The role of RD2 and NOD2 in host-pathogen interactions of the BCG vaccine

By

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Abstract

Bacille Calmette-Guérin (BCG) is the only vaccine currently in use against tuberculosis. Despite its use for nearly a century, the scope of the global tuberculosis pandemic demonstrates that the vaccine is not providing the necessary protection. Improving BCG requires the identification of bacterial determinants of immunogenicity and protection, as well as analysis of how the vaccine stimulates the relevant host pathways.

Work done previously by our laboratory has established that BCG daughter strains have undergone multiple deletions during its ongoing *in vitro* evolution. Following the initial attenuating mutation, the vaccine lost Region of Difference 2 (RD2) during on-going propagation. This is hypothesized to have resulted in the over-attenuation of the vaccine. To test this, we disrupted RD2 in *Mycobacterium tuberculosis* H37Rv and evaluated its phenotype in cellular and animal models. These experiments suggested candidate genes for complementation into BCG strains naturally lacking RD2. Vaccine-challenge studies and immunological assays were used to determine what role RD2 played in vaccine protection. Our findings revealed that RD2 is required for full virulence in *M. tuberculosis* and modulates host innate immunity. However, the presence of RD2 does not increase pulmonary protection in BCG, even though it increases the immunogenicity of the vaccine.

Additionally, previous studies revealed that the host intracellular receptor NOD2 (Nuclear Oligomerization Domain 2) is critical for mediating innate and adaptive immune response to *M. tuberculosis*. Therefore we hypothesized that NOD2 also was important for vaccination. To test this, we vaccinated *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ animals and

then challenged them with an aerosol dose of *M. tuberculosis*. We characterized the effect of *Nod2* disruption on bacterial burden, pathology, and adaptive immunity. Our experiments revealed that the loss of NOD2 resulted in increased pathology in vaccinated animals following infection with *M. tuberculosis*. Furthermore, they suggested that NOD2-mediated control of pathology is accomplished through the action of T-cells.

Overall these studies further our knowledge of the *in vitro* evolution of the BCG vaccine, and how this affects protection. Additionally, our investigation of the role of a host receptor in vaccination highlights the importance of understanding the interactions between host and pathogen for vaccine-induced protection.

Résumé

Le vaccin Bacille de Calmette-Guérin (BCG) est le seul vaccin disponible destiné à protéger contre la tuberculose. Malgré l'usage du vaccin face a la pandemie globale de la tuberculose indique qu'il ne fournit pas la protection requise. Cependant, l'ampleur de l'épidémie mondiale suggère que l'efficacité du vaccin est faible. L'améelioration du vaccin BCG néecessite l'identification de facteurs bactériens de virulence et l'élucidation de la façon dont le vaccin induit une réponse immunitaire dans l'hôte.

Des travaux effectués antérieurement dans notre laboratoire ont établi que les souches filles de BCG ont subi de nombreuses délétions au cours de l'évolution continue *in vitro*. Suite à la première mutation, la Région de Différence 2 (RD2) est perdue pendant la propagation de la bactérie. Nous avons émis l'hypothèse que cela explique l'atténuation élevée du souche. Pour vérifier notre hypothèse, nous avons perturbé RD2 de *M. tuberculosis* H37Rv et évalué son phénotype dans des modèles cellulaires et animaux. Ces expériences identifient des gènes candidats pour la complémentation des souches de BCG naturellement déficientes en RD2. Des études utilisant des provocations par vaccin et des tests immunologiques ont été utilisées pour déterminer le rôle joué par RD2 dans la protection vaccinale. Nos résultats ont révélé que RD2 est nécessaire pour la virulence complète de *M. tuberculosis* et qu'elle module l'immunité innée. Cependant, la présence de RD2 n'augmente pas la protection pulmonaire avec BCG, même si RD2 augmente l'immunogénicité du vaccin.

De plus, des études antérieures ont révélé que NOD2 (Nuclear Oligomerization Domaine 2), un récepteur intracellulaire de l'hôte, est essentiel pour la médiation des réponses immunitaires innées et adaptatives contre *M. tuberculosis*. Donc, nous avons

supposé que NOD2 était aussi important pour la vaccination. Pour confirmer cette hypothèse, nous avons vacciné les souris *Nod2* $^{+/+}$ et *Nod2*$^{-/-}$, et par la suite provoqué ces souris avec une dose aérosol de *M. tuberculosis*. Nous avons caractérisé l'effet d'une perturbation de NOD2 sur la charge bactérienne, la pathologie, et l'immunité adaptative. Nos expériences démontrent que la perte de NOD2 mène à une pathologie aggravée chez les animaux vaccinés suite à une infection par *M. tuberculosis*. De plus, nos résultats suggèrent que le contrôle de la pathologie attribué à NOD2 est réalisé grâce à l'action des lymphocytes T.

Les études réalisées ici améliorent notre compréhension de l'évolution *in vitro* du vaccin BCG et comment ce processus peut affecter la protection antibactérienne. De plus, notre enquête sur le rôle d'un récepteur de l'hôte dans la vaccination souligne l'importance de la compréhension des interactions entre l'hôte et les agents pathogènes pour la protection vaccinale.

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In general the adaptive relationship between microorganisms and host is effective only for the precise circumstances under which adaptation evolved – in circumstances which constitute physiologic normalcy for the host concerned. Any departure from this normal state is liable to upset equilibrium and to bring about a state of disease.

- Rene Dubos, 1961

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Contributions to original knowledge

The following original results are presented in this thesis

1) The observation that the RD2 region is required for the full virulence of *M. tuberculosis* in a mouse model. The loss of this region results in decreased bacterial burden, decreased histopathology, and increased survival time in mice lacking an adaptive immune system.
2) The genes *Rv1979c-Rv1982* partially restore virulence by inhibiting the host apoptotic response.
3) The addition of *Rv1979c-Rv1982* to an RD2-deficient BCG strain increases the immunogenicity of the vaccine.
4) Neither the presence of *Rv1979c-Rv1982* nor of the full RD2 region in the vaccine increases pulmonary protection. This supports the hypothesis that increased immunogenicity does not necessarily result in better protective efficacy.
5) The loss of NOD2 reduces the protective efficacy of the BCG vaccine in a mouse model. The loss of this receptor results in greater pulmonary inflammation and increased pathology.

Contribution of Authors

This thesis was prepared in accordance with the guidelines stated in McGill University's "Guidelines for Thesis Preparation". The format conforms to the "Manuscripts-based thesis" option. All of the studies have been performed in the laboratory of Dr. Marcel Behr under his sole supervision. The contributions of authors are delineated below In this section, authors are designated by their initials (i.e. Robert Kozak, R. K.).

Chapter 1. Review of the Literature

R. K. wrote the literature review

Chapter 2. Kozak R.A., Alexander D.C., Liao R., Sherman D.R., and Behr M.A. Region of Difference 2 contributes to virulence of *Mycobacterium tuberculosis*.

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R.K. and M.A.B analyzed the data and wrote the manuscript. R.K., D.C.A., and M.A.B. designed the experiments. D.A.C., D.S. and R.L. provided the strains. R.K performed the experiments.

Chapter 3. Kozak R.A., and Behr M.A. Divergence of Immunologic and Protective Responses of Different BCG strains in a Murine Model.

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R.K. and M.A.B. designed the experiments, analyzed the data and wrote the manuscript. R.K. performed the experiments.

Chapter 4. Kozak R.A., Divangahi M., and Behr M.A. Loss of NOD2 Reduces Protective Efficacy of the BCG Vaccine.

Journal of Immunology. In Preparation

R.K. and M.A.B. wrote the manuscript. R.K., M.D., and M.A.B. designed the experiments and analyzed the data. R.K. performed all experiments, except the FACS experiments, which were performed by M.D.

Chapter 5. Discussion and Opportunities for Research

R.K. wrote the discussion

Other manuscripts not included in this thesis, but to which a significant contribution was made by the candidate, are listed as follows:

Divangahi M., Mostowy S., Coulombe F., Kozak R., Guillot L., Veyrier F., Kobayashi K.S., Flavell R.A., Gros P., and Behr M.A.
NOD2-deficient mice have impaired resistance to Mycobacterium tuberculosis infection through defective innate and adaptive immunity.

J Immunol. 2008 Nov15;181(10):7157-65.

Gideon H. P., Wilkinson K.A., Rustad T.R., Oni T., Kozak R.A., Sherman D., Meintjes G., Behr M.A., Rangaka M.X., Vordermeier H.M., Young D.B., and Wilkinson R.J. Hypoxia induces a target of Mycobacterium tuberculosis specific memory T cells absent from common BCG vaccines. *PLoS Pathog.* 2010 Dec23;6(12):e1001237.

Objectives and rationale

The work presented in this thesis concentrates on the BCG vaccine, and how host-pathogen interactions determine vaccine protection. The paradigm for a live attenuated vaccine is that the organism must be safe, yet sufficiently virulent to generate a productive infection in the host that results in induction of a protective immune response against a fully virulent strain of the organism.

As will be described in Chapter 1, the *in vitro* evolution of the BCG vaccine resulted in numerous daughter strains that differ genotypically and phenotypically. The first two studies will focus on one particular deletion event, the loss of Region of Difference 2 (RD2), its role in virulence and in vaccine protection. These studies were based on the hypothesis that the prolonged *in vitro* passage of BCG has resulted in the loss of important systems for *in vivo* pathogenesis. We pursued studies of our region of interest in *M. tuberculosis* first. The first study (presented in Chapter 2), hypothesized that the deletion of the region would lead to decreased virulence *in vivo*. Our objective was to formally examine this in a mouse model, and characterize the mechanisms through which genes in this region cause pathogenicity. Building upon this, the second study (presented in Chapter 3) hypothesized that the reinstatement of virulence genes from this region would improve the protective efficacy of the vaccine.

The work presented in the third study (presented in Chapter 4) concentrates on the host side of the paradigm. Chapter 1 will discuss the role of known host genes in susceptibility to mycobacterial disease, and the third study will investigate the role of one such gene, Nuclear Oligomerization Domain 2 (*NOD2*), in vaccination. We hypothesized that a gene whose loss affects the host response to *M. tuberculosis* would also affect the

response to BCG, particularly the generation of protective immunity. Therefore we sought to characterize what effect disruption of *Nod2* would have on the immune response generated by BCG.

List of abbreviations

BCG: Bacille Calmette-Guérin
CARD: Caspase recruitment domain
CFP10: culture filtrate protein 10
CR: Complement Receptor
CFU: Colony forming units
COX-2: Cyclooxygenase 2
DC-SIGN: DC-specific intracellular adhesion molecule-3 grabbing nonintegrin
DNA: Deoxyribose nucleic acid
DOTS: Directly observed treatment, short course
ELISA: Enzyme-linked immunosorbent assay
ESAT6: early secreted antigenic target 6-KDa protein
ESX-1: ESAT-6 system-1
FACS: Fluorescence-activated cell sorting
HIV: Human Immunodeficiency Virus
HSA: Human serum antigen
iciA: Inhibitor of chromosome initiation
IFN-β: Interferon-beta
IFN-γ: Interferon-gamma
IL: Interleukin
IPR1: Intracellular pathogen resistance 1
IRF: Interferon regulatory factor
ISRE: IFN stimulated response elements
Kb: kilobase
LAM: Lipoarabinomannan
LRR: Leucine rich repeat
LTTR: LysR-type transcriptional regulator
mAb: Monoclonal antibodies
MAP: Mitogen activated protein
MARCO: macrophage receptor with collagenous structure
MAVS: Mitochondrial antiviral signaling
MDR-TB: Multi-drug resistant tuberculosis
MGC: Multinucleated giant cell
MOI: Multiplicity of infection
MMP: Matrix metalloproteinase
MTB: *Mycobacterium tuberculosis*
NADPH: Nicotinamide adenine dinucleotide phosphate
NEMO: NF-kappa-B essential modulator
NLR: NOD-like receptor
NOX-2: NADPH oxidase 2
NTM: Non-tuberculosis mycobacteria
ORF: Open Reading Frame
oriC: Origin of replication
PBMC: Peripheral blood mononuclear cell
PDIM: Phthiocerol Dimycocerosates
PGE: Prostaglandin E
PGL: Phenolic Glycolipid
PIM: Phosphatidyl-myo-inositol mannosides
PIN: pilT N-terminal domain
PRR: Pattern Recognition Receptor
RD1: Region of Difference 1
RD2: Region of Difference 2
RIP2: Receptor interacting protein 2
SCID: Severe Combined Immunodeficiency
SOCS: Suppressor of cytokine signaling
STAT: Signal Transducers and Activators of Transcription
TB: Tuberculosis
Tbk1: TANK binding kinase 1
TDM: Trehalose Dimycolate
TGF: Transforming growth factor
TLR: Toll-like Receptor
TNF: Tumor necrosis factor
WHO: World Health Organization
XDR-TB: Extensively drug resistant tuberculosis

CHAPTER 1: Review of the Literature

1. *Mycobacterium tuberculosis*

1.1 The history of tuberculosis

Tuberculosis has been a scourge of humanity for millennia. Paleomicrobiologic study has detected DNA from *Mycobacterium tuberculosis* from one of the first human settlements, indicating that organism has been with us since humans started forming agricultural communities [1]. Over the years, the disease has been referred to by a variety of terms including: phthisis, consumption, the white death and king's evil. Often these terms were based on observed symptoms as the patient would become pale and start wasting away while the disease ran its course.

The disease was described in the 17th century by the physician Richard Morton, who noted "tubercles" were always present in cases of phthisis [2]. Despite this description of these nodules of pathology, which is a hallmark of disease, it was more than a century before Laënnec suggested the term "tuberculosis". The invention of the stethoscope by Laënnec was instrumental in allowing for the diagnosis and characterization of the disease [3]. Although these discoveries advanced our clinical understanding of tuberculosis, the most significant finding regarding its aetiology occurred in 1882, when Robert Koch demonstrated that the *tubercle bacillus*, later renamed *M. tuberculosis*, was the cause of the disease. This ushered in a new era in bacteriological and clinical research on the disease. At the beginning of the 20th century Charles Mantoux developed a diagnostic skin test based on extracts of the organism that now bears his name. By 1921 a vaccine named Bacillus of Calmette and Guérin was developed (to be discussed in greater detail later). In 1948 one of the first randomized clinical trials conducted showed that streptomycin could cure tuberculosis [4] and was

followed soon after by the development of other anti-mycobacterial drugs [5]. Control and elimination of tuberculosis seemed possible through the use of combination therapy against the infection.

The steady decrease in TB cases began to see a reversal in the mid-1980s [6]. This reversal came at a time when interest in tuberculosis was declining, as it was falsely believed resistance to main-line TB drugs would not be a concern [7]. The collapse of the Soviet Union, deteriorating conditions in many developing nations, and the spread of HIV together conspired to turn TB into a global epidemic such that by 1993 the WHO declared TB a global health emergency. This led to the WHO recommendation that its DOTS program (originally named for directly observed therapy short course), which had been implemented by a number of countries already, be adopted worldwide [8]. Despite the success of this program in well-studied settings, recurrence of disease has been shown to vary considerably, further threatening the ability of antibiotic-based TB control to stem the epidemic [8]. As the greatest numbers of TB cases live in resource-poor settings, there can be considerable difficulties in maintaining a treatment regimen consistently for 6 months. Failure to adhere to treatment can result in drug-resistance, and ultimately multi-drug resistant organisms (MDR). This further compounds the problem, as treatment of MDR-TB require additional drugs, and longer treatment periods.

1.2 Current scope of the global epidemic

In 2000, estimates showed that incidence was 8.5 million new cases per year globally, resulting in an estimated 1.8 million deaths per year [7]. The 2007 figures from the WHO do not lend themselves to optimism, as the incidence is approximately 8

million cases, with 1.6 million deaths per year (www.who.int/tb/publications/global_report/2009/). The majority of the global burden comes from the developing world, where health infrastructure is lacking, and HIV is more prevalent (Figure 1). Recently, in South Africa, 52 out of 53 patients infected with XDR-TB died, highlighting not only the difficulties in treating extremely drug resistant forms of the disease, but also the importance of addressing HIV, as the majority of these patients were co-infected with the virus [9]. The infamous "TB traveler", who exposed fellow airline passengers to MDR-TB demonstrates how a transmission threat in country can readily become a multi-national problem. Epidemiologic investigations of TB spread in Montreal have shown that populations where TB incidence are the highest (aboriginals, immigrants) are the groups that are least likely to have interaction with the health system [10]. It can be assumed that this is not a phenomenon unique to Montreal, and this represents a serious challenge to public health.

1.3 Clinical and diagnostic aspects of tuberculosis

Tuberculosis is an airborne infection and pulmonary disease is the most frequent manifestation. The most common symptom is a non-productive cough early in the illness. Chest and pleuritic pain, due to inflammation may also occur. Fever, night sweats, malaise, weight loss are all potential systemic manifestation of the disease that are most likely mediated by cytokines [7]. Primary infection is usually first identified as a middle or lower zone infiltrate often associated with enlargement of lymph nodes at the base of the lungs [7]. Following primary infection the bacterium may remain clinically dormant in the lungs or, in the case of a majority of patients, progress on to active disease. At this

point, a chronic productive cough becomes a common, yet non-specific feature of active TB. It is through chronic cough that the host then expels organisms, leading to new infections and completing the transmission cycle of *M. tuberculosis*. In the pre-HIV era, pulmonary tuberculosis represented approximately 85% of cases, and extra-pulmonary disease made up the remaining 15% [11]. However, since HIV has become more prevalent, the number of extra-pulmonary tuberculosis cases has risen. Pulmonary tuberculosis has a fatality rate of 50% if untreated, whereas extra-pulmonary (or milliary) tuberculosis has near 100% fatality if untreated.

Assuming an individual is not immuno-comprimised, the risk of developing disease is approximately 5-10% [12]. However it must be noted that the estimated rate of progression from infection to disease requires assays for both conditions. Diagnosis of infection by *M. tuberculosis* (called tuberculosis infection to distinguish this condition from active TB) is determined through immunologic testing, classically via the tuberculin skin test, whereby a mycobacterial purified protein derivative is injected intradermally, and the delayed type hypersensitivity reaction is measured 72 hours later. Until recently, the Mantoux test was the only test available to diagnose active or latent infection. However, measurement of IFN-γ release from the lymphocytes of a suspected TB patient using ELISA-based assays shows higher specificity than the skin test because of the use of antigens that are specific to *M. tuberculosis* and not found in other organisms, such as the BCG vaccine [13]. In contrast, diagnosis of TB disease is based on microbiologic testing. Current WHO recommendations advise collection of three sputum samples, which are examined for the presence of mycobacterium using the Ziehl-Nielsen stain followed by microscopy (www.who.int). If these smears are negative but disease is still

suspected X-rays may be used. In developed countries, the sputum samples are subject to culture, which is a more sensitive diagnostic test, but requires greater laboratory infrastructure and results in higher costs to the health care system.

Host factors, both genetic and acquired, are strong determinants of both the skin-test reaction and the risk of clinical disease after infection. In addition, bacteriologic differences also affect the risk of disease. Therefore, the outcome of infection is felt to be governed by a combination of bacterial virulence factors and host susceptibility factors, which will be discussed in greater detail in later sections [11, 14].

1.4 Host pathogen-interactions

M. tuberculosis is a Gram-positive, acid-fast positive facultative intracellular parasite. The bacterium is the causative agent of human tuberculosis, while closely related variants of the organism are the cause of zoonotic forms of tuberculosis (e.g. *M. bovis* in cattle, *M. caprae* in goats). The genome is 4.4 Mb in length, and contains over 4000 open reading frames (ORFs) with a G + C content of 65.6% [15]. Like all mycobacteria, *M. tuberculosis* has a lipophilic wall that is composed of mycolic acids and complex lipids anchored to arabinogalactan, which is anchored to peptidoglycan [16]. This waxy layer serves not only as a barrier to desiccation, but also the complex lipids within it, such as trehalose dimycolate (TDM), phenolic glycolipids (PGL) and phthiocerol dimycocerosate (PDIM), are virulence factors [17]. TDM inhibits the production of IL-12p40 in murine macrophages, and PDIM is required for growth in murine lungs [18, 19]. Interestingly, PGL inhibits the cytokines TNF-α, IL-6, IL-12, and

is produced by several of the hypervirulent strains, such as W-Beijing and HN878 but not by the reference strain, H37Rv [20].

As *M. tuberculosis* is an intracellular pathogen, it is first phagocytosed by an alveolar macrophage, where it resides and, in the case of a productive infection, will replicate. The macrophage is thought to be the primary host-cell for *M. tuberculosis*, although *in vitro* evidence suggests that the bacterium can also infect epithelial cells and dendritic cells [21-23]. The potential role of epithelial cells in the lifecycle of *M. tuberculosis* is currently not settled. It has been suggested that dendritic cells are required for the dissemination of infection from the primary site of bacterial deposition to the regional lymph nodes [24]

Phagocytosis is crucial to bacterial survival; as such, *M. tuberculosis* does not resist uptake, but instead utilizes a myriad of receptors to facilitate this process. The complement receptors CR1, CR3 and CR4 play a major role in phagocytosis of *M. tuberculosis,* and the bacterium can activate complement pathways to facilitate opsonization with C3b and C3bi. Inhibition of phagocytosis using mAbs against CR1 and CR3 reduced uptake by 40% and 39% respectively, and when the antibodies were used in conjunction uptake was reduced to 64% [25]. The utilization of receptors may also be cell-type specific; with blood monocytes *M. tuberculosis* favoured CR1 and CR3, whereas with alveolar macrophages CR4 was the major receptor [26]. Interestingly, infection of CR3-deficient mice did not yield a higher bacterial burden or differences in survival times when compared to wild-type mice. Pathology and innate immune responses were also comparable, demonstrating that *M. tuberculosis* makes use of alternate pathways to enter the cell.

The mannose receptor represents another entry method for mycobacteria. This receptor is a type I transmembrane protein that recognizes mannose, fucose, glucose or *N*-acetylglucosamine residues on glycoproteins [27]. Lipidoarabinomannan, a mycobacterial lipoglycan, is a ligand for this receptor and facilitates phagocytosis of the organism. Coating of microspheres with either LAM from different *M. tuberculosis* strains or non-tuberculosis strains of mycobacteria revealed that LAM from both *M. tuberculosis* strains (Erdman and H37Rv) facilitated microsphere attachment to macrophages better than LAM from non-tuberculosis mycobacteria [28]. The ability of *M. tuberculosis* to facilitate phagocytosis through the mannose receptor is likely a virulence mechanism, since interaction of mannosyl-capped LAM from the bacterium with the mannose receptor activates anti-inflammatory signals [29]. LAM also interacts with the dendritic cell receptor DC-SIGN, and may be the method of entry used by *M. tuberculosis* for infection of these cells [30]. Interestingly, DC-SIGN does not bind LAM from fast-growing, non-pathogenic mycobacteria, such as *M. smegmatis* [30]. Whether this is beneficial to the host cell for the activation of pathogen-specific immune responses or beneficial to the bacterium by providing another niche for infection remains to be determined.

Finally, scavenger receptors, CD14 and Fcγ receptors have all also been implicated in phagocytosis of *M. tuberculosis*, although they may represent alternate or cell specific pathways [31, 32].

1.4.1 Recognition by Host Receptors

Multiple host receptors are involved in the recognition of *Mycobacterium tuberculosis*. Toll-like receptors are transmembrane proteins which recognize pathogen associated molecules, and activate signalling pathways involved in the innate immune response [33]. The toll-like receptors 1, 2, 4 and 9 are all activated by *M. tuberculosis*. The agonists for these receptors are distinct, as TLR2 recognizes 19 kDa lipoprotein, TLR9 recognizes CpG motifs, and TLR4 detects a heat-labile bacterial component (Figure 3) [34-36]. Although these receptors may detect bacterial ligands, their role in mycobacterial resistance is less clear. In one study, neither TLR4 nor TLR2-deficient mice were more susceptible to low dose aerosol infection of *M. tuberculosis* than wild-type mice. However, $TLR2^{-/-}$ mice were more susceptible than wild-type mice when they received an extremely high dose [37]. Loss of TLR2 in macrophages results in decreased secretion of TNF-α, IL-1 and IL-6 following infection with BCG. Interestingly, $TLR2^{-/-}$ mice had higher bacterial burdens of BCG than either $TLR4^{-/-}$ or wild-type mice[38]. TLR9 knock-out mice had a similar phenotype, only showing susceptibility to a high dose infection [36]. Mice that were deficient in both TLR2 and 9 were hyper-susceptible to infection with *M. tuberculosis*, likely due to the decreased production of IL-12[36]. The scavenger receptor MARCO, which recognizes TDM, interacts with TLR2 to induce TNF-α, demonstrating the importance of TLR2 in mycobacterial recognition.

Host receptor induction is not always beneficial for the host and may be subverted to the benefit of *M. tuberculosis*. BCG infection also leads to activation of Notch1 through TLR2 and downstream activation of SOCS3[39]. Activation of SOCS3 inhibits

IFN-γ stimulated STAT1 phosphorylation, and suggests a method through which mycobacteria moderate host TLR2 signalling [40].

Phosphatidyl-myo-inositol mannosides are components of the mycobacterial cell wall, which can trigger Notch1 activation leading to expression of COX-2 and MMP-9. Both MMP-9 and COX-2 are involved in chronic inflammation and pathogenesis associated with tuberculosis [41]. Phosphatidyl-myo-inositol mannosides can also induce SOCS3 through Notch1 signalling [42].

NOD-like receptors have also been found to sensors of mycobacteria, and will be discussed in greater detail in a subsequent section.

