ABSTRACT

Title of Dissertation: RV3165C AND RV3167C ARE TWO NOVEL ANTI-APOPTOTIC GENES OF MYCOBACTERIUM TUBERCULOSIS

Serdar Abidin Gurses, Doctor of Philosophy, 2012

Dissertation Directed By: Associate Professor Volker Briken
Department of Cell Biology and Molecular Genetics

Tuberculosis continues to kill close to two million people every year. This is due to the ease with which the bacteria spreads, the ineffectiveness of the vaccine currently in use, as well as the long drug regimen that is required for treatment of the disease. In order to develop new vaccines or drugs which require shorter treatment regimens, it is important to understand the interaction of the immune system with Mycobacterium tuberculosis (Mtb). One of the mechanisms by which Mtb is thought to survive in the human host is the inhibition of the apoptosis of infected cells. I was able to identify Rv3165c and Rv3167c as two novel anti-apoptotic genes of Mtb. Mtb knock-outs lacking Rv3165c or Rv3167c (∆Rv3165c, ∆Rv3167c) failed to inhibit apoptosis of infected macrophages and the apoptosis of infected macrophages depended on caspase-8 and 3. Inhibiting reactive oxygen species (ROS) production or scavenging ROS inhibited the
apoptosis of ∆Rv3165c or ∆Rv3167c infected macrophages. ∆Rv3165c and ∆Rv3167c failed to upregulate the expression of the only catalase of Mtb, katG. Therefore, it seems that Mtb needs to upregulate katG expression in order to neutralize host cell produced ROS to inhibit apoptosis. Rv3165c was found to reside mainly in the cell envelope while Rv3167c, which is predicted to be a probable transcriptional regulator, was found exclusively in the cytosol of mycobacteria. Rv3165c was required for the upregulation of Rv3167c expression. These data led to a working model in which Rv3165c sits in the plasma membrane of Mtb and detects a signal while Mtb is in the phagosome. This signal detection results in upregulation of Rv3167c expression. Increased Rv3167c expression enables Rv3167c to change the expression of Mtb genes like katG. Rv3167c upregulates expression of anti-apoptotic genes and downregulates the expression of pro-apoptotic genes. RNA sequencing and antibody microarray experiments identified the host cell components that are differentially regulated in ∆Rv3167c infected cells compared to Mtb infected cells. Validation of these targets will help find the host cell pathways Mtb manipulates to inhibit host cell apoptosis.
RV3165C AND RV3167C ARE TWO NOVEL ANTI-APOPTOTIC GENES OF MYCOBACTERIUM TUBERCULOSIS

By

Serdar Abidin Gurses

Dissertation submitted to the Faculty of the Graduate School of the University of Maryland, College Park in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2012

Advisory Committee:

Professor Volker Briken, PhD, Chair
Professor David Mosser, PhD
Professor Kenneth Frauwirth, PhD
Professor Ian Mather, PhD
Professor Philip DeShong, ScD
© Copyright by
Serdar Abidin Gurses
2012
DEDICATION

I dedicate this dissertation to

My family
ACKNOWLEDGEMENTS

I would like to thank Dr. Volker Briken for entrusting me with my current research project and guiding me through it. I would also like to thank my committee members Dr. David Mosser, Dr. Kenneth Frauwirth, Dr. Ian Mather, and Dr. Philip DeShong for their time and support. I would like to thank the previous members of the Briken lab, especially Dr. Kamalkannan Velmurugan and Dr. Jessica Miller for teaching me so many techniques and methods in the lab. I would like to thank all the current members of the Briken lab: Amro, Ben, Gaya, Hanna, Jeff, Lalitha, Swati for being there when I needed them.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................................................................. ii

TABLE OF CONTENTS .................................................................................................................... iii

LIST OF FIGURES ................................................................................................................................. v

LIST OF ABBREVIATIONS ...................................................................................................................... vii

CHAPTER 1 INTRODUCTION .............................................................................................................. 1

1.1 Tuberculosis ...................................................................................................................................... 1
  1.1.1 Prevention of tuberculosis ........................................................................................................ 5
  1.1.2 Treatment of tuberculosis ......................................................................................................... 7
  1.1.3 Mycobacterium tuberculosis ...................................................................................................... 8

1.2 Immune response and mycobacteria ............................................................................................... 9

1.2.1 Immune response to mycobacteria .......................................................................................... 9

1.2.2 Prevention of phagosome maturation by Mtb ........................................................................ 12

1.3 Apoptosis ....................................................................................................................................... 15

1.3.1 Role of ROS in apoptosis ............................................................................................................ 19

1.3.2 Apoptotic response to bacterial pathogens ................................................................................ 19

1.4 Apoptotic response to Mtb ............................................................................................................. 23

1.4.1 Inhibition of apoptosis by Mtb .................................................................................................. 24

1.4.2 Anti-apoptotic genes of Mtb .................................................................................................... 25

1.4.3 Identification of novel anti-apoptotic genes of Mtb ................................................................. 26

1.5 Summary and significance ............................................................................................................. 29

CHAPTER 2 MATERIALS AND METHODS ......................................................................................... 34

2.1 Materials ......................................................................................................................................... 34

2.2 Bacteria and culture conditions ...................................................................................................... 36

2.3 In vitro hydrogen peroxide production ......................................................................................... 36

2.4 Creation of complements of knockouts ...................................................................................... 36

2.5 Creation of Rv3165c-myc and Rv3167c-myc expressing BCG ....................................................... 37

2.6 Electroporation of plasmids ........................................................................................................ 37

2.7 Cell culture maintenance ............................................................................................................... 38

2.8 Infections ....................................................................................................................................... 38

2.9 Detection of apoptosis .................................................................................................................. 39

2.10 Determination of necrotic cell death ........................................................................................... 40

2.11 Determination of the number of live bacteria after infection ....................................................... 40

2.12 Mycobacteria genomic DNA isolation ....................................................................................... 41

2.13 Southern blotting .......................................................................................................................... 41

2.14 Caspase activity detection ............................................................................................................ 42

2.15 Whole cell lysate preparation ...................................................................................................... 42

2.16 Polyacrylamide gel electrophoresis and western blotting ............................................................ 43

2.17 Subcellular fractionation .............................................................................................................. 43

2.18 Culture filtrate preparation .......................................................................................................... 44
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Estimated epidemiological burden of TB in 2010</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Transmission cycle of Mtb</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Apoptosis</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>Extrinsic and intrinsic pathways of caspase activation</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>ROS mediates the balance between the pro-apoptotic and anti-apoptotic</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>phenotypes induced by</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>The J21 cosmid</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>3/4 and 7/10 regions of the J21 cosmid contain anti-apoptotic genes</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>Deletion of the 7/10 region in Mtb reduces its virulence in SCID mice</td>
<td>31</td>
</tr>
<tr>
<td>9</td>
<td>Preliminary experiments indicate $Rv3165c$ and $Rv3167c$ genes as the anti-</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>apoptotic genes in the 7/10 region.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>$\Delta Rv3165c$ and $\Delta Rv3167c$ induce significantly more apoptosis</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>of THP-1 macrophages</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Confirmation of deletion of $Rv3165c$ and $Rv3167c$ by RT-PCR</td>
<td>52</td>
</tr>
<tr>
<td>12</td>
<td>Confirmation of the insertion of the hygromycin resistance gene into the</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>$Rv3165c$ and $Rv3167c$ genes</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Complementation of $\Delta Rv3165c$ and $\Delta Rv3167c$ reverses their</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>pro-apoptotic phenotypes</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Confirmation of complementation of $\Delta Rv3165c$ by southern blot</td>
<td>56</td>
</tr>
<tr>
<td>15</td>
<td>Confirmation of complementation of $\Delta Rv3167c$ by southern blot</td>
<td>57</td>
</tr>
<tr>
<td>16</td>
<td>Apoptosis inhibits growth of $\Delta Rv3167c$ inside macrophages</td>
<td>58</td>
</tr>
<tr>
<td>17</td>
<td>Apoptosis inhibits growth of $\Delta Rv3167c$ inside macrophages</td>
<td>60</td>
</tr>
<tr>
<td>18</td>
<td>A low percentage of $\Delta Rv3167c$ infected THP-1 cells go through</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>necrosis...</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>$\Delta Rv3165c$ and $\Delta Rv3167c$ induce significantly higher levels</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>of caspase-3 and/or 7 and 8 but not caspase-9 activity in THP-1 cells.</td>
<td></td>
</tr>
</tbody>
</table>
Figure 20: Inhibiting ROS production of scavenging ROS abolishes apoptosis induction by $\Delta Rv3165c$ and $\Delta Rv3167c$................................................................................................................................................................. 66

Figure 21: Detection of the amounts of cytokines in the supernatants of uninfected or Mtb or $\Delta Rv3167c$ or $\Delta Rv3167c::C$ infected THP-1 macrophages................................................. 67

Figure 22: Regulation of $CCL3$ and $CCL20$ expression in Mtb and $\Delta Rv3167c$ infected THP-1 macrophages.................................................................................................................................................. 69

Figure 23: ELISA confirms upregulation of $CCL3$ expression in $\Delta Rv3167c$ infected THP-1 macrophages. ................................................................................................................................................. 71

Figure 24: THP-1 macrophage proteins whose expression or phosphorylation have changed 8 hrs or 24 hrs after infection with $\Delta Rv3167c$ compared to Mtb. ............... 73

Figure 25: InterProScan result for prediction of protein domains of $Rv3167c$. .............. 76

Figure 26: $\Delta Rv3165c$ and $\Delta Rv3167c$ do not upregulate katG expression in macrophages. ................................................................................................................................................. 78

Figure 27: Regulation of $Rv3165c$, $Rv3167c$, $katG$, $nuoB$, $nuoG$ in vitro and in macrophages. .................................................................................................................................................. 80

Figure 28: $\Delta Rv3165c$ do not upregulate $Rv3167c$ expression in macrophages. .......... 81

Figure 29: $Rv3165c$ is in the cell envelope and $Rv3167c$ is in the cytoplasm. ................. 83

Figure 30: $\Delta Rv3167c$ is not less virulent than Mtb in mice............................................ 85

Figure 31: $\Delta Rv3167c$ is not less virulent than Mtb in guinea pigs.................................. 87

Figure 32: The working model showing the confirmed localizations of $Rv3165c$ and $Rv3167c$ and their predicted functions. .................................................................................................................. 96
# LIST OF ABBREVIATIONS

- **AP** – Alkaline phosphatase
- **ASK1** – Apoptosis signal-regulating kinase
- **ATP** – Adenosine triphosphate
- **BAK** – Bcl-2 homologous antagonist/killer
- **BAX** – Bcl-2-associated X protein
- **BCG** – Bacillus Calmette-Guérin
- **BCL-2** – B-cell lymphoma 2 protein
- **BFL1** - Bcl-2-related gene expressed in fetal liver
- **BID** – BH3-interacting domain death agonist
- **BMDM** – Bone marrow derived macrophage
- **cAMP** – Cyclic adenosine monophosphate
- **CBA** – Cytometric bead array
- **CCL** – C-C motif chemokine
- **CD** – Cluster of differentiation
- **CD4+ T cells** – Helper T cells
- **CD8+ T cells** – Cytotoxic T cells
- **CE** – Cell envelope
- **CF** – Culture filtrate
- **CFP** – Culture filtrate protein
- **CFU** – Colony forming units
- **CR** – Complement receptor
- **DC** – Dendritic cell
- **DED** – Death effector domain
- **DPI** – Diphenylene iodonium
- **DUOX** – Dual oxidase
- **ELISA** – Enzyme linked immunosorbent assay
- **FADD** – Fas-associated death domain
- **FAP** – Fibronectin attachment protein
- **FCS** – Fetal calf serum
- **FLICA** – Fluorescent Labeled Inhibitor of Caspases
- **FPKM** – Fragment per kilobase of transcript per million mapped reads
- **IL** – Interleukin
- **iNOS** – Inducible nitric oxide synthase
- **LAM** – Lipoarabinomannan
- **ManLAM** – Mannose capped LAM
- **MAP** – Mitogen activated protein
- **MAPK** – MAP kinase
- **MDR** – Multi-drug resistant
- **MHC** – Major histocompatibility complex
- **MKK** – MAP kinase kinase
- **MNC** – Mononuclear cell
- **MOI** – Multiplicity of infection
- **Mtb** – Mycobacterium tuberculosis
- **Myd88** – Myeloid differentiation primary response protein
NADH – Nicotinamide adenine dinucleotide
NADPH – Nicotinamide adenine dinucleotide phosphate
NF-κB – Nuclear factor-κB
NLR – Nucleotide binding domain, leucine rich repeat containing
NOD – Nucleotide binding oligomerization domain containing
NOX – NADPH oxidase
PAMP – Pathogen associated molecular patterns
PBS – Phosphate buffered saline
PBST – PBS-tween
PknE – Protein kinase E
PknG – Protein kinase G
PMA – Phorbol 12-myristate 13-acetate
pNPP – p-Nitrophenyl Phosphate
RD – Region of difference
ROS – Reactive oxygen species
SECA2 – Protein translocase ATPase
SCID – Severe combined immunodeficiency
SOD – Superoxide dismutase
SODD – Silencer of death domains
TB – Tuberculosis
Th1 – CD4+ T helper cell type 1
Th2 – CD4+ T helper cell type 2
TLR – Toll like receptors
TM – Transmembrane
TMHMM – Transmembrane hidden Markov Model
TNF-α – Tumor necrosis factor alpha
TNFR – TNF-α receptor
TRADD – TNFR-associated death domain
TRAF – TNF-α receptor-associated factor
TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling
WT – Wild type
XDR – Extensively-drug resistant
CHAPTER 1 INTRODUCTION

1.1 TUBERCULOSIS

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (Mtb). One third of the world’s population is currently infected with Mtb, but only 3-15% of the infected individuals develop the disease in their lifetime (1, 2). There were nearly 8.8 million new cases of tuberculosis and an estimated 1.4 million people died of tuberculosis in 2010 (3) (Figure 1). People who do not develop the disease but still carry live Mtb are said to have latent TB. This latent TB can turn into active TB following a weakening of the immune system (3, 4). Although the tuberculosis death rate and the number of deaths are declining, the rate of this decline is very slow. Another significant statistic is that among women between the ages of 15-44, tuberculosis is among the three major causes of death (3).

TB is spread via aerosols formed when an infected person coughs or sneezes. A single bacterium is thought to be enough to cause an infection. When an uninfected person inhales droplets carrying Mtb the bacteria gain access to the lung where they are phagocytosed by alveolar macrophages through recognition by receptors on their surfaces. While Mtb is replicating in the alveolar macrophages the macrophages move to the underlying epithelial layer (1). Meanwhile Mtb or dendritic cells (DC) containing Mtb antigens move to the lymph nodes in the lung. These DCs can then activate CD4+ T cells in the lymph nodes. Activated CD4+ T cells then move to the sites of infection in the lung to recruit and activate macrophages (5, 6). Blood vessel development increases (via increased vascular endothelial growth factor), which helps macrophage recruitment (7, 8). The recruited macrophages, monocytes and neutrophils surround the infected
**Figure 1: Estimated epidemiological burden of TB in 2010.**

Numbers in thousands\(^a\).

\(^a\) indicates no estimate available.

\(^a\) Numbers for mortality, prevalence and incidence shown to two significant figures.

\(^b\) Mortality excludes deaths among HIV-positive TB cases. Deaths among HIV-positive TB cases are classified as HIV deaths.

\(^c\) Best, low and high indicate the point estimate and lower and upper bounds of the 95% uncertainty interval.

\(^d\) Estimates for India have not yet been officially approved by the Ministry of Health & Family Welfare, Government of India and should therefore be considered provisional. Adapted from (3).
macrophages to form a granuloma. Macrophages can then differentiate into multi-nucleated giant cells, epithelioid cells, and foamy macrophages. Lymphocytes and a fibrous cuff surround the granuloma (1, 8, 9).

Two to three weeks after infection the mycobacteria are contained and the number of mycobacteria stabilizes. The number of blood vessels entering the granuloma decreases. This helps decrease the levels of oxygen in the center of the granuloma in which the mycobacteria are contained, thus helping to prevent their replication (1). If the immune system of the host is compromised during latent TB, (e.g., because of a decrease in TNF-α secretion) the granuloma can exhibit caseous necrosis and will eventually cavitate. The mycobacteria within the granuloma then can enter the airways of the lungs and can be transmitted from the infected person to others (4) (Figure 2). Since formation of the granuloma and its later disintegration provides a mechanism to keep Mtb alive and then to be transmitted, granuloma formation is suggested to be beneficial for the Mtb (10). When TNF-α was neutralized in mice with chronic infection, they could not maintain granulomas, the number of bacteria increased 10 fold and the mice survived less than 120 days while the control mice lived more than 6 months (11). These data show the importance of TNF-α for the survival of the host.

