ROLE OF *MYCOBACTERIUM TUBERCULOSIS* NUCLEOSIDE DIPHOSPHATE KINASE IN THE PATHOGENESIS OF TUBERCULOSIS

by

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ABSTRACT

*Mycobacterium tuberculosis* (Mtb) evasion of the host immune response leads to its resilient persistence within the macrophage ultimately triggering active tuberculosis, a deadly infectious disease afflicting millions of people globally. To combat this disease, there is an urgent need to reveal the underlying mechanisms of pathogenesis by understanding how mycobacteria interact with the host macrophage. The work of this thesis contributes to this understanding through the identification of Mtb nucleoside diphosphate kinase (Ndk) as a virulence factor that contributes to pathogenesis by interfering with key host small G-proteins essential for proper function of the innate immune response. Combining biochemical interaction assays with GTPase activation assays, this work demonstrated that Mtb Ndk interacts with host Rab5, Rab7, and Rac1 to inactivate their downstream signaling. The consequence of interfering with Rab5 and Rab7 signaling is a dramatic decrease in phagolysosome fusion of mycobacteria phagosomes, while inactivation of Rac1 led to NADPH oxidase assembly defect causing impaired reactive oxygen species production. Additional studies utilizing mycobacteria strains with knocked down Ndk further demonstrated its contribution to virulence. Indeed, strains with knocked down Ndk were substantially more prone to intracellular killing within the macrophage, which correlated with increased levels of phagolysosome fusion, raised levels of macrophage oxidative burst, and an increase in programmed cell death. Consistent with these findings, *in vivo* virulence of Mtb with knocked down Ndk was reduced significantly compared to wild type Mtb when monitoring mice survival. Altogether, the work of this thesis demonstrated that Mtb Ndk is a *bona fide* mycobacterial virulence factor that attenuates host innate immunity.
PREFACE

Parts of this thesis have been published in peer-reviewed journals:


   In this study, I was responsible for experimental design, performing most experiments, data analysis, and writing the manuscript. Deghmane, A.E. assisted in some experimental designs. Hong, T. performed the experiment linked to Figure 2E of the article. Hmama, Z. assisted in editing of the manuscript. This published work is located in sections 3.1 and 4.1.


   In this study, I designed and performed all experiments, and wrote the manuscript. Hmama, Z. assisted in editing the manuscript. This published work is located in section 2.7.1 as a reference cited.


   In this study, I designed the majority of the experiments and contributed to development of the work shown in Figures 1, 2, and 5. Lau, A. performed most of the experiments relating to cloning of destination vectors. Wang, X. and Liao, T.Y. contributed to the development of the work shown in Figures 3 and 4. The manuscript was written as a joint effort by Hmama, Z and me. Hmama, Z. also contributed to some of the experimental design. This published work is located in section 2.4.2 and 3.2.

In this study, I designed and performed all experiments. Wang, X. assisted in work related to Figure 5A. I wrote the manuscript and Hmama, Z. helped with editing of the manuscript. This work is located in sections 3.3 and 4.2.1.


This manuscript has been recently submitted and is under review. In this study, I was responsible for the majority of the experimental design and work. Obregon-Henao, A. and Orme, I.M. contributed to *in vivo* work shown in Figure 1D. I wrote the manuscript with editing and comments by Stokes, R.W. and Hmama, Z. This work is located in sections 3.4 and 4.2.2.

All animal experiments are approved by UBC animal care committee according to protocols (Animal Certificate #A08-0873).
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<th>Full Form</th>
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<tbody>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow derived macrophages</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CFP</td>
<td>culture filtrate proteins</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CM-DCFDA</td>
<td>5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DXT</td>
<td>dextran</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ExoS</td>
<td>exoenzyme S</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP/GTP</td>
<td>guanosine di-/tri-phosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione s-transferase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thio-galactoside</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
<tr>
<td>Ndk</td>
<td>nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>NuoG</td>
<td>NADH ubiquinone oxidoreductase chain G</td>
</tr>
<tr>
<td>OADC</td>
<td>oleic acid albumin dextrose complex</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Phox</td>
<td>phagocyte oxidase</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>RILP</td>
<td>rab7 interacting lysosomal protein</td>
</tr>
<tr>
<td>RLU</td>
<td>relative luminescence units</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SapM</td>
<td>secreted acid phosphatase M</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
</tr>
<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>WCL</td>
<td>whole cell lysate</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>YopE</td>
<td>Yersinia outer membrane virulence protein E</td>
</tr>
</tbody>
</table>
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1.1 Mycobacterium tuberculosis

1.1.1 Disease and infection cycle

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* (Mtb) infection remains a global concern today. Over a third of the world population is latently infected and 2 million people die yearly from this deadly infectious disease afflicting primarily the lungs \cite{1}. Active TB disease differs from latently infected individuals due to the manifestation of clinical symptoms such as persistent cough, fever, and weight loss \cite{2}. In addition, individuals with active disease are highly infectious and thus are positive for sputum smears and cultures. Only 5-10% of infected individuals will develop active disease following initial infection – the majority of which occurs within one to two years \cite{3}. However, these percentages climb dramatically in HIV positive individuals \cite{4}. Other predisposing risk factors related to TB disease include poor nutrition, drug use, alcoholism, and close contact with large populations of people (schools, hospitals, prisons) \cite{2}. A combination of four drugs (rifampin, isoniazid, pyrazinamide, ethambutol or streptomycin) is the most common course of treatment due to development of mycobacterial resistance to a single drug \cite{2}. However, this treatment is difficult due to problems with patient compliance during the long course of the antibiotic regimen which usually lasts from 6 to 9 months. In recent years, increasing rates of co-infection with HIV \cite{4} along with emerging strains of multi- and extensively- drug resistant Mtb (MDR- / XDR-TB) has further increased the global impact of TB \cite{5, 6}. Indeed, MDR-TB and XDR-TB strains have been isolated in areas with higher socioeconomic status than those typically associated with a high TB incidence rate \cite{7-9}. In addition, lack of an effective vaccine and the need for more efficient drugs raise concerns regarding the growing problem of TB, especially in endemic countries. To
truly combat TB, we need to significantly enhance our current knowledge of how the pathogen interacts with the host to circumvent an otherwise robust immune system.

Infection by Mtb occurs by aerosol route and once inhaled, the bacterium is phagocytosed by its principle host, the alveolar macrophage. This is followed by a localized inflammatory response which is highlighted by the formation of the granuloma, a defining pathologic feature of TB (summarized in Fig. 1) \cite{10, 11}. The granuloma is the formation of a mass of cell types around the infected alveolar macrophage, consisting of other macrophages, monocytes, neutrophils, giant cells, foamy macrophages, and epithelioid macrophages \cite{10, 11}. Further, the initiation of the adaptive immune response brings additional lymphocytes to surround the granuloma \cite{11}. This generally occurs 2-3 weeks post infection with extensive vascularization within the granuloma, and is the body’s way of containing the mycobacterial infection \cite{10}. However, the granuloma can reactivate to exhibit extensive pathology indicative of disease progression, and these are commonly correlated to a decrease in the number of blood vessels penetrating the structure, an increase in the number of foamy macrophages \cite{11}, and an increase in the number of necrotic cells which contribute to the formation of the caseous center, a section of necrotic tissue with cheese-like appearance \cite{10}. Ultimately, the active granuloma ruptures and spreads thousands of infectious bacilli into the lung airways \cite{10}, contributing to the development of a persistent cough which further aids in the spreading of TB.

**Figure 1 (next page). Mtb infection cycle.** The following figure illustrates the life cycle of Mtb upon phagocytosis by alveolar macrophages to granuloma formation which controls infection, and ultimately reactivation of the bacteria causing caseation of the granuloma. Reprinted with permission from Nature Publishing Group; Nature Immunology.
1.1.2 Bacteria resistance to host killing

*Mycobacterium tuberculosis* is a rod-shaped aerobic facultative intracellular pathogen, and has unique characteristics such as slow growth rate, high G+C content, and a thick cell wall composed partly of mycolic acids [12]. The success of Mtb as a pathogen can be attributed to its ability to adapt to environmental changes throughout the course of infection. Hostile environments within the host include nutrient deprivation, hypoxia, reactive oxygen and nitrogen species, and intraphagosomal bactericidal enzymes and acidity. For instance, the high mycolic acid content in its cell wall contributes to resistance against dehydration, acidic or alkali conditions, and chemical disinfectants, as well as common antibiotics [13]. There are many properties that contribute to Mtb virulence: specialized mechanism for cell entry [14, 15], detoxification of reactive oxygen and nitrogen radicals [16], antigenic proteins modulating host response [17, 18], phagosome maturation arrest caused by various virulence factors [19-22], various glycolipids altering host signaling pathways [23-25], and inhibition of apoptosis to suppress the adaptive immune response [26-28]. A summary for a few of these host resistance mechanisms relating closely to the work of this dissertation will be discussed in section 1.3.

1.1.3 BCG vaccine as surrogate pathogen

The study of Mtb pathogenicity is hindered by its high virulence, and requires the availability of a BSL-3 laboratory. This is at times inconvenient or cumbersome to satisfy the protocols of certain experiments. One model organism used commonly by researchers in the field, is *M. bovis* Bacillus Calmette-Guérin (BCG). It is currently the only approved vaccine against TB, and is a very suitable surrogate pathogen since it is closely related to Mtb while remaining non-pathogenic in healthy individuals. It is important to note that BCG is derived by serial passages over 13 years from *Mycobacterium bovis* [29], the causative agent of tuberculosis in cattle. At the genomic level, *M. bovis* is >99.95% identical to Mtb and contains no unique
genes \cite{30}. However, key differences revolve around deletion events within the \textit{M. bovis} genome when compared to the Mtb genome. For instance, \textit{M. bovis} contains $\sim$ 50 less coding genes and a large number ($\sim$ 2500) of single nucleotide polymorphisms when compared to Mtb \cite{30}. Furthermore, there are differences in the expression of cell wall components and altered or missing major secreted antigenic proteins which combine to play a central role to mycobacteria physiology and pathogenesis \cite{30}. Ultimately, these differences provide a basis which places Mtb closer to the common ancestor of the tuberculosis complex when compared to \textit{M. bovis}, and that \textit{M. bovis} evolved from a progenitor of the tuberculosis complex \cite{30, 31}. Taking this into consideration, although attenuated, BCG still share a high degree of genomic similarity ($>99\%$) to Mtb, with the key differences attributed to the loss of ten “regions of difference” (RD1-RD10) in BCG \cite{32}. It has been further documented that both RD1 and RD2 contribute to the virulence of Mtb and is a basis for the attenuation observed with BCG \cite{33}. Nevertheless, phagosome maturation arrest and deficient antigen presentation, both key features of Mtb pathogenesis have been confirmed with BCG \cite{34-39}.

1.2 Small GTPase proteins

1.2.1 Overview

The small guanosine triphosphatase (GTPase) Ras superfamily consists of more than 170 proteins whose role is to regulate unique aspects of cellular function \cite{40, 41}. These small guanine nucleotide binding proteins are monomeric with molecular masses in the range of 20-40 kDa \cite{40}. The superfamily has been divided into five major families based on structure and functional similarities: Ras, Rho, Rab, Ran, and Arf \cite{41}. The Ras family regulates gene expression, Rho family regulate both cytoskeletal reorganization and gene expression, Rab and Arf family together regulate intracellular vesicle trafficking, and the Ran family regulates nucleocytoplasmic transport \cite{41}. All small GTPases share the mechanism to act as a molecular
on/off switch. This is possible due to conserved domains responsible for GDP/GTP binding and GTPase activity across all small G-proteins. Indeed, a high degree of amino acid homology is observed amongst Rab and Rho proteins compared to Ras proteins (~30%) [40]. Despite the conserved domains, small GTPases differ in their region of interaction with downstream effectors, and further differentiation occurs during posttranslational modifications with different lipid moieties, which is responsible for proper targeting of the protein to specific cellular organelles or membranes [40]. As mentioned previously, the defining feature of small GTPases is the ability to act as a molecular switch, and this is achieved through the binding of GTP (active) in exchange for GDP (inactive), which then induces a conformation change allowing for interaction with downstream effectors (summarized in Fig. 2) [40]. Other key regulators of small GTPases include guanine nucleotide exchange factor (GEF), which accelerates the release of GDP and its subsequent binding of GTP, and GTPase activating protein (GAP) which is responsible for catalyzing the hydrolysis of the GTP molecule to GDP thereby switching the G-protein back to its inactive state [40]. Meanwhile, a third regulator, GDP dissociation inhibitor (GDI) is able to keep the G-protein in its GDP bound form.
Figure 2. Regulation of small GTPase protein activity. This illustration shows the mechanism of small G-protein cycling and the factors responsible to catalyze different parts of the activation cycle. Reprinted with permission from The American Physiological Society; Physiological Reviews.

1.2.2 Rab family

The Rab GTPase family, the largest branch of the superfamily, is central to intracellular membrane trafficking processes. These processes include exocytosis, endocytosis, and recycling of proteins, macromolecules, and foreign microbes [40]. Transport of these cellular loads occurs from one membrane compartment to another via vesicles. It is the events of vesicle transport that the Rab family controls, including targeting, docking, and fusion processes between the vesicle and the acceptor membrane [40, 42]. Furthermore, vesicle trafficking plays a role in various other functions, including cell polarity, cytokinesis, and cell motility. Another important aspect of Rab function is its localization within the cell. A unique, hypervariable carboxyl terminal domain containing two cysteine residues is post-translationally modified by the attachment of geranylgeranyl moieties in order for correct translocation of Rab proteins to target
membranes\[43,44\]. Ultimately, it is unsurprising that several intracellular pathogens would target host Rabs in order to disrupt vital trafficking processes aimed to destroy the invading microbe. Indeed, Rab modulation on phagosomes by intracellular pathogens has already been shown in Mtb, Coxiella, Helicobacter, Salmonella, and Legionella\[45,46\].

1.2.3 Rho family

Rho, Rac and Cdc42 are three of the most well studied members of the Rho family, which primarily regulate cytoskeletal reorganization. Like Rab proteins, the Rho family are regulated similarly by GEFs and GAPs, and its localization is also affected by lipid modifications\[40\]. Control of the actin cytoskeleton is crucial in cellular functions such as cell shape, motility, adhesion, and cytokinesis. Specifically, Rho proteins regulate stress fiber formation\[40\], while Rac proteins regulate ruffling and lamellipodia formation\[40\], and Cdc42 regulates filopodium formation\[40\]. Together, they are required for many actin cytoskeleton-dependent cellular processes mentioned above. In addition to this, rearrangement of actin filaments is directly responsible for the formation of the phagocytic cup in uptake of particles by professional phagocytes\[47\]. Furthermore, Rac has also been demonstrated to be an essential component of the NADPH oxidase complex, where it functions together with other subunits to play a key role in production of reactive oxygen species (ROS) during pathogen invasion\[48-51\]. Indeed, some pathogens such as Salmonella and Yersinia have already been demonstrated to inhibit this host response\[52-55\], while other pathogens have been shown to steer the cell into phagocytic uptake via a specific pathway to remain hidden to the host\[47,56\]. Thus, it can be seen that the Rho family plays an integral role in the context of cellular functions during pathogen invasion.
1.3 Host pathogen interactions

1.3.1 Phagosome maturation arrest

The ability of Mtb to persist and replicate within the host macrophage is a central factor in the development of TB disease \[^{57}\]. Intracellular survival of Mtb is aided by a combination of factors including a unique cell wall structure, which physically shields the bacterium from bactericidal and hydrolytic enzymes \[^{58}\], and secretion of enzymes to combat host reactive oxygen and nitrogen radicals \[^{59, 60}\]. Although all these factors contribute to Mtb persistence within the macrophage, one recurring and highly important feature of this pathogen is inhibition of normal phagosome maturation process, thereby abrogating physical fusion of phagosomes with lysosomes and ultimately protecting the bacterium from a bactericidal environment \[^{21, 61, 62}\].

Phagosome biogenesis is characterized by a rapid and sequential fusion of vacuoles containing ingested pathogens with various endosomal compartments leading to acidification dependent on recruitment of the vacuolar proton ATPase subunits \[^{62}\]. Thereafter, the acquisition of acidic lysosomal enzymes by the phagosome and their activation results in efficient killing and degradation of invading pathogens \[^{63}\] following which the macrophage can initiate antigen presentation to enable the proper detection of pathogen antigens by effectors of the adaptive immune response \[^{63, 64}\].

Rab GTPases play a major role in the control of normal phagosome biogenesis. Normally, phagosome biogenesis is initiated by fusion with endosomes coated with the small GTPase, Rab5. This step is essential for recruitment of the early endosome antigen 1 (EEA1), which drives the phagosome towards further maturation \[^{65}\]. However, this early maturation event is disrupted by Mtb and the closely related vaccine strain \(M. bovis\) BCG, both of which exclude EEA1 from their phagosomes \[^{66}\]. As the phagosome matures into more advanced stages,
another prominent member of late phagosome markers, the GTPase Rab7 is recruited and serves as a docking site for RILP (Rab7-interacting lysosomal protein) (summarized in Fig. 3). RILP possesses two distinct domains: one that binds to the GTP-bound form of Rab7 and another that recruits the dynein/dynactin complex \cite{67, 68}. By simultaneously associating with both targets, RILP promotes the interaction of vesicles bearing active Rab7 with lysosomes \cite{67}. 
Figure 3. A model of the role of Rab7-RILP in phagolysosome fusion. This figure illustrates the mechanism of interaction between Rab7 and RILP which leads to phagolysosome formation. MT: microtubule; MTOC: microtubule organization center. Reprinted with permission from the American Society for Microbiology; Molecular and Cellular Biology.

In this context, several mycobacterial factors have been demonstrated to interfere with the host network of Rabs, leading to attenuation of phagosome maturation (summarized in Fig. 4). For instance, the mycobacterial glycolipid ManLAM, together with the lipid phosphatase SapM inhibit proper generation of essential PI(3)P, which is controlled by Rab5 on the phagosome membrane, necessary for docking and fusion of downstream Rabs \[^{25,69}\]. In the late stage phagolysosome fusion events, Mtb protein tyrosine phosphatase (PtpA) dephosphorylates host vacuolar sorting protein VPS33B (a component of the complex responsible for activating Rab7) \[^{70}\], contributing to a significant decrease in phagolysosome fusion events that correlated
with enhanced survival of Mtb in human macrophages [70]. In addition, PtpA has further been demonstrated to inhibit phagosome acidification through interaction with subunit H of the macrophage vacuolar H⁺-ATPase machinery, leading to a defect in intracellular killing of Mtb [22]. Collectively, emerging evidence clearly show a critical role for mycobacterial virulence factors which hijack the Rab signaling network.