1.4.2 Modification of apoptosis

Following phagocytosis, the bacterium utilizes multiple strategies to inhibit phagolysosome fusion, thereby providing itself with its niche for replication and persistence [43, 44]. Faced with a persistent intracellular infection, the host cell response then dictates the ultimate outcome of infection, and whether this involves spread of bacteria to other host cells or the recruitment of a protective immune response. In the context, apoptosis of the infected macrophage is believed to represent a primary method for controlling *M. tuberculosis* infection. Evidence for this comes from the observation that suppression of apoptosis is critical to bacterial persistence, and is correlated with mycobacterial virulence [45, 46]. However, confusing this model is the suggestion that *M. tuberculosis* activates apoptosis by several pathways. The activation of p38 and apoptosis-signalling kinase 1 by TNF-α occurs as a result of infection [47]. The bacterium has also been shown to induce lipoxin A4, which prevents PGE2 synthesis,

which is involved in membrane repair. This latter data argues that the organism does not so much induce apoptosis after infection, but rather shift the balance from apoptosis to necrosis, which is favourable for the bacterium by providing a mechanism of escape to neighbouring cells without the induction of a protective immune response [48]. Another consideration in this model is that the apoptosis pathway depends upon bacterial burden. At a multiplicity of infection (MOI) ≥ 25, programmed cell death occurs through caspase-independent pathways that involve lysosomal proteases, but not TNF-α, MyD88 or TLR4 [49]. At lower MOIs, TNF-α is involved, as are MyD88 and numerous caspases [49-51]. TLR4 and TLR2 induction by mycobacterial ligands, such as the 19 kDa antigen in the case of TLR2, can also trigger apoptosis [49, 52]. For the experiments that follow below, we have aimed for an MOI well below 25, as it is not thought that a macrophage infected with > 25 bacteria represents a natural level of infection encountered in the host.

As the host has multiple apoptotic triggers, *M. tuberculosis* has evolved multiple mechanisms to counteract this. Macrophage infection studies comparing virulent H37Rv to the avirulent H37Ra reveal that H37Rv induced expression of Mcl-1, a host anti-apoptotic factor, although it was not determined which bacterial factor was responsible [53]. H37Rv can also prevent the cross-linking of annexin-1 by proteolysis of the amino terminal domain of the protein. Annexin-1 expression on the surface of a macrophage would normally promote pro-apoptotic mechanisms, however its degradation instead favours necrosis [54].

The secreted proteins encoded by *Rv3654c* and *Rv3655c* interact with the host factors PSF and ALO17 [55]. PSF is involved in post-translational modifications of caspases, and *Rv3654c* may cleave the protein to prevent its function [55]. The *nuoG*

gene is a bacterial NADH dehydrogenase that decreased apoptosis in both a mouse and macrophage when complemented into *M. kansasii* [56]. Studies with deletion mutants demonstrate that *nuoG* prevents phagosomal accumulation of reactive-oxygen species and may inhibit the activity of NOX-2 [57]. The *sodA* gene encodes a superoxide dismutase that protects *M. tuberculosis* from oxidative attack within the macrophage [58]. Similar to *nuoG*, this inhibition of reactive-oxygen species also prevents apoptosis [59]. The observation that there is a negative correlation between expression of the antigenic protein MPT64, and the expression of host TNF-α, as well as other pro-apoptotic factors in human biopsies implicate this antigen in moderating apoptosis [60, 61]. Suppression of TNF-α expression, paired with induction of IL-10 by the bacterium is also a strategy to evade apoptosis, as the role of TNF-α in inducing apoptosis is well established[51, 62].

The apoptotic response is also important for the induction of the immune response. Inhibition of apoptosis decreases cross-presentation of antigens by dendritic cells to T-cells, thereby reducing the adaptive immune response [63, 64]. Clinical data have shown an inverse correlation between IFN-γ production by PBMCs and induction of apoptosis in patients suffering severe TB; IFN-γ is a key mediator of anti-mycobacterial immunity [51, 65]. Despite extensive ongoing research, the mechanisms through which *M. tuberculosis* induces and/or prevents apoptosis remains to be completely elucidated.

1.5 Animal models for mycobacterial virulence

Animal models have been integral to understanding tuberculosis, since Robert Koch first used guinea pigs to demonstrate his famous postulates of causality [66]. The development of the BCG vaccine was made possible by repeated testing of the passaged vaccine in guinea pigs, until it was deemed sufficiently attenuated [67], a prediction that was shown true through decades of experience with the vaccine in human populations. Modern usage of the guinea pig model focuses on its use in vaccinology. A noteworthy example was the discovery that BCG complemented with RD1 enhanced vaccine protection [68]. Infection of guinea pigs with *M. tuberculosis* generates pathology, specifically necrotic granulomas, similar to that seen in humans, that fail to contain the bacterium [69].

The rabbit is another animal model that was used in early studies. Experiments by Lurie in the first half of the 20th century illustrated the pathology of the disease in rabbits, and demonstrated that genetic factors play a role in susceptibility to infection [70, 71]. The rabbit model can be used to distinguish *M. bovis* from *M. tuberculosis*. These animals are more sensitive to infection with *M. bovis* than *M. tuberculosis,* likely due to the ability of *M. bovis* to disseminate better [72]. Additionally, the rabbit model demonstrated that the CDC1551 strain had comparable virulence to H37Rv, but may be more easily transmitted [73]. Pathologically, infection in rabbits resembles that seen in humans with cavitation and caseous necrosis [74]. While both the rabbit and the guinea pig models present valuable features of human TB, the difficulties in containment of these animals combined with paucity of immunological reagents limit their use for mechanistic research.

Macaques are the most common non-human primates used for studies of the disease. Tuberculosis in a macaque is pathologically similar to humans, particularly the granulomas [69]. Necrosis and calcification of granulomas is observed, and the animal can develop active or latent disease [75]. Since the full spectrum of disease is possible within this model, it is particularly useful for evaluative research, such as testing vaccines and drugs prior to human trials. Additionally, this is the only animal model that allows for the study of HIV-TB co-infection, as it is possible to infect macaques with SIV [76]. However, the cost of these animals, paired with the difficulties in containment and the paucity of reagents for mechanistic research (e.g. knock-out strains) limit the use of this model.

While these experiments using these animals have contributed significantly to TB research, it is the mouse that is the most common model in use today. It has the advantages over other models of being relatively cheap, and easy to house, which allows for the use of large numbers of animals in experiments. It also serves as a good model for testing both drugs and vaccines [69] and offers tremendous advantages in testing both pathogen and host determinants of disease through the availability of immunologic reagents and genetically modified strains of mice.

1.5.1 Mouse Model of Mycobacterial Virulence

Early pioneering work by Dubos and colleagues helped demonstrated value of a mouse model for tuberculosis. The experiments revealed that survival times in *M. tuberculosis* infected NCS and C57BL/6 mice differed from Swiss mice [77, 78]. This work implied a role for host genetics in resistance to *M. tuberculosis*, and helped

establish the utility of the C57BL/6 mouse. Subsequent work has revealed a that resistance profiles differ amongst the more commonly used mouse strains, with BALB/c and C57BL/6 mice being considered the most resistant [79].

The use of *Rag1*$^{-/-}$ mice in virulence studies has also been valuable in understanding mycobacterial pathogenesis. The RAG1 protein is involved in recombination activities that are essential for the maturation of B and T-cells. Consequently, *Rag1*$^{-/-}$ mice lack B and T-cells, and can only control mycobacterial infections though innate mechanisms [80]. The advantage of this model is that adoptive transfer of B and T-cells are possible, and the role of these subsets in the control of infection can be evaluated. Feng *et al.* (2000) first used this adoptive transfer model to demonstrate that FACS-purified $CD4^+$ and $CD8^+$ T-cells were involved in controlling an aerosol infection of BCG [81]. The role of T_{reg}-cells in suppressing $CD4^+$ T-cell mediated protection, and Th17 in conferring protection in vaccination have also been demonstrated using these mice [82, 83].

Another genetically susceptible mouse has applications in virulence studies. SCID (severe combined immunodeficiency) mice are highly susceptible to infection, yet their greatest strength lies in their usage for testing the virulence of vaccine candidates. The safety profile of both BCG complemented with RD1, and an *M. tuberculosis* strain lacking *phoP* have been evaluated in SCID mice [68, 84], because it is expected that a new TB vaccine will occasionally be given unwittingly to an infant who suffers from SCID. Interestingly, a recent study used these mice to determine virulence genes of *M. tuberculosis* involved in apoptosis. The complementation of *nuoG* into *M. kansasii*,

increased virulence of the bacterium in a SCID mouse, and suggested a novel mechanism for virulence screening [56].

Unlike larger animal models (such as primates), mice allow for infection by a variety of means, including aerosolization, intravenous, intratracheal, oral and intraperitoneal. Aerosolization with a low-dose of *M. tuberculosis* (~ 100 CFU) is the most frequent method of infection, as it is the most physiologically relevant [69]. Following the implantation of *M. tuberculosis* in the lungs there is uncontrolled exponential growth for the first 2 weeks as only innate immune mechanisms are available to contain the bacteria. By 3-4 weeks post infection pulmonary bacterial burden reaches a peak, as the adaptive immune response is fully activated. Dissemination to the lymph nodes occurs after 9 to 11 days, which is the site where adaptive immunity is induced. Mycobacterial specific T-cells are detectable 2 or 3 days later. Dissemination to the spleens and liver also occurs, and bacteria can be detected by 11 to 14 days post-infection [85]. Comparison of C57BL/6 mice, where dissemination is more rapid, to C3H mice where dissemination takes longer suggest that bacterial spread may be beneficial to the host, as it results in the induction of adaptive immunity at an earlier time-point, and hence, to an earlier control of the bacterial infection [85].

After 4 weeks bacterial numbers stabilize, and remain stable during the chronic phase of infection. Infection experiments using *M. tuberculosis* H37Rv engineered to contain an unstable plasmid, pBP10, allow a window into bacterial replication during this phase of infection, based on the premise that loss of the plasmid is expected to correlate with chromosomal replication of the bacteria (and conversely that retention of the plasmid will then signal a state of non-replication). In these experiments, it was revealed

that stable bacterial numbers observed during this period of infection is not because of the absence of replication, but rather that *M. tuberculosis* replication is balanced by bacterial killing by the immune system [86]. However, with time, ongoing bacterial replication and the associated slowly progressing pathology result in death of the host, between 6 months and 1 year after infection in a C57BL/6 mouse. These different phases of infection allows for the association of bacterial gene expression with different phases of infection. Infection of mice with transposon mutagenesis libraries determined 197 genes essential for *in vivo* survival of *M. tuberculosis* [87]. Additionally, due to the relative ease of processing infected organs, it is possible to determine tissue-specific virulence factors. An example of this was the use of transposon mutants to demonstrate the requirement of PDIM for bacterial growth in the lungs [18].

The relative ease through which genetic knock-outs can be generated is another advantage of this animal model. Deletion studies in mice have demonstrated that TLR2 and TLR9 are important for anti-mycobacterial immunity, but that TLR4 is redundant [36, 37]. Similar investigations into the role of NOD1 and NOD2 revealed NOD2 to be crucial for the innate and adaptive immune response to mycobacteria [29, 88, 89]. Furthermore mouse models have shown that the loss of IFNγ, TNFα and IL-12 increase susceptibility to *M. tuberculosis* [90-92], paralleling observations from children who harbour natural mutations in these cytokines or their receptors. As more cytokines are discovered, the use of knock-out mouse models helps understand their role in the immune response to *M. tuberculosis*. IL-23 and IL-17 have both been shown to be important for vaccine-induced immunity, and IL-27-deficient mice were more resistant to infection [93, 94]. The requirements of CD4 and CD8 T-cells in protection against infection were also

shown using knock-out models[95, 96]. However, $CCR2^{-/-}$ mice were found only to be susceptible to high dose infections, demonstrating that knock-out mice do not always give clear phenotypes [97].

The latency model of infection is substantially different than the human form, as bacterial burden and pathology are significantly higher [69, 98]. Currently, the model used to simulate latency is the Cornell model, which involves intravenous infection, followed by incomplete (i.e. non-sterilizing) treatment with isoniazid and pyrazinamide [99]. In this model, reactivation of disease is induced at a later point, enabling one to test the bacterial determinants of survival during this latent phase of infection. One weakness is that this model lacks a standardized protocol in terms of strain usage, inoculum, route of infection, and length of drug-free period [98]. Variation in length of treatment affected whether reactivation could be induced through TNF-α or NOS2 inhibition, and limits the usefulness of the model [98].

In addition to differences in latency, there are other limitations to the mouse model of TB. The progression of infection in mice differs somewhat from that seen in humans. Human infection occurs with a low dose of bacteria that results in granuloma formation, minimal pathology, and clinical disease in only a subset of individuals. In contrast, in mice there is steady growth of the bacterium, no latent phase, and gradual increasing pathology that leads to death [89]. Granulomas, which represent a hallmark of the disease, also vary between mice and humans. Infection in humans can result in a range of granulomas, including those that have become necrotic, calcified, fibrotic, or developed into cavities. However, granulomas in a mouse lung are much more homogeneous and caseous necrosis and cavitation do not occur [69].

At a more general level, the immune systems of mice and humans differ in aspects that are relevant to modeling TB. Expression of inducible NO synthase occurs in mouse macrophages, and is induced by IFN-γ, whereas expression of NO by human macrophages is not certain. Expression of TLRs, ICAMs, CD molecules and other receptors differ in levels, and by cell types [100]. Lymphocyte percentages in the blood are higher in mice than humans, and expression of the MHC class II molecule also differs [100]. CD8 T-cells, which are believed to be required for clearance of the bacteria, do not produce granulysin in mice, a feature not shared with human T-cell [101]. As the importance of new T-cell subsets becomes more evident, so do the differences in the murine model. Induction of Th17 cells requires TGF-β in mice but in humans the role of this cytokine is less clear [102]. Mounting evidence suggests that effector and central memory T-cells are important for life-long immunity to *M. tuberculosis* [103, 104]. However, it may not be possible to generate this required subset due to the short lifespan of mice. Despite these limitations, it is expected that the adaptability of this model for setting up different experimental conditions will keep it in the forefront of tuberculosis research.

2. The evolution of the BCG vaccine

2.1 History of the BCG vaccine

The current vaccine recommended by the WHO against *M. tuberculosis* is the BCG vaccine (www.who.int). The vaccine arose as a result of serial passaging of *Mycobacterium bovis*, which is the causative agent of tuberculosis in cattle. From 1908 to 1921, Albert Calmette and Camille Guerin performed 230 serial passages of the bacterium on potato bile and Sauton media, as it was noted that these ingredients decreased bacterial clumping and virulence respectively [67, 105]. During this time, successive intravenous animal infections demonstrated that attenuation had occurred, and by 1921 vaccination began in human subjects. From 1924 onwards, BCG underwent a second phase of attenuation, with the vaccine being distributed by the Pasteur Institute to 34 laboratories by 1926, and subsequently being further distributed by these countries to others [106, 107]. The growth conditions in which the vaccine was propagated generally involved sub-culturing on potato-bile media, Sauton media, or use of a deep culture method, often with slight variations between laboratories [106, 107]. This resulted in the vaccine being under continued *in vitro* selective pressure, that had produced its initial attenuation, for nearly five decades [108]. By the time lyophilization technology allowed for the creation of standardized seed lots of vaccine in the 1960s, over 50 sub-strains existed [67, 107]. This complicated history of the derivation and propagation of early BCG strains has given rise to considerable phenotypic and genotypic diversity among BCG vaccines in use today [108-116].

2.2 Genomic and phenotypic differences between strains

Even prior to genomic analysis it was realized that BCG strains were not a solitary entity but rather a collection of strains, differing in biochemical and immunological characteristics (Figure 2). For example, examining the delayed type hypersensitivity to tuberculin in vaccinated individuals, it was noted that BCG Prague differed considerably from other strains, implying some sort of intrinsic difference [117]. Early biochemical comparisons focused on differences in protein production, colony morphology and lipid content [106, 117, 118]. BCG strains could be dichotomized based on the production of the secreted antigens MPB70, MPB83 and MPB64, with some strains producing all three in great quantities, while other strains did not produce these at all [116, 117, 119]. For some time, it was incorrectly believed that these variations were a result of diverse culturing and production methods, rather than being due to intrinsic differences between strains [117]. This notion was formally addressed by genetic and genomic studies.

Comparative genomics of BCG strains revealed numerous deletions, insertions and single nucleotide polymorphisms that distinguished BCG from *M. bovis*, and also distinguished BCG strains from each other [110, 115, 120, 121]. Vaccine strains were grouped into “early” or “late” strains based on the presence or absence of these genomic elements. Early strains include BCGs Russia, Japan (Tokyo), Sweden, Moreau and Birkhaug, all of which produce MPB70, MPB83 and MPB64. BCG Russia can be considered the most ancestral strain, and closest to the original BCG initially attenuated by Calmette and Guérin. This strain has a single nucleotide insertion in its *recA* gene, resulting in a frameshift mutation. RecA is involved in homologous recombination in

other bacteria, and its inactivation provides an explanation of why BCG Russia has remained genetically stable [122]. When comparing the immunostimulatory ability of early and late strains, early strains induced higher levels of TNF-α, IL-1beta and IL-12 [123]. Likely the presence of key antigens is responsible for this phenotype. Late strains include Pasteur, Prague, China, Glaxo, Danish, Tice, Phipps and Frappier, all of which fail to produce MBP70, MPB83 and MPB64. Common features of this group also include the deletion of region of difference 2 (RD2) and point mutations in *sigK*, *crp* and *mmaA3* [110, 111, 114]. While these genomic differences allow for the easy grouping of BCG, daughter strains within each group also have strain specific mutations, which complicates the investigation of phenotype-genotype links for a given BCG strain [115].

2.2.1 Over-attenuation of BCG

Early reports by vaccinologists note that BCG underwent a decrease in virulence after 1927 [124]. Subsequent analysis of vaccine trials determined that protection ranged between 0 and 80%, with one explanation being that differences between BCG strains may be the cause of this variation [125].

The advent of technologies for genomic analysis, combined with studies in animals has not only determined that certain BCG strains are over-attenuated, but also which mutations and deletions are responsible. One such example is BCG Japan (BCG Tokyo), where a 22-bp deletion in *Rv3405c* prevents biosynthesis of PDIM and PGLs, both of which are important for virulence [113]. A comparative study examining the protection provided to mice by five different BCG strains found the bacterial burden in BCG Japan-vaccinated mice was similar to mice vaccinated with PBS [126]. Similarly,

BCG Glaxo does not produce PDIM and PGLs; the mutation responsible has not yet been characterized, although it is likely a deletion affecting the *fadD26* and *pps* genes [113, 115]. This vaccine strain was also reported to be severely attenuated, as guinea pigs that received it had almost one-log higher lung burden of *M. tuberculosis* than those immunized with other BCG strains [127]. BCG Prague has a frame shift mutation in *phoP*, a transcriptional regulator of a two component system involved in mycobacterial virulence [115]. Deletion of *phoP* in *M. tuberculosis* results in attenuation [84, 128] providing a mechanistic explanation of why BCG Prague might provide inferior protection in animal models [126]. These three examples support the hypothesis that *in vitro* conditions created a competitive environment where an attenuated mutant that could out-grow a virulent wild-type bacterium would be selected [108]. BCG Glaxo, BCG Japan and BCG Moreau are deficient in PDIM and PGL biosynthesis, yet they are not related strains, and were cultivated under different conditions [107, 110, 113]. Therefore *in vitro* growth in general, rather than a specific growth condition, appears to be responsible for the ongoing evolution of BCG strains, which is hypothesized to have resulted in overly attenuated vaccines [108].

The vaccine underwent 53 years of serial passaging, equivalent to approximately 15 000 generations (based on a doubling time of 20-24h). The loss of genomic material during decades of *in vitro* passaging rivals the amount of DNA deleted from strains of *M. tuberculosis* that have been circulated in humans for millennia [108]. This is suggestive that the forces of selection acted in an accelerated manner once this intracellular pathogen was exposed to the completely different conditions of *in vitro* lab culture. Further

investigation will be required to determine which other deletions in BCG have resulted in over-attenuation.

2.3 RD1: the attenuating mutation

Genomic comparison of BCG to *M. tuberculosis* and *M. bovis* helped uncover a candidate mutation responsible for the original attenuation of BCG [105, 129]. Subtractive genomic hybridization revealed that a 9.5 kb region, region of difference 1 (RD1), that was present in *M. bovis* three *M. tuberculosis* strains (H37Rv, H37Ra and Erdman) and absent from three BCG strains [129]. Further investigation reported RD1 to be absent from 23 of 23 tested BCG strains, and present in all of the 129 *M. tuberculosis* complex isolates they tested [105]. The RD1 locus is composed of 9 ORFs spanning *Rv3871* to *Rv3879c* (www.tuberculist.org), and is part of secretion system for the T-cell antigens ESAT-6 and CFP-10, that is important for bacterial uptake by the macrophage, and cell lysis [130-132]. The role of RD1 in virulence has subsequently been established in both cell lines and murine models [132-134]. Infection of mice with an RD1-deficient strain of H37Rv (H37RvΔRD1) resulted in decreased pulmonary and splenic bacterial burden, decreased pathology and increased survival times compared to *M. tuberculosis* [135]. Interestingly, BCG complementation with RD1 grew better, and were more virulent than their parental BCG strain in BALB/c and SCID mice, yet failed to achieve the full virulence of *M. tuberculosis* [134]. In a closely related experiment, Sherman and colleagues reported that H37RvΔRD1 was more virulent than BCG Russia as it produced greater inflammation and higher bacterial burdens in a long term murine infection [133]. These data suggested that regions other than RD1 were also important for full

mycobacterial virulence, and hence, that the attenuation of current BCG strains occurred as the result of a complex, multi-step process.

3. Region of Difference 2 (RD2)

3.0 RD2

The RD2 region is 10.8 kb and is composed of the genes *Rv1978* to *Rv1988* [110]. This locus is consistently present in virulent strains of the *M. tuberculosis* complex, as it was detected by hybridization in *M. bovis* and was found in 80 out of 80 clinical isolates of *M. tuberculosis* [129]. Interestingly, deletions encompassing *Rv1978-Rv1979c* and *Rv1983-Rv1984c* have been detected in clinical isolates of *M. tuberculosis* and *M. africanum* [136, 137]. The RD2 locus has direct repeats positioned at either end of the region, thereby permitting the formation of secondary structures and suggesting a mechanism for its deletion [129]. While this observation suggests a mechanism for the molecular event, the exact selective pressure that favoured the deletion of RD2 remains unknown. Remarkably, *in vitro* studies comparing RD1-deficient H37Rv to wild-type H37Rv reported a down regulation of the RD2 genes *nrdF1* and *Rv1982* [138]. Additionally, Raghavan and colleagues observed that the RD1-secreted protein EspR regulated transcription of the putative operon containing these same genes [139]. These studies provide a conceptual link between the RD1 region and RD2 for virulence. The precise function of RD2 is not known, but microarray analysis and transposon site hybridization suggest a role for in virulence [140-143]. Infection of bone-marrow derived macrophages from C57BL/6 mice with a library of transposon mutants revealed that the loss of *Rv1978* decreased bacterial growth in macrophages [140]. In contrast, microarray

analysis of similar naïve and IFN-γ activated macrophages did not note a significant change in the expression of *Rv1978*. Instead, Schnappinger and colleagues reported up-regulation of *nrdF1, Rv1985c*, *Rv1986* and *Rv1988* in naïve macrophages, and *Rv1985c, Rv1986* and *Rv1988* in activated macrophages [141]. The RD2 region is also implicated in latency, as during hypoxic conditions designed to mimic latency, *Rv1986, Rv1987* and *mpt64* undergo changes in expression [142, 143]. Epidemiologic evidence from countries that have made changes to their vaccination programs suggests that RD2-containing vaccines may be more virulent than those that have lost this region. In Finland it was noted that switching from a RD2-containing vaccine to one missing RD2 led to a decrease in osteitis [144]. Similarly, in 1981 when Czechoslovakia changed their vaccine from BCG Prague to BCG Russia an increase in this complication occurred [145]. However, these strain switches do not directly implicate RD2, because RD2-deleted strains are mutated for *sigK*, *crp* and *mmaA3*, while RD2-containing strains are wild-type for these genes. For this reason, isogenic mutants of RD2 are required to formally assess its role in virulence. The sections below will discuss in greater detail specific genes within this region.

3.1 *Rv1979c*

The predicted function of *Rv1979c* is an amino acid permease, based on its similarity to the *gabP* gene of *E. coli* (www.tuberculist.org, [129]). Indirect evidence to support this comes from growth experiments comparing BCG strains in Sauton media where single amino acids served as the sole nitrogen source [112]. BCG Japan, which has RD2, and therefore *Rv1979c*, was able to grow in media where arginine, histidine, lysine

or proline was the sole amino acid. However, BCG Pasteur, which lacks the RD2 region, was unable to grow under similar conditions [112]. Therefore, *Rv1979c* may be a transporter for one of these amino acids. *Rv1979*c may also be a T-cell antigen in both human and cattle infections of tuberculosis [146-148]. Cockle *et al* (2002) noted that not only are peptides from *Rv1979c* immunogenic, but they also offer promise for a subunit vaccine [148].

3.2 *Rv1981c/nrdF1*

Rv1981c/nrdF1 encodes a class Ib R2-subunit of a ribonucleotide reductase [15, 149]. Ribonucleotide reductases catalyze the reaction of converting ribonucleotides into deoxyribonucleotides, a reaction essential for cellular functions such as DNA repair and replication. These enzymes are divided into two groups, class I that are oxygen dependent, and class II which are oxygen independent. In mycobacteria, class I ribonucleotide reductases are composed of two homodimers in a $\alpha_2\beta_2$ subunit structure [150]. *M. bovis* and *M. tuberculosis* are distinct from other mycobacteria in that they not only encode a class Ib ribonucleotide reductase, but also encode an alternate class Ib R2-subunit, *Mb2003c* and *Rv1981c*/nrdF1 respectively [15, 149-151]. The presence of this alternate gene suggests that the function of *nrdF1* is unique to the pathogenesis of *M. tuberculosis*. Biochemical evidence demonstrated that *nrdF1* does not complement for the loss of the other R2 subunit, *nrdF2*, thereby indicating its function is not a redundant pathway [149].

Treatment *in vitro* of *M. tuberculosis* with fluoroquinolones or novobiocin resulted in upregulation of *nrdF1*, but not other genes that encode ribonucleotide reductases [152].