In the mouse model, following infection the number of Mtb increases in a logarithmic fashion until 3-4 weeks and then reaches a stationary level. The leveling of the number of Mtb is thought to be due to the onset of adaptive immunity. However, even the immunocompetent mice strains succumb to TB (4). Mtb that has been delivered to the lungs of the mice can disseminate to the liver and spleen as early as 11 days. The numbers of Mtb in these organs increase and reach a stationary level, which is typically
Figure 2: Transmission cycle of Mtb. Live Mtb is inhaled into the lungs where it is phagocytosed by alveolar macrophages. The infected macrophages travel into the epithelial layer. Blood vessels start to develop around the infected cells and more macrophages are recruited. The recruited macrophages, monocytes and neutrophils surround the infected macrophages to form a granuloma. Macrophages can then differentiate into multi-nucleated giant cells, epithelioid cells, and foamy macrophages. Upon weakening of the immune system the granuloma can exhibit caseous necrosis and will eventually cavitate. The mycobacteria within the granuloma then can enter the airways of the lungs and can be transmitted from the infected person to others. Adapted from (1).
lower than the number in the lungs (4, 12). Following aerosol infection of immunocompetent C57BL/6 mice Mtb infects different types of phagocytic cells in the lungs. Although it has been long believed that macrophages are the primary niche for Mtb, it has been reported that the highest percentage of Mtb infected cells in the lungs and the lymphoid nodes were myeloid dendritic cells (DC). The next highest infected cell population comprised recruited interstitial macrophages (6). Interestingly, the myeloid dendritic cells were among the poorest stimulators of Mtb specific CD4$^{+}$ T cells whereas alveolar macrophages were the best stimulators (6). Therefore not only macrophages but also other phagocytic cell types must be taken into consideration when trying to understand the immune response against Mtb.

1.1.1 Prevention of Tuberculosis

The vaccine currently in use against TB is Mycobacterium bovis Bacillus Calmette–Guérin (BCG), which was generated by passaging a strain of M.bovis for 13 years (between 1908 and 1921) on potato slices soaked in glycerol (13). After BCG was shown to be useful and safe as a vaccine, it was distributed to other countries which then maintained and passaged their own strains. For many years the reason for attenuation of BCG was not known. When the BCG genome was compared to the genome of parental strain M.bovis, several regions of difference were found (14–17) but only “region of difference 1” (RD1) was found to be lacking in all of the BCG strains (18, 19). RD1 contains 9 genes; Rv3871, Rv3872 (PE35), Rv3873 (PPE68), Rv3874 (esxB), Rv3875 (esxA), Rv3876, Rv3877, Rv3878 and Rv3879c. These genes do not have any known functions except esxB, which encodes for the 10kDa culture filtrate protein (CFP10) and
esxA, which encodes for the 6kDa early secreted antigenic target (ESAT-6). Deletion of RD1 from Mtb leads to decrease in virulence and produces a phenotype similar to BCG, indicating the involvement of the RD1 genes in virulence of Mtb (20).

BCG is effective against the disseminated form of TB in children (21). However the efficacy of BCG in protecting adults from pulmonary TB varies from 0 - 80% and varies between countries (22). In developing countries the protection efficacy is the lowest. The variability of the protective efficacy of BCG might be due to geographical, environmental and genetic factors (22, 23). Interestingly, the protective efficacy of BCG is lower closer to the equator. One of the reasons for this is thought to be the increased concentration of ultraviolet radiation (UVR) towards the equator. The evidence for this comes from experiments in which exposure to UVR reduced the development of the immune response by BCG in mice (possibly due to increased IL-10 production) and BCG vaccinated guinea pigs had reduced resistance against Mtb if they were exposed to UVR before or after vaccination (24–26). Another reason is thought to be previous or simultaneous infection with parasites leading to a Th2 (Type II helper T cell) immune response that prevents an effective response against Mtb. A Th1 (Type I helper T cell) response is required to achieve immunity against Mtb, but the presence of a Th2 response increases the severity and the onset of the disease (27, 28). Therefore it is suggested that a successful vaccine would have to diminish the Th2 responses in addition to inducing Th1 responses (29). Prior exposure to live environmental mycobacteria greatly decreases the effectiveness of BCG. This is thought to be due to prevention of BCG multiplication and therefore production of a weak immune response (30). Pre-sensitization of guinea pigs
and mice with prior exposure to environmental mycobacteria leads to some protection against Mtb (31–33).

A different explanation for the variable effectiveness of BCG comes from the fact that the strains that each country received from Institut Pasteur were passaged many times. Studies have shown that the various strains that are used around the world have deletions, insertions and SNPs different from each other. On the basis of these differences BCG vaccines were divided into the early strains, represented by BCGs Japan, Birkhaug, Sweden, and Russia and the late strains, including BCGs Pasteur, Danish, Glaxo, and Prague. The major difference between the early and late strains is the lack of RD2 in the late strains (16, 17, 34, 35). A recent study showed that the late strains of BCG had more genetic variability than the early strains, when compared to the late strain BCG Pasteur. Referring to other research done, Brosch et al. suggests that the early BCG vaccine strains might have higher protective efficacy than the late strains (36).

In conclusion, the low efficacy of BCG in preventing tuberculosis has led researchers to try to develop new vaccines. The production of effective and low cost vaccines for use in areas affected most by tuberculosis is much needed and will save many lives. To achieve this goal a better understanding of failure of BCG and new strategies to produce effective vaccines are required.

1.1.2 Treatment of tuberculosis

The treatment of tuberculosis involves a six month minimum drug regimen involving 4 drugs, isoniazid, rifampin, pyrazinamide and ethambutol (37). Unfortunately strains of Mtb that are drug resistant have emerged through the years due to inconsistent
or inappropriate use of drugs. There are multi drug resistant (MDR) Mtb strains that are resistant to more than one of the major drugs against Mtb. The estimated number of MDR tuberculosis cases in 2010 was 650,000 and 150,000 deaths were caused by MDR tuberculosis in 2008 (3). According to estimates, 3.3% of all new tuberculosis cases were MDR tuberculosis in 2009. In 2010 in some areas of the world the percentage of MDR tuberculosis has been reported to be 28% of new tuberculosis cases (3). The patients who have MDR Mtb have to be treated with a second line of drugs that are not only more expensive but also can lead to more side effects in the patients. Treatment of MDR Mtb can last up to two years, which also increases the cost of the treatment. In addition to MDR Mtb, extensively drug resistant (XDR) Mtb strains have emerged. These strains are resistant to the second line of drugs as well as the first line. Fifty eight countries reported XDR tuberculosis cases (3). This evidence shows the requirement for improved and cheaper drugs as well as new vaccine strain(s) (38, 39).

1.1.3 Mycobacterium tuberculosis

Mtb is a rod shaped bacillus that has a length between 1-4µm and width between 0.3-0.6µm (40). In terms of replication time, mycobacteria are divided into slow growers (give colonies on solid media in more than 7 days) and fast growers (give colonies on solid media in less than 7 days). M.tuberculosis and M.bovis are slow growers. M.fortuitum and M.smegmatis are fast growers (41, 42). The mycobacterial colonies on solid media are usually rough, crinkled and white to cream colored (41). The replication time of Mtb is around 24 hrs in vitro and in vivo in the lungs of mice (43). The genome of Mtb is 4,411,529 bp and contains an estimated 4,000 genes. The genome has a high G+C
content of 65.6% (44). The Mtb genome contains many repetitive DNA sequences including duplicated housekeeping genes and insertion sequences (44). The cell envelope of Mtb consists of a cell membrane and a complex cell wall. The cell wall contains layers of peptidoglycan, arabinogalactan, mycolic acids and lipids on the outer part. There are lipoarabinomannans and phosphotidylinositol mannosides in the cell wall with their lipid moieties inserted in the cell membrane. Due to this complex cell wall, Mtb is resistant to many chemicals and therapeutic agents (45). Components of the Mtb cell wall are involved in diminishing the immune response (discussed below in 1.2.2). It has been shown that the outer cell wall, consisting of mycolic acids and lipids, actually forms a symmetrical lipid bilayer (46). It is because of this outer membrane that Mtb can not be Gram stained.

1.2 IMMUNE RESPONSE AND MYCOBACTERIA

1.2.1 Immune response to mycobacteria

Upon entering the human host, mycobacteria are phagocytosed by macrophages through recognition by surface receptors. Human monocyte complement receptors CR1 and CR3 are involved in the phagocytosis of Mtb. Complement component C3 in the serum binds to Mtb and is required for adherence of Mtb to human monocytes and heat inactivation of serum significantly reduces Mtb adherence to monocytes (47, 48). Mtb Erdman and H37Rv strains require the presence of mannose receptors on the macrophage surfaces for adherence (49). Lipoarabinomannan (LAM) of Mtb has been discovered as a ligand for the mannose receptor (50). Surfactant protein A (Sp-A), a collectin, increases
Mtb attachment to macrophages and its uptake but it is not required for an effective
immune response against Mtb in a murine infection model (51–53).

Humans with active TB produce a Th1 response to Mtb and develop granulomas
to contain the bacteria but cannot kill all the bacteria. However the Th1 responses in
active TB patients help to restrict the replication and spread of Mtb. In AIDS patients
Mtb cannot be contained in granulomas and the bacteria replicate and spread from the
lungs to other organs due to decreased number of CD4\(^+\) T cells and downregulation of the
Th1 response (4, 54, 55).

Toll-like receptors (TLRs) are pattern recognition receptors (PARPs) which detect
pathogen-associated molecular patterns (PAMPs). TLRs reside in the cell membrane and
inside the cell, detect ligands and induce immune responses through induction of
cytokines such as TNF-\(\alpha\), IL-1, IL-6 and IL-12. Upon PAMP recognition, TLRs form
dimers. Through adaptors like myeloid differentiation primary response gene 88
(MyD88) and TRIF-related adaptor molecule (TRAM) they activate protein kinases like
interleukin-1 receptor-associated kinase 1 (IRAK1), leading to changes in gene
expression through NF-\(\kappa\)B (56, 57).

TLR2 recognizes 19-kDA lipoprotein, lipomannan and phosphatidyl-myoinositol
mannoside of Mtb while TLR4 recognizes heat-labile components of the cell wall. The
19-kDa lipoprotein of Mtb induces apoptosis in macrophages through TLR2 in a caspase-
8 dependent manner (58). Although TLR2 and TLR4 recognize many Mtb components
and TLR2 knock-out mice show a 50-75\% reduction in TNF-\(\alpha\) production while TLR4
knock-out mice show a 30-40\% reduction, TLR2 or TLR4 knock-out mice have little
enhanced susceptibility to Mtb infection. (59). However mice lacking Myd88 (one of the
signal transducers for TLRs) were highly susceptible to Mtb infection (60). These data indicate potential involvements of other TLRs and/or other Myd88 dependent receptors in the immune response against Mtb.

Intracellular recognition of mycobacteria by host cells depends on intracellular proteins called NLRs (Nucleotide binding domain, leucine rich repeat containing) that include NODs (Nucleotide binding oligomerization domain containing). NLRs are involved in activation of caspase-1 that converts pro-IL-1β and pro-IL-18 to active IL-1β and IL-18 (57). NOD1 recognizes γ-D-glutamyl-meso-diaminopimelic acid (ie-DAP) from peptidoglycans of Gram negative bacteria and NOD2 recognizes muramyl dipeptide (MDP) from peptidoglycans from Gram negative and Gram positive bacteria. When peritoneal macrophages from NOD2 knock-out mice were infected with Mtb there was a 40 to 50% reduction in TNF-α production and a 20 to 25% reduction when peritoneal macrophages from NOD1 knock-out mice were used. Blocking the internalization of Mtb, which prevents its recognition by NODs also leads to a 40 to 50% decrease in TNF-α production. When mononuclear cells (MNCs) from patients defective for NOD2 were infected with Mtb there was a 65 to 80% reduction in TNF-α secretion. As another evidence for the synergistic interaction of TLRs and NLRs, it was shown that addition of TLR2 ligand 19-kDa lipoprotein together with NOD2 ligand MDP increased TNF-α production from MNCs much more than the total of the ligands by themselves (59).

While it was shown that macrophages and DCs lacking NOD2 were impaired in their ability to produce inflammatory cytokines (TNF-α and IL-12) and NO upon infection with Mtb, it was also found that NOD2 deficient mice were no more susceptible to Mtb infection than wild-type mice and had similar bacterial counts in the lungs up to 8
weeks after infection (61). However, it was also reported that NOD2 deficient mice have significantly higher bacterial counts by 6 months and succumb to death earlier than wild-type mice after Mtb infection (62). So, although in vitro NOD2 plays a role in cytokine production, especially synergistically with TLR2 it seems to be dispensable for the immune response against Mtb and the control of infection during the early stages of infection. In the case of TNF-α, depletion of TNF-α by a monoclonal antibody in C57BL/6 mice causes mice to succumb to death faster following infection with Mtb. Similarly, infection of C57BL/6 mice lacking TNF-α receptor with Mtb causes them to succumb to death faster (63). Therefore TNF-α is required for a successful immune response against Mtb in mice. The fact that NOD2 deficient mice are not more susceptible to Mtb during early stages of infection although NOD2 deficient macrophages produced significantly less TNF-α upon Mtb infection in vitro suggests two possibilities. One possibility is that even though TNF-α production by macrophages is also decreased in vivo, it is still enough for an effective immune response. The other possibility is that the in vitro result does not reflect conditions in vivo and NOD2 knock-out mice can produce similar levels of TNF-α in response to Mtb, at least during the early stages of infection. It is also possible that TNF-α production by another cell type in NOD2 knock-out mice is not affected and that is enough for the host response.

1.2.2 Prevention of phagosomal maturation by Mtb

Mtb prevents the fusion of the phagosome with lysosomes and thus prevents phagosomal maturation (64, 65). Pathogenic mycobacteria utilize different strategies to prevent the phagosomal maturation in order to survive, replicate in the phagosome, cause
necrosis of the infected cells and disseminate (66, 67). The strategies adapted by pathogenic mycobacteria involve manipulation of the host cell signaling that leads to the fusion of the lysosome with the phagosome.

Coronin 1 is a protein identified in the membranes of phagosomes that contain live mycobacteria after infection of murine macrophages. Coronins are thought to be involved in the regulation of the actin cytoskeleton (68). When macrophages were infected with live mycobacteria, coronin 1 was present on phagosomes but when the macrophages were infected with dead mycobacteria, coronin 1 left the phagosomes, which was followed by the fusion of phagosomes with lysosomes and the degradation of the mycobacteria (69). Coronin 1 is dispensable for regulation of the actin cytoskeleton but activates the Ca$^{2+}$-dependent phosphatase calcineurin. Active calcineurin then blocks the fusion of phagosomes with lysosomes (70). Kupffer cells in the liver do not express coronin1. Following infection of Kupffer cells all mycobacteria were located in phagolysosomes and were then degraded. Expression of coronin 1 in a nonmacrophage cell line was sufficient to prevent lysosomal fusion of the mycobacterial phagosome, allowing survival of mycobacteria (69). These experiments showed the ability of mycobacteria to recruit coronin 1 to phagosomes to prevent their fusion with lysosomes.

Mannose capped lipoarabinomannan (ManLAM) of Mtb is a glycolipid that is a component of the Mtb cell wall. ManLAM from Mtb prevents phagosome maturation, probably by blocking the generation of phosphatidylinositol 3-phosphate (PI3P) and its recruitment to the phagosomal membrane (71–73). PI3P is involved in the recruitment and functioning of PI3P binding domain containing proteins that are involved in membrane trafficking and phagosome maturation (74). ManLAM also inhibits the
activation of mitogen-activated protein kinases (MAPK) in humans (75). MAPK activation elicits the synthesis of TNF-α and induction of iNOS (inducible nitric oxide synthase) (76–78). By blocking MAPK in monocytes, ManLAM prevents the production of TNF-α and nitric oxide. TNF-α is a major cytokine that promotes apoptosis in mycobacteria infected macrophages (79). These data indicate that the ManLAM of Mtb indirectly inhibits apoptosis. Pathogenic mycobacteria also have strategies to block the bactericidal effects of NO and the recruitment of the iNOS to the phagosome (80). Mtb uses its proteasome to defend itself against the destructive effects of NO and mutants with transposon insertions in proteasome associated genes are susceptible to acidified nitrite (81, 82).

Mycobacteria also have proteins that prevent phagosome maturation. One of them is the mycobacterial protein kinase G (pknG). A pknG knock-out of Mycobacterium bovis BCG colocalized with lysosomes and could not resist destruction by macrophages, unlike wild type M. bovis BCG. pknG was present in both the lumen of the phagosomes and the cytosol of the infected macrophages and inhibited phagosome lysosome fusion (83). The presence of pknG in the host cytosol suggests it might interfere with host cell signaling in order to prevent phagosome maturation. pknG autophosphorylates but this does not affect its kinase activity. Autophosphorylation of pknG prevents fusion of Mtb containing phagosomes with lysosomes (84). Expression of PknG in M. smegmatis increases the persistence of M. smegmatis in mice (85). PknG mutant of Mtb is less virulent in SCID mice (86). This evidence suggests that by preventing phagosome lysosome fusion, pknG increases the virulence of Mtb.
1.3 APOPTOSIS

Apoptosis is a form of programmed cell death, characterized by shrinkage of cytoplasm, condensation of chromatin, fragmentation of chromosomal DNA, formation of cell membrane blebs and formation of apoptotic bodies (87) (Figure 3). Apoptosis is the predominant method for the removal of unwanted cells in the immune and central nervous systems. Removal of certain cells during embryonic development or upon damage is also achieved through apoptosis (88). Apoptosis is preferred over necrosis in these situations because necrosis can damage other healthy cells and cause inflammation, whereas the apoptotic bodies that are formed are taken up by neighboring phagocytes and thus damage is prevented (89, 90). Certain mutations can lead to decreased levels of apoptosis and therefore promote survival of cancer cells (88). Mutations of caspase genes that inhibit their activity or downregulation of caspase gene expression are common in certain cancers (91, 92). On the other hand, flaws in the removal of apoptotic bodies can lead to autoimmune diseases (93).