**Figure 4. Mycobacterial manipulation of intracellular trafficking.** This schematic illustrates a model of intracellular Mtb interaction with host GTPases Rab5 and Rab7. Potential mycobacterial virulence factors are labelled in red. Reprinted with permission from John Wiley and Sons; Cellular Microbiology.

1.3.2 NADPH oxidase mediated killing

The phagocyte NADPH oxidase (NOX2) complex consists of two constitutively associated transmembrane proteins, gp91phox and gp22phox and four cytosolic subunits: p40phox, p47phox, p67phox, and Rac1, a small GTPase (summarized in Fig. 5) [71, 72]. Fully functional NOX2 requires membrane translocation of p40phox, p47phox, active Rac1 (GTP-bound form) and p67phox,
and their assembly around \( \text{gp91}^{\text{phox}} \) and \( \text{gp22}^{\text{phox}} \) subunits \cite{71, 72}. NOX2 assembly leads to \( \text{gp91}^{\text{phox}} \) activation to generate superoxide through a redox chain by transferring electrons from cytosolic NADPH to phagosomal oxygen \cite{71}. The production of superoxide is in turn converted into several other microbicidal molecules, such as hydrogen peroxide and hydroxyl radicals, along with peroxynitrite when combined with nitric oxide radicals \cite{71}.

**Figure 5. NADPH oxidase assembly.** This figure illustrates the subunits of the phagocyte oxidase and how they interact to form a functioning complex responsible for ROS production. Reprinted with permission from Nature Publishing Group; Cell Death & Disease.

Reactive oxygen species (ROS) produced by NOX2 were classified 30 years ago as powerful microbicidal agents against many intracellular pathogens \cite{73, 74}. *In vivo* evidence for the contribution of NOX2 to the innate immunity arsenal was deduced from field observations of high susceptibility of chronic granulomatous disease (CGD) patients to opportunistic pathogens \cite{75, 76}. Such observations were experimentally confirmed in mouse models of CGD \cite{77, 78}. Recent years have seen a growing body of evidence to suggest a crucial role for ROS in the control of mycobacterial infection \cite{77}. In particular, one group has recently identified Mtb \textit{nuoG}
as a potential virulence factor operating at the level of NOX2 by mechanisms yet to be defined \cite{79}. Mtb also possesses several resistance mechanisms to detoxify ROS, further cementing the role of phagocytes respiratory burst in antimicrobial defense against mycobacteria. For instance, Mtb catalase KatG mediates decomposition of hydrogen peroxide into water and oxygen \cite{80}. It was demonstrated that Mtb lacking \textit{katG} failed to grow in wild type macrophages, but grew normally in macrophages from NOX2 deficient mice, and this virulence was confirmed in the mouse model \cite{16}. However, a separate study showed that KatG was dispensable for the survival and growth of \textit{M. intracellulare} in the mouse despite mediating resistance to hydrogen peroxide \cite{81}. Consistent with this, the growth defect of Mtb\textit{ΔkatG} was only observed between 2 to 4 weeks after infection in the mouse and beyond 4 weeks, the growth was comparable to wild type Mtb \cite{80}. Possible explanations for this are that there are additional factors which are involved in the protection against the oxidative burst or simply that the oxidative burst alone is not enough to fully eradicate the bacteria. An additional function of KatG within the mycobacterium is activation of the tuberculosis drug isoniazid, which disrupts mycolic acid synthesis required for the mycobacterial cell wall \cite{82}. This was first observed from clinical strains which acquired resistance to isoniazid coupled with a loss of catalase-peroxidase activity \cite{83}. Although paradoxical, this may explain the observed virulence by KatG knockout strains in the chronic phase of mouse infections as well as natural knockout strains from clinical isolates. One explanation for the paradoxical requirement of KatG in virulence is that loss of the gene is accompanied by increased expression of an alkyl hydroperoxide reductase, AhpC, which can compensate for the detoxifying role of KatG \cite{84}. Additional antioxidant mechanisms employed by Mtb include the superoxide dismutase genes (\textit{sodA} and \textit{sodC}), where they catalyze the conversion of superoxide anions to hydrogen peroxide \cite{16}. While the role of NOX2 in innate immunity is well established, recent reports suggest that it might act beyond the control of
intracellular infections to trigger macrophage apoptosis \cite{85, 86}, a central event that paves the road to adaptive immunity \cite{87-89}. Indeed, a connection has been established with ROS production and apoptosis \cite{90-92}. Relevant to this is the documented ability of Mtb to manipulate macrophage apoptosis \cite{26, 27, 93}, and instead promote macrophage necrosis and cellular lysis leading ultimately to the spread of the infection \cite{28, 94}. This phenotype has been recently contrasted by the identification of attenuated Mtb mutants that induce apoptosis instead of necrosis \cite{79, 87}.

1.4 Aims of study

The overall objective of this thesis is to characterize the mechanism of mycobacterial persistence within the phagosome through identification of potential virulence factors which interfere with various aspects of the innate immune response. Specifically, the initial goal was to probe whether mycobacteria interfered with late phagolysosome fusion events governed by the small GTPase Rab7 and its downstream effector RILP. Later, once the connection between mycobacteria mediated Rab7 disruption and phagolysosome fusion arrest had been made, the aim was to identify the potential virulence factor responsible and elucidate the exact mechanism of action. Based on preliminary work investigating this link, it was found that a mycobacterial protein involved with nucleotide metabolism, nucleoside diphosphate kinase (Ndk) had the potential to act as a virulence factor in the context of phagosome modulation. Ndk exhibits autophosphorylation and phosphotransfer capabilities which assist to balance intracellular nucleotide pools within mycobacteria \cite{95}. Furthermore, it has been previously demonstrated that Ndk possesses GAP activity \textit{in vitro} for Rho-GTPases \cite{96}. Thus, the working hypothesis for this project is: Mtb Ndk interferes with pathways of the innate immune response to Mtb infection through modulation of host small G-proteins. In order to investigate this hypothesis, mycobacterial strains with knocked down Ndk expression were used to assess for various aspects of phagolysosome fusion and intracellular survival. Combined with a recombinant protein
coated latex bead model of infection, I was able to examine the mechanism of Mtb persistence in the phagosome that would be directly mediated by Ndk. Overall, the study has provided significant insight into the mechanism of innate immune response evasion by Mtb. The complete elucidation of the mechanism underlying Ndk-mediated virulence in Mtb will further enhance our knowledge towards the complete story of host-pathogen interaction, and perhaps lead to new therapeutics to combat this global disease.
CHAPTER 2: MATERIALS AND METHODS

2.1 Reagents and chemicals

2.1.1 Commercial reagents

Endotoxin-free mammalian cell culture reagents were from StemCell Technologies (Vancouver, British Columbia, Canada). Protease inhibitor mixture, PMSF, trypsin-EDTA, and glutathione-agarose beads were purchased from Sigma-Aldrich (St. Louis, MO). Protein A-agarose beads were from Bio-Rad laboratories (Hercules, CA). TALON polyhistidine-Tag purification resin was purchased from Clontech (Mountain View, CA). DMEM, Fetal calf serum (FCS), OPTI-MEM and HBSS were purchased from Gibco Laboratories (Burlington, Ontario, Canada). Lipofectamine 2000, Dextran (Texas Red, Cascade Blue and fluorescein; 10,000 MW), Luminol, CM-DCFDA, Annexin V-488, and CellMask™ Deep Red were obtained from Invitrogen (Burlington, Ontario, Canada). Aldehyde/sulfate latex beads (diameter, 4 µm) were obtained from Interfacial Dynamics (Portland, OR). [γ-32P]-Guanosine 5’-triphosphate, was purchased from Perkin Elmer (Boston, MA). Peptide inhibitor to gp91phox and its scrambled version were synthesized by GenScript (Piscataway, NJ). Restriction enzymes, Calf intestine alkaline phosphatase (CIAP), Taq DNA polymerase, and T4 DNA ligase were purchased from Fermentas (Burlington, ON, Canada). Gateway recombination vector conversion system reagents were purchased from Invitrogen. Luria-Bertani (LB) broth and LB agar were from Fisher Scientific (Pittsburgh, PA). Oleic acid, dextrose and catalase complex (OADC) and 7H9 and 7H10 agar culture media were from Difco Laboratories (Detroit, MI). Chloramphenicol was purchased from Sigma-Aldrich and kanamycin, hygromycin and zeocin were from Invitrogen. MinElute PCR purification and plasmid purification Mini and Midi Kits were from Qiagen (Mississauga, ON, Canada).
2.1.2 Antibodies

Rabbit Rab5 and Rab7 antibodies, mouse monoclonal antibody to Glutathione-S-Transferase (GST), and rabbit or mouse anti-GFP were purchased from Sigma-Aldrich. Rabbit RILP antibody was described previously \cite{98}. Mouse 6x histidine antibody was purchased from Genscript. Goat cathepsin S and rabbit 47\textsuperscript{phox} antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and was purchased from Sigma-Aldrich. Rac1, RhoA, Cdc42 antibodies were purchased from Millipore (Temecula, CA). p67\textsuperscript{phox} antibody was purchased from BD Transduction Laboratories (Mississauga, ON, Canada). Rabbit Ndk antibody was generated by GenScript, using KLH conjugated ELASQHYAEHEGK peptide fragment corresponding to amino acids 44 to 56 of Mtb Ndk. Rabbit cleaved caspase-3 (Asp175) antibody was purchased from Cell Signaling (Danvers, MA). Mouse Ndk antibodies were generated by injection of full-length recombinant Mtb Ndk (100 µg) solubilized in 150 µl Imject Alum (Pierce, Rockford, IL) adjuvant in FVB mice. Thereafter, the mice were boosted twice with 50 µg Ndk-Alum mixture after intervals of 14 days. Ten days after final injection, cardiac puncture was performed, and the titer of Ndk antiserum was determined by ELISA. The animal husbandry and immunization protocol were approved by the Animal Care Office at the University of British Columbia (Certificate number: A08-0873). Secondary peroxidase conjugated antibodies, monoclonal anti-rabbit IgG native-peroxidase, and FITC-conjugated anti-rabbit and anti-mouse IgG were purchased from Sigma-Aldrich. Alexa Fluor 647-conjugated anti-rabbit IgG was purchased from Invitrogen.

2.2 Bacteria

2.2.1 Strain maintenance and generation

\textit{M. bovis} BCG (Pasteur 1173P2), \textit{M. tuberculosis} H37Rv and their derivative strains were grown in Middlebrook 7H9 broth (BD Diagnostic Systems, Mississauga, ON, Canada)
supplemented with 10% (v/v) OADC (oleic acid, albumin and dextrose solution; BD Diagnostic Systems) and 0.05% (v/v) Tween 80 (Sigma-Aldrich) at 37°C on a rotating platform (50 rpm) for BCG, and standing culture for Mtb.

Mtb Ndk-AS and Ndk-S were generated using our integrative pJAK1.A plasmid (selection marker, kanamycin) \[^{99}\] encoding the full length \textit{ndkA} gene in sense and anti-sense orientations as described \[^{20,99}\]. To generate red-fluorescent bacteria, Ndk-S, Ndk-AS, and wild type BCG or Mtb strains were transformed with pSMT3 vector (a gift from Dr. M. Abdallah, VU Medical Centre, Amsterdam, the Netherlands) encoding the DsRed protein under the control of the Hsp60 promoter (pSMT3-DsRed). Killed bacteria were prepared by 2 h incubation at 37°C in the presence of 50 µg/ml gentamicin. \textit{Salmonella enterica} serovar \textit{typhimurium} (SL 1344) and the corresponding sifA mutant were provided by Dr. Brett Finlay (University of British Columbia, Vancouver, B.C.) and grown as described previously \[^{100}\].

2.2.2 Bacterial preparation for infection

For macrophage infection, bacteria in mid-log phase were harvested by 5 min centrifugation at 14,000 \(x\ g\). Where necessary, bacteria were stained by FITC or Texas Red (Sigma-Aldrich) at a final concentration of 10 µg/ml at 37°C for 1 h. They were subsequently washed three times with 7H9 plus 0.05% Tween 80 and passed through 25 gauge needles several times prior to infection.

2.2.3 Mycobacterial lysis

Mycobacterial lysates were prepared by resuspending cell pellets in \(350 \mu l\) of 50 mM Tris, 5 mM EDTA, 0.6% SDS, 0.05% NaN\(_3\), 1 mM PMSF. The cells were then mixed with 100 mg of glass beads and shaken in a bead beater for 15 second intervals for 10 times. Thereafter, lysates were separated from insoluble fractions and cell debris by centrifugation at 12,000 \(x\ g\) at 4°C for 30 min.
2.2.4 Mycobacteria transformation

Competent cells were prepared using Mtb grown to an OD$_{600}$ between 0.6 to 0.8. Bacteria were washed three times with 10% glycerol with 0.05% Tween 80, and finally resuspended in $1/10^{th}$ the original culture volume with the same wash buffer. Competent cells (400 µl) were then mixed with 1 µg of DNA and transferred to an electroporation cuvette of 0.2 cm diameter (Bio-Rad, Hercules, CA). Cells were electroporated with 2.5V, and allowed to recover in 7H9 supplemented with 10% OADC in the absence of antibiotics for 2 h or overnight for *M. smegmatis* and Mtb/BCG respectively. Cells were then plated on 7H10-OADC in the presence of appropriate antibiotic.

2.3 Cell culture and transfection

J774A.1 and RAW 264.7 murine macrophage cell lines (American Type Culture Collection, Manassas, VA) were maintained in 10 cm diameter culture dishes (Corning Inc., Corning, NY) at a density of ~ $10^5$ per cm$^2$ in DMEM containing 5% FCS and 1% each of L-glutamine, HEPES, non-essential amino acids (100 X solution, StemCell). Bone marrow derived macrophages (BMDM) were obtained by flushing out femurs and tibias of 6-8 week old female C57BL/6 (Jackson Laboratory, Sacramento, CA) according to protocols approved by the University of British Columbia Animal Care and Use Committees. Cells were then maintained in complete DMEM containing 10 ng/mL M-CSF for 6 days.

Prior to transfection, RAW macrophages were washed extensively and harvested by scraping. Cells were seeded at a density of 80-90% confluency on culture plates of varying size, and allowed to adhere overnight. Cells were then transfected with the various constructs using the Lipofectamine 2000 reagent (Invitrogen) as per manufacturer’s recommendation. Twenty-four h post-transfection, cells were washed and treated as required based on the experiment.
2.4 Vectors and DNA manipulation

2.4.1 Vector constructs

Plasmid vector expressing recombinant GST fusion protein to RILP, Rab7wt, Rab7Q67L (constitutively active) or Rab7T22N (dominant negative) were previously described [98, 101]. Plasmid vector expressing Rab7 and Rab5a in his-tag expression vector pET16b were previously described [102, 103]. Vectors for mammalian cell transfection: Rab5-GFP, Rab7wt-GFP, Rab7Q67L-GFP, Rab7T22N-GFP, GFP-EEA1 and GFP-2xFYVE were generated as previously described [98, 104, 105]. RILP-DsRed plasmid was provided by Dr. Brett Finlay. Ndk-DsRed plasmid was generated by inserting PCR-amplified ndkA between the XhoI and HindIII sites of pDsRed2-N1 (Clontech).

Mtb Rv2445c (ndkA) was amplified from genomic DNA using the forward primer, TTG GGC CAT ATG ACC GAA CGG ACT CTG, containing an NdeI site, and reverse primer CAC CCG AAG CTT GCC GGG AAA CCA, containing a HindIII site. *M. smegmatis* ndkA was amplified from its genomic DNA using the forward primer TTG GGC CAT ATG ACT GAG CGG ACC CTC and the reverse primer GAA TTG AAG CTT GCC GGT GGC CTC GCC GGG, containing a NdeI site and HindIII site, respectively. The amplified genes were inserted into pET22b vector using the same restriction sites to generate a C-terminal his-tag fusion protein. Human rilp gene was amplified from pGEX-4T3-RILP [98], using the forward primer TTT CAT ATG GAG CCC AGG AGG GCG GCG, containing a NdeI site, and the reverse primer TTT AAG CTT GGC CTC TGG GGC GGC TGA, containing a HindIII site. The amplified insert was cloned into pET22b for His-tag expression. All plasmid constructs were subsequently verified by sequencing (Macrogen Co, South Korea).
2.4.2 Gateway cloning

Construction of mycobacterial destination vectors. pMV261 and pMV361 vectors (gift from Dr. William Jacobs Jr., Albert Einstein College of Medicine, Bronx, NY) were linearized by the blunt cut restriction enzyme PvuII within their multi-cloning sites. The vectors were then treated with CIAP for 30 min at 37°C, followed by inactivation at 85°C for 15 min to dephosphorylate the 5’ end in order to prevent self-ligation. The linearized vectors were then ligated with the appropriate blunt reading frame cassette (attR1-cam-ccdB-attR2) with T4 DNA ligase overnight at 16°C. The ligated product was then transformed into ccdB Survival T1 E. coli, and positive clones in the correct orientation of the cassette were screened by PCR using specific primers.

PCR. Pfx50 DNA polymerase and PCR primers were obtained from Invitrogen and were used according to the manufacturers' instructions. Sources of DNA templates and primer sequences are shown in Table 1. Thermocycling conditions were as follows: 3 min at 94°C for initial denaturation of the DNA, 30 cycles of 1 min at each of the following temperatures: 94°C (denaturing), 55-58°C (annealing), 72°C (extending), followed by 10 min at 72°C for a final extension.

Table 1. Primer list used in Gateway cloning study.
**Clonases.** BP Clonase, containing Int and IHF, catalyzes the integrative (BP) reaction \((attB\times attP \rightarrow attL+attR)\). LR Clonase, containing Xis, Int, and IHF, catalyzes the excisive (LR) reaction \((attL\times attR \rightarrow attB+attP)\). Both clonases and other recombinational cloning materials are available from Invitrogen, as part of the Gateway Cloning System.