This class of antibiotics binds DNA gyrase and topoisomerase IV resulting in double stranded DNA breaks and bactericidal activity. The induction of *nrdF1* suggests it has a unique role in the response to this specific form of DNA damage *[152]*. This hypothesis is supported by the fact that *nrdF1* is not part of an operon of ribonucleotide reductase genes, and therefore may be regulated by different stresses. However, another study reported that a strain of H37Rv where *nrdF1* had been deleted was no more susceptible to novobiocin than wild-type H37Rv [153]. Additionally, loss of *nrdF1* did not affect aerobic growth, sensitivity to the DNA cross-linking reagent mitomycin C or sensitivity to hydroxyurea. *In vivo* growth in the lungs of B6D2/F_1 was similarly unaffected [153]. However, this group did not report if growth in a macrophage or if growth in other organs was affected by deletion of this gene. Additionally, recent reports implying a role for the virulence factor EspR in regulating expression of *nrdF1* suggest it may have an as of yet undetermined role in pathogenesis [139].

3.3 *Rv1982*

Rv1982 is predicted to encode a toxin-antitoxin system of the VapBC family, one of a predicted 88 present in *M. tuberculosis* [154]. This family of TA systems contains a PIN domain, which is predicted to degrade RNA through ribonuclease activity[155] Orthologues for this gene were found in *M. africanum, M. bovis, M. canetti* and *M. microti,* and a homologue is present in the closely-related non-tuberculous mycobacteria *M. kansasii* [154]. It has been demonstrated that other VapC toxins inhibited translation through ribonuclease activity; it is tempting to speculate that *Rv1982* may also inhibit growth. This gene is dysregulated by the loss of the RD1 region or the secreted virulence

factor EspR [138, 139]. As EspR functions early during infection, likely during phagosome acidification, it supports the idea that *Rv1982* inhibits growth under conditions that are unfavourable to the bacterium.

3.4 *Rv1980/mpt64*

The presence of antigenic protein MPB64 in the culture filtrate of BCG strains was one of the first methods used to distinguish different sub-strains of BCG [119]. As the sequence of *mpb64* (from *M. bovis*) is identical to the sequence of *mpt64* (from *M. tuberculosis*), there is some confusion in literature as to the name of this protein; conventionally, experiments using *M. bovis* (or *M. bovis* BCG) refer to MPB64 while experiments looking at *M. tuberculosis* call the same protein MPT64. The protein is a secreted antigen found abundantly in the culture filtrate [116]. The NMR solution structure reveals MPT64 to be a B-grasp family protein, with multiple predicted protein-protein interaction domains [156].

This antigen contains multiple B and T-cell epitopes [157, 158]. Therefore, it has been shown to be highly immunogenic, generating humoral and cell mediated responses in mice, guinea pigs and humans [159-165]. Due to this property, MPT64 has been considered a promising candidate for improving diagnostic tests, as one study noted 82% sensitivity in detecting active TB [166]. The immunogenic properties of this antigen have also advanced MPT64 as a candidate subunit vaccine or as a gene that can be added to select BCG strains to increase its antigenic repertoire [161, 167]. In addition, in several instances MPT64 has been combined with another antigen to form a fusion protein [159, 168, 169]

The function of MPT64 is unknown, and the antigen has no homology to known proteins. Immunohistochemistry revealed an inverse correlation between the presence of MPT64, and markers of apoptosis in patient lymph node biopsies [60, 61]. Mustafa also demonstrated that the antigen may decrease TNF-α production in the cells within a granuloma [60]. MPT64 is only secreted by actively dividing cells, and it is hypothesized to be involved in protein-protein interaction [156]. These facts make it tempting to speculate that MPT64, perhaps in concert with other RD2 proteins, may interact with the host cell to modulate TNF-α levels and/or apoptosis in an as yet uncharacterized way.

3.5 *Rv1983/PE_PGRS35* and *Rv1984c/cfp21*

Mycobacterium tuberculosis contains numerous genes that are part of the PE multi-gene family, whose gene products are identified by their proline-glutamic acid amino acid sequence at the amino terminal of the protein [170]. *Rv1983* (alias *PE_PGRS35*) is one of 67 PE_PGRS genes found in the bacterium [171]. The exact function of *Rv1983* remains unknown, however this family of proteins has been suggested to be a method by which *M. tuberculosis* generates antigenic diversity[170].

Rv1984c (also referred to as *cfp21*) encodes a secreted antigen that is found in the culture filtrate, and is highly immunogenic [172, 173]. CFP21 possesses numerous T-cell epitopes, which are able to stimulate IFN-γ release [174, 175]. Consequently, its potential has been demonstrated as a diagnostic antigen, and as a booster for co-administration with BCG [161, 176]. CFP21 is predicted to be a cutinase, and studies using the recombinant form of the enzyme demonstrate it preferentially hydrolyzes short and medium chain lipids, suggesting it may help provide carbon sources for bacterial growth

[177]. Interestingly, genomic analysis of 100 clinical isolates of *M. tuberculosis* revealed one isolate where *Rv1983* and *Rv1984c* where deleted, suggesting these genes to be dispensable for full virulence [136].

3.6 *Rv1985c*

The RD2 region contains a predicted LysR-family transcriptional regulator (LTTR); encoded by *Rv1985c*. LTTR proteins represent the largest family of bacterial regulators, and are involved in numerous functions including cell growth, stress response and virulence [178-180]. Regulatory proteins of this family have great conservation in their structural regions, and contain an N-terminal helix-turn-helix motif that interact with DNA, as well as a C-terminal co-factor binding domain [181]. Amino acid sequence comparisons suggest *Rv1985c* may be homologous to another LTTR-protein found in *E. coli* ArgP. *In silico* examination of the *Rv1985c* crystal structure demonstrate that lysine, arginine and canavanine bind the regulator. Consequently, it has been proposed that these amino acids may regulate the function of this protein [182]. Several recent reports implicate *Rv1985c* as a regulator involved in latency [143, 183]. Biochemical studies demonstrated that increased concentrations of recombinant *Rv1985c* (referred to as *iciA*) decreased DNA replication *in vitro*. The IciA protein binds A+T rich regions within *oriC*, and thereby prevents chromosomal unwinding, and replication. Interestingly, its function is similar to that of *iciA* found in *E. coli*, despite only 35.8% homology. It is proposed that this is a mechanism through which *M. tuberculosis* prevents replication during latency [183]. Additionally, *Rv1985c* was upregulated 4 and 7 days following the induction of hypoxia *in vitro* [143]. As these conditions are meant to mimic latency, it

corroborates the hypothesis that this gene plays a role in latency. Likely the role of *Rv1985c* may function to activate genes involved in amino acid transport and replication during conditions when nutrients are plentiful, but during limiting metabolic conditions it is involved in inhibiting bacterial growth [182].

3.7 *Rv1986*

Rv1986 is one of two predicted *lysE* putative membrane transporters found in the *M. tuberculosis* genome, that may have arisen through a gene duplication event [184]. The gene is homologous to *lysE* found in *C. glutamicum* which is a transporter for L-lysine and L-arginine [185]. In this bacterium *lysE* is regulated by the an adjacent gene, *lysG*, which encodes an LTTR protein [185]. The similarities in genomic topology between *lysG/lysE* in *C. glutamicum* and *Rv1985c/Rv1986* suggest that *Rv1985c* may be a regulator for *Rv1986*. *In vitro* studies in *M. smegmatis* indicate a role for Rv1986 for growth in limiting conditions. Transposon mutagenesis of a Rv1986 homologue found in this bacteria resulted in an impaired ability of *M. smegmatis* to survive during stationary phase growth [186]. This report suggests that this transporter is important for bacteria by balancing out intracellular lysine and arginine levels. Work done in our lab supports this theory, as lysine rich media was bacteriostatic for BCG Pasteur (which lacks RD2), but not for the RD2-containing BCG Russia. (Behr lab, unpublished data). This work would indicate that the function of Rv1986 is an amino acid exporter. However, *Rv1985c/Rv1986* is homologous to the *attR/attX* virulence system found in *R. fascians* [187]. The compound secreted by this transporter is required for full virulence, and is hypothesized to be a β-lactam containing structure that may be derived from arginine

[187]. If a parallel situation exists in *M. tuberculosis*, *Rv1986* may have the capacity not only to export amino acids, but also an as-of-yet unknown virulence factor.

A role for this gene in virulence is suggested by bacterial expression profiles in macrophages [141]. In both naïve, and IFN-γ activated macrophages *Rv1986* was reported to be significantly upregulated at 24h and 48h post-infection [141]. While the function of *Rv1986* remains to be fully elucidated, its gene product is a potent antigen. This protein was identified, along with the RD2-proteins *Rv1983* and *Rv1979c*, as immunogenic in cattle infected with *M. bovis* [148]. Its antigenic potential has also been demonstrated in human subjects suffering from either latent or active TB, where it is a potent inducer of IL-2 secretion in memory T-cells. (Gidgeon et al. – in submission). Paradoxically, recognition by T-cells may also be important to bacterial virulence, particularly for the establishment of latent infection [188].

3.8 *Rv1987 and Rv1988*

Rv1987 is a potential membrane anchored chitinase, which has shown potential as a diagnostic antigen in several studies [148, 189]. *Rv1988* is predicted a methyltransferase, that has been demonstrated to mediate resistance to macrolides, and is under control of the putative transcriptional activator *whiB7* [190, 191].

4. Host susceptibility to mycobacterial infection

4.1 Vaccine induced protection to *M. tuberculosis* in a mouse

Following aerosol infection, *M. tuberculosis* survives within the phagosome of macrophages (and dendritic cells), which express pro-inflammatory cytokines and chemokines, such as TNFα, IFN-γ and IL-12 [192]. Initially, infected macrophages are surrounded by foamy macrophages and neutrophils, which will form the core of the granuloma. Much of what goes on during the early phase of infection remains unknown, however bacterial numbers increase implying that there is exponential growth until this is held in check by nascent adaptive immune response. Therefore, rapid induction of this response is crucial to control and limit bacterial growth and increase survival.

The induction of adaptive immunity coincides with dissemination of the bacteria to the lymph nodes approximately 9-11 days post-infection, where the generation of effector T-cells occurs [85]. Vaccination produces mycobacterial specific IL-17 producing $CD4^+$ T-cells that serve as sentinel cells that will recruit effector $CD4^+$ T-cells to the lungs [193]. Mycobacterial antigens are presented to $CD4^+$ and $CD8^+$ T-cells by MHC class I and class II molecules respectively. Apoptosis of infected macrophages leads to cross presentation of antigens to dendritic cells for priming $CD8^+$ T-cells [194]. The recruitment of lymphocytes to the lungs occurs after 15 to 20 days, $CD4^+$ T-cells traffic inside the granuloma as well to form a mantle around it [192, 195, 196]. The exact function of these cells *in vivo* is not known. It is hypothesized that they activate macrophages, and it is known that $CD4^+$ T-helper cells release cytokines to help polarize a protective Th1 response (reviewed in [192]). However recent studies suggest that the number of IFN-γ producing T-cells is a poor indicator of protection, and that protection

correlates best with a robust $CD8^+$ T-cell response [197]. Evidence suggests that other T-cell subsets are also play a role in protection. Regulatory T-cells have been shown to prevent clearance of *M. tuberculosis*, and are detrimental to protection, whereas Th17 cells can convey moderate protection even in the absence of IFN-γ [82, 83].

The discovery of more T-cell subsets (i.e. Th22) indicates that immunity to mycobacterial infection is a complex process, and the role of these subsets in vaccination remains to be determined.

4.2 Monogenic control of mycobacterial disease

The notion that host factors played a role in susceptibility to TB is not a new concept. An early 20th century study comparing sets of fraternal and identical twins noted that 70 percent of the identical twins had similar "behaviour" towards tuberculosis, whereas in fraternal twins the correspondence was only 25% [198]. Early animal studies identified that strains of mice and rabbits differed in their susceptibility to mycobacterial infection [70, 77]. Lurie noted differences not only in survival times, but also disease development between Race III and FC strains of rabbits infected with *M. bovis*, suggesting a role for host factors in disease severity [70]. Research over the last three decades has lead to the discovery of numerous host-susceptibility genes. Genetic control of diseases can be through monogenic traits or complex traits. Monogenic factors controlling resistance to mycobacteria were identified in patients suffering disseminated forms of mycobacterial disease, often from organisms that would normally be considered only weakly virulent. These rare mutations were grouped together into a syndrome, now referred to as mendelian susceptibility to mycobacterial disease (MSMD). Even though

these mutations occur in only a paucity of the population, they have illustrated key pathways involved in resistance to mycobacteria (Figure 4).

4.2.1 Mutations in the interferon response pathway

Interferon-gamma is a cytokine that is critical to host defense against mycobacteria. As a result, the discoveries of mutations at different levels of its associated signalling pathway are associated with susceptibility to mycobacteria. Deficiencies in the interferon gamma receptor render the cell insensitive to IFN-γ, and this was first identified in children who developed fatal cases of disseminated BCG infection [14, 199-201]. Two interferon regulatory factor genes *Irf-1* and *Irf-8* are also resistance factors to mycobacterial infection [202, 203]. Deletion of the *Irf-1* gene results in macrophages with reduced levels of iNOS mRNA and NO production. Infection of IRF-$1^{-/-}$ mice with BCG results in extensive infection and with *M. tuberculosis* in rapid dissemination, and death [202, 204, 205]. Infection of BXH-2 mice (which lack *Irf8* gene) with *M. tuberculosis* results in rapid dissemination, reduced granuloma formation, and decreased survival times [203]. IRF-1 and IRF-8 binds ISRE (Interferon-sensitive response element) elements, and through interaction with each other, or other transcription factors, leads to activation of genes such as *Il12-p40, IL1* and *Tlr4* [203, 206]. Loss of either factor results in an impaired Th1 response [207]. This is due to decreased production of IL-12p40 and IFN-γ in lymphocytes, leading to impaired T-cell priming. It has also been noted that the promoter of *Nramp1* (discussed below) has IRF-8 binding sites, suggesting a synergy between these resistance factors [208].

4.2.2 IL-12 deficiencies

IL-12 is another cytokine that mediates anti-mycobacterial immunity, and is critical for generating a Th1 response. Mutations are found both in IL12B1 and IL12B, which code for the first chain of the receptor, and the IL-12p40 subunit, making this the only known inherited cytokine deficiency [206, 209, 210]. IL-12 is important for dendritic cell migration, the maturation of $CD4^+$ T-cells and for inducing IFN-γ. IL-$12p40^{-/-}$ mice are unable to mount an effective Th1 response, and cannot contain *M. tuberculosis* [91, 211]. A similar phenotype is seen in human patients, where this immunodeficiency often results in BCGosis, disseminated NTM infections, and increased risk of tuberculosis [210, 212]. IL-12 deficiency is less severe than mutations affecting the interferon gamma receptor, as treatment with IFN-γ can sometimes counter-balance the loss of IL-12 [211].

4.2.3 STAT1 and NEMO

Mutations affecting transcription factors or regulators also result in susceptibility to bacterial and viral infections. STAT1 and NEMO are both involved in downstream signalling in response to cytokine stimulation, and important for host-resistance to mycobacteria [206, 213]. STAT1 is a transcription factor that activates gene expression in response to type-I and type-II interferons. STAT1 dimerizes and binds gamma interferon-activating sequences to induce transcription of genes [214]. NEMO is a protein kinase that phosphorylates the inhibitor of NF-κB, facilitating its activation [215]. Loss of function of either protein results in defective cytokine production and decreased expression of pro-inflammatory genes [206].

4.3 Complex genetic control of mycobacterial disease

Complex genetic factors of resistance are more difficult to detect, as the effect is often not as strong as a monogenic trait. Furthermore, the frequency of the susceptibility allele in the population is much higher than that of monogenic traits. However, the pathways illustrated by monogenic traits can provide a scaffolding upon which more complex genetic factors can be placed. Two well-studied examples are *Nramp1* (alias *Slc11a1*) and *Ipr1*.

Nramp1 (Natural resistance-associated macrophage protein 1) is a membrane protein expressed primarily in macrophages that acts to transport divalent metal ions in a pH- dependent manner [216, 217]. The exact mechanism through which *Nramp1* restricts mycobacterial growth is not known. Macrophages from *Nramp1* deficient mice showed impaired vacuolar-ATPase activity and decreased phagosomal acidification [218]. Additionally the transporter may limit access to metal ions such as iron and manganese required by the bacterium [218]. *Nramp1* was the first gene linked to susceptibility to BCG infection in mice, but does not affect growth of *M. tuberculosis* [219, 220]. Despite this lack of phenotype in mice, Nramp1 has been linked to tuberculosis susceptibility in humans, including paediatric TB and multi-drug resistant TB [221-225].

The *Ipr1* gene was first discovered in mice to mediate resistance to BCG and *M. tuberculosis* [226, 227]. Loss of this gene increased bacterial burden and pathology in the lungs, and decreased survival [226]. In addition to mouse studies, polymorphisms in the human homologue, SP110, have been linked to tuberculosis susceptibility in a West African cohort [228]. The bactericidal effect exerted by this gene involves production of reactive oxygen species and is macrophage specific, as T-cells from knock out mice were

not functionally affected [226, 229]. Interestingly, the loss of this gene also reduces the protective efficacy of BCG vaccination [230].

4.4 Nuclear Oligomeriztion Domain 2 (NOD2)

Recent studies have proposed *NOD2* as a mycobacterial susceptibility gene, as it has been identified in genome wide association studies for leprosy and Crohn's disease [231, 232]. The etiological agent of leprosy is *Mycobacterium leprae*, and evidence suggests Crohn's disease may be due to infection with *Mycobacterium avium paratuberculosis*. *NOD2* has also been associated with a risk of TB in African-Americans, although a study in South Africa failed to associate polymorphisms in this gene with increased disease risk [233, 234]. That *NOD2* has been associated with leprosy, combined with the demonstration that it is a susceptibility factor in mice strongly suggests a role in human disease [89]. This gene will be examined in more detail in the following section.

NOD2 (CARD15 in mice) is a member NLR family, of which there are 22 known members in human and 34 in mice. Like the other members of this family NOD2 has a central NACHT domain, a carboxy-terminal LRR region and an amino-terminal CARD domain [235]. It is mainly expressed in monocytes, macrophages and dendritic cells, although it is also present in epithelial cells, intestinal paneth cells and T-cells [236]. NOD2 is an intracellular pattern recognition receptor that detects the muramyl dipeptide (MDP) component of bacterial peptidoglycan bacteria [237]. This receptor has been implicated in the detection of both extracellular and intracellular bacteria [29, 238-240]. NOD2 is a cytosolic protein, although it has been found to be associated with the plasma

membrane in intestinal epithelial cells [241]. Localization of MDP to the cytosol involves clathrin-mediated endocytosis and the phagosomal transporter PEPT2 [242]. Transport by PEPT2 relies on a proton gradient, and requires the action of a vacuolar ATPase [243]. Upon detection of MDP, NOD2 interacts with the serine/threonine kinase RIP2 via CARD domains forming a complex that results in polyubiqitination of NEMO leading to activation of NF-κB and MAP kinases [237, 244, 245]. Ubiquitination of RIP2 is required for optimal activation of the NOD2 pathway, and multiple host ubiqitinases have been suggested to be mediate this process [245-247]. This signalling pathway has been found to function independently of MyD88 [246].

Interestingly, NOD2 expressing-HEK 293 activated IRF3 and IFN-β in response to single-stranded viral RNA, and the LRR domain of NOD2 was shown to interact with MAVS [248]. Pull down experiments in THP-1 cells have demonstrated NOD2 interacts with another protein involved in anti-viral response, 2'-5'-oligoadenylate synthetase type 2 [249]. This protein activates RNase-L, which plays a role in the host cell response to both intracellular bacteria and viruses [249]. The specific role of NOD2 in viral recognition remains an avenue for further investigation.

Studies investigating *Nod*2-deficient mice, or mice with the Crohn's-associated *Nod2* variant have demonstrated a clear role for this receptor in innate immunity. Activation by MDP results in expression of pro inflammatory cytokines, chemokines, Type-I interferons and antimicrobial peptides [236, 247, 250-253]. NOD2 may also induce caspase-1 activation and IL-1β, possibly through interactions with NLRP1, however evidence also suggests this caspase-1 activation is NOD2 independent [247]. There is also evidence of cooperation between NOD2 and other PRRs. Stimulation of

dendritic cells with the TLR4 agonist LPS and MDP yielded higher levels of IL-6, IL-12 and TNF-α than with MDP alone [253]. Enhanced cytokine expression was also seen when macrophages were stimulated with MDP and TDM, suggesting crosstalk between NOD2 and MARCO [250].

Evidence indicates that NOD2 may serve as a pathway for pathogen detection under conditions where the host cell could become desensitized to TLR stimulation. Macrophages treated with LPS developed a tolerance to TLR stimulation, but became re-sensitized when stimulated with MDP. This effect was lost in *Nod2*$^{-/-}$-deficient cells [254, 255].

Recent investigations have discovered NOD2 is also involved in mediating autophagy. This process utilizes the lysosomal pathway to degrade cellular components and foreign material during instances of cell starvation, infection or programmed cell death. Treatment of dendritic cells with siRNA followed by MDP demonstrated a requirement for NOD2 in autophagy. Furthermore, this process was required for antigen presentation and also required ATG16L1 [256]. NOD2 functioned to recruit ATG16L1 to the plasma membrane at the site of bacterial entry and to facilitate autophagy [257]. Defects in this pathway due to a truncation of the NOD2 protein have been linked to the pathogenesis of Crohn's disease [237, 241].

In addition to its role in innate immunity, NOD2 is also involved in the adaptive immune response. Prior to the discovery of NOD2, influential studies by Freund demonstrated that antigens combined with mycobacterial cell wall in a water and oil immersion could induce cell-mediated and humoral immunity [258, 259]. It was later determined that the active component in Freund's Complete Adjuvant was the MDP

[260, 261]. Experiments in mice demonstrate the link between adjuvancy and adaptive immunity, as *Nod2*$^{-/-}$ mice immunized with HSA (human serum albumin) and MDP had lower antigen specific IgG1 titers compared to their wild-type counterparts [262]. NOD2 may aid the induction of adaptive immunity through multiple mechanisms. MDP stimulation of monocytes and lymphocytes revealed increased surface expression of numerous co-stimulatory molecules involved in adaptive immunity [263]. This receptor has also been shown to produce cytokines that are required not only in the transition from innate to adaptive immunity, but also for the development of Th17 cells [89, 264]. Additionally, downstream interactions may also be important, as *Rip2*$^{-/-}$ T-cells were defective in their ability to differentiate into Th1 cells, and to proliferate under TCR stimulation [265]. However, other groups have shown that the loss of RIP2 does not affect T-cell proliferation. Infection studies with the parasite *T. gondii* demonstrated NOD2 interacts with c-Rel and facilitate *Il2* transcription [266]. This suggests NOD2 may have both MDP- dependent and independent functions in adaptive immunity.

As this PRR plays a pivotal part in innate and adaptive immunity, it comes as no surprise that its deletion results in susceptibility to numerous extracellular and intracellular bacteria [236]. The role of this receptor during infections involving BCG and *M. tuberculosis* will be examined in the following section.

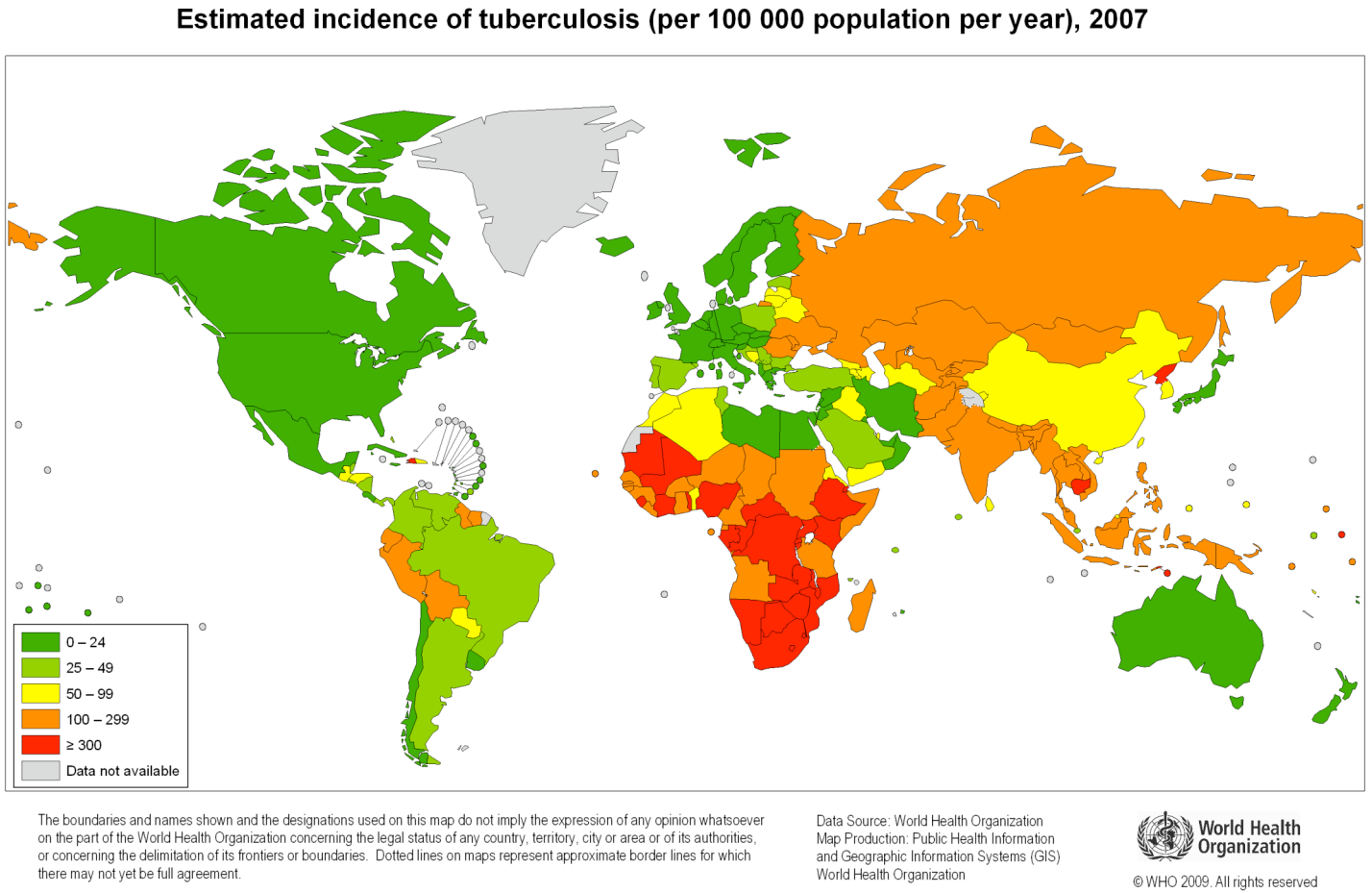
4.5 Recognition of *M. tuberculosis* by NOD2

NOD2 is a non-redundant system of recognition by macrophages for *M. tuberculosis* that synergizes with TLR2 to induce production of IL-1β, IL-6 and TNF-α [29, 267]. Interestingly while TLR2 and NOD2 synergy improves cytokine response, there is no influence of the NOD2 pathway on apoptosis, even though apoptosis can be induced by the 19-kDa antigen through TLR2 [268]. Infection with the bacterium results in NOD2 recognition followed by polyubiquitination of RIP2, and expression of IFNα/β, and RANTES via a pathway involving Tbk1 and Irf5 [239, 246, 251]. This activation is dependent on the ESX-1 secretion system, suggesting there is a requirement for translocation of bacterial products into the cytosol [246, 251].

