Functional analysis of nucleoside diphosphate kinase from *Mycobacterium avium* subspecies *paratuberculosis*

by

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Abstract

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University of Guelph, 2017

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Dr. Lucy Mutharia

*Mycobacterium avium* subspecies *paratuberculosis* (*Map*), the causative agent of Johne’s disease, is an intracellular pathogen capable of surviving and replicating within macrophages for extended periods with minimal activation of the innate immune response. Mycobacterial secreted protein, nucleoside diphosphate kinase (Ndk), altered signalling pathways for macrophage activation, subverting host innate immune responses, disrupting maturation of membrane-bound phagosomes, and enabling the intracellular survival of mycobacteria within macrophages. Rab small GTPase proteins play a major role in phagosome maturation, facilitating the transition from early phagosomes to phagolysosomes, where bacteria are degraded. Nucleoside diphosphate kinase (Ndk) from *Mycobacterium tuberculosis* inactivated Rab proteins, leading to evasion of innate immunity by interfering with phagosome maturation. Ndk is also a housekeeping enzyme, important in the maintenance of nucleoside triphosphate (NTP) pools, with histidine at position 117 (H117) involved in autophosphorylation and phosphotransfer activity, and synthesis of NTPs. In my thesis research, I have cloned and expressed putative recombinant *Map* Ndk (*Map rNdk*) in *E. coli* and generated anti-Ndk polyclonal antibodies in rats. Native *Map* Ndk was detected by immunoprecipitation using anti-Ndk antibodies. The interaction of *Map rNdk* with bovine macrophage proteins involved in phagosome maturation (Rab5 and Rab7) and other *Map* proteins, as well as its immunogenicity during natural infection were examined. In contrast to studies with *M. tuberculosis*, no interactions were observed between *Map rNdk* and Rab5 or Rab7. *Map rNdk-H117* catalytic mutants were constructed and will be discussed.
Acknowledgments

I would like to thank my advisor Dr. Lucy Mutharia for giving me this opportunity to be a member of her lab and taking me on as a graduate student to contribute to research on *Mycobacterium paratuberculosis* and Johne’s disease. Dr. Mutharia has helped me tremendously over the course of my project and always challenged me to think critically. I would also like to thank Dr. Joseph Lam for offering his guidance and expertise. Their leadership and knowledge have allowed me to flourish in my field and greatly enhanced my capabilities as a researcher and scientist.

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Finally, I would like to thank my family for their unwavering support, love, and encouragement.
Declaration of Originality

I hereby confirm that I am the sole author of the written work here enclosed and that I have compiled it in my own words. I have cited and acknowledged all persons who were significant facilitators of the work.

I carried out and completed all experimental procedures, data analyses, composition of figures and tables, and writing of the manuscript with the following exceptions:

- Serum from *Map*-infected cattle (cattle with Johne’s disease) was generously provided by Dr. David F. Kelton, Department of Population Medicine, OVC, University of Guelph
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>BoMAC</td>
<td>Bovine macrophage cell line</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>eATP</td>
<td>Extracellular ATP</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>Elongation factor thermo unstable</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibronectin-attachment protein</td>
</tr>
<tr>
<td>FcR</td>
<td>Immunoglobulin receptor</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GEC</td>
<td>Gingival epithelial cells</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HOPS</td>
<td>Homotypic protein sorting</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat-shock protein</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IS</td>
<td>Insertion sequence</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-associated membrane protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LSP</td>
<td>Large sequence polymorphism</td>
</tr>
<tr>
<td>M cells</td>
<td>Microfold cells</td>
</tr>
<tr>
<td>Man-LAM</td>
<td>Mannosylated lipoarabinomannan</td>
</tr>
<tr>
<td>Map</td>
<td>Mycobacterium avium subspecies paratuberculosis</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophages</td>
</tr>
<tr>
<td>Mtb</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>Ndk</td>
<td>Nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>NDP</td>
<td>Nucleoside diphosphate</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleoside triphosphate</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS containing Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCR-RE</td>
<td>Polymerase chain reaction-restriction endonuclease</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PI3P</td>
<td>Phosphatidylinositol-3-phosphate</td>
</tr>
<tr>
<td>P2X7-R</td>
<td>P2X7 purinergic receptor</td>
</tr>
<tr>
<td>RILP</td>
<td>Rab7-interacting lysosomal protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar adenosine triphosphatase</td>
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</table>
Chapter 1: Literature review

1.1 Introduction to Mycobacterium avium subspecies paratuberculosis

*Mycobacterium* is the single genus within the family Mycobacteriaceae and contains more than 150 species that are mostly saprophytic/nonpathogenic and a small proportion that are opportunistic pathogens that can infect humans and animals. Mycobacteria are identified as belonging to the high G+C Gram-positive bacteria based on 16S ribosomal RNA sequence comparison, with a GC content in their genomes in the range of 62-70% (Rastogi et al., 2001; Fu and Fu-Liu, 2002). However, the cell wall of mycobacteria, like the Nocardia, stain Gram-variable using the Gram stain (they are weakly Gram positive) and are identified as acid-fast using the Ziehl-Neelsen stain (Berd, 1973; Trifiro et al., 1990). Mycobacteria are aerobic, non-motile, rod-shaped actinomycetes with a lipid-rich cell envelope (constituting up to ~60% of the dry cell mass depending on species) that provides a highly effective permeability barrier to hydrophilic and antibiotics (Jarlier and Nikaido, 1990; Jarlier and Nikaido, 1994; Brennan and Nikaido, 1995; Rastogi et al., 2001).

Mycobacteria are classified into three groups based on phenotypic characteristics and pathogenicity: saprophytes, such as *M. smegmatis* and *M. phlei*; opportunistic pathogens, such as *M. simiae, M. xenopi,* and subspecies within the *M. avium* complex; and obligate pathogens, such as *M. avium subsp. paratuberculosis,* *M. leprae,* and the *M. tuberculosis* complex (e.g., *M. tuberculosis, M. bovis*) (Rastogi et al., 2001). Growth rate on laboratory medium was used to differentiate between saprophytes and opportunistic pathogens. The former group shows visible colonies in 3-7 days, while the latter group are slow-growing opportunistic and obligate pathogens, with colonies becoming visible in weeks to months. For this literature review, I will focus on *Mycobacterium avium* subspecies *paratuberculosis* biology, Johne’s disease, host
innate immune responses during infection, and the role of bacterial nucleoside diphosphate kinase (Ndk) in virulence.

The *M. avium* complex includes two species, *M. avium* and *M. intracellulare* (Turenne et al., 2007). The *M. avium* species includes four subspecies, *M. avium* subsp. *hominissuis* (*Mah*), *M. avium* subsp. *avium* (*Maa*), *M. avium* subsp. *silvaticum* (*Mas*) and *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Although very closely related (<95% nucleotide sequence similarity across their genomes), species within the *M. avium* complex are distinct and represent a wide variety of environmental and animal-associated organisms that vary significantly in host preference, pathogenicity, and distribution in the environment (Paustian et al., 2005; Turenne et al., 2007). *Map* is an obligate intracellular, infectious enteric pathogen and the causative agent of Johne’s disease in ruminant animals (Harris and Barletta, 2001). *Map* bacteria grow extremely slowly (generation time of 22-26 h or more), are mycobactin auxotrophs requiring mycobactin J supplementation for *in vitro* culture, and are characterized by the presence of insertion sequences (e.g. *IS900*, *IS1311*, *ISMAP02*), large sequence polymorphisms (e.g. LSPA 18, LSPA 20), and conserved *hspX*, and *hsp65* genes (Francis et al., 1953; Collins et al., 1989; Collins et al., 1990; Thorel et al., 1990; Whittington et al., 1998; Bannantine et al., 2003). Although *Map* bacteria cannot replicate in the environment, fecal-shed bacteria persisted for long periods of time in manure and soil by entering a state of dormancy and forming a spore-like structure (Whittington et al., 2004; Lamont et al., 2012a). Furthermore, *Map* can enter and replicate within free-living environmental amoebae, which provide an ecological niche outside the animal host and may enhance the virulence of the organism (Cirillo et al., 1997; Whan et al., 2006).

Three *Map* subspecies are distinguished based on genetic differences such as genome size, insertion sequences, growth characteristics and nutritional requirements in vitro, and host
tropism (Collins et al., 1990; Whittington et al., 1998; Whittington et al., 2001; Sohal et al., 2010, Stevenson, 2015). The faster growing cow (C) type or type II, sequenced by Li et al. (2005) and Wynne et al. (2010), was isolated from a broad range of domestic and wild species. Domestic animals include cattle, sheep, goats, camels, and farmed deer (Collin et al., 1990; Godfroid et al., 2000; Begg et al., 2005; de Juan et al., 2005; Alharbi et al., 2012). Some of the wild species, which may serve as a reservoir of infection against hosts include bison, rabbits, wild boar, feral cats, and free-ranging birds (Buergelt et al., 2000; Beard et al., 2001; Alvarez et al., 2005; Palmer et al., 2005; Corn et al., 2005). The C type was also isolated from non-human primates and has been linked to human Crohn’s disease (Uzoigwe et al., 2007; Singh et al. 2011). Several studies have detected Map bacteria by PCR and culture methods from a proportion of patients with Crohn’s disease (Collins et al., 2000; Ikonomopoulos et al., 2000; Ryan et al., 2002; Autschbach et al., 2005). Although there is still a debate over the role of Map in Crohn’s disease, the possible involvement has raised significant public concern (Hermon-Taylor, 2001; Bannantine et al., 2004; Grant, 2006). The slower-growing, fastidious sheep (S) type or type I/III, has a narrow host range that includes sheep, camels, and goats (de Juan et al., 2005; Biet et al., 2012; Ghosh et al., 2012). Bison type is isolated from bison and is the predominant Map strain isolated from animals, including human beings, in India. (Sohal et al., 2010).

1.2 Johne’s disease, diagnosis, and treatment

Johne’s disease is a contagious infection, characterized by chronic granulomatous inflammation in the small intestine and is recognized as a serious problem worldwide due to its significant economic impact for the dairy cattle industry (Benedictus et al., 1987; Kormendy et al., 1989; Ott et al., 1999; Tiwari et al., 2006). Clinical symptoms, including diarrhea, decreased milk
production, and weight loss, were first described by Johne and Frothingham (1895) but the requirements for Koch’s postulates were not realized until Twort and Ingram (1912) successfully cultured *Map* and transmitted the infection to healthy calves. The majority of cattle with Johne’s disease are infected in the early stages of life, where newborn calves less than 6 months of age are most susceptible (Taylor, 1953; Payne and Rankin, 1961; Windsor and Whittingston, 2010). Factors contributing to individual animals being able to control the infection include host genetics, environmental conditions, infectious dose, and genetic diversity of *Map* strains (Koets et al., 2000; Begg et al., 2005; Sweeney et al., 2006; Gollnick et al., 2007; Verna et al., 2007; Singh et al., 2009; Singh et al., 2012). Johne’s disease can be divided into four stages based on symptoms, pathology, and host immune responses (Whitlock and Buergelt, 1996; Tiwari et al., 2006; Rosseels and Huygen, 2008). The silent stage is the period immediately following ingestion of live *Map* bacteria when the host immune response either clears or limits pathogen growth; animals in this phase are not detected by clinical, immunological or molecular-based tests. The subclinical or preclinical stage is characterized by a protective cell-mediated immune response with intermittent and low fecal shedding of *Map* bacteria. Animals in this phase may be detected by fecal PCR of *Map* DNA but not by current antibody-based tests. The late subclinical phase is characterized by moderate to heavy fecal *Map* shedding and seroconversion to an antibody-mediated response, where bacteria can be detected by culture, PCR, and by ELISA tests. The clinical stage is characterized by chronic diarrhea, weight loss, and eventual death (Whitlock and Buergelt, 1996; Tiwari et al., 2006; Rosseels and Huygen, 2008).

There are currently no effective vaccines available for Johne’s disease and diagnosis, particularly at the asymptomatic stage (preclinical and subclinical), remains challenging (Sweeney et al., 2006). Serodiagnosis by ELISA was recommended as the primary methodology
used to detect *Map*-infected animals (Collins et al., 2006). Thus far, all the current, commercially available ELISAs have high specificity (90-99%) but suffer from poor sensitivity (13-42%) (Sweeney et al., 2006). As there is no effective treatment, the recommended control of Johne’s disease at the herd level involves the culling or removal of infectious animals to limit contact with calves and animal husbandry practices (van Roermund et al., 2007; Collins et al., 2010).

1.3 Economic impact and prevalence of Johne’s disease

Financial losses associated with Johne’s disease are due to decreased milk production, premature culling, lower slaughter value due to body-weight losses, fertility problems, and an increased susceptibility to other diseases (Benedictus et al., 1987; Kormendy et al., 1989; Ott et al., 1999; McKenna et al., 2006; Nielsen and Toft, 2009). In the U.S., 68% of dairy herds have been shown to have at least one cow infected with *Map* (Ott *et al.*., 1999; NAHMS, 2008). In Canada, dairy herds with at least one cow infected with *Map* ranges from 33% in Prince Edward Island to 74% in Alberta, with estimated annual costs in economic losses of up to CDN$15 million (Mckenna *et al.*, 2006; Tiwari *et al.*, 2006). These figures were based on ELISA tests, all of which suffer from poor sensitivity. Therefore, there are uncertainties of the true prevalence of infection and economic losses associated with Johne’s disease may be higher (Sweeney *et al.*, 2006).

1.4 Transmission and host adaptive immune response

*Map* is most commonly transmitted via the fecal-oral route, where it is shed in feces from infected animals, further contaminating the environment and spreading the infection to susceptible, usually young, animals (Yayo Ayele *et al.*, 2001; Sweeney, 2011). In utero infection (vertical mode of transmission) is likely to occur in a proportion of animals (Sweeney *et al.*, 1992b; Sweeney *et al.*, 2012). Infected animals can also shed *Map* in milk or colostrum, leading
to direct transmission from dam to calf (Sweeney et al., 1992a; Streeter et al., 1995). If the
infection is not cleared during the silent phase, animals progress to the subclinical stage lasting
up to 10 years or more (Doyle and Spears, 1951, Larsen et al., 1975). Adaptive immune
responses during the subclinical stage were initially associated with a dominant Map-specific T-
helper 1 (Th1) cell-mediated response, characterized by high levels of interferon-gamma (IFN-
γ), with a switch to a dominant Th2 humoral (antibody) response in the clinical stages,
characterized by high levels of antibodies (Clarke et al., 1996; Sweeney et al., 1998; Perez et al.,
1999; Stabel, 2000). However, observations from several studies have questioned the classical
Th1/Th2 switch, as the switch may occur earlier than previously reported and both T helper cell
types may be present during early infection (Waters et al., 2003; Robinson et al., 2008; Begg et
al., 2010; Mortier et al., 2014). These results highlighted the limitations in the current
understanding of the regulation of adaptive immune responses during Map infection and whether
a protective role of antibodies in the control of mycobacterial diseases exists. As such, it will be
important to identify Map-specific antigens that induce early responses to improve diagnosis of
infected animals before fecal shedding of Map bacteria and to limit the spread the infection to
susceptible animals (Begg and Griffin, 2005; Sohal et al., 2008; Facciolo et al., 2013; Facciulo et
al., 2016).