**BP reactions for cloning PCR products.** The \(attP\) Entry plasmid pDONR221/Zeo (150 ng) was mixed with 1-3 µl of each purified PCR product in reactions (10 µl) that contained 2 µl BP Clonase in 25 mM Tris HCl at pH 7.5, 22 mM NaCl, 5 mM EDTA, 5 mM spermidine HCl, 1 mg/mL BSA. After 2 h incubation at 25°C, proteinase K (2 µg in 1 µl) was added, and each reaction was incubated at 37°C for 10 min. Aliquots (2 µl) of each reaction were transformed into *E. coli* TOP10 and plated on zeocin LB plates (50 µg/mL). Positive clones were screened by colony PCR and the subsequent mini-prepped DNA were sequenced by Macrogen Co (South Korea).

**LR Reactions for subcloning in destination vectors.** Aliquots containing 150 ng of each miniprep DNA of Entry clones were incubated with 150 ng of the appropriate Destination Vector in 10 µl reactions containing 2 µl of LR Clonase, 50 mM of Tris HCl at pH 7.5, 50 mM of NaCl, 0.25 mM of EDTA, 2.5 mM of spermidine HCl, and 0.2 mg/mL of BSA. Then proteinase K (2 µg in 1 µl) was added, and reactions were incubated at 37°C for 10 min. Aliquots (2 µl) of each reaction were transformed into *E. coli* TOP10 and plated on kanamycin (50 µg/mL) LB plates. Positive clones were screened by colony PCR and sequenced by Macrogen Co.

**2.4.3 Antisense mRNA knock-down**

*Mtb ndkA* gene was PCR amplified using the forward primer CCG AAG CTT GTG ACC GAA CGG ACT CTG GTA, and reverse primer CCG AAG CTT CTA GGC GCC GGG AAA CCA GAG, both using the restriction site *HindIII*. The 411 bp insert was cloned into pMV261
cut at *Hind*III of the multicloning site. Prior to ligation, the pMV261 was treated with CIAP to remove the 5’ phosphate and prevent self-ligation. Positive clones were then checked by digestion with *Pvu*II. Clones with *ndkA* inserted in the sense orientation would give a fragment of 4800 bp and 60 bp, while clones of *ndkA* inserted in the anti-sense orientation would give fragment sizes of 4400 bp and 360 bp. The plasmids were then electroporated into competent BCG and plated on 7H10 media supplemented with OADC and containing 25 µg/ml kanamycin.

To generate stable antisense expressing vector, the converted mycobacterial destination vector pJAK1.A (derivative of integrative pMV361) was used to clone in full length Mtb *ndkA* gene in sense and antisense directions by use of *attB* adapters. Thereafter, the plasmids were transformed into competent Mtb H37Rv to generate Mtb Ndk-AS (antisense, Ndk knockdown) and Mtb Ndk-S (sense, control).

### 2.5 Protein expression and purification

#### 2.5.1 Expression of recombinant proteins in *E. coli*

GST fusion recombinant proteins were expressed by transformation of plasmids into *E. coli* strain BL21 and grown to an OD<sub>600</sub> of 0.6 at 37°C and expression was induced with 0.2 mM IPTG at 22°C overnight. After centrifugation, bacteria were resuspended in PBS containing 1 mM DTT, 0.1 mM PMSF, 1 mg/ml lysozyme for 30 min and lysed by sonication. Bacterial lysates were clarified by high-speed centrifugation and then purified on glutathione-agarose resin (Sigma-Aldrich). Fusion proteins were eluted by 10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.5.

To generate 6x-His tagged recombinant protein, plasmids were transformed into *E. coli* strain BL21 and grown to an OD<sub>600</sub> of 0.8 at 37°C and expression was induced with 0.2 mM IPTG at 22°C overnight. After centrifugation, bacteria were resuspended in PBS containing 1 mM PMSF, 1 mg/ml lysozyme for 30 min and lysed by sonication. Bacterial lysates were
clarified by high-speed centrifugation and then purified on TALON polyhistidine-Tag purification resin. Fusion proteins were eluted in 250 mM imidazole.

2.5.2 Expression of recombinant proteins in M. smegmatis

Miniprep DNAs of expression clones in pJAK2.D (6x-His amino fusion) were transformed into competent M. smegmatis by electroporation and plated at 37°C on 7H10 plates containing 30 µg/mL kanamycin. Positive colonies were transferred into 7H9 media containing 0.05% Tween 80, 30 µg/mL kanamycin and supplemented with 2% OADC. After growth at 37°C to an OD₆₀₀ of 1.0, the cells were spun-down at 5000 x g for 10 min and resuspended in minimal 7H9 Tween media supplemented with kanamycin and 0.2% acetamide and expression was continued at 25°C for 24 h. Bacteria were harvested by centrifugation at 5000 x g for 10 min and lysed by sonication in 50 mM sodium phosphate, 300 mM NaCl, pH 7, in the presence of 1 mg/mL lysozyme and 1 mM PMSF. Protein purification was carried out by affinity chromatography using TALON polyhistidine-Tag purification resin (Clontech, Mountain View, CA) according to the manufacturer’s recommendations.

2.6 Immunoprecipitation assays

Transfected and/or infected macrophages were lysed in extraction buffer (25 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100, 0.5% NP-40, 0.2 mM PMSF and protease inhibitor cocktail) for 20 min at 4°C and debris were removed by high speed centrifugation. Soluble lysates were mixed with irrelevant or specific antibody to the protein to be pulled-down (1:100) and incubated for 2 h at 4°C. Pre-blocked protein A-agarose beads with 3% BSA were added to the mixtures and the samples were incubated for additional 30 min at 4°C. Agarose beads were washed extensively and protein complexes were solubilized in 2 X Laemmeli buffer and subjected to SDS-PAGE and Western blotting with specific antibody to detect associated protein to the immunoprecipitate.
2.7 Small GTPase activation assay

2.7.1 Rab activation assay

The lack of commercial availability of a Rab GTPase activation assay kit prompted the need to devise an efficient and quantitative method to assess the amount of active Rab (GTP bound) proteins from infected macrophages. This culminated in the publication of an article in *Methods Mol Biol.* 531:57-69, titled Detection of Activated Rab7 GTPase with an Immobilized RILP Probe. In this article, we describe a novel, alternative, nonradioactive assay to measure Rab7 activity which takes advantage of the specific binding of activated Rab7 to its effector RILP. Active Rab7 bound to immobilized recombinant RILP on latex beads can be detected quantitatively by either classical Western blotting or flow cytometry.

2.7.2 Rho/Rac/Cdc42 activation assay

Confluent RAW cells seeded on 6 cm plates were infected by Mtb strains for 1 h at a MOI of 20:1. Thereafter, cells were incubated in the presence of 200 ng/ml LPS for 15 min to induce Rho GTPase activation. Subsequently, cells were lysed in cold buffer containing 30 mM HEPES (pH 7.2), 100 mM NaCl, 10% glycerol, 1% Triton X100, 1 mM EDTA, 10 mM MgCl$_2$, and 1 mM PMSF. Soluble protein fractions were analyzed for levels of active Rho-GTPases by using a Rac/Rho/Cdc42 activation assay kit (Millipore).

2.8 GTPase activating protein activity assay

**Dot Blot method.** Rab5 or Rab7 (1 µg) were loaded with [$\gamma$-32P]GTP by incubation with 10 µCi of [$\gamma$-32P]GTP in 100 µl of reaction buffer (50 mM HEPES at pH 7.4, 50 mM NaCl, 0.1 mM DTT, 5 mM EDTA and 1 mg/ml BSA) for 10 min at 37°C. 10 mM MgCl$_2$ was then added (to terminate the reaction) and incubated on ice for 10 min. Thereafter, 5 µl of each reaction mixture were spotted onto nitrocellulose membrane. Unbound material was removed with extensive washing with cold wash buffer (25 mM HEPES at pH 7.4, 50 mM NaCl, 1 mM
MgCl₂, 1 mM DTT) and membrane squares were then incubated in the presence or absence of 10 µg/ml Mtb or *M. smegmatis* Ndk for 2 h at room temperature. After 3 washes with cold wash buffer, membrane-associated radioactivity was determined by autoradiography and quantification was done by measuring membrane counts in a scintillation counter.

**SDS-PAGE method.** Rab7-GST was resolved by 12% SDS-PAGE and transferred to nitrocellulose membrane. Thereafter, membranes were incubated in renaturation buffer (50 mM HEPES at pH 7.2, 5 mM MgAc, 100 mM KAc, 3 mM DTT, 10 mg/ml BSA, 0.1% Triton X-100, 0.3% Tween 20) overnight at 4°C with gentle agitation. Membrane strips were then loaded with [γ-³²P]GTP by incubation with 10 µCi of [γ-³²P]GTP in 100 µl of reaction buffer (50 mM HEPES at pH 7.4, 50 mM NaCl, 0.1 mM DTT, 5 mM EDTA and 1 mg/ml BSA) for 10 min at 37°C. Unbound radioactivity was then washed extensively with cold reaction buffer and membrane strips were then incubated in the presence of bacterial culture supernatants and protease inhibitor cocktails for 4 h at room temperature. After 3 washes with cold buffer, membrane-associated radioactivity was determined by autoradiography.

**2.9 Coating of latex microspheres with proteins**

Latex beads were coated with proteins according to manufacturer’s protocol (Invitrogen). In brief, 10⁸ beads were washed twice with 25 mM MES buffer (pH 5.8) and resuspended in 500 µl of the same buffer containing 250 µg/ml of protein. After overnight incubation at room temperature on a shaker, latex beads were washed 3 times with PBS and resuspended in 1 ml of PBS containing 1% BSA. Based on the difference between protein concentration of the coating solution before and after incubation with beads, the coating was estimated to be about 0.2-0.3 pg protein per bead. Protein coating was also routinely verified prior to each experiment by immunostaining with specific antibodies and FACS analysis.

To generate red fluorescent beads, BSA-beads were labelled with the PKH26 red
Fluorescence linker kit (Sigma-Aldrich). In brief, beads were incubated in PKH solution (1:500) for 10 min at 37°C. Beads were then washed three times and resuspended in PBS.

2.10 Digital confocal microscopy

2.10.1 Intracellular staining.

Immunofluorescence staining was performed on cells seeded on coverslips. Treated or infected cells were first fixed in 2.5% paraformaldehyde in PBS for 20 min at 37°C, followed by extensive washes with PBS. Subsequently, cells were permeabilized in PBS buffer containing 0.2% saponin and 1% normal goat serum (permeabilization buffer) for 15 min at room temperature. Specific antibody to the protein of interest was then used at 10 µg/ml in permeabilization buffer to stain the cells for 20 min at room temperature. Thereafter, cells were washed three times with PBS and incubated in either secondary FITC or Texas Red-conjugated antibody for 20 min. Cells were then washed 4 times with PBS and mounted for fluorescent microscopy analysis.

2.10.2 Microscopy

Transfected or immunostained cells adherent to coverslips were mounted on microscope slides in FluorSave™ (Calbiochem-Novabiochem, La Jolla, CA) to minimize photobleaching. Slides were then examined by digital confocal microscopy using an Axioplan II epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY) equipped with 63x/1.4 Plan-Apochromat objective (Carl Zeiss Inc). Images were recorded using a CCD digital camera (Retiga EX, QImaging, Burnaby, BC, Canada) coupled to the Northern Eclipse software (Empix Imaging, Inc., Mississauga, ON, Canada).
2.11 ROS detection assay

Macrophages were cultured in complete DMEM in 96 well white plates (Corning) at $10^5$ per well. Prior to ROS assay, cell media was replaced with DMEM without phenol red and luminol was added to a final concentration of 50 $\mu$M. Wells were then infected with Mtb strains or coated beads. Thereafter, plates were loaded into a Tropix TR717 microplate luminometer (Applied Biosystems, Bedford, MA) adjusted to 37°C and relative luminescence was then measured at 60 sec intervals over 60 min. Intracellular detection of ROS was achieved by incubating adherent macrophages to cover slips with 10 $\mu$M CM-DCFDA for 30 min at 37°C prior to infection with Mtb strains expressing DsRed. Cells were then analyzed by confocal microscopy.

2.12 Apoptosis assay

Adherent RAW cells on coverslips were infected with Mtb strains. At 48 h post phagocytosis, cells were washed twice with cold PBS and then incubated in Alexa Fluor 488 Annexin V (1:20, Invitrogen) in staining buffer containing 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl$_2$, for 20 min at room temperature. Coverslips were then analyzed by confocal microscopy. Alternatively, infected cells were scraped and fixed in PBS plus 2% paraformaldehyde for 15 min at room temperature. Cells were then washed with PBS and incubated with anti-cleaved caspase-3 (1:250) in permeabilization buffer (0.1% Triton X100 and 1% BSA in PBS) for 20 min at room temperature. Thereafter, cells were washed and stained with Alexa Fluor 647-conjugated goat anti-rabbit IgG (1:200) for 20 min at room temperature, washed again and analyzed by FACS.
2.13 Mycobacterial survival assays

2.13.1 Macrophage intracellular survival

RAW macrophages or BMDM were infected with BCG or Mtb strains at an MOI of 10:1. Cells were washed thrice 2 h post infection to remove extracellular bacteria. Thereafter, cells were lysed in 0.025% SDS at the specific post-infection time points (24-72 h). Serial dilutions of recovered Mtb were then plated on solid 7H10 media supplemented with 10% OADC and appropriate antibiotics where necessary, and CFU counts were performed after 3 weeks incubation at 37°C.

2.13.2 Animal studies

C57BL/6 SCID mice (4- to 6-week old females, Jackson Laboratory) were injected subcutaneously in the scruff of the neck with $10^6$ of each of the indicated Mtb strains and then monitored for survival. All animals were maintained in accordance with protocols approved by the Colorado State University Animal Care and Use Committees.
CHAPTER 3: RESULTS

3.1 BCG mediated inactivation of host Rab7 leads to arrest of phagolysosome fusion

3.1.1 The Rab7-RILP interaction in macrophages

To demonstrate the presence of the Rab7-RILP interaction system in macrophages, we co-transfected the RAW 264.7 macrophage cell line with GFP-tagged Rab7wt (wild type form) or Rab7T22N (inactive form) and DsRed-tagged RILP constructs and when cells expressed detectable signal for both proteins, they were exposed to latex beads to induce phagosome biogenesis. At 4 h post-phagocytosis, samples were fixed and examined by digital confocal microscopy. Consistent with data previously obtained with epithelial cell system models [98], the images shown in Fig. 6 clearly demonstrated a recruitment of the lysosomal protein RILP to latex bead phagosomes, which uniformly co-localized with phagosomes in Rab7wt-transfected cells. In contrast, in cells transfected with Rab7T22N, RILP did not reach the phagosome despite a significant phagosomal surrounding with inactive Rab7 (Fig. 6). Thus, although recruitment of Rab7 to the phagosomal membrane is independent of its activation, RILP recruitment implies that Rab7 is in the active, GTP-bound form as shown earlier in transfected HeLa cells [98, 106].
Figure 6. Rab7-RILP interaction in macrophages. A. RAW cells were co-transfected with RILP-DsRed and Rab7-GFP or Rab7T22N-GFP as described in Materials and Methods then exposed to 4 μm latex beads for 1 h at 37°C. Partially attached, non-ingested beads were removed by a 5 min treatment with trypsin-EDTA and extensive washing with HBSS and cells were replenished with culture medium and cultured at 37°C. At 4 h post-phagocytosis, cells were fixed and analyzed by digital confocal microscopy. The yellow signal corresponds to colocalization of Rab7 (green fluorescence) with RILP-DsRed (red fluorescence). B. Quantification of the confocal data shown in A. Values are the mean ± SD of positive phagosomes in 50-80 cells from three independent experiments.

3.1.2 Live BCG disrupts the intracellular Rab7-RILP interaction

The data presented above demonstrated that the Rab7-RILP interaction is contributing to the phagolysosome fusion process in macrophages. This suggested that the block of phagosome maturation by mycobacteria in macrophages might involve inhibition of fusion with lysosomes dependent on Rab7-RILP interaction. To examine this hypothesis, we infected RAW macrophages that were transfected with RILP-DsRed and examined them by confocal microscopy (Fig. 7A and 7C). In cells infected with killed BCG, there was an abundant colocalization of RILP with bacterial phagosomes (Fig. 7A-a) indicating phagolysosomal fusion. Additional experiments examining intracellular distribution of Rab7 in infected cells showed recruitment of Rab7 to a large majority (> 80%) of phagosomes containing killed BCG (Fig. 7A-b) and this was expected. In contrast, cells infected with live BCG, showed almost no recruitment of RILP to the phagosomes (Fig. 7A-c). However, since Rab7 recruitment was
observed in a large proportion (> 60%) of live BCG phagosomes (Fig. 7A-d), the lack of RILP recruitment cannot be totally attributed to the lack of Rab7 recruitment to the phagosomes.

**Figure 7. Live BCG disrupts phagosomal recruitment of RILP.**  A. Panel a and c – Adherent RAW cells on coverslips were transfected with RILP-DsRed and then infected with either live or gentamicin-killed GFP-BCG as described in Materials and Methods. Cells were then washed extensively, fixed and examined by confocal microscopy. Panel b and d – RAW cells were infected with live or killed GFP-BCG then fixed/permeabilized and stained for intracellular Rab7 as described in Materials and Methods. Samples were then examined by digital confocal microscopy. The yellow signal in panels a, b and d corresponds to colocalization of RILP (red fluorescence) with BCG (green fluorescence) and Rab7 (red fluorescence) with BCG (green fluorescence) respectively. B. RILP-transfected RAW cells were loaded with CB-DXT (0.5 mg/ml) for 2 h, washed and chased in culture medium for an additional 2 h period. The DXT-loaded cells were then infected with either live or gentamicin-killed GFP-BCG as described in Materials and Methods. Cells were then washed extensively, fixed and examined by confocal microscopy. The white signal in the top panel (see also the magnification insert a) corresponds
to colocalization of RILP (red fluorescence) with BCG (green fluorescence) and dextran (blue fluorescence). **C and D.** Quantification of the confocal data shown in A, B, respectively. Values are the mean ± SD of positive phagosomes in 50-80 cells from three independent experiments. **E.** RAW cells were transfected with Rab7-GFP and RILP-DsRed as indicated then infected with live BCG, killed BCG, *S. typhimurium* (wild-type), or *S. typhimurium* (∆SifA). At 4 h post-phagocytosis, cells were lysed and immunoprecipitation with anti-GFP was performed as described in Material and Methods.