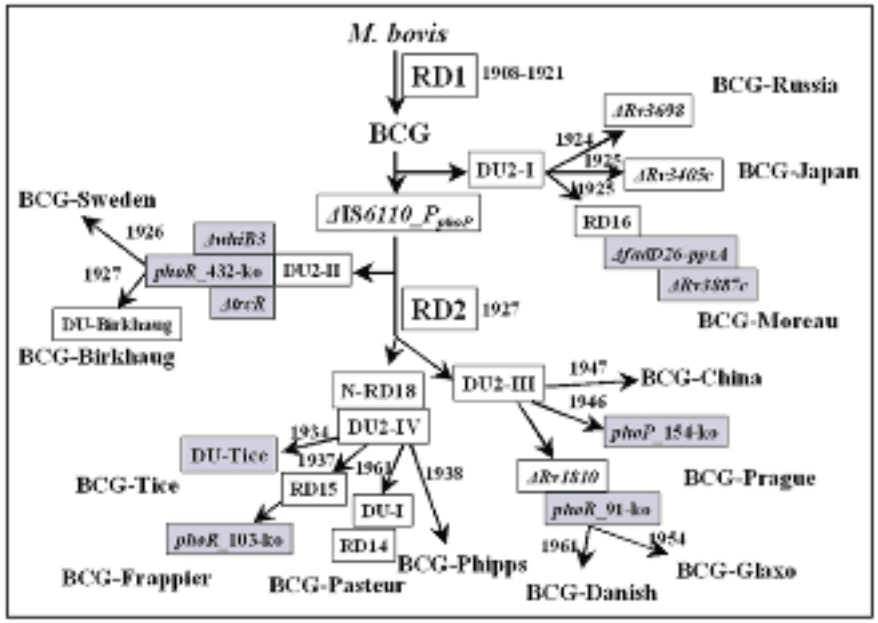
The peptidoglycan present in *M. tuberculosis* contains two structurally different variants of MDP, *N*-acetyl MDP and *N*-glycolyl-MDP. Studies revealed *N*-glycolyl MDP to be unique to selected Actinomycetes, of which *M. tuberculosis* is a member. Additionally *N*-glycolyl MDP was a more potent inducer of both pro-inflammatory cytokines and RIP2 activation than the acetylated variant of MDP [250]. Whether *N*-glycolyl MDP levels differ between *M. tuberculosis* and other mycobacteria, as well as the effect of this on *in vivo* infection remain unknown.

Experiments in mice reveal that in the early stages of disease loss of NOD2 results in a decreased pro-inflammatory cytokines, but similar bacterial burdens [88, 89]. *Nod2*$^{-/-}$ mice were found to be deficient in the IFN-γ/IL-12 axis of innate immunity and also had an impaired adaptive immune response, as measured by mycobacteria-specific T-cell responses. This decreased innate and adaptive immunity was not confined to the lungs, but was systemic, and consequently survival times were decreased in these animals

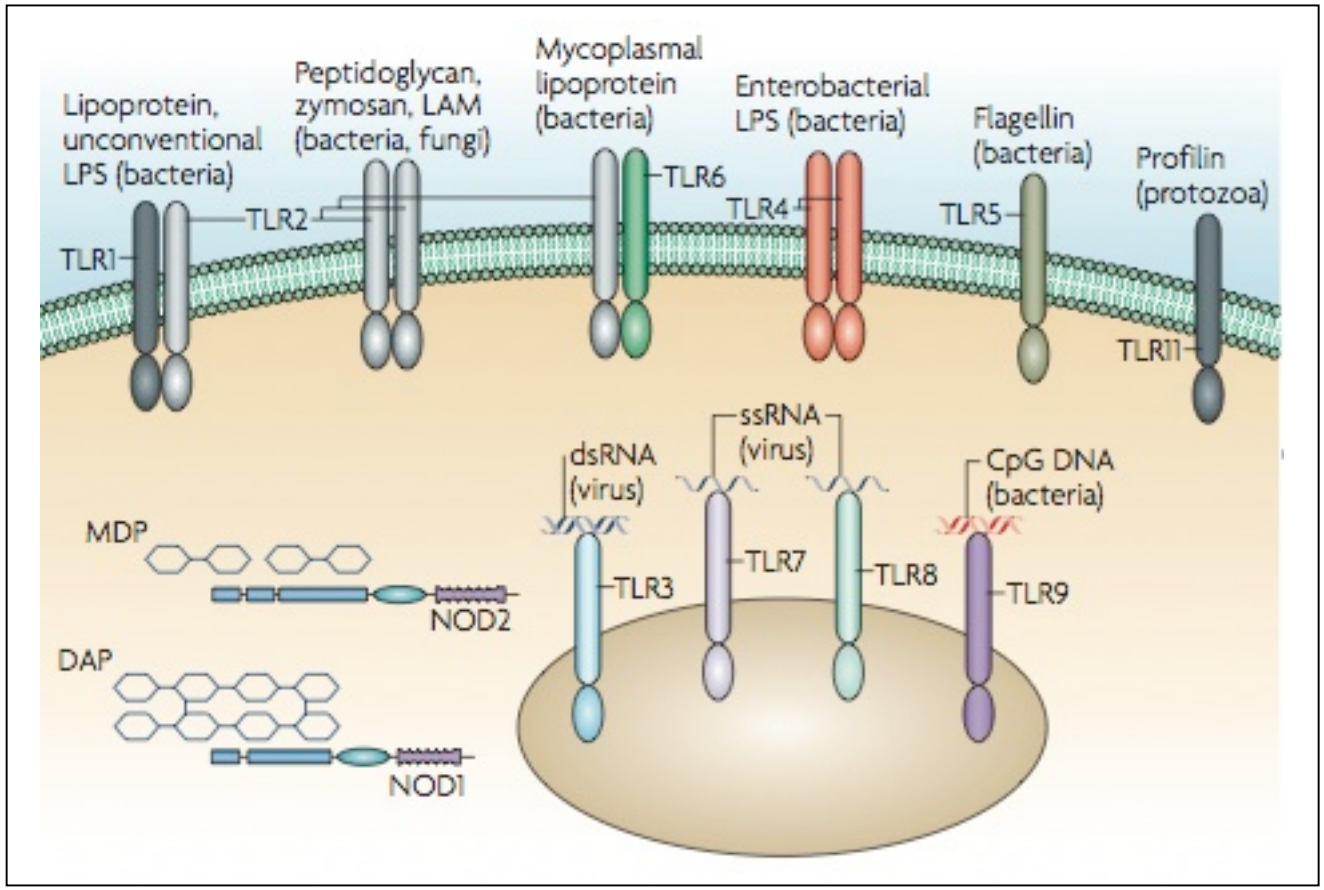
[89]. Collectively these data demonstrate the importance of NOD2 in anti-mycobacterial immunity, and the role of NOD2 in BCG vaccination will be one of the major foci of this thesis.



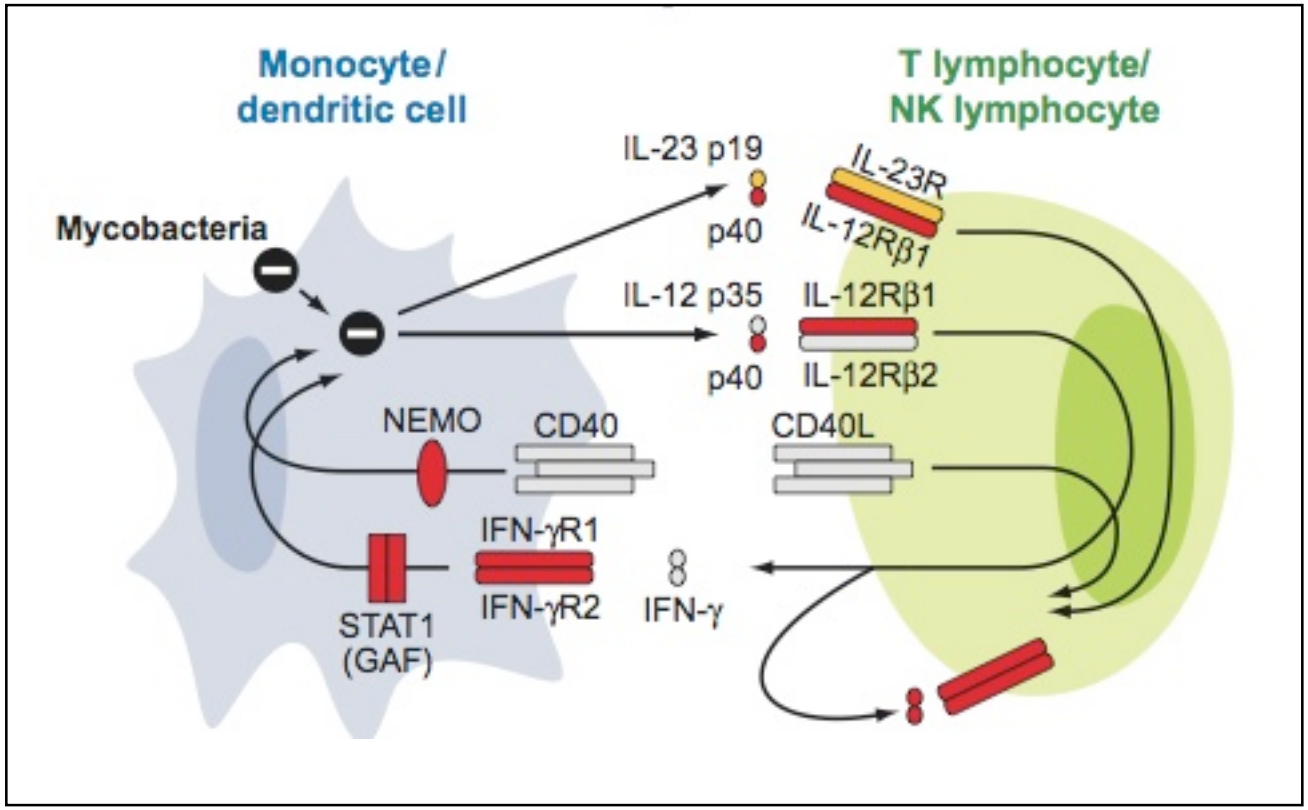
Chapter 1 - Figure 1. Estimated incidence of tuberculosis.
Most recent estimates of global tuberculosis incidence according to the World Health Organization. This figure is taken from www.who.int.



Chapter 1 - Figure 2. Genealogy of BCG strains.
Schematic representation of the genealogy of BCG vaccines showing known mutations and duplications. Grey boxes represent mutations known to affect virulence. This figure was reproduced from [121]



Chapter 1 - Figure 3. Common pattern recognition receptors.
Common TLR and NLR pattern recognition receptors and their ligands. Reproduced from Kauffman S.H., *The contribution of immunology to the rational design of novel antibacterial vaccines.* Nat Rev Microbiol, 2007 Jul;**5**(7):491-504



Chapter 1 - Figure 4. Monogenic control of mycobacterial disease.
Mendelian susceptibility to mycbacterial disease genes and the immune pathways they affect. Reproduced from [206].

Preface to Chapter 2

As was revealed in Chapter 1, the *in vitro* evolution of the BCG vaccine resulted in the loss of RD2. This chapter is dedicated to addressing the first aim of the thesis. We have used an isogenic deletion mutant to evaluate the role of RD2 in virulence. Deletion studies using the mouse model of infection is an established method for evaluating virulence genes. This manuscript evaluates our hypothesis that the loss of RD2 reduces virulence of *M. tuberculosis*.

Chapter 2: Region of Difference 2 contributes to virulence of *Mycobacterium tuberculosis*

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1. Abstract

Mycobacterium bovis BCG strains are live, attenuated vaccines generated through decades of *in vitro* passage. Because *in vitro* growth does not select for interaction with the host, it has been hypothesized that genetic loci lost from BCG code for virulence determinants that are dispensable for growth in the laboratory, as exemplified by Region of Difference 1 (RD1), lost during the original derivation of BCG between 1908 and 1921. Region of Difference 2 (RD2) was lost during ongoing propagation of BCG between 1927 and 1931, a time that coincides with reports of ongoing attenuation of the vaccine. In this study, RD2 has been disrupted in *M. tuberculosis* H37Rv, to test whether its loss contributed to further attenuation of BCG. Deletion of RD2 did not affect *in vitro* growth; in contrast, the mutant manifested a decrease in pulmonary and splenic bacterial burdens and reduced pathology in C57BL/6 mice. This attenuated phenotype was complemented by re-introducing the genes *Rv1979c-Rv1982* (including *mpt64*), but not *Rv1985c-Rv1986*. In RAW 264.7 macrophages, H37Rv:ΔRD2 showed a decreased proliferation and impaired modulation of the host innate immune response; both observations were complemented with *Rv1979c-Rv1982*. To test the effect of RD2 disruption on innate immunity, *Rag* -/- mice were infected; H37Rv:ΔRD2 had increased survival times compared H37Rv. These findings support the notion that the safety profile of certain BCG vaccines stems from multiple attenuating mutations, with the RD2 deletion resulting in a less virulent organism through impaired bacterial manipulation of the host innate immune response.

2. Introduction

Tuberculosis (TB) has been, and continues to be one of the most widespread bacterial infections worldwide (www.who.int/tb/publications/global_report/2009/). On an individual level, management of TB cases can be achieved in settings where prompt microbiologic diagnosis and appropriate treatment are provided. However, on a global public health level, this approach has had limited success, indicating a need to consider alternative strategies for TB control, including immunization.

For nearly a century, *Mycobacterium bovis* BCG strains have been used as vaccines against TB. The efficacy observed in a number of different BCG trials has provided proof-of-principle data that TB should, in theory, be a disease that is preventable through vaccination. However, variable estimates of efficacy have diminished the enthusiasm for BCG immunization. While the effectiveness of BCG vaccines has been the subject of controversy, their safety profile has generally not been disputed; about one in 100,000 vaccinees suffers disseminated BCG disease, and those with BCG-osis typically have severe immunologic defects, either genetic (e.g. SCID) or acquired (e.g. AIDS)[14, 201, 269]. Thus, BCG vaccines provide an example of a profound attenuation, enabling researchers to uncover virulence determinants that are present in *M. tuberculosis* and virulent *M. bovis*, but absent from BCG strains.

The study of TB pathogenesis through BCG vaccines is supported by theoretical considerations and experimental observations. In theory, an intracellular host-adapted bacterium like *M. tuberculosis* is equipped with virulence determinants critical for interaction with the eukaryotic cell in which it resides. By propagating the bacteria in the laboratory without host selection for over five decades, as was the case with BCG

Pasteur, mutants are expected to emerge based on enhanced fitness *in vitro*, with no pressure to maintain bacterial factors required during infection. Previous work has confirmed this prediction, through the study of Region of Difference 1 (RD1), a 9.8 kb genomic deletion that occurred during the original derivation of BCG between 1908 and 1921[129]. This deletion was shown to contribute to the attenuation of BCG by both gene disruption [135] and complementation experiments [134]. Interestingly, complementation of BCG Pasteur with the RD1 region did not fully restore pathogenicity in a murine model, thus, it was hypothesized that other genes may be involved in virulence [68]. BCG Pasteur not only lacks RD1 but also has incurred other mutations, including Region of Difference 2 (RD2), lost between the years 1927 and 1931, a time when vaccinologists reported further attenuation of the BCG vaccine [124, 270]. To evaluate the role of RD2-associated virulence, we engineered a targeted knock-out within the *M. tuberculosis* reference strain H37Rv and conducted a series of *in vitro* and *in vivo* investigations. Here, we demonstrate that loss of RD2 leads to a reduction in virulence, providing evidence that the safety profile of BCG Pasteur stems from the cumulative effect of multiple attenuating mutations.

3. Methods

3.1 Bacterial strains

Mycobacterium tuberculosis strains H37Rv, H37Rv:ΔRD2, H37Rv:ΔRD2::pMV306, H37Rv:ΔRD2::p7982 , H37Rv:ΔRD2::p8586 and BCG Russia were cultured in Middlebrook 7H9 liquid medium (Becton Dickinson) containing 10% ADC (albumin dextrose complex; Becton Dickinson), 0.5% glycerol and 0.08% Tween-

80 (Sigma-Aldrich) or Middlebrook 7H10 agar containing 10% OADC (oleic acid plus ADC; Becton Dickinson), 0.5% glycerol and PANTA (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin, Becton Dickinson). Hygromycin or kanamycin (Sigma-Aldrich) was added to the media when needed.

3.2 Mutant construction and complementation

The *M. tuberculosis* H37Rv:ΔRD2 strain was prepared using homologous recombination and sucrose counter-selection as previously described [135]. Specifically, the RD2 deletion in BCG Pasteur is separated by only 335 bp from a region present in *M. tuberculosis* but deleted from virulent *M. bovis*, known as RD6 [271]. Therefore, to delete the RD2 region from *M. tuberculosis*, we amplified DNA flanking the region (*Rv1977-Rv1978* and *Rv1988-Rv1989*) into the suicide vector pKO for introduction into H37Rv, selection for hygromycin-resistant mutants and then counter-selection on sucrose to screen out clones harboring the intact *sacB* gene. Details on construct design and the primers utilized are provided in Table 1.

To assess complementation with selected portions of the RD2 region, the integrating plasmid pMV306-kan was used (gift of P. Domenech) [272]. Inserts were amplified with AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen) and PCR products were confirmed by sequencing at the McGill University and Génome Québec Innovation Centre, Montreal Canada. Primers were designed to span *Rv1979c-Rv1982* (plasmid designated p7982) and *Rv1985c-Rv1986* (plasmid designated p8586) from H37Rv genomic DNA (Figure 1), with the empty vector used as a complementation control, as has been previously described [114]. Primers used in this study are listed in

Table 1. Plasmids were electroporated into *M. tuberculosis* H37Rv:ΔRD2, kanamycin resistant clones were selected and the presence of the plasmid assessed by PCR as has been previously described [273].

3.3 Verification of complementation

For H37Rv:ΔRD2::p7982, we verified expression of the genes by testing production and secretion of MPT64, coded for by *Rv1980* (Supplemental Figure. 1). Immunoblotting of the culture filtrate was performed as described by Charlet *et al.* [114]. The procedure was carried out with the following modification: after bacteria were grown in containment in Middlebrook 7H9 media, the supernatant was separated by centrifugation and filter-sterilized using a 0.22μm filter (Millipore) prior to separation under reducing conditions on a 12% SDS-PAGE gel. Gel transfer and blotting were then performed following established protocols.

For H37Rv:ΔRD2::p8586, we determined expression of both *Rv1985c* and *Rv1986* in the wild-type and complemented strain using quantitative reverse transcription-PCR (qRT-PCR), following procedures described elsewhere [273]. Briefly, RNA was extracted from cultures in log-phase growth (optical density 600nm of 0.4-0.6) using a modified phenol-chloroform extraction method, which has been described previously [114] and levels of *Rv1985c* and *Rv1986* were normalized to the amount of *sigA* RNA (Supplemental Figure 1). Primers used to in the qRT-PCR experiments are listed in Table 1.

3.4 In vitro studies of mutants

To investigate the presence or absence of phthiocerol dimycocerosate (PDIM) of the H37Rv parental strain, and the RD2-deletion mutant, bacteria were grown in Middlebrook 7H9 media, and radiolabelled with [1-^{14}C] propionic acid or [1-^{14}C] acetic acid in the manner described by Reed and colleagues [20]. Once bacteria had reached log-phase growth, whole lipids were extracted and separated by thin-layer chromatography (TLC) as previously described [20]. To examine if the deletion of RD2 lead to the dysregulation of other genes outside this region, bacterial RNA from culture at log-phase growth was analyzed by microarray. RNA was isolated and analyzed using established lab protocols [108].

3.5 Macrophage preparation

Macrophages were prepared in a manner similar to that described by Divangahi *et al*. [89]. RAW 264.7 cells were pelleted and resuspended in RPMI 1640 media plus 10% fetal bovine serum (vol/vol) (Wisent), HEPES (Wisent) and 100 U/ml penicillin, 100μg/ml streptomycin (Wisent). Macrophages were added into 6 or 12-well culture plates and allowed to adhere overnight at 37°C with 5% CO_2.

3.6 Macrophage Infection

Bacteria were grown to mid-log phase before being pelleted and resuspended in RPMI 1640 media plus 10% fetal bovine serum and HEPES. Macrophages ($1x10^6$ cells per well) were infected with different bacterial strains at a multiplicity of infection of 5 (MOI=5) for 4 h. Following this, cells were washed three times with warm RPMI, and

fresh media was added. Cells were incubated at 37°C with 5% CO_2. At desired time points, triplicate wells for each bacterial strain were examined. Supernatant was removed from the wells and added to a conical tube containing PBS+1% triton X-100 (Sigma) in order to lyse dead cells containing mycobacteria. Following this, the remaining macrophages were lysed with three washes of PBS+1% triton X-100 then pelleted and resuspended in Middlebrook 7H9 media. Bacterial numbers were determined through plating on Middlebrook 7H10 with PANTA and colony counts determined after 3 weeks.

3.7 Cytokines

RAW 264.7 macrophages ($2x10^5$ cells per well) were infected with the strains at a multiplicity of infection of 1 and 5 (MOI 1:1 and 5:1) as previously described [89]. The macrophages were incubated in the presence of the bacteria for 24 h at 37°C in 5% CO_2. After 24 h the culture supernatant from three wells was pooled and collected, then centrifuged in 0.22μm durapore tubes (Millipore) in order to ensure removal of bacteria and cells, and then assayed using enzyme-linked immunosorbent assays for TNF-α (ELISA; R&D Systems). For each strain, OD readings were taken from three wells and averaged.

3.8 Apoptosis

In vitro apoptosis was measured using enzyme-linked immunosorbent assay (Cell Death Detection ELISA^{Plus}, Roche Applied Science). Cytoplasmic histone-associated-DNA-fragments were quantified following the instructions provided by the manufacturer. Relative apoptosis was calculated as a ratio of the absorbance of infected macrophages to the absorbance of uninfected macrophages.

3.9 Animal Studies

Mice were aerosolized with ~150 colony forming units (CFU) of the various bacterial strains mentioned above in accordance with methods previously described [20]. Prior to infection, bacterial cultures were adjusted to mid-log phase growth and stored in 20% glycerol at -80°C. For aerosol infection these stocks were diluted 1:100 in PBS/Tween-20 (0.05%). Eight-week-old C57BL/6 mice (Harlan Laboratories) were aerosolized for a total of 10 min using a Lovelace Nebulizer, model 01-100 (In Tox Products). This method resulted in the implantation of approximately 150-200 CFU per lung, confirmed at 24 hr post-infection by homogenizing lungs from three mice per group in 7H9/ADC and determining bacterial counts. Mice were sacrificed at 1, 2, 3 and 6 weeks after infection, at which point lungs and spleens were extracted, homogenized, serially diluted and plated to evaluate CFU present in each organ. Mice aerosol infected with complemented strains were sacrificed at 3 weeks. For the time-to-death study, 7-8 week-old $Rag^{1tm1Mom\ Tg(TIE2GFP)287Sato/J}$ mice (Jackson Laboratory) were infected via aerosolization and bacterial burdens at 24 hr post-infection were determined as described above. Mice were weighed weekly and sacrificed if a veterinarian blinded to their infecting strain judged that they manifest the following symptoms: lethargy, trembling or weight loss more than 15% of their body weight. Lungs and spleens were harvested prior to fixation in 10% neutral buffered formalin for histopathological analysis.

3.10 Histomorphology

Immediately following collection, lung and spleen tissue was preserved in 10% neutral buffered formalin and processed (RIC, Plateforme d'Histologie, Universite de

Montreal). To evaluate tissue morphology, sections were cut 4-5μm thick from paraffin-embedded sections and underwent routine hematoxylin and eosin staining. Representative slides were evaluated for pathology and granuloma size by a reader blinded to the bacterial strain and rated semi-quantitatively from most severe to least severe pathology.

3.11 Figures and Statistics

Differences in Gaussian data collected were tested for significance using the student's t-test, with a p-value ≤ 0.05 considered statistically significant. Differences in semi-quantitative data (histopathology) were tested for significance using the rank-sum test. Survival data were assessed using the log-rank test. Figures were generated using GraphPad Prism 4.0b.