1.5 Map invasion of the intestinal mucosal barrier

After oral ingestion, Map bacteria travel through the ruminant digestive system, where the acidic,
hypoxic, and high osmolality conditions can increase invasion and virulence. These conditions
increased the binding between cell wall proteins and extracellular matrix host proteins to mediate
attachment and entry into intestinal epithelium, and facilitated macrophage invasion (Secott et
al., 2002; Secott et al., 2004; Patel et al., 2006; Wu et al., 2007). Microfold (M) cells overlying the regions of ileal and jejunum Peyer’s patches were identified as the main route of entry enabling Map to invade the intestinal epithelial barrier of calves (Momotani et al., 1988), although uptake through epithelial cells was also demonstrated (Lamont et al., 2012b). The uptake by M cells in mice depended on Map cell wall-associated fibronectin-attachment protein (FAP), which binds to host extracellular matrix fibronectin proteins and mediates adhesion via α5β1 integrin receptors on the apical surface of M cells (Secott et al., 2002, 2004). The uptake of Map was equally efficient across both the jejunum and ileum epithelium (Ponnusamy et al., 2013; Facciuolo et al., 2016). Sigurdardóttir et al. (2005) showed that Map bacteria entered the intestinal mucosa through both M cells and enterocytes in regions with and without Peyer’s patches. However, Ponnusamy et al. (2013) showed the level of uptake in regions that do not contain Peyer’s patches were relatively low, suggesting the preferential uptake by M cells rather than by enterocytes. Interestingly, the uptake of Map by enterocytes induced pro-inflammatory responses, which were induced by engaging microbial pattern recognition receptors (PRRs), such as the membrane-bound Toll-like receptor 9 (TLR9) and the cytosolic nucleotide-binding oligomerization domain protein 1 (NOD1) receptor (Pott et al., 2009; Bermudez et al., 2010; Lamont et al., 2012b). Uptake by murine intestinal epithelial cells and bovine epithelial cells induced the secretion of chemokine macrophage inflammatory protein 2 (MIP-2) and pro-inflammatory cytokine interleukin-1 beta (IL-1β), respectively, which recruit tissue-resident macrophages (Pott et al., 2009; Lamont et al., 2012b). Uptake by M cells rather than epithelial cells may be more advantageous since M cells rarely induce a host response to invading pathogens (Kucharzik et al., 2000).
1.6 *Map* infection of macrophages and phagosome maturation

After crossing the intestinal epithelial barrier, *Map* is phagocytosed by subepithelial macrophages in the lamina propria. Entry of *Map* into macrophages is mediated by complement receptors (CD11b/CD18), integrin receptors (CD11/CD18), mannose receptors, CD14, and the immunoglobulin receptor (FcR) (Hostetter et al., 2005; Souza et al., 2007). Phagocytes, such as macrophages, are important cells of the innate immune system and the host’s first line of defense against invading bacterial pathogens (Smith et al., 2011). Particulate materials internalized by phagocytes become sequestered in a plasma membrane-derived, membrane-bound vacuole, called the phagosome (Allen and Aderem, 1996). Phagosome maturation is a dynamic process that involves fusion and fission events with subcompartments of the endocytic pathway in a sequential ordered manner (Fig. 1) (Desjardins et al., 1994a, b; Desjardins et al., 1997; Jahraus et al., 1998). The ‘kiss and run’ hypothesis suggests that phagosomes make a transient contact (‘kiss’) and connection with early endosomes is initiated by the formation of a fusion pore, followed by the fission (run) of the organelles, allowing the transfer of luminal contents and exchange of membrane markers at the site of contact. The acquisition of later markers together with loss of early markers by maturing phagosomes allows subsequent fusion of phagosomes with later endosomes (Desjardins, 1995). Acidification of phagosomes, like endosomes and lysosomes, is regulated by vacuolar H+ ATPase (V-ATPase) trafficked to the membrane of the maturing phagosome along with cathepsins (a family of acidic proteases) and lysosomal hydrolases (Swallow et al., 1998; Lukacs et al., 1990; Tapper et al., 1995; Fratti et al., 2003). Rab5 and Rab7 are small GTPase proteins important in regulating membrane trafficking and are commonly used as markers for the early endosome and late endosome, respectively (Gorvel et al., 1991; Bucci et al., 1992; Feng et al., 1995).
Small GTPases are monomeric GTP-binding proteins with a molecular mass of ~20-40 kDa and known to regulate a variety of cellular functions by interacting with multiple distinct downstream effectors (Takai et al., 2001, Wennerberg et al., 2005). Small GTPases act as molecular switches that cycle between the cytosolic, inactive GDP-bound state, and the membrane-associated, active GTP-bound state (Wennerberg et al., 2005). However, they possess low intrinsic GTP hydrolysis activity and GDP/GTP exchange capabilities are regulated by a guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP). GEF activates small GTPases by promoting the exchange of GDP for GTP whereas GAP inactivates them by accelerating intrinsic GTP hydrolysis and converts active GTP-bound form to inactive GDP-bound form (Schmidt and Hall, 2002; Bernards and Settleman, 2004). Another negative regulator, guanine nucleotide dissociation inhibitor (GDI), is a feedback mechanism able to keep G proteins in the inactive GDP-bound form (Seabra and Wasmeier, 2004). The GDP to GTP switch induces a conformational change in the protein allowing for interaction with downstream effectors. Rab5 functions by interacting with multiple effectors, such as the protein kinase p150, human vacuolar protein sorting 34 (hVPS34), and early endosome autoantigen 1 (EEA1) (Simonsen et al., 1998; Callaghan et al., 1999; McBride et al., 1999). The adaptor protein p150 possesses a kinase domain that recruits hVPS34, a class III phosphatidylinositol-3-kinase (PI3K), which in turn phosphorylates phosphatidylinositol (PI) to generate phosphatidylinositol-3-phosphate (PI3P) (Christoforidis et al., 1999; Vieira et al., 2001). The effector protein EEA1, which directly interacted with Rab5, was recruited to the phagosomal membrane by binding to PI3P through its FYVE domain and was anchored by PI3P on the cytosolic side of the phagosome to mediate fusion with the early endosome and development of the late endosome (Patki et al., 1997; Gaullier et al., 1998). The transferrin receptor is another early endosomal
marker and is transported to the cell surface following synthesis in the endoplasmic reticulum and passage through the Golgi complex (Daurty-Varsat et al., 1983; Klausner et al., 1983). These receptors bind iron that is bound to transferrin and are internalized to fuse with early endosomes, where acidification of this endosomal compartment would release the iron from transferrin and both the transferrin and receptor are recycled back to the cell surface (Daurty-Varsat et al., 1983; Klausner et al., 1983). The exchange of Rab5 with Rab7 was mediated by the homotypic and vacuole protein sorting (HOPS) complex, and Rab7 was then able to recruit Rab7-interacting lysosomal protein (RILP), mediating fusion of the late endosome with the lysosome (Rink et al., 2005; Poteryaev et al., 2010). RILP possesses a domain that binds to GTP-bound Rab7 and another domain that recruits the dynein/dynactin complex, a microtubule-associated complex, facilitating the fusion with late endosomes (Jordens et al., 2001; Harrison et al., 2003).

Lysosomal-associated membrane protein (LAMP) is a late maturation marker found on late endosomes and lysosomes. Phagosomes then fused with and acquired late maturation markers from lysosomes and transformed into phagolysosomes, identified by the presence of hydrolytic proteases (cathepsin D) and a very acidic pH reported to be as low as 4.5 (Botelho et al., 2000). At low pH, cathepsin became activated and degraded phagocytosed particles (Diment and Stahl, 1985; Lennon-Duménil et al., 2002). Phagocytosed peptides were loaded onto MHC-II and activated effectors of the adaptive immune response (Houde et al., 2003; Ramachandra et al., 2008; Byun et al., 2012).

During phagocytosis, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) of macrophages was activated by pattern recognition of microbial structures, which assembled and fused with the phagosomal or plasma membrane (Borregaard et al., 1993; Garcia and Segal, 1984; Jancic et al., 2007). Activation occured by recognition of microbial
Figure 1. Stages of phagosome maturation. After phagocytic uptake of bacteria, membrane-bound phagosomes undergo several fusion and fission events with subcompartments of the endocytic pathway in a sequential ordered manner. Bacterial pathogen-associated molecular patterns (PAMPs) activate macrophages, inducing NOX2 and V-ATPase to assemble and fuse with the plasma and phagosomal membrane. The nascent phagosome then fuses with the early endosome and acquires early maturation markers, transferrin receptor and Rab5. GTP-bound Rab5 recruits the downstream effector VPS34 kinase, which phosphorylates PI to generate PI3P. The effector protein EEA1 is recruited to the phagosomal membrane, binds to PI3P and interacts directly with Rab5. The early phagosome can then fuse with the late endosome and acquire late maturation marker, Rab7, which in turn recruits RILP to mediate fusion with the lysosome and formation of the phagolysosome, characterized by the late maturation marker, LAMP. The increased acquisition of V-ATPases by the maturing phagosome significantly decreases the pH as indicated. The acidic protease procathepsin D is also acquired and undergoes proteolytic processing to produce the mature, active enzyme. Cathepsin D is active at low pH and begins to degrade phagocytosed particles. Cathepsin D also plays a role in activating MHC-II onto which peptides are loaded for antigen presentation. The macrophage then switches to the function of antigen presentation as they migrate to lymph nodes for proper detection by effectors of the adaptive immune response. Figure was modified and adapted from Poirier and Av-Gay (2012).
structures called pathogen-associated molecular patterns (PAMPs), such as Man-LAM and LPS, that interacted with pattern recognition receptors (PRRs) of macrophages (e.g. TLRs and NOD-like receptors) (Schlesinger et al., 1994; Medzhitov and Janeway, 2000; Ferwerda et al., 2007). NOX2 is an enzyme complex composed of 2 transmembrane proteins, gp91phox and gp22phox, and four cytosolic proteins: p40phox, p47phox, p67phox, and the small GTPase, GDP-bound Rac1 (Parkos et al., 1997; Clark et al., 1990; Abo et al., 1991; Wientjes et al., 1993). This enzyme is involved in the production of reactive oxygen species (ROS), such as superoxide (O2\(^{-}\)), hydrogen peroxide (H₂O₂), and hydroxyl radicals (Roos et al., 1984; Bannister et al., 1992 Karakousis et al., 2004, Flannagan et al., 2009). Activation of macrophages also resulted in the production of nitric oxide (reactive nitrogen species or ROS) by inducible nitric oxide synthase (iNOS), which was recruited to the phagosomal membrane (Schmidt et al., 1992; Vodovoz et al., 1995). Production of free radicals are an important part of the innate immune system and essential to defend against invading pathogens by targeting microbial proteins, lipids, and DNA (de Zwart et al., 1999).

In order for intracellular bacteria to survive within macrophages, the process of phagosome maturation must be disrupted to avoid exposure to free radicals and proteolytic hydrolysis by the acidic enzymes of the lysosome. The ability of Map bacteria to resist intracellular killing by blocking phagosome maturation was reported, although the mechanism has not been fully defined (Clemens and Horwitz, 1995; Kuehnel et al., 2001; Cheville et al., 2001; Woo et al., 2007). Kuehnel et al. (2001) showed that phagosomes with live Map bacteria were only mildly acidified (~pH 6.3) following infection of a murine macrophage cell line compared to heat-inactivated Map (~pH 5.2), and was related to the lack of recruitment of vacuolar H-ATPase (Sturgill-Koszycki et al., 1994; Xu et al., 1994). There were increased levels
of the early maturation marker, transferrin receptor, and decreased levels of late maturation marker, LAMP, on phagosome containing live *Map* bacteria relative to those containing heat-inactivated *Map*, again indicated a block in phagosome maturation (Kuehnel et al., 2001; Hostetter et al., 2003). Woo et al. (2007) showed poor phagosome-lysosome fusion in *Map*-infected bovine monocytes with no significant production of ROS and RNS. These results collectively showed that *Map* bacteria were able to survive within macrophages and subvert host innate immune responses.

### 1.6.1 Inflammation and the role of extracellular ATP

Inflammation is an important homeostatic response managed by a complex network of pathways that determine the level, intensity, and localization of inflammation (Chen and Nunez, 2010). It is a component of host innate defences facilitating control of tissue trauma and infection by pathogens through activation of complement and recruitment and activation of phagocytes. Host-pathogen interactions result in pro-inflammatory responses and can lead to cell death by apoptosis (programmed cell death) or necrosis that produce different morphological and physiological outcomes. The formation of granulomatous lesions (as manifested in Johne’s disease, tuberculosis, and bovine tuberculosis) is characterized by a localized collection of epithelioid cells, macrophages, and lymphocytes, originating from circulating monocytes recruited to the site of inflammation (Sheffield, 1990). Infection of peripheral blood mononuclear cells with *Map* bacteria induced formation of microgranulomas in vitro (Abendaño et al., 2014) and lambs orally fed *Map* strain K-10 bacteria developed granulomas *in vivo* (Dukkipati et al., 2016). In immunodeficient hosts, granuloma formation results in minimal tissue necrosis and apoptosis, characterized by multibacillary granulomata (Molloy et al., 1994; Lammas et al.,
In contrast, immunocompetent hosts develop paucibacillary granulomata with a central core of dying macrophages (necrosis) surrounded by macrophages undergoing caspase-dependent apoptotic cell death in contact with activated lymphocytes. Apoptotic cells are rapidly cleared by recruited phagocytes facilitating tissue repair (Elliot et al., 2009). Viable bacilli released from host macrophages as a result of cell death by necrosis or apoptosis are phagocytosed by blood-derived monocytes migrating to the site of inflammation/infection. Recruited phagocytes are less permissive for intracellular growth and infected cells reinitiate apoptosis, facilitating clearance of the pathogen (Molloy et al., 1994).

The purinergic pathway, comprised of extracellular ATP (eATP) and other nucleotides, as well as its metabolite adenosine, is one such pathway regulating inflammatory responses (Dubyak and El-Moatassim, 1993; Di Virgilio et al., 2001; Bours et al., 2011). The release of ATP, which can be metabolized by cell surface-associated ecto-enzymes (e.g. ectoATPase, ecto-5’-nucleotidase, adenosine deaminase), can signal through a broadly expressed family of membrane-bound receptors expressed throughout body tissues on a wide variety of immune and non-immune cells. P2 purinergic receptors for ATP and related nucleotides (e.g. ADP) and P1 purinergic receptors for adenosine (Fig. 2). The release of ATP and other intracellular signalling molecules (e.g. uric acid and heat shock proteins) are known as damage-associated molecular patterns (DAMPs) that serve as danger signals for host cells that respond to changes in their external environment and play a critical role in inflammation (Quintana and Cohen, 2005; Chen and Nunez, 2010; Kono et al., 2010). These molecules act in concert to regulate a variety of cellular processes and to control the nature, intensity, and resolution of the inflammatory response. Extracellular ATP induces maturation of dendritic cells, secretion of the proinflammatory cytokine IL-1β in macrophages leading to ROS production, chemotaxis of
neutrophils, and stimulated effectors of the adaptive immune response (Freyer et al., 1988; Oryu et al., 1996; Ferrari et al., 1997a, b; Wilkin et al., 2001; Corriden et al., 2008; Bours et al., 2011). These danger signal responses complemented and further activated innate defences (phagosome maturation, production of free radicals), both of which effectively link innate immune responses with adaptive immune responses required to destroy pathogens.

P1 receptors contribute to immunosuppressive responses that act in later stages as immunoregulatory feedback mediators (Di Virgilio and Vuerich, 2015). P2Y receptors are G-protein-coupled receptors that are responsive to ATP, ADP, UDP, and UTP, which induced migration/recruitment and maturation of circulating cells (e.g. monocytes, neutrophils) (Aliteri et al., 1990; Fredholm et al., 1994). P2X receptors are ATP-gated ion channels that induced the formation of nonselective membrane cation transport pores in mouse macrophages with rapid Na\(^+\) and Ca\(^{2+}\) influx and K\(^+\) efflux (Buisman et al., 1988; Steinberg et al., 1987a, b; Surprenant et al., 1996; Rassendren et al., 1997; Ferrari et al., 1999). Seven P2X (P2X\(_1-7\)) subtypes are currently known, with P2X-7 being the most studied, and will be the focus of my review (Di Virgilio and Vuerich, 2015). ATP-induced P2X-7 responses in mouse macrophages caused depolarization of the membrane, which was reversed upon removal of extracellular ATP (Steinberg et al., 1987a). However, sustained activation by exogenous eATP caused cell death of mouse macrophages by apoptosis (Steinberg et al., 1987a). Although ATP is present in millimolar concentrations (3-10 mM) in the cytosol, its presence extracellularly was much lower (~500 nM range in plasma), where eATP levels are kept extremely low by minimal permeation across the lipid bilayer and ecto-enzymes that rapidly hydrolized ATP (Born and Kratzer, 1984; Ryan et al., 1996; Bours et al., 2011; Di Virgilio and Vuerich, 2015). Thus, release of ATP most
Figure 2. Role of endogenous extracellular ATP in innate immune responses. Specific physiological or pathological conditions are stimuli for the release of intracellular ATP. ATP can accumulate in the extracellular space by vesicular or granular release, active transport by plasma membrane channels (e.g. ATP-binding cassette transporter or ABC transporter, such as P glycoprotein and cystic fibrosis transmembrane conductance regulator), or released in massive amounts accompanying necrotic cell death. Extracellular ATP acts on P2Y and P2X purinergic receptors to induce immunomodulatory responses, and its metabolite adenosine acts on P1 receptors (not shown) to induce immunosuppressive responses, ATP metabolism is achieved by cell surface-associated ecto-enzymes (e.g. ecto-ATPases). Figure was modified and adapted from Bours et al. (2011).
frequently occurs by leakage after cell death by necrosis, or accidental cell death, at sites of infection and inflammation. However, ATP was also released by active transport via plasma membrane channels (e.g. P glycoprotein, pannexin channel, cystic fibrosis transmembrane conductance regulator) or packaged within secretory granules or vesicles and released extracellularly from platelets of humans and other animals (e.g. cat, cattle, horse, pig), human neutrophils, and human mast cells (Meyers et al., 1982; Born and Kratzer, 1984; Cockcroft and Stutchfield, 1989; Abraham et al., 1993; Jiang et al., 1998; Pelgrin and Surprenant, 2006; Sandilos et al., 2012).

