P<0.05$, **$P<0.01$, ***$P<0.001$).

Individual knockdown of miR-132 or miR-26a in MDMs by miRNA inhibitors resulted in an increase in p300 protein levels when compared to the inhibitor negative control, especially at 24 h after transfection (Fig. 3.2A). However, combined knockdown of miR-132 and miR-26a in MDMs resulted in a more robust increase in p300 protein levels relative to the negative control than the single knockdowns (Fig. 3.2B).
Figure 3.2: Knockdown of miR-132 and miR-26a alone or in concert increases p300 protein expression in human macrophages.

After miR-132 and/or miR-26a knockdown by transfection with miRNA inhibitors, p300 protein levels were analyzed by Western blot of protein-matched whole cell lysates from (A) single miRNA knockdown or (B) double miRNA knockdown MDMs (left panels). Blots were re-probed with actin as a loading control. Densitometry analysis of the immunoblot results shows an increase in p300 levels with miR-132 and miR-26a single or double knockdown (right panels). Shown are representative Western blots and representative densitometry for the single knockdown (n = 2) and cumulative densitometry data representing data from four individual experiments for the double knockdown (mean ± SEM, ***P<0.001).

Conversely, over-expression of miR-132 or miR-26a alone by miRNA mimics led to reduced p300 protein levels when compared to the corresponding mimic negative control at some time points (Fig. 3.3A). Double over-expression of miR-132 and miR-26a in combination demonstrated stronger and more consistent decreases in p300 protein levels.
in MDMs (Fig. 3.3B). Based on these data, we conclude that p300 is also a target of miR-132 in human macrophages and further, demonstrate the novel finding that miR-26a also targets p300.

Figure 3.3: Over-expression of miR-132 and miR-26a alone or in concert decreases p300 protein expression in human macrophages.

After over-expression of miR-132 and/or miR-26a by transfection by miRNA mimics, p300 protein levels were analyzed by Western blot of protein-matched cell lysates from (A) single miRNA over-expressing or (B) double miRNA over-expressing MDMs (left panels). Blots were re-probed with actin as a loading control. Densitometry analysis of the immunoblot results shows a decrease in p300 levels with miR-132 and miR-26a single or double over-expression (right panels). Shown are representative Western blots with representative densitometry analysis for the single over-expresser (n =2), while the double over-expresser densitometry is cumulative data from three individual experiments (mean ± SEM, *P<0.05, ***P<0.001).
3.3.3 miR-132 and miR-26a knockdown regulates transcriptional and translational macrophage responses to IFN-γ

Since p300 is an important transcriptional mediator of IFN-γ responses, we hypothesized that knockdown of miR-132 and miR-26a could improve IFN-γ responses in human macrophages via increased p300 levels. We assessed the expression of six known IFN-γ responsive genes encompassing a variety of macrophage functions, including FcγRI/CD64, CYBB, IRF1, HLA-DRα, CXCL10, and B.7.2/CD86, by qRT-PCR in miR-132 and miR-26a knockdown MDMs (24 h transfection) after treatment with recombinant IFN-γ for 6 h or 24 h (34). Our results demonstrate increased transcriptional expression of all six genes upon IFN-γ stimulation in miR-132 and miR-26a knockdown cells when compared to the transfection control cells (also treated with IFN-γ) (Fig. 3.4A-F).
Figure 3.4: miR-132 and miR-26a knockdown improves macrophage transcriptional responses to IFN-γ.

miR-132 and miR-26a were knocked down in MDMs by transfection with miR-132 and miR-26a inhibitors and then stimulated with 40ng/mL recombinant human IFN-γ. Cells were lysed at 6 h or 24 h to assay for transcription of known IFN-γ-responsive genes (A) CD64/FcγRI (n = 5), (B) CYBB/p91-PHOX (n = 5), (C) IRF-1 (n = 4), (D) HLA-DRα (n = 4), (E) CXCL10 (n = 3), and (F) CD86/B7.2 (n = 4) by qRT-PCR. Shown are cumulative data from multiple donors, normalized to β-actin, each performed in triplicate (mean ± SEM, **P<0.01, ***P<0.001).

In addition to increased mRNA levels we observed increased cell surface protein expression of FcγRI/CD64 and HLA-DR (Fig. 3.5A-B) in knockdown MDMs by confocal microscopy at 48 h after IFN-γ treatment, indicating that there was also increased translational expression. Therefore, we demonstrate that knockdown of miR-132 and miR-26a in human macrophages can improve transcriptional responses to IFN-γ (via increased expression of p300), and these increased mRNA levels correspond to increases in protein expression of IFN-γ induced genes.
Figure 3.5: miR-132 and miR-26a knockdown improves macrophage translational responses to IFN-γ. Transfected MDMs on coverslips were stimulated with IFN-γ for 48 h and stained with antibody for (A) FcγRI or (B) HLA-DR and examined by confocal microscopy. Shown are representative sections from one of three independent experiments performed on triplicate coverslips. MFI was calculated from at least 10 cross-sectional images and normalized to the number of nuclei then plotted as fold increase relative to the transfection control group (n=3, mean ± SEM, *P<0.05, **P<0.001).

To demonstrate that the previously observed increases in transcriptional responses to IFN-γ are a result of miR-132 and miR-26a targeting of p300 and not off-target effects of these miRNAs on other IFN-γ pathway proteins, we assessed effects of miR-132 and miR-26a knockdown on integral downstream IFN-γ signaling proteins. Binding of IFN-γ to its receptor results in recruitment of JAK1 and JAK2, which phosphorylates STAT1 leading to its homodimerization and translocation to the nucleus to associate with p300. Western blot analyses of STAT1, JAK1, and JAK2 (Fig. 3.6A-C) after miR-132 and miR-26a knockdown in MDMs show no significant changes in expression of these
proteins relative to the negative control group, indicating that miR-132 and miR-26a do not affect expression of these IFN-γ pathway proteins.

Figure 3.6: miR-132 and miR-26a knockdown does not alter other proteins in the IFN-γ signaling pathway or JAK-STAT-independent genes. After knockdown of miR-132 and miR-26a by transfection with miRNA inhibitors, (A) STAT1, (B) JAK1, and (C) JAK2 protein levels were analyzed by Western blot of protein-matched whole cell lysates (top panels). Blots were re-probed with actin as a loading control. Cumulative densitometry analyses of immunoblot results from two independent experiments show no significant changes in expression levels of these proteins with miR-132 and miR-26a knockdown (bottom panels). To assess expression of (continued on next page)
The IFN-γ induced JAK-STAT independent genes (D) CCL3, (E) CCL4, and (F) CEBPβ, miR-132 and miR-26a were knocked down in MDMs by transfection with miR-132 and miR-26a inhibitors and then stimulated with 40 ng/mL recombinant human IFN-γ. Cells were lysed at 6 h or 24 h to assay for transcription by qRT-PCR. Shown are cumulative data from two individual donors, normalized to β-actin, each performed in triplicate (mean ± SEM).

In addition, to further demonstrate that the increased transcriptional responses after miR-132 and miR-26a knockdown are mediated by increased p300 protein levels and not a result of widespread increases in transcription due to miRNA knockdown, we assessed transcriptional responses of IFN-γ induced JAK-STAT-independent genes. These genes include CCL3/MIP-1α, CCL4/MIP-1β, and CEBPβ, which are known to be induced by IFN-γ but whose signaling after IFN-γ stimulation are not mediated through the JAK-STAT signaling pathway, and so do not require association of STAT1 homodimers with p300 to initiate their transcription (143,144). After miR-132 and miR-26a knockdown in MDMs and treatment with IFN-γ transcriptional levels of CCL3/MIP-1α, CCL4/MIP-1β, and CEBPβ were assessed by qRT-PCR. Transcription of CCL3/MIP-1α, CCL4/MIP-1β, and CEBPβ did not significantly increase or decrease in the miR-132 and miR-26a knockdown MDMs compared to the control knockdown group, which also received IFN-γ treatment (Fig. 3.6D-F). Together these data provide further support that the increased transcriptional responses to IFN-γ previously observed are mediated through increased protein levels of p300, as a result of decreased miR-132 and miR-26a expression.
Figure 3.7: Over-expression of miR-132 and miR-26a does not alter transcriptional responses to IFN-γ.

miR-132 and miR-26a were over-expressed in MDMs by transfection with miR-132 and miR-26a mimics and then stimulated with 40ng/mL recombinant human IFN-γ. Cells were lysed at 6 h or 24 h to assay for transcription of known IFN-γ-responsive genes (A) CD64/FcγRI, (B) CYBB/p91-PHOX, (C) IRF-1, (D) HLA-DRα, (E) CXCL10, and (F) CD86/B7.2 by qRT-PCR. Shown are cumulative data from three individual donors, normalized to β-actin, each performed in triplicate (mean ± SEM).