4.Results

4.1 *In vitro* assessment

In vitro growth kinetics were similar for H37Rv and H37Rv:ΔRD2 (Figure 2A) indicating that the loss of RD2 did not lead to an evident growth defect in the organism, consistent with the original RD2 deletion having occurred *in vitro*. Complemented strains also showed similar growth patterns (data not shown). TLC analysis indicated that the H37Rv parent strain and the RD2 deletion mutant were both lacking PDIM (Figure 2B). As both H37Rv and the RD2 deletion mutant were negative for PDIM, we concluded that any subsequent differences in virulence were not associated with loss of this lipid. Microarray analysis, to look for the potential effect of RD2 disruption on expression of genes outside of RD2, did not uncover any genes reproducibly dysregulated outside of RD2 (data not shown).

4.2 Analysis of bacteria burden and histopathology in murine infections

In a first experiment, we tested for whether RD2 disruption had a measurable effect on *in vivo* virulence in the murine model. The murine aerosol infection model allows for the evaluation of lung bacterial burden, an indicator of TB pathogenesis and splenic burden, an indicator of bacterial dissemination from the site of infection [135]. After aerosol infection of C57BL/6 mice with H37Rv and H37Rv:ΔRD2, we observed steady bacterial growth in the H37Rv-infected mice for the first 3 weeks followed by a plateau, consistent with the findings reported by other labs [135]. By comparison, H37Rv:ΔRD2 showed a 0.75 log reduction in bacterial levels in the lungs, and a 0.6 log reduction in the spleen throughout week 2 and 3. Notably, by week 6, H37Rv:ΔRD2 had

achieved a bacterial burden comparable to H37Rv (Figure 3A and 3B), as has been observed previously in the case of H37Rv:ΔRD1[135].

4.3 Complementation experiments

To complement H37Rv:ΔRD2, we considered the 11 genes within this region as candidates and selected those most likely to have a role in virulence based largely on considerations from published literature [116, 141, 156, 163]. We excluded *Rv1988* from consideration as this gene has been shown to play a role in resistance to macrolides [191]. Tsolaki and colleagues have demonstrated that *Rv1983-84c* were deleted in a clinical strain of *M. tuberculosis*, demonstrating that these genes are dispensable for full virulence to humans [136]. While *Rv1978* and *Rv1987* were considered as candidates, we decided not to pursue the study of these genes as their predicted functions (hypothetical unknown and hypothetical chitinase) did not suggest an obvious role in virulence, and because their topology in the genome suggested the need for complementation of individual genes. From this, we were left with two candidate intervals: *Rv1979c-82*, which includes the gene coding for the antigen MPT64, and *Rv1985c-86*, a putative LysR-like transcriptional regulator and adjacent LysE-like transporter. Since the most marked difference in bacterial burden was observed 3 weeks post-infection, we selected this time-point to test our complemented strains. As shown in Figures 4A and 4B, lung bacterial burden was partially restored in mice infected with either with H37Rv:ΔRD2::p7982, or with H37Rv:ΔRD2::p8586, however partial restoration of splenic bacterial numbers was only seen with H37Rv:ΔRD2::p7982. As we had also noted a difference in histopathology between H37Rv and H37Rv:ΔRD2, we examined the effect these differently

complemented strains had on our pathology phenotype. As shown in Figure 4C, mice infected with H37Rv:ΔRD2::p7982 exhibited a pathological profile that was indistinguishable from that seen in H37Rv-infected mice. Conversely, mice infected with H37Rv:ΔRD2::p8586 showed reduced pathology, as seen with H37Rv:ΔRD2 (data not shown). These findings, combined with the observation of splenic CFU suggested that *Rv1985/1986c* do not contribute to full virulence early after infection, perhaps because these genes have a role in the more chronic phase of infection, as has been suggested by study of the enduring hypoxic response [143]. This strain was subsequently set aside for future investigations. As our results indicated that complementation with *Rv1979c-Rv1982* restored most of the attenuation phenotype, subsequent experiments were carried out using this strain alone.

4.4 Bacterial growth in macrophages

As the phenotype was seen early after infection, when adaptive immune responses have had little opportunity to modulate the course of infection, we tested whether presence of RD2 affects interaction between *M. tuberculosis* and its host cell. To investigate this, we infected RAW 264.7 cells at a multiplicity of infection (MOI) of 5:1 and evaluated bacterial growth over a seven day time period. As a control for severe attenuation, we infected macrophages in parallel with BCG Russia, based on published observations on this strain in macrophages [135]. Compared to BCG Russia, a strain of BCG in which the RD2 region is intact, each strain of *M. tuberculosis* grew better, with no distinguishable differences during the first days of infection. However, by day seven,

there was a modest relative decrease in H37Rv:ΔRD2 compared to H37Rv, whereas H37Rv:ΔRD2::p7982 bacterial numbers were similar to H37Rv (Figure 5).

4.5 Cytokine expression in RAW macrophages

Our data suggested that RD2 may modulate the innate immune response to *M. tuberculosis.* Early control of mycobacterial infection is achieved through the innate immune response, whereby reduced or absent levels of TNF-α or other cytokines have been associated with increased mycobacterial virulence [45, 62, 274, 275]. To evaluate cytokine production, a mouse macrophage cell line, RAW 264.7, was used as host cells. These macrophages were infected at an MOI =1 and cell supernatant was harvested after 24 h. H37Rv produced significantly lower levels of TNF-α compared to H37Rv:ΔRD2. However, in macrophages infected with H37Rv:ΔRD2::p7982, TNF-α levels were reduced to a level comparable to the wild-type strain H37Rv (Figure 6A). These results demonstrate that the loss of RD2 impairs the ability of the bacterium to modulate host cytokines important for innate defense against mycobacteria.

4.6 Apoptosis

Previously, Mustafa and colleagues described an inverse association between MPT64 expression and apoptosis in biopsy samples from mycobacterial lymphadenitis [60]. Our finding that H37Rv:ΔRD2 manifest decreased survival in macrophages combined with the elevated levels of TNF-α led us to suspect that RD2 may decrease host-cell apoptosis, in which case, presence of this element might be associated with a deviation away from an apoptotic host cell towards necrosis [48]. To investigate

differences in apoptosis, we measured cytoplasmic histone-associated-DNA-fragments by ELISA. As a control for apoptosis, BCG Russia was used to infect macrophages, as it is known to induce higher levels of apoptosis than H37Rv [46]. At 48 h post-infection, levels of apoptosis were comparable between cells infected with the different strains (data not shown). However, at 4 days post-infection the levels of relative apoptosis were higher in cells infected with H37Rv:ΔRD2 (Figure 6B). Cells infected with H37Rv:ΔRD2::p7982 showed levels of apoptosis that were lower than H37Rv:ΔRD2, however this failed to achieve statistical significance. The lower bacterial burden seen at the subsequent time-point was likely due to higher levels of apoptosis, and the loss of RD2 reduced the ability of the bacteria to prevent programmed cell-death.

4.7 Survival

As another experiment to test whether deletion of RD2 affects the innate immune response to *M. tuberculosis*, we aerosol infected H37Rv, H37Rv:ΔRD2 and H37Rv:ΔRD2::p7982 into *Rag*^{1tm1Mom} Tg(TIE2GFP)287Sato/J mice that lack an adaptive immune response. As shown in Figure 7, infection with H37Rv resulted in a median survival of 79 days and no H37Rv-infected animals survived to the termination of this experiment after 28 weeks. In contrast, only 37.5% of mice infected with H37Rv:ΔRD2 had succumbed to infection by week 28, and therefore no median survival time could be assigned. The H37Rv:ΔRD2::p7982 presented an intermediate phenotype, with a median survival time of 125 days.

5. Discussion

The genomic differences between BCG strains are well established [110, 115]. This has led to the hypothesis that the ongoing evolution of BCG has resulted in the loss of regions involved in virulence, and has caused the vaccine to become over-attenuated [108]. Despite this plethora of genomic data, the phenotypic consequence of mutations in BCG strains has not been convincingly demonstrated, beyond the original loss of the RD1 region [133-135]. Intriguingly, Raghavan and colleagues demonstrated that transcription of genes within the RD2 region is influenced by the RD1-associated secreted virulence factor EspR, presenting a conceptual link between the deletion of these two elements from BCG [139]. Our data demonstrate that RD2 plays a role in mycobacterial virulence, and that its deletion from *M. tuberculosis* leads to a decrease in bacterial growth in both a macrophage and a murine model. Furthermore, infection of macrophages resulted in increased levels of TNF-α and increased apoptosis with RD2 disruption, suggesting that a portion of RD2 containing the gene coding for MPT64 has an anti-apoptotic effect on the host cell.

These findings are consistent with, and expand upon, previous investigations that have explored the role of certain portions of the RD2 region. Mustafa and colleagues demonstrate that the antigen MPT64, (encoded for within RD2), may decrease TNF-α production in the cells within a granuloma [60]. MPT64 is only secreted by actively dividing cells, and it is hypothesized to be involved in protein-protein interaction [156]. These facts make it tempting to speculate that MPT64, perhaps in concert with other RD2 proteins, may interact with the host cell to modulate TNF-α levels and/or apoptosis in an as yet uncharacterized way. If MPT64 is involved in suppressing TNF-α production

within the granuloma, and given that TNF-α is crucial for granuloma formation [276, 277], this would fit well with our observation of decreased pathology and reduced bacterial burden in mice infected with H37Rv:ΔRD2.

The attenuation seen in the RD2 knock-out strains could be due not only to an inability of the bacterium to suppress host responses, but also due to difficulties in growth within the hostile environment of the macrophage. *Rv1982c* is predicted to encode a toxin-antitoxin of the VapBC family; this family of proteins has been shown to inhibit translation through ribonuclease activity [154]. It has been proposed that toxin-antitoxin systems function in *M. tuberculosis* to slow growth during unfavourable conditions [155]. Thus it is tempting to speculate that the loss of *Rv1982c* may prevent H37Rv:ΔRD2 from arresting growth within the phagosome that might otherwise promote its survival.

Since tuberculosis is a disease of multiple stages (i.e. initial infection, early logarithmic bacterial growth, establishment of persistent infection, eventual induction of active disease, transmission to a new host), it is likely that different genes of the organism have their greatest importance at different phases of the disease. Therefore, it follows that complete virulence requires a collective effect of many genes, and that the attenuation seen when 11 genes are deleted is unlikely to be reversed fully with the introduction of a few. Consistent with this idea, neither of our complemented strains fully restored all aspects of virulence. It has been shown previously that *Rv1985c* is upregulated as part of the response to the hypoxic conditions meant to mimic latency [143]. It is possible that these genes are involved in virulence at a later time-point, or in some manner not revealed by our experiments. Additionally, other genes within the region may also be involved in virulence, and warrant further study. For example, the second antigen within

this region, CFP21, has been shown to be strongly immunogenic in mice, and may well play a role in infection [278].

Finally, *Rv1979c* is predicted to be an amino acid transporter, although the substrate is unknown (http://genolist.pasteur.fr/TubercuList/). Chen *et al.* (2002) demonstrated that the *Rv1979c*-containing BCG Japan was able to grow in media where arginine, histidine, lysine or proline, were the sole nitrogen sources, while BCG Pasteur was unable to grow under the same conditions [112]. *Rv1979c* may therefore be a transporter for one or more of these amino acids, and the loss of this gene may restrict growth in the macrophage.

Our results suggest that multiple genes within the RD2 region contribute to virulence, and are consistent with the view that multiple genomic deletions contributed to the safety profile associated with contemporary BCG vaccines. Ongoing work is now assessing whether the addition of these genes into a natural RD2 mutant, BCG Pasteur, increase in the immunogenicity and protective efficacy of the vaccine.

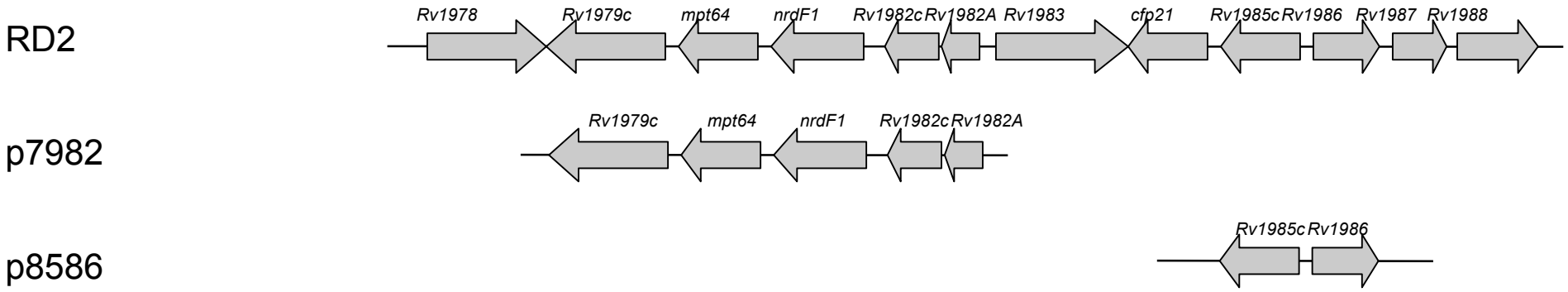
6. Acknowlegdements

The authors thank the members of the Behr, Reed and Schurr laboratories for their input. The authors thank Pilar Domenech and Vlad Kamenski for their invaluable assistance during animal experiments. R. K. was supported through a MUHC studentship. M.B. is a Chercheur National of the FRSQ and a William Dawson Scholar of McGill University. Work was funded by an operating grant from the Canadian Institutes for Health Research, MOP-79309.

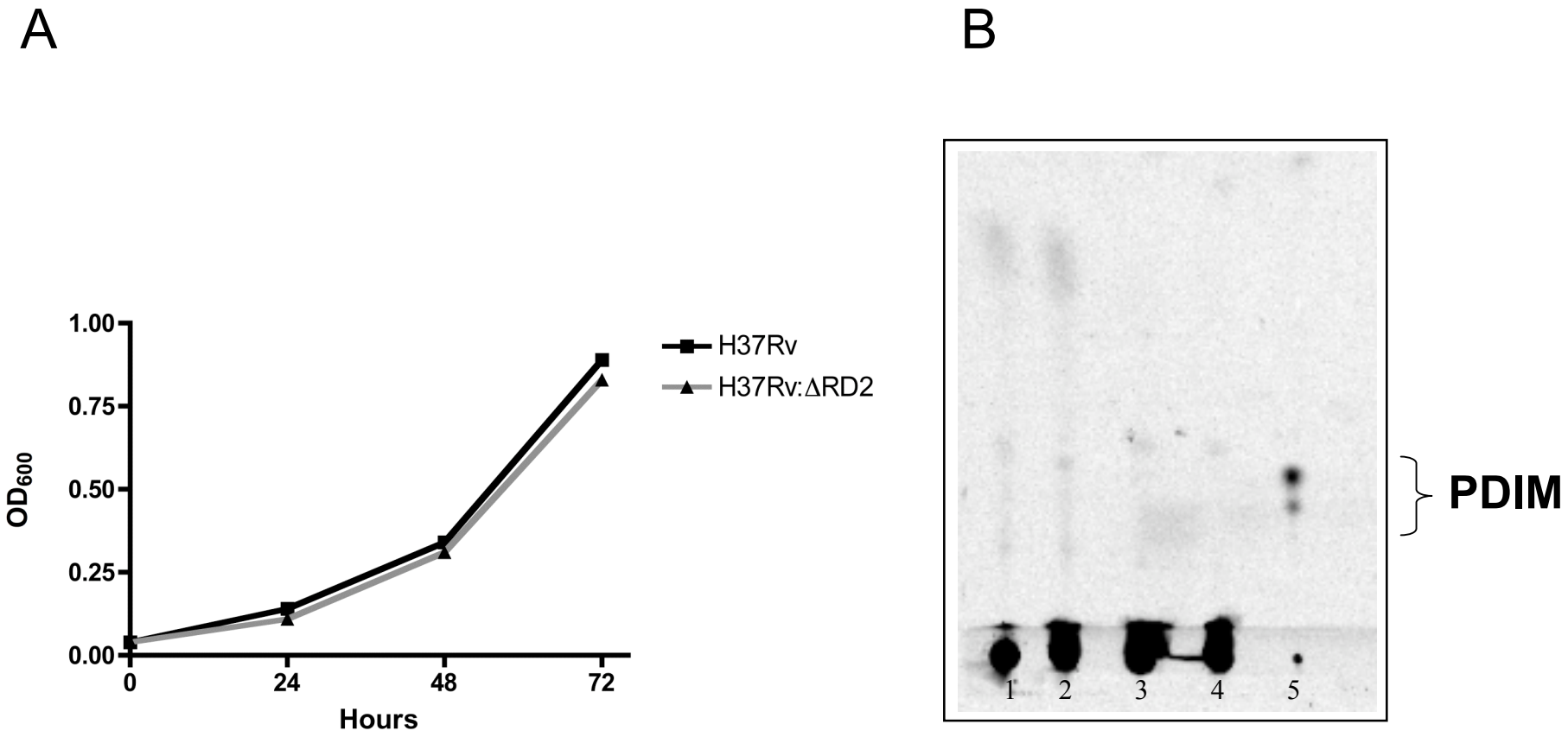
7. Figures and tables

Table 1: Oligonucleotide primers used in study

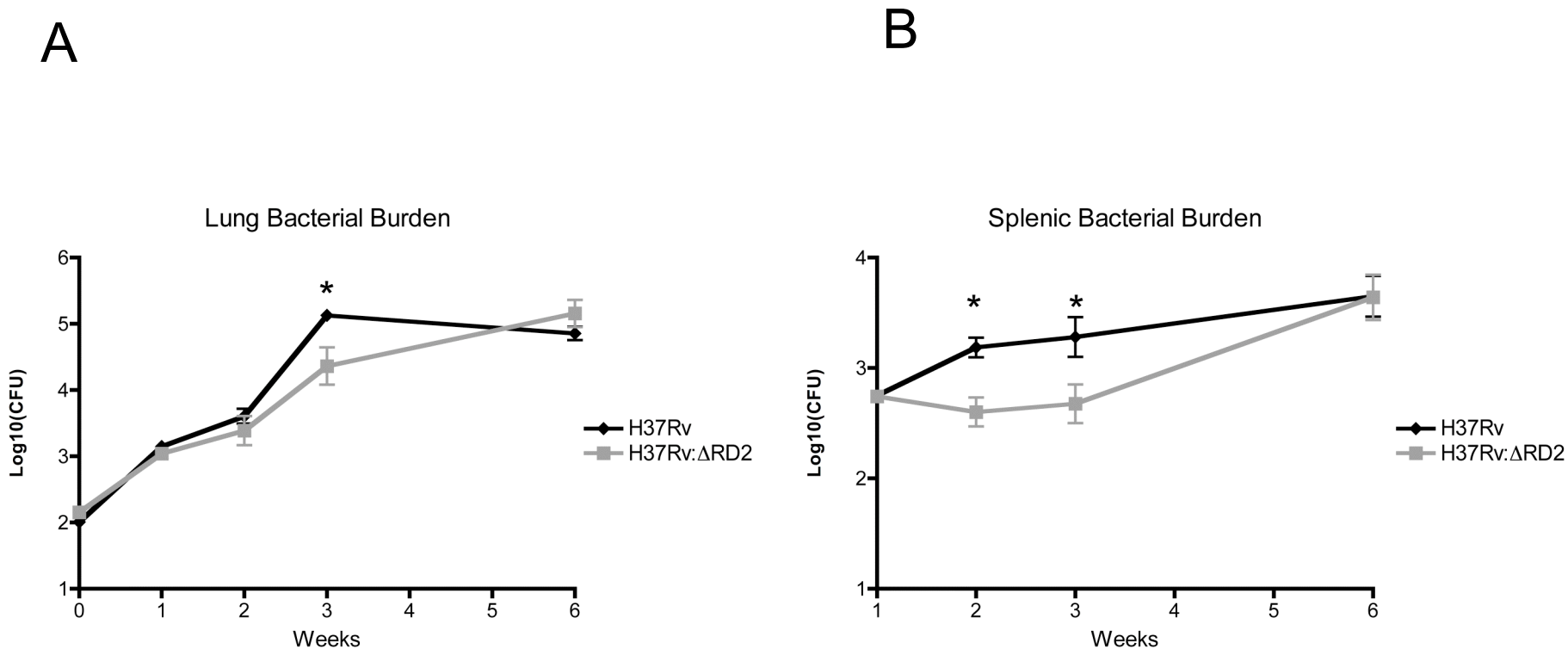
Primer	Sequence
SigAF	tgcagtcggtgctggaca
SigAR	cgcgcaggacctgtgagc
Rv1985cF	gaaatgcgctacctaccagtg
Rv1985cR	gtgaacccgtcggatagatg
Rv1986F	accgtcgtgttgctaggc
Rv1986R	ataccgcactggctgtgac
7982F	gtaagcttcactacgtgcctacagtccc
7982R	cattctagagctgaccgattgatattc
KanF	aacaagccagggatgtaacg
KanR	ccataaaaccgcccagtcta
rpl 21	ggctatgccaaggtgatagc
rpl 22	ccgatgatcttctgtttgac
rpl 23	ggtggtcgccgtggagttgc
rpl 24	agttcggcgagacgcagcag



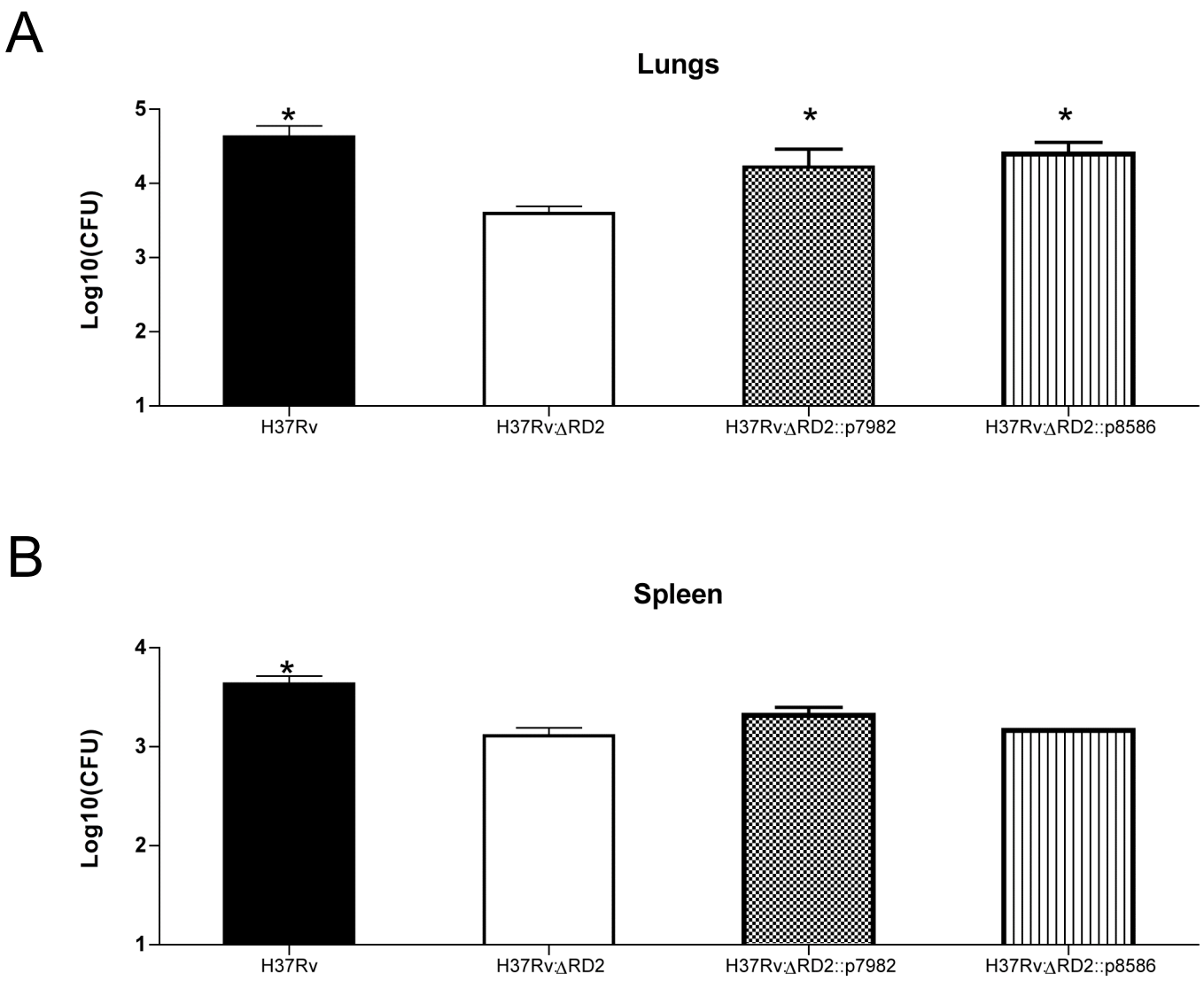
Chapter 2 - Figure 1. RD2 Region and Constructs for Complementation.
RD2 region encompasses genes *Rv1978-Rv1988*. Amplicons of *Rv1979c-Rv19*82 and *Rv1985c-Rv1986* were cloned into the integrative plasmid pMV306 for complementation, and were designated p7982 and p8586 respectively.