Apoptosis is carried out by caspases, which are zymogenic proteases. They cleave various cellular substrates that in turn lead to the phenotypes seen during apoptosis (94, 95). There are two pathways for caspase activation, an extrinsic and an intrinsic pathway (88) (Figure 4). The extrinsic pathway involves the binding of ligands to cell surface receptors, which is followed by recruitment and binding of cellular proteins to the cytoplasmic side of the receptors, thus leading to activation of initiator caspases 2, 8, 9, and 10 and effector caspases 3, 6, and 7 (96, 97). TNF-α and Fas-L are two of the ligands which bind to their receptors, TNF-α receptor-1 (TNFR1) and Fas respectively, in this pathway (98). Both of these receptors have death domains on their cytoplasmic sides,
Figure 3: Apoptosis. Progression of apoptosis and its phenotypes.
Figure 4: Extrinsic and intrinsic pathways of caspase activation. Adapted from (100).
which allow them to interact with other proteins that have death domains. TNFR1 interacts with TNFR-associated death domain (TRADD) and Fas interacts with Fas-associated death domain (FADD) (98). FADD has another domain called death effector domain (DED), which allows it to interact with caspase-8 (caspase-8 also has DED). This interaction causes the activation of caspase-8 which then leads to the activation of downstream caspases such as caspase-3 (87).

The TRADD that interacts with TNFR1 can also interact with FADD and lead to the activation of caspases (99). TRADD can also interact with RIP1 and TRAF2 to activate anti-apoptotic signals. TNF-α can interact with another receptor, TNFR2 and this interaction leads to activation of NF-κB, which leads to production of anti-apoptotic proteins (101). Fas can also interact with Daxx to activate it, which then activates apoptosis signal-regulating kinase 1 (ASK1). ASK1 is a MAPKKK that activates c-jun N-terminal kinase and p38 MAPK signaling pathways followed by apoptosis (102–104).

The intrinsic pathway of caspase activation involves the release of cytochrome c from the mitochondria after the permeabilization of its outer membrane upon apoptotic stimuli. The release of cytochrome c is regulated by the Bcl-2 protein family, some of which promote and some of which inhibit the release of cytochrome c (105). Depending on the cell type, cytochrome c is usually proapoptotic. The release of cytochrome c in some cases is required for events like cellular differentiation (106). The released cytochrome c, together with Apaf-1 and pro-caspase-9, forms a complex that has been called the apoptosome. The apoptosome then activates the downstream effector caspases (107, 108).
There is crosstalk present between the two caspase activation pathways. Caspase-8, which is a part of the extrinsic pathway can cleave and activate the protein Bid which is a Bcl-2 protein family member. The activation of Bid promotes the release of cytochrome c from mitochondria and activation of caspase-9 (108). Therefore it is possible to observe caspase-9 activation following extrinsic caspase activation.

### 1.3.1 Role of ROS in apoptosis

Reactive oxygen species (ROS) are used by the cells in both defense against pathogens and regulation of cell signaling (109–111). ROS are produced by mitochondria as well as Nox and Duox enzymes that are on the membrane, using NADPH and oxygen (112–114). As signaling molecules, ROS can induce prolonged JNK activation and TNF-α induced apoptosis in certain cells. As another strategy, NF-κB activation can be blocked by ROS and this prevents transcription of survival genes and promotes cell death (Figure 5) (115). ROS are able to inhibit the activity of some NF-κB activators since they contain specific cysteines that can be oxidized and lose their activity afterwards (110). Thus, increased levels of ROS promote apoptosis by inducing prolonged JNK activation and blocking NF-κB activation.

### 1.3.2 Apoptotic response to bacterial pathogens

Some pathogens induce apoptosis while others inhibit apoptosis. Pathogens can employ toxins and virulence factors for these purposes (116, 117). Pathogens also affect host cell apoptosis through manipulation of cell survival pathways that include NF-κB and MAPK. One of these pathogens is *Chlamydia pneumoniae*, which can activate NF-
Figure 5: ROS mediates the balance between the pro-apoptotic and anti-apoptotic phenotypes induced by TNF-α. Increased levels of ROS can induce prolonged JNK activation that leads to apoptosis and blocks NF-κB activation to promote apoptosis. Adapted from (115).
κB to inhibit apoptosis. *Chlamydia* also inhibits apoptosis by preventing cytochrome c release and thus promotes its own survival (118, 119). *Chlamydia pneumoniae* causes an increase in expression of the anti-apoptotic host gene *mcl-1* in infected cells (120).

*Rickettsia rickettsii* is another pathogen that inhibits host cell apoptosis by increasing NF-κB activation to promote its own survival. Inhibition of NF-κB activation in infected cells results in activation of caspases 8, 9 and 3, followed by an increase in apoptosis (121).

*Salmonella typhimurium* uses its protein AvrA to inhibit apoptosis in macrophages (122). AvrA is a 33 kDa protein secreted by *Salmonella typhimurium* that has been characterized as an acetyltransferase that can inhibit JNK activation in host cells (123).

*Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis* have plasmids that contain genes for a type III secretion system (TTSS) that is necessary for their virulence. TTSS is responsible for secretion of Yop proteins (124). YopJ of *Yersinia pseudotuberculosis* inhibits MAPK and NF-κB pathways, and induces apoptosis of infected macrophages (125–128). In the meantime, the actions of other Yop proteins affect the cell signaling network and the organization of the cytoskeleton host cell to prevent phagocytosis of the bacteria (124). These actions of YopJ and other Yop proteins are thought to help the survival of the bacteria by inhibiting the innate immune response of the host cells (128).

*Bordetella pertussis*, the bacterium that causes whooping cough, secretes many adhesins to attach to the host ciliated cells and toxins to cause lesions and host cell death after it arrives in the host respiratory tract (129). *Bordetella pertussis* induces apoptosis of J774A.1 cells and murine alveolar macrophages. This mechanism is thought to benefit *Bordetella pertussis* by eliminating macrophages that can kill it in the lungs so that it can
infect the epithelial cells and replicate. The pro-apoptotic phenotype of *Bordetella pertussis* depends on one of the secreted toxins, adenylate cyclase-hemolysin (AC-Hly) (130). AC-Hly consists of an adenylate cyclase domain and a pore-forming repeat in a toxin hemolysin domain. AC-Hly is secreted by a type I secretion mechanism (131). Upon entrance into the cell, the adeylate cyclase domain binds to calmodulin and is activated to catalyze the synthesis of cAMP from ATP (132). Increased amounts of cAMP disrupt cellular functions and induce apoptosis (130).

*Streptococcus pneumoniae* induces apoptosis of alveolar macrophages which leads to the clearance of the bacteria from the lungs. Inhibition of caspase activation in the *S. pneumoniae* infected host cells decreased the rate of apoptosis and inhibited the killing of *S. pneumoniae* (133). *S. pneumoniae* inhibits the upregulation of expression of the anti-apoptotic protein Mcl-1 and blockage of this inhibition results in decreased apoptosis and killing of *S. pneumoniae*. Infection of Mcl-1 transgenic mice with *S. pneumoniae* led to decreased apoptosis of alveolar macrophages and better survival of the bacteria compared to infection of nontransgenic mice (134).

As shown in the examples above, inhibition of apoptosis by bacterial pathogens promotes their survival and inhibition of the innate immune response. Inhibition of apoptosis is achieved by different strategies, including activation of NF-κB, inhibition of JNK activation and increasing expression of host anti-apoptotic proteins. Promotion of apoptosis is also employed by bacterial pathogens to remove cells that can kill them. Therefore, the outcome of apoptosis can be either beneficial or detrimental for the host.
1.4 APOPTOTIC RESPONSE TO MTB

Apoptosis of infected macrophages is suggested to be a way to eliminate suitable growth environments for Mtb (79, 135). Virulence of the mycobacterial strains has an inverse relationship with their ability to induce apoptosis. Virulent strains of mycobacteria, such as *M. tuberculosis* H37Rv, Erdman and *M. bovis* wild type were shown to induce significantly less apoptosis in macrophages than avirulent strains, including *M. tuberculosis* H37Ra, *M. bovis* BCG and *M. kansasii* (135, 136). The relatively high rate of apoptosis of macrophages by avirulent strains was not due to higher intracellular growth. On the contrary while the virulent strains increased in number, the avirulent strains decreased in number, which suggests that the apoptosis of macrophages may help decrease the number of intracellular mycobacteria (135).

Induction of apoptosis by Fas ligand (FasL) aids in the killing of intracellular Mtb by human blood monocyte derived macrophages (137).

Inside the phagosomes of macrophages, the mycobacteria are safe from being presented to T cells through MHCI and CD1. Apoptosis is a way for the host to overcome this problem. Following apoptosis, the apoptotic blebs are taken up by uninfected macrophages and dendritic cells. The apoptotic blebs contain mycobacterial proteins and cell wall lipids (138). The dendritic cells that have taken up the apoptotic blebs then present the enclosed mycobacterial antigens to CD8⁺ T cells via MHCI and CD1. This pathway of cross-presentation leads to the activation of CD8⁺ T cells (138–140). CD8⁺ T cells are important in the immune response against Mtb as mice lacking CD8⁺ T cells are more susceptible to infection with Mtb. CD8⁺ T cells are also responsible for macrophage activation via production of IFN-γ and killing of mycobacteria infected cells (138, 141).
Vaccination of mice with apoptotic blebs from mycobacteria-infected macrophages was also shown to protect against tuberculosis (140).

1.4.1 Inhibition of apoptosis by Mtb

Mtb can inhibit apoptosis that is induced by other factors. FasL by itself is enough to induce apoptosis in macrophages. It was shown that when Mtb infected macrophages were treated with FasL the percentage of apoptotic cells had decreased compared to uninfected cells. It was also found that in Mtb infected macrophages, the expression of Fas on the surface is down regulated, which can explain the decrease in apoptosis following FasL treatment (137).

TNF-α can also induce apoptosis. Infection of macrophages with both virulent and avirulent Mtb were shown to induce TNF-α secretion from the cells. However, infection with virulent Mtb also led to production of soluble TNF-α receptor 2 (sTNFR2) (142). This process can decrease the amount of TNF-α interacting with TNFR, which would lead to inhibition of apoptosis of Mtb infected cells.

Infection of cells with mycobacteria results in the up-regulation of anti-apoptotic genes of the Bcl-2 family, such as mcl-1 (143) and bfl-1 (144). Reducing Mcl-1 protein expression in the infected cells by using antisense oligonucleotides leads to an increase in apoptosis of the cells infected with virulent Mtb. Reducing Mcl-1 amount also leads to decreased survival of virulent Mtb inside the cells (143). In mice, after infection with Mtb, the expression of the anti-apoptotic gene bcl-2 increased, whereas the expression of pro-apoptotic gene bax decreased (145). BCG infection can inhibit S-nitroso-N-acetylpenicillamine induced apoptosis in macrophages from wild type mice by more than
50% but cannot inhibit apoptosis induced in macrophages from A1 knock-out mice, which lack the anti-apoptotic gene bfl-1 (144). These data show that mycobacteria can manipulate regulation of both the host genes and proteins to increase anti-apoptotic gene expression and/or decrease pro-apoptotic gene expression in order to inhibit apoptosis and thus survive inside the host cell. Since it was shown that avirulent mycobacteria induced more apoptosis, these strains would be expected to lead to higher cellular immunity via cross-presentation to CD8\(^+\) T cells. Production of new avirulent vaccine strains of mycobacteria that induce high levels of apoptosis can be achieved by identification of mycobacterial anti-apoptotic genes that are responsible for inhibition of apoptosis in host cells. Identification of anti-apoptotic mycobacterial genes can also reveal new targets for the use of antimycobacterial compounds.

### 1.4.2 Anti-apoptotic genes of Mtb

Previous work by our group and others has identified several anti-apoptotic genes of Mtb. *nuoG* (encodes NuoG subunit of the type I NADH (nicotinamide adenine dinucleotide)-dehydrogenase of Mtb), *secA2* (encodes a protein required for the secretion of superoxide dismutase A [SodA], Alpha-crystallin [Acr] and Rv0390) and *pknE* (protein kinase E, a serine threonine protein kinase) genes are required for inhibition of apoptosis by Mtb (146–148). NuoG inhibits the accumulation of phagosomal ROS, prevents an increase in TNF-\(\alpha\) secretion and inhibits host cell apoptosis (149). SecA2 functions in the secretion of proteins SodA, Acr, Rv0390 and KatG. *SecA2* mutant of Mtb is less virulent in SCID mice (150). The anti-apoptotic property of SecA2 seems to come from its involvement in secretion of the protein SodA, since re-establishment of SodA...
secretion in secA2 knock-out Mtb reversed the pro-apoptotic phenotype of the secA2 knock-out (146). KatG secretion is also defective in secA2 knock-out Mtb (150), but KatG was not involved in apoptosis inhibition as re-establishment of SodA secretion was enough to reverse the pro-apoptotic phenotype of secA2 mutant strain (146). SodA enzyme converts superoxide radicals to hydrogen peroxide and oxygen, thereby protecting the bacteria against oxidative damage (151). An Mtb mutant that had reduced SodA levels is pro-apoptotic and less virulent than wild-type Mtb (151). The pknE promoter is induced when the cells are exposed to nitric oxide donors and pknE is involved in regulating the host cell arginase pathway by increasing arginase 1 and arginase 2 activities for survival of Mtb (152). Increased arginase 1 and arginase 2 activities lead to decreased NO production as they compete with iNOS for L-arginine (153). pknE is also involved in inhibiting expression of several TLRs (152).

The common point of these three anti-apoptotic genes is their role in neutralizing or preventing ROS or NO species. This highlights the involvement of ROS and NO in promoting apoptosis. On the other hand, Mtb has proteins working to inhibit apoptosis by neutralizing or preventing ROS or NO species.

### 1.4.3 Identification of novel anti-apoptotic genes of Mtb

In order to identify Mtb genes that are responsible for the inhibition of apoptosis, a gain-of-function genetic screen was performed using a strain of non-pathogenic M. smegmatis (147). The mc²155 strain that was used has the ability to grow fast and is also easily transformable (154). The human monocyctic leukemia cell line THP-1 was used as host cells for this screen. Early studies showed that infection of THP-1 cells with
*M.smege*mati*is* led to a much higher level of apoptosis (~ 40%) compared to uninfected (less than 5%) or BCG infected cells (less than 10%) just 16 hr after infection (147). For the gain of function screen, 312 Mtb genomic fragments were used to transform *M.smege*mati*is*. The 312 *M.smege*mati*is* clones were then used to infect THP-1 cells. Five clones with reduced apoptosis were identified. The cosmids that enabled *M.smege*mati*is* to inhibit apoptosis were extracted from the clones and were used to transfect *M.smege*mati*is* again. This was done to confirm that the decreased apoptosis was due to the presence of the cosmids in *M.smege*mati*is*. The decrease in apoptosis was reproducible for two of the five clones (147).

Although the 2 cosmids were able to reduce the pro-apoptotic phenotype of *M.smege*mati*is*, *M.smege*mati*is* clones with the cosmids still induced high levels of apoptosis (between 15 and 25%) (147). This was probably due to the ability of *M.smege*mati*is* to strongly induce apoptosis faster than other mycobacteria. To better assess the effects of the cosmids in inhibition of apoptosis induction, *M.kansasi*ii, a facultative pathogenic species was used. *M.kansasi*ii induces high levels of apoptosis but is slower in killing the host cells (significant levels of apoptosis induced after 3 days). The 2 cosmids that showed consistent decrease of apoptosis in *M.smege*mati*is* as well as the empty cosmid vector were transformed into *M.kansasi*ii. The clones with these cosmids induced significantly lower amounts of apoptosis compared to the empty cosmid containing *M.kansasi*ii (147).

To find smaller regions inside one of the cosmids (named J21) that are responsible for the inhibition of apoptosis, four sections within J21 were knocked out in Mtb using phage transduction (Velmurugan K, unpublished) (Figure 6). In addition to the
Figure 6: The J21 cosmid. The J21 cosmid that has been identified as a region that contains anti-apoptotic genes has the Mtb genomic sequence from 3511000bp to 3546000bp (44). J21 region was shown to contain anti-apoptotic genes (147).
discovery of anti-apoptotic gene *nuoG*, the 7/10 region was found to contain anti-apoptotic genes (Velmurugan K, unpublished) (Figure 7). As expected from an apoptosis inducing knock-out strain, ∆7/10 was shown to be less virulent than wild type Mtb in a survival experiment using SCID mice (Velmurugan K, unpublished) (Figure 8). The 7/10 region contains five complete genes: *MoxR3, Rv3167c, Rv3165c, Rv3166c* and *Rv3163c*. *Rv3163c* is a secreted protein that previous data suggests is not involved in apoptosis (29; Velmurugan K, unpublished). Individual knock outs of *MoxR3, Rv3167c, Rv3165c, and Rv3166c* were made via phage transduction and were used in THP-1 infections. Initial data showed increased apoptosis upon infection with ∆*Rv3165c* and ∆*Rv3167c* (Miller J, unpublished) (Figure 9). Therefore, the preliminary data suggested that *Rv3165c* and *Rv3167c* were the anti-apoptotic genes in the 7/10 region. The research that is presented here was performed to confirm that ∆*Rv3165c* and ∆*Rv3167c* were pro-apoptotic, to find the pathways involved in apoptosis of ∆*Rv3165c* and ∆*Rv3167c* infected cells and to characterize *Rv3165c* and *Rv3167c*.