3.3.4 miR-132 and miR-26a over-expression does not alter transcriptional responses to IFN-γ

We previously demonstrated that by over-expressing miR-132 and miR-26a in human macrophages using miRNA mimics we could reduce cellular levels of p300. Therefore, we hypothesized that reduced responses to IFN-γ stimulation would be observed in miR-132 and miR-26a over-expressing MDMs, since less p300 would be available to associate with STAT1 in the nucleus to mediate transcription at GAS elements. We assessed transcriptional responses of miR-132 and miR-26a over-expressing MDMs to IFN-γ by
assaying for mRNA levels of the six known IFN-γ induced genes previously studied
(FcγRI/CD64, CYBB, IRF1, HLA-DRα, CXCL10, and B.7.2/CD86) at either 6 h or 24 h
after recombinant IFN-γ stimulation by qRT-PCR. Contrary to our hypothesis, we did not
observe any changes in mRNA levels of these six IFN-γ-responsive genes in the miR-132
and miR-26a mimic transfected MDMs compared to the negative control transfected
MDMs, which also received IFN-γ treatment (Fig. 3.7A-F). Since transcriptional
responses to IFN-γ have been studied as early as 1 h after treatment in macrophages, we
also assessed transcriptional responses of these six genes 1 h after IFN-γ stimulation of
miR-132 and miR-26a over-expressing macrophages, and again observed no significant
changes in transcriptional levels of IFN-γ-induced genes (data not shown).

This surprising result led us to explore possible explanations for the lack of observed
changes in transcriptional responses after miR-132 and miR-26a over-expression. First
we assessed possible off-target effects of the miRNA mimics on IFN-γ pathway proteins
including STAT1, JAK1, and JAK2. After transfection of miR-132 and miR-26a mimics
we assessed STAT1, JAK1, and JAK2 protein levels at various times by Western blot.
Both Western blot results and their densitometric analysis indicate that over-expression
of miR-132 and miR-26a does not change STAT1, JAK1, and JAK2 protein levels in
human macrophages (Fig. 3.8A-C).
Figure 3.8: Altering expression of miR-132 and miR-26a levels does not significantly change STAT1, JAK1, JAK2, or CBP protein levels. After over-expression of miR-132 and miR-26a by transfection with miRNA mimics, (continued on next page)
(Fig. 3.8 continued)
(A) STAT1, (B) JAK1, and (C) JAK2 protein levels were analyzed by Western blot of protein-matched whole cell lysates (top panels). Blots were re-probed with actin as a loading control. Densitometry analyses of immunoblot results show no significant changes in expression levels of these proteins with miR-132 and miR-26a over-expression (bottom panels). CBP protein levels were assessed by Western blot after miR-132 and miR-26a (D) knockdown or (E) over-expression in protein-matched whole cell lysates (top panels). Blots were re-probed with actin as a loading control. Cumulative densitometry analyses of immunoblot results from two independent experiments show no significant changes in CBP expression during either miR-132 and miR-26a knockdown or over-expression (bottom panels).

Expression of downstream IFN-γ signaling proteins appears to be intact after transfection of miR-132 and miR-26a mimics, yet miR-132 and miR-26a over-expression can reduce cellular levels of p300. Therefore, we hypothesized that there may be other compensatory factors for p300 function that prevent a drop in transcriptional responses to IFN-γ.

CREB-binding protein (CBP) is another transcriptional co-activator with similar structure and overlapping functions as p300 (145,146). miRNA target prediction algorithms identify CBP as a potential target of miR-26a, but not miR-132. We assessed CBP protein levels in MDMs after miR-132 and miR-26a knockdown and over-expression to determine if CBP levels were affected by alterations in miRNA expression (Fig. 3.8D-E). We observed no significant differences in CBP protein expression under either miR-132 and miR-26a double knockdown or over-expressing conditions as compared to the appropriate negative controls.
3.3.5 Knockdown of miR-132 and miR-26a improves IFN-γ mediated macrophage function

As we previously determined that modulation of miR-132 and miR-26a expression regulates macrophage transcriptional and translational responses to IFN-γ, we next analyzed effects of these two miRNAs on IFN-γ regulated macrophage function. We assayed for function of the increased cell surface FcγRI/CD64 levels by IgG-mediated phagocytosis of sheep red blood cells (SRBC). MDMs transfected with miR-132 and miR-26a inhibitors for 24 h and treated with IFN-γ for 48 h were incubated with IgG-coated SRBC. We found increased numbers of phagocytosed SRBC in miR-132 and miR-26a knockdown MDMs compared to the transfection control MDMs (Fig. 3.9A). Thus, together our data provide evidence that suppression of miR-132 and miR-26a expression improves the IFN-γ induced activation and function of human macrophages and suggests that activation of these two miRNAs by M.tb may be an effective pathogen survival mechanism.
miR-132 and miR-26a inhibitor or control transfected MDMs were incubated with IgG-coated sheep red blood cells (SRBC). (A) Phagocytosis was measured by counting the total number of SRBC ingested by 100 MDMs (phagocytic index). Three independent experiments were performed and scored (mean ± SEM, *P<0.05). Protein matched cell lysates from MDMs with and without M.tb infection (72 h) were probed for (B) FcγRI and (C) HLA-DR by Western blot and then re-probed for actin as a loading control. Shown are representative data from two independent experiments with cumulative densitometry results (mean ± SEM, *P<0.05, **P<0.01).

3.3.6 Expression of HLA-DR and FcγRI are decreased during M.tb infection, correlating with increased miR-132 and miR-26a levels

To further support our hypothesis that increased expression of miR-132 and miR-26a after M.tb infection dampens macrophage immune functions, we assayed for expression of FcγRI and HLA-DR in M.tb-infected MDMs by Western blot. We observed decreased expression of both FcγRI and HLA-DR (Fig. 3.9B-C) 72 h post-infection as compared to the time-matched non-infected lysates. These decreases in protein levels correlate with
the increase in miR-132 and miR-26a expression observed during our initial screen of miRNAs altered during *M.tb* infection. Together, our data indicate that increased host miRNA expression of miR-132 and miR-26a down-regulate key macrophage host defense functions and provide another mechanism employed by *M.tb* to ensure its survival.

Figure 3.10: Knockdown of miR-132 alone or in conjunction with miR-26a does not affect the monolayer but can decrease bacterial burden in MDMs. (A) MDM monolayer cell numbers were assessed at 72 h after nucleofection to ensure equal numbers of cells between negative control siRNA and single and double miRNA inhibitor transfected cells. Nuclei in cell lysates were enumerated after Napthol blue black staining. Shown are cumulative data obtained from four independent experiments each performed in triplicate (mean ± SEM); no significant differences between groups were determined by one-way ANOVA. (B) MDMs nucleofected with miR-132 inhibitor was infected with *M.tb*-Lux at MOI 5:1 and lysed at various times for determination of (continued on next page)
Luciferase activity to analyze intracellular \( M. tb \) growth. Both control siRNA and miR-132 inhibitor groups were treated with 40 ng/mL recombinant IFN-\( \gamma \) at 48 h after initial infection. Shown is a representative experiment (mean \( \pm \) SD of triplicate wells in each test group, \( *P<0.05 \)) of three independent experiments. LTX delivery of miR-132 and miR-26a inhibitors allows for miRNA knockdown 24 h after \( M. tb \)-Lux infection. (C) At 72 h after initial infection cells were lysed and assayed for luciferase activity. Shown is mean \( \pm \) SD of triplicate wells in each test group (\( n = 1 \), \( *P<0.05 \)).