To demonstrate the relevance of RILP in lysosome fusion with BCG phagosomes, RILP-transfected macrophages were pulse-chased with Cascade Blue dextran (CB-DXT) in order to label lysosomes and then exposed to killed or live bacteria. DXT is a non-degradable, cell permeable probe that accumulates in lysosomes [35, 107]. The confocal images obtained and their quantification (**Fig. 7B and 7D**) showed that phagosomes containing killed BCG (green fluorescence) colocalized with both RILP (red fluorescence) and DXT (blue fluorescence) leading to a marked white signal in the merged images (see magnification insert **B-a**). Conversely, live BCG was totally secluded from both markers (see magnification insert **B-b**). Additionally, the merge images showed that RILP colocalized uniformly with DXT-loaded lysosomes (magenta signal) except in the area surrounding live BCG phagosomes.

To seek additional evidence to support disruption of Rab7-RILP interaction in BCG-infected macrophages, RAW cells were simultaneously transfected with Rab7-GFP and RILP-DsRed prior to infection with mycobacteria. Cell lysates were then prepared and subjected to Rab7 pull-down with anti-GFP antibodies and Western blotting with anti-RILP. Comparison was made to infection with *S. typhimurium*, which was reported to secrete an effector, SifA (*Salmonella* induced filaments), responsible for uncoupling Rab7 from RILP in epithelial cells [108]. The results obtained (**Fig. 7E**) showed significant pull-down of Rab7 associated RILP in control non-infected cells and in cells infected with killed BCG or SifA defective *Salmonella*. In contrast, there was no detectable Rab7-RILP association in cells infected with live BCG and
wild-type *S. typhimurium*. These findings were consistent with the immunofluorescence data (Fig. 7A) and demonstrated that live BCG disrupts the Rab7-RILP interaction in macrophages.

### 3.1.3 Live BCG inactivates Rab7

The failure of RILP recruitment by phagosomes containing live mycobacteria suggested that Rab7 on phagosomes is likely inactive, i.e., in the GDP-locked form. To address this hypothesis, we took advantage of the findings that RILP interacts only with active Rab7 (GTP bound form) (Fig. 6 and [98, 106]) and developed a novel Rab7 activation assay based on Rab7 pull-down with RILP-coated latex beads. In order to validate this assay, lysates were prepared from RAW cells transfected with GFP-tagged constructs of either wild-type Rab7, Rab7Q67L (constitutively active) or Rab7T22N (constitutively inactive) and incubated with RILP-coated latex beads overnight at 4°C. Beads were then extensively washed and subjected to Western blotting analysis with anti-GFP antibodies. Results in Fig. 8A showed that only the wild-type and constitutively active mutant interacted with RILP-beads, whereas the inactive Rab7T22N did not. The specificity of Rab7-RILP association was demonstrated by the absence of Rab7 pull-down with the control GST coated beads.
Figure 8. Live BCG inactivates Rab7. A. Raw cells were transfected with wild-type Rab7-GFP (WT), Rab7Q67L-GFP (constitutively active), or Rab7T22N (dominant negative). Cells were lysed 24 h post-transfection and incubated with GST or GST-RILP coated latex beads. Attached material to the beads was then subjected to SDS-PAGE and Western blotting with anti-GFP antibodies as described in Material and Methods. Membranes were then stripped and reprobed with anti-RILP antibodies. Lower panel shows 10% of whole-cell lysate (WCL) from each sample used in the assays to show that similar levels of Rab7 constructs were subjected to pull down with anti-GFP antibodies in each treatment sample. B. J774 and mouse bone marrow macrophages (BMDM) were infected with M. smegmatis, gentamicin-killed BCG, or live BCG at MOI 20:1. At 4 h post phagocytosis, cells were lysed and soluble proteins were mixed with RILP-beads overnight at 4°C to pull down endogenous active Rab7. The beads were then washed and attached material was subjected to Western analysis with anti-Rab7 antibodies (top panel). Membrane was then stripped and reprobed with anti-GST to control for the amounts of RILP-beads used in each treatment sample (middle panel). In the bottom panel, 10% of WCL used in pull-down assay were submitted to Western analysis with anti-Rab7 antibodies to ensure that equal amounts of Rab7 were subjected to pull down with RILP-beads in each treatment sample. IB: immunoblot. C. J774 cells were infected with M. smegmatis, gentamicin-killed BCG, or live BCG at MOI 20:1. At 4 h post phagocytosis, cells were washed extensively with HBSS and detached with cold PBS plus 5 mM EDTA and fixed with 2% paraformaldehyde. Samples were then subjected to FACS analysis along with the control non-infected cells to determine auto-fluorescence. Results are expressed as histograms of fluorescence intensity. The percentages of phagocytosis were determined with the WinMDI 2.8 software.
The validated assay was then used to examine the activation state of endogenously expressed Rab7 in infected J774 cells. Results in Fig. 8B showed that RILP-beads precipitated Rab7 from control non-infected cells and from cells infected with killed BCG. In contrast Rab7 was not detected in pull-down assays with lysate from cells infected with live BCG, indicating that this protein was in an inactivated state. Similar inhibitory effects of live BCG on Rab7 pull-down was observed in bone-marrow derived macrophages. Additional controls included assays with cells infected with the non-pathogenic species *M. smegmatis*. As expected, this species which fails to survive within the macrophage [34, 35, 109] had no effect on Rab7 activation (Fig. 8B). Parallel Western blotting experiments examining Rab7 in whole cell lysates showed similar levels of endogenous Rab7 in different treatment groups. These control experiments indicated that reduced pull down of Rab7 from BCG-infected samples was not due to a decrease in the levels of endogenous Rab7. The absence of detectable Rab7 in pull-down assays with RILP-beads from live BCG-infected cells correlated with high levels of bacterial uptake (> 90%) consistently observed with both J774 and RAW cells (Fig. 8C). Taken together, these data demonstrated that the failure of phagosomes containing live BCG to recruit RILP is due to interference with Rab7 activation.

### 3.1.4 Live BCG produces a Rab7 inactivating factor

The observations that RILP recruitment to phagosomes in macrophages is dependent on Rab7 activation (switch of GDP form into GTP form) ([106] and Fig. 6) along with the failure of RILP to interact with Rab7 from cells infected with live, but not killed, BCG suggested that metabolically active bacteria secrete an inhibitor of Rab7 activation. To examine this possibility, J774 cell lysates (source of endogenous Rab7) were incubated with culture filtrate proteins (CFP) of exponentially growing mycobacteria, in the presence of protease inhibitors, and then assayed with RILP-beads. The results obtained (Fig. 9A) showed that BCG CFP completely inhibited
the binding of Rab7 to immobilized RILP. In contrast, *M. smegmatis* CFP and the control culture medium alone had no effect on Rab7-RILP interaction. These results suggested that mycobacterial CFP might contain a GAP-like activity that catalyzes the conversion of Rab7-GTP into inactive GDP form. To examine this hypothesis, Rab7 inactivation was further examined with a GAP activity assay using $[^\gamma]^{32}\text{P-GTP}$. Recombinant Rab7-GST was resolved by SDS-PAGE, transferred to nitrocellulose membranes and renatured. Membrane strips were then loaded with $[^\gamma]^{32}\text{P-GTP}$ and incubated with bacterial CFPs as described in Materials and Methods. Consistent with the RILP pull-down data, the results shown in Fig. 9B and 9C indicated that CFP from BCG and Mtb, but not *M. smegmatis*, contain a factor that catalyses about 75% hydrolysis of the $\gamma$-phosphate from Rab7-GTP on the membrane. Taken together, these results demonstrated that the block of Rab7-RILP interaction in infected macrophages is dependent, at least in part, on a GAP-like activity.

**Figure 9.** BCG secretes a Rab7 inactivating factor. **A.** Equal amounts of proteins from a pooled source of J774 cell lysates were incubated 1 h at room temperature with either BCG or *M. smegmatis* culture filtrate proteins (CFP) or culture media (7H9-Tween) alone or left untreated (control). The mixtures were then incubated with GST or GST-RILP coated latex beads overnight at 4°C. The beads were washed and attached material was subjected to Western analysis with anti-Rab7 antibodies (upper panel). Membranes were then stripped and reprobed.
with anti-GST to control for the amounts of coated beads used in each treatment sample (lower panel). B. Rab7-GST was resolved by SDS-PAGE and transferred to nitrocellulose membrane. Rab7 protein bands were then renatured and loaded with 10 μCi of [γ-32P]-GTP at 37°C. Membranes were then extensively washed and either left untreated (control) or incubated with mycobacterial CFP in the presence of protease inhibitors at room temperature for 4 h. Membranes were then washed dried and exposed to X-ray film. After film development, membranes were probed with anti-Rab7 antibodies to ensure equal loading of Rab7 protein (lower panel). C. Band intensities in B were quantified by densitometry using the ImageJ software (http://rsb.info.nih.gov/ij/) and shown as percent of bound GTP relative to the control untreated Rab7wt strips.

3.1.5 Rab7 inactivating factor is diffusible within the macrophage

In order to examine the extent of Rab7 inactivation in BCG-infected macrophages, experiments were performed to examine RILP recruitment to phagosomes containing inert particles in the vicinity of the bacterium. Thus, RILP-transfected RAW cells were co-infected with both latex beads and live or killed BCG then subjected to confocal microscopy analysis. The images obtained (Fig. 10A and 10B) showed a total absence of RILP on bead-phagosomes in macrophage containing live BCG. In contrast, control experiments where cells were infected with killed BCG revealed an abundant recruitment of RILP to bead-phagosomes. Collectively, these data demonstrated clearly that live BCG expresses a diffusible Rab7 inactivating factor capable of trafficking beyond the phagosome to target other Rab7 positive compartments.

Figure 10 (next page). Rab7 inactivating factor is diffusible. A. RAW cells were transfected with RILP-DsRed and exposed to live or killed BCG (MOI 20:1) mixed with 4 µm latex beads, particle to cell ratio: 2:1. Non-ingested bacteria and beads were removed by extensive washing with HBSS and cells were replenished with culture medium and cultured at 37°C. At 4 h post-phagocytosis, cells were fixed and analyzed by digital confocal microscopy. The yellow signal in the top panel corresponds to colocalization of BCG (green fluorescence) with RILP-DsRed (red fluorescence). The red signal (RILP-DsRed) is seen on bead containing phagosomes of cells infected with killed but not live BCG. B. Quantification of the confocal data shown in A. Values are the mean ± SD of positive bead phagosomes in 50-80 cells from three independent experiments.
3.2 Generation of a broad range of recombination cloning vectors in mycobacteria

3.2.1 Background

A common pathway in the study of mycobacterial host pathogen interactions involves cloning genes of interest for various applications such as cytosolic overexpression, secretion of expressed gene products, as well as expression of reporter-gene fusion proteins to allow for tracking. Each of these is highly dependent upon recombinant DNA technology to rapidly manipulate the large sets of relevant genes currently available from mycobacterial genome data \[110-112\]. For years, researchers in the TB field relied on using cloning methods in which DNA inserts are outfitted with restriction sites to make them compatible with mycobacteria–E. coli shuttle vectors derived from mycobacteriophage based expression systems \[113\]. However, these methods are time-consuming and lack flexibility and uniformity and thus represent a major hindrance to the advance in TB research. An alternative to conventional cloning that is proving to be highly efficient and relatively simple is recombination cloning (RC), which allows high efficiency cloning without restriction enzymes or ligases \[114, 115\]. This strategy is currently widely used in several organisms, from E. coli to insects and mammals and thus represents an attractive approach to overcome cloning limitations in mycobacteria.

RC uses engineered site-specific recombination sites derived from bacteriophage \(\lambda\) \[115\]. Four types of sites are involved in two reactions as follows: i) the BP reaction \((\text{attB} \times \text{attP} \rightarrow \text{attL} + \text{attR})\) accomplishes the directional cloning of a PCR product or gene of interest (GOI) flanked with the \text{attB} sites (\text{attB1-GOI-attB2}) into an entry clone and ii) the LR reaction \((\text{attL} \times \text{attR} \rightarrow \text{attB} + \text{attP})\) for the subsequent subcloning of the GOI into a destination vector designed for a specific purpose (Fig. 11).
Figure 11. In vitro site-specific recombination cloning with mycobacterial destination vector. A. The gene of interest is PCR amplified with 5’ attB extended specific primers. B. Thereafter, the attB-PCR product is subjected to BP clonase reaction that facilitates recombination of attB sites with the attP sites of pDONR221 to create an attL-containing entry clone. C. The recombination of attL sites with the attR sites of mycobacterial converted destination vectors is catalyzed by LR clonase reaction.

In this study we converted the widely used extrachromosomal pMV261 and integrative pMV361 mycobacterial expression vectors \cite{116,117} into various destination vectors, thus creating a universal RC gateway in mycobacteria that allows rapid one-step cloning of mycobacterial and foreign genes for different purposes. We have selected pMV261 and pMV361 expression vectors because they represent an outstanding advance in *E. coli*-mycobacteria shuttle vector design as they permitted for the first time stable expression of foreign genes into *M. smegmatis* and BCG \cite{117}. Common elements in these 2 vectors include the Tn903-derived *aph* gene (kanamycin resistance), and an origin of replication functional in *E. coli* (*oriE*) derived from pUC19 \cite{118}. The expression cassette contains BCG *hsp60* gene promoter region, ribosome-binding site, and the first six Hsp60 codons (MAKTIA) as well as a multicloning site and the *E.*
coli rrnAB11 transcriptional terminator. The vectors differ by inclusion of either a mycobacterial plasmid replication origin (oriM) (pMV261) or the attP and int genes of mycobacteriophage L5 (pMV361).

3.2.2 Recombination cloning destination vectors for cytosolic expression in mycobacteria

To engineer destination vectors for cytosolic protein overexpression, pMV261 and pMV361 were first linearized by restriction enzyme PvuII followed by subsequent ligation of an Invitrogen reading frame cassette flanked with the attR1 and attR2 recombination sites (attR1–cam–ccdB–attR2). This construct allows subsequent subcloning of GOI in frame with sequence encoding the first six amino acids (MAKTIA) of Hsp60 in destination vectors (Fig. 12A). The resulting vectors were named pJAK1.A (converted pMV361) and pJAK2.A (converted pMV261). To examine whether pJAK.1A and pJAK.2A would support RC of foreign genes and their expression in mycobacteria, we PCR amplified ORFs encoding fluorescent proteins (EGFP and mCherry) and luciferase with attB1 and attB2 flanked forward and reverse primers. A standard BP reaction was then performed to clone ORFs into entry vector pDONR221 as described in the Materials and Methods section. Thereafter, we performed a one-step LR reaction between pDONR221 carrying ORFs and pJAK destination vectors to obtain pJAK.1A and pJAK.2A expressing EGFP, mCherry and luciferase (Fig. 12B). Vectors were then electroporated into competent M. bovis BCG. Figures 13A and 13B showed that mycobacteria transformed with pJAK.1-EGFP and pJAK.1-mCherry stably expressed green and red fluorescence respectively. As well, transformation of BCG with pJAK.1-luciferase led to bioluminescent mycobacteria detectable within living animals by bioimaging (Fig. 13C). Similar results were obtained with converted pMV261 (data not shown). Of particular note, this is the first demonstration of in vivo bioimaging with slow growing mycobacteria. It holds promise for extension to analysis of infection with virulent Mtb and thus offers clear advantages
over current methods, which rely on the recovery of pathogenic mycobacteria based upon colony counts. Given the extremely slow growth rate of mycobacteria (3 to 4 weeks to form colonies on agar plates) and the risk of contaminations, this approach has proved to be tedious and onerous.

Figure 12. Engineering of mycobacterial destination vectors for in vitro recombination cloning. **A.** To generate mycobacterial destination vectors for cytosolic gene expression, pMV261 and pMV361 vectors were linearized by the blunt cut restriction enzyme PvuII downstream of the hsp60 promoter within their multi-cloning sites, and subsequently CIAP treated prior to ligation with the Invitrogen reading frame cassette C.1 in order to allow subsequent cloning in frame with sequence encoding MAKTIA. **B.** pDONR221 carrying attL flanked EGFP, mCherry or luciferase was subjected to LR clonase reaction with pJAK1.A and pJAK2.A, as described in Material and Methods, to obtain cytosolic expression of green and red fluorescent protein and luciferase respectively under hsp60 promoter.
Figure 13. Validation of recombination cloning with mycobacterial destination vectors. Competent BCG organisms were electroporated with pJAK1.A expressing mCherry, EGFP or luciferase and positive clones were selected on 7H10 agar plates supplemented with kanamycin. Positive clones were grown in 7H9-Tween 80 plus kanamycin and aliquots of BCG expressing mCherry (A) and EGFP (B) were examined by fluorescent microscopy. C. BCG cultures expressing luciferase were adjusted to $5 \times 10^7$ and $5 \times 10^8$ CFUs in 100 µl PBS and inoculated subcutaneously into BALB/c mouse. Thirty minutes later animals were anesthetised and injected intraperitonially with 3 mg luciferase in 300 µl PBS then subjected to \textit{in vivo} bioluminescence imaging.

3.2.3 Recombination cloning vectors for improving the BCG vaccine

One of the goals of gene expression in mycobacteria is the development of improved recombinant BCG (rBCG) vaccines expressing genes that would increase its immunogenicity and by doing so its protective efficacy against TB. Whether overexpressing mycobacterial genes or introducing foreign genes, it is essential to have systems for stable expression and secretion of gene products for optimal exposure to and stimulation of immune system effectors. pAL5000-derived replicative and integrative plasmids bearing the mycobacterial \textit{hsp60} promoter have been widely used and fusion of ORFs with the signal sequence of secreted mycobacterial proteins was shown to optimize immunogenicity by targeting expressed proteins to the cell membrane for secretion \cite{38,119,120}. 
To ensure rapid and successful expression of selected genes for secretion, we converted pMV261 and pMV361 into a RC destination vector for one-step LR reaction to express ORFs in fusion with a signal sequence. Thus, the gene segment encoding the ribosomal binding site and signal sequence from antigen 85B (FbpBSS) was PCR amplified and cloned in pMV261 and pMV361. Subsequently, Invitrogen reading frame cassette B was inserted into the PvuII site in order to allow cloning of GOI in frame with the start codon ATG of FbpBSS (Fig. 14A). FbpBSS is commonly used for optimal secretion of mycobacterial and foreign proteins by recombinant BCG strains [38, 119]. The resulting destination vector pJAK1.B (converted pMV361) was tested for RC by performing a LR reaction with pDONR221-GFP as described in Fig. 12B. Thereafter, competent M. smegmatis organisms were transformed with pJAK1.B-EGFP and selected positive clones were shown to efficiently secrete EGFP by measuring fluorescence signal in culture media (Fig. 14B) and Western blot analysis with anti-EGFP antibodies (Fig. 14C). Similar results were obtained with pJAK2.B vector.