1.5 SUMMARY AND SIGNIFICANCE

Mtb continues to kill millions of people every year (3). One of the reasons of failure in the eradication of Mtb is the low success rate of the current BCG vaccine (22, 23). Another reason is the lack of drugs that can kill Mtb in weeks compared to the current drugs that take months to be effective (37). That is why it is necessary to develop new and effective prevention and treatment methods against TB. To achieve this goal it is necessary to understand the mechanisms by which Mtb survives in the host. One of these mechanisms might be the prevention of apoptosis of infected host cells. A better
Figure 7: 3/4 and 7/10 regions of the J21 cosmid contain anti-apoptotic genes. Mtb and knock-outs lacking the 3/4 or 5/8 or 7/10 regions were used to infect THP-1 macrophages. Mtb knock-outs lacking the 3/4 or 7/10 regions were found to induce significantly more apoptosis compared to Mtb. Data are the mean ± SEM of a representative experiment performed in duplicate. UT, untreated; ns, not significant; ***, 0.0001 < p < 0.001 (Experiment performed by Kamalakannan Velmurugan).
Figure 8: Deletion of the 7/10 region in Mtb reduces its virulence in SCID mice. Five SCID mice were infected by injection of $10^6$ Mtb or ∆7/10 in the tail and monitored. Mtb infected mice died on the 14$^{th}$ and 16$^{th}$ days, while ∆7/10 infected mice died on 32$^{nd}$ and 33$^{rd}$ days. Log-rank test found the curves to be significantly different. **, $0.001 < p < 0.01$ (Experiment performed by Kamalakannan Velmurugan).
Figure 9: Preliminary experiments indicate \textit{Rv3165c} and \textit{Rv3167c} genes as the anti-apoptotic genes in the 7/10 region. THP-1 macrophages were infected with Mtb and the knock-outs for 5 days. The cells were collected and stained with TUNEL to determine the % of apoptotic cells. Data are the mean ± SEM of a representative experiment performed in duplicate. **, 0.001 < p < 0.01 (Experiment performed by Jessica Miller).
understanding of this mechanism will help in the development of vaccine strains that do not prevent host cell apoptosis and hence induce a better immune response. Also, identification of Mtb components that inhibit apoptosis can reveal new targets for drugs. Drugs that target anti-apoptotic components of Mtb would help the immune system to kill the intracellular Mtb that is hard to target with other drugs. Previously a genomic DNA region of Mtb was identified to contain anti-apoptotic genes (147). The goals of my research were to identify some of the genes in this region, characterize the genes and identify the mechanisms by which the products of these genes inhibit host cell apoptosis.
CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

SCID mice (BALB/c background) were from Jackson Laboratories (Bar Harbor, ME). The anti-myc antibody (9E 10) developed by [Bishop, J.M.] was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology (Iowa City, IA). Cleaved caspase-8, cleaved caspase-9, cleaved caspase-3 antibodies were purchased from Cell Signaling Technology® (Danvers, MA). Anti-actin antibody was purchased was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GroEL, anti-LAM, anti-DnaK, and anti-FAP antibodies were purchased from Colorado State University through the TB Vaccine Testing and Research Materials Contract as part of NIH, NIAID Contract No. HHSN266200400091C (Fort Collins, CO). Horse radish peroxidase-conjugated goat anti-mouse IgG, horse radish peroxidase-conjugated goat anti-rabbit IgG, horse radish peroxidase-conjugated donkey anti-goat IgG, alkaline phosphatase conjugated streptavidin (Streptavidin-AP) were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Cytometric Bead Array kit for human inflammatory cytokines was purchased from BD Biosciences (San Jose, CA). N-acetyl cysteine, and Hygromycin B were purchased from EMD Chemicals (Philadelphia, PA). FLICA™ kits were purchased from ImmunoChemistry Technologies LLC (Bloomington, MN). In Situ Cell Death Detection Kit, Fluorescein for TUNEL and LightCycler® 480 SYBR Green I Master for qPCR, PhosSTOP phosphatase inhibitor cocktail tablets and cOmplete, Mini, EDTA-free protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Ribo-zero™ and ScriptSeq™ mRNA-Seq Library Preparation kits for RNA
sequencing were purchased from Epicentre (Madison, WI). Human CCL3/MIP-1 alpha DuoSet and Human CCL20/MIP-3 alpha DuoSet ELISA kits were purchased from R&D Systems® (Minneapolis, MN). Lysis buffer for protein microarray was supplied by Kinexus (Vancouver, Canada). Electroporation cuvettes were purchased from VWR (Bridgeport, NJ). Fetal calf serum was purchased from Thermo Scientific (Logan, UT). Human serum was purchased from Gemini Bio-Products (West Sacramento, CA). Gentamicin, and TRIzol® Reagent were purchased from Invitrogen™ (Grand Island, NY). Three well slides were purchased from Electron Microscopy Sciences (Hatfield, PA). BrightStar® Psoralen-Biotin Kit and BrightStar® BioDetect™ Nonisotopic Detection Kit for Southern blots, and TURBO DNA-free™ were purchased from Ambion (Grand Island, NY). AlkPhos Direct™ hybridization buffer was purchased from Amersham (Piscataway, NJ). Centriplus® YM-3 centrifugal filters were purchased from Millipore™ (Billerica, MA). Thermo Scientific SuperSignal West Femto Substrate, Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific Pierce BCA™ Protein Assay Kit, Costar 96-Well EIA/RIA plates for ELISA, Triton® X-100, Phorbol 12-myristate 13-acetate (PMA), kanamycin, streptomycin, Tween®-80, and Thermo Scientific Phusion® RT-PCR Kit for reverse transcription were purchased from Fisher (Pittsburgh, PA). Proteinase K was purchased from Fermentas (Glen Burnie, MD). Staurosporine, xanthine oxidase, xanthine, glutathione, DPI, lysozyme, protease inhibitor cocktail were purchased from Sigma (St. Louis, MO).
2.2 BACTERIA AND CULTURE CONDITIONS

*M. tuberculosis* H37Rv (ATCC 25618) was obtained from the American Type Culture Collection (Manassas, VA). Middlebrook 7H9 medium supplemented with 0.5% glycerol, 0.05% Tween-80, and 10% ADS (Albumin, 5% w/v, dextrose, 2% w/v, NaCl, 0.85% w/v) or ADC (Albumin, 5% w/v, dextrose, 2% w/v, catalase, 0.003% w/v, NaCl, 0.85% w/v) was used to grow Mtb strains (155). Mycobacteria containing plasmids with antibiotic resistance genes were grown in the presence of 50µg/ml hygromycin or 40µg/ml kanamycin or 20µg/ml streptomycin.

2.3 IN VITRO HYDROGEN PEROXIDE PRODUCTION

Bacteria were collected by centrifugation and resuspended in PBS to adjust their concentration to $10^6$ bacteria/ml. To produce hydrogen peroxide *in vitro*, 250µM xanthine and 0.1U/ml xanthine oxidase were added to the bacteria and the bacteria were shaken for 90 min at 37 °C.

2.4 CREATION OF COMPLEMENTS OF KNOCKOUTS

For complementation of the ∆Rv3165c knockout, the sequence from 200bp upstream of *Rv3165c* to 110bp downstream of *Rv3165c* was cloned into the integrating vector pDB60 that contains a streptomycin resistance gene. The insertion of the correct sequence was confirmed by plasmid sequencing. The plasmid was electroporated into ∆Rv3165c and colonies were grown and selected as mentioned below. For complementation of ∆Rv3167c knockout, the sequence from 60bp upstream of *Rv3167c* to the 3’ end of *Rv3167c* was cloned into the extrachromosomal expression vector
pmv261. The plasmid was electroporated into ΔRv3167c and colonies were grown and selected as mentioned below.

2.5 CREATION OF RV3165C-MYC AND RV3167C-MYC EXPRESSING BCG

To make Rv3167c-myc, the sequence from 60bp upstream of Rv3167c to the 3’ end of Rv3167c with the myc tag sequence was cloned into the extrachromosomal expression vector pmv261. The plasmid was electroporated into BCG and colonies were grown and selected as mentioned below. To make Rv3165c-myc, the sequence from 50bp upstream of Rv3165c to the 3’ end of Rv3165c with the myc tag sequence was cloned into the extrachromosomal expression vector, pmv261. The plasmid was electroporated into BCG and colonies were grown and selected as mentioned below.

2.6 ELECTROPORATION OF PLASMIDS

To electroporate plasmids, 5ml of late log phase culture was centrifuged at 2000g for 10 min and the cells were washed three times with an equal volume of wash solution (10% glycerol with 0.05% Tween-80). The final pellet was resuspended in 1/10th the original volume of wash solution. Bacteria (400µl) were added to a 2mm electroporation cuvette containing 2-5µl of plasmid. Bacteria were electroporated at 2.5kV; 25µFD; 1000Ω in a BioRad GenePulser Xcell™ electroporator. 7H9 (1ml) medium was added to the bacteria, which were allowed to recover for 3hr for M. smegmatis or 24hr for slow growers (Mtb, BCG, M. kansasii). The cells were then plated on 7H10 agar plates containing the appropriate selective medium and incubated for 3-4 days (M.smeagmatis) or 3 weeks (Mtb, BCG) at 37 °C. Resulting colonies were grown in 7H9 medium with
appropriate antibiotic and the cultures were screened by PCR, Southern or western blot to check for uptake of the plasmid.

2.7 CELL CULTURE MAINTENANCE

Each vial of human acute monocytic leukemia cell line THP-1 (ATCC™ TIB-202™) was thawed at 37 °C in a water bath and the cells were washed with RPMI-1640 medium and resuspended in 20ml RPMI-1640 supplemented with heat inactivated 10% fetal calf serum. Cells were grown in a 37 °C incubator in the presence of 5% CO₂. THP-1 cells were passaged before their concentration reached 0.8 x 10⁶ cells/ml.

2.8 INFECTIONS

For infections, 0.6-0.9 million THP-1 cells per ml were plated into 24 well plates in the presence of 20ng/ml phorbol 12-myristate 13-acetate and incubated for 20hr. Before infection, cells were washed three times with magnesium/calcium-free phosphate buffered saline (PBS) and 0.5ml of infection medium (RPMI-1640, 10% heat inactivated FCS, 5% human serum) was added to each well. Bacteria to be used in the infections were washed once with PBS containing 0.05% Tween®-80 (PBST). A sample of bacteria was mixed in equal ratio with 10% formalin in a cuvette and the OD was measured. Cells were infected at a multiplicity of infection (MOI) of 3:1 by adding three times the amount of bacteria per cell and incubating the plates for 4hr at 37 °C in the presence of 5% CO₂. Infection media was removed after 4hr and the wells were washed two times with PBS and then 1ml chase medium (RPMI-1640, 10% heat inactivated FCS, 100µg/ml gentamicin) was added to each well. Cells were then incubated for the indicated times. At
the end of infections, the chase media in the wells were placed in 15ml tubes and PBS with 5mM ethylenediaminetetraacetic acid (EDTA) was added to the wells to lift the cells. The lifted cells were added to the 15ml tubes and centrifuged. The cell pellets were resuspended in 4% paraformaldehyde overnight at 4°C to fix cells and kill intracellular Mtb. For infections on slides, 120,000 cells were plated into each well of 3-well slides and incubated as mentioned before. When glutathione, N-acetyl cysteine, diphenylene iodonium were used they were included both in the infection and the chase media.

2.9 DETECTION OF APOPTOSIS

To detect apoptosis in THP-1 cells, the In Situ Cell Death Detection Kit, Fluorescein was used. This kit labels the free 3’-OH termini of DNA with fluorescein-dUTP using the enzyme terminal deoxynucleotidyl transferase (TdT). The reaction is called TdT-mediated dUTP-X nick end labeling (TUNEL). Before TUNEL, cells were washed two times with PBS and permeabilized with permeabilization solution that contained 0.1% Triton® X-100 and 0.1% sodium citrate on ice for 3 min. Cells in each sample were incubated with 50µl TUNEL solution that is a mix of the enzyme, nucleotides and the buffer. After 1hr at 37 °C the cells were washed two times and resuspended in PBS. Flow Cytometric analysis of cells was done using either a Becton Dickinson FACSCalibur™, FacsCanto™ II, FacsAria™ or Accuri® C6. For measuring apoptosis of cells on slides, after the last wash of the cells, wells were left to dry, ProLong® Gold antifade reagent with DAPI was added to the wells and coverslips were placed. Slides were left overnight at room temperature and images of cells were taken.
using a Zeiss LSM 710 laser scanning microscope and processed using AxioVision LE software.

2.10 DETERMINATION OF NECROTIC CELL DEATH

To quantify the levels of necrosis in infected cells, ToxiLight® BioAssay Kit (Lonza, Rockland, ME) was used. Briefly, 20 µl of cell supernatant from each well was added to the wells of a white bioluminescence compatible 96 plate in triplicate and mixed with 100 µl of Adenylate Kinase detection reagent (reconstituted in assay buffer). After 5 min of incubation the luminescence was measured by a BioTek® Synergy microplate reader.

2.11 DETERMINATION OF THE NUMBER OF LIVE BACTERIA AFTER INFECTION

To determine the effect of apoptosis on the intracellular growth of Mtb, \( \Delta Rv3165c \), and \( \Delta Rv3167c \), for each time point 3 wells of differentiated THP-1 macrophages were infected with those strains. Following infection, THP-1 cells were lysed at 0 hr, 1 day and 2 day time points for 3:1 MOI infections and 0 hr, 1 day, 2 day, 3 day, 4 day and 6 day time points for 0.5:1 MOI infections using 0.1% Triton X-100 in PBS. No gentamicin was used in the chase media in order to keep any extracellular mycobacteria that might be released after host cell death alive. The lysates were diluted in PBST \( 10^4 \), \( 10^5 \), \( 10^6 \) for time points up to 3 day and \( 10^5 \), \( 10^6 \) and \( 10^7 \) times after 3 day. 10µl from each dilution was spotted three times on square 7H10 plates. That resulted in 9 spots for each well of infection. After 14 days the numbers of colonies in the spots were
counted and all of the 27 counts for the 3 wells were averaged (The numbers were less in case of contaminations or mixing of spots).

2.12 MYCOBACTERIA GENOMIC DNA ISOLATION

Bacteria (10ml) were centrifuged at 2000 g for 10 min and resuspended in 1ml lysis buffer (25mM Tris-HCl, 10mM EDTA, 50mM glucose) and centrifuged again. The pellet was resuspended in 450µl lysis buffer. Lysozyme (50µl, 10mg/ml) was added, mixed gently and the sample was incubated at 37°C overnight. Sodium dodecyl sulfate (100µl) and proteinase K (50µl, 10mg/ml) were added to the sample, mixed and incubated at 55°C for 30 min. NaCl (200µl, 5M) was added to the sample followed by 160µl 65 °C preheated CTAB (Cetrimide saline solution: 0.1g/ml cetrimide, 0.04g/ml NaCl). The sample was incubated at 65°C for 10 min. An equal volume (~ 1ml) chloroform-isoamyl alcohol (24:1) was added to the sample, mixed gently and the sample was centrifuged at 12,000 g for 5 min. The aqueous phase was placed in a new tube and mixed with 0.7X volume isopropanol and mixed gently. The sample was incubated at room temperature for 5-10 min and centrifuged at 16,000 g for 10 min. The pellet was washed with 1ml 70% ethanol, centrifuged at 16,000g for 5 min and air dried. The pellet was resuspended in 50µl water.

2.13 SOUTHERN BLOTTING

The probe DNA for southern blots of \textit{Rv3165c} was made by PCR using the primers that were used to amplify the 5’ end of \textit{Rv3165c} for knockout production. The probe DNA for southern blots of \textit{Rv3167c} was made by PCR using the primers that were
used to amplify the 3’ end of \textit{Rv3167c} for knockout production. The probes were labeled with biotin using BrightStar® Psoralen-Biotin Kit. Genomic DNA isolated from the clones was digested with XhoI for \textit{Rv3165c} southern blots and with EcoRI for \textit{Rv3167c} southern blots. The DNA fragments were separated by agarose gel electrophoresis, transferred to charged nylon membrane, and denatured with 0.4N NaOH. The probe was denatured at 90 °C for 10 min in the presence of 10mM EDTA and hybridized to the membrane at 55 °C for 16 hr in hybridization buffer (AlkPhos Direct™ hybridization buffer with 0.5M NaCl). The membrane was washed and the probe was detected using a BrightStar® BioDetect™ Nonisotopic Detection Kit.

2.14 CASPASE ACTIVITY DETECTION

Caspase activity was detected by Fluorescent-Labeled Inhibitor of Caspases (FLICA™). A working solution of the FLICA™ reagent was added to the cells and incubated for 1hr at 37 °C. Cells were washed two times and resuspended with PBS. Analysis was accomplished using flow cytometry.

2.15 WHOLE CELL LYSATE PREPARATION

Cells were lysed using lysis buffer (for mycobacteria: 50mM Tris-HCl [pH 7.5], 5mM EDTA, 0.6% SDS, 10mM Na₃PO₄, prokaryotic protease inhibitor cocktail; for THP-1 cells 50mM Tris-HCl, 150mM NaCl, 1% Triton® X-100, eukaryotic protease inhibitor cocktail). To lyse mycobacteria, glass beads were added to the tubes and the cells were lysed for 10 min using a Biospec Mini-Beadbeater-8. Samples were centrifuged at 10,000g for 5 min at 4 °C and the supernatants were filtered through
Costar® Spin-X® centrifuge tube filters to remove whole bacteria. Thermo Scientific Pierce BCA™ Protein Assay Kit was used to determine protein concentrations (156).