3.3.7 Knockdown of miR-132 and miR-26a can decrease intracellular bacterial load of \( M. tb \)-infected MDMs

As we previously demonstrated that knocking down miR-132 and miR-26a in MDMs can improve macrophage responses to IFN-\( \gamma \), an important cytokine for control of \( M. tb \), we expected that knockdown of miR-132 and miR-26a in conjunction with IFN-\( \gamma \) could decrease macrophage intracellular bacterial load after \( M. tb \) infection. We knocked down miR-132 alone, miR-26a alone, or miR-132 and miR-26a in combination by the Lonza nucleofector system then infected with \( M. tb \)-Lux 24 h after transfection as previously described. Intracellular survival and growth of \( M. tb \) were determined by measuring luciferase activity at various times before and after treatment with recombinant IFN-\( \gamma \). We observed a significant decrease in intracellular bacterial load in the miR-132 single knockdown group as compared to the negative control transfected group both before (\( n = 1 \), data not shown) and after (\( n = 3 \)) IFN-\( \gamma \) treatment (Fig. 3.10B). However, this result was not consistently reproducible in all donors tested and not observed for miR-26a single knockdown or combined miR-132 and miR-26a knockdown (data not shown).
ensure that any differences in bacterial load observed between groups were not the result of differences in cell number due to transfection, we enumerated nuclei in single and double knockdown MDMs by napthol blue black staining. The results from Fig. 3.10A show that there were no significant differences in cell number between the transfection groups, including the inhibitor negative control. To address the inconsistency in reproducibility of the bacterial burden results we used the Lipofectamine LTX with Plus Reagent system to transfect adherent MDM monolayers. The Lipofectamine LTX system allows us to initiate transfection after M.tb-Lux infection, which also allows the outcome of transfection to remain in effect for longer duration after M.tb infection. Results from Fig. 3.10C show that even without IFN-γ stimulation at 72 h after M.tb-Lux infection there was reduced bacterial load in the miR-132 and miR-26a knockdown MDMs, suggesting that there may be an IFN-γ-independent effect on human macrophages with miR-132 and miR-26a knockdown. However, these data were also inconsistent from donor to donor.

3.4 Discussion

Our data demonstrate that through a single miRNA, or multiple miRNAs acting in concert, an intrinsic macrophage activation pathway can be tuned, in this case to the benefit of the invading pathogen. In identifying p300 as a target of up-regulated miRNAs miR-132 and miR-26a during M.tb infection, we have uncovered another layer to the conundrum of IFN-γ function against human tuberculosis. It has long been understood that IFN-γ is an essential mediator of host defenses against tuberculosis, but the historical
data on IFN-\(\gamma\) effects in mice and man have not always been congruent. Murine models of tuberculosis do not mimic the clinical and pathological hallmarks of the course of human disease and IFN-\(\gamma\) seemingly plays a stronger role in defenses against \(M. tb\) in murine models than in humans. Not only has high naturally occurring levels of IFN-\(\gamma\) been detected in human tuberculosis, but treatment of human tuberculosis with IFN-\(\gamma\) has demonstrated little therapeutic benefit. These observations suggest that there exist endogenous braking mechanisms against a continual high IFN-\(\gamma\) stimulus, which may also be detrimental to the host.

Yet in the case of human tuberculosis, the existing level of macrophage activation upon \(M. tb\) infection is often not enough to eradicate the pathogen. This may be a result of either host-initiated mechanisms to prevent overt activation, or through pathogen co-option of endogenous host regulatory pathways. The data from Ting, et al. demonstrating a reduction in human macrophage IFN-\(\gamma\) responses only in the presence of \(M. tb\) suggest that it is the latter. Building upon the work of Ting, et al. that \(M. tb\) infection results in decreased association of p300 at GAS elements after IFN-\(\gamma\) treatment, our work supports the role of miRNAs in mediating this effect, at least in part, providing a mechanism for their observation (39). This work supports the concept that \(M. tb\) modulates miR-132 and miR-26a, in addition to other host miRNAs, which can attenuate host immune responses to promote its own survival.
While we were able to demonstrate an effect in modulation of miR-132 and miR-26a on p300 protein levels in human macrophages, and subsequently show that knockdown of miR-132 and miR-26a can improve transcriptional, translational, and functional responses to IFN-γ, we were unable to show a suppression of IFN-γ effects during over-expression of these miRNAs. We explored some possible explanations for this effect, including alterations in the downstream IFN-γ signaling proteins JAK1, JAK2, and STAT1, as well as compensation of p300 functions by the homologous CBP. Levels of JAK1, JAK2, and STAT1 remained relatively unchanged during miR-132 and miR-26a over-expression, as was the case with miRNA knockdown. Protein levels of CBP also did not show significant changes with either miR-132 and miR-26a knockdown or over-expression. Compensation of p300 functions by the unaltered levels of CBP may account for the lack of change in IFN-γ responses during miRNA over-expression. Also, although we have no experimental data to support or refute this hypothesis, decreased translocation of p300 to the nucleus may be occurring through miR-132 and/or miR-26a targeting of transporter components. In addition, other compensatory mechanisms that may come into play with non-physiological levels of miRNA over-expression may also affect IFN-γ responses in human macrophages.

We also observed an inconsistent effect of miRNA knockdown on intracellular bacterial load. While we observed that miR-132 knockdown does decrease intracellular bacterial survival and growth of M.tb-Lux with some donors, other donors did not demonstrate a significant difference in bacterial load with either miR-132 knockdown alone or also with
miR-26a knockdown. Since our experiments are conducted with primary human macrophages, historically we have observed significant donor to donor variability in responses to both infection and transfection efficacy, which may have affected our results. We addressed the issue of transfection by utilizing a different transfection system that allowed us to deliver miRNA inhibitors to adherent MDMs after *M.tb*-Lux infection, thereby allowing for longer efficacy of the inhibitors, especially at 72 h after infection when miR-132 and miR-26a expression levels were observed to increase. However, we again saw inconsistent results between donors. This may be a limitation of the *in vitro* infection model we utilized; with our pure MDM culture system we have historically observed significant monolayer loss by 96 h after virulent *M.tb* infection (monolayer loss can occur earlier or later depending on the donor). Significant monolayer loss may prevent accurate quantification of bacterial numbers, especially since the increase in miR-132 and miR-26a during *M.tb* infection does not occur until 72 h after infection. Additionally, at times decreases in intracellular bacterial survival and growth appeared to be independent of IFN-γ treatment. This observation may be a result of IFN-γ-independent but p300-dependent basal transcription of some immune response genes, such as HLA-DR (147,148).

It stands to reason that miR-132 and miR-26a may normally function as negative regulators of macrophage activation by IFN-γ. In the case of *M.tb* pulmonary infection induction of these two miRNAs in alveolar macrophages may serve to limit the immune response and damage to the delicate alveolar tissue space. However, whether the
induction of miR-132 and miR-26a is initiated by *M. tb* or through internal host pathways was not addressed in this study and will be the focus of future work. Additionally, the question of whether or not this phenomenon is specific to virulent mycobacterial strains also requires further study. Regardless of whether it is the host or the pathogen that drives up-regulation of miR-132 and miR-26a expression, we have assigned a novel role for miR-132 and miR-26a in human macrophages as regulators of the IFN-γ induced macrophage activation pathway. This knowledge can lead the way to therapeutic regulation of macrophage activation via miRNAs for diseases of overt Th1-type IFN-γ mediated macrophage activation, or as adjunct therapy with IFN-γ for the treatment of tuberculosis.
Chapter 4: \textit{Mycobacterium tuberculosis} infection decreases macrophage Tpl-2 protein levels and alters responses to TLR agonists

4.1 Introduction

The first innate immune responders during \textit{Mycobacterium tuberculosis} (\textit{M.tb}) infection are macrophages, which utilize pathogen recognition receptors (PRR) to identify pathogens based on broad classes of pathogen associated molecular patterns (PAMP) (149). Ligation of PRRs such as the toll-like receptors (TLR) by PAMPs results in the initiation of immune defense programs such as cellular activation and production of cytokines to mount a coordinated immune response to the pathogen (150). TLRs are a conserved family of transmembrane PRRs that can detect a wide range of both intracellular and extracellular microbial and viral products (151).