Figure 14. Recombinational cloning and expression of secreted proteins. A. Mycobacterial destination vector for expression of secreted proteins was made by insertion of the signal

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sequence of antigen 85 (-40 bp to +150 bp region) of the \textit{fhpB} gene (\textit{fhpBSS}) from Mtb into the \textit{BamHI} site within the multi-cloning site of pMV261/361. The correct orientation of insertion was verified by specific PCR primers and sequencing. The \textit{fhpBSS} containing vector was then linearized at \textit{PvuII} restriction site, which is directly downstream of \textit{BamHI}, and treated with CIAP prior to ligation of reading frame cassette B for the final destination vector. **B.** Competent 	extit{M. smegmatis} organisms were electroporated with pJAK1.B-EGFP or control empty vector pMV361. A selected positive \textit{M. smegmatis} clone expressing EGFP was grown in 7H9-Tween 80 and absorbance and fluorescence were measured for the indicated time. **C.** Supernatants from 24 h cultures were examined for secretion of EGFP by Western blotting with anti-EGFP antibodies.

The destination vector pJAK1.B would represent an important tool for vaccine development. Indeed, it can be used to stably express many individual human or mouse ORFs (cytokines, apoptosis inducers or transcription factors) already subcloned in Entry vectors compatible with RC (http://orf.invitrogen.com). Testing the protective efficacy of rBCG secreting protein products of selected ORFs in animal models might lead to identification of host factors that would enhance the efficacy of the current TB vaccine.

### 3.2.4 Recombination cloning system for expression of fluorescence-tagged protein in mycobacteria

To engineer a destination vector for expression of EGFP-tagged protein, we PCR amplified the gene segment encoding EGFP using pEGFP-N1 (Clontech) as template and specific primers with \textit{EcoRI} (Forward primer) and \textit{HindIII} (reverse primer) enzyme restriction sites (Table 1). Thereafter, the PCR product was ligated in pMV261 digested with the same enzymes followed by insertion of an Invitrogen reading cassette upstream of \textit{egfp} and within the \textit{PvuII} restriction site (Fig. 15A). The new vector was named pJAK2.C. To test RC with pJAK2.C, we amplified the full length ORF encoding Ag85B and its signal sequence with primers bearing \textit{attB} adapters (Table 1). Thereafter, the PCR product was transferred into pDONR221 by the BP reaction and then into pJAK2.C by the LR reaction. Competent \textit{M. smegmatis} was transformed with pJAK2.C-Ag85B and selected positive clones were transferred to liquid media. **Figure 15B**
shows increasing level of fluorescent signal, as a function of time in culture supernatants, and secretion of Ag85B was further confirmed by Western blotting with anti-EGFP antibodies (Fig. 15C).

**Figure 15. Recombinational cloning and expression of EGFP-tagged recombinant proteins.**

A. To generate a vector for expression of EGFP-tagged protein we first inserted EGFP encoding gene between the EcoRI and HindIII sites within the multicloning site of pMV261. Subsequently, the egfp containing vector was linearized at the PvuII site upstream of egfp within the multicloning site, and treated with CIAP. The reading frame cassette C.1 was then introduced by ligation within this PvuII site to generate the final destination vector. B. *M. smegmatis* was transformed with vector expressing Ag85 and its signal peptide in fusion with EGFP (pJAK2.C-Ag85B) or empty vector pMV261 (control). Selected positive *M. smegmatis* clone expressing Ag85B-EGFP was grown in 7H9-Tween 80 and absorbance and fluorescence were measured for the indicated time. C. Supernatants from 24 h culture of were examined for secretion of EGFP by Western blotting with anti-EGFP antibodies.

EGFP expresses a reliable signal that can be assayed by epifluorescence microscopy, immunoblotting or spectrofluorometry and thus has been widely used for analysis of distribution and trafficking of endogenous mammalian proteins. Therefore the RC system for expression of fluorescent proteins described here would be very useful for microscopic visualization of
mycobacterial proteins trafficking within the host macrophage and subsequent interaction with potential host cell partners expressed as fusion proteins with a complementary fluorescent tag.

3.2.5 Expression of recombinant protein in mycobacteria

A major obstacle associated with overexpression of many recombinant mycobacterial proteins in *E. coli* is the production of insoluble proteins. One strategy to overcome the formation of inclusion bodies is to use the fast growing *M. smegmatis* as an expression host that is more closely related to Mtb and BCG from which the proteins are derived\(^{[120,121]}\). Therefore, we adapted the pMV261 vector for the expression of inducible 6x-histidine-tagged recombinant proteins in the fast-growing *M. smegmatis*. We first modified vector pMV261 by replacement of the *hsp60* promoter with the inducible *M. smegmatis* acetamidase promoter in frame with a sequence encoding 6x histidines. The acetamidase promoter and 6x His segment was cut with *Xba*I and *Bam*HI restriction enzyme from pALACE plasmid \(^{[122]}\) and cloned in pMV261 linearized with the same enzymes. Then, we inserted a reading cassette *attR1−cam−ccdB−attR2* that allows RC in frame with the 6x histidine gene segment (Fig. 16A). The resulting destination vector pJAK2.D was tested for the expression of recombinant Ag85B known to produce inclusion bodies in *E. coli* expression systems \(^{[123,124]}\). Thus, Ag85B was subcloned into pJAK2.D by a LR reaction using a pDONR221-Ag85B as an entry clone. The resulting pJAK2.E-Ag85B was used to transform competent *M. smegmatis*.* A positive clone was then transferred into liquid medium and Ag85B was expressed after induction of gene expression with acetamide as described in the Material and Methods section. A large amount of soluble recombinant Ag85B was purified from bacterial lysate (Fig. 16B). The pJAK2.D expression system will also be extremely useful for mammalian proteins that are exclusively expressed as inclusion bodies in the *E. coli* and thus extremely difficult to refold as functional native proteins. For instance cathepsin S is expressed exclusively as inclusion bodies in *E. coli* \(^{[125-127]}\), but when
expressed in our pJAK2.D/M. smegmatis system, a large proportion of recombinant protein was found in the soluble fraction of bacterial lysate (Fig. 16C).

Figure 16. Recombinational cloning and expression of histidine-tagged recombinant proteins. A. To generate a destination vector for expression of His-tagged recombinant protein, we first cut the acetamidase promoter in fusion with a segment gene encoding 6x histidine from pALACE plasmid with XbaI and BamHI restriction enzymes. We then used the same enzymes to cut the hsp60 promoter from pMV261. The acetamide-6x histidine insert was then ligated into cut pMV261 vector and the modified vector was transformed into TOP 10 E. coli. Positive clones were screened by double digestion with XbaI and BamHI. The resulting plasmid was then converted into destination vector by insertion of Invitrogen reading cassette B within the PvuII site. The B cassette allows RC in frame with the 6x histidine. M. smegmatis was electroporated with pJAK2.D expressing Ag85B (B) or cathepsin S (C). Positive clones were grown in 7H9-Tween 80. After induction with acetamide, bacteria were lysed as described in Materials and Methods. Samples of total proteins from non-induced and induced M. smegmatis and corresponding soluble and insoluble fractions were subjected to SDS-PAGE and Western blotting with anti-6x histidine tag or anti-CatS antibodies.
One concern raised in a publication by Belfield et al., 2007 [128] demonstrated that the Gateway recombination site \textit{attrB1} contains a potential translational frameshift site where expression of a supposed -1 deletion mutant protein would result in a fully functional protein. Despite this, it was demonstrated that the expression and activity of the wild-type protein was unaffected since these levels were comparable or higher than the -1 deletion mutant. Thus, these observations pose no major implications on expression of full-length proteins using the Gateway system.

\textbf{Figure 17. Mycobacterial destination vectors for recombination cloning.} Features of (A) integrative and (B) replicative mycobacterial destination vectors derived from mycobacterial-\textit{E. coli} shuttle vectors pMV361 and pMV261.

In summary, we have generated a series of mycobacterial destination vectors for site-specific recombination \textit{in vitro} that represent new tools of a broad interest for TB research with their major features shown in \textbf{Figure 17} above. Over 2000 Entry clones of Mtb ORFs
compatible with RC are currently available at the Pathogen Functional Genomics Resource Center (J. Craig Venter Institute, http://pfgrc.jcvi.org/). Thus a gene of interest can be transferred by a simple LR reaction into our mycobacterial destination vectors, which serve a multitude of functional genomic studies. RC with mycobacterial destination vectors provides increased speed, versatility, and efficiency over current conventional restriction enzyme–based cloning methods. The time and cost saving are dramatic to the advance of our ongoing research project and we believe that many other TB researchers will also benefit from this cloning strategy.
3.3 Mycobacterial nucleoside diphosphate kinase blocks phagosome maturation

3.3.1 Ndk from pathogenic mycobacteria deactivates Rab5 and Rab7 GTPases

Based upon a previously published work showing that secreted Mtb Ndk manipulates the Rho GTPase regulatory cycle \[96\] and our recent observation that pathogenic mycobacteria express GAP activity towards Rab7 GTPase \[129\], we over-expressed and purified recombinant Mtb Ndk to homogeneity and examined its interaction with both Rab7, and the closely-related molecule, Rab5. Recombinant Ndk from the non-pathogenic *M. smegmatis* was also included in these experiments. We first examined Ndk binding to Rab molecules. Recombinant Mtb or *M. smegmatis* Ndk were coated onto flat bottom 96 well plates and exposed to increasing concentrations of Rab5 or Rab7. As illustrated in Figure 18, both *M. smegmatis* and Mtb Ndk bind to Rab5 (Fig. 18A) and Rab7 (Fig. 18B) in a dose-dependent manner. No difference in binding characteristics was observed between *M. smegmatis* and Mtb Ndk. In other experiments, soluble Rab7 and Rab5 were incubated with Ndk then subjected to immunoprecipitation with anti-Ndk antibodies and protein A agarose beads. SDS-PAGE and Western blotting analysis of pulled-down material with specific antibodies showed that Ndk interacts effectively with both Rab5 and Rab7 GTPases (Fig. 18C and 18D), thus confirming the solid phase binding assays.

We next examined whether bound Ndk expresses GAP activity towards Rab molecules. Rab5 and Rab7 proteins were preloaded with radioactive GTP as described in the Material and Method section. GTP-bound Rab proteins were then spotted onto nitrocellulose membrane squares and incubated in the presence and absence of Mtb or *M. smegmatis* Ndk and were washed. After exposure to X-ray films, membranes were probed with either anti-Rab5 or anti-Rab7 antibodies. The blotting analysis confirmed equal loading of Rab5 and Rab7 proteins on membrane squares (Fig. 18E and 18F, lower panels) and the radioactive signal (Fig. 18E and 18F, top panels) clearly demonstrated that Mtb Ndk is able to dephosphorylate the γ-phosphate of bound GTP to
Rab5 and Rab7. Quantification of the radioactivity showed 95% and 90% depletion of gamma phosphate from both Rab5 and Rab7, respectively. In contrast, *M. smegmatis* Ndk dephosphorylated Rab5 only partially (35% reduction of GTP) (Fig. 18F) and had almost no effect on Rab7 (Fig. 18E) despite efficient binding to both GTPases (Fig. 18A and 18B). These findings demonstrated that Ndk from pathogenic mycobacteria expresses strong GAP activity towards Rab5 and Rab7 GTPases.

**Figure 18.** Ndk interacts with and deactivates Rab5 and Rab7 GTPases. A and B. ELISA microplates were coated with 10 µg/ml Mtb or *M. smegmatis* Ndk or control BSA, and incubated for 1 h with increasing concentrations of Rab5 or Rab7, previously loaded with 1 mM GTP in
reaction buffer (50 mM HEPES at pH 7.4, 50 mM NaCl, 0.1 mM DTT, 5 mM EDTA and 1 mg/ml BSA) for 10 min at 37°C. Following 3 washes, attached Rab5 or Rab7 was probed with primary rabbit anti-Rab5 or Rab7, followed by secondary anti-rabbit-HRP conjugate. Thereafter, the interaction was visualized at absorbance 450 nm after addition of TMB substrate. Values from control BSA were subtracted. Results (mean ± SEM) are from 3 independent experiments.

C and D. Recombinant Ndk (3 µg) and GTP-loaded Rab5 or Rab7 (3 µg) were incubated in PBS buffer for 1 h at 4°C. Thereafter, anti-Ndk antibodies (1:100) were added (1 h at 4°C) and subjected to immunoprecipitation with protein A agarose beads for 30 min at room temperature. Samples were washed three times then analyzed by SDS-PAGE and Western blot with anti-Ndk and anti-Rab5 or anti-Rab7 followed subsequently by monoclonal anti-rabbit IgG, native-peroxidase. Pulled down Rab7 and Rab5 are shown as the 25 kDa and 27 kDa protein bands respectively, while the 15 kDa protein band corresponds to Ndk. E and F. Recombinant Rab5 and Rab7 were loaded with [γ-32P]-GTP and spotted onto nitrocellulose membranes. After extensive washes, membranes were either left untreated or incubated with recombinant Mtb or M. smegmatis Ndk at room temperature for 2 h. Membranes were washed, dried and exposed to X-ray film (upper panel). The radioactive signal observed depicts remaining active GTP-Rab7 or -Rab5 on membranes and values are quantification of bound [γ-32P]-GTP relative to control untreated samples as determined by radioactive count in a liquid scintillation counter. After film development, membranes were probed with anti-Rab7 or anti-Rab5 to ensure equal spotting of Rab proteins (lower panel).

3.3.2 Ndk from pathogenic mycobacteria inhibits phagolysosome fusion

Rab5- and Rab7-regulated endosomal trafficking in macrophages is known to be dependent on GTP binding [42, 45]. Therefore, the in vitro data showing that Rab5-GTP and Rab7-GTP are potential substrates for Ndk GAP activity (Fig. 18) suggested that Ndk might disrupt maturation of phagosome-containing pathogenic mycobacteria. To verify this hypothesis, we used the latex bead model for protein and glycolipid delivery into phagosomes [69, 70, 130] and examined the effect of Ndk on phagosome-lysosome fusion in RAW 264.7 macrophages. Thus, 4 µm latex beads were coated with Mtb or M. smegmatis Ndk or with BSA (control). The efficiency of coating was regularly examined by SDS-PAGE and Coomassie Blue staining, and FACS analyses of beads labelled with specific antibodies (data not shown). RAW cells were first pulse-chased with FITC-labelled dextran (FITC-DXT) and exposed to coated beads for 20 min at 4°C. Synchronous uptake was induced by temperature shift to 37°C and cells were incubated for
2 h to allow for phagosome-lysosome fusion to occur. Cells were then fixed and examined by
digital confocal microscopy. DXT is a non-biodegradable compound that accumulates in the
lysosome and is commonly used to directly visualize fusion of phagosomes with lysosomes\textsuperscript{135, 131}. The results illustrated in Fig. 19A show that vacuoles containing BSA-coated beads were
uniformly surrounded with green-fluorescent vesicles indicative of fusion with lysosomes. In
contrast, the images clearly demonstrated that phagosomes containing Mtb Ndk-coated beads did
not reach lysosomes. Consistent with these findings, \textit{M. smegmatis} Ndk, which has only a minor
effect on Rab GTPases, did not affect bead phagosome fusion with lysosomes.

\textbf{Figure 19 (next page). Ndk contributes to phagosome maturation arrest.} \textit{A.} RAW 264.7
cells were pulse-chased overnight with FITC-DXT (0.5 mg/ml) and allowed to ingest control
BSA-coated, Mtb Ndk-coated, or \textit{M. smegmatis} Ndk-coated latex beads. Two hours post-
phagocytosis, cells were washed and fixed for analysis by confocal microscopy. \textit{B.}
Quantification of the confocal data shown in panel A. \textit{C.} RAW 264.7 cells were loaded with
FITC-DXT overnight and allowed to ingest a mixture (1:1) of either PKH-labelled BSA-beads
and unlabelled BSA-beads (upper panel, control) or PKH-labelled BSA-beads and unlabelled
Ndk-beads (lower panel). Two hours post-phagocytosis, cells were trypsinized, washed, and
fixed for analysis by confocal microscopy. In the top panel (BSA control), the yellow signal
reflects a colocalization of the PKH-BSA-beads with the lysosomal marker dextran. In the
bottom panel dotted circles indicate the position of Ndk-beads, while the red fluorescent signal
(PKH) shows the location of BSA-beads and a significant decrease of dextran colocalization
with distant PKH-BSA-beads. \textit{D.} Quantification of the confocal data shown in panel C. Values
in B and D are the mean ± SD of phagosome colocalization of with FITC-DXT in 50-80 cells
from three independent experiments.
To examine whether Ndk dissociates from beads and exits the phagosomal membrane toward the cytosol to inhibit fusion with lysosomes, we analyzed FITC-DXT-loaded macrophages co-infected with red fluorescent (PKH-labelled) BSA-beads and unlabelled Ndk-beads (or BSA beads, control). The expectation was that protein released from Ndk-bead phagosomes would act on distant vacuoles containing red-fluorescent BSA-beads. Indeed, confocal images of cell co-infected with Ndk-beads and PKH-beads, (Fig. 19C, lower panel)
showed that PKH-beads almost completely excluded FITC-DXT vesicles from their phagosomes indicating a block of fusion with lysosomes. In contrast, an abundant green fluorescent signal surrounded PKH-phagosomes in cells co-infected with control beads coated with BSA (Fig. 19C, upper panel) indicative of fusion with DXT-loaded lysosomes. The observation of Ndk-mediated down-modulation of phagosome maturation cannot be attributed to a global toxicity of the host cell maturation. Indeed, the viability, morphology and adherence of RAW cells infected with Ndk-beads over 24 h culture period were similar to that of the control non-infected cells.

Collectively, these experiments suggest that secretory Ndk released from pathogenic mycobacteria within the phagosome might have access to the cytosolic face of the phagosomal membrane to interact with and inhibit effectors of phagosome maturation.