2.16 POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

Sample protein (≤ 50 µg) was mixed with sample buffer (250mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 10% β-mercaptoethanol and 0.01% bromophenol blue) at a 4:1 ratio and proteins were denatured at 95 °C for 5 min and placed on ice (155). Samples were pipetted into the wells of 4-20% polyacrylamide gradient gels and separated at 125V using Tris-HEPES-SDS running buffer (Thermo Scientific). Proteins were transferred to a 0.45µm PVDF membrane using Genie® Blotter (Idea Scientific). Membranes were blocked using 5% non-fat dry milk in PBS for 1hr at room temperature and incubated with primary antibodies diluted in the recommended buffer. After secondary antibody incubation, the chemiluminescent signal was produced using SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Substrate.

2.17 SUBCELLULAR FRACTIONATION

For each fractionation, 50ml cells were centrifuged at 3000g for 15 min. The pellets were washed with PBS, centrifuged again and resuspended in breaking buffer (PBS, 1% protease inhibitor cocktail, 20 µg/ml soybean trypsin inhibitor, 25µg/ml DNase, 50µg/ml RNase). Cells were lysed using a French® Press. Unbroken cells were pelleted at 3000g for 20 min. The supernatants were centrifuged at 100,000g for 2hr to
pellet the cell wall and the cell membrane (cell envelope). The supernatants that contained cellular cytoplasm were transferred to fresh tubes and the pellets were washed with PBS. The pellets were resuspended in the breaking buffer without DNase and RNase.

2.18 CULTURE FILTRATE PREPARATION

To prepare culture filtrates (CF), bacteria were grown in 7H9 medium and passaged three times in Sauton’s liquid medium (155), centrifuged and the supernatant was filtered (0.22µm). CFs were concentrated at least 20 times using Centriplus® YM-3 centrifugal filters. Protease inhibitor cocktail (1% final concentration) and soybean trypsin inhibitor (20 µg/ml final concentration) were added to the CFs.

2.19 RNA ISOLATION

Cells were lysed using TRIZOL® Reagent and pipetting. To lyse mycobacteria, 200µl zirconia beads were added to the tubes and the cells were lysed using Biospec Mini-Beadbeater-8. After 5 min at room temperature, chloroform (0.2 volume) was added to the samples and the samples were shaken vigorously for 20 sec and incubated at room temperature for 3 min. Samples were centrifuged for 15 min at 12,000 g at 4 °C and the aqueous phase was transferred to a new tube. The chloroform extraction was repeated and the final aqueous phase was mixed with an equal volume of isopropanol. After the samples were gently mixed, they were incubated at room temperature for 10 min and spun for 10 min at 12,000 g at 4 °C. The pellets were washed with 70% ethanol, air dried
and resuspended in RNase free water. TURBO DNA-free™ was added to RNA samples to degrade DNA.

2.20 REVERSE TRANSCRIPTION AND REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

cDNA was produced from RNA using the Thermo Scientific Phusion® RT-PCR Kit. For each reaction, cDNA was produced from 500ng RNA using random hexamers. Triplicates were processed for qPCR using a LightCycler® 480 SYBR Green I Master kit and the Bio-Rad CFX96™ real-time PCR machine. qPCR reactions included 0.5µM of each primer and 0.5µl cDNA.

2.21 SAMPLE PREPARATION FOR RNA SEQUENCING

Ribosomal RNA (rRNA) was removed from 5µg RNA samples using Ribo-zero™ kits. To remove bacterial rRNA from infected THP-1 RNA samples Gram(+) Bacterial Ribosomal Removal Solution was included in the reactions. ScriptSeq™ mRNA-Seq Library Preperation kit was used to prepare a RNA sequencing library from these rRNA depleted RNA samples.

2.22 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

CCL3 and CCL20 concentrations in the cell culture supernatants were measured using DuoSet ELISA kits. Briefly, 96-well plates were coated with 2µg/ml (CCL3) or 0.4µg/ml (CCL20) capture antibody. After 16 hr incubation, wells were washed three times with 400µl PBST and blocked with 300µl reagent diluent (1% BSA in PBS) for 1hr
at room temperature. Wells were washed and 100µl of triplicates of sample or standards were added to the wells. After 2hrs at room temperature, wells were washed and 100µl detection antibody (36µg/ml) in reagent diluent was added to the wells for 2hrs at room temperature. Wells were washed and 100µl 1X Streptavidin-AP was added to the wells for 20 min at room temperature. Wells were washed and 100µl substrate solution (1mg/ml $p$-nitrophenyl phosphate disodium salt dissolved in 1.02M diethanolamine, pH 9.8) was added to the wells. The absorbance of samples at 405 nm was measured after 5-10 min.

2.23 MEASUREMENT OF HUMAN INFLAMMATORY CYTOKINE LEVELS IN SUPERNATANTS USING A CYTOMETRIC BEAD ARRAY KIT

BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kits were used for measuring the levels of IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 cytokines in the supernatants 48 hr after infection. 50µl mixed capture beads were mixed with 50µl PE detection reagent and with either 50µl standards or samples. After 3 hr at room temperature, 1ml wash buffer was added and samples were centrifuged. The pellets were resuspended in 300µl wash buffer and samples were analyzed using a flow cytometer.

2.24 INFECTION OF SCID MICE AND DETERMINATION OF THE BACTERIAL LOAD

To infect the mice with mycobacteria, 7 mice were placed in the basket of the Glas-Col® Inhalation Exposure System (Model No A 4212), the lid was closed and clamped. A sterile nebulizer was attached to the machine. $5 \times 10^6$ wild type Mtb in 5ml
PBST was injected into the nebulizer with a syringe. A standard program was employed: 1800 seconds nebulizing time, 1800 seconds cloud decay time, 900 seconds decontamination time. After the UV lights turned off, the mice were removed and placed into their cages. The nebulizer was placed into a solution of vesphene for sterilization. The basket and the inside of the machine were cleaned with vesphene and 70% ethanol. A new sterile nebulizer was attached to the machine to infect the mice with Δ3167c and the same protocol was followed.

To determine the bacterial load, three mice per condition were sacrificed the next day. Mice were sedated by placing cotton balls that had 1ml isoflurane into the containers and then killed via cervical dislocation. The thoracic cavity was opened and the lungs were removed. A small piece of the left lung was cut and placed in 10% buffered formalin for histopathology. The rest of the lung was cut into very small pieces with scissors. The pieces were placed in a Stomacher® bag and 4.5ml PBST was added. The bag was sealed and placed in another bag. The bag was then placed in a Stomacher® 80 Biomaster for mashing the lung pieces for 5 min on high setting. The homogenate was filtered through a strainer into a 50ml tube. The bacteria were pelleted by centrifuging for 15 min at 2000g. The pellets were resuspended in 200μl PBST and plated on 7H10 plates. The plates were checked for colonies after 3-4 weeks of incubation at 37 °C. The numbers of colonies were counted and the numbers were graphed. Mice were observed daily and disease progression was scored as agreed by the University of Maryland Institutional Animal Care and Use Committee (IACUC) until they were in a moribund state and had to be sacrificed.
2.25 STATISTICAL ANALYSIS

Unless otherwise noted, one way analysis of variance (ANOVA) with Tukey post-test was performed using GraphPad Prism® 5 to find the significance of difference between the data values. The graph bars show the mean of the data while the error bars show the standard deviation. Significance is shown as: ns, not significant; *, 0.01< p <0.05; **, 0.001< p < 0.01; ***, 0.0001< p < 0.001.
CHAPTER 3 RESULTS

3.1 RV3165C AND RV3167C ARE ANTI-APOPTOTIC GENES

3.1.1 ∆rv3165c and ∆rv3167c induce high levels of apoptosis of THP-1 macrophages

The 7/10 region of the J21 cosmid was shown to contain anti-apoptotic genes of Mtb (Velmurugan, K. unpublished) (Figure 7). Dr. Jessica Miller created single gene deletions of the genes in this region in Mtb H37Rv (∆MoxR3, ∆Rv3165c, ∆Rv3166c, ∆Rv3167c) and her preliminary experiments suggested that Rv3165c and Rv3167c were the anti-apoptotic genes in the 7/10 region. In order to confirm this, Mtb with single gene deletions (∆MoxR3, ∆Rv3165c, ∆Rv3166c, and ∆Rv3167c) were used to infect PMA-differentiated THP-1 macrophages. THP-1 cells were incubated 4 days after infection and the percentage of apoptotic cells was determined with the TUNEL assay. ∆Rv3165c and ∆Rv3167c induced significantly higher levels of apoptosis (36.36% and 43.73%) compared to Mtb (11.81%), while other deletions (∆MoxR3 [14.20%] and ∆3166c [16.53%]) did not (Figure 10). Thus, it was confirmed that the ∆Rv3165c and ∆Rv3167strains that were used in the infection experiments are pro-apoptotic.

3.1.2 ∆rv3165c and ∆rv3167c are confirmed to be knock-outs for Rv3165c and Rv3167c

To confirm that ∆Rv3165c and ∆Rv3167c were knockouts, genomic DNA was isolated from the bacterial cultures and used in PCRs that amplify a 150 bp region in the middle of the genes. Separation of PCR products on an agarose gel confirmed the lack of Rv3165c and Rv3167c in the ∆Rv3165c and ∆Rv3167c knockout strains respectively.
Figure 10: \( \Delta Rv3165c \) and \( \Delta Rv3167c \) induce significantly more apoptosis of THP-1 macrophages. PMA-differentiated THP-1 macrophages were untreated (UT) or infected with Mtb or the knock-out strains for 4 days. The cells were collected and stained with TUNEL to determine the % of apoptotic cells. Data are the mean ± SEM of three independent experiments performed in duplicate (n=6). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test: ns, not significant; **, 0.001< p < 0.01; ***, 0.0001< p < 0.001.
Genomic DNA was also digested with restriction enzymes to give different sizes of DNA fragments around the genes. Southern blots were performed to show the size difference of the bands that hybridized to the probes. Wild type Mtb should give a band of 2003bp with the probe after XhoI digestion of its genomic DNA, while ΔRv3165c should give a 6422bp band (Figure 12A). Wild type Mtb should give a band of 6045bp with the probe after EcoRI digestion of its genomic DNA, while ΔRv3167c should give a 2620bp band (Figure 12C). Southern blots confirmed the insertion of the hygromycin fragment inside the genes in the knock-out bacteria (Figure 12B, 12D). These experiments confirmed the lack of Rv3165c in ΔRv3165c strain and lack of Rv3167c in ΔRv3167c strain.

3.1.3 ΔRv3165c and ΔRv3167c complementation reverses their pro-apoptotic phenotypes

To show that the pro-apoptotic phenotype of ΔRv3165c and ΔRv3167c resulted from the lack of the genes and no other additional genetic mutation or epigenetic factor, the knockouts were complemented by reintroducing the knocked-out gene. Following the complementations the bacteria were used to infect PMA-differentiated THP-1 cells for 2 days. Both knockouts were complemented as the 65.18% apoptosis rate induced by ΔRv3165c went down to 19.85% which is not significantly different than the 10.49% induced by Mtb (Figure 13A). Similarly, the 83.41% apoptosis rate induced by ΔRv3167c went down to 8.75% which is not significantly different from the 15.75% induced by Mtb (Figure 13B). The difference in the apoptosis of THP-1 macrophages infected by ΔRv3165c and ΔRv3167c in the screening of 7/10 region (Figure 10) versus the later
Figure 11: Confirmation of deletion of *Rv3165c* and *Rv3167c* by RT-PCR. cDNA was synthesized from RNA samples isolated from wild-type Mtb and the knock-out strains. Primers amplifying 235 and 281 bp regions in the middle of *Rv3165c* and *Rv3167c* respectively were used in PCR reactions.

1: No cDNA control
2: Mtb RNA
3: ∆*Rv3165c* RNA
4: ∆*Rv3167c* RNA
5: Mtb cDNA
6: ∆*Rv3165c* cDNA
7: ∆*Rv3167c* cDNA
8: 100 bp DNA ladder
9: No cDNA control
10: Mtb cDNA
11: ∆*Rv3165c* cDNA
12: ∆*Rv3167c* cDNA
13: No cDNA control
14: Mtb cDNA
15: ∆*Rv3165c* cDNA
16: ∆*Rv3167c* cDNA
Figure 12: Confirmation of the insertion of the hygromycin resistance gene into the \textit{Rv3165c} and \textit{Rv3167c} genes. The theoretical sizes of fragments that would be detected with the probes after digestion of \textit{Mtb} and \textit{\Delta Rv3165c} genomic DNA with XhoI (A) or digestion of \textit{Mtb} and \textit{\Delta Rv3167c} genomic DNA with EcoRI (C). Arrows represent the genes, green regions inserted in the genes represent hygromycin genes. Southern experiments confirmed the sizes of the DNA fragments in \textit{Mtb} and the knock-outs (B, D).
Figure 13: Complementation of ∆Rv3165c and ∆Rv3167c reverses their pro-apoptotic phenotypes. THP-1 cells were infected with Mtb, ∆Rv3165c and its complement or with ∆Rv3167c and its complement for 2 days. The cells were collected and stained with TUNEL to determine the % of apoptotic cells. Data are the mean ± SEM of three independent experiments performed in duplicate (n=6). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test: ns, not significant; ***, 0.0001< p < 0.001.
experiments (Figure 13) is attributed to the use of different stocks of THP-1 cells and/or batches of heat inactivated fetal calf serum. Although in the later experiments infected cells go through higher rates of apoptosis in a shorter amount of time, this phenotype has stayed consistent for all of the experiments.

To confirm that $\Delta Rv3165c::C$ and $\Delta Rv3167c::C$ were complements, genomic DNA was isolated from the bacterial cultures and digested with restriction enzymes to give different size fragments of DNA around the genes. Southern blots were performed to show the size differences of the bands hybridized to the probes. Southerns confirmed the presence of an extra band from the complementation plasmid in the complements (Figure 14, 15). Thus, it was confirmed that $\Delta Rv3165c::C$ and $\Delta Rv3167c::C$ were complements and that through complementation the pro-apoptotic phenotypes of $\Delta Rv3165c$ and $\Delta Rv3167c$ were reversed. It was also confirmed that the pro-apoptotic phenotypes of $\Delta Rv3165c$ and $\Delta Rv3167c$ were due to lack of $Rv3165c$ and $Rv3167c$ respectively.

### 3.1.4 Apoptosis of infected cells inhibits the intracellular growth of $\Delta Rv3167c$

Apoptosis of infected macrophages reduces the number of phagocytosed mycobacteria (135, 157). To determine the effect of apoptosis on the intracellular growth of $\Delta Rv3165c$ and $\Delta Rv3167c$, THP-1 macrophages were infected with Mtb, $\Delta Rv3165c$ and $\Delta Rv3167c$ at an MOI of 3:1. Infected THP-1 cells were lysed right after infection, and 1 day and 2 days post infection, and the numbers of live intracellular bacteria were determined. No significant differences were observed between Mtb and the knockout strains for the three time points (Figure 16A). In order to find if there were live extracellular mycobacteria in the chase media and if their number would make a
Figure 14: Confirmation of complementation of ΔRv3165c by southern blot. The theoretical sizes of fragments that would be detected with the probes after digestion of Mtb, ΔRv3165c and ΔRv3165c::C genomic DNAs with Xhol (A). Southern experiment showed the presence of the expected extra band in the complement of ΔRv3165c (B). The integrating plasmid used for ΔRv3165c complementation produced a 7609 bp band.
Figure 15: Confirmation of complementation of ΔRv3167c by southern blot. The theoretical sizes of fragments that would be detected with the probes after digestion of Mtb, ΔRv3167c and ΔRv3167c::C genomic DNAs with EcoRI (A). Southern experiment showed the presence of the expected extra band in the complement of ΔRv3167c (B). The episomal plasmid used for ΔRv3167c complementation produced a 736 bp band.
Figure 16: Apoptosis inhibits growth of ∆Rv3167c inside macrophages. THP-1 macrophages were infected with Mtb or ∆Rv3165c or ∆Rv3167c at an MOI of 3:1 with (A) or without (B) gentamicin in the chase media (3 wells per condition). Cells were lysed right after 4 hr of infection (0 day), 1 and 2 day after infection. Dilutions were made and spotted on 7H10 plates. Numbers of colonies were counted 14 days after incubation at 37°C. Data are the mean ± SEM of two independent experiments performed in triplicates (n=6). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. *, 0.05< p <0.01. See materials and methods for a detailed protocol.
difference in the total number of live mycobacteria, gentamicin was omitted in the chase media. When gentamicin was omitted from the chase media and the experiment was repeated for ΔRv3167c the number of ΔRv3167c was 30% less than the number of Mtb 2 day post infection indicating that the difference is due to the extracellular mycobacteria (Figure 16B). From there on no gentamicin was used in the chase media for those infections. The presence of gentamicin-sensitive mycobacteria suggests that some of the intracellular mycobacteria that were released via apoptosis got out of the apoptotic bodies and/or some of the intracellular mycobacteria got out of the cells via other ways (158, 159). When the infections were done with an MOI of 0.5:1 no significant difference was observed between Mtb and ΔRv3167c for the three time points (Figure 17A). However when the numbers of bacteria were determined 6 day post infection the number of ΔRv3167c was 56% less than the number of Mtb, showing that apoptosis of ΔRv3167c infected cells inhibited the growth of ΔRv3167c while Mtb continued to grow (Figure 17B). A preliminary experiment performed with ΔRv3167c::C in addition to Mtb and ΔRv3167c did not reveal any significant difference between the numbers of bacteria up to 3 day post infection but the experiment has to be repeated with further time points to be comparable to the previous experiments (Figure 17C). In order to find the rate of necrosis of THP-1 macrophages infected with Mtb and ΔRv3167c and confirm that it is apoptosis that inhibits the growth of ΔRv3167c in infected cells, the release of intracellular adenylate kinase (AK) into cell culture supernatant was measured by means of the ToxiLight® bioluminescence Assay 2 day post infection (Figure 18). At an MOI of 3:1, cells infected with ΔRv3167c were significantly less necrotic (12%) compared to cells infected with Mtb (25.6%). At an MOI of 0.5:1, cells infected with ΔRv3167c were not
Figure 17: Apoptosis inhibits growth of ΔRv3167c inside macrophages. THP-1 macrophages were infected with Mtb or ΔRv3167c or ΔRv3167c::C at an MOI of 0.5:1 (3 wells per condition). Cells were lysed right after 4hr of infection (0 day) and 2 day after infection (A) or 0 day, 2 day, 4 day and 6 day after infection (B) or 0 day, 1 day, 2 day and 3 day after infection (C). Dilutions were made and spotted on 7H10 plates. Numbers of colonies were counted 14 days after incubation at 37°C. Data are the mean ± SEM of two independent experiments performed in triplicate (n=6) (A and B) or the mean ± SEM of one experiment performed in triplicate (n=3) (C). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. ***, 0.0001< p < 0.001. See materials and methods for a detailed protocol.
Figure 18: A low percentage of ∆Rv3167c infected THP-1 cells go through necrosis. AK release assay was performed 2 day post infection of THP-1 macrophages at MOIs of 3:1 and 0.5:1. Staurosporine was added to the cells as positive control. Data are means of triplicates of one representative experiment (n=3). Error bars represent SEM. Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. ns, not significant; ***, 0.0001< p < 0.001.
more necrotic (0.8%) compared to cells infected with Mtb (2.3%). The significantly higher rate of necrosis for Mtb at MOI of 3:1 but not at 0.5:1 suggests that Mtb induces necrosis at higher MOIs. These results also suggest that the increased numbers of Mtb seen 2 day post infection following 3:1 MOI infection is due to Mtb replicating inside THPM1 cells, causing necrosis and getting out of the cell. The experiments with gentamicin strengthen this idea since the presence of gentamicin kills extracellular bacteria and neither an increase in numbers of Mtb nor a difference compared to ΔRv3167c were seen (Figure 16A).