Activation signals from TLRs are mediated downstream through a number of signaling pathways including the MAP kinases (MAPK) (152). The serine/threonine kinase Tpl-2, also known as Cot or MAP3K8, is a MAP kinase kinase kinase (MAP3K) that activates the MAPK extracellular signal-regulated kinases 1 and 2 (ERK1/2) through phosphorylation and activation of their activator MAPK kinase (MEK) (153). Tpl-2 was originally identified as a proto-oncogene (154), but has since been observed to be the
major mediator of ERK1/2 activation downstream of most, if not all TLRs, in macrophages and other immune cells (155).

ERK1/2 activation as a result of TLR signaling induces the activation of MAPK-dependent transcription factors such as activator protein 1 (AP-1), which activates transcriptional programs for the production of pro-inflammatory cytokines and other immune mediators (155). The importance of Tpl-2 in mediating TLR signals has been observed from studies of Tpl-2 knockout macrophages and other immune cells (156). LPS stimulation of Tpl-2−/− BMDDMs demonstrated reduced levels of ERK1/2 phosphorylation and decreased pro-inflammatory cytokine TNF-α production (157), as well as other defects in immune responses. Additionally, loss of Tpl-2 has been demonstrated to enhance host susceptibility to two intracellular pathogens, Listeria monocytogenes and M.tb (158,159). Therefore, Tpl-2 stands as an important macrophage immune mediator and an excellent host target for regulation by mycobacterial immune evasion mechanisms. We provide evidence in this study that Tpl-2 levels can be modulated by virulent M.tb infection in human macrophages, resulting in altered downstream MAPK signaling that may favor bacterial survival within the host, as well as providing an immune-depressed environment for subsequent invasion by other pathogens.
4.2 Materials and Methods

4.2.1 Buffers and reagents

Dulbecco’s PBS without Ca$^{2+}$ and Mg$^{2+}$, RPMI medium 1640 with L-glutamine, HEPES buffer, and TRIzol were purchased from Invitrogen. 7H11 agar plates were prepared with Bacto Middlebrook 7H11 from Difco Laboratories, oleic acid-albumin-dextrose-catalase enrichment medium, and glycerol. Human serum albumin was purchased from CSL Behring.

4.2.2 Bacterial strains

Lyophilized *Mycobacterium tuberculosis* H$_{37}$R$_{v}$ (ATCC #25618) and *Mycobacterium bovis* BCG Pasteur (ATCC #35734) were obtained from the American Tissue Culture Collection, reconstituted, and used as described (114). Briefly, *M.tb* and BCG were grown for 9-14 days on Middlebrook 7H11 agar plates and scraped into a 2 mL polypropylene tube containing two sterilized 3 mm glass beads in 1 mL RPMI. The samples were pulse vortexed 5 times and the resulting suspension settled for at least 30 minutes to remove bacterial clumps. The settled suspension was removed to a second tube for an additional 10 minutes to help further settle out any bacterial clumps. The concentration of bacteria and the degree of clumping ($\leq 10\%$) in the final suspension were determined by Petroff-Hausser chamber counting; bacteria prepared in this fashion are $\geq 90\%$ viable by CFU assay. Heat killed *M.tb* was prepared by incubating the single cell suspension at 95º C for 1 h.
4.2.3 Isolation and culture of human monocyte-derived macrophages (MDM)

Blood obtained from healthy, Purified Protein Derivative negative human volunteers using a protocol approved by The Ohio State University Institutional Review Board (IRB) was processed as described (23,115). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood on a Ficoll-Paque cushion (GE Healthcare) and cultured in Teflon wells (Savillex) in RPMI 1640 medium containing 20% autologous human serum for five days at 37°C/5% CO₂ for differentiation of monocytes to monocyte-derived macrophages (MDM). On day 5 MDM were harvested from Teflon wells and used for transfection experiments.

4.2.4 Mycobacterial infection of human macrophages

Six day old MDM were incubated with M.tb or BCG at a multiplicity of infection (MOI) of 5:1 in RHH medium (RPMI 1640 medium with 10mM HEPES and 0.4% human serum albumin) for 30 min at 37°C in 5% CO₂ on a platform shaker for equal dispersion of bacteria followed by an additional 90 min incubation without shaking. MDM were washed three times with warm RPMI and repleted with RPMI containing 2% autologous human serum for the remainder of the experiment, unless receiving further treatments.

4.2.5 Western blotting

MDM monolayers were washed once in PBS and lysed in TN-1 lysis buffer (50 mM Tris, 10 mM EDTA, 10 mM Na₄PO₇, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM
Na$_3$VO$_4$, 10 µg/mL each aprotinin and leupeptin) at 4ºC for 10 min and centrifuged at 10,000 x g for 10 min at 4ºC to remove cell debris (117). Protein concentrations of the cleared lysates were measured by the BCA-protein assay kit (Pierce). Protein-matched cell lysates were separated by SDS-PAGE and analyzed by Western blot using the following antibodies: Cot/Tpl-2 and β-actin (Santa Cruz); p44/42 MAPK (ERK1/2), Phospho-p44/42 MAPK (Thr202/Tyr204), MEK1/2, and Phospho-MEK1/2 (Ser217/221) (Cell Signaling). The protein band intensities were measured using the online ImageJ software provided by the NIH. Background intensity was subtracted from each sample and then normalized to the β-actin loading control.

4.2.6 Tpl-2 expression studies by qRT-PCR

*M.tuberculosis*-infected MDM in triplicate wells were lysed in TRIzol reagent and total RNA was isolated according to manufacturer’s instructions. RNA quantity and quality were evaluated by NanoDrop 2000 spectrophotometer. Total RNA (100 ng) was reversed transcribed to cDNA by SuperScript II reverse transcriptase (Invitrogen) and expression of Tpl-2 was determined by qRT-PCR using Taqman Gene Expression Assays (Applied Biosystems) and normalized to β-actin as a housekeeping gene. Negative controls in all qRT-PCR included no reverse transcriptase and no template (cDNA) groups in the reactions. Triplicate samples were analyzed in duplicate wells in each experiment.
**4.2.7 Stimulation of M.tb infected MDMs with TLR agonists**

MDM monolayers were stimulated with various TLR ligands at 72 h after *M.tb* infection. Bacteria were prepared and incubated with Day 6 MDM monolayers as previously described. At 72 h after initial infection, monolayers were washed twice with warm RPMI and then stimulated with 5 µg/mL lipomannan from virulent *M.tb* Erdman strain, 100 ng/mL LPS (TLR4 ligand), 5 µg/mL Pam₃Cys-Ser-(Lys)₄ (Pam₃CysSK₄) (TLR2 ligand), or 1 µM CpG oligodeoxynucleotides (ODN) (TLR9 ligand) or control ODN in RPMI. LPS was obtained from Sigma, Pam₃CysSK₄ was obtained from Calbiochem, and CpG ODN and control ODN were purchased from InvivoGen.

**4.2.8 Statistical analysis**

Most experiments were performed at least two independent times using two different donors and yielded similar results. The magnitude of the response from each independent experiment varied among the donors; however, the pattern of experimental results was the same from donor to donor. To account for this variability, we normalized the data to an internal control in each experiment. A ratio of experimental results to control was obtained, and the mean ratio was then tested for a significant difference from one using *t* statistics. In most instances an unpaired one-tailed Student’s *t*-test was used to analyze differences between groups. *P*-value < 0.05 was considered significant.
4.3 Results

4.3.1 Tpl-2 protein expression is decreased during virulent M.tb infection

A recent report identified Tpl-2 as a mycobacterial resistance factor using a Tpl-2 knockout mouse model (159). Therefore, we assessed Tpl-2 protein expression during M.tb infection in human macrophages over time, including at an early time point of 24 h, as well as at a later time point of 72 h (the doubling time of M.tb is approximately 24 h). The results show that the Tpl-2 protein levels decrease during the course of M.tb infection. In comparison to the time-matched non-infected control groups we observed a significant reduction in Tpl-2 protein expression, especially at 72 h after infection (Fig. 4.1A). Since expression of Tpl-2 is important for mediating host resistance to virulent M.tb, we hypothesized that there may be differential regulation of Tpl-2 expression between virulent and attenuated vaccine strains of mycobacteria. Therefore, we compared Tpl-2 protein expression by Western blot in MDMs after M.tb infection to that after BCG infection. Again we observed a decrease in Tpl-2 expression with M.tb infection over time; in contrast, Tpl-2 protein levels increased at 24 h after BCG infection and remained elevated above resting levels for the entire period under observation (Fig. 4.1B). Since the observation of decreased Tpl-2 expression was strongest at 72 h after M.tb infection, which is the time at which M.tb is undergoing early rounds of replication in our model, we assessed whether loss of Tpl-2 expression requires replicating M.tb. We compared Tpl-2 expression in non-infected MDMs with those either infected with live M.tb or treated with heat killed M.tb. The results in Fig. 4.1C show that treatment of MDMs with
heat killed *M. tb* also leads to decreased Tpl-2 expression at 72 h, indicating that replication is not required for the effect observed. However, the heat killed *M. tb* was not able to induce as robust of a decrease in Tpl-2 expression as live *M. tb* compared to the non-infected control groups at earlier time points.