3.3.3 Mtb Ndk inhibits recruitment of Rab5 effectors to phagosomes

Membrane acquisition of EEA1 effector is an essential molecular event for phagosomal maturation [65]. Endosomal recruitment of EEA1 occurs via binding to active (GTP bound) Rab5 [42]. Given that pathogenic mycobacteria exclude EEA1 from their phagosomes [69] and that Mtb Ndk deactivates Rab5 (Fig. 18F) we examined whether Ndk interferes directly with the process of phagosomal recruitment of EEA1. To do so, we transiently transfected RAW macrophages with a chimera consisting of GFP fused to EEA1 then subjected them to phagocytosis of coated latex beads. Cells were examined by confocal microscopy 20 min after bead attachment to the cell membrane (Fig. 20A). In cells ingesting BSA-coated beads (control) about 65% bead phagosomes were surrounded by abundant green fluorescent signal reflecting normal recruitment of Rab5 effector EEA1. In contrast, macrophages infected with Ndk-coated beads showed almost no recruitment of EEA1 to the phagosomes. EEA1 is recruited to endosomal membranes via binding of its FYVE domain to phosphatidylinositol 3-phosphate (PI3P), which results from phosphatidylinositol (PI) phosphorylation by the class III
phosphoinositide 3-kinase enzyme hVPS34 (human vacuolar protein sorting 34) \cite{132}. Therefore we examined whether inhibition of EEA1 recruitment in the presence of Ndk is related to reduced PI3P formation on phagosomal membrane. RAW macrophages were transfected with a 2xFYVE-GFP construct, which is commonly used as a fluorescent probe for in situ detection of PI3P on endosomal membranes \cite{132,133}. Cells were then allowed to ingest coated latex beads and were examined by confocal microscopy. The images obtained (Fig. 20C) showed abundant recruitment of the FYVE domain to about 55% of phagosomes containing BSA-coated beads, reflecting a membrane enrichment in PI3P product, while most (95%) of Ndk-bead phagosomes excluded the fluorescent probe most likely due to a failure of PI phosphorylation by hVPS34. Given that hVPS34 binds to and is seemingly activated by GTP-bound (active) Rab5 \cite{133,134}, our findings strongly suggest that Mtb Ndk interrupts hVPS34 recruitment to the phagosomes via dephosphorylation of Rab5-GTP.
Figure 20. Ndk inhibits EEA1 recruitment to phagosomes. A. RAW cells were transfected with EEA1-GFP and thereafter allowed to ingest either control BSA- or Ndk-coated latex beads. The green signal shows presence of EEA1 on the phagosome containing control beads (upper panel), while the lack of signal around the phagosome (lower panel) shows diminished recruitment of EEA1 to Ndk-bead containing phagosomes. B. Quantification of the confocal data shown in panel A. C. Raw cells were transfected with 2xFYVE-GFP, and thereafter allowed to phagocytose either control BSA- or Ndk-coated latex beads. 2xFYVE is a specific marker for PI3P. The green signal seen in the upper control panel indicates an abundance of PI3P generated on the phagosome, while the lack of signal on Ndk-bead phagosomes indicate absence of PI3P. D. Quantification of confocal data shown in panel C. Values in B and D are the mean ± SD of phagosome colocalization with EEA1-GFP and 2xFYVE-GFP respectively in 50-80 cells from three independent experiments.

3.3.4 Mtb Ndk inhibits recruitment of RILP to late phagosomes

Fusion of late phagosomes with lysosomes is dependent upon interaction of Rab7 molecules with effector molecules RILP [67]. We have recently demonstrated that macrophage
infection with live BCG inhibited RILP recruitment despite acquisition of detectable amounts of Rab7 on the phagosome. Given that phagosomal recruitment of RILP occurs via binding to active (GTP bound) Rab7 \[98, 106\] and the observation made here that Ndk catalyzes the GTP/GDP switch on recombinant Rab7 molecules (Fig. 18), it is likely that ablation of Rab7-RILP interaction in infected macrophages results from the export of Ndk by mycobacterium within the phagosome. To verify this prediction, we double transfected RAW macrophages with Rab7-GFP and RILP-DsRed and generated phagosomes with BSA- or Ndk-coated beads. The results obtained from confocal analyses (Fig. 21A) showed a strong colocalization of red and green signals on a large number (75%) of phagosomes containing BSA-beads, indicating normal recruitment of Rab7 and its effector molecule RILP. In contrast, most Ndk-bead-containing phagosomes (> 80%) excluded RILP from their membranes despite a substantial acquisition of Rab7 molecules (Fig. 21A). These findings established a correlation between phagolysosome fusion arrest observed above (Fig. 19A and 19C) and Ndk-dependent disruption of Rab7-RILP interaction.

Figure 21. Ndk disrupt Rab7-RILP interaction. A. RAW cells were double transfected with Rab7-GFP and RILP-DsRed as described in Materials and Methods. Thereafter, cells were allowed to ingest either control BSA- or Ndk-coated latex beads. The yellow signal seen (upper panel) shows colocalization of Rab7 and RILP on the phagosome. The green signal (lower panel) shows phagosomes positive for Rab7 but no recruitment of RILP. B. Quantification of the
confocal data shown in panel A. Values in B are the mean ± SD of phagosome colocalization with RILP-DsRed in 50-80 cells from three independent experiments.

3.3.5 Antisense inhibition of Ndk expression attenuates survival of BCG in macrophages

To assess directly the contribution of Ndk to mycobacterial virulence in the context of phagosome maturation arrest, we created a BCG strain with knocked-down expression of Ndk and examined its fate in RAW macrophages. Thus, mycobacterial shuttle vector pMV261 was engineered to express the Ndk gene in sense (S-Ndk) and anti-sense (AS-Ndk) directions in BCG. This resulted in a recombinant strain that expresses high levels of Ndk antisense mRNA leading to a significant decrease of Ndk protein expression as shown by Western blot analysis (Fig. 22A). Additionally, compared to its parental strain, the Ndk knocked-down BCG strain showed no differences in its in vitro growth in culture media (Fig. 22B). To examine the contribution of Ndk to mycobacterial survival within the host, we infected RAW macrophages with wild-type BCG, or BCG expressing sense or antisense Ndk. Cells were then lysed and serial dilutions of recovered bacteria were plated on agar media plates at 24 h and 48 h post infection. The CFU counts (Fig. 23A) showed significantly decreased intracellular survival of BCG AS-Ndk strain. Specifically, at the 48 h time point, we observed a 1.5 log_{10} decrease of BCG AS-Ndk CFUs relative to CFUs obtained from macrophages infected with wild-type and S-Ndk strains. Both control strains showed comparable survival rates.
**Figure 22. Generation of recombinant BCG with knocked down Ndk expression.** A. Wild-type BCG, BCG transformed with pMV261-S-Ndk (sense, overexpression), and BCG transformed with pMV261-AS-Ndk (antisense, knockdown) were lysed as described in Materials and Methods, and subjected to 15% SDS-PAGE, followed by Western blot with anti-Ndk antibodies. Mycobacterial lipoamide dehydrogenase C (LpdC) was used as an internal control for equal loading. B. Growth curve of the BCG strains shown in panel A expressed as BCG/ml converted from absorbance at 600 nm.

The reduction of BCG survival by inhibiting Ndk expression in the macrophage further strengthens our findings that Ndk functions within host cells to inhibit phagolysosome fusion. Indeed, confocal analyses of macrophages loaded with FITC-DXT and infected with BCG strains showed a substantial number (~40%) of BCG AS-Ndk phagosomes that fuse with lysosomes, whereas virtually no phagosome containing wild-type BCG showed interaction with the lysosomal compartments (Fig. 23B and 23C). Taken together, these data demonstrate that Ndk contributes significantly to successful long-term survival of pathogenic mycobacteria within the macrophage.

**Figure 23. BCG AS–Ndk has decreased intracellular survival correlating with increased phagolysosome fusion.** A. RAW macrophages were infected with BCG strains (MOI of 10:1). Then culture media was supplemented with 50 µg/ml gentamicin to kill extracellular non-ingested mycobacteria. 2 h (0 h time point), 24 h, and 48 h post infection, cells were washed three times in PBS and lysed in 0.025% SDS. Serial dilutions of recovered bacteria were then plated on solid 7H10 media supplemented with 10% OADC. CFU counts were performed after 3 weeks incubation at 37˚C. Bars, mean ± SEM (three independent experiments). B. RAW cells adherent to cover slips were loaded with 0.5 mg/ml Texas Red-Dextran overnight and then
infected with FITC-labelled BCG strains (wild-type: WT, expression of sense (S-), or antisense (AS-) Ndk) at an MOI of 20:1. At 4 h post-phagocytosis, cells were fixed with 2.5% paraformaldehyde and mounted onto slides for confocal analysis. Bright field and merged fluorescent images are shown with an outline of the cell boundaries. Green signal indicates BCG that are not colocalized with dextran, while yellow signal shows colocalization of BCG with lysosomes. C. Quantification of data shown in panel B. Values are the mean ± SD of positive phagosomes in 50-80 cells from three independent experiments.
3.4.1 Ndk contributes significantly to Mtb virulence

We recently showed that mycobacterial Ndk plays an essential role in intracellular survival of the attenuated M. bovis BCG strain by a mechanism dependent on phagosome maturation arrest [20]. To examine whether Ndk also contributes to survival of virulent Mtb, we first attempted to generate an Ndk mutant in the Mtb strain H37Rv using various methods, including a gene disruption approach utilizing transducing mycobacteriophages [135]. Unfortunately, ndkA gene disruption resulted in a phenotype of growth attenuation to the extent that the use of the mutant in our studies was severely hindered. We therefore opted for protein knock down with mRNA antisense, the only approach developed so far to study essential genes in Mtb [20,136-140]. To do so, we used our integrative vector pJAK1.A [99], to express a stable full length antisense (or sense, control) mRNA sequence to ndkA and generated a strain (Mtb Ndk-AS) in which Ndk protein expression was undetectable by Western blot (Fig. 24A). Fortunately, Mtb Ndk-AS displayed a similar growth profile to that of wild type Mtb and the control sense strain (Mtb Ndk-S) (Fig. 24B). However, knock down of Ndk affected significantly the ability of Mtb to persist in RAW 264.7 macrophages to the extent that at 72 h post infection, Mtb Ndk-AS lost greater than 2 log\(_{10}\) colony-forming units (CFUs), relative to the wild type or Ndk-S strains (Fig. 24C). These findings suggested that the Ndk protein might contribute to Mtb virulence \textit{in vivo} and this was verified in C57BL/6 SCID mice. In this regard, Kaplan Meier survival analysis (Fig. 24D) demonstrated that the control strain expressing Ndk sense mRNA caused death of the immuno-deficient mice at similar rate seen in mice infected with wild type strain. In contrast, animals infected with Mtb Ndk-AS survived significantly longer (P<0.0001, Kaplan Meier Log-rank test). Taken together, these data demonstrated clearly that Ndk
contributes to Mtb virulence through mechanisms that we have attempted to elucidate.

Figure 24. Knock down of Ndk attenuates the survival of Mtb in the host. A. Mtb Ndk-AS along with wild type and Ndk-S strains were lysed in 50 mM Tris, 5 mM EDTA, 0.6% SDS, 0.05% NaN3 in the presence of protease inhibitors and 0.1 mm glass beads as described \(^{[20]}\). Samples were then subjected to SDS-PAGE and Western blot analysis with Ndk and 19 kDa lipoprotein antibodies. The latter was used to confirm loading of equal amounts of proteins. B. Growth curves comparing wild type, Ndk-S and Ndk-AS strains expressed as absorbance at 600 nm. C. RAW 264.7 macrophages were infected with Mtb strains at a MOI of 10:1 and then washed thrice 2 h post infection to remove extracellular bacteria. Cells were subsequently lysed in 0.025% SDS at the indicated post-infection time points. Serial dilutions of recovered Mtb were then plated on solid 7H10 media supplemented with 10% OADC and CFU counts were performed after 3 weeks incubation at 37°C. Results (mean CFU ± SEM) correspond to 2 independent experiments. D. C57BL/6 SCID mice were infected subcutaneously with 10^6 wild type, Ndk-S or Ndk-AS Mtb (n =10 per group) and time to morbidity was monitored over four months.

3.4.2 Recombinant Mtb Ndk binds specifically to Rac1 GTPase

Our recent findings that Ndk expresses GAP activity towards Rab5 and Rab7 \(^{[20, 129]}\) suggested that this activity might extend to other host GTPases. Therefore, we examined
whether Ndk targets macrophage Rho GTPases, known to play essential roles in early events of innate immunity against intracellular pathogens [47, 50, 141]. To do so, macrophages were allowed to ingest Ndk-coated latex beads. Then cell lysates were subjected to immunoprecipitation with Ndk antibody. Proteins associated with Ndk released from latex beads were analyzed by Western blot with Rac1, Rho, or Cdc42 antibodies. The results obtained showed that only Rac1 was interacting with Ndk within the macrophage (Fig. 25A). Rac1 binding to Ndk was further confirmed with reverse pull down experiments using Rac1 antibody and Western blotting with Ndk antibody, which demonstrated either a direct or indirect physical association between Ndk and Rac1 (Fig. 25B).

Figure 25. Mtb Ndk binds to Rac1 within the macrophage. A. Lysates from RAW macrophages ingesting Ndk-coated beads were incubated with Ndk or irrelevant (Irr.) antibody and protein A agarose beads. Pulled down Ndk and any associated proteins were analyzed by Western blot with antibodies to Rac1, Rho, or Cdc42. Rac1 (top set), but not Cdc42 (middle set) or Rho (bottom set), was found to be associated with Ndk intracellularly B. Similar immunoprecipitation experiments as in A, but using Rac1 or irrelevant antibody to pull down Rac1 and any associated proteins. Top panel indicates total amount of pulled down Rac1, while bottom panel shows amount of Ndk associated to Rac1. Data are representative of three independent experiments.
3.4.3 Mtb uses Ndk as GAP activity towards macrophage Rac1

The results shown above (Fig. 25) suggested that Mtb Ndk crosses the phagosomal membrane to bind to and inactivate specifically Rac1. To verify this hypothesis, we examined the activation level of Rho GTPases in infected macrophages with pull down experiments using binding domains derived from Rac1 and Cdc42 interacting proteins (PAK-1 PBD), or Rho interacting protein (Rhotekin RBD). These binding domains interact only with GTP-bound forms of Rho GTPases [142]. Mtb infected RAW cells were exposed to LPS in order to activate the Rho GTPases, then cell lysates were examined for the amount of active Rac1, Rho, or Cdc42. Western blot analyses with Rac1, Cdc42 and Rho antibodies demonstrated that Mtb inhibits significantly the level of LPS-induced Rac1 activation (Fig. 26A, top panel). In contrast, Mtb had no apparent effect on Cdc42 and Rho activation. This GAP activity was also observed in macrophages ingesting Ndk-coated beads, instead of Mtb organisms, demonstrating a specific Ndk GAP activity on Rac1 (Fig. 26A, lower panel).
Figure 26. Mtb Inactivates Rac1 in infected macrophages. A. RAW macrophages were allowed to ingest Mtb wild type (WT) (MOI 10:1) or coated latex beads (5:1) then treated with LPS (500 ng/ml) for 15 min to activate Rho GTPases. Lysates were then prepared and examined for levels of active (GTP-bound) Rho GTPases by pull down with specific probes (Millipore kit) and Western blot with Rac1, Rho, or Cdc42 antibodies (top panel). In the bottom panel, similar Western blot analyses were applied to equal aliquots of untreated whole cell lysates to confirm that equal amounts of total Rho proteins were used in the pull down assay. B. RAW macrophages were infected with Mtb WT or Mtb Ndk-AS and cell lysates were assayed for Rac1 activation 0 min, 15 min, and 60 min post phagocytosis. Top panels indicate amount of active Rac1 detected in cell lysates and bottom panels indicates levels of Rac1 in untreated whole cell lysate aliquots. Data are representative of three independent experiments.

To further examine Mtb effects on Rac1 and the phagosomal events it regulates, we performed a time-course Rac1 activation assay with macrophages infected by Mtb Ndk-AS and wild type Mtb. The results obtained showed a dramatic reduction of active Rac1 levels 15 min post infection and undetectable levels 1 h later in macrophages infected with wild type Mtb (Fig. 26B, left panel). In contrast, levels of active Rac1 remained unchanged in macrophages infected with Mtb Ndk-AS (Fig. 26B, right panel). Taken together, these data clearly demonstrated that Mtb Ndk expresses GAP activity on both basal and induced Rac1-GTP levels in the macrophage.

3.4.4 Ndk disrupts phagosomal recruitment of p67phox

Active Rac1 (GTP bound form) has been shown to translocate to early phagosomes in order to facilitate recruitment of its binding partner, the NOX2 subunit p67phox [72]. Given that Ndk expresses GAP activity towards Rac1 (GTP into GDP switch), we examined whether Mtb interferes with phagosomal recruitment of p67phox. We first applied intracellular staining and confocal microscopy to estimate the proportion of Rac1 and p67phox positive phagosomes in
Mtb-infected RAW cells. Results obtained (Fig. 27A and B) showed a substantial reduction of Rac1 and p67phox positive phagosomes (13% and 29% respectively) in cells infected with live Mtb relative to those infected with killed Mtb (86% Rac1 and 88% p67phox positive phagosomes, respectively). In contrast, recruitment of p47phox to live Mtb phagosomes was comparable to that of phagosomes containing killed Mtb, consistent with previous findings that phagosomal recruitment of p47phox is independent of Rac1 or p67phox [143]. To demonstrate that the NOX2 assembly defect is related to Ndk GAP activity, we applied similar confocal analyses to cells infected with Mtb Ndk-AS. The images clearly showed a restoration of Rac1 and p67phox recruitment to Mtb Ndk-AS containing phagosomes to a level similar to those observed in cells infected with killed Mtb (~ 76% and 72% positive phagosomes, respectively) (Fig. 27C and D). As expected, much lower numbers of Rac1 and p67phox positive phagosomes (13% and 30% respectively) were observed in cells infected with control strain Mtb Ndk-S (Fig. 27C and D).