Several studies have demonstrated the release of ATP from mouse and human cells infected with pathogens or pathogen-derived immunomodulatory molecules (e.g. LPS). Ferrari et al. (1997a) showed ATP was released from human macrophages stimulated with LPS. Extracellular ATP was significantly higher (~1300 pM) in human epithelial cells infected with pathogenic E. coli strain compared to uninfected cells (~500 pM) (Säve and Persson, 2010). ATP released from mouse macrophages infected with clinical strains of E. coli and S. aureus were demonstrated in vitro (Xiang et al., 2013). Primary mouse macrophage cells infected with E. coli and S. aureus released significantly higher ATP (~4000 pM for E. coli infected-macrophages and ~7000 pM for S. aureus infected-macrophages) compared to uninfected macrophage cells (~200 pM). Choi et al. (2013) demonstrated the release of intracellular ATP from primary gingival epithelial cell cultures infected with P. gingivalis. Moreover, ATP was released from murine cancer tumour cells dying after exposure to cytotoxic agents (Ghiringhelli et al., 2009).

The interaction of extracellular ATP with P2X7 receptor induced the secretion of the pro-inflammatory cytokine IL-1β from human macrophage cell lines exposed to LPS, which was blocked with a P2X7 inhibitor (Ferrari et al., 1997b). Moreover, mouse macrophages obtained
from a P2X7 receptor-deficient mouse line did not secrete IL-1β after treatment with LPS, clearly demonstrating this plasma membrane protein was responsible for ATP-induced release of IL-1β (Solle et al., 2001). Exposure of human macrophages to ATP induced the activation of caspase-1, -3, and -8 by acting on P2X7, and apoptotic DNA fragmentation (Ferrari et al., 1999).

Extracellular ATP activated the NOD-like receptor family member (Nalp3), which associated with caspase-1 and other proteins to activate the inflammasome, inducing the secretion of the proinflammatory cytokine IL-1β and production of ROS by mouse macrophage (Mariathasan et al., 2006; Cruz et al., 2007). ATP-induced apoptotic cell death in murine and human macrophages resulted in DNA fragmentation, membrane blebbing, shrinkage of cytoplasm, and chromatic condensation (Molloy et al., 1994; Ferrari et al., 1999; Verhoef et al., 2003).

1.6.2 Effects of extracellular ATP on mycobacteria-infected macrophages

Molloy et al. (1994) showed ATP-induced apoptosis enhanced intracellular killing of avirulent *M. bovis* BCG in monocyte-derived macrophages (MDMs) isolated from healthy donors. In contrast, H2O2-induced necrosis of *M. bovis* BCG-infected human macrophages had no effect on bacilli viability, suggesting the reduction in *M. bovis* BCG viability was linked to host cell apoptosis and not necrosis based on differences in morphological criteria between these two types of cell death. Lammas et al. (1997) also demonstrated enhanced killing of *M. bovis* BCG-infected human MDMs after ATP-induced apoptosis. ATP-induced responses involved in killing bacilli were mediated by P2X7 purinergic receptors, and were blocked by P2X7 antagonists (Lammas et al., 1997). Moreover, infected macrophages with discrete vacuoles containing single bacilli became swollen with multiple bacilli following exogenous eATP treatment of human MDMs, which indicated the formation of the phagolysosome and increased bacterial killing.
(Molloy et al., 1994; Lammas et al., 1997). Human MDMs infected with virulent strains of *M. tuberculosis* induced phagosome-lysosome fusion and increased killing of intracellular bacilli following eATP treatment compared with untreated *M. tuberculosis*-infected MDMs (Kusner and Barton, 2001). ATP-mediated killing of *M. bovis* BCG-infected primary mouse macrophages was abolished in macrophages with a P2X7 gene disruption (Fairbain et al., 2001). Furthermore, a loss-of-function polymorphism in the human P2X7 receptor abolished ATP-induced killing of *M. bovis* BCG-infected human MDMs (Saunders et al., 2003).

In contrast to studies with *M. tuberculosis* and *M. bovis* BCG, bovine monocytes isolated from peripheral blood infected with the virulent *Map* strain K-10 were not killed following eATP treatment (Woo et al., 2007). Extracellular ATP killed *Map*-infected and uninfected monocytes equally but did not affect intracellular *Map* viability (Woo et al., 2007). Woo et al. (2009) later showed that culture supernatants of *Map*-infected and uninfected bovine monocytes had equal concentrations of endogenous eATP, which is in contrast to previous studies demonstrating release of ATP after infection with pathogens or stimulation with bacterial antigens (Ferrari et al., 1997a; Säve and Persson, 2010; Xiang et al., 2013). The release of ATP by macrophages infected with mycobacteria has not been reported. Interestingly, removal of endogenous eATP from *Map*-infected bovine MDMs by apyrase, which hydrolyzed ATP, decreased survival of intracellular bacilli while addition of exogenous ATP increased survival of bacilli (Woo et al., 2009). Again, this is in contrast to previous studies where addition of exogenous eATP increased killing of *M. tuberculosis* and *M. bovis* BCG in murine and human macrophages (Molloy et al., 1994; Lammas et al., 1997; Fairbairn et al., 2001; Kusner and Barton, 2001; Saunders et al., 2003). The differences in the viability of intracellular mycobacteria following activation with eATP can be attributed to pathogen genetic and pathogenic properties, interactions with the
specific (bovine vs. human/mouse) host cells and the experimental approaches used (e.g. ATP detection assays, multiplicity of infection) (Woo et al., 2007; Woo et al., 2009). Kabara and Coussens (2012) showed that Map-infected bovine MDMs were less apoptotic than uninfected bovine MDMs after H₂O₂-induced apoptosis. Therefore, apoptosis was higher in uninfected cells, supporting the notion that Map bacteria inhibits host cell death, creating a replicative protected niche. Periasamy et al. (2013) showed that apoptosis of bovine macrophage was dependent on the pathogen burden, where apoptosis was induced at a MOI (multiplicity of infection) of 10 (Map:macrophage) but not at a MOI of 1. At a MOI of 50, both apoptosis and necrosis were induced (Periasamy et al., 2013). Thus, maintaining a balance between immunomodulatory and immunosuppressive responses via the purinergic pathway is critical during host-pathogen interactions. Disorders in this and other pathways leads to disease.

Ndk from *M. tuberculosis*, *M. bovis* BCG, and other bacterial pathogens interfered with eATP and P2X₇ purinergic pathways, and inactivated small GTPases (Rac1, Rab5, and Rab7) involved in ROS production and phagosome maturations. For these reasons, I decided to investiage the role of Map Ndk as a virulence factor.

1.7 Nucleoside diphosphate kinase: catalytic mechanism and function

Nucleoside diphosphate kinase (Ndk) is an evolutionarily conserved, multifunctional housekeeping enzymes that play a major role in maintenance of nucleoside triphosphate pools (Ray and Mathews, 1992, Lascu and Gonin, 2000a). Ndk can be cytosolic, membrane-associated, and secreted extracellularly into culture supernatants with no known signal leader sequence and has not been assigned a specific secretion pathway (Kimura and Shimada, 1988; Sundin et al., 1996; Shankar et al., 1997a; Chopade et al., 1997; Mukhopadhyay et al., 1997; Zaborina et al.,
The enzyme catalyzed the transfer of the 5’ terminal phosphate group from nucleoside triphosphates (NTPs) to nucleoside diphosphates (NDPs) (Lascu and Gonin, 2000a). The reaction was reversible and proceeded through a transient covalent bond of an autophosphorylated histidine intermediate (Ndk-His-P*) in the conserved histidine-glycine-serine-aspartic acid (HGSD) motif, to generate the corresponding NTP or dNTP (Sundin et al., 1996; Arumugam and Ajitkumar, 2012):

\[
\begin{align*}
N_1TP + Ndk + N_2DP & \rightleftharpoons N_1DP + \textbf{-Ndk-His-P}* + N_2DP \quad (1) \\
N_1DP + \textbf{-Ndk-His-P}* + N_2DP & \rightleftharpoons N_1DP + Ndk + N_2TP \quad (2)
\end{align*}
\]

Ndk removed the phosphate group from the ribose or deoxyribose forms of purine and pyrimidines and was nonspecific with respect to the acceptor and donor nucleotides (Sundin et al., 1996; Chakrabarty, 1998; Lascu and Gonin, 2000a). Therefore, Ndk exhibited NTPase activity when removing the phosphate group and NTP synthesizing activity by transfer of the phosphate group. The quaternary and three-dimensional structure of Ndk was determined in several prokaryotes and eukaryotes (Janin et al., 2000; Lascu et al., 2000b). Although all known eukaryotic Ndks are hexamers while some bacterial Ndks are tetramers, both are built from the same dimer; hexamers are composed of three dimers while the tetramer is composed of a pair of dimers (Janin et al., 2000; Lascu et al., 2000). The X-ray structure of Ndk from M. tuberculosis formed a hexamer (Chen et al., 2002). The histidine (His) residue 117 in the HGSD motif was important for Ndk’s biochemical activities (Tiwari et al., 2004; Arumugam and Ajitkumar, 2012). In M. smegmatis, replacement of the His-117 to glutamine in recombinant Ndk significantly impaired its NTPase, autophosphorylation, and phosphotransfer activities (Arumugam and Ajitkumar, 2012). NTPase and autophosphorylation activities were examined.
by incubating recombinant *M. smegmatis* Ndk mutant with a radiolabeled gamma-phosphate ATP ([γ-32P]-ATP), and analyzing samples by autoradiography. The NTPase activity was determined by the hydrolysis of [γ-32P]-ATP and quantifying the release of 32Pi while the autophosphorylation activity was examined through the detection of phosphorylated recombinant Ndk following incubation with [γ32P]-ATP. The NTP synthesising activities were examined by incubating recombinant Ndk with [γ-32P]-ATP and NDPs (e.g. GDP, CDP), and detecting the corresponding gamma-labeled NTP (Arumugam and Ajitkumar, 2012). In all cases, NTPase, autophosphorylation, and phosphotransfer activities of the recombinant Ndk-His117 mutant were impaired in comparison with wild-type recombinant Ndk. Tiwari et al (2004) also showed impairment in autophosphorylation and phosphotransfer activities using *M. tuberculosis* recombinant Ndk, in which the His-117 residue in the HGSDD motif was replaced with glutamine (Tiwari et al., 2004).

### 1.7.1 Role of bacterial Ndk in ATP-induced apoptotic cell death

Zaborin a et al. (1999a) identified Ndk and another nucleotide-utilizing enzyme (ATPase) from the culture supernatant of avirulent *M. bovis* BCG. The addition of exogenous eATP induced cell death in mouse peritoneal macrophage cultures. However, the addition exogenous of eATP to concentrated culture filtrates of *M. bovis* BCG prevented ATP-induced death of mouse macrophages (Zaborina et al., 1999a). The authors suggested that Ndk and ATPase hyrolyzed and sequestered ATP molecules from the P2X7 receptor, interfering with the induction of ATP-induced purinergic cell death pathways (Zaborina et al., 1999a). In another study, Zaborina et al. (1999b) showed that Ndk, adenylate kinase, ATPase, and 5’-nucleotidase were present in the culture filtrates of a *P. aeruginosa* mucoid strain isolated from patients with cystic fibrosis were
effective in increasing ATP-induced cell death of mouse macrophages compared to eATP alone (Zaborina et al., 1999b). The response was dependent on P2X\(_7\) receptors as addition of an irreversible inhibitor of P2X\(_7\) abolished ATP-induced cell death (Zaborina et al., 1999b).

Punj et al. (2000) identified Ndk, adenylate kinase, and 5’- nucleotidase in the culture filtrates of a virulent strain of *Vibrio cholerae*, an extracellular Gram-negative bacterium. The addition of exogenous ATP induced cell death in mouse macrophage cell lines. However, the addition of culture filtrates of *V. cholerae* and exogenous ATP to mouse macrophage cell lines increased ATP-induced apoptosis compared to eATP alone, and pretreatment with a P2X\(_7\) antagonist abolished ATP-induced cell death (Punj et al., 2000). Addition of exogenous ATP to human gingival epithelial cells (GECs) also induced cell death, and GECs infected with an avirulent strain of *Porphyromonas gingivalis*, an intracellular pathogen, prevented ATP-induced cell death compared to eATP alone (Yilmaz et al., 2008). Moreover, an ndk-deficient mutant was no longer able to inhibit ATP-induced apoptosis of infected gingival epithelial cells, suggesting Ndk hydrolyzed and sequestered ATP, abolishing P2X\(_7\)-dependent responses. The increase in ATP-induced cell death in *P. gingivalis*-infected cells were blocked by a P2X\(_7\) antagonist, again demonstrating ATP-induced cell death was dependent on P2X\(_7\) purinergic membrane receptors (Yilmaz et al., 2008).

Culture filtrates of avirulent *M. tuberculosis* (strain H37Ra) contained secreted Ndk among other secretome proteins (Dar et al., 2011). Addition of *M. tuberculosis* culture filtrates with exogenous ATP decreased ATP-induced death of mouse macrophage cell lines compared to eATP alone (Dar et al., 2011). ATP-induced cell death was reduced when a P2X\(_7\) antagonist was used, again demonstrating the response to be mediated by the P2X\(_7\) receptor. Dar and colleagues (2011) also detected Ndk in the secretome of a virulent strain of *Salmonella* typhimurium, an
intracellular Gram-negative bacterium, and a virulent strain of *Vibrio cholerae*, and extracellular Gram-negative bacterium. The addition of exogenous ATP with culture filtrates of virulent *S. typhimurium* decreased ATP-induced cell death compared to eATP alone, which was similar to the decrease in ATP-induced cell death from avirulent *M. tuberculosis* and avirulent *M. bovis BCG* (Zaborina et al., 1999a). In contrast, the addition of exogenous ATP with culture filtrates of virulent *V. cholerae*, containing secreted Ndk and other nucleotide-utilizing enzymes (e.g. adenylate kinase, ATPase), increased ATP-induced cell death compared to eATP alone (Dar et al., 2011). In a later study, Choi et al. (2013) showed that ATP-induced activation of the P2X7 receptor in human gingival epithelial cells (GECs) isolated from gingival tissue resulted in ROS production through P2X7 receptors. Moreover, addition of ATP to GECs infected with an avirulent strain of the Gram-negative, intracellular opportunistic pathogen *P. gingivalis* decreased ROS production and increased bacterial survival while GECs infected with Ndk-deficient *P. gingivalis* mutant were unable to prevent ROS production and decreased bacterial survival (Fig. 3).

Collectively, these studies suggested that bacterial pathogens have developed divergent strategies to modulate ATP-dependent host cell death depending on their mode of survival during infection. In extracellular pathogens, the secreted Ndk, perhaps in combination with other extracellular nucleotide-utilizing enzymes, enhanced ATP-induced cell death of phagocytes via P2X7 receptors, further blocking phagocyte-mediated killing in order to establish infections. In contrast, the secreted Ndk of the intracellular pathogens depleted eATP, blocking activation of P2X7 receptors and ATP-induced death of the parasitized host cell. The hydrolysis of eATP by ecto-enzymes can further modulate macrophage function. These signalling molecules, receptors and pathways act in concert and provide different mechanisms to modulate macrophage function
Figure 3. Mycobacterial Ndk blocks eATP-induced responses acting on P2X7 receptors.
Infected or drying cells (e.g. phagocytes) release endogenous ATP. Extracellular ATP acts on P2X7 receptors, inducing production of ROS, activation of the inflammasome, and secretion of pro-inflammatory cytokine IL-1β. Pathogenic mycobacteria (e.g. M. tuberculosis) secrete Ndk extracellularly, which crosses the phagosomal membrane towards the cytosol. Microbe-derived Ndk and IL-1β both lack secretory signal peptides. Once in the cytosol, Ndk can be released to the extracellular space by several mechanisms. (A) Movement of secreted Ndk through host cell plasma membrane channels along with IL-1β to the extracellular space. (B) Simultaneous incorporation of IL-1β and microbe-derived Ndk within secretory vesicles that fuse with host cell membrane and release contents extracellularly. (C) Association of microbe-derived Ndk with host ER-network and subsequent export via classical host-cell secretion pathways. Once in the extracellular space, the NTPase activity of Ndk can metabolize ATP, hydrolyzing ATP from the P2X7-R and modulate signalling pathways. ROS, reactive oxygen species; P2X7-R, P2X7 receptor; Mtb, M. tuberculosis. Figure was modified and adapted from Spooner and Yilmaz (2012).
with different outcomes for the pathogen and host (Dar et al., 2011).