Figure 4.1: Tpl-2 protein levels are decreased during *M. tb* infection. Tpl-2 protein levels were analyzed by Western blot of protein-matched whole cell lysates (continued on next page)
(Fig. 4.1 continued)

after (A) virulent *M. tb* infection and in time-matched non-infected MDMs (left panel, representative of n = 4). Densitometry analysis of the immunoblot results shows decreased Tpl-2 expression with *M. tb* infection (right panel). (B) Western blot analysis of protein-matched whole cell lysates for Tpl-2 expression in MDMs after infection by *M. tb* or BCG over time (left panel, representative of n = 3). Densitometry analysis of the immunoblot shows decreased Tpl-2 expression with virulent *M. tb* compared to vaccine strain BCG (right panel). (C) Tpl-2 protein expression detected by Western blot after live *M. tb* infection or heat killed *M. tb* (left panel, representative of n = 2). Densitometry analysis shows that heat killed *M. tb* also suppresses Tpl-2 levels relative to non-infected control, but to a reduced extent compared to live *M. tb* (right panel). All blots were re-probed with actin as a loading control.

### 4.3.2 Tpl-2 mRNA levels increase during *M. tb* infection and are predicted to be targeted by *M. tb*-induced miRNAs

We next assessed Tpl-2 mRNA expression in human macrophages during *M. tb* infection by quantitative real-time PCR (qRT-PCR). Contrary to the previous observations of decreasing Tpl-2 protein expression over time, we saw increased Tpl-2 mRNA expression by qRT-PCR during *M. tb* infection. Both non-infected MDMs and *M. tb* infected MDMs showed an increasing trend in Tpl-2 mRNA expression over the time period observed; however, Tpl-2 mRNA levels were significantly increased with *M. tb* infection when compared with the time-matched non-infected controls (Fig. 4.2A). This pattern of increasing mRNA expression coupled with decreased protein production suggests involvement of mechanisms of post-transcriptional or post-translational regulation, such as miRNAs. To assess potential regulation of Tpl-2 by miRNAs, we queried target prediction algorithms with the previously generated list of miRNAs differentially expressed during *M. tb* infection (see Chapter 2) to identify miRNAs that may target Tpl-2. Since miRNAs are negative regulators of protein production and Tpl-2
mRNA expression is increased but this increase is not reflected in protein, we looked for miRNAs increased during *M.tb* infection that are predicted to target Tpl-2. Of the 31 significantly up-regulated MDM miRNAs at 72 h after *M.tb* infection only four were identified by more than one prediction algorithm as putative miRNA regulators of Tpl-2: miR-132, miR-21, miR-146b-5p, and miR-26a.

![Graph](image)

**Figure 4.2:** Tpl-2 protein levels decrease, but mRNA expression increases during *M.tb* infection.

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(Fig. 4.2 continued)

(A) *M. tb* infected and non-infected MDMs were lysed at various times to assay for Tpl-2 mRNA expression by qRT-PCR. Shown are cumulative data from three individual donors, normalized to β-actin, each performed in triplicate (mean ± SEM, *P*<0.05, **P**<0.01). miRNAs previously identified as being significantly up-regulated after *M. tb* infection and predicted to target Tpl-2 were knocked down by transfection with (A) miR-21 inhibitor and (C) miR-132 inhibitor prior to *M. tb* infection and Tpl-2 protein levels were assessed by Western blot. Blots were re-probed with actin as a loading control (top panels, n = 1). Densitometry analysis of the immunoblot results do not show substantial increases in Tpl-2 expression in the miRNA knockdown groups compared to the negative control (bottom panels).

We investigated the effects of miR-21 and miR-132 single knockdown on Tpl-2 expression during *M. tb* infection. miRNAs are negative regulators of translation, so we predicted an increase in Tpl-2 expression after miR-132 or miR-21 knockdown if these miRNAs are true negative regulators of Tpl-2 in human macrophages. MDMs were transfected with miR-21 or miR-132 inhibitor by the Lonza nucleofector system prior to infection with *M. tb*. At various times after *M. tb* infection transfected cells were lysed and assessed for Tpl-2 protein levels by Western blot. The results of a single experiment with each inhibitor show no changes in Tpl-2 protein expression relative to the negative control that would be congruent with knockdown of a miRNA regulator of Tpl-2 (Fig. 4.2B-C).

### 4.3.3 Effects of *M. tb* infection on Tpl-2 regulator p105

A mechanism of post-translational regulation for Tpl-2 is control of Tpl-2 protein stability through association with NF-κB1 p105. Binding of Tpl-2 with the C-terminal portion of p105 is required to maintain steady state levels of Tpl-2 as well as prevent
premature activation of Tpl-2 (153). BMDM from Nfkb1−/− mice are deficient in p105 and show low levels of Tpl-2 as well as reduced activation of MEK and ERK in response to LPS stimulation (160). We hypothesized that reduced levels of p105 protein expression during M.tb infection could lead to a decrease in half-life of Tpl-2 with reduced levels of Tpl-2 protein.

To further explore this mechanism of Tpl-2 post-translational regulation we examined p105 expression in MDMs during M.tb infection. Protein levels of p105 were assessed by Western blot in cell lysates at various times after live M.tb infection or heat killed M.tb treatment. Compared to the time-matched non-infected controls, there was a small decrease in p105 expression during live M.tb infection (Fig. 4.3A) and a small increase in p105 levels after treatment with heat killed M.tb (Fig. 4.3B). These results suggest that loss of p105 to stabilize Tpl-2 during infection with live M.tb may be contributing to the reduced Tpl-2 levels observed, but is unlikely to be the only mechanism responsible for this finding. Our early results suggest differences in the host response in terms of p105 levels for heat killed M.tb versus live M.tb.
Figure 4.3: p105 protein levels are not substantially decreased to a small degree in MDMs after infection with live *M. tb* but not heat killed *M. tb*. p105 protein levels were determined by Western blot in (A) *M. tb*-infected and (B) heat killed *M. tb* infected MDMs along with the time-matched non-infected controls. Blots were re-probed with actin as a loading control (left panels, representative of n = 2 for live *M. tb* and n = 1 for heat killed *M. tb*). Densitometry analysis of the immunoblot results shows only small differences in p105 expression after infection with live or heat killed *M. tb* (right panels).

4.3.4 *M. tb* infection suppresses MEK and ERK1/2 activation after stimulation with *M. tb*

*Erdman lipomannan*

Downstream of Tpl-2 signaling are the MAP2K MEK and the MAP kinases ERK1/2, which serve to activate an array of pro-inflammatory genes in response to TLR activation. Suppression of Tpl-2 expression in human macrophages by prior *M. tb*
infection may alter macrophage responses to subsequent infections or inflammatory stimuli. To investigate the effects of inflammatory stimuli on macrophage responses during *M.tb* infection we treated MDMs at 72 h after *M.tb* infection with *M.tb* Erdman lipomannan (ErdLM). Lipomannans are mycobacterial lipoglycans that induce strong inflammatory activity; while it is known that lipomannan activates TLR2 and not TLR4, it has been reported that lipomannan can activate a number of other macrophage receptors, including the complement receptor CR3 (161). *M.tb*-infected MDMs were lysed after ErdLM treatment for times up to 1 h and assessed for MEK and ERK1/2 activation by Western blot. Tpl-2 expression was also determined by Western blot to confirm suppression of Tpl-2 by previous *M.tb* infection (Fig. 4.4A). The results in Fig. 4.4B show that ErdLM induced decreased levels of phosphorylated MEK in the *M.tb*-infected MDMs, which is congruent with the observation of decreased Tpl-2 levels. Consequently, we determined that there were decreased levels of phosphorylated ERK1/2 after ErdLM treatment in the *M.tb* infected MDMs (Fig. 4.4C). Unexpectedly, we also observed that *M.tb* infection decreased the amount of total ERK1/2 present in the cells.
Figure 4.4: *M. tb* infection suppresses MEK and ERK1/2 activity after *M. tb* Erdman lipomannan stimulation.