Figure 27 (next page). Decreased recruitment of Rac1 and p67phox to Mtb phagosomes. A. RAW macrophages were infected with live or gentamicin killed (50 µg/ml, 1 h) Mtb expressing DsRed for 1 h. Cells were then fixed/permeabilized and stained with Rac1, p47phox or p67phox antibodies and corresponding FITC-conjugated secondary antibodies. Cells infected with killed Mtb showed clear yellow signals indicating colocalization of the bacterial phagosome with all three phox subunits. Conversely, both Rac1 and p67phox, but not p47phox, were absent on phagosomes containing live bacteria. B. Quantification of the confocal data shown in panel A. C. Macrophages were infected with Ndk-AS and Ndk-S Mtb strains then immunostained as in A. The images show recruitment of Rac1 and p67phox to phagosomes containing Ndk-AS but not Ndk-S bacteria. D. Quantification of the confocal data shown in panels C. Values in B and D are the mean ± SD of positive phagosomes in 50-80 cells from three independent experiments. E. Fluorescence histograms of phagosomal levels of Rac1 and p67phox in infected macrophages obtained by FACS analysis approach described in Fig. 28.
Figure 28. FACS analysis of Mtb phagosomes. Macrophage cell surface was labelled with CellMask™ Deep Red (detectable by FL4 channel) at 0.2 µg/ml for 5 min at 37°C prior to infection with DsRed mycobacteria (FL2). Then cells were treated with Trypsin-EDTA to remove non-ingested but partially attached bacteria. Thereafter, cells are homogenized in 20 mM HEPES buffer, pH 7.4 containing 0.25% sucrose, 0.1% BSA, and 0.5 mM EGTA. Homogenates were then centrifuged at 400 x g for 2 min to remove nuclei and intact cells and the upper fractions were collected and centrifuged at 3000 x g for 10 min at 4°C. The pellets correspond to crude phagosome preparations where bacteria included in cell membrane-derived vacuoles (double FL2/FL4 positive events) are readily distinguished from both cell debris and free bacteria released from disrupted vacuoles. Thus, phagosome preparations can be stained with specific antibodies followed by FITC-conjugated secondary antibodies and levels of phagosomal markers (FL1 histograms) can be easily determined by FACS.

As an alternative approach, a previously developed quantitative FACS analysis method [35] was used to assess the level of NOX2 components on individual phagosomes. To adapt this method to mycobacterial phagosomes, macrophage plasma membrane was stained with CellMask™ Deep Red (detectable by FL4 channel), and then cells were infected with Mtb strains expressing fluorescent DsRed protein (FL2). Following cell disruption, mycobacteria included in cell membrane-derived vacuoles (double FL2/FL4 positive events) were readily identified by FACS (Fig. 28). Phagosome preparations were then stained with Rac1 or p67phox antibodies and FITC-conjugated secondary antibodies (FL1). Samples were subjected to FACS analysis and mean fluorescence intensities (MFI) were deduced from fluorescence histograms. Results obtained (Fig. 27E) showed higher recruitment of Rac1 and p67phox to phagosomes.
containing Mtb Ndk-AS (MFI: 49.6 and 42.6 respectively) relative to phagosomes containing Mtb Ndk-S (MFI: 25.3 and 21.8 respectively) or Mtb wild type (MFI: 23.9 and 15.7 respectively). To establish a direct link between Ndk GAP activity and defective NOX2 assembly, additional FACS analyses were applied to phagosomes containing coated beads (Fig. 29) and showed a marked decrease of Rac1 and p67phox recruitment to the Ndk bead phagosomes (MFI: 8.1 and 3.8 respectively) relative to control phagosomes containing BSA-beads (MFI: 14.6 and 7.3 respectively). Taken together, these findings showed for the first time that Mtb uses Ndk GAP activity to disrupt phagosomal assembly of NOX2 via interference with Rac1-dependent recruitment of p67phox.

![Figure 29. Rac1 and p67phox levels on Ndk-bead phagosomes.](image)

CellMask-labelled RAW cells were allowed to ingest BSA or Ndk coated 3 µm magnetic beads for 1 h. Bead containing phagosomes were then isolated by a magnet from crude preparations obtained as described in Fig. 28. Purified phagosomes were stained with Rac1 or p67phox antibodies or irrelevant (control) antibody and FITC-conjugated secondary antibody. Samples were then washed and analyzed by FACS to quantify levels of FL1 signal on gated FL4 positive events, which correspond to true phagosomes. FL1 histograms showed decreased levels of Rac1 and p67phox on Ndk-bead phagosomes relative to control BSA-bead.

### 3.4.5 Mtb Ndk inhibits macrophage oxidative burst

Previous findings that Rac1 and p67phox subunits are essential for NOX2 assembly and
activation of gp91phox to generate superoxide \cite{72, 144} suggested that disruption of Rac1/p67phox translocation to the phagosome by Ndk would affect NOX2-dependent ROS production. To verify this hypothesis, we applied a luminol-dependent chemiluminescence assay to assess ROS production in Mtb infected cells. Luminol is a membrane diffusible reagent commonly used for quantitative detection of superoxide anion radicals and hydrogen peroxide molecules \cite{145, 146}. Bone marrow derived macrophages (BMDM) from C57BL/6 mice were infected with Mtb strains and assayed for kinetics of chemiluminescence production over a period of 60 min. Relative luminescence profiles obtained (Fig. 30A) revealed that cells infected with Mtb Ndk-AS induced significantly higher amounts of ROS production (peak value = 256 RLU) compared to those infected with wild type Mtb or Mtb Ndk-S (peak value ~ 120 RLU). Thereafter, we confirmed the apparent inhibitory effect of Ndk with experiments using coated beads (Fig. 30B), which showed minor ROS responses to Ndk beads (peak value = 32 RLU) relative to ROS production induced by BSA beads (peak value = 76 RLU). Additionally we applied confocal microscopy to visualize intracellular accumulation of ROS using CM-DCFDA, a cell-permeable probe that is nonfluorescent until oxidized within the cell \cite{147, 148}. Thus, in RAW cells infected with Mtb Ndk-AS, the confocal images showed a strong colocalization of oxidized CM-DCFDA (green fluorescence) with bacterial phagosomes (red fluorescence) indicating accumulation of large amounts of ROS around Mtb Ndk-AS (Fig. 30C and 30D). Conversely, green signal was totally absent in cells infected with either wild type Mtb or Mtb Ndk-S. Such an effect of Ndk on ROS production was reproduced when switching the cell system model with BMDM (Fig. 31). Collectively, these experiments demonstrated that the macrophage oxidative response to Mtb is marginal and that knock down of Ndk converts the bacterium into a potent inducer of the ROS response.
Figure 30. ROS production is inhibited in the presence of Mtb Ndk. A. BMDM were stimulated with 100 ng/ml LPS for 48 h then exposed to the indicated Mtb strains (A) or coated latex beads (B) in the presence of 50 µM luminol. Thereafter, luminescence was quantified using a Tropix TR717 microplate reader. Results are expressed as Relative Luminescence Units (RLU) as a function of time C. RAW macrophages were loaded with 10 µM CM-DCFDA for 30 min at 37°C and subsequently infected by Mtb strains expressing DsRed at a MOI for 30 min. Thereafter, cells were washed and fixed with 2.5% PFA and mounted for analysis by confocal microscopy. Yellow signal indicates colocalization of phagosomes containing Mtb (red fluorescence) with oxidized CM-DCFDA (green fluorescence), indicative of ROS production. D. Mean ± SD of positive phagosomes observed in 50-80 cells from three independent experiments.
Figure 31. Mtb Ndk inhibits ROS production in BMDM. A. Adherent cells on cover slips were stimulated with LPS then infected with Mtb strains expressing DsRed in presence of CM-DCFDA as described in Fig. 30C. Cells were then fixed and examined by confocal microscopy. Yellow signal (indicative of ROS production) is visible on phagosomes containing Mtb Ndk-AS but absent on those containing wild type and Ndk-S strains. B. Mean ± SD of positive phagosomes observed in 50-80 cells from three independent experiments.

3.4.6 Inhibition of NOX2 activity impairs apoptosis response to in Mtb infection

Mtb is known to inhibit macrophage apoptosis [27, 94] by mechanisms yet to be clarified. Based upon previous findings that NOX2 activity might extend beyond intracellular killing to induce apoptosis [79, 87] we examined whether Mtb uses Ndk to disrupt the NOX2-apoptosis link. First, we applied Annexin V cell surface staining, a popular approach for detection of phosphatidylserine (PS) translocation to the extracellular membrane leaflet, which reflects early steps of apoptosis events [149]. Adherent RAW cells on coverslips were infected with Mtb strains for 48 h then stained with Alexa Fluor 488 conjugated Annexin V and examined by confocal microscopy. The images showed very low numbers of Annexin V positive cells in samples infected with wild type Mtb and Mtb Ndk-S (5% and 6% positive, respectively). In contrast, a
higher number of Annexin V positive cells (44%) was observed in samples infected with Mtb Ndk-AS (Fig. 32A, top panel). To establish a direct link between ROS and apoptosis in infected cells, Annexin V staining was repeated on macrophages exposed to Mtb Ndk-AS in the presence of a specific gp91phox peptide inhibitor (gp91 INH) or its control scrambled version (gp91 SCR) [97]. The results obtained showed clearly that gp91 INH, but not gp91 SCR, reversed completely Mtb Ndk-AS-induced PS translocation to the cell surface (5% Annexin V positive, Fig. 32A, bottom panel). The effect of the gp91phox inhibitor was confirmed with experiments showing that gp91 INH completely inhibited ROS production in cells infected with Mtb Ndk-AS, which was normally elicited in the presence of gp91 SCR (Fig. 33). In a complementary series of experiments, we analyzed caspase 3 activation, which occurs during the final stages of apoptosis [26]. Thus, infected macrophages were subjected to intracellular staining with antibody to cleaved (i.e. active) caspase 3 and Alexa Fluor 647 conjugated secondary antibody, then analyzed by FACS. Results obtained (Fig. 32B) showed high numbers of apoptotic macrophages in sample tests infected by Mtb Ndk-AS (11.8% positive events) relative to those infected with wild type Mtb or Mtb Ndk-S (~ 4.6%). Not surprisingly, the wild type and Ndk-S strains rather inhibited the spontaneous apoptosis observed in control non-infected cells (7.3% positive cells). As expected, Mtb Ndk-AS-induced caspase 3 cleavage was abolished in the presence of the gp91phox inhibitor. Collectively, these data demonstrated that Mtb blocks macrophage apoptosis by a mechanism dependent, at least in part, on Ndk-mediated attenuation of NOX2 activity.
Figure 32. Mtb Ndk inhibits ROS dependent macrophage apoptosis. A. RAW macrophages were infected for 48 h with the indicated Mtb strains. To block ROS production, gp91\textsuperscript{phox} peptide inhibitor (gp91 INH) or its scrambled version (gp91 SCR) were added at 50 µM 1 h prior to infection by Mtb Ndk-AS. Cells were then stained with Alexa Fluor 488-Annexin V and analyzed by confocal microscopy. Images are a merge of bright field, red fluorescence (Mtb DsRed strains), and green fluorescence (Annexin V). Values in the top right corner indicate the percentage of apoptotic cells. B. Macrophages were infected as described in A, and then stained for cleaved caspase 3 as described in materials and methods. Cells with active caspase 3 were identified and quantified by FACS. Data are representative of two independent experiments.
Figure 33. Validation of gp91phox inhibitor peptide. RAW macrophages were incubated in the presence of gp91phox inhibitor peptide (gp91 INH) or its scrambled version (gp91 SCR) at a final concentration of 50 µM. Cells were infected 1 h later with Mtb Ndk-AS in the presence of 50 µM luminol and chemiluminescence was monitored as described in Fig. 30. The results obtained showed strong inhibition of ROS production by gp91 INH.

3.4.7 Ndk knock down increases Mtb susceptibility to ROS-mediated intracellular killing

Results presented above (Fig. 32) together with initial experiments showing attenuated Mtb Ndk-AS survival in RAW macrophages (Fig. 24) suggested that Ndk-mediated inhibition of ROS reduces the macrophage killing capability. To verify this hypothesis, we repeated the survival assay using primary murine macrophages in which ROS production was blocked with gp91 INH. At 72 h post-infection, control experiments showed a significant reduction (~ 1.5 log10) in CFU counts when infecting with Mtb Ndk-AS relative to wild type or Ndk-S (Fig. 34A). However, in the presence of gp91 INH, Ndk-AS survival was restored to a level comparable to that of Mtb wild type at every time point measured (Fig. 34B). These observations were validated with assays in the presence of control scrambled peptide, which did not affect Ndk-AS survival. Taken together, these experiments clearly demonstrated that down modulation of ROS production by Ndk contributes significantly to Mtb persistence in the macrophage.
Figure 34. Inhibition of ROS production prevents intracellular killing of Mtb Ndk-AS.  
A. BMDM (5 x 10^5/well) were infected with the indicated Mtb strains and washed thrice 2 h post 
infection to remove extracellular bacteria. Thereafter, cells were lysed in 0.025% SDS at the 
indicated post-infection time points. Serial dilutions of recovered Mtb were then plated on solid 
7H10 media supplemented with 10% and CFU counts were performed after 3 weeks incubation 
at 37°C.  
B. Similar experiment as in A, in the presence or absence of 50 µM gp91phox inhibitor 
peptide (or its scrambled version) added 1 h prior to infection with Mtb Ndk-AS. Results (mean 
CFU ± SEM) correspond to 2 independent experiments.
CHAPTER 4: DISCUSSION

4.1 Mycobacterial GAP activity

The initial study examined the biogenesis of mycobacterial phagosomes and revolved around the contribution of the small GTPase Rab7 in this process. Based on observations reported earlier, Rab7 is first recruited by phagosomes in a GDP bound form, and then a GEF (guanine nucleotide exchange factor) catalyzes the exchange of GDP for GTP\[^{150}\]. The GTP-bound form of Rab7 on the phagosomal membrane allows full activation of the G protein, which then contributes to later stages of phagosome development\[^{151}\]. As part of the process, normal Rab7 inactivation and recycling is mediated by GAP, which catalyzes the GTP switch into GDP on the Rab7 molecule\[^{152}\].

One of the well-characterized downstream effector of Rab7 is the RILP molecule\[^{68, 98}\]. Accumulated data using epithelial cell line models showed that RILP interacts with activated Rab7 in order to bridge phagosomes with dynein-dynactin, a microtubule-associated motor complex\[^{67}\]. The motors displace phagosomes in the centripetal direction as well as promote the extension of phagosomal tubules toward late endocytic compartments such as lysosomes\[^{67}\].

The results presented in this study demonstrated that the Rab7-RILP interaction is also occurring on latex phagosomes in the macrophage when Rab7 is bound to GTP. However, this interaction is disrupted in macrophages infected with live \textit{M. bovis} BCG or wild-type \textit{Salmonella enterica typhimurium}. These data recapitulated previous observations of RILP exclusion from \textit{Salmonella} phagosomes in infected epithelial cells\[^{108}\]. The same study showed normal acquisition of active Rab7 and explained RILP exclusion by direct interaction between \textit{Salmonella} virulence factor SifA\[^{108}\] and Rab7, resulting in competitive displacement of RILP. Our observations that BCG phagosomes recruit Rab7 but fail to recruit the marker RILP along with absence of fusion with DXT-loaded lysosomes are consistent with the findings of Clemens.
et al. [153] who reported that in Rab7 transfected HeLa cells, phagosomes containing live Mtb acquired little or no LAMP-1, a strong lysosomal marker [154, 155], despite their tendency to carry more Rab7 than phagosomes of heat-killed Mtb. Our data also show that beyond the BCG phagosome, the RILP molecule colocalizes quite uniformly with lysosomes identified with CB-DXT in agreement with earlier studies showing abundance of RILP on lysosomes in HeLa cells [98].

The results reported in this study suggested that Rab7 is likely converted into Rab7-GDP but remains attached to the membrane of phagosomes containing live BCG indicating that cytosolic Rab-GDI [43, 156] are unable to completely deplete Rab7-GDP from the phagosome. These findings are consistent with the observation of significant colocalization of Rab7-T22N (GDP locked form) with latex bead phagosomes (Fig. 6). Furthermore, the ability of Rab-GDI to extract Rab7-GDP from phagosomes has been shown to be regulated by other chaperone factors such as Hsp90 [44, 157]. Therefore, in our model, the possibility of BCG interference with Rab-GDI regulators is an additional mechanism that might contribute to the retention of Rab7-GDP on the phagosome membrane.

The findings presented in this study differ markedly—but do not entirely contradict—from those of Via et al. [158], who reported that Rab7 levels are significantly reduced on BCG phagosomes isolated from J774 macrophages. We believe that Rab7-GDP (low affinity binding GTPase) dissociated easily from the phagosomal membrane during cell homogenization and phagosome purification by Via et al. Indeed, these authors showed high concentrations of Rab7 (presumably GTP locked form and thus high affinity binding GTPase) on latex bead phagosomes. Consistent with this hypothesis, a previous study from our group reported a reduced Rab7 staining on phagosomes containing latex beads mechanically isolated from cells that were exposed to mycobacterial lysates [35].
The earlier observation that mycobacteria arrested phagosome maturation despite the presence of constitutively active mutant Rab7Q67L \[153\] suggested that Rab7 GTPase is not the only key regulator of phagosome maturation. In fact, Rab5 recruitment has been shown to play a prominent role in phagosome maturation \[^{69,159}\], as well as VPS34, which is a common effector of both Rab5 and Rab7 \[^{160}\]. In this context, the finding that anti-Rab7 activity is trafficking beyond the mycobacterial phagosome suggested the possibility of extended activity towards Rab5 and other GTPases within the host cell. Nevertheless, this should not decrease the significance of the Rab7-RILP interaction in macrophages, which is clearly demonstrated here as an important mechanism regulating phagosome biogenesis targeted by pathogenic mycobacteria to block fusion with lysosomes.

The finding that live, but not killed, BCG reproduces the effect of Salmonella suggested the possibility of an active inhibitory mechanism mediated by expression of a SifA-like protein by Mycobacterium. However, our search in BCG and Mtb genome data bases did not reveal any predicted SifA ortholog. This was not entirely unexpected as, in contrast to Salmonella \[^{161}\], Mycobacterium does not induce filament formation within the host cell. Meanwhile, our Rab7 activation assay based on affinity binding of Rab7-GTP to RILP-beads clearly demonstrated the predominance of inactive Rab7 (GDP bound form) in macrophages infected with live but not killed BCG. This inactive form of Rab7 cannot associate with and recruit RILP intracellularly \[^{98}\]. Such findings suggested that live mycobacteria express a GAP-like activity inhibiting the function of Rab7 which has been recruited to the phagosome. Indeed, analysis of BCG CFP revealed a secreted activity that catalyzes the hydrolysis of the $\gamma$-phosphate from GTP leading to the switch of GTP into GDP on the Rab7 molecule. The likelihood of this mechanism within the host cell is supported by previous findings that several pathogenic bacteria secrete proteins that act as either GAP or GEF, and eventually facilitate their pathogenesis \[^{162}\]. For
instance secreted ExoS cytotoxin by *Pseudomonas aeruginosa* disrupts the actin cytoskeleton by acting as GAP for Rho-GTPases \(^{163}\). Similarly, *Yersinia pseudotuberculosis* secretes a cytotoxic factor, YopE, which depolymerizes the actin stress fiber, through its GAP activity for Rho-GTPases \(^{164}\). In this line of investigations, a recent study identified a nucleoside diphosphate kinase (Ndk) in mycobacteria, which acts as a GAP for Rho-GTPases \(^{96}\).