1.7.2 Effects of bacterial Ndk on phagosome maturation

As previously discussed (section 1.6), the activity of small GTPase proteins is regulated by GTPase-activating proteins or GAPs (deactivates the proteins to their inactive GDP-bound state) and guanine nucleotide exchange factors or GEFs (activating the proteins to their active—GTP bound state) (Schmidt and Hall, 2002; Bernards and Settleman, 2004). Rho GTPases (Rho, Rac, and Cdc42) are important regulators of actin cytoskeleton organization required for phagocytosis (Xu et al., 1994a, b; Cox et al., 1997; Caron and Hall, 1998; Hoppe and Swanson, 2004). The Rac small GTPase is also an essential component of NOX2 involved in generating ROS (Xu et al., 1994a). A study by Chopra et al. (2004) initially demonstrated the GTPase-activating protein (GAP) activity of extracellularly secreted Ndk on Rho small GTPases from virulent M. tuberculosis (strain H37Rv). In this study, $[\gamma^{32}\text{P}]$-GTP-labelled RhoA, Rac1 and Cdc42 proteins were spotted onto nitrocellulose membrane and incubated with or without culture filtrates of M. tuberculosis. The addition of culture filtrates resulted in hydrolysis of $[\gamma^{32}\text{P}]$-GTP as demonstrated by decreased radioactivity. Pre-incubation of anti-Ndk antibodies with the culture filtrates of M. tuberculosis significantly decreased GAP activity by 70%, demonstrating Ndk was mainly responsible for GAP activity in the culture filtrates of M. tuberculosis. The influence of Ndk GAP activity was further substantiated using recombinant Ndk instead of M. tuberculosis culture filtrates, where heat-inactivated Ndk failed to hydrolyze $[\gamma^{32}\text{P}]$-GTP-labelled Rho GTPases (Chopra et al., 2004). The interaction between recombinant M. tuberculosis Ndk and $[\gamma^{32}\text{P}]$-GTP-labelled Rho GTPases was demonstrated using a pull-down experiment. GTP-labelled RhoA, Rac1 and Cdc42 coupled to Sepharose beads were incubated with or without
Labeled Rho GTPases were eluted, resolved by thin-layer chromatography, and visualized by an autoradiogram. *M. tuberculosis* Ndk interacted and inactivated all GTP-labelled Rho GTPases, and no inactivation was observed for the control experiment.

Sun et al. (2010) demonstrated the interaction of recombinant *M. tuberculosis* Ndk with recombinant Rab5 and Rab7 small GTPases preloaded with GTP. Rab5 and Rab7 play a major role in phagosome maturation (Fig. 1). ELISA microplates were coated with recombinant Ndk from virulent *M. tuberculosis* or BSA as a control, and were incubated with increasing concentrations of Rab5 and Rab7. Bound Rab5 and Rab7 were probed with primary rabbit anti-Rab5 or Rab7. Recombinant *M. tuberculosis* Ndk interacted with Rab5 and Rab7 in a dose-dependent manner. The same results were obtained for recombinant Ndk from nonpathogenic *M. smegmatis*. These results were further substantiated by pull-down experiments. Recombinant Rab5 and Rab7 were incubated with recombinant Ndk from *M. tuberculosis* or *M. smegmatis* and subjected to co-immunoprecipitation with anti-Ndk antibodies. Pulled-down proteins were analyzed by Western blotting using anti-Ndk and anti-Rab5 or anti-Rab7 antibodies, showing that Ndk from both *M. tuberculosis* and *M. smegmatis* interacted with Rab5 and Rab7. The GTPase activity of Ndk was then examined by incubating the Ndk protein with [γ-32P]-GTP-labelled Rab5 and Rab7. Interestingly, *M. smegmatis* Ndk had very little or no GTPase activity towards GTP-bound Rab5 and Rab7, respectively, while *M. tuberculosis* Ndk showed 90% or higher depletion of the γ-phosphate of GTP-bound Rab5 and Rab7. These results indicated that, despite equal binding to Rab5 and Rab7, Ndk from pathogenic *M. tuberculosis* expresses strong GAP activity compared to that from the non-pathogenic *M. smegmatis*.

Using fluorescence microscopy, Sun et al. (2010) showed that *M. tuberculosis* Ndk, and not *M. smegmatis* Ndk, was able to disrupt phagosome maturation. Purified Ndk protein from *M.
tuberculosis and M. smegmatis was adsorbed onto latex beads and incubated with mouse macrophage cell lines to induce phagocytic uptake of the beads. The latex bead-containing phagosome has been widely used as a model system to study phagosome maturations (Desjardin et al., 1994a, b; Desjardin et al., 1997; Jahraus et al., 1998). In this experiment, mouse macrophage cell lines were loaded with green fluorescent-labeled dextran and incubated with M. tuberculosis or M. smegmatis Ndk-coated beads. Control assays used BSA-coated latex beads. Dextran is a non-biodegradable compound that is trafficked to the lysosome and is used to analyze fusion of phagosomes with lysosomes (Hmama et al., 2004). Phagosomes containing BSA-beads were surrounded with green-fluorescent vesicles, indicating fusion with lysosomes. In contrast, phagosomes containing M. tuberculosis Ndk-beads did not reach lysosomes, indicating a block in phagosome maturation. Like BSA-beads, phagosomes containing M. smegmatis Ndk-beads, were also surrounded with green-fluorescent vesicles, indicating fusion with lysosomes. This was consistent with the inability of M. smegmatis Ndk to hydrolyze [γ-32P]-GTP-labelled Rab5 and Rab7.

Sun et al. (2010) also showed that M. tuberculosis Ndk crossed or was exported across the phagosomal membrane to the cytosol in order to interact with GTP-bound Rab5 and Rab7 (active state) on the cytosolic side of the membrane. In this experiment, green fluorescent dextran-loaded macrophage cells were co-incubated with red-fluorescent BSA-beads and unlabelled Ndk-beads or BSA beads (control). Confocal images showed that macrophage cells co-incubated with unlabelled Ndk beads and red-labelled BSA beads excluded green-labeled vesicles from their phagosomes, indicating a block in phagosome-lysosome fusion. In contrast, red-labelled phagosomes were surrounded with green fluorescence when macrophage cells were co-incubated with unlabelled BSA beads and red-labelled BSA, indicating fusion of phagosomes
with lysosomes. Furthermore, macrophage cells were transfected with downstream effector EEA1-GFP (green fluorescent protein) or FYVE-GFP and incubated with *M. tuberculosi*s Ndk—or BSA-coated beads. EEA1 is recruited to the phagosomal membrane to interact with PI3P through its FYVE domain (section 1.7). Phagosomes with Ndk-coated beads showed no recruitment of EEA1-GFP or FYVE-GFP. In contrast, macrophages incubated with BSA-coated beads were surrounded by abundant green fluorescent signal. These experiments were not examined with *M. smegmatis* Ndk. However, these results suggested that Ndk inactivated Rab5, decreasing recruitment of hVPS34 necessary to phosphorylate PI and to generate PI3P, resulting in a failure in recruiting EEA1 to PI3P through its FYVE domain (section 1.7). Inactivation of Rab7 and decreased recruitment of RILP effector resulting in arrest of fusion between phagosomes and lysosomes by *M. tuberculosi*s Ndk was also demonstrated (Sun et al., 2010). Green-labeled Rab7 and red-labeled RILP were co-transfected in mouse macrophages and allowed to phagocytose *M. tuberculosi*s Ndk-beads or BSA-beads. Confocal images showed colocalization of Rab7 and RILP on phagosome when BSA-beads were used, which was not observed with *M. tuberculosi*s Ndk-beads. In summary, these results suggested that pathogenic *M. tuberculosi*s Ndk possesses GAP activity and is trafficked within mouse macrophage cells beyond phagosomes, leading to inhibition of phagosome biogenesis processes dependent on Rab5 and Rab7. Therefore, *M. tuberculosi*s Ndk contributes to intracellular survival and establishment of infection. Another study by Sun et al. (2013) examined the interaction between *M. tuberculosi*s Ndk and Rho small GTPases (Rho, Rac1, Cdc42). Whole cell lysates of macrophage cell lines incubated with *M. tuberculosi*s Ndk-beads or BSA-beads were subjected to co-immunoprecipitation with anti-Ndk antibodies. Pulled-down proteins were analyzed by Western blotting using anti-Rho, anti-Rac, or anti-Cdc42 antibodies. *M. tuberculosi*s Ndk
interacted with and inactivated only Rac1, and not Rho or Cdc42, while BSA did not interact with any of the Rho GTPases. This in in contrast to the result by Chopra et al. (2004) that showed \textit{M. tuberculosis} Ndk interacted with Rac, Rho, and Cdc42. The authors suggested that the cellular context was significant in the interaction of Ndk with specific small GTPases and that Rac1 and not Rho or Cdc42 was the cellular target of the bacterial protein. Using fluorescence microscopy, they found that recombinant \textit{M. tuberculosis} Ndk decreased recruitment of p67\textsuperscript{phox} (a subunit of NOX2). Confocal images showed macrophages ingesting recombinant \textit{M. tuberculosis} Ndk-coated beds crossed the phagosomal membrane to the cytosol using primary Ndk antibody and fluorescent secondary antibody. Finally, \textit{M. tuberculosis}-infected macrophages inhibited ROS production whereas cells infected with \textit{M. tuberculosis} containing a disruption in the \textit{Ndk} gene and allowed to ingest BSA-coated beads did not.

1.7.3 Proteins that modulated the specificity of Ndk

Ndk from \textit{M. smegmatis} was shown to interact with other \textit{M. smegmatis} cellular proteins that modulated the specificity of NTP synthesis (Shankar et al., 1997a) that was also previously demonstrated with \textit{P. aeruginosa} Ndk (Kavanaugh-Black et al., 1994; Shankar et al., 1996; Chopade et al., 1997; Mukhopadhyay et al., 1997). \textit{M. smegmatis} Ndk was purified by ammonium sulfate precipitation and coupled to Sepharose bead matrix. \textit{M. smegmatis} total cell extract was then passed through the matrix and interacting proteins eluted. Four proteins named according to molecular mass, P$_{50}$, P$_{60}$, P$_{65}$, and P$_{70}$, interacted with \textit{M. smegmatis} Ndk. Except for P65, the N-terminal amino acid sequences of the proteins were identified. P$_{70}$ had homology to \textit{E. coli} and \textit{P. aeruginosa} pyruvate kinase; P$_{60}$ shares homology to a cell-wall protein from \textit{M. tuberculosis} and \textit{M. leprae}, and to Map heat shock protein 65 (Hsp65); and P$_{50}$ shares homology
to the elongation factor Tu (EF-Tu) from *M. leprae*. Shankar et al. (1997a) performed NTP synthesizing assays by incubating *M. smegmatis* Ndk with eluates containing P<sub>50</sub>, P<sub>60</sub>, P<sub>65</sub>, and P<sub>70</sub> each separately with [γ<sup>32</sup>P]-ATP and a mixture of NDPs. Protein complexes of P<sub>50</sub> and P<sub>70</sub> with Ndk generated predominantly GTP, while Ndk-P<sub>60</sub> and Ndk-P<sub>65</sub> generated predominantly CTP and UTP, respectively.

In *P. aeruginosa*, Ndk had been reported to exist in two forms with equally efficient autophosphorylation activity; the 16-kDa cytoplasmic Ndk and the truncated membrane-associated 12-kDa Ndk, generated by the proteolytic action of periplasmic elastase (Shankar et al., 1996, Kamath et al., 1998). Cytoplasmic Ndk was nonspecific in its phosphorl transfer activity whereas the membrane-associated Ndk was shown to generate [γ<sup>32</sup>P]-GTP when incubated with UDP, CDP, or GDP and [γ<sup>32</sup>P]-ATP in an NTP synthesizing assay. Moreover, the membrane-associated Ndk formed a complex with pyruvate kinase to predominantly generate GTP, proposed to be important in the alginate biosynthetic pathway for the production of GDP-mannose (Sundin et al., 1996; Chakarabarty, 1998). Complex formation of Ndk from *P. aeruginosa* with EF-Tu to predominantly synthesize GTP was also reported (Mukhopadhyay et al., 1997). EF-tu is a GTP-binding protein involved in protein biosynthesis and it was proposed that the Ndk-EF-Tu complex provides a significant source of GTP during translational elongation (Kaziro et al., 1991; Mukhopadhyay *et al.*, 1997). By purifying *P. aeruginosa* ribosomes and performing an in vitro translational assay with [γ<sup>32</sup>P]-ATP and NDPs, GTP was shown to be predominantly synthesized (Mukhopadhyay *et al.*, 1997). When anti-Ndk antibodies were added, GTP synthesis was inhibited whereas anti-EF-Tu antibodies changed the specificity from GTP to nonspecific NTP generation. Moreover, Ndk was shown to associate with the 30S ribosomal subunit, whereas EF-Tu was found to be associated with the 50S ribosomal subunit.
(Mukhopadhyay et al., 1997). *P. aeruginosa* Ndk also co-purified with succinyl CoA synthetase (Kavanaugh-Black et al., 1994). The co-purified 33 kDa α-subunit of succinyl CoA synthetase was shown to undergo autophosphorylation. Although succinyl CoA synthetase, an enzyme involved in the citric acid cycle, was shown to possess Ndk-like activity with a strong preference for purine nucleotides (e.g. formation of $[\gamma^{32}\text{P}]$-GTP from $[\gamma^{32}\text{P}]$-ATP), the Ndk-succinyl CoA synthetase complex generated both purine and pyrimidine nucleotides (e.g. $[\gamma^{32}\text{P}]$-GTP, $[\gamma^{32}\text{P}]$-CTP, and $[\gamma^{32}\text{P}]$-UTP from $[\gamma^{32}\text{P}]$-ATP), which indicated the influence of Ndk on the activity of succinyl CoA synthetase (Murakami et al., 1972; Kavanaugh-Black et al., 1994).

### 1.8 Research rationale

Ndk from several pathogenic bacteria is known to be extracellularly secreted and has been identified as a virulence factor interfering with macrophage signalling pathways and bactericidal innate host defences. *M. tuberculosis* recombinant Ndk inactivated small GTPases Rac1, Rab5, and Rab7 (Sun et al., 2010; Sun et al., 2013). Furthermore, the Ndk protein has been shown to cross the phagosomal membrane into the cytosol suggesting that when released from the phagosome by the viable bacteria, it facilitates the pathogen’s establishment of an intracellular niche by inactivating small GTPases and inhibiting phagosome maturation. Ndk from the non-pathogenic *M. smegmatis* and the opportunistic pathogen *P. aeruginosa*, copurified and interacted with several proteins that modulated the specificity of Ndk, mostly to GTP (Mukhopadhyay et al., 1997; Shankar et al., 1996; Shankar et al., 1997b; Kavanaugh-Black et al., Chopade et al., 1997; Mukhopadhyay et al., 1997). The histidine residue in the HGSD motif was shown to be critical for the Ndk’s NTPase, autophosphorylation, and phosphotransfer activities (Tiwari et al., 2004; Arumugam and Ajitkumar, 2012).
I hypothesize that Ndk from \textit{Map} is a secreted virulence factor that can interact with Rab5 and Rab7 small GTPases and other \textit{Map} proteins. Initial objectives were to recombinantly express \textit{Map} Ndk in \textit{E. coli} and generate polyclonal IgG antibodies in rats (rat anti-Ndk antibodies) using the purified His-tagged Ndk protein as the immunogen. \textit{Map} Ndk was examined for its immunogenicity during infection in uninfected and naturally \textit{Map}-infected cattle. \textit{Map} rNdk-His-117 mutants were generated and results will be discussed. Secretion of Ndk by \textit{Map} was then examined by Western blotting and immunoprecipitation using rat anti-Ndk antibodies. In order to examine if \textit{Map} Ndk interacted with Rab proteins, pull-down experiments were employed using two different methods. In the first method, I used \textit{Map} Ndk immobilized to TALON resin followed by incubation of whole cell lysates of bovine macrophage cell lines, BoMAC (Stabel and Stabel, 1995). In the second method, BoMAC cells were incubated with recombinant \textit{Map} Ndk-coated latex beads, the cells lysed, and pull-down assays performed using rat anti-Ndk antibodies and Protein G-conjugated magnetic beads. Finally, \textit{Map} rNdk was examined for its interactions with other \textit{Map} proteins using a pull-down experiment by immobilizing Ndk to TALON resin followed by incubating with \textit{Map} lysate or culture filtrate proteins.
Chapter 2: Materials and Methods

2.1 Bacterial strains and growth conditions

*Mycobacterium avium* subspecies *paratuberculosis* strains gc86 were isolated in our laboratory in December 2001 from the feces of cows from dairy herds in southern Ontario (Facciulo et al., 2013). The strains were characterized by mycobactin J-dependency during culture, by PCR amplification of *IS900*, *hspX*, *ISMAP02*, and *hsp65*, and shown to be cattle-type by *IS1311* PCR-restriction endonuclease (Collins et al., 1989; Ellingson et al., 1998; Whittington et al., 1998; Thorel et al., 1990; Turenne et al., 2006)

*Map* cultures were initiated by inoculating a 1-mL frozen ‘seedlot’ containing $10^8$ CFU/ml into 50 mL Middlebrook 7H9 medium (Difco) supplemented with 5 g/L glycerol, 1 g/L casitone, OADC (oleic acid, albumin, dextrose, catalase), and 2 mg/L mycobactin J. *Map* cells were grown to mid-logarithmic phase (OD$_{600} = \sim 0.9$), harvested by centrifugation (4000 × g, 15 min, 4°C), washed with 10 mM sterile phosphate-buffered saline (PBS, pH 7.4), and resuspended in 200 mL of Watson-Reid medium (pH 6.0) supplemented with 2 mg/L mycobactin J (Allied Monitor), 4.1 g/L sodium pyruvate, and 0.075 g/L ferric ammonium citrate (Merkal and Curran, 1974). Cultures were grown to mid-log phase at 37°C and harvested as described above.