At 72 h after *M. tb* infection MDMs or time-matched non-infected controls were stimulated with 5 µg/mL ErdLM and lysed at varying times for analysis of MEK and ERK1/2 activation by Western blot of protein-matched whole cell lysates. (A) Tpl-2 expression was decreased by *M. tb* infection throughout the timeframe under study. The Tpl-2 immunoblot was re-probed for actin as a loading control (top panel immunoblots, bottom panel densitometry). (B) Levels of phosphorylated MEK were determined by Western blot; blot was re-probed for total MEK as a loading control (top panel). Densitometry of immunoblots shows decreased MEK activation in *M. tb*-infected cells (bottom panel). (C) Levels of phosphorylated ERK1/2 were determined by Western blot; blot was re-probed with total ERK1/2 and actin as loading controls (left panel). Densitometry of the immunoblot for phosphorylated ERK1/2 normalized to total ERK1/2 shows a decrease in active ERK1/2 (middle panel) and decreased total ERK1/2 expression normalized to actin in *M. tb*-infected cells (right panel). Shown are representative Western blots and densitometry data representing data from two individual experiments.
4.3.5 *M.tb* infection does not decrease ERK1/2 activation but does decrease MEK activation after pure TLR agonist stimulation

In previous experiments we observed decreased Tpl-2 expression in MDMs during *M.tb* infection, and consequently reduced phosphorylated MEK and phosphorylated ERK1/2 activity in infected cells after ErdLM treatment. ErdLM is a known agonist of TLR2 but can also activate other macrophage receptors with mixed pro- and anti-inflammatory effects. Therefore, we investigated ERK1/2 activation in *M.tb*-infected MDMs after stimulation with the TLR4 agonist LPS, the TLR2 agonist Pam$_3$CysSK$_4$, and the TLR9 agonist CpG, which are all known inducers of ERK1/2 activation in macrophages.

MDMs infected with *M.tb* for 72 h or time-matched non-infected MDMs were incubated with 100 ng/mL LPS, 5 µg/mL Pam$_3$CysSK$_4$, 1 µM CpG ODN or the control ODN for 15 minutes, 30 minutes, or 1 h. Whole cell lysates were collected and analyzed for ERK1/2 activation by Western blotting of the phosphorylated ERK1/2 (Figure 4.5A-C). Under basal conditions without TLR agonist there appears to be low level, relatively stable phosphorylated ERK1/2 activity in *M.tb*-infected cells compared to the non-infected cells. After treatment with LPS, Pam$_3$CysSK$_4$, or CpG ODN we detected similar or elevated phosphorylation of ERK1/2 in *M.tb*-infected MDMs relative to the TLR agonist treated non-infected MDMs. These increases in ERK1/2 phosphorylation were observed despite slightly reduced expression of total ERK1/2 with *M.tb* infection in the Pam$_3$CysSK$_4$ and CpG ODN stimulation experiments. These findings with ERK1/2 phosphorylation stand in contrast to the previous observations of decreased ERK1/2 activation after ErdLM stimulation in *M.tb*-infected MDMs, which suggests that there are
alternative pathways either suppressing phosphorylation of ERK1/2 activated by ErdLM or activation of other ERK1/2 phosphorylation pathways mediated by pure the TLR agonists.

Figure 4.5: Activated ERK1/2 and total ERK1/2 expression after TLR2, TLR4, and TLR9 agonist stimulation in *M. tb*-infected MDMs
At 72 h after *M. tb* infection MDMs or time-matched non-infected controls were stimulated with 100 ng/mL LPS, 5 µg/mL Pam3CysSK4, or 1 µM CpG ODN and lysed at varying times for analysis of ERK1/2 activation by Western blot of protein-matched whole cell lysates. (A) Levels of phosphorylated ERK1/2 after LPS stimulation were determined by Western blot. (B) Levels of phosphorylated ERK1/2 after Pam3CysSK4 stimulation were determined by Western blot. (C) Levels of phosphorylated ERK1/2 after CpG ODN compared to control ODN stimulation were determined by Western blot. All blots were re-probed for total ERK and actin as loading controls (top panels).

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(Fig. 4.5 continued)
Densitometry of the immunoblots are shown for phosphorylated ERK1/2 normalized to total ERK1/2 (middle panels) and total ERK1/2 expression normalized to actin (bottom panels). White bars are non-infected cells, blue bars are non-infected cells with agonist, black bars are M.tb infection alone, and red bars are M.tb infection plus agonist. Shown are representative Western blots and densitometry data representing data from two individual experiments for LPS and Pam3CysSK4 and one experiment for CpG ODN.

In order to address the likelihood of alternative ERK1/2 phosphorylation pathways activated by pure TLR agonists, we examined MEK phosphorylation in M.tb-infected MDMs after LPS and Pam3CysSK4 treatment as MEK activation is a more proximal step to Tpl-2 activity, since phosphorylation of MEK is a direct function of Tpl-2. Western blotting of phosphorylated MEK showed decreased MEK activity during M.tb infection without agonist treatment compared to the unstimulated non-infected control (Fig. 4.6A-B). As in the case of ErdLM stimulation, we observed decreased MEK phosphorylation with M.tb infection after Pam3CysSK4 treatment compared to the Pam3CysSK4 stimulated non-infected control (Fig 4.6B). However, with LPS treatment there was slightly increased MEK activation in M.tb infected groups at initial times studied; this was primarily due to decreased levels of total MEK detected during M.tb infection (Fig. 4.6A). Together these data suggest the possibility of alternative pathways for ERK1/2 phosphorylation outside of MEK activity during treatment with TLR agonists, especially Pam3CysSK4. Decreases in MEK phosphorylation during M.tb infection correlate with decreases in Tpl-2, which has MEK kinase activities, yet ERK1/2 phosphorylation remains at the same or elevated levels compared to the non-infected controls. Therefore,
non-Tpl-2/MEK mediated mechanisms of ERK1/2 phosphorylation may be occurring during activation of TLRs by pure agonists including Pam3CysSK₄ and LPS.

Figure 4.6: Activated MEK and total MEK expression after TLR2 or TLR4 agonist stimulation in M.tb-infected MDMs
At 72 h after M.tb infection MDMs or time-matched non-infected controls were stimulated with 100 ng/mL LPS or 5 µg/mL Pam3CysSK₄ and lysed at varying times for analysis of MEK activation by Western blot of protein-matched whole cell lysates. (A) Levels of phosphorylated MEK after LPS stimulation were determined by Western blot. (B) Levels of phosphorylated MEK after Pam3CysSK₄ stimulation were determined by Western blot. All blots were re-probed for total MEK as a loading control (top panels). Densitometry of the immunoblots is shown for phosphorylated MEK normalized to total MEK (bottom panels). White bars are non-infected cells, blue bars are non-infected cells with agonist, black bars are M.tb infection alone, and red bars are M.tb infection plus agonist. Shown are representative Western blots and densitometry data representing data from two individual experiments.
4.4 Discussion

Through studies in knockout mice the MAP3K Tpl-2 has been established as an important immune regulator and host resistance factor against intracellular pathogens, including *M. tb* (158,159). Yet its pattern of expression and regulation in human macrophages during *M. tb* infection remains unknown. We examined Tpl-2 protein and mRNA expression during virulent *M. tb* infection of MDMs and discovered incongruence between message and protein levels. Tpl-2 protein levels decreased over time in *M. tb*-infected MDMs while mRNA expression for Tpl-2 increased over the same time period. These findings suggest a role for post-transcriptional and/or post-translational regulation of Tpl-2 during infection. Furthermore, we explored Tpl-2 expression patterns in infection with attenuated vaccine strain BCG and during treatment with heat killed *M. tb* and discovered that decreases in Tpl-2 expression appears to be a specific characteristic of virulent *M. tb* infection that is not dependent on replication.