In summary, this study provides evidence that mycobacteria actively disrupt Rab7-RILP association as one of several mechanisms used to prevent phagosome maturation to fusion with lysosomes. The results suggest that mycobacteria express within the macrophage a diffusible GAP-like protein for Rab7 GTPase and experiments are in progress to examine whether mycobacterial Ndk or another specific GAP is deactivating Rab7.

**4.2 Role of Ndk in Mtb pathogenesis**

**4.2.1 Phagosome maturation arrest**

Earlier observations that arrest of phagosome maturation occurs only in macrophage ingesting live mycobacteria \(^{165}\) suggested a mechanism dependent on active secretion of virulence factors capable of crossing the phagosomal membrane and deactivating critical regulators of phagosome biogenesis. Ndk (~ 15 kDa) is one of many secreted mycobacterial proteins \(^{166, 167}\) and the present study examined its effects on the regulation of phagosome biogenesis in the context of macrophage infection with mycobacteria.

Our hypothesis that Ndk acts as a potential inhibitor of phagosome maturation was supported by i) our recent findings that live mycobacteria express a GAP-like activity on Rab7 that has been recruited to the phagosome \(^{129}\) and ii) by the concomitant demonstration that mycobacterial Ndk acts as a GAP for Rho-GTPases \(^{96}\). Furthermore, several pathogens have been shown to secrete proteins that act as GAP and facilitate their pathogenesis. For instance, *Pseudomonas aeruginosa* ExoS cytotoxin disrupts the actin cytoskeleton by acting as GAP for
Rho-GTPases \cite{163} and \textit{Yersinia pseudotuberculosis} cytotoxic factor, YopE, depolymerizes the actin stress fiber through its GAP activity for Rho-GTPases \cite{164}. Similarly \textit{Legionella pneumophila} virulence factor LepB exhibits GAP activity towards host cell Rab1 GTPase to disrupt proper membrane cycling and activation \cite{168}.

The current study used purified recombinant Ndk adsorbed on latex beads in order to mimic intraphagosomal expression of proteins occurring during mycobacterial infection. In fact, a major advance in phagosome biology was made possible by using the latex bead system for analyses of many phagosome functions \cite{169} and the option of coating these beads has been successfully used for examining modulation of phagosome biogenesis by several bacterial products \cite{170, 171}. Thus, we have demonstrated that latex bead-mediated intracellular delivery of Ndk blocks phagosome fusion with FITC-DXT-loaded lysosomes as a result of exclusion of the Rab7 downstream effector RILP from the phagosomal membrane. These findings corroborate our observation of direct binding of Ndk to Rab7 \textit{in vitro} and the subsequent dephosphorylation of the γ-phosphate of Rab7-GTP leading to inactive GDP-bound molecules.

The earlier observation that mycobacteria arrest phagosome maturation despite the presence of constitutively active mutant Rab7Q67L \cite{153} suggested that Rab7 GTPase is not the only key regulator of phagosome maturation. In fact, membrane recruitment of another small GTPase, Rab5 was found to mediate EEA1-dependent phagosome fusion with early endosomes \cite{69}. EEA1 is recruited to phagosomal membrane in the presence of the hVPS34 product PI3P and active Rab5 (GTP bound form) \cite{42}. Binding of the EEA1 FYVE domain to PI3P stabilizes the interaction between Rab5 and EEA1 \cite{172}. As EEA1 was shown to be excluded from Mtb and BCG phagosomes by a mechanism dependent on mycobacterial lipid phosphatase SapM \cite{173}, we examined an alternate mechanism mediated by mycobacterial GAP activity towards Rab5 GTPase. This hypothesis was confirmed by the demonstration of direct binding of
Ndk to Rab5-GTP and its dephosphorylation, consistent with the observation of reduced recruitment of the Rab5 interacting effector EEA1 to phagosomes containing Ndk-coated latex beads. Thus, while SapM decreases EEA1 recruitment through hydrolysis of phagosomal PI3P \[173\], Ndk is acting through Rab5-GTP deactivation and attenuation of its interaction with hVPS34 leading ultimately to diminished phosphorylation of PI on the phagosomal membrane. This conclusion is consistent with previous findings that hVPS34 catalytic activity begins after binding to GTP-bound Rab5 \[134\].

Figure 35. Sequence alignment of Mtb, BCG, and M. smegmatis. There is 100% homology between Mtb and BCG Ndk, which decreases to ~80% when compared to M. smegmatis Ndk. Residues of difference that could potentially be important in the catalytic GAP activity are highlighted.

Of particular note is the finding that recombinant Ndk from M. smegmatis has a minor effect on Rab5 and no effect on Rab7 GTPase, consistent with its failure to block fusion of bead-containing phagosomes with lysosomes. These observations are in agreement with earlier reports showing that M. smegmatis fails to block phagosome maturation and are unable to ensure successful long term survival within the macrophage \[37\]. Comparison of amino acid sequence (Fig. 35) showed high homology (~80%) between Mtb/BCG and M. smegmatis Ndks, including the conservation of the key catalytic histidine 117 (H117). However, the finding that mutation of the H117 residue does not affect GAP activity, despite significantly reduced functions in autophosphorylation, ATPase and GTPase activities \[96,166\] suggest a specific domain –present in Mtb and BCG Ndks, but absent from that of M. smegmatis– involved in GAP catalytic activity.
yet to be identified. Based on the crystal structure of Mtb Ndk, there is a distinct difference near the C-terminal region of the protein compared to that of *M. smegmatis*[^174]. In particular, alanine 136, which is responsible for closing the polypeptide chain on itself with a salt bridge to arginine 4, is substituted with glutamic acid in *M. smegmatis* Ndk. Furthermore, residues 95-99 show dissimilarities between the two proteins, and this is of importance due to it being an integral part of the conserved ‘Kpn’ loop. Both of these differences suggest that the two Ndks differ in folding patterns, subunit stability and quaternary structure in ways that might affect functional activities of Ndk from *M. smegmatis*.

The ability to construct mutant strains of mycobacteria and test individual gene products for specific functions has significantly advanced discovery of virulence factors and our understanding of TB pathogenesis. To demonstrate the relevance of Ndk-mediated deactivation of Rab5 and Rab7 to the virulence of mycobacteria, we knocked-down Ndk gene expression in BCG using established antisense strategy[^175, 176]. We observed increased fusion of phagosomes containing BCG AS-Ndk with lysosomes along with a significant decrease in bacterial survival within the macrophage. Thus Ndk appears to contribute to bacterial survival at early stages of infection. It is likely that mycobacteria continue to export more Ndk for alternate activities beyond the vicinity of the phagosome. Indeed, Mtb Ndk was shown to localize into the nucleus of the host cell and cause superoxide radical-mediated DNA cleavage[^177]. Taken together, these findings suggest that Ndk contributes to the survival of Mtb at least by two independent mechanisms: i) arrest of phagosome maturation and subsequent establishment of the infection and ii) interference with host gene expression via DNase activity.

Our finding that attenuated BCG AS-Ndk bacteria show normal growth is highly relevant to the development of live TB vaccines. Thus far, two basic strategies are being employed in the development of novel live mycobacterial vaccines. The first strategy is to improve the
immunogenicity of the existing BCG vaccine \cite{119, 178} and the second is to use attenuated Mtb itself in order to mimic natural infection \cite{87, 179}. Our demonstration of decreased survival of BCG AS-Ndk and promotion of phagolysosome fusion provides a rational and straightforward basis for extension to Mtb attenuation. By combining BCG AS-Ndk with additional mutations that disable virulence-promoting functions but preserve normal growth in culture media, it may be possible to develop a new generation of safe and effective attenuated Mtb vaccine strains that will have greater protective efficacy than BCG.

In summary, our findings suggest that mycobacterial Ndk possesses GAP activity that is trafficking within the host cell beyond phagosomes leading to inhibition of phagosome biogenesis processes dependent on Rab5 and Rab7. In doing so, Ndk contributes to intracellular survival and subsequent establishment of mycobacterial infection.

4.2.2 Disruption of NADPH oxidase assembly and activity

Because 20 years ago CGD neutrophils were found to be capable of destroying Mtb \cite{180}, functional NOX2 in the macrophage was not considered to be a major contributor to the control of mycobacterial infection. However, many field studies suggested that patients with CGD who live in TB endemic areas are more susceptible to TB than others \cite{181, 182}, which is consistent with studies demonstrating increased persistence of Mtb in the lungs of mice with defective NOX2 \cite{77}. In the current study, we used a protein knock down approach to demonstrate that Ndk enhances Mtb virulence by a mechanism dependent, at least in part, on inhibition of NOX2-mediated ROS production. In particular, we provided direct evidence that Ndk acts as a GAP to inactivate Rac1 and block phagosomal recruitment for both Rac1 and its partner molecule NOX2 subunit p67\textsubscript{phox}.

Ndk is a ubiquitous small protein (~ 15 kDa) found in virtually all organisms, from eukaryotes to prokaryotes. In Mtb, Ndk catalyzes the production of nucleoside triphosphates as
precursors for RNA, DNA and polysaccharide synthesis, which are critical for normal bacterial physiology \cite{95}. This possibly explains why our attempts to knock out the Ndk gene in Mtb were unsuccessful, suggesting that Ndk is probably essential for Mtb growth. Contrasting with this hypothesis, an effort to comprehensively identify all genes required for Mtb growth using the transposon site hybridization (TraSH) technique suggested that the Ndk gene is not essential for Mtb growth \cite{183}. However, as cautioned by the authors of that study, TraSH is simply a screening tool and therefore cannot provide a definitive conclusion about gene essentiality. Indeed, several genes known to be essential for Mtb growth, such as ideR \cite{184}, rmlD \cite{185} and whiB2 \cite{186} have not been identified as essential by the TraSH approach. Thus, whether or not Ndk is essential, is a research question that is still open for further investigation; but it remains beyond the scope of our current study, which rather focused on deciphering the mechanisms by which Ndk promotes Mtb survival in the host.

Mycobacterial Ndk has been shown to interact with and inactivate recombinant Rho, Cdc42 and Rac1 proteins \cite{96}. Our study was inspired by these findings and confirmed a part of them. In fact, we found that within the macrophage, both Mtb and recombinant Ndk (delivered on the surface of latex beads) interact with and inactivate native Rac1, but not Rho or Cdc42. This suggests that results obtained from cell free systems do not always reflect host-pathogen interactions in the whole cell system. Not surprisingly, this type of discrepancy has been observed with other pathogens that use GAP activities as a mechanism of virulence. For instance, secreted YopE from \textit{Yersinia}, and SptP from \textit{Salmonella} were shown to have GAP activity towards all three Rho GTPases extracellularly. However, YopE acts only on Rac1 and RhoA \cite{53,54}, whereas SptP inactivates Rac1 and Cdc42, but not RhoA \cite{187,188} in cultured cells. In the case of \textit{Yersinia}, a recent study established a direct link between YopE-mediated inactivation of Rac1 and inhibition of ROS production \cite{55}, consistent with our findings that
selective Ndk GAP activity towards Rac1 is sufficient to block ROS production in the macrophage. Inhibition of ROS production in nascent phagosomes has also been reported in macrophages infected with the protozoan *Leishmania*, an intracellular pathogen that is structurally and metabolically distinct from Mtb, which interferes with NOX2 by a mechanism independent of GAP activities [51, 189]. Indeed, *Leishmania* was shown to use its abundant surface lipophosphoglycan to restrict phagosomal recruitment of both p47phox and p67phox but not Rac1. Conversely, our study showed that Ndk disrupts the recruitment of Rac1 and p67phox but not p47phox consistent with previous findings that phagosomal recruitment of p67phox, but not p47phox, is stabilized by active Rac1 [72].

The overall emerging picture from ongoing studies of phagosome remodelling by Mtb suggests that more than one virulence determinant might act in concert to modulate a single event of phagosome biogenesis. For instance, the cell wall glycolipid lipoarabinomannan, which blocks the Ca^{2+} signaling pathway [25], synergizes with the acid phosphatase SapM, which hydrolyzes PI(3)P [173], to abolish phagosome maturation processes that are dependent on recruitment of early endosomal antigen 1. Such a synergism appears to also be the case for mycobacterial interference with NOX2 activity on the phagosomal membrane. Indeed, a recent study showed that the NuoG subunit of the type I NADH dehydrogenase also promotes Mtb interference with NOX2 activity, as evidenced by increased levels of ROS on Mtb ΔnuoG phagosomes [79]. However, the finding that NuoG is not secreted raises a question about the mechanistic connection between distant NuoG, contained within the mycobacteria, to NOX2 components, on the cytosolic face of the phagosome membrane. Conversely several different groups [166, 167, 190-192] have shown that Mtb Ndk is secreted, suggesting that Ndk translocates to the cytosolic surface of the vacuole where it interacts with Rac1. Such a hypothesis is supported by previous findings that live Mtb exports a variety of proteins and glycolipids intracellularly [37].
and the demonstration that many of them cross the phagosomal membrane towards the host cell cytosol to interact with and inhibit critical regulators of phagosome biogenesis \cite{37, 173, 195}.

A highly relevant finding from the present study was that Ndk knock down converted virulent Mtb into an attenuated strain that lost resistance to the hostile environment in the host both \textit{in vitro} and \textit{in vivo}. Indeed, Mtb Ndk-AS infected cells were able to generate NOX2-dependent ROS production and also to undergo apoptosis thus ensuring maximal restriction of bacterial proliferation. In contrast, virulent Mtb strains were shown to down-modulate apoptosis in favor of necrosis \cite{196, 197}, which releases viable intracellular bacilli for further spreading of the infection and tissue damage during active tuberculosis disease. The link between ROS production, apoptosis and intracellular killing demonstrated in our study is consistent with earlier findings that intracellular oxidative stress induces phosphatidylserine externalization and increased caspase 3 activity \cite{198, 199}, and that apoptosis induced by the Fas ligand attenuates Mtb survival within the macrophage \cite{200}. In addition to restricting the niche for mycobacterial replication, macrophage apoptosis contributes indirectly to the initiation of adaptive immunity mediated by dendritic cells. Indeed, infected macrophages undergoing apoptosis shed vesicles loaded with bacterial material (or apoptotic blebs) that prime dendritic cells for enhanced presentation of mycobacterial antigen to T cells \cite{87-89}.

In summary, while the role of Ndk in physiological processes has been intensively investigated, its contribution to Mtb pathogenesis has been completely ignored. Our investigations have extended our knowledge of the biological effects of Ndk, to include inactivation of macrophage NOX2 and apoptosis, therefore highlighting a novel strategy used by Mtb to circumvent host innate immunity.
4.3 Conclusion and future directions

This thesis describes the involvement of Ndk in Mtb virulence, and further discerns the mechanisms underlying its interference to host signaling at the cellular level. Ndk GAP activity in relation to host macrophage Rab5, Rab7, and Rac1 is a potent strategy employed by Mtb to subvert the host innate immune response (summarized in working model Fig. 36), ultimately leading to increased intracellular survival and potential down modulation of the adaptive immune response through inhibition of apoptosis. Based on work thus far, this project has the potential to divert into two different areas of study; the molecular level of Ndk mediated pathogenesis and animal models for *in vivo* relevance.

**Figure 36. Model for Mtb Ndk interactions within the macrophage.** This model illustrates the intracellular targets that Ndk interacts with and inactivates via its GAP activity. Multiple downstream effector interference and subsequent deactivation of the host innate response mechanisms are indicated.
To study the GAP activity of Ndk at the molecular level, the domain responsible must be identified. This is possible through the use of truncation mutants of recombinant Ndk subjected to GAP activity assays with its substrates. Then to narrow down specific residues involved, point mutations in recombinant Ndk can be generated and assessed. In addition, it would be interesting to perform crystallography analysis on the interaction between Ndk and its substrates. From there, information about the active site and substrate specificity can be gathered. Overall, results obtained from these studies could open possibilities of specific inhibitors to these domains that could be used to combat TB. The likelihood of developing a successful inhibitor to Mtb Ndk is impacted by its homology to the human gene product at the amino acid level (47%). Nevertheless, if key residues or small domains responsible for GAP activity can be defined, there is a chance it would be specific for the Mtb gene product.

An alternative study to build on work from this thesis is to further substantiate the role of Ndk \textit{in vivo}. A more comprehensive study involving bacterial load assessment in organs of infected mice would strengthen our conclusions. Further, it would be prudent to demonstrate the survival of Mtb Ndk-AS compared to wild type Mtb in mice lacking NOX2 function. We would expect that in these NOX2 deficient mice, the survival of wild type and Ndk antisense strains would be similar, thus further validating the link between ROS mediated killing in the absence of Ndk. Lastly, the observed increase in apoptosis could be further studied to assess the impact on the adaptive immune response. To study this, both cytokine production and antigen presentation can be examined in mice infected with Mtb strains. We would expect enhanced cytokine production and antigen presentation in mice infected by Mtb Ndk-AS compared to wild type Mtb. If this is the case, an argument can be made to assess the potential of Mtb Ndk-AS as a vaccine candidate. Furthermore, it is imperative to generate a vaccine which is avirulent for the safety of administration. In this context, although relatively safe, the traditional BCG vaccine remains
partially virulent in immunocompromised individuals, thus hindering its use in a large subset of
target patients who are commonly co-infected with HIV. Thus far, we have established the
reduced virulence of Mtb Ndk-AS in mice; however, this must be improved further to remove
virulence altogether, which can be possible with simultaneous deletion or knock down of an
additional virulence gene. Once such an avirulent strain has been generated, further vaccination
studies in mice or guinea pigs can be performed.

4.3.1 Significant highlights

- GAP activity towards Rab7 found present in supernatant from Mtb and BCG
- Mtb Ndk responsible for GAP activity towards host Rab7 as well as Rab5 leading to
  inhibition of phagolysosome fusion
- Mtb Ndk inactivates Rac1 resulting in disruption of NOX2 assembly and activation
- Knockdown of Ndk in Mtb/BCG reversed effects of phagolysosome fusion block and
  inhibition of NOX2 activity, ultimately leading to a significant decrease of survival
  within the macrophage
REFERENCES