2.1.1 Preparation of *Map* whole cell lysate and culture filtrate

*Map* cultures (40-45 mL = 0.7g) were centrifuged at 3000 × g, 4°C for 30 min. Pellets were resuspended in homogenization buffer containing sterile PBS, glycerol, phenylmethylsulfonyl (PMSF), and ethylenediaminetetraacetic (EDTA, pH 8.0) and 1 mL fractions transferred to a microcentrifuge tube containing 0.5 g of 0.1 mm zirconia/silica beads (BioSpec Products). Tubes were shaken in a Mini-Bead Beater cell disruptor (BioSpec Products) for five 30 s pulses, with 1
Cellular debris and zirconia beads were removed by centrifugation at 10,000 \times g for 10 min, and the whole cell lysate was stored at –20°C. Culture supernatants were filtered through a 0.2 µm filter to remove cell debris, and the filtrate dialyzed against PBS using Amicon Ultra-15 centrifugal filter units with 3 kDa nominal molecular weight limit (NMWL; EMD Millipore), and concentrated ~100-200-fold. Protein concentration was quantified using a bicinchoninic acid (BCA) kit (Sigma Aldrich).

### 2.2 Cloning of putative Map 2268c gene (nucleoside diphosphate kinase)

The DNA sequence for the open reading frame (ORF) of putative Map strain K-10 2268c (Ndk) (Kegg paratuberculosis) was chemically synthesized as one dsDNA linear gBlock (Integrated DNA Technologies). The gBlock gene fragment was designed with a carboxyl-terminal hexa histidine-tag (6xHis-tag) sequence and ~25-bp overlapping terminal sequences (Appendix Fig. S1) with the linearized pET30a expression vector possessing a kanamycin selective marker (Novagen). The gene of interest in this system is cloned behind a T7 promoter recognized by the phage T7 RNA polymerase (Rosano and Ceccarelli, 2014). The pET30a plasmid was linearized with NdeI and BamHI restriction sites (FastDigest; Thermo Scientific). The His-tagged recombinant Map Ndk (Map rNdk) gBlock insert was cloned into the pET30a expression plasmid using the isothermal assembly method (Gibson et al., 2009). Briefly, 25 femtomoles of the gBlock was added to 75 femtomoles of linearized pET30a plasmid and the reaction was incubated for 1 h at 50°C. One tenth of the reaction was added to chemically competent E. coli HB101 host cells (Agilent). Host cells transformed with pET30a containing Map rNdk (pET30a-Map rNdk) insert were selected for on Lysogeny Broth (LB) (BD Difco) agar medium containing 50 µg/mL kanamycin. The pET30a-Map rNdk plasmids were purified from E. coli HB101 host cells using the QIAprep Spin Miniprep Kit (Qiagen) as specified by the manufacturer and the
plasmid submitted for sequencing of both strands of the cloned DNA using the forward and reverse primer for the T7 promoter and T7 terminator (Thermo Scientific). All sequencing was performed by the Genomics Facility at the University of Guelph’s Advanced Analysis Centre. The pET30a-Map rNdk plasmids (10 ng) were transformed into E.coli BL21- CodonPlus(DE3) host cells (Agilent), and transformants selected on LB agar medium supplemented with 35 µg/mL chloramphenicol and 50 µg/mL kanamycin. The E.coli BL21- CodonPlus(DE3)-RIPL host cells encode the T7 RNA polymerase, placed in the bacterial genome in a prophage (λDE3), under the control of the lacUV5 promoter and can be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (Studier and Moffat, 1986). These cells also contain extra copies of genes that encode the tRNAs that most frequently limit translation of heterologous proteins in E. coli (e.g. arginine, isoleucine, leucine, proline) and a plasmid that confers chloramphenicol resistance.

2.2.1 Expression and purification of recombinant Map Nucleoside diphosphate kinase

E.coli BL21-CodonPlus(DE3) host cells containing pET30a-Map rNdk plasmids were cultured at 37°C in LB containing 50 µg/mL kanamycin and 35 µg/mL chloramphenicol until an OD600 of 0.6 was reached. Induction of protein expression was carried out with 0.1 mM IPTG and cultures were incubated for 2 h at 37°C. Bacterial cultures (40 mL) were centrifuged (3000 × g, 4°C, 20 min) and the cell pellet and culture supernatants kept on ice for processing. The cells were resuspended in 5 mL ice-cold lysis buffer (50 mM sodium phosphate buffer and 300 mM NaCl at pH 8.0, 10 mM imidazole, 1 mg/mL lysozyme, and 1 mM PMSF). The cell suspension was sonicated (five 30 s pulses with 30 s rest), and centrifuged (10,000 × g, 4°C, 20 min) to separate the soluble protein fraction (supernatant) from the cellular debris (pellet). The culture supernatant was concentrated and dialyzed against PBS using Amicon Ultra-15 centrifugal filter
units with 3 kDa NMWL. The His-tagged Map rNdk proteins were purified by immobilized metal affinity column chromatography (IMAC) with 0.5 mL of TALON-cobalt resin (Clontech Laboratories, Inc.). The lysate (1 mg) was added to the resin and incubated with gentle mixing for 1 h at 4°C to allow binding of His-tagged proteins to the cobalt. An imidazole gradient (5 mM, 10 mM, 20 mM, 50 mM, 100 mM, 150 mM, and 200 mM) was used to elute bound proteins with 4 column volumes of elution buffer (50 mM sodium phosphate buffer and 300 mM NaCl, pH 8.0). The fractions eluted with 100-200 mM imidazole were pooled, concentrated, and dialyzed against PBS using Amicon Ultra-15 centrifugal filter units with 3 kDa NMWL.

2.2.2 Bioinformatic analysis of Map Ndk

The protein sequences were obtained from UniProt (accession number Q73XP1). SignalP (version 4.1) and Secretome P (version 2.0) were used to identify putative secretory signal peptides. Hydrophilicity/Hydrophobicity analysis was determined using GenScript’s Peptide Property Calculator. The isoelectric point (pI) was determined using the Isoelectric Point Calculator (Kozlowski, 2016). ExPASy SIM was used for amino acid sequence alignments.

2.2.3 Mutagenesis of Map rNdk histidine residue at position 117

The codon for histidine at position 117 (H117; codon CAC) of Map Ndk DNA sequence was mutated by PCR using the QuikChange method based on overlapping oligonucleotide primers (Zheng et al., 2004). The pET30a-Map Ndk plasmid was used as template and H117 was replaced to alanine (H117A; CAC → GCC), glutamic acid (H117E; CAC → GAA), glycine (H117G; CAC → GGC), glutamine (H117Q; CAC → CAA), and tyrosine (H117Y; CAC → TAC) (Appendix Fig. S2). The forward and reverse primers (Table S1) were chemically synthesized with 5’ phosphate (Integrated DNA Technologies). To 50 ng of pET30a-Map rNdk
template, 2 units of Phusion High-Fidelity DNA polymerase (Thermo Scientific), 10x Phusion Buffer (Thermo Scientific), 0.5 µM forward and reverse primers, and 200 µM dNTPs (Thermo Scientific) were added in a 50 µL reaction in four PCR tubes. The PCR amplifications were carried out with MJ Mini Personal Thermal Cycler (Bio-Rad). The extension reaction was carried out by pre-heating the reaction mixture to 94°C for 3 min followed by 16 cycles of 94°C for 1 min, 54°C for 1 min, 68°C for 12 min (2 min/kilobase (kb); pET30a = 5.42 kb), and a final incubation at 68°C for 1 h. Amplified PCR products were pooled and purified using the GeneJet Gel Extraction Kit (Thermo Scientific). To purify unmethylated PCR products, the methylated template was digested with one unit of DpnI and incubated for 16 h at 37°C. PCR products were sequenced using the T7 forward primer and replacement of His residue 117 confirmed. PCR products (50 ng) were transformed into E. coli HB101 cells and selected for on LB agar containing 50 µg/mL kanamycin. Plasmids were purified from selected colonies cultured for 16 h at 25°C using the QIAprep Spin Miniprep Kit (Qiagen), transformed into E. coli BL21-CodonPlus(DE3) host cells, and selected for on LB agar containing 35 µg/mL chloramphenicol and 50 µg/mL kanamycin. Affinity purification of Map rNdk-H117 mutant proteins was performed as described above.

2.3 SDS-PAGE

Protein samples were prepared with SDS sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 30% glycerol, 0.05% bromophenol blue) containing 5% β-mercaptoethanol and heated to 95°C for 5 min. Electrophoresis was carried out at 90 V for 1.5 h in a 15% w/v SDS-polyacrylamide gel and proteins were detected by Coomassie Brilliant Blue G-250 staining (Simpson, 2010) or silver staining (Chevallet et al., 2006).
2.4 Western blotting

Proteins were transferred to a nitrocellulose membrane (BioTrace NT; Pall Corporation) using a semi-dry transfer system (Trans-Blot SD Transfer Cell, Bio-Rad Laboratories) at 15 V for 40 min. Membranes were blocked for 2 h at room temperature with PBS containing either 5% w/v nonfat dry milk for the detection of Rab proteins or 2.5% Tween-20 for the detection of Map rNdk. Membranes were incubated for 2 h at 25°C in primary antibody diluted in PBS-T (1:1000 or 1:2000 for rat polyclonal antibodies or 1:200 for bovine serum polyclonal antibodies) or PBS-T with 5% w/v BSA (1:1000 rabbit Rab5 or Rab7 monoclonal antibody). The Rab Family Antibody Sampler Kit was purchased from Cell Signaling Technology (New England Biolabs Ltd., Whitby, Ontario). Secondary antibody was diluted in PBS-T (1:4000 for HRP-conjugated goat anti-rat IgG and HRP-conjugated mouse anti-bovine IgG and 1:5000 for HRP-conjugated goat anti-Rab IgG). Membranes were washed 3 times for 10 min in PBS-T after primary and secondary antibody incubations. Colour development was performed using diaminobenzidine as described previously (Nadkarini and Lindhardt, 1997). Immunoblotting using a His-probe (Thermo Scientific) was performed using the SNAP i.d 2.0 Protein Detection System (Millipore). After transfer, 30 mL of blocking solution (TBS: 50 mM Tris-Cl, 150 mM NaCl, pH 7.6; 0.05% Tween-20; 0.5% nonfat dry milk) was added to the membrane and incubated for 15 min followed by vacuum application. His-probe was then added (3.36 units) for 30 min, followed by vacuum application. The membrane was washed three time with 35 mL of TBS. Colour development was performed using diaminobenzidine.

2.5 Preparation of antigen-specific rat polyclonal serum antibodies

A 1:1 emulsion of 100 µg of purified Map rNdk and TitreMax gold adjuvant (Sigma-Aldrich) was injected intramuscularly into two Sprague-Dawley rats in sterile 10 mM PBS (pH 7.4)
(Mutharia and Lam, 2007). Subsequently, four bi-weekly immunizations were administered, which consisted of 25 µg of Map-Ndk emulsified in Freund’s incomplete adjuvant (Sigma-Aldrich). Four days after the last immunization, rats were euthanized and whole blood was collected. The cell-free serum was prepared by allowing the blood to clot for 1 h at 30°C and then centrifuged at 1000 × g for 10 min. The serum was then removed by pipetting and stored at -20°C. All protocols involving animal handling, immunizations, and blood collections were performed by animal care technicians at the University of Guelph’s Central Animal Care Facility. The treatment and immunization protocols of all animals in this study were approved by the University of Guelph Animal Care and Use Committee and described in an Animal Utilization Protocol by Dr. Lucy M. Mutharia (Department of Molecular and Cellular Biology).

Bovine sera was obtained from Map-infected cows, that tested positive by commercial IDEXX tests for Map culture from cow feces and serum antibodies by ELISA (IDEXX Laboratories, Inc.), and negative-control sera obtained from uninfected calf and ELISA-negative cows. The control sera were a gift from Dr. Niel Karrow, Department of Animal Biosciences, University of Guelph. Calf serum was collected from 2-month-old Holstein calves that had been removed within 24 h after birth and maintained in an animal isolation facility. Sera from uninfected cows were kindly provided by Dr. David F. Kelton, Department of Population Medicine, OVC, University of Guelph, from a herd with no reported cases of Johne’s disease and were confirmed seronegative by ELISA (IDEXX Laboratories, Inc.) (Facciulo et al., 2013).

2.5.1 Serum Absorption

E. coli BL21 lysates with no gene inserts in pET30a plasmids (600 µg), and Mycobacterium smegmatis cell extract (300 µg) and culture filtrates (300 µg) were diluted in PBS and incubated with nitrocellulose membranes (3 x 3 cm) for 2 h at room temperature. The membranes with
immobilized proteins were briefly washed with PBS and blocked with PBS-2% (w/v) non-fat dry
milk powder for 1 h at room temperature. The nitrocellulose membrane was washed briefly in
PBS-T. To remove serum antibodies reacting with Ndk from *E. coli* and environmental
mycobacteria, the cow sera were diluted (1:300) in 5 mL PBS-T and incubated with the
membrane for 16 h at 15°C. The serum was collected in 15 mL conical tubes and this process
was repeated 3 times. Aliquots of the absorbed serum were stored at -20°C.

### 2.6 Bovine macrophage cell culture

Bovine macrophage (BoMAC cell line) cells (previously obtained by Stabel and Stabel, 1995)
were cultured in RMPI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS),
2.0 mM L-glutamine, 25 mM HEPES buffer, 100 unit/mL of penicillin, 100 µg/mL of
streptomycin, and 0.25 µg/mL of amphotericin B (Invitrogen). After reaching confluence (~80%)
as determined by light microscopy, the BoMACs were detached with 0.05% trypsin-EDTA
(Invitrogen) and the cells were seeded into 6-well culture plates at a density of 1x10^6 cells per
well and incubated at 37°C with 5% CO₂ for 2 h to allow for adhesion. BoMAC cells were then
stimulated with 100 ng/mL of *E. coli* serotype K235 lipopolysaccharide (Sigma) for 15 min to
activate small GTPases and coated latex beads were added to the cultures at a bead-to-cell ratio
of 5:1 for 2 h (Sun et al., 2013). The cultures were washed three times with PBS and BoMAC
cells were lysed with ice-cold lysis buffer (30 mM HEPES pH 7.2, 100 mM NaCl, 10% glycerol,
1% v/v Triton X-100, 1 mM EDTA, 10 mM MgCl₂, 1 mM PMSF, and protease cocktail inhibitor
(Roche) as specified by the manufacturer, using a cell scraper to lift and detach cellular
components. The BoMAC lysates were transferred to a microcentrifuge tube, 5 units DNase
(Thermo Scientific) added, rested on ice for 30 min, and stored at -80°C.
2.6.1 Coating of latex beads with Map rNdk

Aldehyde/sulfate latex beads (4% w/v, 4 µm) (Thermo Scientific) were coated with purified proteins as described by Sun et al., (2010, 2013). The beads are formed from amorphous polymer (polystyrene), providing a hydrophobic surface in which the charged groups at the ends of the polymer chains provide the colloidal stability of latex beads to prevent them from aggregating. Adsorption of antigens to aldehyde/sulfate latex occurs via passive adsorption based on hydrophobic interaction, Van der Waal’s forces, and ionic interactions (Cantarero et al., 1980; Wood and Gadow, 1983; Thermo Scientific).