We investigated two possible mechanisms of Tpl-2 regulation in human macrophages during *M. tb* infection, one that is post-transcriptional and one that is post-translational. We identified four miRNAs that are significantly up-regulated during *M. tb* infection that are predicted post-transcriptional regulators of Tpl-2 and carried out experiments using miR-132 and miR-21 knockdown to establish biological evidence for targeting of Tpl-2 by these two miRNAs. In very early studies we observed no significant increases in Tpl-2 protein levels after transfection with the miRNA inhibitors. However, these results do not
preclude a miRNA role for regulation of Tpl-2 during *M.tb* infection as more experiments are needed to confirm the findings of these initial studies. Two additional miRNAs, miR-26a and miR-146b-5p, must also be tested as putative Tpl-2 regulators both alone and in combination with other potential miRNA regulators, since a single miRNA alone may not have a significant impact on a target, but multiple miRNAs acting in concert can may produce additive or synergistic effects on suppression of an mRNA target.

We also explored the possibility of post-translational regulation of Tpl-2 by p105. We hypothesized that loss of p105 during *M.tb* infection could lead to destabilization of Tpl-2 and thereby a reduction in steady state levels of Tpl-2. Our results did not support a decrease in p105 during *M.tb* infection; however, loss of Tpl-2 stabilization by p105 could still be occurring through decreased association between Tpl-2 and p105 in the cytoplasm. We are currently in the process of carrying out co-immunoprecipitation experiments to address association between Tpl-2 and p105 in MDMs during *M.tb* infection. Although we have studied two possible mechanisms for control of Tpl-2 expression, other methods of protein regulation are also possible, such as increased ubiquitination of Tpl-2 and subsequent proteasomal degradation during *M.tb* infection. These other means of Tpl-2 regulation may also be at play; possibly in combination with one or both of the previously studied regulatory measures, and will be studied as part of future experiments.
In our model of human macrophages we also investigated downstream effects of Tpl-2 suppression during *M.tb* infection after TLR activation by studying MEK and ERK1/2 phosphorylation. We observed decreased MEK phosphorylation and ERK1/2 phosphorylation in *M.tb* pre-infected macrophages after treatment with the TLR2 agonist ErdLM, which was expected with decreased Tpl-2 expression during infection. However, ERK1/2 phosphorylation was not significantly decreased in *M.tb* pre-infected macrophages after treatment with the classical TLR2 agonist Pam3CysSK4, TLR4 agonist LPS, and TLR9 agonist CpG ODN, suggesting that alternative ERK1/2 phosphorylating pathways are also activated with these ligands. Preliminary studies of the more proximal event of MEK phosphorylation appear to support this hypothesis, since with Pam3CysSK4 there remained decreased MEK phosphorylation during *M.tb* infection. 

A confounding finding during these studies was the decrease in total ERK1/2, and sometimes total MEK during *M.tb* infection. Consequently, it appears that in addition to modulating Tpl-2 levels, *M.tb* infection may also suppress protein expression of other components of the MAP kinase signaling pathway through unknown mechanisms. There is ample evidence that *M.tb* can manipulate MAPK pathways leading to NF-κB activity in several ways to enhance its survival in macrophages (162-165). It is important to point out that ErdLM is known to be recognized by other surface receptors on macrophages besides TLR2 which may explain its unique behavior in these assays (166).

Previous reports have established the importance of Tpl-2 in mediating host resistance to the intracellular pathogens *M.tb* and *L. monocytogenes* (158,159). However, through
these studies we have demonstrated that pathogens such as virulent *M. tb* do not remain defenseless in the face of macrophage immune responses; instead, *M. tb* has the capacity to suppress expression of host resistance factors such as Tpl-2 to promote its own survival within the macrophage. Although the mechanism through which Tpl-2 expression is decreased during *M. tb* infection is as yet unknown, there are several promising leads. As *M. tb* is primarily a chronic infection, existing in latent form in most infected individuals, suppression of host microbial resistance factors such as Tpl-2 by *M. tb* infection can have important consequences for host responses to secondary infections. We have shown that *M. tb* pre-infection can alter macrophage signaling to some TLR agonists, especially mycobacterial lipomannan. Additional studies of biological function such as cytokine production or bacterial growth assays are still needed to demonstrate a functional consequence of alterations in MAP kinase signaling, but there exists substantial evidence to indicate that *M. tb* is able to subvert macrophage immune responses through Tpl-2.
Chapter 5: Synthesis

The lungs represent a major interface between our bodies and the outside environment and as a result are the battleground between the first-line innate immune defenses and airborne pathogens like M.tb. Among the first immune responders in the lung is the alveolar macrophage, which has the ability to phagocytose and kill invading pathogens. However, M.tb has co-evolved with humans and is well-adapted to overcoming macrophage defenses, even utilizing the macrophage as its cellular host. The work in our laboratory is focused on the early interface between M.tb and the macrophage, as these initial interactions between host and pathogen will determine establishment of infection and course of disease.

In recent years the global health community has faced a concerning rise in rates of multidrug-resistant and extensively drug-resistant tuberculosis. Yet, we still possess nearly the same arsenal of antibiotics for TB treatment as we did half a century ago. There is dire need for new lines of therapies against novel targets in both the host and pathogen, in addition to generation of new compounds for antibiotics. It has become evident that existing drugs and treatment regimens are inadequate and new combination regimens will be required to curb this global health crisis (167). Therefore, of special
interest for study are macrophage molecules that may be modulated by host-directed therapies for better killing of *M. tb*, such as miRNAs and kinase signaling pathways.

In order to study the host molecules that regulate macrophage responses in tuberculosis we utilized a primary human macrophage model of MDMs. MDMs resemble alveolar macrophages in certain fundamental ways; for example, both cell types express many of the same receptors that detect *M. tb* (20,117,168,169). MDMs are also an accepted model for tissue macrophages infected by *M. tb* in the course of disseminated disease, since tissue macrophages predominantly arise from differentiation of circulating monocytes in the blood. Thus, the MDM is recognized as an excellent model for studying human macrophage responses to *M. tb* infection.

Prior to our studies there were little data on miRNA functions in human macrophages during virulent *M. tb* infection and the miRNA profile of primary human macrophages during *M. tb* infection had never been elucidated. Previous work predominantly studied miRNA expression in mixed cell populations or serum, which likely contributed to the lack of consensus in miRNAs identified between studies as well as difficulties in identifying and validating mRNA targets downstream of the miRNAs. As *M. tb* is a uniquely human pathogen, we believed that it was important to study miRNA expression in human macrophages since there are known differences in immunity between humans and murine models, especially in regards to *M. tb* pathology. To date, our study is the only
one to report miRNA expression profiling results in primary human cells, which are biologically more reflective of the natural cellular host for *M. tb*.

In order to determine the miRNAs altered during *M. tb* infection of primary human macrophages we performed miRNA expression profiling by NanoString. There are a number of approaches to miRNA expression profiling each with a different set of strengths and pitfalls including but not limited to coverage, reproducibility, cost, ease of analysis, and accessibility. Among the most commonly used miRNA expression profiling techniques are highly parallel PCR-based methods, hybridization-based platforms like microarray, and next-generation sequencing (170). PCR-based techniques have long been utilized in laboratories for studies of RNA expression, but can be time consuming to optimize due to differences in primer hybridization conditions. We opted to use qRT-PCR for validation of selected expression profiling results. Hybridization-based methods like microarray are among the first massively parallel expression profiling techniques and are still in wide use today; however, due to the specific labeling chemistry used microarrays may have imperfect specificity and lack the ability to provide absolute quantification of miRNAs, instead being more suited for detecting relative abundance of miRNAs. Next-generation sequencing has the ability to detect novel miRNA species, but is often costly and requires significant computational and analysis efforts (171).