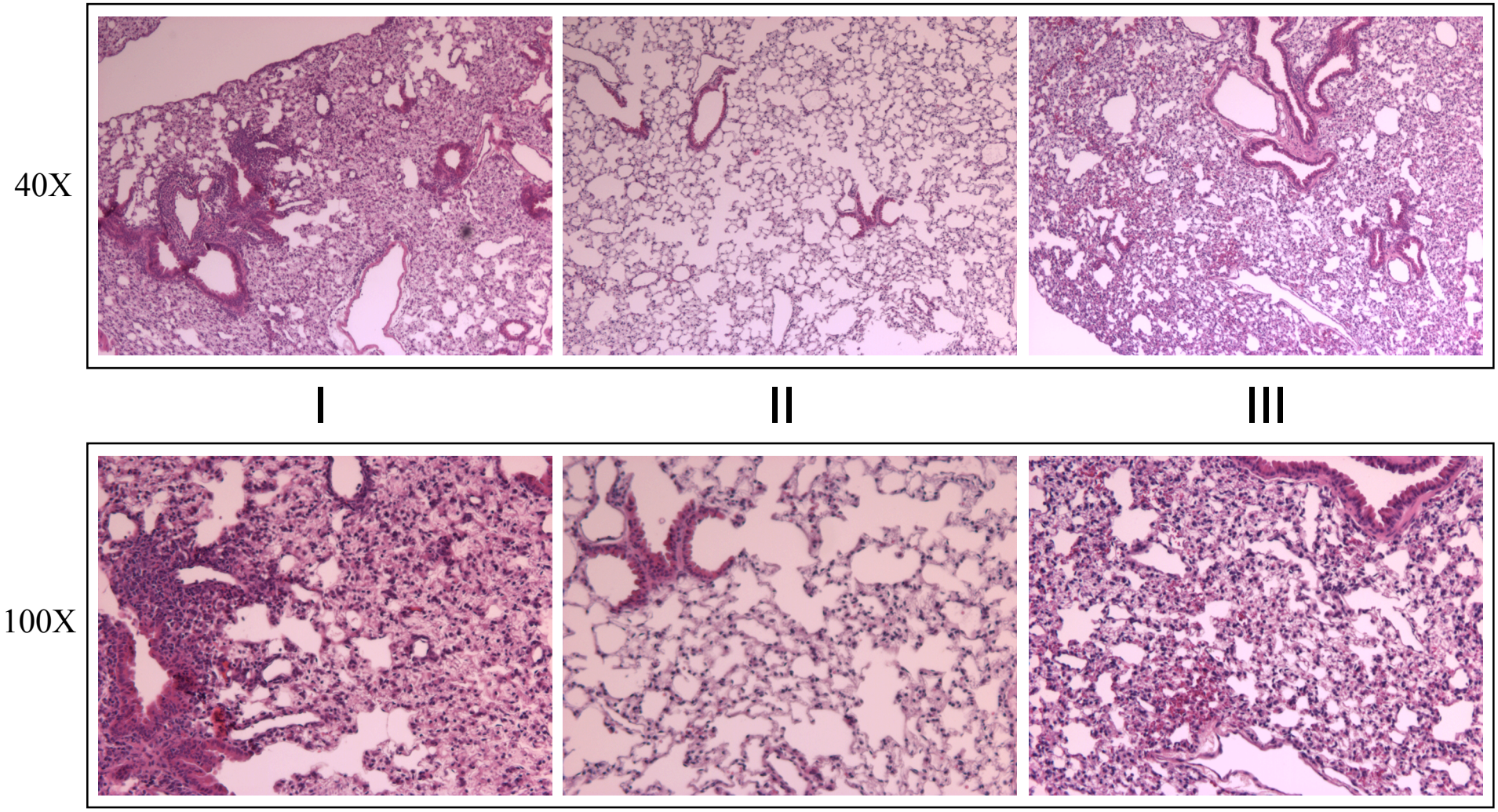
Chapter 2 - Figure 2. *In vitro* characterization of the deletion mutant.
In vitro growth kinetics of the H37Rv and H37Rv:ΔRD2 as determined by OD_{600} readings (A). Experiment was repeated in triplicate, and data is from one representative experiment. Thin-layer chromatography plate of apolar lipids from H37Rv and H37Rv:ΔRD2. Lanes 1 and 3 represent lipids from the H37Rv strains used in this experiment, labeled with [1-^{14}C] proprionic acid and [1-^{14}C] acetic acid respectively. Lanes 2 and 4 represent lipids from H37RvΔRD2, labeled with [1-^{14}C] proprionic acid and [1-^{14}C] acetic acid respectively. Lane 5 is the apolar lipids labeled with [1-^{14}C] proprionic acid from a strain of H37Rv known to produce PDIM (B).



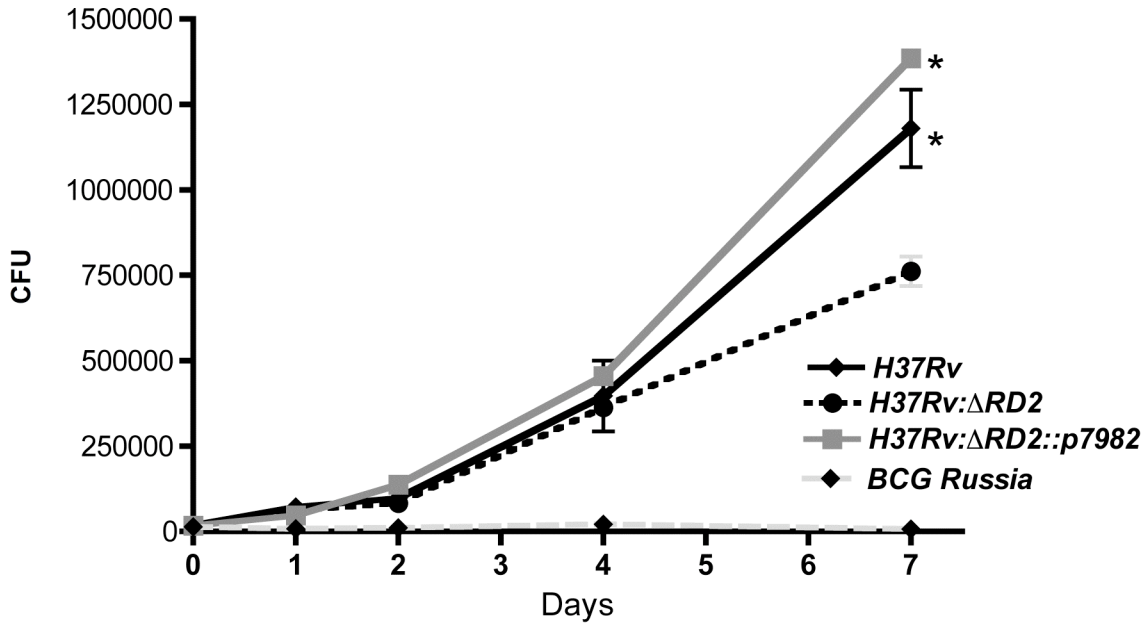
Chapter 2 - Figure 3. *In vivo* assessment of virulence.
Bacterial growth in the lungs (A) and spleens (B) of C57BL/6 mice. 6-8 week old mice were used, and were infected via aerosolization with about 150 CFU of either H37Rv (black line) or H37Rv:ΔRD2 (grey line). Data at each time point is the mean ± SEM of 10 mice per strain from 2 separate infections, where * indicates $p < 0.05$.



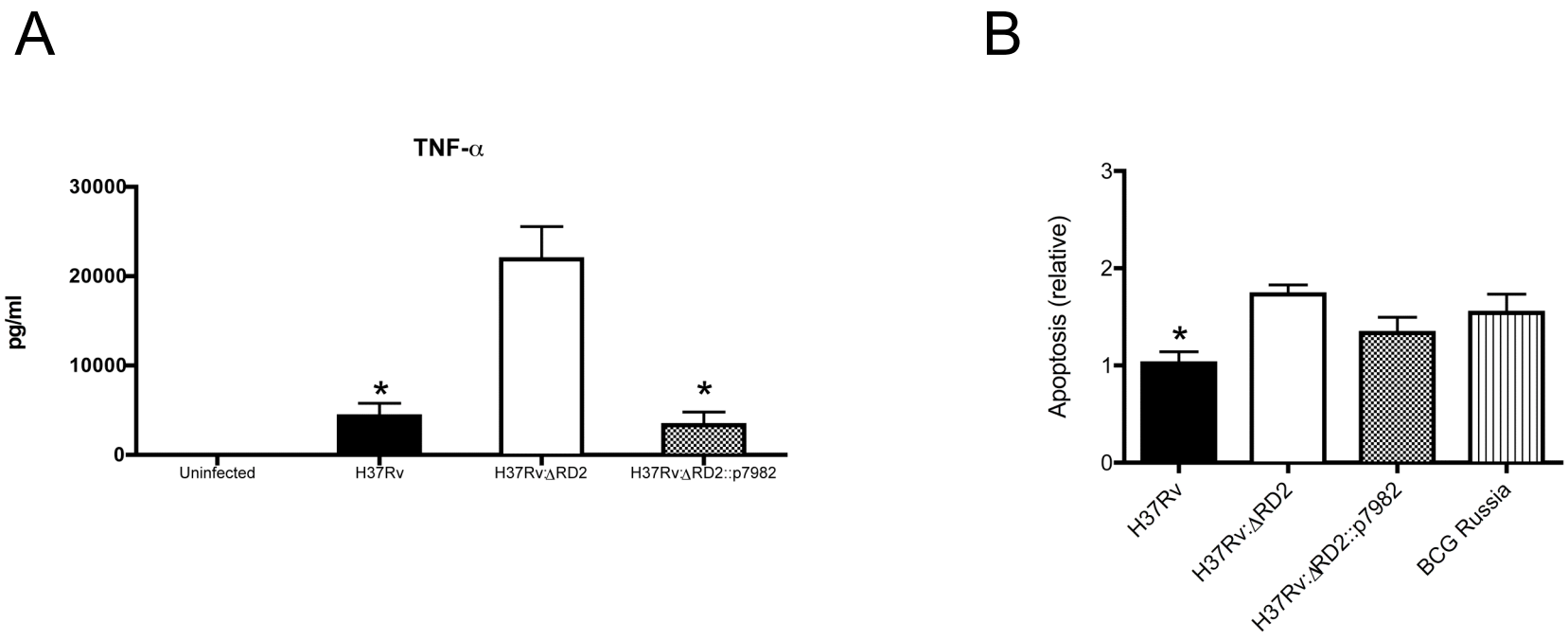
Chapter 2 - Figure 4. Complementation of virulence phenotype.
Bacterial burden in the lungs (A) and spleen (B) at 3 weeks post-infection. 6-8 week old C57BL/6 mice were aerosol infected with H37Rv (black bar), H37Rv:ΔRD2 (white bar), H37Rv:ΔRD2::p7982 (grey bar) or H37Rv:ΔRD2::p8586 (striped bar). Data represents the mean ± SEM of 6 mice, where * indicates $p < 0.05$.



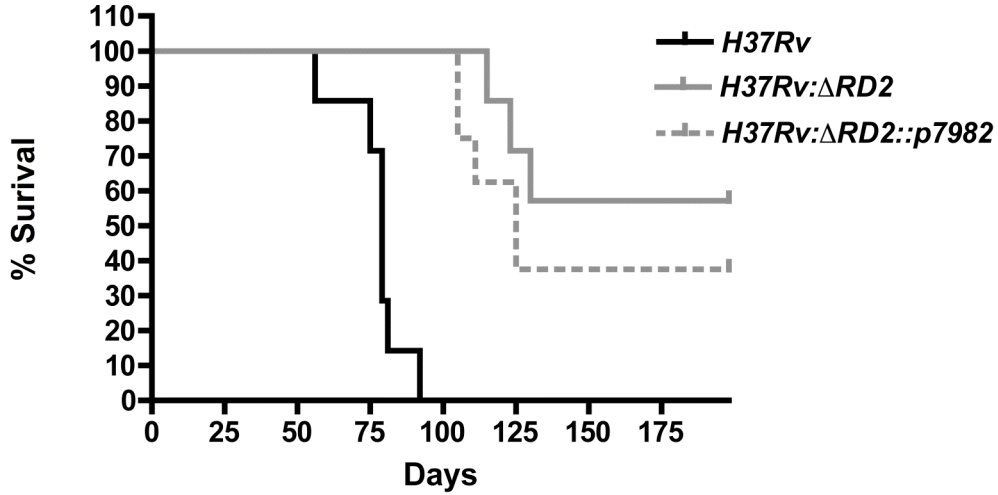
Chapter 2 - Figure 4. Complementation of virulence phenotype.
Histology of lung (C) lesions from infected mice with H37Rv (i), H37Rv:ΔRD2 (ii), or H37Rv:ΔRD2::p7982 (iii) at 3 weeks post infection. 5 mice per group were used and lungs were stained with H & E. Micrographs (magnification, 40X and 100X) shown are representative lung sections from each group.



Chapter 2 - Figure 5. Characterization of bacterial mutants during macrophage infection.
Bacterial growth in murine macrophages over a seven-day period. RAW 264.7 macrophages were infected with H37Rv (black line), H37Rv:ΔRD2 (dashed black line), H37Rv:ΔRD2::p7982 (solid grey line), or BCG Russia (dashed grey line). Data from each time is taken from the mean of wells infected in triplicate, and the experiment was performed twice. Error bars represent ± SEM, where * indicates $p < 0.05$.

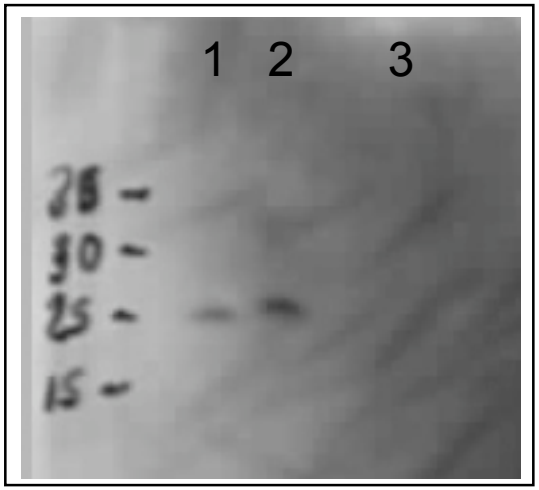


Chapter 2 - Figure 6. Effect of RD2 deletion on interaction with macrophages.
RAW 264.7 macrophages were infected with H37Rv, H37Rv:ΔRD2 or H37Rv:ΔRD2::p7982 at an MOI=1 and supernatant was harvested 24h post-infection for TNF-α determination by ELISA (A). Data represent the mean ± SEM. Relative apoptosis levels in RAW 264.7 macrophages at 96h post-infection (B). Macrophages were infected and an MOI = 5 for 4 h with H37Rv (black bar), H37Rv:ΔRD2::p7982 (grey bar) H37Rv:ΔRD2 (white bar), or BCG Russia (striped bar). Wells were infected in triplicate and apoptosis was evaluated by ELISA kit. Error bars represent mean ± SEM, where * indicates $p \leq 0.05$.

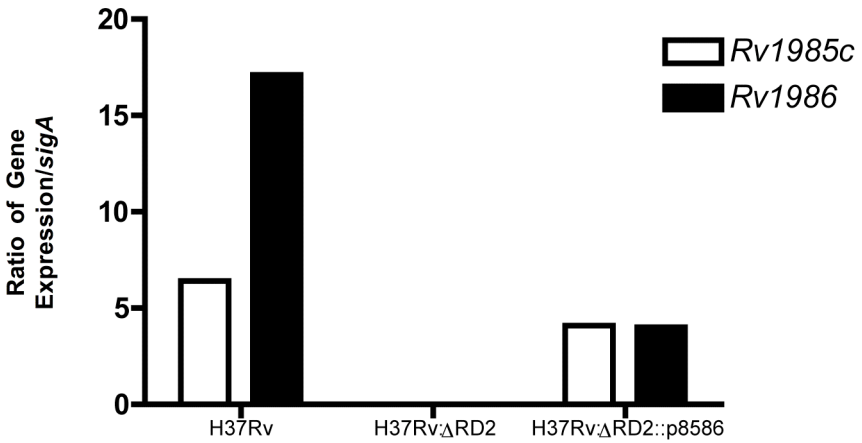


Chapter 2 - Figure 7. Survival Experiment.
Survival in C57BL/6 mice infected with different *M. tuberculosis* strains. Mice were aerosol-infected with H37Rv (black line), H37Rv:ΔRD2 (grey line) or H37Rv:ΔRD2::p7982 (dashed line). Approximately 200 CFU were implanted per lung. H37Rv had a median survival of 79 days, H37Rv:ΔRD2::p7982 had a median survival of 125 days, and H37Rv:ΔRD2 failed to achieve a median survival time point. 8 mice per group were used. This is a representative figure of two independent experiments.

A



B



Chapter 2 - Supplemental Figure 1. *In vitro* assessment of complementation.
SDS-PAGE and immunoblotting with anti-MPT64 antibody of culture filtrate from the strains used in this study. Strains used were as follows: 1 = H37Rv, 2 = H37RvΔRD2::*Rv1979c-82*, 3 = H37RvΔRD2. Band at approximately 24 kDa represents secreted MPT64 (A). Expression of *Rv1985c* (white) and *Rv1986* (black) in *M. tuberculosis* strains. All values were normalized to levels of *sigA* (B).

Preface to Chapter 3

As was reviewed in Chapter 1, complementation of genes lost during vaccine propagation would be predicted to enhance the efficacy of BCG. In Chapter 2 the role for RD2 in the full virulence was established, and candidate genes for suggested for addition to BCG. The following chapter examines the role of RD2 in vaccine immunogenicity and protective efficacy.

Chapter 3: Divergence of Immunologic and Protective Responses of Different BCG strains in a Murine Model

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1. Abstract

The ongoing evolution of BCG after its derivation between 1908-1921 resulted in strains that differ genetically and phenotypically. The second deletion event following the original attenuating mutation (loss of Region of Difference 1) was loss of Region of Difference 2 (RD2). While other groups have compared various BCG strains, the role of RD2 in vaccine protection remains unknown. In this study, the RD2-containing BCG Russia, BCG Pasteur (which is a natural mutant for RD2), and BCG Pasteur complemented with RD2-genes *Rv1979c-Rv1982c* were compared through various *in vitro* and *in vivo* assays. We determined that the presence of RD2 did not affect vaccine persistence, but led to increased immunogenicity, as measured by ELISpot. Additionally, T-cells from animals immunized with BCG Russia and the complemented BCG Pasteur (BCG Pasteur::*Rv1979c-82*) were shown to be more effective at killing *M. tuberculosis* in macrophages than T-cells from animals immunized with BCG Pasteur. These findings suggested that BCG Russia and BCG Pasteur::*Rv1979c-82* would convey greater protection in a mouse vaccine-challenge model. Interestingly, all BCG strains conveyed similar lung protection, in terms of *M. tuberculosis* burden and histopathology at 4, 8 or 12 weeks post-infection. Our data demonstrate that the loss of RD2 did not reduce BCG protection in the lungs and revealed a dissociation between immunogenicity conferred by BCG strains and protection against disease.

2. Introduction

At present, tuberculosis remains one of the most serious bacterial infections, and a significant global health concern. The increase in drug-resistant strains, combined with the difficulties in diagnosing new and latent infections highlight the need for an effective vaccine. The current vaccine, *Mycobacterium bovis* BCG, has been administered to over 4 billion individuals, and appears to protect infants from disseminated tuberculosis (TB) [279]. Unfortunately, the efficacy of BCG against adult, pulmonary disease is uncertain, with estimates ranging from 0 to 80% across clinical trials [125].

The evolution of the BCG vaccines used provides one hypothesis for why such variation in protection may have been observed. Following the original attenuating mutation, BCG underwent over five decades of *in vitro* passaging, often under a variety of laboratory conditions, all over the world. This created an array of BCG strains that differed both genetically and phenotypically [109, 111-115]. The lack of standardization of BCG vaccines poses a practical challenge to programs that currently immunize children against TB. Moreover, because the loss of genomic material during decades of *in vitro* passaging rivals the amount of DNA deleted from strains of *M. tuberculosis* that have been circulated in humans for millennia, it has been proposed that BCG has become overly attenuated, to the point of impotence [108].

A critical divergence in the evolution of BCG was the loss of the 10.8 kb Region of Difference 2 (RD2). This lead to "early" (RD2 present) and "late" (RD2 absent) BCG strains [110]. Whether this division of BCG strains has affected the protective efficacy of BCG is unclear because: 1) no placebo-controlled trials of an early BCG vaccine were conducted, and 2) no randomized trials directly comparing an early BCG strain and a late

BCG strain have been performed. However, epidemiologic evidence from countries that have made changes to their vaccination programs suggests that RD2-containing vaccines may be more immunogenic than those that have lost this region. In Finland, it was noted that switching from a RD2-containing vaccine to one missing RD2 lead to a decrease in osteitis [144]. Similarly, in 1981 when Czechoslovakia changed their vaccine from BCG Prague (a natural RD2 mutant) to BCG Russia (which contains RD2) an increase in this complication occurred [145].

Due to the difficulties in performing a direct comparison of vaccine immunogenicty in human trials, numerous groups have made use of the animal model for the comparison of different BCG strains [126, 127, 280]. The limitations of these studies are twofold. First, differences between existing strains are numerous, making it difficult to attribute increased or decreased protection to a specific element, such as RD2. Second, in studies comparing RD2-containing strains to RD2-deleted strains, there is no complementation of the missing region, a necessary technique to determine the specific role of RD2 in vaccine protection. Previous work done by our lab has demonstrated that the loss of RD2 leads to a decrease in virulence in *M. tuberculosis* (Kozak et al. in submission). The specific role of RD2 in vaccine efficacy has not yet been determined, and is the subject of this report.

3. Methods

3.1 Bacteria

BCG strains Russia, BCG Pasteur::pMV306, BCG Pasteur::pMV306:*Rv1979c-Rv1982 (*BCG Pasteur::*Rv1979c-82*) and *M. tuberculosis* H37Rv were grown in 7H9 liquid medium (Becton Dickinson) supplemented with glycerol, 0.05% Tween 80 (Sigma-Aldrich) and 10% ADC (albumin, dextrose, catalase, Becton Dickinson) or 7H10 agar containing 10% OADC (oleic acid plus ADC, Becton Dickinson), 0.5% glycerol and PANTA (Becton Dickinson) at 37°C. 25μg/ml kanamycin (Sigma-Aldrich) was added when necessary.

3.2 Complementation of BCG Pasteur

The integrating plasmid pMV306-kan was used to complement BCG Pasteur (gift of P. Domenech). Primers were designed spanning the *Rv1979c-Rv1982* insert from H37Rv genomic DNA (TubercuList). Inserts were amplified with AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen). The PCR products were confirmed by sequencing at the McGill University and Genome Quebec Innovation Centre, Montreal Canada. Inserts were ligated into digested plasmids as has been previously described [114]. The primers used in this study are listed in Table 1.

The plasmids generated were then electroporated into BCG Pasteur [273]. The empty plasmid vector was used as a complementation control. Kanamycin resistant clones were selected and tested for by PCR as has been previously described [273].

3.3 Verification of complementation

For BCG Pasteur::*Rv1979c-82*, verification of gene expression was done by testing for production and secretion of MPT64, coded for by *Rv1980*. Immunoblotting of the culture filtrate was performed as described by Charlet *et al.* [114] with the following modifications: after bacterial growth in 7H9 media, the supernatant was separated by centrifugation and filter-sterilized using a 0.22μm filter (Millipore) prior to concentration and separation under reducing conditions on a 12% SDS-PAGE gel. Gel transfer and blotting were then performed following established protocols.

3.4 Peritoneal macrophages

Peritoneal macrophages were harvested from C57BL/6 by flushing the peritoneal cavity with RPMI 1640 media supplemented with 10% fetal bovine serum, 10 mM HEPES and penicillin/streptomycin (Wisent). Macrophages were resuspended in fresh media, and counted, then added to 6 well plates ($2x10^5$ cells/well) and allowed to adhere overnight at 37°C in 5% CO_2.

3.5 ELISpot

Splenocytes were harvested and enumerated from vaccinated mice at 28 days post-infection and cultured in RPMI 1640 media with 10% BSA and 0.02% HEPES. Splenocytes were treated with ACK lysis buffer to remove red blood cells, incubated in media overnight at 37°C to remove monocytes by adherence, then seeded into a 96-well plate pre-coated with IFN-γ and IL-17 capture antibodies. The ELISpot assay was performed in accordance with manufacturer's instructions (R&D Systems). Cells were

incubated for 24 and 48h with or without stimulation by *M. tuberculosis* culture filtrate (10μg/ml).

3.6 T-cell cytotoxcity assay

T-cell cytotoxicity was evaluated by co-culturing T-cells from vaccinated animals with *M. tuberculosis* infected macrophages. Four weeks after vaccination, spleens were harvested and digested in a manner similar to that described by Divangahi and colleagues [89]. Briefly, spleens were removed asceptically, passed through a 100μm filter (BD Biosciences) to generate single-cell suspensions, and incubated in ACK lysis buffer for 5 minutes to remove red blood cells. Splenocytes were resuspended in RPMI plus 10% FBS, penicillin, streptomycin and HEPES. Splenocytes were incubated overnight at 37°C in 5% CO_2 to remove macrophages by adherence. CD90.2-positive cells were purified by positive selection in a MACS column. Peritoneal macrophages were infected with *M. tuberculosis* at an MOI of 5 for 4 hours at 37°C in 5% CO_2. Cells were washed 3 times with media, and then fresh RPMI was added to each well. CD90.2-positive cells were added to the macrophages at a ratio of 5:1. After 96h supernatant was removed and added to 1% Triton X-100. Macrophages in wells were lysed with 3 washes of 1% Triton X-100. Bacterial CFU were determined by plating. Plates were counted after 3-4 weeks.

3.7 Mice

Six to eight week-old mice C57BL/6 mice were purchased from Jackson Laboratories and were maintained at the McGill University Health Centre. All mouse

experiments were conducted in accordance with the guidelines established by McGill University.