3.2 ANALYSIS OF EFFECTS OF ΔRV3165C OR ΔRV3167C INFECTION ON THE HOST CELLS

3.2.1 ΔRv3165c and ΔRv3167c induce high levels of caspase-3 and/or 7 and 8 activities in THP-1 cells

Apoptosis of macrophages infected with mycobacteria is mediated by the effector caspases 3 and 7 (79, 160, 161). In order to find if caspases are involved in the pro-apoptotic phenotype of ΔRv3165c and ΔRv3167c in 24hr infected cells, cells were stained with FLICA™ reagent. FLICA™ reagent consists of a fluorescent dye attached to a caspase inhibitor, which is a peptide sequence that binds to caspases. When the FLICA™ reagent enters the cell it binds to active caspase and is retained in the cell during the washes. The number of cells that contain activated caspase is then determined by flow cytometry. When caspase-3 and 7 FLICA™ reagent was used it was found that a higher percentage of THP-1 cells infected with ΔRv3165c or ΔRv3167c had caspase-3 and/or 7
activity (17.21% and 17.89% respectively) compared with Mtb infected cells (8.30%) (Figure 19A). Similarly, when caspase-8 FLICA™ reagent was used it was found that a higher percentage of THP-1 cells infected with ΔRv3165c or ΔRv3167c had caspase-8 activity (27.29% and 28.45% respectively) compared to Mtb infected cells (11.80%) (Figure 19B). However staining of the 24hr infected THPM1 cells with caspaseM9 FLICA™ reagent showed that a similar percentage of THPM1 cells infected with Mtb, ΔRv3165c or ΔRv3167c had caspase-9 activity (7.84%, 11.1%, 13%) respectively (Figure 19C). These experiments showed that caspases-3 and/or 7 and 8 were activated in ΔRv3165c and ΔRv3167c infected THPM1 cells and that apoptosis of ΔRv3165c and ΔRv3167c infected cells was likely a result of the extrinsic pathway of caspase activation.

3.2.2 Inhibiting ROS production or scavenging ROS leads to loss of the pro-apoptotic phenotype of ΔRv3165c and ΔRv3167c

Increased levels of ROS can promote apoptosis by inducing prolonged JNK activation and blocking NF-κB activation (115). Previous work in our lab showed that the pro-apoptotic phenotype of ΔnuoG was abolished in the presence of ROS inhibitor or scavengers and that the phagosomes of cells infected with ΔnuoG had higher levels of ROS (149). Therefore, in order to investigate the involvement of host cell produced ROS in the apoptosis of ΔRv3165c and ΔRv3167c infected cells, ROS production was blocked by NADPH oxidase inhibitor diphenylene iodonium (DPI). Alternatively ROS scavengers were added to the infection and chase media to block the effects of ROS. Blocking ROS production by DPI led to loss of the pro-apoptotic phenotypes of ΔRv3165c and ΔRv3167c. 88.51% and 91.81% apoptosis seen in the ΔRv3165c and
Figure 19: ΔRv3165c and ΔRv3167c induce significantly higher levels of caspase-3 and/or 7 and 8 but not caspase-9 activity in THP-1 cells. Caspase activities were detected by staining cells 24hr after infection with the FLICA™ reagent. For + control staurosporine (1µM) was added to uninfected cells. Data are the mean ± SEM of two independent experiments performed in duplicate (n=4). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. ns, not significant; **, 0.001< p < 0.01; ***, 0.0001< p < 0.001.
ΔRv3167c infected cells was reduced to 15.98% and 17.76% respectively compared to 13.63% apoptosis in Mtb infected cells (Figure 20A). Addition of glutathione to the media led to a decrease of apoptosis from 24.13% and 18.48% for ΔRv3165c and ΔRv3167c infected cells to 1.05% and 0.51% respectively. Addition of NAC decreased the apoptosis levels to 1.03% and 1.84% (Figure 20B). Removal of ROS by these two methods and the loss of the pro-apoptotic phenotype of the knock-outs showed the involvement of ROS in the apoptosis of infected cells.

3.2.3 Secreted cytokine profile of cells infected with Mtb versus ΔRv3167c or ΔRv3167c::C

To identify any possible differences in the levels of IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 after infection in the supernatants of THP-1 macrophages, the BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit was used. This kit was chosen as it could detect Th1 cytokines IL-12 and TNF-α, pro-inflammatory cytokines IL-1β and IL-6, and IL-10, which down regulates Th1 responses. No significant difference was found in the levels of these cytokines in the supernatants of THP-1 macrophages infected for 2 days with Mtb, ΔRv3167c or ΔRv3167c::C (Figure 21). ΔRv3167c and its complement were chosen in this experiment due to sample limitations of the kit. However, in some of the following experiments Rv3165c was omitted due to the complexity and hardship of working with two strains compared to one. The lack of difference in the levels of cytokines tested indicates that the pro-apoptotic phenotype of ΔRv3167c is not due to an increase or decrease in the secretion of these cytokines by the host cells.
Figure 20: Inhibiting ROS production of scavenging ROS abolishes apoptosis induction by ΔRv3165c and ΔRv3167c. Infected THP-1 cells were incubated with DPI (A) or Glutathione or NAC (B) and were collected 2 days (A) or 36hrs (B) after infection and stained with TUNEL to determine the % of apoptotic cells Data are the mean ± SEM of two independent experiments performed in duplicate (n=4) (A) or the mean ± SEM of a representative experiment with four replicates per group (n=4) (B). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. ns, not significant; ***, 0.0001 < p < 0.001.
Figure 21: Detection of the amounts of cytokines in the supernatants of uninfected or Mtb or ΔRv3167c or ΔRv3167c::C infected THP-1 macrophages. A BD CBA kit was used to measure the amounts of IL-8 (A), IL-1β (B), IL-6 (C), IL-10 (D), TNF-α (E) and IL-12 p70 in the supernatants of uninfected THP-1 macrophages or THP-1 macrophages infected with Mtb or ΔRv3167c or ΔRv3167c::C. Data are the mean ± SEM of two independent experiments performed in duplicate (n=4). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test versus Mtb. ns, not significant.
3.2.4 Deletion of Rv3167c changes the expression of host genes in vivo

In order to better understand how ΔRv3167c induces apoptosis of THP-1 macrophages, the host gene expression profile was determined by RNA sequencing. RNA was isolated from uninfected THP-1 macrophages and from those that were infected with Mtb or ΔRv3167c for 16hr. rRNA was removed from the total RNA samples and a RNA sequencing library was prepared. Sequencing of this library showed that there were 2332 differentially regulated genes in THP-1 macrophages infected with Mtb vs. ΔRv3167c. 1223 genes were upregulated in Mtb infected THP-1 macrophages compared to 1109 genes in ΔRv3167c infected THP-1 macrophages. There were few bacterial transcripts that were detected in these samples and comparison of bacterial gene expression could not be made. Real time qPCR (RT-qPCR) was used for selected transcripts to validate the results of RNA sequencing. CCL20 was chosen as it is upregulated in Mtb infected monocytes. In addition, CCL20 downregulates ROS production and inhibits apoptosis of monocytes infected with mycobacteria (162). CCL3 was chosen as it is induced in macrophages after infection with Mtb and inhibits intracellular growth of Mtb (163). In addition since both molecules are secreted, ELISA could be employed to find their concentrations in the supernatants of infected cells.

According to RNA sequencing, Mtb infected cells had 10 fold more CCL3 FPKMs (fragment per kilobase of transcript per million mapped reads) than untreated cells and ΔRv3167c infected cells had 62 fold more (Figure 22A). RT-qPCR result was similar to RNA sequencing result for CCL3 expression (CCL3 was 48.85 fold upregulated in Mtb infected cells and 316.11 fold upregulated in ΔRv3167c infected cells) (Figure 22B).

According to RNA sequencing, Mtb infected cells had 351 fold more CCL20 FPKMs
Figure 22: Regulation of CCL3 and CCL20 expression in Mtb and ΔRv3167c infected THP-1 macrophages. CCL3 and CCL20 expression levels were determined in Mtb and ΔRv3167c infected THP-1 macrophages 16 hr after infection by RNA sequencing (A and C) or by real-time qPCR (B and D). Plotted values are fold change in FPKMs for CCL3 (A) or CCL20 (C) relative to untreated cells. For B and D data are the mean ± SEM of one experiment performed in triplicate (n=3) and the results are depicted as fold change relative to Mtb infected cells and normalized for expression of the housekeeping gene GAPDH. Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. ***, 0.0001< p < 0.001.
then untreated cells and ∆Rv3167c infected cells had 26 fold more (Figure 22C). RT-qPCR showed a different trend compared to RNA sequencing result for CCL20 expression (CCL20 was 32.32 fold upregulated in Mtb infected cells and 104.4 fold upregulated in ∆Rv3167c infected cells) (Figure 22D). These results confirmed the upregulation of CCL3 following infection with Mtb.

In order to find which of the results was correct for CCL20 and confirm CCL3 results at the protein level, ELISA was used to assay for CCL3 and CCL20. ELISA for CCL3 showed that there was significantly more CCL3 in the supernatants of ∆Rv3167c infected THP-1 macrophages (227 pg/ml) than Mtb infected ones (184 pg/ml) 24 hrs after infection. ELISA also showed that 48 hr after infection, there was significantly more CCL3 in the supernatants of ∆Rv3167c infected THP-1 macrophages (267 pg/ml) than Mtb infected ones (69 pg/ml) (Figure 23A). The decrease in CCL3 levels in untreated and Mtb infected cells between 24 and 48 hr is thought to be due to degradation. Cancer cell lines are implicated in degradation of CCL3 (164) and THPM1 cell line can also be degrading CCL3 in the supernatant. The lack of a decrease for ∆Rv3167c might be due to high levels of CCL3 translation in the cells that hides a decrease in CCL3 levels in the supernatant. While ELISA confirmed CCL3 expression differences that were seen both in RNA sequencing and qPCR, CCL20 protein levels were slightly lower in the supernatants of ∆Rv3167c infected THP-1 macrophages (540 pg/ml) than in the supernatants of Mtb infected ones (737 pg/ml) 24 hrs after infection and also similar in the supernatants of ∆Rv3167c infected THP-1 macrophages (676 pg/ml) compared to the supernatants of Mtb infected ones (760 pg/ml) 48 hrs after infection (Figure 23B). Therefore ELISA of CCL20 did not validate the RNA sequencing or qPCR result. These
Figure 23: ELISA confirms upregulation of CCL3 expression in ΔRv3167c infected THP-1 macrophages. CCL3 and CCL20 protein concentrations in the cell culture supernatants were measured using DuoSet ELISA kits. Data are the mean ± SEM of two experiments performed in triplicate (n=6). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test versus Mtb. ns, not significant; *, 0.05< p <0.01; ***, 0.0001< p < 0.001.
results show that a biological replicate of the RNA sequencing experiment is necessary and that the results need to be confirmed by qPCR. Also it is important to show that the differences in gene expression lead to differences in protein expression since it is mostly the difference in protein levels (or activation or deactivation of proteins) that can make a difference in the phenotype.

3.2.5 Deletion of \( \text{Rv3167c} \) changes the expression of host pro-apoptotic and anti-apoptotic proteins \textit{in vivo}

In order to find if the differences in the gene expression levels led to differences in protein expression and phosphorylation, the expression and phosphorylation state of some of the host proteins was determined. Whole cell lysates of THP-1 macrophages infected with Mtb or \( \Delta \text{Rv3167c} \) were prepared 8 and 24hrs after infection. 100\( \mu \)g of samples were sent to Kinexus Bioinformatics Corporation, which used the protein samples in antibody microarray experiments. For the antibody microarray, proteins in the whole cell lysates were attached to fluorescent dye. Control and treated proteins were loaded on the same microarray side by side. After incubation, slides are washed and scanned. Analysis of the results showed that 33 proteins were differentially regulated in cells infected with Mtb vs. \( \Delta \text{Rv3167c} \) for 8hr (Figure 24A). Twenty four proteins were differentially regulated in cells infected with Mtb vs. \( \Delta \text{Rv3167c} \) for 24hr (Figure 24B). After 8hrs of infection several pro-apoptotic proteins like ABL1, AIFM1, BID, ASK1, and BAK were upregulated in \( \Delta \text{Rv3167c} \) infected THP-1 macrophages. Anti-apoptotic proteins like RAF1, MAPKK5, RB1, PPP5C, AKT3, SMAD1 were down regulated in \( \Delta \text{Rv3167c} \) infected THP-1 macrophages compared to Mtb. After 24 hr of infection
Figure 24: THP-1 macrophage proteins whose expression or phosphorylation have changed 8 hrs or 24 hrs after infection with ΔRv3167c compared to Mtb. 100µg of whole cell lysates of THP-1 macrophages infected with Mtb or ΔRv3167c were prepared 8 (A) and 24hr (B) after infection and used in antibody microarray experiments. Z scores are calculated by subtracting the overall average intensity of all spots within a sample from the raw intensity for each spot, and by dividing it by the standard deviations (SD) of all of the measured intensities within each sample. Z ratios are further calculated by taking the difference between the averages of the observed protein Z scores and dividing by the SD of all of the differences for that particular comparison. A Z ratio of ±1.2-1.5 is inferred as significant. Pink color indicates upregulation in ΔRv3167c infected cells, blue color indicates downregulation in ΔRv3167c infected cells.
expression of pro-apoptotic proteins like RIPK4, AK2, ASK1 and AIFM1 was upregulated in ΔRv3167c infected THP-1 macrophages. At the same time expression of anti-apoptotic proteins like SODD, MKK1 and MLK3 was downregulated in ΔRv3167c infected THP-1 macrophages.

Activation of ABL1 (Abelson tyrosine-protein kinase 1) leads to JNK and p38 MAPK activation followed by induction of apoptosis (165). ASK1 (Apoptosis signal regulating protein-serine kinase, MAP3K5) activation also induces apoptosis through JNK and p38 MAPK (166). ATF2 (Activating transcription factor 2) is a transcriptional activator that gets activated by p38 MAPK and induces apoptosis (167, 168). Increased expression or phosphorylation of these proteins suggests that the apoptosis of ΔRv3167c infected THP-1 macrophages might be regulated via JNK and/or p38 MAPK. According to preliminary experiments, inhibiting p38 MAPK activity reduced the apoptosis levels of ΔRv3167c infected human MDM’s to the level of wild-type Mtb infected cells (Srinivasan L, unpublished data).

Some of the proteins that were found to be regulated are involved in caspase-9 activation. Cleaved BID (BH3-interacting domain death agonist) leads to release of cytochrome c from mitochondria and caspase-9 activation (169). BAK (Bcl-2 homologous antagonist/killer) works against the anti-apoptotic function of BCL2 and promotes cytochrome c release and caspase-9 activation (170). Knocking down AKT3 leads to increased caspase-9 activity and apoptosis (171). In addition, caspase-9 was found to be upregulated in the antibody microarray. However, as shown in figure 19 ΔRv3167c infected cells did not have significantly higher levels of active caspase-9.
Therefore, we believe that either these results are false positives or caspase-9 levels go down after 8 hr.

3.3 EFFECT OF DELETION OF RV3165C AND RV3167C ON MYCOBACTERIAL GENE EXPRESSION

3.3.1 Rv3165c and Rv3167c are required for the increase of katG expression inside host cells

The involvement ROS in the pro-apoptotic phenotype of the knock-out strains suggested that the bacteria might lack mechanisms that are able to counter the effects of ROS. Mtb only has one catalase gene (katG) that can neutralize the ROS hydrogen peroxide, which led to the idea that katG might be regulated by Rv3165c and/or Rv3167c.