Latex beads (10^8) were prepared as specified by the manufacturer. The beads were washed twice with 500 µL ice-cold 25 mM MES buffer (pH 5.8), resuspended in 500 µL of the same buffer containing 250 µg/mL of Map rNdk or bovine serum albumin (BSA, Roche) as a control, and incubated for 16 h at room temperature. Beads were then washed 3 times with 500 µL PBS and resuspended in 1 mL of PBS with 0.1% BSA to block the remaining active sites on the latex beads and reduce non-specific binding. The efficiency of coating was examined by SDS-PAGE and Coomassie Blue staining (Appendix Fig. S7). The coating of proteins was estimated to be between 0.18-0.25 pg of protein per bead based on the difference between the protein concentration before adsorption and after incubation with latex beads. To check for contamination, LB media was incubated with or without (negative control) coated-latex beads or inoculated with E. coli (positive control) for 24 h at 37°C. Beads were stored at 4°C.

2.7 Pull-down assays

For pull-downs using Talon Resin, his-tagged Map rNdk (2 µg) was immobilized on 100 µL of Talon resin in a microcentrifuge tube and used as bait. BoMAC whole cell lysate, Map whole cell lysate, or Map culture filtrate proteins were mixed with Map rNdk in PBS at a final volume
of 500 µL for 3 h at room temperature or 16 h at 4°C with gentle mixing. The resin was allowed to settle by gravity and the supernatant transferred to a microcentrifuge tube. The resin was then washed three times with 500 µL of PBS containing 20 mM imidazole with gentle mixing for 5 min. *Map* rNdk and interacting proteins were eluted with 20 µL PBS containing 200 mM imidazole.

Immunoprecipitation of native *Map* Ndk and pull-down assays to determine interaction between *Map* rNdk and Rab small GTPase proteins from BoMAC lysates were performed using SureBeads Protein G magnetic beads (Bio-Rad). Protein G beads bind to immunoglobulin G (IgG) antibodies primarily through their Fc regions and bind weakly to Fab fragments (Kato et al., 1995). Beads (0.3 mg) were prepared using a mini magnetic rack (Bio-Rad) as specified by the manufacturer. Protein G beads have a moderate binding affinity to rat IgG polyclonal antibodies (Bio-Rad). The beads were washed three times with 500 µL PBS-T and incubated with rat anti-*Map* rNdk polyclonal antibodies (1:500) for 10 min at room temperature with gentle mixing. To reduce non-specific binding to Protein G beads, BoMAC lysates and *Map* lysates or culture filtrate proteins were pre-cleared by pre-incubation with 0.1 mg of Protein G for 30 min. The Protein G-rat IgG antibody complex was washed three to four times with 500 µL PBS-T and then incubated with pre-cleared lysates and culture filtrate proteins for 1 h at 4°C with gentle mixing. The Protein G-rat IgG antibody complex was washed three times with 500 µL PBS and target antigen eluted with 20 µL of 20 mM glycine (pH 2.0) directly into a microcentrifuge tube containing 1 µL of 1 M Tris base to neutralize the eluent. Samples were prepared for analysis by SDS-PAGE and Western blotting as described above.
Chapter 3: Results

3.1 Map Ndk shows high homology to Ndk from other mycobacteria

The amino acid sequences of Ndk from Map strain K-10 was compared with Ndk of M. tuberculosis H37Rv, M. bovis BCG Pasteur 1173P2, M. smegmatis MC2 155, and M. avium strain 104 (Fig. 4). High amino acid sequence identity of >80% was found when Ndk of Map was compared to Ndk of M. smegmatis, M. tuberculosis, and M. bovis BCG (Ndk of M. tuberculosis and M. bovis BCG share 100% sequence identity). Map Ndk showed the highest amino acid sequence identity with M. avium (Paustian et al., 2005). Amino acid sequence identity between E. coli and Map was 45%. The conserved catalytic histidine 117 in the histidine-glycine-serine-aspartic acid (HGSD) motif was found in all species as expected. The proteins had similar molecular mass of ~15 kDa and number of amino acid residues varied from 136 for Map, M. avium, M. tuberculosis, and M. bovis BCG, to 139 for M. smegmatis, and 143 for E. coli (Fig. 4). There were no putative N-terminal Sec-dependent or twin arginine translocase signal peptides found.

3.2 Expression and detection of recombinant Map Ndk

Map rNdk was successfully expressed in E. coli BL21 (Fig. 5 A and B) and the His-tagged protein was purified to near homogeneity by affinity chromatography using Co-TALON resin (Fig. 5 C and D). The His-probe detected rNdk in lysates of the induced but not uninduced E. coli cells (Fig. 5 B). Following purification and concentration of rNdk, the protein was detected by Coomassie Blue staining from both the uninduced (Fig. 5 C) and IPTG-induced (Fig. 5 D) E. coli cells. The apparent molecular mass of the His-tagged protein, at ~15 kDa, corresponded to
Map  MTERTLVLIKPDGVQRQLVGEIIGRIERKGLTLVALELRHVSQD 44
E. coli  MAIERTFSIIKPNAVAKNVIGNIFARFEAAGFKIVGTKMLHLTVAI 44
M. avium  MTERTLVLIKPDGVQRQLVGEIIGRIERKGLTLVALELRHAEHE 44
M. smegmatis  MTERTLVLIKPDGVQRQLVGEIIGRIERKGLTLVALELRHVSQD 44
Mtb / M. bovis BCG  MTERTLVLIKPDGVQRQLVGEIIGRIERKGLTLVALELRHVSQD 44

Map  LAAQHYAEHEKPFASLLEFITSGPVVAAIVEGPRAILAARQL 88
E. coli  EQARGFYAEHDGKPFFASLLEFITSGPVVAAIVEGPRAILAARQL 88
M. avium  VSQDLAAQHYAEHDGKPFFASLLEFITSGPVVAAIVEGPRAILAARQL 88
M. smegmatis  LARQHYAEHADKPFASLLEFITSGPVVAAIVEGPRAILAARQL 88
Mtb / M. bovis BCG  LASQHYAEHDPFASLLEFITSGPVVAAIVEGTRAAILAARQL 88

Map  AGGTDPVEKAIPGTIRGDFLETQNLVHGSDSVEASKREIAL 131
E. coli  LLGATNPANALAGTLRADYADSLTENHTGSDSVEASAIREAY 131
M. avium  AGGTDPVEKAIPGTIRGDFLETQNLVHGSDSVEASKREIAL 131
M. smegmatis  AGGTDPVEKAIPGTIRGDFLETQNLVHGSDSVEASKREIAL 131
Mtb / M. bovis BCG  AGGTDPVEKAIPGTIRGDFLETQNLVHGSDSVEASKREIAL 131

<table>
<thead>
<tr>
<th>Amino acid identity</th>
<th>With Map Ndk</th>
</tr>
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<tbody>
<tr>
<td>Map</td>
<td>WFPDA--------  136</td>
</tr>
<tr>
<td>E. coli</td>
<td>FFGGEVCPTR      143  45.0%</td>
</tr>
<tr>
<td>M. avium</td>
<td>WFPDA--------  136</td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>WFPGEATA------ 139  82.6%</td>
</tr>
<tr>
<td>Mtb / M. bovis BCG</td>
<td>WFPGEA-------- 139</td>
</tr>
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Figure 4. Protein sequence alignment comparing amino acid identity of *Map* Ndk to E. coli and other mycobacterial species. The red coloured amino acids represent the conserved histidine-glycine-serine-aspartic acid (HGSD) motif which contains the catalytic His-117 residue. *Map* Ndk shares 45% identity with *E. coli*, 82.6% identity with *M. smegmatis*, 83.8% identity with *M. tuberculosis* (Mt) and *M. bovis BCG*, and 99.3% identity with *M. avium*. *Mt* and *M. bovis BCG* share 100% identity.
Figure 5. Expression and purification of Map rNdk. Coomassie stained SDS-PAGE (A) and Western blot with His-probe (B) of 5 µg each of lysates of the E.coli BL21 cells with empty plasmids (lane 2), and the E.coli cells carrying pET-Map rNdk plasmid before (lane 3) and after (lane 4) induction with IPTG. Lane 1 corresponds to protein standards (PS). The arrow shows the His-tagged recombinant Map rNdk (lane 4, A) was detected by the His-probe antibody (lane 4, B). Lysates (1 mg) of the uninduced (C) and IPTG induced (D) E.coli cells carrying the recombinant plasmids were loaded on TALON-IMAC resin columns. The unbound proteins were collected in the flow-through (FT) fraction. An imidazole gradient of 5-200mM was used to elute proteins bound to the resin and 1 mL fractions were collected. Samples (10 µl) were resolved on a 15% (w/v) SDS-polyacrylamide gel and stained by Coomassie Brilliant Blue G-250. Arrow head shows position of His-tagged Map rNdk. LY, lysate.
the theoretical molecular mass of 14.76 kDa (UniProt). Histidine has a molecular mass of 155 Da, which may have increased the mass of His-tagged rNdk. Basal, or ‘leaky’, expression of Map rNdk was observed in the lysate of uninduced *E. coli* cells (Fig. 5 C). Leaky expression of proteins was consistent with gene expression systems based on bacteriophage T7 RNA polymerase, due to initiation of the T7 promoter by T7 RNA polymerase in the absence of induction (Studier and Moffat, 1986). Map rNdk eluted from the resin at an imidazole concentration of 50 mM from uninduced cells and 20 mM from induced cells. A protein band of slightly lower molecular mass than Map rNdk was detected with 5 mM and 10 mM imidazole but not with 20-200 mM imidazole.

### 3.2.1 Antigen specificity of the rat polyclonal serum antibodies

Polyclonal serum from rats immunized with Map rNdk was used in Western blot analyses against *E. coli* BL21 lysates and the affinity purified Map rNdk (Fig. 6 B and C). The polyclonal sera was used at a dilution of 1:2000, which was previously determined to be optimal for detection of 0.5 µg of affinity purified Map rNdk (Appendix Fig. S3). Following induction with IPTG, the *E. coli* cells showed overexpression of an ~15-kDa protein corresponding to the affinity purified rNdk protein (Fig. 5 and 6). Another overexpressed protein of slightly lower molecular mass (Fig 5A and 6A) and with low binding affinity to the TALON-IMAC resin was also detected in lysates of IPTG-induced and uninduced *E.coli* (Fig. 5 C and D). Both these protein bands reacted with the polyclonal antibodies against Map rNdk (Fig. 6 B). Depletion of cross-reactive rat polyclonal antibodies against shared epitopes by serum absorption did not significantly reduce reactivity to the two proteins suggesting that Map Ndk and the *E. coli* protein, presumptively native *E. coli* Ndk, have shared epitopes (Fig. 6 C). Interestingly, the
polyclonal serum was absorbed with cell lysates of *E. coli* BL21 and *M. smegmatis* which should have significantly depleted the serum of antibodies recognizing shared epitopes. In contrast to the overproduced presumptive native *E. coli* Ndk protein in the lysates of *E. coli* with empty plasmids, no overproduced protein could be detected in the culture filtrates of *E. coli* with empty plasmids (Fig. 6 D). However, *Map* rNdk was detected in the culture filtrates of uninduced and IPTG-induced *E. coli* cells carrying pET-*Map* rNdk plasmid (Fig. 6 E) and reacted with the polyclonal antibodies against *Map* rNdk (Fig. 6 F).

### 3.2.2 Immunogenicity of *Map* rNdk during infection

To examine whether *Map* Ndk induces an antibody-mediated immune response in cattle, serum from cows naturally infected with *Map* bacteria and serum from uninfected calves were reacted with purified r*Map* Ndk and lysates of *E. coli* with empty plasmids and plasmids expressing *Map* rNdk (Fig. 7). The unabsorbed and absorbed serum from the uninfected cows (Fig. 7 B and C) reacted with the purified *Map* rNdk and showed extensive cross-reactivity with *E. coli* cellular proteins. The unabsorbed and absorbed serum from cattle that has tested positive for *Map* infection using the IDEXX JD-ELISA and PCR tests (Fig. 7 D and E) also reacted with the *Map* rNdk protein, with a protein band corresponding to the presumptive *E. coli* Ndk and with a few other *E. coli* cell proteins. There was little to no observable difference to the strength of reactivity of purified *Map* rNdk between absorbed and unabsorbed antibodies from both uninfected and *Map*-infected cows (compare Fig. 7 B-E, Appendix Fig. S4). As Ndk is a conserved housekeeping enzyme, these results further demonstrated the conservation of protein epitopes in Ndk from environmental bacteria.
Figure 6. Antigen specificity of rat polyclonal antibodies against *E. coli* lysates and *Map rNdk*. (A-C) Cells of *E. coli* BL21 lysates (10 µg) with empty plasmid, with plasmids carrying *Map rNdk* before and after IPTG induction, and the purified *Map rNdk* protein, were resolved on a 15% (w/v) SDS-polyacrylamide gel and stained by Coomassie Brilliant Blue G-250 (A), then transferred to blots and reacted with 1:2000 dilution of rat polyclonal antibodies that were unabsorbed (B) and absorbed (C) with lysates of *E. coli* and *M. smegmatis* cells. The rat polyclonal antibodies reacted with a 15 kDa protein corresponding to the affinity purified *Map rNdk* and with an overproduced native *E. coli* protein of slightly lower molecular mass (B and C). In (D), *E. coli* lysates and culture filtrates (2.5 µg) with empty plasmid were analyzed by SDS-PAGE. In contrast to the presumptive native *E. coli* Ndk observed in the lysate, this band was not detected in the culture filtrate. *Map rNdk* in the culture filtrates (5 µg) of uninduced and induced *E. coli* cells (E) reacted with unabsorbed rat polyclonal antibodies (F). Arrows indicate position of *Map rNdk* and arrowhead indicates presumptive native *E. coli* Ndk. PS, protein standards; LY, lysate; CF, culture filtrate.
Figure 7. Antigen specificity of bovine polyclonal antibodies against *E. coli* lysates and *Map* rNdk. Cells of *E. coli* BL21 lysates (10 µg) with empty plasmid, with plasmids carrying *Map* rNdk before and after IPTG induction, and the purified *Map* rNdk protein were resolved on a 15% (w/v) SDS-PAGE and stained by Coomassie Brilliant Blue G-250 (A). Samples from (A) were analyzed by Western blotting using unabsorbed (B and D) and absorbed (C and E) bovine serum (1:300) from uninfected cows (B and C) and *Map*-infected cows (D and E). Arrows indicate position of *Map* rNdk. PS: protein standards; LY, lysate.
3.2.3 Generation of *Map* rNdk-H117 catalytic mutants

The histidine residue at position 117 of *Map* rNdk in the HGSD motif was shown to be required for the biochemical activities of recombinant Ndk in *M. smegmatis* and *M. tuberculosis* (section 1.7) (Tiwari et al., 2004; Arumugam and Ajitkumar, 2012). *Map* rNdk-H117 catalytic mutants were prepared by performing site-directed mutagenesis based on overlapping PCR as described in the materials and methods. H117 residues were mutated to alanine (a polar to hydrophobic substitution), glutamic acid (a polar to polar substitution), glutamine (a polar to polar substitution), glycine (a polar to nonpolar substitution), and tyrosine (a polar to polar substitution). The *Map* rNdk-His117 mutants were examined for their reactivity with absorbed rat polyclonal antibodies and absorbed bovine polyclonal antibodies from *Map*-infected cattle (Fig. 8). The H117Y mutant showed decreased interactions with the rat polyclonal antibodies (Fig. 8 B). Compared to rat polyclonal antibodies (Fig. 8 B), the absorbed bovine serum from *Map*-infected cattle reacted less strongly with the purified *Map* rNdk proteins and especially with the H117G, H117Q, and H117Y catalytic mutants (Fig. 8 C), suggesting that mutations may be located in the antigenic determinant and alter affinity to avidity of antibody-epitope interactions (Benjamin and Perdue, 1996; Dougan et al., 1998; Frank, 2002).