3.8 Vaccination and aerosol infection

BCG growth was determined by optical density (OD_{600}), followed by dilution in sterile PBS. Mice were vaccinated with approximately $6x10^5$ CFU (colony-forming units) subcutaneously. To evaluate dissemination of BCG, five mice were sacrificed at 2 and 4 weeks post-vaccination. Lungs, spleen and mediastinal lymph nodes were homogenized and plated to determine colony counts after 3 weeks. To assess vaccine protection, mice were challenged with a moderate dose (200 CFU) of *M. tuberculosis* strain H37Rv by aerosol using methods previously described [20]. Briefly, prior to infection, bacterial cultures were adjusted to mid-log phase growth and stored in 20% glycerol at -80°C. For infection these stocks were diluted in PBS/Tween-20 (0.05%). Mice were aerosolized for a total of 10 min, which resulted in the implantation of approximately 200-250 CFU per lung. The inoculum was empirically determined at 24h post-infection by homogenizing and plating lung tissue from three mice. Otherwise, mice were sacrificed at 4, 8 and 12 weeks after infection, at which point lungs and spleen were extracted, homogenized, serially diluted and plated to evaluate bacterial burden present in each organ.

3.9 Histopathology

Immediately following collection, lung and spleen tissue was preserved in 10% neutral buffered formalin and processed (RIC, Plateforme d'Histologie, Universite de

Montreal). To evaluate tissue morphology, 4-5μm thick sections were obtained from the paraffin-embedded tissues and underwent routine hematoxylin and eosin staining.

3.10 Figures and statistics

Figures were generated using GraphPad Prism 4.0b. Differences in Gaussian data were considered statistically significant based on a student's t-test p-value of < 0.05 when testing a single hypothesis. For complex experiments with multiple time-points and therefore multiple hypothesis-testing, we employed a more conservative threshold of $p \leq 0.01$ to judge a finding to be statistically significant. All significant experiments reported were independently replicated.

4. Results

4.1 Complementation of BCG Pasteur and *in vitro* growth of strains

To complement BCG Pasteur, we selected the genes *Rv1979c-Rv1982*. These genes compose a predicted operon that contains *Rv1980*, which encodes for the highly immunogenic protein MPT64, which possesses both B and T-cell epitopes [116, 157] (Table 2). Moreover, work done by our group has shown that the deletion of RD2 results in decreased virulence of *M. tuberculosis* and that this attenuation of virulence can in part be complemented by the introduction of the genes *Rv1979c-1982*, but not other genes of RD2 (Kozak *et al*., submitted). These facts made this a potential candidate for increasing the immunogenicity of the vaccine. Additionally, *Rv1979c* is a predicted amino acid transporter (http://genolist.pasteur.fr/TubercuList/), which suggested it might allow the bacterium to grow under limiting *in vivo* conditions.

BCG Russia, BCG Pasteur, and BCG Pasteur::*Rv1979c-82* were grown in 7H9 liquid media and growth kinetics were similar for all strains (Figure 1). The culture filtrate was removed from BCG Pasteur::*Rv1979c-82* for western blotting to verify expression of MPT64 (Figure 1), serving as an indicator that the *Rv1979c-Rv1982* operon was expressed in this strain.

4.2 *In vivo* persistence of BCG strains

The ability of the vaccine to persist within the host, and thereby generate an immunologic response, is important for vaccine efficacy. Additionally, more rapid dissemination to the lymph nodes has been shown to be important for the induction of the immune response to mycobacteria [85, 126, 281]. In order to evaluate persistence and

dissemination, mice were vaccinated sub-cutaneously with either BCG Russia, BCG Pasteur::*Rv1979c-82* or BCG Pasteur. At 15 and 28 days post-vaccination, bacterial burden was examined in the lungs, mediastinal lymph nodes, and spleens. At day 15 after immunization, mice that had received BCG Russia had higher bacterial numbers in the spleens ($p = 0.003$), but not the lungs or the lymph nodes, compared to mice vaccinated with BCG Pasteur (Figure 2). This effect was partially restored when BCG Pasteur::*Rv1979c-82* was used. At 28 days post-vaccination, there was no difference in bacterial numbers in the lung and lymph nodes; no bacteria were detected in the spleens at this time-point, for any of the strains (Figure 3). Comparing bacterial burdens at day 15 and day 28, we note that BCG numbers decreased in the lungs and spleen, but remained relatively stable over time in the lymph nodes. Together, these data demonstrated that the presence of RD2 increased dissemination of the vaccine from the site of vaccination, but did not affect persistence of the vaccine in the host at one month after immunization.

4.3 Loss of RD2 reduces immunogenicity

BCG vaccination results in the generation of adaptive immunity, particularly interferon-gamma (IFN-γ) lymphocytes, which are protective against *M. tuberculosis* infection [282, 283]. Therefore, we evaluated the ability of the different vaccines to elicit an adaptive immune response. Our data has shown similar levels of persistence for all three vaccine strains at day 28 after immunization. Therefore, we assessed adaptive immunity at that time point in order to test for the effect of bacterial differences. This was accomplished by enumerating interferon-gamma (IFN-γ) and IL-17 producing cells in splenocytes incubated with culture filtrate protein from *M. tuberculosis* (MTB CF). After

24h and 48h incubation, all vaccine strains elicited greater numbers of IFN-γ and IL-17 splenocytes than animals that had received PBS instead of a vaccine (Figure 4). Additionally, mice that had received either BCG Russia or BCG Pasteur::*Rv1979c-82* ($p \leq 0.01$ for both strains) had significantly higher numbers of these cells compared to BCG Pasteur at 24h. A similar effect was seen after 48h. These results suggest that the loss of RD2 decreases the immunogenicity of the BCG vaccine.

4.4 Growth inhibition assay

The increased immunogenicity shown by BCG Russia and BCG Pasteur::*Rv1979c-82* in our ELISpot data suggested that T-cells from vaccinated mice might manifest greater inhibition of intracellular bacterial growth, compared to those from BCG Pasteur. The generation of antigen specific T-cells by a vaccine is important for the control of *M. tuberculosis*, likely due to their ability to produce IFN-γ, and generate a Th1 response [284]. It has been shown, by Parra and colleagues, that the ability of splenocytes from vaccinated mice to inhibit bacterial intracellular growth is associated with vaccine protection [285]. To test the different BCG strains, peritoneal macrophages were infected with *M. tuberculosis* and washed after 4h. To these macrophages we added CD90.2-positive T-cells isolated from the spleens of mice that had been immunized with the different BCG strains 28 days before. After 96h, the addition of T-cells from PBS treated animals provided no measurable benefit in controlling intracellular bacterial growth, as compared to no cells being added. In contrast, T-cells from vaccinated animals contributed to control of *M. tuberculosis* growth, when compared with macrophages that received T-cells from PBS-vaccinated

mice (Figure 5). Among the vaccines given, T-cell mediated killing of infected macrophages was more pronounced when T-cells from BCG Russia or BCG Pasteur::*Rv1979c-82* ($p \leq 0.01$ for both) vaccinated mice were used when compared to T-cells BCG Pasteur.

4.5 Evaluating vaccine pulmonary protection in a murine model

The critical role of BCG is to provide protection against pulmonary TB, by reducing bacterial burden and decreasing pathology. Our *in vitro* and *ex vivo* data showed that the presence of RD2 resulted in a more immunogenic vaccine, thus we hypothesized that the presence of RD2 could be associated with a greater level of protection against *M. tuberculosis*. To investigate this, mice were immunized with one of the BCG strains or with PBS. Four weeks later, mice were infected with a low dose of *M. tuberculosis* H37Rv, and at 4, 8, and 12 weeks post-infection organs were harvested to determine bacterial burden. At all time points, vaccination with any of the BCG strains conveyed greater protection than mice that had received PBS (Figure 6). At the 4-week time point all BCG strains provided equal protection, each showing an approximate decrease of about one $\log_{10}$ ($p < 0.05$). At the 8-week time point there appeared to be a trend towards BCG Russia-vaccination resulting in a lower CFU ($p = 0.02$), but this apparent effect was transient, as no differences were observed 12 weeks after infection. Lung pathology was also examined at each time point. At 4, 8 and 12 weeks post-infection, vaccination with any strain reduced lung pathology compared to vaccination with PBS. Nonetheless, there were no discernable differences in the amount of pathology between the different vaccine strains (Supplemental Figure 1).

4.6 Protection against bacterial dissemination

In addition to providing pulmonary protection, BCG reduces bacterial dissemination from the lungs and may therefore have its greatest utility in preventing miliary and meningeal TB in infants. Therefore we decided to evaluate whether the presence of RD2 would affect protection against disseminated disease by measuring splenic CFU. At all time points, splenic bacterial numbers in vaccinated mice were lower than the PBS vaccinated group. Furthermore, all BCG strains showed the same level of protection at 4 weeks (Figure 6). However, by 8 weeks BCG Russia-vaccinated mice showed 0.37 $\log_{10}$ fewer bacteria in the spleens compared to BCG Pasteur ($p = 0.02$). While this failed to achieve our statistical cut-off value, it nonetheless demonstrated a trend towards RD2 protecting against dissemination. This same effect was seen in mice vaccinated with BCG Pasteur::*Rv1979c-82* ($p = 0.01$). This phenotype was also present at the 12-week time point, where BCG Russia ($p \leq 0.01$) and BCG Pasteur::*Rv1979c-82* ($p \leq 0.01$) vaccination again resulted in lower splenic bacterial burden compared to vaccination with BCG Pasteur. These data suggested that the loss of RD2 impairs the ability of the BCG vaccine to prevent dissemination.

5. Discussion

Vaccinologists observed that the BCG vaccine became further attenuated between the years of 1927 and 1931 [110, 286]. During this time it has also been documented that the vaccine lost the RD2 region [110]. These two observations have led to the hypothesis that early, RD2-containing BCG strains would provide better protection against infection with *M. tuberculosis* [120]. Our data reveals that the loss of RD2 in BCG leads to a less immunogenic vaccine, but this did not translate into an evident effect on protection against pulmonary disease, in a murine model.

Recent reports have demonstrated that early BCG strains are more immuno-stimulatory than late BCG strains in mouse and human cell lines [123]. One potential reason for the increased immunogenicity, which we also observed, may be the presence of MPT64. While the exact function of MPT64 is unknown, its role in stimulating the immune system is well documented. This protein is a B and T-cell antigen, and it also increases immunogenicity when added to BCG as part of a fusion protein with antigen-85B [116, 157, 159, 168]. As MPT64 is a potent inducer of delayed-type hypersensitivity reactions in guinea pigs and humans [287-289], the higher numbers of IFN-γ and IL-17 producing cells observed 4-weeks after vaccination may be due in part to priming by this antigen. Furthermore, the higher levels of IFN-γ and IL-17 generated by vaccination with BCG Russia and BCG Pasteur::*Rv1979c-82* may explain the increased intracellular bacterial killing in infected macrophages, given that IFN-γ enhances the anti-mycobacterial activity of macrophages and IL-17 can recruit both monocytes and T-cells [193, 290, 291]. Lagranderie demonstrated that BCG Russia vaccination produces higher levels of IL-2 in mice compared to vaccination with BCG Pasteur [126]. IL-2 is

important for T-cell activation, and it is likely that this cytokine is also playing a role in enhancing the anti-mycobacterial response in our *ex vivo* assay [292, 293].

Despite the increased immunogenicity, the results of our animal experiments demonstrated that vaccination with BCG Russia or BCG Pasteur::*Rv1979c-82* did not provide additional protection against pulmonary TB as compared to BCG Pasteur. Similar results have been obtained in several studies comparing "early" and "late" BCG strains [126, 127, 280] in which panels of vaccines were compared, each possessing strain specific mutations [108, 115, 127, 280]. While the loss of RD2 did not affect pulmonary protection, its loss resulted in increased dissemination of *M. tuberculosis*. This is not consistent with the findings previously mentioned. Lagranderie and colleagues observed that vaccination with BCG Pasteur lead to increased elimination of splenic bacteria than did vaccination with BCG Russia [126]. However, it is important to note that they did not challenge with *M. tuberculosis* by aerosol, but instead used an intravenous challenge with a recombinant BCG strain. Horwitz *et al* (2009) compared early and late strains BCG strains in guinea pigs, and detected no discernable difference in protection from dissemination [127]. Unlike our study, Horwitz and colleagues selected BCG Tokyo as their early strain, and challenged intravenously with *M. tuberculosis* [127]. BCG Tokyo is mutated for *Rv3405c* and therefore does not produce phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs), which have both been associated with full virulence of *M. tuberculosis* [113]. Therefore this mutation may have selectively affected the protective efficacy afforded by this vaccine in a manner independent of RD2.

Despite the increase in the IFN-γ response observed with the partial complementation of RD2 into BCG Pasteur, there was no corresponding decrease in bacterial burden. This supports the hypothesis that increased immunogenicity does not necessarily result in better protective efficacy. In unpublished work, BCG Pasteur complemented with wild-type *sigK* induces a strong IFN-γ response to the antigen MPB70, yet, protection against *M. tuberculosis* (in guinea pigs) and *M. bovis* (in cattle) was not enhanced when compared to BCG Pasteur (Behr, Izzo and Gordon, unpublished observations). Our current study looking at MPT64 provides data consistent with this observation, demonstrating that IFN-γ and IL-17 production by T-cells do not necessarily predict vaccine protection by different BCG strains [197]. As BCG strains offer comparable protection despite antigenic differences, the significance of these markers as surrogates or correlates of protection may need to be reconsidered [294].

This study has demonstrated that presence of the RD2 does not alter the efficacy of the vaccine. As recent data have pointed to the role of innate immune responses in the induction of protection against *M. tuberculosis*, it is possible that the most important role of BCG is to set off an innate immune response through the pathogen associated molecular patterns it contains, and that the antigens that elicit adaptive immune responses are less critical for BCG-induced protection. Given that BCG strains produce *N*-glycolyl muramyl dipeptide, shown to be critical for induction of NOD2-mediated responses [250], ongoing research is evaluating whether the innate recognition of BCG, rather than adaptive immune responses, is important in conferring protection against challenge by *M. tuberculosis*.

6. Acknowledgements

The authors thank the members of the members of the Behr, Reed and Schurr laboratories for their input. The authors thank Vlad Kamenski for his invaluable assistance during animal experiments and Maziar Divangahi for his suggestions and advice. R. K. was supported through a MUHC scholarship. M.B. is a Chercheur National of the FRSQ and a William Dawson Scholar of McGill University. Work was funded by an operating grant from the Canadian Institutes for Health Research, MOP-79309.

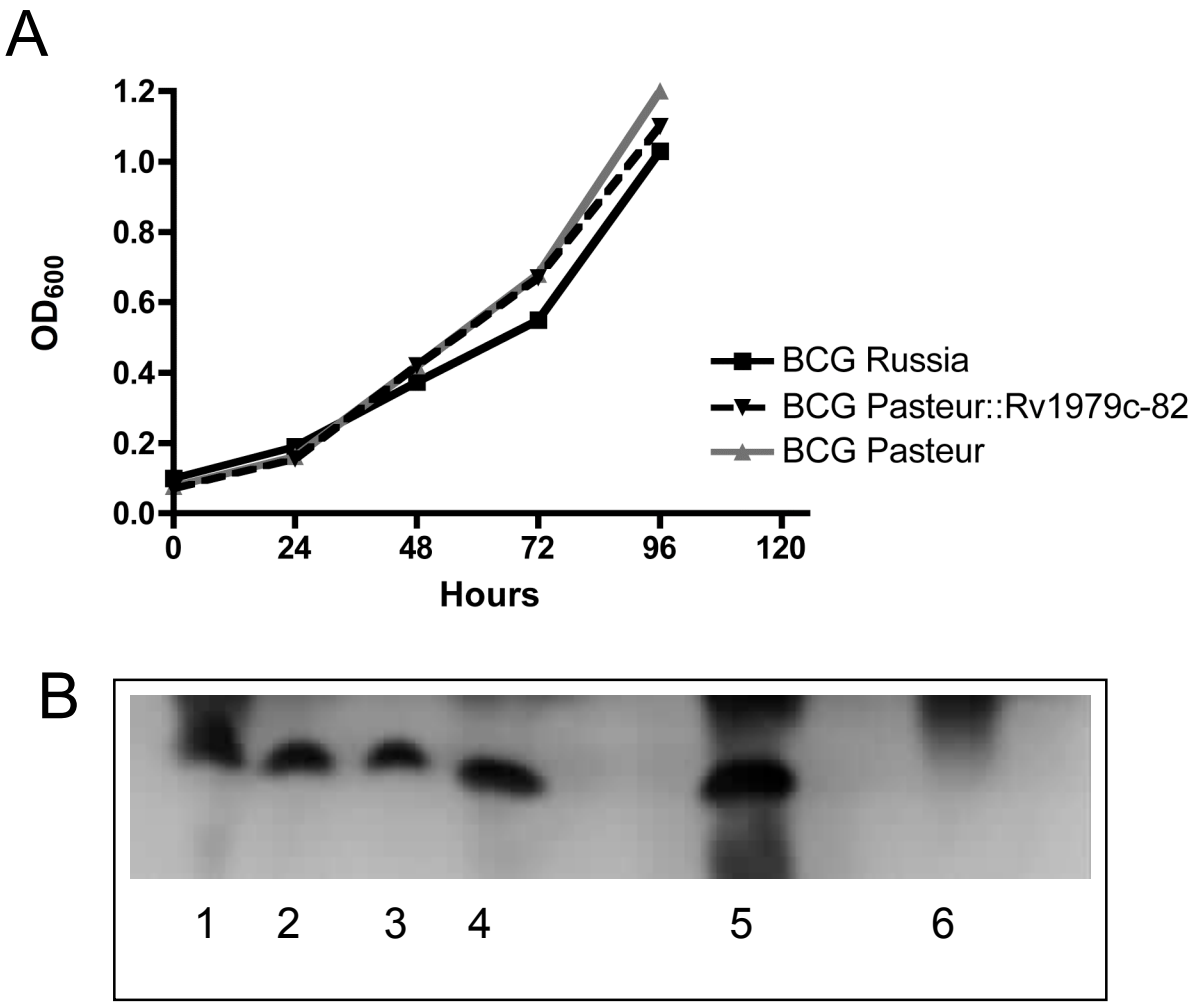
7. Figures and tables

Table 1: Oligonucleotide primers used in this study

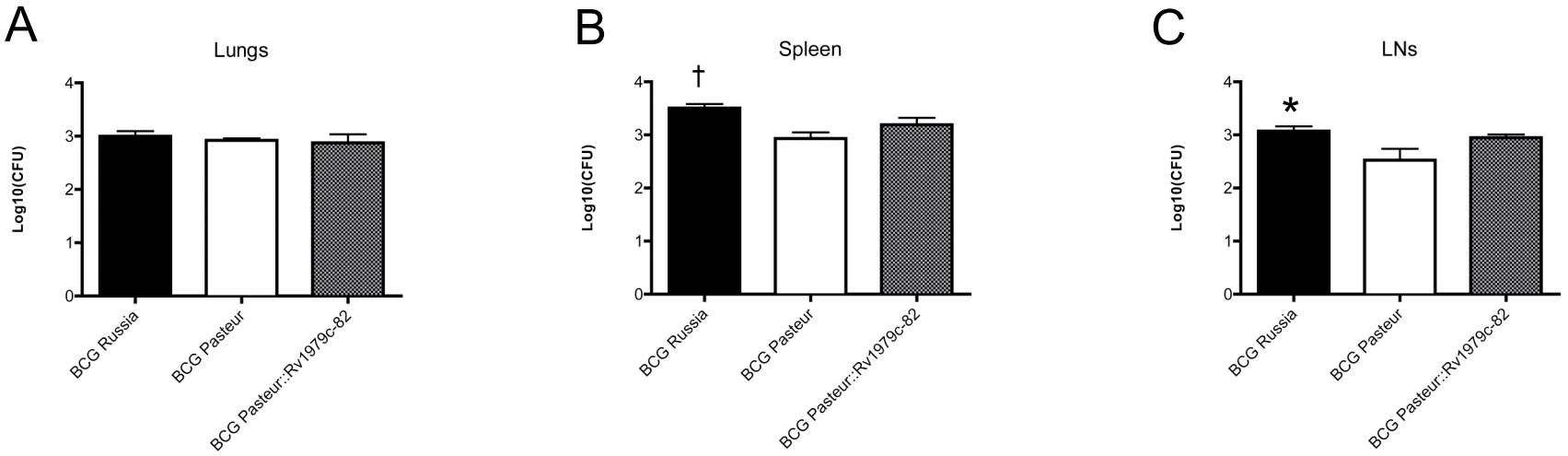
Primer	**Sequence**
7982F	gtaagcttcactacgtgcctacagtccc
7982R	cattctagagctgaccgattgatattc
KanF	aacaagccagggatgtaacg
KanR	ccataaaaccgcccagtcta

Table 2: BCG strains used in this study

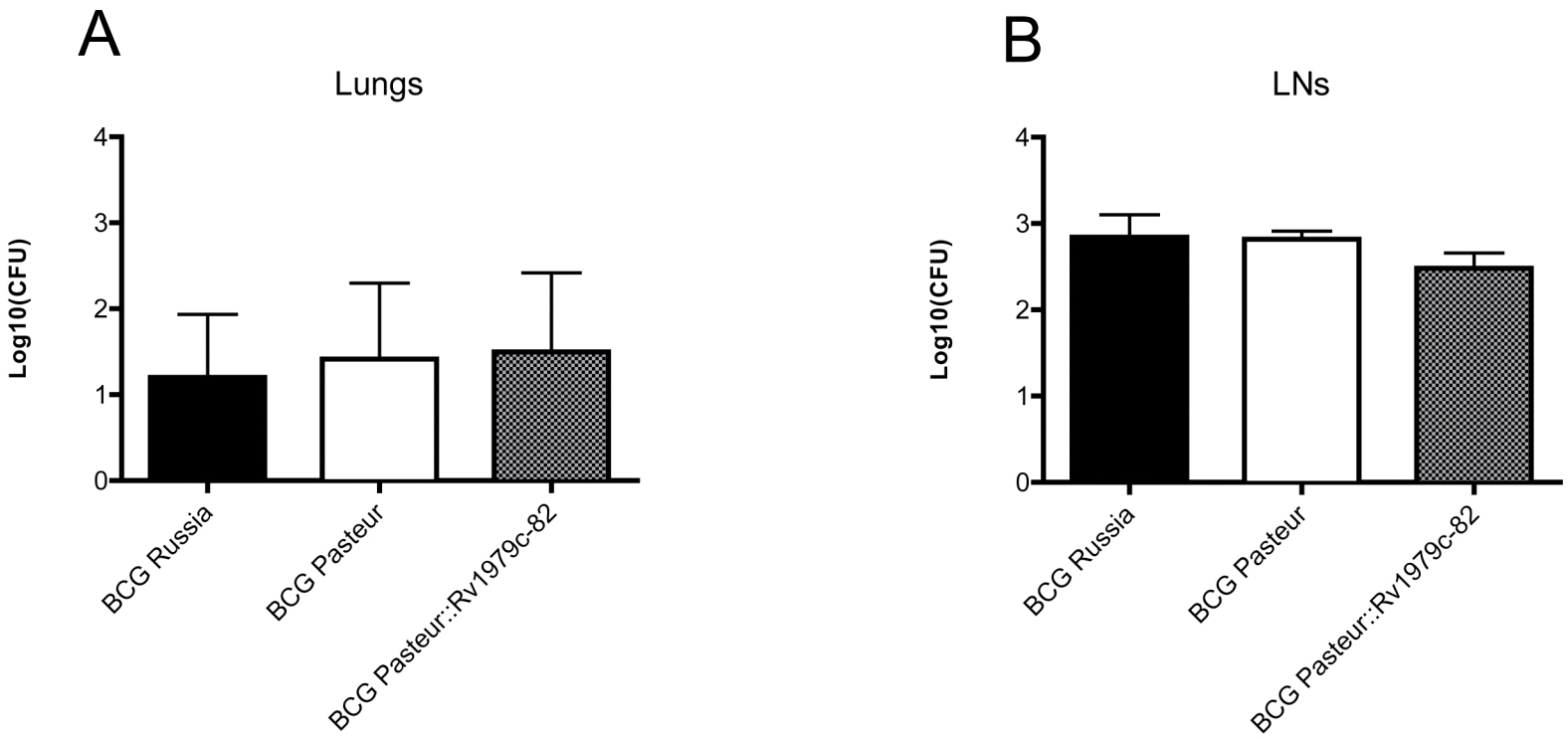
Strain	**Presence of entire RD2 region**	**Presence of MPT64**
BCG Russia	+	+
BCG Pasteur	-	-
BCG Pasteur::*Rv1979c-82*	-	+



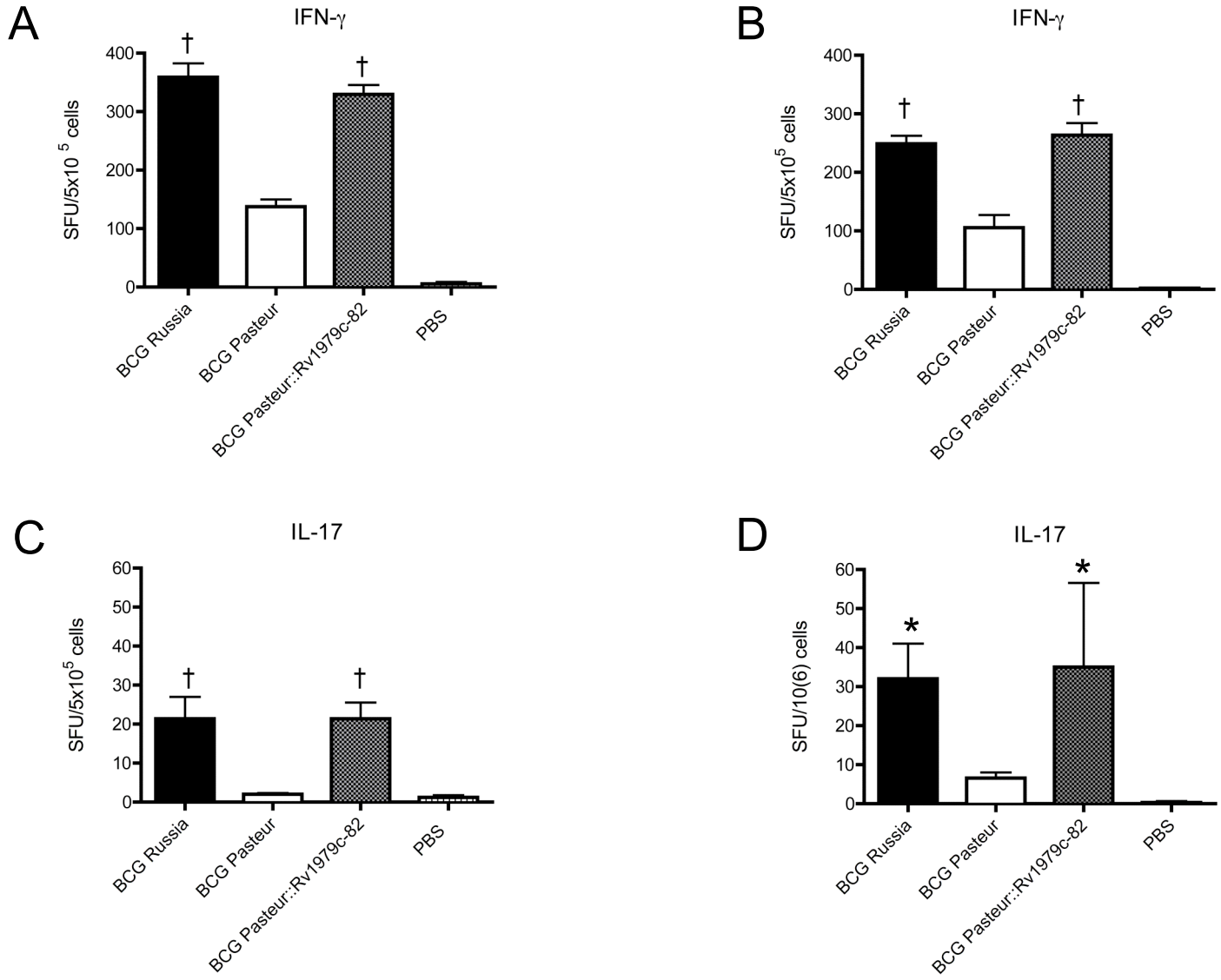
Chapter 3 - Figure 1. *In vitro* characterization of BCG strains.
In vitro growth kinetics of BCG Russia, BCG Pasteur, and BCG Pasteur::*Rv1979c-82* as determined by OD_{600} readings (A). Experiment was repeated in duplicate and data is from one representative experiment. SDS-PAGE and immunoblotting with an anti-MPT64 antibody from the strains used in this study. Antigen MPT64 is approximately 25 kDa. Lane 1 is culture filtrate from BCG Russia, lanes 2-5 are BCG Pasteur::*Rv1979c-82* and lane 6 is BCG Pasteur.