Rv3167c is a probable transcriptional regulatory protein that might belong to the TetR family of transcription factors (TFRs) (44) and this predicted function raised the possibility that Rv3167c might regulate the expression of other genes. According to InterProScan, a software that can identify protein domains, Rv3167c contains an N-terminal TetR-type DNA-binding (DNB) helix-turn-helix domain and a C-terminal TetR-like transcriptional regulator domain that includes ligand-binding and dimerization (LBD) domain (Figure 25) (172). Most of the TFRs repress transcription of genes when they bind to DNA through their DNB domain. Upon binding of a ligand to their C-terminal (LBD) domain, TFRs detach from DNA (173). There is evidence that some TFRs acting as activators. SczA of Streptococcus pneumoniae is a member of TFRs and it is involved in the upregulation of genes associated with Zn$^{2+}$ stress (174). MdoR of Mycobacterium sp. Strain JC1, was shown to be a TetR-like transcriptional activator,
Figure 25: InterProScan result for prediction of protein domains of Rv3167c.
which is implicated in the upregulation of genes necessary for the oxidation of methanol (175).

RNA was isolated from Mtb, ΔRv3165c and ΔRv3167c grown in vitro to log phase. Following reverse transcription, real time qPCR was performed to measure katG expression. katG expression levels were similar in ΔRv3165c (1.31 fold over Mtb) and ΔRv3167c (1.18 fold over Mtb) compared to Mtb and the difference was not statistically significant (Figure 26A).

To check for the effect of ROS on katG expression in vitro, xanthine and xanthine oxidase were added to Mtb, ΔRv3165c and ΔRv3167c in vitro cultures to induce hydrogen peroxide formation after they were resuspended in PBS. After 90 min of incubation, RNA was isolated from the bacteria. Following reverse transcription, real time qPCR was performed to measure katG expression. katG expression was found to increase in not only Mtb (23.59 fold over Mtb in vitro) but also in ΔRv3165c and ΔRv3167c (33.88 and 33.96 fold over Mtb in vitro) (Figure 26B). To determine if ΔRv3165c and ΔRv3167c can upregulate katG expression in macrophages as well, THP-1 cells were infected with Mtb, ΔRv3165c and ΔRv3167c for 16hr and RNA was isolated from infected cells. Following reverse transcription, real time qPCR was performed to measure katG expression. Although in macrophages Mtb upregulated katG expression 12.25 fold over Mtb in vitro, ΔRv3165c and ΔRv3167c upregulated katG 5.62 and 8.44 fold less than Mtb in macrophages respectively (2.18 and 1.45 fold upregulation over Mtb in vitro) (Figure 26C). These results suggest that Mtb requires Rv3165c and Rv3167c to upregulate katG expression during infection of macrophages. As katG encodes for catalase, upregulation of katG is thought to be required to neutralize ROS during an
Figure 26: ΔRv3165c and ΔRv3167c do not upregulate katG expression in macrophages. RNA was isolated from untreated in vitro cultures (A) or xanthine and xanthine oxidase treated in vitro cultures (B) or THP-1 cells infected with mycobacteria for 16 hr (C). cDNA was synthesized from RNA and used in real time qPCR experiments. Data are of one representative experiment with three replicates per group (n=3). Error bars represent SEM. Results are depicted as fold change relative to Mtb under the same conditions and normalized for expression of the housekeeping gene SigA. Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. ns, not significant; ***, 0.0001 < p < 0.001.
infection and lack of upregulation of katG in ΔRv3165c and ΔRv3167c can explain involvement of ROS in the pro-apoptotic phenotypes of those mutants.

3.3.2 **Rv3165c is required for Rv3167c expression inside host cells**

As Rv3167c is predicted to be a probable transcriptional regulatory protein and Rv3165c and Rv3167c are close to each other in the Mtb genome, RT-qPCR was performed to determine if Rv3165c and Rv3167c regulate each others’ mRNA expression. To determine if Rv3165c and Rv3167c exert their anti-apoptotic activity through nuoG, its expression was measured as well. The expression of nuoB was determined as a control since it is a part of the operon that includes nuoG (147). The lack of Rv3165c or Rv3167c did not affect the expression levels of any of the genes investigated *in vitro* (Figure 27A). NuoB and nuoG expression levels were not affected by the lack of Rv3167c in macrophages either (Figure 27B).

Real time qPCR was used to measure the expression of Rv3167c in ΔRv3165c, and Rv3165c in ΔRv3167c. Rv3165c expression in ΔRv3167c was 1.17 fold increased compared to Mtb in macrophages but that was not a significant difference (Figure 28A). While Mtb upregulated Rv3167c expression significantly in macrophages (11.48 fold) compared to Mtb grown in 7H9, ΔRv3165c did not upregulate Rv3167c expression significantly in macrophages (1.43 fold over Mtb) (Figure 28B). Therefore, it was concluded that Rv3165c was required for induced Rv3167c expression in macrophages.
Figure 27: Regulation of *Rv3165c, Rv3167c, katG, nuoB, nuoG in vitro* and in *macrophages*. RNA was isolated from *in vitro* Mtb, ∆*Rv3165c* and ∆*Rv3167c* cultures and from THP-1 cells infected with Mtb or ∆*Rv3167c* for 16 hr. cDNA was synthesized from the RNA and used in real time qPCR experiments. Data are the mean ± SEM of three (n=9) (A) or two (B) independent experiments performed in triplicate (n=6). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. Results are depicted as fold change relative to Mtb *in vitro* (A) or relative to Mtb in macrophages (B) and normalized for expression of the housekeeping gene *SigA*. 
Figure 28: ΔRv3165c do not upregulate Rv3167c expression in macrophages. RNA was isolated from *in vitro* Mtb, ΔRv3165c and ΔRv3167c cultures and from THP-1 cells infected with these strains for 16 hr. cDNA was synthesized from the RNA and used in real time qPCR experiments. Data are of a representative experiment (out of 3) with three replicates per group (n=3). Error bars represent SEM. Results are depicted as fold change relative to Mtb in macrophages (A) or Mtb *in vitro* and normalized for expression of the housekeeping gene *SigA*. Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. ns, not significant; **, 0.001< p < 0.01.
3.4 ANALYSIS OF CELLULAR LOCALIZATIONS OF RV3165C AND RV3167C

To obtain information about the cellular localizations and better understand the functions of Rv3165c and Rv3167c, myc-tagged Rv3165c and Rv3167c were expressed in BCG. Subcellular fractions were prepared and the distributions of the myc-tagged proteins in those fractions were analyzed. Additionally possible presence of the myc-tagged proteins in the culture filtrates was investigated.

3.4.1 Rv3165c is in the cell envelope, whereas Rv3167c is in the cytoplasm

TMHMM Server v. 2.0 (software for prediction of transmembrane helices in proteins) predicts that Rv3165c is a transmembrane protein (Figure 29A) whereas Rv3167c is secreted (176). Rv3165c is a hypothetical unknown protein whereas Rv3167c is a probable transcriptional regulatory protein. For Rv3167c to be involved in transcriptional regulation it must be in the cytoplasm. To confirm predicted cellular localizations, myc-tagged versions of Rv3165c and Rv3167c were produced in BCG using the extrachromosomal expression vector pMV261. Subcellular fractions of BCG cultures expressing either Rv3165c-myc or Rv3167c-myc were prepared. Equal percentages of cell envelope and cell cytoplasm fractions were separated by PAGE and proteins were detected by western blotting. Anti-myc antibody was used to detect myc-tagged proteins. Rv3165c was localized mostly to the cell envelope whereas Rv3167c was solely located in the cytoplasm (Figure 29B). This result confirmed the predicted locations of the proteins. LAM and DNAK were used as cell fraction markers. Mtb LAM is present solely in the cell envelope fraction and DNAK (probable chaperone protein) is present solely in the cytosolic fraction.
Figure 29: Rv3165c is in the cell envelope and Rv3167c is in the cytoplasm.
Prediction of transmembrane helices of Rv3165c by TMHMM (A). BCG clones expressing myc tagged Rv3165c or Rv3167c were grown to late log phase, lysed using a French® Press and the cell envelope fraction was separated from the cytosolic fraction. Fractions were separated by PAGE for western blotting. Anti-myc antibody was used for detection of myc tagged proteins. Anti-LAM and anti-DNAK antibodies were used for detection of LAM and DNAK in the fractions (B). BCG clones expressing myc tagged Rv3165c or Rv3167c were grown in Sauton’s medium to late log phase, the cells were collected and the culture filtrate was concentrated. Equal percentages of the whole cell lysate and the culture filtrate fractions were loaded in the gel. Anti-FAP and anti-GroEL antibodies were used for detection of FAP and GroEL in the fractions (C).
3.4.2 Rv3165c and Rv3167c are not secreted

To check if either of the two proteins was secreted, culture filtrates (CF) of BCG expressing the myc-tagged proteins were prepared and concentrated. CF samples and whole cell lysates (WCL) were separated by PAGE and proteins were detected by western blotting. Anti-myc antibody was used to detect myc-tagged proteins. Both of the proteins were only detected in the WCL, showing that they were not secreted (Figure 29C). Anti-FAP antibody was used to show the presence of FAP (fibronectin attachment protein) mostly in the CF fraction (some FAP is present in the cell membrane, which is why it is detected in the WCL fraction). Anti-GroEL antibody was used to show the presence of GroEL (probable chaperone protein) solely in the WCL fraction and that there was no cellular death and lysis that would have released proteins to the CF.

3.5 VIRULENCE OF ΔRv3167C KNOCK-OUT STRAIN

In order to determine whether knocking out Rv3167c in Mtb changed its virulence especially because of its increased pro-apoptotic phenotype, eight SCID mice per condition were infected with Mtb and ΔRv3167c via aerosol route. 24hr later three mice per condition were sacrificed and the bacteria from their lungs were plated. The levels of bacteria in the lungs were similar (264 ± 33 colony forming units of Mtb vs. 297 ± 23 colony forming units of ΔRv3167c) (Figure 30A). The remaining infected mice were observed and scored until they were in a moribund state and had to be sacrificed (Figure 30B).

All of the ΔRv3167c infected mice died on the 28th day of infection while Mtb infected mice died between 33rd and 39th days of infection. A log-rank test found the
Figure 30: ΔRv3167c is not less virulent than Mtb in mice. 8 SCID mice were aerosol infected with a low dose of Mtb or ΔRv3167c. A) Colony forming units (CFU) in the lungs were shown to be not significantly different between Mtb and ΔRv3167c (3 mice per condition were sacrificed. Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test). B) The remaining 5 mice per condition were monitored. All of the ΔRv3167c infected mice died on the 28th day of infection while Mtb infected mice died between 33rd and 39th days of infection. Statistical significance was determined by a log-rank test. **, 0.001< p < 0.01.
survival curves to be significantly different indicating possibly that $\Delta Rc3167c$ is more virulent than Mtb. This experiment has to be repeated to confirm this phenotype of $\Delta Rv3167c$ in SCID mice.

According to a study designed and supervised by our collaborator Dr. Petros C. Karakousis at Johns Hopkins University School of Medicine, $\Delta Rv3167c$ is not less virulent than Mtb in guinea pigs. Guinea pigs were infected with Mtb, $\Delta Rv3167c$, $\Delta Rv3167c::C$ or $\Delta NuoG$ strains by aerosol infection at a low-dose and the numbers of mycobacteria in the lungs were determined 1, 28 and 56 day post-infection (Figure 31A). Although the number of $\Delta Rv3167c$ was significantly higher than the number of wild-type Mtb by day 28, they were similar by day 56. The body weights of the guinea pigs were measured before they were sacrificed and found to be similar for all of the strains at the three time points (Figure 31B). The experiment in mice together with the experiment in guinea pigs suggest that $\Delta Rv3167c$ is not less virulent than wild-type Mtb.

3.6 SUMMARY

Mtb inhibits apoptosis of infected host cells and this is thought to enable Mtb to survive and prevent a successful immune response from the host. I was able to identify $Rv3165c$ and $Rv3167c$ as two novel anti-apoptotic genes of Mtb. THP-1 macrophages infected with Mtb knock-out strains lacking $Rv3165c$ or $Rv3167c$ ($\Delta Rv3165c$, $\Delta Rv3167c$) had high levels of apoptosis. Apoptosis depended on caspase-8 and 3 and/or 7, but not on caspase-9 in THP-1 macrophages, suggesting the involvement of extrinsic but not intrinsic caspase activation. Inhibiting ROS production or scavenging ROS inhibited the apoptosis of $\Delta Rv3165c$ or $\Delta Rv3167c$ infected macrophages. ROS is thought to promote
**Figure 31: ΔRv3167c is not less virulent than Mtb in guinea pigs.** Guinea pigs were infected with Mtb, ΔRv3167c, ΔRv3167c::C or ∆NuoG (12 guinea pigs per group; n=12) by aerosol infection at a low-dose (less than 100 bacteria per guinea pig) and four guinea pigs were sacrificed per strain per time point for determination of number of mycobacteria in the lungs (A). The body weights of the guinea pigs were measured before they were sacrificed (B). Statistical significance was determined by one way analysis of variance (ANOVA) with Tukey post-test. *, 0.05< p <0.01. (Experiment designed and supervised by our collaborator Dr. Petros C. Karakousis at Johns Hopkins University School of Medicine.)
the pro-apoptotic cell signaling pathways, but not start them (103). However, the signal that activates the extrinsic caspase activation in ΔRv3165c or ΔRv3167c infected macrophages is not known at this point.

Preliminary RNA sequencing and antibody microarray experiments identified host cell components that may be differentially regulated in ΔRv3167c infected cells compared to Mtb infected cells. Confirmation of these targets will help identify the host cell pathways that Mtb manipulates to inhibit host cell apoptosis and qPCR and other methods will be used to confirm Mtb genes that are involved in apoptosis inhibition.

ΔRv3165c and ΔRv3167c failed to upregulate the expression of the only catalase of Mtb, katG. Therefore, this suggests that Mtb may need to upregulate katG expression in order to neutralize host cell produced ROS to inhibit apoptosis. However, there is also evidence contradicting these results, which is mentioned in the discussion. Rv3165c was found to reside mainly in the cell envelope while Rv3167c, which is predicted to be a probable transcriptional regulator, was found to be exclusively in the cytosol of mycobacteria. Rv3165c was found to be required for upregulation of Rv3167c expression.

An experiment using SCID mice as well as another one using guinea pigs suggest that ΔRv3167c is not less virulent than wild-type Mtb as predicted. It was expected that this pro-apoptotic mutant would be less virulent as it was shown for the other pro-apoptotic mutants of Mtb. Further experimentation is necessary to confirm this phenotype of ΔRv3167c.
CHAPTER 4 DISCUSSION

The presented data established the requirement of \( Rv3165c \) and \( Rv3167c \) for apoptosis inhibition by Mtb in THP-1 macrophages. In addition to THP-1 macrophages, experiments using human blood monocyte derived macrophages and guinea pig alveolar macrophages confirmed the pro-apoptotic phenotype of \( \Delta Rv3165c \) and \( \Delta Rv3167c \) (Hurley, B. unpublished). The complementation of \( \Delta Rv3167c \) was done with the myc-tagged \( Rv3167c \) and it is thought that the tag does not affect the structure and function of \( Rv3167c \) since the N-terminus of TetR-like transcriptional regulators is surface-exposed (173). In addition, \( \Delta Rv3167c \) was successfully complemented and this suggests that myc-tagged \( Rv3167c \) was functional.

Infection of THP-1 macrophages either at 3:1 or 0.5:1 MOI inhibits growth of \( \Delta Rv3167c \) (Figure 16, 17). The difference was observed in 3:1 MOI infection when gentamicin was omitted in the chase media, indicating that the difference is due to extracellular mycobacteria. From there on no gentamicin was used in the chase media for those infections. The increasing trend of the graph for Mtb after day 1 in the absence of gentamicin compared to the decreasing trend in the presence of gentamicin suggests that there are many extracellular Mtb that got out of the cells alive, presumably via necrosis. When the infections were done at an MOI of 0.5:1 no difference in the numbers of bacteria were observed up to 4 day after infection. This was expected since the apoptotic phenotype is observed around 4-5 day after infection at that MOI. When the infections with different MOIs are compared, 0.5:1 MOI infection has higher inhibitory effect on the growth of \( \Delta Rv3167c \). This is thought to be due to the presence of uninfected
bystander macrophages which can take up the apoptotic blebs resulting from the apoptosis of infected macrophages and kill the mycobacteria inside better (177). A recent study however suggests that the effect of uninfected macrophages was contact independent and they produced a soluble molecule that acted on the infected macrophages to increase their antibacterial activities (178).

Apoptosis of infected cells is reported to kill the intracellular mycobacteria (79, 135) and our data shows that apoptosis prevented ΔRv3167c from replicating as much as Mtb. This suggests that in our infection system, either apoptosis kills ΔRv3167c less effectively than what has been shown before or apoptosis diminishes the replicating niche for ΔRv3167c, thereby prevent ΔRv3167c from replicating as much as Mtb but it does not necessarily kill it. The number of total mycobacteria (live and dead) can be determined by microscopy after infection to better analyze the effect of apoptosis on ΔRv3167c. Apoptosis rate was higher in ΔRv3167c infected cells, whereas the rate of necrosis was significantly lower compared to Mtb infected cells. This is consistent with the reports suggesting that Mtb uses necrosis to break free from the cell, unharmed and spreads to other uninfected cells (66, 67). Although the necrosis rate is high for Mtb infected cells, the percentage of viable cells that can accommodate Mtb is higher, which can lead to increased numbers of Mtb. The higher numbers of Mtb is also probably due to the ability of Mtb to replicate to bigger numbers inside the cell without causing apoptosis compared to pro-apoptotic mutants.