3.2.4 Determining the localization of native Ndk from cultured *Map* cells

Cloning, expression and functional analyses of the putative Ndk from *Map* has not been previously reported. Cell lysates and culture filtrates of *Map* stain gc86 were examined by Western blotting with rat polyclonal antibodies. Although Ndk has no known signal secretion peptide sequence, previous studies have reported the extracellular secretion of NdK in a number of pathogens, including *M. bovis BCG* and *M. tuberculosis* (Zaborina et al., 1999a, b; Punj et al.,
2000; Zaborina et al., 2000, Chopra et al., 2003; Yilmaz et al., 2008; Dar et al., 2011). In this study, Map rNdk was shown to be secreted extracellularly in the culture filtrates of uninduced and induced E. coli cells (Fig. 6 E and F).

Native Map Ndk was not detected in the lysate and culture filtrate when using a 1:1000 dilution of rat polyclonal antibodies (Appendix Fig. S5 B). This polyclonal antibody at a dilution of 1:1000 and 1:500 detected as little as 3.12 µg and 0.78 µg of Map rNdk, respectively (Appendix Fig. S3). Cellular Map Ndk was also undetected in Western blot analyses using 20 µg and 50 µg proteins of Map lysate with an antibody dilution of 1:500 (Appendix Fig. S5 C and E), suggesting the protein is present in extremely small quantities in lysates and culture filtrates of cultured Map cells. Native Map Ndk was detected by immunoprecipitation from Map gc86 cell lysates and culture filtrates by SDS-PAGE and Western blotting (Fig. 9 A-D, lane 7). Based on the intensity of reactions with the rat polyclonal antibody, native Map Ndk was present in relatively higher concentrations in the lysates compared to the culture filtrates (Fig. 9 A-D, lane 7). There was no discernible difference observed in the molecular mass between native Map Ndk and Map rNdk (Fig. 9 B and D, lanes 7 and 9). In addition to Map Ndk, the elution fraction analyzed by SDS-PAGE contains a large number of unidentified proteins that interacted with the antibody-coupled Protein G beads (Fig. 9 A and B, lane 7). In this assay, the antibodies were not covalently linked to the Protein G magnetic beads, which resulted in the detachment and elution of the 150-kDa rat IgG antibodies in the low pH glycine buffer (Janeway et al., 2001). The IgG heavy chain (~50 kDa) and light chain (~25 kDa) were detected on the blots by reactions with the HRP-conjugated goat anti-rat IgG secondary antibodies (Fig. 9 A-D, lane 7). The faintly labelled band in lane 2 (Fig. 9 B) was not identified, and likely an artifact.
Figure 8. Antigen-specificity of the rat and bovine polyclonal antibodies against purified *Map* rNdk and *Map* rNdk H117 catalytic mutants. Histidine at position 117 of purified *Map* rNdk was replaced with alanine (H117A), glutamic acid (H117E), glycine (H117G), glutamine (Q), and tyrosine (H117Y) as described in the materials and methods. (A) Purified *Map* rNdk and H117 catalytic mutants (0.5 µg) analyzed by SDS-PAGE and Coomassie Blue stained. (B) Samples from (A) analyzed by Western blotting with absorbed rat polyclonal antibodies (1:2000). (C) Samples from (A) analyzed by Western blotting using absorbed bovine polyclonal antibodies (1:300) from *Map*-infected cows. PS, protein standards.
Figure 9. Localization of Native Map Ndk by immunoprecipitation. Rat polyclonal antibodies against Map rNdk protein were non-covalently bound to protein G magnetic beads. Protein G binds IgG antibodies. Immunoprecipitation of Ndk was performed by addition of the protein to the pre-cleared Map lysate (A and B) and culture filtrate (C and D) proteins (0.5 mg each). Protein binding to the immobilized antibodies were eluted with 20 µl of 20 mM glycine (pH 2.0), neutralized with Tris base and 10 µl loaded per lane of 15% (w/v) SDS-PAGE. Gels were silver-stained (A and C) and analyzed Western blotting (B and D) using 1:1000 dilution of rat anti-Ndk polyclonal antibodies. Lane 1 (PS), protein standards; Lane 2 (S), supernatants containing proteins not bound to the the protein G beads; Lanes 3-6 (W1-W4), the proteins eluting in the protein G washes; E, the proteins binding to the protein G-IgG beads and eluted with glycine buffer. The arrows indicate position of purified Map rNdk (0.5 µg), which was used as a reference (lane 9). Native Map Ndk in the elution (E) fractions (lane 7) is highlighted in red. The faintly detected native Map Ndk in the culture filtrate (D) is indicated by the asterisk, and was seen with a slightly higher intensity in the original blot.
These results showed native Map Ndk to be mainly a cellular protein that is also secreted extracellularly. In this study, this was also true for E. coli, where the overproduction of the presumptive native E. coli Ndk was detected in the lysate and not in culture filtrates (Fig. 6 D), indicating the enzyme to be mainly intracellular.

3.3 Interactions between Map rNdk and Rab small GTPases

The interaction of recombinant Ndk from M. smegmatis and M. tuberculosis with Rab5 and Rab7 small GTPase proteins was previously reported (Sun et al., 2010). In order to determine if Map rNdk binds to Rab5 and Rab7, a pull-down experiment was employed. Map rNdk was immobilized to TALON-IMAC resin, incubated with lysates of a cultured bovine macrophage BoMAC cell line, and after washing with buffer to remove unbound proteins, the proteins interacting with the rNdk were eluted with imidazole as described in the materials and methods. Rab5 and Rab7 proteins were detected in BoMAC lysates using commercial anti-Rab5 and anti-Rab7 antibodies (Fig. 10). The imidazole concentration used at 200 mM was determined to be optimal for purification of Map rNdk (Fig. 5 C and D). In this assay, BoMAC Rab5 and Rab7 did not interact with the Map rNdk (Fig. 11 D). Assays using different concentrations of BoMAC lysates and rNdk incubated for 16 h at 4°C were also tested (Appendix Fig. S6). No interactions between Map rNdk and BoMAC Rab5 or Rab7 were detected.

The interaction between Map rNdk and Rab5 or Rab7 proteins was further examined by incubating BoMAC cells with Map rNdk- or BSA-coated latex beads as described in the materials and methods. Lysates of untreated cells (control), cells incubated with BSA-coated latex beads (control), and cells incubated with Map rNdk-coated latex beads were added to the Protein G-rat IgG complex and interacting proteins eluted and analyzed by silver staining (Fig.
Figure 10. Detection of Rab5 and Rab7 small GTPase proteins by Western blotting. (A) BoMAC whole cell lysate (10 µg) was resolved on a 15% (w/v) SDS-polyacrylamide gel and stained by Coomassie Brilliant Blue G-250. (B) Rab5 (~25 kDa) and Rab7 (~23 kDa) proteins in BoMAC lysate (10 µg) were analyzed by Western blotting using Rab5 and Rab7 rabbit monoclonal antibodies (1:1000). PS, protein standards.
Figure 11. Pull-down assays to investigate interactions between Map rNdk and Rab small GTPase proteins, Rab5 and Rab7. (A) Affinity purified Map rNdk (2 µg) was immobilized to TALON resin and serves as control. (B) BoMAC lysates (50 µg) added to TALON resin. (C) BoMAC lysates (50 µg) added to Map rNdk immobilized to TALON resin as described in (A). All reactions were incubated for 3 h at room temperature, washed with 20 mM imidazole buffer, and the bound proteins eluted with 20 µl 200mM imidazole. Ten microliters of the eluates were resolved by SDS-PAGE and silver-stained (A-C). (D) Elution fraction 1 (E1) was analyzed by Western blotting with rat polyclonal antibodies (1:2000) as shown in lane 2, as well as Rab5 and Rab7 rabbit monoclonal antibodies (1:1000) as shown in lanes 3 and 4, respectively. Arrows indicate Map rNdk. Similar results were obtained for elution fraction 2 (E2, data not shown). PS, protein standards; S, supernatant; W, wash; E, elution.
Figure 12. Pull-down assays to investigate interactions between Map rNdk and Rab small GTPase proteins, Rab5 and Rab7. BoMAC cells were incubated with Map rNdk- (BoMAC-rNdk) or BSA- (BoMAC-BSA, control) coated latex beads at a ratio of 1:5 for 2 h at 37 or left untreated (control). Lysates from BoMAC cells were incubated with rat anti-Ndk antibody (1:1000) and Protein G beads as described in the materials and methods. Elution fractions (20 µl) were analyzed by silver-staining (A) and Western blotting (B and C) using rat polyclonal antibodies (B, 1:2000) and Rab5 or Rab7 rabbit monoclonal antibodies (C, 1:1000). Map rNdk is highlighted in red and the heavy chain (~50 kDa) and light chain (~25 kDa) of rat IgG antibodies are highlighted in green. Purified Map rNdk (200 ng) was used as a reference (B). PS, protein standards.
12 A) and Western blotting (Fig. 12 B and C). As previously found (Fig. 9), the IgG heavy chain (~50 kDa) and the light chain (~25 kDa) were observed in the elution fractions as the rat IgG antibodies were not crosslinked to Protein G, causing them to elute with the target antigens (Fig. 12 B). Other unidentified proteins of ~17 and > ~78 kDa coeluted in the bound fraction indicating either non-specific binding to the beads or to rat IgG antibodies (Fig. 12 B). The unidentified ~17 kDa band was observed only in the elution fractions of untreated cells and cells incubated with BSA-coated latex beads, but not from cells incubated with Map rNdk-coated latex beads (Fig. 12 B). Rab5 or Rab7 proteins, however, were not detected. (Fig. 12 C).

3.4 Interactions between Map rNdk and other Map proteins

M. smegmatis Ndk interacted with other cellular M. smegmatis proteins, such as EF-Tu and pyruvate kinase (Shankar et al., 1997a). These interactions modulated the specificity of NTP synthesis, which was also reported in P. aeruginosa (Kavanaugh-Black et al., 1994; Shankar et al., 1996; Chopade et al., 1997; Mukhopadhyay et al., 1997). Interestingly, Map EF-Tu was identified in the cell-wall fraction (He and De Buck, 2010) and in the culture filtrate (Facciulo et al., 2013). In this thesis, Map gc86 cell lysates and culture filtrates were incubated with TALON resin on which Map rNdk was immobilized. Proteins interacting with the rNdk protein were eluted with imidazole as described in the materials and methods. No specific rNdk-interacting proteins could be detected in Map lysate or culture filtrate proteins (Fig. 13 and 14). The same results were obtained when Map rNdk was incubated with up to 1mg of Map lysate proteins (data not shown).
Figure 13. Pull-down assays investigating interactions between *Map* rNdk and *Map* lysate proteins. *Map* rNdk (2 µg) was immobilized to TALON resin, incubated with 100 µg (B) or 200 µg (C) of *Map* lysate for 16 h at 4°C, and eluted with 20 µl of 200 mM imidazole as described in the materials and methods. (A) *Map* lysate control (100 µg) incubated with TALON resin alone. Samples (20 µl) were analyzed by SDS-PAGE and silver-stained. PS, protein standards; S, supernatant; W, wash; E, elution. Arrows indicates *Map* rNdk.
Figure 14. Pull-down assays investigating interactions between Map rNdk and Map culture filtrate proteins. Map rNdk (5 µg) was immobilized to TALON resin, incubated with 250 µg (C) of Map culture filtrate for 16 h at 4°C, and eluted with 20 µl of 200 mM imidazole as described in the materials and methods. (A) Ndk control (5 µg) immobilized to TALON resin alone. (B) Map culture filtrate control (250 µg) incubated with TALON resin alone. Samples (20 µl) were analyzed by SDS-PAGE and silver-stained. S, supernatant; W, wash; E, elution. Arrows indicate Map rNdk.
Chapter 4: Discussion

In this thesis, the 15-kDa putative Map Ndk protein was successfully expressed in induced E.coli cells. Basal, or ‘leaky’, expression of Map rNdk was also observed in the lysate of uninduced E. coli cells. This was not unexpected as basal expression of recombinant proteins using gene expression systems based on bacteriophage T7 RNA polymerase is known to occur (Studier and Moffat, 1986), where the T7 RNA polymerase initiates the T7 promoter in the absence of induction. The rat polyclonal antibodies reacted on Western blots with two protein bands, the recombinant Map Ndk and an E. coli protein of slightly lower MW suggesting the presence of shared epitopes and presumptively identifying the lower MW protein as native E. coli Ndk. The rat polyclonal antibodies absorbed with lysates of E.coli and M. smegmatis retained almost undiminished reaction with the E. coli and Map proteins. Although Map Ndk shared 45% and 82.6% amino acid identity with E. coli and M. smegmatis Ndk (Fig. 4), respectively, failure to deplete antibodies to the shared epitopes suggested that these epitopes were not accessible on the non-denatured E. coli or M. smegmatis proteins used for serum absorption, the epitopes were exposed on the proteins after being denatured for analysis by SDS-PAGE, and the epitopes were linear not conformational epitopes. Since the absorbent was not denatured, antibodies against linear epitopes not exposed on the surface of the native proteins would not be depleted and would react with the denatured protein on blots.

E. coli Ndk was reported to have an experimental molecular mass of 16.5 kDa and has a deduced molecular mass of 15.5 kDa (Hama et al., 1991). The presumptive native E. coli Ndk in this study was overproduced (Fig. 6 D), consistent with the study by Hama et al. (1991) that showed the overproduction of E. coli Ndk (at a level of approximately 25% of total cellular proteins). As expected, the E. coli putative Ndk protein had a much lower affinity for the
TALON-IMAC column which can be attributed to the lack of the six extra histidine residues present in the recombinant Map Ndk protein. However, I have not confirmed the protein to be E. coli Ndk. The difference in MW could be due to several reasons. Hama and colleagues (1991) used a different E. coli strain, E. coli JM83 (pKT8P3), and the proteins were resolved on a 20% SDS-polyacrylamide gel as opposed to the 15% used in this study. In this study, Map rNdk was observed to migrate in the gel to a location that showed an apparent molecular mass of ~15.5-16 kDa using a 20% gel and slightly lower (~ 14 kDa) using a 12% gel (data not shown). Moreover, the histidine residues in the polyhistidine tag, which have a molecular mass of 155 Da, could also have accounted for the differences observed.

As Ndk from Map shared 45% to >80% aminoa acid sequence identity with the Ndk of E. coli and other mycobacterial species, respectively, this suggested that proteins would have shared or common epitopes as well as specific epitopes; thus animals exposed to environmental bacteria would have antibodies reacting with the Map Ndk as observed in this thesis (Fig. 7). The reactivity of bovine polyclonal antibodies from non-infected cows and Map-infected cows against Map rNdk were more or less equally reactive (Fig. 7 and Appendix Fig. S4). This indicated prior exposure to Ndk, which was shown to be antigenic. Interestingly, polyclonal antibodies from sera of uninfected cows were shown to be more reactive to E. coli antigens than sera from Map-infected cows (Fig. 7), indicating that cows without Johne’s disease were exposed to more environmental E. coli bacteria than cows with Johne’s disease.

Map rNdk and His-117 mutants were all more or less equally reactive with polyclonal antibodies from Map-infected cattle (Fig. 8C). Serum polyclonal antibodies from rats, however, were less reactive to H117G and H117Y mutants (Fig. 8 B). The entire surface of an antigens has many epitopes, with many overlapping domains that antibodies can discriminate as distinct
epitopes (Schroer et al., 1983; Benjamin et al., 1984). Approximately 15 amino acids represent an epitope, which are discontinuous in the primary sequence but are brought together upon folding of the protein (Benjamin and Purdue, 1996). Polyclonal antibodies are raised against many epitopes on the surface of an antigen (Frank, 2002). Therefore, cross-reactivity decreases linearly with the number of amino acid substitutions as each exposed amino acid contributes a small amount to the total binding, as the surface of an antigen would appear continuous due to the many overlapping set of epitopes. By contrast, a monoclonal antibody usually binds to a single epitope on the antigen surface and cross-reactivity declines nonlinearly with the number of amino acid substitutions in the target epitope because only a small number of amino acids control the binding. By using absorbed rat polyclonal antibodies, mutations to H117 residues were shown to be less reactive to H117G and H117Y (Fig. 9 B). Site-directed mutagenesis have been used to create epitopes in which only a single amino acid is mutated, as in this study, allowing the measurement of the relative binding of the antibody caused by an amino acid substitution. Although absorbed antibodies were used to reduce cross-reactivity to E. coli and M. smegmatis Ndk, the relative binding of Map rNdk and His-117 mutants with a purified, monoclonal antibody will allow better determination of the effects of these mutations; considering His-117 is part of the epitope recognized by the monoclonal antibody.