We began with microarray in our early efforts to determine the human macrophage miRNA expression profile after *M. tb* infection, but initial results proved to be
inconsistent and difficult to reproduce by qRT-PCR. Next we used NanoString for miRNA expression profiling of *M.tb*-infected human primary macrophages. NanoString is also a hybridization-based method for expression profiling, but unlike microarray it is able to discriminate between similar miRNA species with great accuracy. Additional benefits for NanoString are ease of data analysis and cost. We were able to determine expression of approximately 600 mature miRNA species by NanoString. Although coverage of miRNA species detectable by NanoString was not as extensive as that for microarrays, ultimately the NanoString results were much more consistent and reproducible by qRT-PCR.

Through miRNA expression profiling of *M.tb*-infected MDMs we observed that miR-132 and miR-26a are up-regulated in human macrophages at 72 h after *M.tb* infection (Fig. 2.3B) and that these miRNAs can down-regulate protein expression of the transcriptional activator p300 (Fig. 3.3), which serves to activate transcription of IFN-γ responsive genes. Based on these data our hypothesis is that *M.tb* co-opts host miRNAs to promote its own survival advantage. However, expression of these miRNAs may not be exclusive to virulent *M.tb* and may also be induced by other mycobacteria. Thus, we are interested in identifying *M.tb*-specific miRNA responses that may play a role in *M.tb* virulence.

The attenuated *M. bovis* BCG has been used as a live vaccine for over five decades to control the spread of *M.tb* with variable efficacy (172). While widely utilized as a vaccine, BCG can still result in complications including disseminated infection,
especially among the immunocompromised (173). *M. tb* and BCG are over 99% identical in genome; however, analysis of the genomic differences between *M. tb* and BCG indicate three main regions of difference. Only region of difference 1 (RD1) is consistently present in clinical and laboratory *M. tb* strains and missing in all BCG substrains (174). Two secreted proteins encoded in RD1, culture filtrate protein of 10-kD (CFP-10) and early secretory antigenic target of 6-kD (ESAT-6), have been extensively studied as potential *M. tb* virulence factors (175, 176); however, the precise attenuating mechanism behind BCG remains unknown. We hypothesize that by comparing the miRNA expression profile of *M. tb*-infected MDMs with that of BCG-infected MDMs in the future, it will be possible to identify miRNAs that are specifically regulated by *M. tb* to mediate virulence, thus providing an important piece of knowledge regarding the source of attenuation for BCG as well as providing mechanisms for virulence in *M. tb*.

After we identified and confirmed p300 as a target of the *M. tb* infection-induced miRNAs, miR-132 and miR-26a, we studied effects of these two miRNAs on macrophage IFN-γ responsiveness, since p300 is an important mediator of IFN-γ signals in human macrophages. We used miR-132 and miR-26a specific miRNA inhibitors delivered by nucleofection to knock down miR-132 and miR-26a levels in MDMs, which increased p300 protein levels (Fig. 3.2), and stimulated these cells with IFN-γ. The miRNA knockdown MDMs demonstrated improved transcriptional (Fig. 3.4), translational (Fig. 3.5), and functional (Fig. 3.9A) responses to IFN-γ. However, we observed only limited effects of miR-132 and/or miR-26a knockdown on reducing
intracellular \(M.tb\) burdens (Fig. 3.10B-C). Another puzzling result we observed was that miR-132 and miR-26a over-expression by transfection of miRNA mimics in MDMs did not alter transcriptional responses to IFN-\(\gamma\) (Fig. 3.7), perhaps due to compensation in transcriptional co-activator activity by CBP, whose expression was unchanged by miRNA over-expression (Fig. 3.8D-E). Overall, these findings suggest that during human \(M.tb\) infection there is decreased capacity for macrophages to be activated by IFN-\(\gamma\) and perform microbicidal functions. When these results are considered in conjunction with observations that there are high levels of IFN-\(\gamma\) detected in TB patients they suggest that vaccination efforts to improve IFN-\(\gamma\) production by lymphocytes will not be the most effective strategies in clearing \(M.tb\). Instead, for example, adjunctive therapies targeting miR-132 and miR-26a along with antibiotics may be a more effective means of controlling \(M.tb\) growth in macrophages.

In addition, we have shown that the expression of the MAP3K Tpl-2, which was previously identified as a host defense molecule against \(M.tb\) in a murine knockout model, is down-regulated during \(M.tb\) infection in human primary macrophages (Fig. 4.1A). Interestingly, mRNA levels of Tpl-2 were increased while Tpl-2 protein levels decreased (Fig. 4.2A), suggesting a role for miRNAs or another post-transcriptional regulatory mechanism. Several miRNAs up-regulated during \(M.tb\) infection were predicted to target Tpl-2, of which we studied miR-21 and miR-132 experimentally (Fig. 4.2B-C). We also investigated post-translational regulation of Tpl-2 by p105 during \(M.tb\) infection (Fig. 4.3). Early studies did not support or preclude either miRNAs or p105 for
loss of Tpl-2 in MDMs; further studies are needed to elucidate the mechanism behind Tpl-2 suppression during *M. tb* infection.

We also reported on the downstream signaling effects of *M. tb*-induced suppression of Tpl-2 in human macrophages by studying ERK and MEK activation after stimulation with the TLR ligands ErdLM, LPS, Pam₃CysSK₄, and CpG ODN. ErdLM stimulation of 72 h *M. tb*-infected macrophages showed decreased activation of ERK1/2 and MEK, congruent with decreased Tpl-2 levels after *M. tb* infection (Fig. 4.4). However, the ERK1/2 activation did not significantly decrease in *M. tb*-infected MDMs after treatment with the pure TLR agonists LPS, Pam₃CysSK₄, and CpG ODN (Fig. 4.5), although decreased MEK phosphorylation was observed (Fig. 4.6), suggesting that alternative ERK1/2 activating pathways are also activated with the pure TLR ligands but not with ErdLM. Additionally, ErdLM is known to be recognized by other surface receptors on macrophages besides TLR2 and *M. tb* is known to suppress other MAPK pathways. This raises the possibility that suppression of Tpl-2 and ERK1/2 activation may be specific effects of *M. tb* outer wall molecules like LM. Further studies are required to elucidate the contributions of alternative pathways to ERK1/2 activation and differences in downstream signaling between mycobacterial LM and pure TLR agonists.

Although induction of miR-132 and miR-26a, and suppression of Tpl-2 expression appear to benefit *M. tb* survival within human macrophages, we do not have the evidence to support that these effects are directly initiated by *M. tb*. An alternative hypothesis is
that up-regulation of miR-132 and miR-26a, as well as down-regulation of Tpl-2, is the result of internal host braking mechanisms to tune inflammatory responses, thereby maintaining a balance between microbial killing and host damage. There is some evidence that Tpl-2 expression is most decreased with \textit{M.\text{\textit{tb}} (Fig 4.1B)}, suggesting that this is a specific virulence effect and not the result of a negative feedback mechanism, but no such evidence exists for miR-132 and miR-26a. The mechanism for initiation of these host responses were not addressed in these studies and should be the focus of future work.

In summary, the studies in this dissertation have uncovered two immunoregulatory mechanisms at work in primary human macrophages during virulent \textit{M.\text{\textit{tb}}} infection. Among the numerous ways that \textit{M.\text{\textit{tb}}} has adapted to survival within the human macrophage by subverting the host’s anti-microbial mechanisms are decreasing macrophage IFN-\(\gamma\) responses through increased miR-132 and miR-26a expression and disruption of MAPK signaling pathways by down-regulating expression of the host-protective kinase Tpl-2. In addition, we have determined the miRNA expression profile of resting primary human macrophages as well as that of virulent \textit{M.\text{\textit{tb}}}-infected primary human macrophages, and identified a subset of miRNAs differentially expressed with \textit{M.\text{\textit{tb}}} infection. This work has furthered previous studies by providing a mechanism for past observations as well as by bringing studies of miRNAs and Tpl-2 into primary human macrophages, thereby providing new evidence for the importance of these molecules in human disease. The results of these studies indicate that miR-132, miR-26a,
and Tpl-2 may be excellent targets of host-directed therapies for TB and the knowledge gained here will benefit future studies to these ends.
Bibliography


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