Apoptosis induced by ΔRv3165c and ΔRv3167c was found to involve caspases. Since only caspase 8 but not caspase 9 activity was increased in the knock-out infected cells, the extrinsic pathway of caspase activation was activated. TNF-α, FasL and TRAIL
(TNF-related apoptosis-inducing ligand) induce apoptosis through caspase-8 activation (87, 179). TNF-α secretion was not affected in the knock-out infected cells but the involvement of FasL or TRAIL in caspase-8 activation is unknown at this point.

The pro-apoptotic phenotype of ΔnuoG is abolished in the presence of ROS inhibitor or scavengers and the phagosomes of cells infected with ΔnuoG have higher levels of ROS (149). In order to investigate the involvement of ROS in the pro-apoptotic phenotype of ΔRv3165c and ΔRv3167c, ROS production was inhibited or ROS was scavenged. As in the case of ΔnuoG both of these methods abolished the pro-apoptotic phenotypes of ΔRv3165c and ΔRv3167c. The amounts of apoptosis induced by ΔRv3165c and ΔRv3167c in the experiments done on the slides were lower since the time point was 36 hr instead of 48 hr and some of the apoptotic cells were not adherent at the end of infection and were washed off. These experiments indirectly showed that there was an increase in ROS production in the ΔRv3165c and ΔRv3167c infected cells. ROS detection reagents will be employed to test for differences in the levels of ROS produced by infected cells. This will directly show if ΔRv3165c and ΔRv3167c infected cells have higher levels of ROS, presumably in the phagosome. The source of ROS can be mitochondrial or phagosomal so experiments will be done with PLB XR-CGD cells, a cell line that lacks the cybb gene (no Nox2 expression) to find if the source of ROS is phagosomal or not (180).

The expression of 2332 host genes was found to be significantly affected due to the lack of Rv3167c in Mtb via RNA sequencing. RT-qPCR and ELISA were performed to check the expression of two genes, CCL3 and CCL20. Although the CCL3 result was confirmed, RT-qPCR of CCL20 gave the opposite result of RNA sequencing and ELISA
of CCL20 gave a result different than RNA sequencing and RT-qPCR. According to these results, it is imperative to repeat RNA sequencing and confirm its results via another technique like RT-qPCR. A biological replicate of RNA sequencing will reduce the number of false positives from the initial data. However, the gene transcription data have to be carefully interpreted since they do not necessarily reflect how the translation is affected. The antibody microarray gave a smaller number of host targets to look at as it analyzed the expression of only 812 proteins and phosphorylation of 262 amino acids. The regulation of these targets has to be confirmed with western blots since only 30 to 45% of the changes in the protein levels detected by the antibody microarray can be reproduced by western blots according to Kinexus.

Although the expression of *katG* increased *in vitro* in ROS treated knock-outs, the knock-outs were defective in upregulating *katG* expression in macrophages. One explanation for these results is that the ROS produced *in vitro* are much more than the amount in macrophages and thus can upregulate *katG* expression through different mechanisms that do not require the presence of Rv3165c or Rv3167c. If the amount of ROS added *in vitro* is dosed down, a difference in *katG* expression might be observed similar to the difference in macrophages. Another explanation is that Mtb needs Rv3165c and Rv3167c to respond to ROS in macrophages but not *in vitro*. This could be due to the presence of other factors in macrophages, such as pH and ingredients of the phagosome that only allow Rv3165c and Rv3167c to respond to ROS.

Our lab previously tested a *katG* mutant to see its effect on host cell apoptosis. THP-1 macrophages infected with a *katG* mutant had significantly higher levels of apoptosis compared to wild type Mtb (149). However, this mutant (INH34 strain) also
lacked the neighboring genes \textit{Rv1907c, furA, Rv1910c, lppc,} and \textit{fadB5} (181). A mutant strain that lacks only \textit{katG} had a similar apoptotic phenotype as wild-type Mtb (Srinivasan L, unpublished data). These findings indicate that \textit{katG} by itself is not essential for apoptosis inhibition by Mtb. In line with this idea, another graduate student in our lab, Jeff Quigley, showed that in addition to \textit{katG}, other genes around \textit{katG} mentioned above are not upregulated in Mtb in the absence of Rv3167c in macrophages. Therefore it is likely that Rv3167c acts as a regulator of \textit{katG} and the other genes around it to prevent apoptosis. Quigley also showed that three sigma factors \textit{sigB, sigE} and \textit{sigH} were upregulated by Rv3167c \textit{in vivo}. Interestingly, \textit{katG} and the genes around it that are regulated by Rv3167c contain putative binding sites for the same sigma factors regulated by Rv3167c according to the software Virtual Footprint (182). According to recent research \textit{ΔsigH} infected macrophages were more apoptotic and had higher caspase-3 and 7 activity compared to Mtb infected cells (183). These data suggest that Rv3167c regulates certain sigma factors (or regulators of sigma factors), which in turn regulate the other genes that are involved in inhibition of apoptosis.

Although \textit{ΔkatG} inhibits apoptosis of THP-1 macrophages just like Mtb, \textit{ΔkatG} was less virulent compared to Mtb when they were used in infecting immunocompetent C57BL/6 mice (184). Complementation of \textit{katG} restored the virulent phenotype in the same mice. When gp91 knock-out mice (no phagocyte superoxide production) were infected with Mtb and \textit{ΔkatG}, both strains showed similar virulence, suggesting that \textit{katG} was involved in neutralizing peroxides produced by phagocyte NADPH oxidase and thus increasing the persistence of Mtb (184, 185). \textit{ΔkatG} did not replicate in murine bone marrow derived macrophages (BMDM) as well as Mtb but showed similar replication in
mice during the early stages of infection (184). This difference is probably due to the simplicity of one system versus the complexity of the other one that involve many different cell types. The seemingly conflicting results for katG deletion mutants may be due to different cell types used in vitro (THP-1 versus murine BMDM). It is possible that ΔkatG gives a phenotype that is different than Mtb only in murine cells. The apoptotic phenotype of ΔkatG in the mouse lungs needs to be analyzed to further understand the decreased virulence of ΔkatG. As mentioned before, although in addition to other proteins KatG secretion is also defective in secA2 knock-out Mtb (150), re-establishment of SodA secretion was enough to reverse the pro-apoptotic phenotype of secA2 mutant strain (146). This result also suggests that KatG is not involved in apoptosis inhibition.

The requirement of Rv3165c for the upregulation of Rv3167c in macrophages indicates a possible mechanism whereby Mtb upregulates Rv3167c expression to possibly inhibit apoptosis and that this requires the presence or activity of Rv3165c to do so. Rv3165c might be interacting directly with Rv3167c. As another possibility Rv3165c might be interacting with another protein to increase the amount of Rv3167c. A detailed predicted model is discussed below.

A working model for Rv3165c and Rv3167c emerged after the cellular localizations of these proteins were determined. Previous evidence showed that Rv3165c was required for Rv3167c expression, that both Rv3165c and Rv3167c were required for katG upregulation in infected macrophages and that ROS inhibition led to decreased apoptosis of ΔRv3165c and ΔRv3167c infected cells. In addition, Rv3167c is predicted to be a transcriptional regulatory protein. These results suggest the following working model, whereby Rv3165c resides in the plasma membrane and Rv3167c is in the
cytoplasm of Mtb. Without a signal that Rv3165c senses, the expression of Rv3167c is low (Figure 32A). When Rv3165c senses an extracellular signal that is thought to be either ROS or an oxidized molecule, it relays the signal directly or indirectly to Rv3167c. This relay causes an increase in Rv3167c expression (Figure 32B). Rv3167c then acts as a transcriptional regulator and upregulates expression of bacterial genes that inhibit apoptosis and downregulates the expression of bacterial genes that can induce apoptosis. Using either one or both of these strategies Mtb inhibits host cell apoptosis (Figure 32C).

Bacteria employ protein sensors for ROS detection. Some of these sensors contain cysteine (C) in C-X-X-C (X is any amino acid) motifs. As the oxidation sensitive thiol groups of cysteines are oxidized, disulphide bonds are formed between the two cysteines in the motif, causing a conformational change of the protein structure and a change in the activity of the protein. Other bacterial sensors detect oxidative stress through iron via the Fenton reaction (Fe$^{2+}$ + H$_2$O$_2$ → Fe$^{3+}$ + ‘OH + ‘OH). Following detection of oxidative stress, these sensors go through modifications like oxidation of histidines, dityrosine formation or carbonylation (186). An example of these sensors is PerR, which is an iron-dependent oxidative stress sensor of Bacillus subtilis that binds to iron or manganese in order to repress its target genes. Following a peroxide dependent stress, one of the histidines in the regulatory binding site of PerR becomes oxidized. This causes the release of iron from PerR followed by derepression of its target genes (187). Although Rv3165c does not contain a C-X-X-C motif, one of its extracellular amino acids predicted by TMHMM Server v. 2.0 is a histidine (Figure 29A). Histidine is one of the amino acids that is sensitive to attack and oxidation by ROS and this oxidation can lead
Figure 32: The working model showing the confirmed localizations of Rv3165c and Rv3167c and their predicted functions. Rv3165c is localized to the cell envelope and predicted to sense a signal and relay this signal to Rv3167c. This relay of the signal causes an increase in Rv3167c transcription and translation. Increased amounts of Rv3167c causes upregulation of bacterial components that inhibit host cell apoptosis.
to a change in the three dimensional structure of the protein (188, 189). Therefore, it is possible that, like the iron-dependent oxidative sensors, ROS production by the host cell changes the structure of Rv3165c through oxidation of the extracellular histidine and this change in the structure either enables Rv3165c to interact with Rv3167c or with another protein that relays the signal to Rv3167c. Complementation of ΔRv3165c mutant with Rv3165c lacking the extracellular histidine will show the importance of that amino acid in signal sensing.

One of the other mechanisms of signal detection by Rv3165c may be via another membrane protein that interacts with Rv3165c and has an extracellular signal sensing domain. This other protein can sense the signal through ligand binding or another mechanism and relay the signal to Rv3165c. Identifying proteins that can interact with Rv3165c through techniques like bacterial two-hybrid system will help validate this signal detection pathway. The points to investigate to prove this model include showing a direct or an indirect interaction between Rv3165c and Rv3167c, showing that Rv3167c regulates expression of other bacterial genes to inhibit host cell apoptosis and that Rv3167c binds to genomic DNA of Mtb to regulate gene expression. TMHMM Server v. 2.0 predicts that for the two-component regulatory system sensors mprB, mtrB, phoR, prrB, senX3, trcS the number of extracellular amino acids varies from 273 to 504 (compared to 3 for Rv3165c) (190–195). The lack of such a big extracellular amino acid stretch of Rv3165c suggests a different mechanism for signal detection by Rv3165c. In addition, according to InterProScan Rv3165c does not have a predicted histidine kinase domain (172) like the sensors of two-component systems (196, 197).
According to InterProScan, Rv3167c lacks any sensor or kinase domain and contains an amino terminal TetR-type DNA-binding helix-turn-helix domain and a carboxy terminal TetR-like transcriptional regulator domain. Lack of a kinase or sensor domain indicates that Rv3167c is not a member of Ser/Thr/Tyr protein kinases or one-component systems that are responsible for many signal transduction pathways (198, 199). All TetR family transcriptional regulators are homodimers and most of them repress transcription of genes (173). As such, demonstration of homodimer formation by Rv3167c will further confirm its classification as a transcriptional regulator. The sequence Rv3167c binds to needs to be identified via methods like chromatin immunoprecipitation in order to characterize it as a repressor or activator of gene transcription. Predicted DNA binding sequences for Rv3167c can be used in electrophoretic mobility shift assays to determine if Rv3167c binds to those sequences. However, the conditions under which Rv3167c binds to DNA may not be replicated in vitro and this might lead to false negative results.

The preliminary data from the SCID mice infection experiment suggest that \(\Delta Rv3167c\) is not less virulent than Mtb and the guinea pig infection experiment data suggest that \(\Delta Rv3167c\) and \(\Delta nuoG\) are not less virulent than Mtb. Both of these experiments need to be repeated in order to confirm these results. The number of \(\Delta Rv3167c\) and its apoptotic phenotype in the lungs of the SCID mice have to be determined to make a better correlation between its apoptotic phenotype and virulence. It is possible that \(\Delta Rv3167c\) does not have a pro-apoptotic phenotype in the phagocytic cells of mice and this leads to a similar virulence as wild-type Mtb. Our lab has identified a DNA region of Mtb that was required for inhibition of apoptosis in THP-1.
macrophages, whereas lack of this region did not lead to loss of virulence of Mtb in mice (Velmurugan, K. unpublished data). However, the lack of 7/10 region from Mtb led to decreased virulence in SCID mice (Figure 8). On the other hand, that was a single experiment and infection experiments in SCID mice and guinea pigs for ∆Rv3167c together present a stronger case. If the decreased virulence of ∆7/10 and similar virulence of ∆Rv3167c (and ∆Rv3165c) compared to Mtb are confirmed, then this would suggest that the other genes in the 7/10 region may be involved in the virulence of Mtb.

In contrast to the guinea pig infection results, ∆nuoG is less virulent in immunocompromised SCID and immunocompetent BALB/c mice (147). But when immunocompetent C57BL/6 mice were infected via aerosol route with Mtb and ∆nuoG, ∆nuoG did not show any decreased virulence for up to 7 weeks as measured by bacterial counts (Miller J. and Gurses S., unpublished data). Keeping in mind that the guinea pig experiment was also done by aerosol infection whereas the SCID and BALB/c mice infections were done intravenously via a lateral tail vein, the physiologically more relevant experiment would be the one done by aerosol route since that allows Mtb to gain access to the lungs first. In addition, the guinea pig is considered to be a better model than the mouse to study Mtb infection (200, 201). The mouse infections that showed that ∆7/10, ∆sodA and ∆katG were less virulent than wild-type Mtb were also done by injection of mycobacteria intravenously via a lateral tail vein (151, 184). It is possible that injection of mycobacteria into the animals, especially mice, is not a good model to study virulence of pro-apoptotic mutants and the differences that were observed in the virulence of the pro-apoptotic mutants were due to characteristics of tail vein injections (e.g., high dose of mycobacteria and a different route and spread pattern of mycobacteria
inside the animal). The different set of cells that the pro-apoptotic mutants encounter following a tail injection might be more affective in mounting a response against them than the cells they encounter following an aerosol infection, which might explain why pro-apoptotic Mtb mutants would seem to be less virulent in infections via tail vein injections. Although pro-apoptotic mutants might show differences in various terms in infections of cultured cells or cell lines compared to Mtb, they might not make a difference in vivo. To compare the two ways of infection, either ∆Rv3167c can be used for infection of mice via tail vein injection or the pro-apoptotic mutants that were shown to be more virulent can be used for infection of mice or guinea pigs via aerosols. If ∆Rv3167c is found to be more virulent or the other pro-apoptotic mutants are found to be similarly virulent as Mtb in these experiments, it would suggest that mouse infections via tail vein injection do not give accurate results in terms of the virulence of pro-apoptotic Mtb mutants. This would also suggest that Mtb pro-apoptotic mutants are not good candidates for new vaccines and other phenotypes of Mtb that can elicit a better immune response have to be studied.

Apoptosis of cultured murine and human macrophages following infection decreases the viability of mycobacteria (135, 137, 143). However, a recent study found that during the initial stage of an in vivo infection the viability of pro-apoptotic ΔnuoG mutant did not decrease after apoptosis of infected cells. The ΔnuoG mutant spread to a higher number of lung myeloid cells but was present in a lower number in each cell following infection of immunocompetent C57BL/6J mice (202). ΔnuoG infection lead to an earlier activation of CD4+ T cells compared to Mtb. Therefore the seemingly more virulent phenotype of the ∆Rv3167c mutant in the SCID mice might be due to the spread
of \( \Delta Rv3167c \) to a higher number of cells through apoptosis and lack of the adaptive immune response. The number of \( \Delta Rv3167c \) bacteria was higher than the number of Mtb in the guinea pig lungs by day 28 but they were not significantly different by day 56. Compared to Mtb, \( \Delta Rv3167c \) infected guinea pig alveolar macrophages go through higher rates of apoptosis (Hurley B., unpublished data). Therefore, it is possible that \( \Delta Rv3167c \) replicated and possibly spread better than Mtb through apoptosis in the early stages of infection followed by a better control of mycobacterial infection by the guinea pigs through adaptive immunity that led to a decrease in the number of bacteria.

In conclusion, I identified two novel anti-apoptotic genes of Mtb that will be added to a short list of anti-apoptotic genes. Several effects of deleting these genes were identified in Mtb as well as in THP-1 macrophages. However, the infection experiments showed that the virulence of one of the mutants, \( \Delta Rv3167c \), was not less than wild-type Mtb as expected from a pro-apoptotic mutant. Although this lack of difference in the virulence phenotype might be specific for \( \Delta Rv3167c \), it is also possible that infection experiments done to show the virulence of other pro-apoptotic mutants were flawed. Confirmation of virulence phenotypes of previously found pro-apoptotic mutants via aerosol infection of mice and/or guinea pigs is suggested, as that method of infection is the more physiologically relevant one. On the other hand, if the decreased virulence of pro-apoptotic mutants is not confirmed through aerosol infections, this could mean that apoptosis inhibition by Mtb is irrelevant in terms of its virulence.