Although Ndk does not contain a signal secretion peptide sequence, previous studies have detected secreted Ndk by Western blotting using anti-Ndk antibodies (Chopra et al., 2004; Dar et al., 2011). An attempt to identify native Map Ndk in the culture filtrate or lysate by this method was unsuccessful in this study (Appendix Fig. S5). In contrast to the endogenously overproduced cellular E. coli Ndk at a level of approximately 25% of total cellular proteins, as reported by Hama et al. (1991) and the presumptive native E. coli Ndk in this study), this indicated cellular
Map Ndk was present in very low amounts. Why E. coli overproduces cellular Ndk while mycobacteria (Map and M. smegmatis in this study) do not remains to be determined. The difficulties in identifying native Map Ndk was overcome by immunoprecipitation, which allowed us to increase the amount of lysate and culture filtrate proteins and reduce the amount of rat serum required for its detection. Map Ndk was shown to be more abundant in the lysate than in the culture filtrate (Fig 10), indicating that Ndk is mainly an intracellular protein, which was also observed for the presumptive native E. coli Ndk protein in this study (Fig. 7 D). This was not unexpected given the housekeeping function of Ndk and the importance of the enzyme in the maintenance of intracellular nucleotide triphosphate pools (Chakrabarty, 1998)

In contrast to the study by Sun et al. (2010) that showed M. tuberculosis and M. smegmatis Ndk binding to Rab5 and Rab7, interactions between Map Ndk and Rab proteins were not detected in this study. Sun and colleagues (2010) used ELISA and pull-down experiments to demonstrate the interaction between recombinant Ndk and recombinant Rab proteins in a cell-free system. In this study, two methods using pull-down assays were used to identify interactions between recombinant Ndk and Rab proteins in a whole-cell system (Fig. 11 and 12, Appendix Fig. S6). The difference between using cell-free systems and whole-cell systems may provide an explanation as previous reports have shown (Chopra et al., 2004; Sun et al., 2013). GTPase-activating protein (GAP) activity of recombinant M. tuberculosis Ndk by Chopra and colleagues (2004) showed that recombinant M. tuberculosis Ndk interacted with Rho, Cdc42, and Rac1 preloaded with [γ32P]-GTP in a cell-free system. Moreover, Ndk possessed GTPase-activating protein (GAP) activity that inactivated the Rho, Cdc42, and Rac1 proteins preloaded with [γ32P]-GTP. However, Sun and colleagues (2013) used a whole-cell system approach to examine GAP activity, where mouse macrophages were allowed to ingest Ndk-coated latex beads and cell
lysates were subjected to immunoprecipitation with anti-Ndk antibody. In contrast to the study by Chopra et al. (2004), the authors showed recombinant *M. tuberculosis* interacted with only Rac1, and not Rho or Cdc42. These results clearly demonstrate the differences between using cell-free systems and whole-cell systems.

I employed the same whole-cell based experiment using latex beads (Fig. 12) to identify interactions between *Map* Ndk and Rab proteins using the same method by Sun et al. (2013). In this study, the interactions between *Map* rNdk and Rab5 or Rab7 were not observed. There could be several reasons for this. Firstly, the interaction between *M. tuberculosis* Ndk and Rab5 or Rab7 was demonstrated using a cell-free system by ELISA (Sun et al., 2010). Secondly, it was previously demonstrated that Ndk may not directly interact with GTP-binding proteins but rather influence the levels of unbound free nucleotides by hydrolyzing GTP through its GTPase activity to the point where free GDP nucleotides compete more effectively with GTP (Randazzo et al., 1992). Thus, direct inactivation without nucleotide exchange may be unlikely as X-ray crystallography showed the guanine nucleotide bound to small GTPases were buried in the protein, making it more difficult for a buried nucleotide to access the same binding site on Ndk as a free nucleotide (Kim et al., 1988; de Vos et al., 1990; Pai et al., 1990; Tong et al., 1991; Randazzo et al., 1992, Zhu et al., 2004). Nevertheless, the small GTPase Rac1 was shown to directly interact with Ndk by Sun et al. (2013) using a whole-cell system with latex beads. Interestingly, Ndk from non-pathogenic *M. smegmatis* was impaired in its GTPase activities towards Rab5 and Rab7 when compared to Ndk from pathogenic *M. tuberculosis* (Sun et al., 2010). These results were in contrast to the study by Arumugam and Ajitkumar (2012) that showed *M. smegmatis* Ndk possessing NTPase activity, although the NTP was not bound to small GTPases and may account for the differences observed. Additionally, despite absolute
sequence identity, Ndk with a mutated His residue in the HGSD motif from pathogenic *M. tuberculosis* did not show phosphotransferase activity to ATP (Tiwari et al., 2004) whereas the same mutant from nonpathogenic *M. tuberculosis* had no effect on phosphotransferase activity (Chopra et al., 2003). The differences in activity of Ndk between non-pathogenic and pathogenic mycobacteria remain to be determined. As shown by Dar et al. (2011), Ndk from extracellular pathogens (e.g. *V. cholerae*) was cytotoxic to mouse macrophages after treatment with exogenous ATP whereas Ndk from intracellular pathogens (*S. typhimurium*) decreased ATP-induced cell death. Interestingly, an Ndk-deleted strain of *S. typhimurium* was able to prevent ATP-induced cell death when complemented with Ndk from *V. cholerae*, despite exhibiting 68% amino acid sequence identity. This indicated that the intrinsic activities of Ndk are indistinguishable, irrespective of their mode of manifestation between intracellular and extracellular pathogens.

An attempt to identify interactions between *Map* Ndk and other *Map* lysate or culture filtrate proteins was not met with success (Fig. 14 and 15). The study by Shankar et al. (1997a) demonstrating interactions between *M. smegmatis* Ndk and other cellular *M. smegmatis* proteins was performed by immobilizing Ndk onto Sepharose matrix and passing the total cell-free extract with a concentration in proteins 50-fold higher than the amount used to immobilize Ndk. However, Ndk was purified from *M. smegmatis* by ammonium sulfate precipitation (Kavanaugh-Black et al., 1994) and was not produced with a polyhis-tag as *Map rNdk* this study. As such, our experiment in determining interactions by immobilizing his-tagged *Map rNdk* to TALON resin was similar, although differences observed due to different methodologies may explain the discrepancies. Furthermore, it may well be that Ndk from pathogenic *Map* may not bind to other *Map* proteins as was demonstrated for Ndk from non-pathogenic *M. smegmatis*. However, these
experiments will require further testing. The study by Mukhopadhyay et al. (1997) showed the predominant generation of GTP upon the interaction between *P. aeruginosa Ndk* and and EF-Tu using the same method as Shankar et al. (1997). However, Mukhopadhyay and colleagues added GTP to the Sepharose matrix. Therefore, it would be interesting to see if the addition of nucleotides may have an effect. Interestingly, EF-Tu was identified in the culture filtrate (Facciuolo et al., 2013) and in membrane vesicles (Martin and Mutharia, 2015, unpublished results) in our lab, as well as a cell-wall associated protein (He and De Buck, 2010). It would be interesting to further examine its interactions with Ndk as GTP-binding proteins involved in a variety of signalling events may further modulate bacterial interference with eukaryotic hosts during invasion. Mammalian heterotrimeric G-protein-like GTP-binding proteins involved in transmembrane signalling were identified in mycobacteria (*M. tuberculosis* and *M. smegmatis*) and shown to interact with Ndk to primarily generate GTP, providing another potential mechanism in evading the innate immune system through altered signalling (Shankar et al., 1997b).
Chapter 5: Future Directions

As Ndk was observed in Map lysates and culture filtrates, it would be interesting to determine if Ndk is also associated with the membrane. Future improvements to the pull-down experiments to determine interactions between Map Ndk and Rab proteins will be required. Incubation of BoMAC cell lysates with Ndk immobilized to Talon resin may require the addition of nucleotides, although free nucleotides are expected to be present in the whole cell lysate. Cell-free systems can also be employed (Chopra et al., 2004; Sun et al., 2010) to determine the GTPase activity of Map Ndk either by ELISA using recombinant Ndk and recombinant Rab protein (Sun et al., 2010) or radioactivity (Chopra et al., 2004). However, as demonstrated by these two studies, results observed from in vitro studies do not always reflect what is observed in vivo. Other methods include Far Western blotting using biotinylated Ndk, BoMAC lysates, or primary antibody, which may significantly increase sensitivity for detecting protein complexes. Similarly, interactions between Map Ndk and other Map proteins will require further optimization and the use of different methods, such as immobilization of Ndk to Sepharose matrix and scaling up, which may prove to be beneficial (Shankar et al., 1997a). Furthermore the addition of nucleotides, such as GTP, may also be required (Mukhopadhyay et al., 1997), although this was not required previously (Shankar et al., 1997a). Once these experiments are met with success, Map Ndk-H117 mutants can be analyzed for their activities. It would be interesting to determine if Map Ndk coated to latex beads can detach from the beads and cross the phagosomal membrane towards the cytosol by fluorescence microscopy as previously demonstrated for M. tuberculosis Ndk (Sun et al., 2010; Sun et al., 2013).

The ability of Ndk and other nucleotide-utilizing enzymes (e.g. pyruvate kinase, ATPase) from P. aeruginosa and M. tuberculosis to alter ATP-induced apoptosis should also be examined.
further. As previously discussed, a limitation in these studies was that only concentrated culture supernatant fractions were assayed, making it difficult to directly link specific bacterial effectors with host-cell death or survival. However, gingival epithelial cells infected with *P. gingivalis* that had a disruption in the *Ndk* gene (Choi et al., 2013) showed increased ATP-induced cell death and increased bacterial survival, indicating that Ndk plays a major role in ATP-induced death. The ability of cells to undergo apoptosis after infection by the release of endogenous ATP promoted the killing of intracellular *M. bovis* BCG in infected macrophages by inducing acidification, phagolysosome fusion, and fragmentation of nuclear chromatin, which can lead to the genetic material of the bacilli being compromised (Molloy et al., 2004). It would be interesting to determine the individual effects of *Map* Ndk and other nucleotide-utilizing enzymes on ATP-induced cell death in uninfected and *Map*-infected bovine macrophages, as it was previously shown that exogenous ATP treatment killed macrophage cells but had no effect on the viability of intracellular *Map* bacilli (Woo et al. 2007). Moreover, the effects of ATP on *Map*-infected macrophages were different to those previously reported for pathogenic *M. tuberculosis*, non-pathogenic *M. bovis* BCG and other pathogenic intracellular bacteria (Woo et al., 2009). Finally, recombinant Ndk from *M. tuberculosis* was able to localize to the nucleus in mammalian cells (HeLa and COS 1 cells), bind the pyrimidine-rich strand of the nuclease-hypersensitive element of the c-myc promoter, and induce cleavage (Saina et al., 2004; Kumar et al., 2005). These results suggested that *M. tuberculosis* Ndk may damage DNA, helping with bacterial dissemination, particularly in later stages of infection. Nuclease activity from *Map* Ndk will provide further information on mycobacterial tactics to evade host immune responses and disseminate.
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Appendix:

5'-GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG ACC GAA CGT
ACC CTG GTG CTG ATT AAA CCG GAT GGC GTA CAG CGC CAG CTG GTT GGC GAA ATC
ATC GGC CGC ATT GAA CGC AAG GGC CTG ACG CTG GTA GCG CTG GAA CTG CGC CAT
GTT AGC CAG GAT CTG GCT GCT CAA CAT TAC GCT GAA CAC GAA GGT AAG CCG TTC
TTC GCC TCT CTG CTG GAA TTC ATT ACT AGC GGT CCG GTT GTA GCA GCG ATC GTT
GAA GGC CCT CGC GCT ATT GCC GCC TTC CGT CAG CTG GGC GGT GGT ACC GAC CCG
GTT GAA AAA GCT ATC CCG GGC ACC ATC CTG GGT GAC TTT GGT CTG GAA ACC CAG
TTC AAC CTG GTC CAC GGT AGC GAC TCT GTT GAA AGC GCG AAA CGC GAA ATT GGT
CTG TGG TTT CCA GAT GCG CAC CAC CAC CAC CAC CAC TGA GGA TCC

GAA TTC GAG CTC C GTC GAC AAG CTT

3’

Figure S1. Map 2268c nucleotide sequence. The putative nucleoside diphosphate kinase sequence (Map 2268c) was obtained from UniProt and chemically synthesized (Integrated DNA Technologies). The overlaps to the pET30a plasmid are indicated by underlined nucleotides. Red nucleotides indicate the Nde1 restriction site (CATATG), which includes the start codon (ATG). Grey nucleotides indicate the polyhistidine tag (CAC). Pink nucleotides indicate the stop codon (TGA) and the green nucleotides indicate the BamHI restriction site (GGATCC). The catalytic histidine residue at position 117 (CAC) is highlighted in yellow and was replaced with different amino acids as described in the materials and methods (Appendix Fig. S2 and Table S1).
Figure S1. Primer design for site-directed mutagenesis. The sequence shown is a section of Map rNdk DNA sequence from which the forward and reverse primers were designed. The catalytic H117 residue (CAC) is shown by red nucleotides. Underlined nucleotides represent the forward and reverse primers, designed with at least one G or C base at each end. Overlaps are at the 5’-terminus and non-overlaps at the 3’-terminus with the targeted mutation in the centre of the overlaps (H117). H117 was mutated to alanine (H117A), aspartic acid (H117E), glycine (H117G), glutamine (H117Q), and tyrosine (H117Y).
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Table S1. Forward and reverse primers for Map rNdk His-117 mutations.
Figure S3. Titre of rat anti-Map rNdk serum polyclonal antibodies. Purified Map rNdk protein was serially diluted and analyzed by Western blotting using different dilutions of rat anti-Map rNdk polyclonal antibodies. The 1:4000 dilution can detect as low as ~125 ng of Map rNdk. The 1:2000 dilution can detect as low as ~15.6 ng of Map rNdk. The 1:1000 dilution can detect as low as ~0.78-1.56 ng of Map rNdk, and the 1:500 dilution can detect as low as ~0.19-0.39 ng of Map rNdk. Only a minor difference was observed between the 1:500 dilution and the 1:250 dilution.
Figure S4. Reactivity of *Map* rNdk with bovine polyclonal antibodies from uninfected and *Map*-infected infected cattle. *Map* rNdk (1.5 µg) analyzed by Western blotting using bovine polyclonal antibodies from unabsorbed serum (A) and absorbed serum (B) from uninfected and *Map*-infected cattle as indicated. Arrows indicate purified *Map* rNdk. PS, protein standards.
Figure S5. Identification of putative Map Ndk. Map lysate and culture filtrate protein (5 µg) analyzed by SDS-PAGE and silver staining (A). Map lysate and culture filtrate proteins (20 µg) analyzed by Western blotting using rat polyclonal antibodies at a 1:1000 dilution (B) and 1:500 dilution (C). Map lysate (50 µg) analyzed by SDS-PAGE and Coomassie Blue staining (D), and Western blotting (E) with rat polyclonal antibodies (1:500). Purified Map rNdk was used as a reference; 200ng for A-C and 100 ng for D and E. PS, protein standards.
Figure S6. Pull-down assays to investigate interactions between *Map rNdk* and Rab small GTPase proteins, Rab5 and Rab7. *Map rNdk* was immobilized to Talon resin, incubated with BoMAC lysate proteins for 3 h at room temperature (A and B) or 16 h at 4°C (C and D), and eluted with 20 µl of 200 mM imidazole as described in the materials and methods. Samples (20 µl) were analyzed by silver staining (A and C) and Western blotting (B and D). (A) BoMAC lysate (100 µg) mixed with immobilized *Map rNdk* (2 µg). (B) Elution fraction 1 (E1) analyzed by Western blotting using Rab5 or Rab7 rabbit monoclonal antibodies (1:1000). (C) BoMAC lysate (250 µg) mixed with immobilized purified *Map rNdk* (5 µg). (D) Elution fraction 1 (E1) analyzed by Western blotting using Rab5 or Rab7 rabbit monoclonal antibodies (1:1000). Similar results were obtained for elution fraction 2 (E2) analyzed by Western blotting as shown in (B) and (D) (data not shown). PS, protein standards; S, supernatant; W, wash; E, elution. Arrows indicate *Map rNdk*. 
Figure S7. Coating of latex beads with Map rNdk and BSA. (A) Latex beads (5 µl = ~100 ng) coated with purified Map rNdk (Beads-Map rNdk, 15 kDa), BSA (Beads-BSA, ~68 kDa), or left uncoated were analyzed by SDS-PAGE (B) Map rNdk-coated latex beads and purified Map rNdk (100 ng) analyzed by Western blotting using rat polyclonal antibodies (1:2000). PS, protein standards.