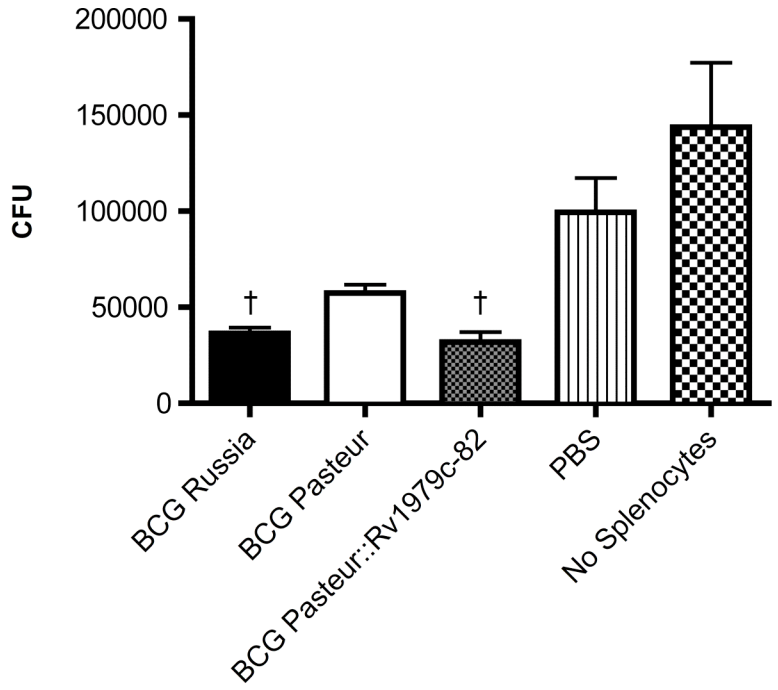
Chapter 3 - Figure 2. *In vivo* assessment of vaccine dissemination.
Bacterial burden in the lungs (A), spleen (B) and lymph nodes (C) at 15 days post-vaccination. 6-8 week old C57BL/6 mice were sub-cutaneously vaccinated with BCG Russia (black bar), BCG Pasteur (white bar) or BCG Pasteur::*Rv1979c-82* (grey bar). Data represents the mean ±SEM of 5 mice, where * indicates $p < 0.05$ and † indicates $p \leq 0.01$.



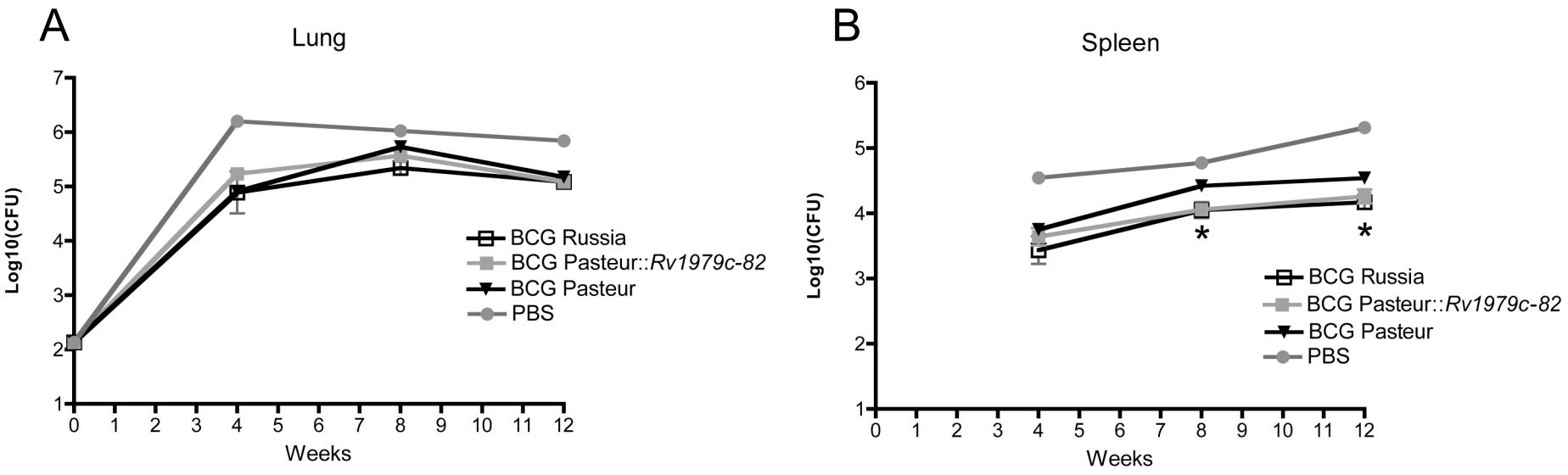
Chapter 3 - Figure 3. *In vivo* persistence of BCG strains.
Bacterial burden in the lungs (A), and lymph nodes (B) at 28 days post-vaccination. 6-8 week old C57BL/6 mice were sub-cutaneously vaccinated with BCG Russia (black bar), BCG Pasteur (white bar) or BCG Pasteur::*Rv1979c-82* (grey bar). Data represents the mean ±SEM of 5 mice, where * indicates $p < 0.05$ and † indicates $p \leq 0.01$.



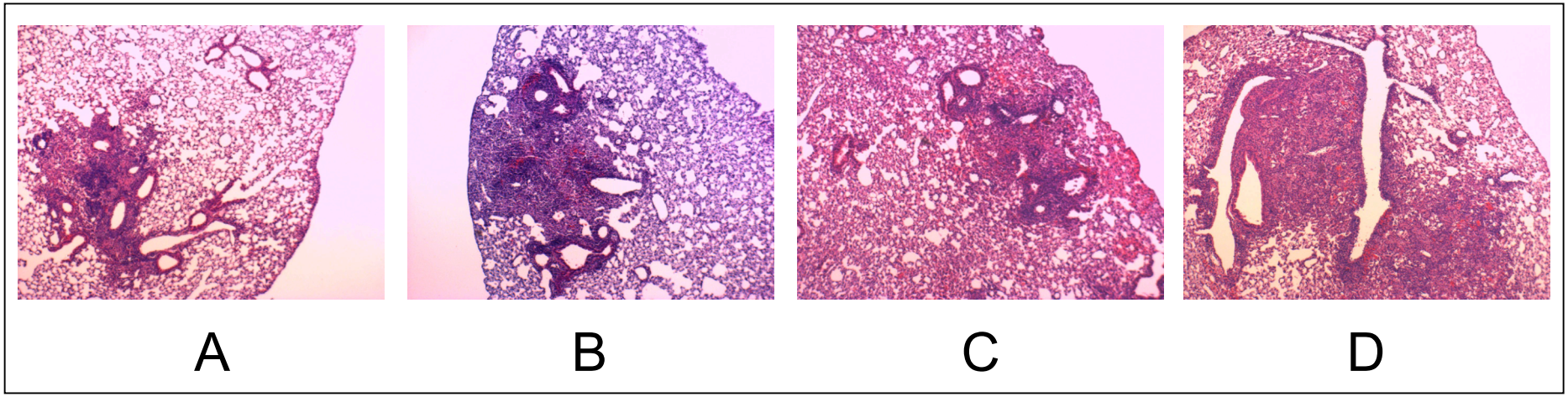
Chapter 3 - Figure 4. Antigen specific T-cell response to M. tuberculosis.
Splenocytes isolated from vaccinated mice after 28 days were cultured in the presence of MTB culture filtrate. ELISpot was used to determine frequency of IL-17 and IFN-γ after 24 (A & C) and 48 h (B & D). Data represents ±SEM of 5 mice, where * indicates $p < 0.05$ and † indicates $p \leq 0.01$.



Chapter 3 - Figure 5. Suppression of *M. tuberculosis* replication by T-cells from vaccinated animals.
T-cells from vaccinated mice were added to peritoneal macrophages infected with *M. tuberculosis* (MOI=5). Bacterial CFUs were determined after 96h. Data represents ±SEM of 4 mice, where * indicates $p < 0.05$ and † indicates $p \leq 0.01$.



Chapter 3 - Figure 6. Vaccine-induced protection in a murine model.
C57BL/6 mice were vaccinated 4 weeks prior to aerosol infection with ~ 200 CFU of *M. tuberculosis*. A. Bacterial burdens in the lungs were evaluated at 4, 8 and 12 weeks post-challenge. B. Splenic bacterial burdens were evaluated at 4, 8 and 12 weeks post-challenge. At the 4 and 8-week time points 7 mice per group were used, and at the 12- week time point 6 mice per group were used. Data represents ±SEM, where * indicates $p < 0.05$and † indicates $p \leq 0.01$.



Chapter 3 - Supplemental Figure 1. Histopathological comparisons of different vaccines.
Histology of lung sections from vaccinated mice at 4 weeks post-challenge. Pathology was similar for all strains at 8 and 12 weeks post-infection. Strains used were as follows: A = BCG Russia, B = BCG Pasteur::*Rv1979c-82*, C = BCG Pasteur, D = PBS (sham vaccination). One mouse was used per group was used and lungs were stained with H & E. Micrographs (magnification, 40X) shown are representative lung sections from each group.

Preface to Chapter 4

A requirement of an efficacious vaccine is that it induces a protective immune response in the host. In Chapter 1 the role of numerous genes in susceptibility to *M. tuberculosis* was delineated. Prior studies have indicated NOD2 to be important for immunity to *M. tuberculosis*, and suggest it may play a role in BCG vaccination. Therefore, we examined the role of NOD2 using knock-out and wild-type mice in vaccine-challenge experiments. This chapter illustrates the interaction between vaccine and host required for vaccine efficacy.

Chapter 4: NOD2 mediates protective efficacy of BCG vaccination

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1. Abstract

NOD2/CARD15 is an intracellular receptor that recognizes muramyl dipeptide (MDP), a component of the mycobacterial cell wall found in the BCG vaccine. The importance of this receptor in mediating innate and adaptive immune response to *M. tuberculosis* has been previously described. Host receptors moderate responses to BCG vaccination, therefore *Nod2* is likely important for this process. In this study we found that the loss of *Nod2* did not affect the burden of *M. tuberculosis* in BCG vaccinated animals. However, there was increased pathology in $Nod2^{-/-}$animals compared to $Nod2^{+/+}$ controls. We examined T-cell numbers within the lungs, as they are critical for the containment of mycobacterial infections within the granuloma. Immunohistochemisty and flow cytometry revealed that the levels of $CD4^+$ and $CD8^+$ T-cells were comparable in vaccinated $Nod2^{+/+}$ and $Nod2^{-/-}$ mice, however the number of mycobacteria-specific IFN-γ and IL-17 producing cells was reduced by *Nod2* disruption. Moreover, $Nod2^{+/+}$ splenocytes showed greater control of intracellular *M. tuberculosis* in infected macrophages *ex vivo*. This demonstrates that while the loss of *Nod2* does not affect the overall number of T-cells in vaccinated animals, it does reduce the cellular response responsible for anti-mycobacterial immunity. Thus, NOD2 mediates the protective efficacy of the BCG vaccine, suggesting a critical role of host genetics in vaccine-induced immunity.

2. Introduction

The vaccine against *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG (BCG), has existed for nearly a century. However, the correlates of protective immunity remain poorly understood. The efficacy of the vaccine has ranged from 0-80% [125]. There are multiple factors suggested as to why BCG is ineffective, including the influence of environmental mycobacteria and the substantial genomic variability in BCG strains [115, 295, 296]. The discovery of mycobacterial-susceptibility genes, such as *nramp1* and *sst1*, implies that host factors also influence immune responses to the vaccine [79, 230].

Recently, a role for nuclear oligomerization domain 2 (NOD2) was shown for innate and adaptive immune responses to *M. tuberculosis*, demonstrating that the optimal generation of host immunity to mycobacterial depends on intracellular recognition [29, 89]. NOD2 is a pattern recognition molecule (PRM) that is sensitive to *N*-glycolyl muramyl-dipeptide (MDP), found in the cell wall of mycobacteria and related organisms [250]. Experimental evidence in mice demonstrates a role for NOD2 in the recognition of, and response to *M. tuberculosis*. Specifically, the loss of this PRM results in decreased expression of various pro-inflammatory cytokines and chemokines, including TNF-α, IL-12p40, IL-6, IL-1β and RANTES [29, 88, 267, 297, 298]. Additionally, NOD2 is important for mediating the transition from innate to adaptive immunity during mycobacterial infection [89].

The goal of BCG vaccination is to generate a robust adaptive immune response that can counteract subsequent infection by *M. tuberculosis,* in order to reduce bacterial burden and limit pathology. Comparison of BCG protection against nine different clinical and laboratory strains of *M. tuberculosis* demonstrated that pathology differed even in

instances where bacterial burden was comparable [299]. This suggests a partial uncoupling of these outcomes, in which limiting pathology is the more crucial function of the BCG vaccine. The containment of bacterial-induced pathology is facilitated by the formation of a granuloma, and is dependent on the release of key cytokines and chemokines by macrophages and T-cells [195]. In particular, IFN-γ and IL-17 are important cytokines for mediating granuloma development, and the production of these cytokines is decreased in *Nod2*-deficient cells [89, 264, 282, 300].

We hypothesized that loss of *Nod2* would attenuate activation of cell-mediated immunity, and result in a decreased efficacy of BCG vaccination. To investigate this possibility, we have examined bacterial burden, immunopathology, cellular recruitment to the lungs, T-cell mediated killing and adaptive immune responses in $Nod2^{+/+}$ and $Nod2^{-/-}$ mice.

3. Materials & Methods

3.1 Bacteria strains and growth conditions

Mycobacterium bovis BCG and *M. tuberculosis* H37Rv were grown on a standing platform at 37°C in 7H9 liquid medium (Difco Laboratories) supplemented with glycerol, 0.05% Tween 80 (Sigma-Aldrich) and 10% albumin-dextrose-catalase (Becton Dickinson and Co.). For growth on solid media 7H10 agar containing 10% OADC (oleic acid plus ADC, Becton Dickinson), 0.5% glycerol and PANTA (Becton Dickinson) was used, and plates were incubated at 37°C.

3.2 Mice

Nod2 $^{+/-}$ males backcrossed on a C57BL/6 background were obtained from the Congenics Facility at Yale University. These were bred with C57BL/6 mice purchased from Harlan Laboratories to establish a *Nod2*$^{-/-}$ breading colony at the McGill University Health Centre and in parallel, a colony of *Nod2*$^{+/+}$ mice derived from wild-type litter mates. Mice 6-8 weeks old were used. All experiments were conducted in accordance with the guidelines of animal research ethics boards of McGill University.

3.3 Vaccination

BCG growth was determined by optical density (OD 600nm), and strains were diluted in sterile PBS. Mice were vaccinated sub-cutaneously with approximately $6x10^5$ CFU. BCG innoculum was plated on 7H10 plates to verify the vaccination dosage. To evaluate persistence of the BCG vaccine, four mice from each group were sacrificed at 4 weeks

post-vaccination, and their lungs, spleen and axillary lymph nodes were homogenized and plated. Plates were counted after 3 weeks.

3.4 *M. tuberculosis* infection of vaccinated mice

To assess the role of NOD2 in vaccine protection, *Nod2*+/+ and *Nod2*-/- mice were challenged with a moderate dose (approximately 200 CFU) of *M. tuberculosis* strain H37Rv by aerosol in accordance with methods previously described [20]. Briefly, prior to infection bacterial cultures were adjusted to mid-log phase growth and stored in 20% glycerol at -80°C. For aerosol infection these stocks were diluted in PBS/Tween-20 (0.05%). Mice were aerosolized for a total of 10 minutes, which resulted in the implantation of approximately 200-250 CFU per lung, confirmed at 24h post-infection by homogenizing lungs from three mice per group in 7H9/ADC and determining bacterial counts. At 4 weeks post-infection mice were sacrificed, the lungs and spleen were harvested, serially diluted, homogenized and plated. Bacteria on the plates were enumerated after 3 weeks.

3.5 Histopathology, immunohistochemistry and flow cytometry

Immediately following collection, lung and spleen tissue was preserved in 10% neutral buffered formalin and processed (IRIC, Plateforme d'Histologie, Universite de Montreal). To evaluate tissue morphology, 4-5μm thick sections were obtained from the paraffin-embedded tissues and underwent routine hematoxylin and eosin staining. A blinded reader evaluated pathology, and the area of granulomas was calculated as a percentage of the field of vision occupied by the lesion. Sections also underwent routine

immunohistochemical staining for the presence of CD3, and were read and enumerated by a trained pathologist (RIC, Plateforme d'Histologie, Universite de Montreal). Flow cytometry was performed in the manner described by Divangahi et al. [89].

3.6 Peritoneal macrophages

Peritoneal macrophages were harvested from C57BL/6 by flushing the peritoneal cavity with RPMI 1640 media supplemented with 10% fetal bovine serum, 10mM HEPES and penicillin/streptomycin (Wisent). Macrophages were resuspended in fresh media, counted, then added to 6 well plates ($2x10^5$ cells/well) and allowed to adhere overnight at 37°C in 5% CO_2.

3.7 Macrophage infection and T-cell cytotoxicity

T-cell cytotoxicity was evaluated by co-culturing T-cells from vaccinated animals with *M. tuberculosis*-infected macrophages. Four weeks after vaccination, spleens were harvested and digested in a manner similar to that described by Divangahi et al. [89]. Briefly, spleens were removed aseptically, passed through a 100μm filter (BD Biosciences) to generate single-cell suspensions, and incubated in ACK lysis buffer for 5 minutes to remove red blood cells. Splenocytes were resuspended in RPMI plus 10% FBS, penicillin, streptomycin and HEPES. They were then incubated in a culture flask overnight at 37°C in 5% CO_2 to purify by adherence. The following day CD90.2-positive cells were purified by positive selection, according to the manufacturers instructions using a DynaMag-2 magnetic bar (Invitrogen). Peritoneal macrophages were infected with *M.*

tuberculosis at an MOI of 5 for 4h at 37°C in 5% CO_2. Cells were washed 3 times with media, and then fresh RPMI was added to each well. CD90.2-positive cells were added to the macrophages in a ratio of 5:1. In certain wells anti-mouse IFN-γ, and mouse IgG2a isotype controls (R&D Systems) were added (5μg/ml) following the addition of splenocytes. After 96h supernatant was removed and added to 1% Triton X-100. Macrophages in wells were lysed with 3 washes of 1% Triton X-100. Bacterial CFU were determined by plating. Plates were counted after 3-4 weeks.

3.8 ELISpot

Splenocytes were harvested and enumerated from vaccinated mice at 28 days post-infection and were cultured in RPMI 1640 media with 10% BSA and 0.02% HEPES. Splenocytes were treated with ACK lysis buffer to remove red blood cells, and incubated in media overnight at 37°C to remove monocytes by adherence. Splenocytes were seeded into 96-well plate pre-coated with IFN-γ and IL-17 capture antibodies. The ELISpot assay was performed in accordance with the manufacturer's instructions (R&D Systems). Cells were incubated for 24 and 48h with or without stimulation by *M. tuberculosis* culture filtrate (MTB CF) (10μg/ml).

3.9 Long-term *M. tuberculosis* infection

To assess the role of NOD2 in vaccine protection to *M. tuberculosis* infection we delivered an elevated dose (400-500 bacteria) of *M. tuberculosis* H37Rv by aerosol in vaccinated *Nod2*+/+ and *Nod2* -/- mice. Mice were monitored bi-weekly and were

sacrificed when they had lost 15% of their body mass from the maximum weight attained during the experiment, or were deemed suffering severe TB disease symptoms: lethargy, trembling and weight loss by a trained veterinarian blinded to the study.

3.10 Figures and Statistics

Differences in Gaussian data collected were tested for significance using the student's t-test, with a p-value < 0.05 considered statistically significant. Differences in semi-quantitative data (histopathology) were tested using the rank-sum test. Survival data was assessed using the log-rank test. Figures were generated using GraphPad Prism 4.0b.

4. Results

4.1 *Nod2* does not affect vaccine persistence

Bacterial persistence within the host is necessary to generate an immunologic response required for protection [85]. At the time of *M. tuberculosis*, 4 weeks post-vaccination, the number of BCG colonies in the lungs, spleen and lymph nodes was comparable between $Nod2^{+/+}$ and $Nod^{-/-}$ mice (Figure 1), indicating that any observed differences in immunity would not be attributable to differences in the vaccine burden.

4.2 *M. tuberculosis* bacterial burden is independent of Nod2 status in vaccinated mice

The primary functions of BCG vaccination are to lower bacterial burden and reduce lung pathology following infection with *M. tuberculosis*. As loss of *Nod2* increased susceptibility to infection with *M. tuberculosis* [89], we hypothesized that vaccination of *Nod2*-deficient mice with BCG would provide less protection compared to wild-type mice. Four weeks after aerosol infection with *M. tuberculosis*, regardless of genotype, BCG-vaccinated mice had fewer bacteria in both organs compared to mice that had received PBS as a vaccine control. These data are consistent with previous studies in BCG vaccination at this time point [88, 89]. Notably, the presence or absence of *Nod2* did not affect pulmonary or splenic bacterial burden (Figure 2).

4.3 The loss of *Nod2* increases pulmonary pathology in vaccinated

Despite comparable bacterial burdens, when lung histophathology was examined, *Nod2*-deficient mice had more severe pathology than *Nod2* wild-type mice. Specifically,

vaccinated *Nod2*-deficient mice had larger more diffuse granulomas and greater inflammation when compared to vaccinated *Nod2*$^{+/+}$ controls (Figure 3A). To quantify the difference in granuloma size between groups, a granuloma representing the most severe pathology was selected from each sample, and the area of the lesion was determined. Comparison of areas from each group confirmed that the vaccinated *Nod2*$^{-/-}$ mice had larger granulomas than wild-type mice (Figure 3B).

4.4 Lymphocyte recruitment is not affected by NOD2

Granuloma formation is dependent upon T-cell recruitment to the site of infection [95]. This suggests that the differences in pathology might be due to defective lymphocyte recruitment or function. To test if lymphocyte recruitment differed, we performed immunohistochemical staining for $CD3^+$ cells on lung sections (Figure 4A). Cell counts were similar for both groups (Figure 4B). The comparable numbers of $CD3^+$ cells observed in both groups led us to quantify $CD4^+$ and $CD8^+$ T-cell numbers, but again no significant differences were detected between vaccinated *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ mice (Figure 5). These data demonstrate that differences in pathology, in *Nod2* knock-out mice, were not due to decreased lymphocyte recruitment, but rather a result of the decreased anti-mycobacterial activity of the T--cells.

4.5 Vaccinated *Nod2* knock-out mice have reduced Th1 and Th17 cytokine production

BCG vaccination results in the generation of mycobacterial specific T-cells, notably Th1 and Th17 cells, which are part of the adaptive immune response [83, 301].

Our pathology data show that *Nod2*$^{-/-}$ mice had a higher absolute number of inflammatory cells, but similar numbers of T-cells compared to *Nod2* wild-type mice. This suggested the possibility that the proportions of mycobacterial-specific T-cells would differ between *Nod2*$^{+/+}$ than in *Nod2*$^{-/-}$ mice. Following stimulation of splenocytes with *M. tuberculosis* culture filtrate for 24h, IFN-γ and IL-17 producing cells were reduced in knock-out mice compared to *Nod2*$^{+/+}$ controls (Figure 6). These cytokines serve as markers for Th1 and Th17 cells, thus our data reveal that the loss of *Nod2* decreased the proportions of mycobacterial-specific T-cells.

4.6 NOD2 plays a role in T-cell restriction of intracellular growth

The generation of antigen specific T-cells by a vaccine is important for the containment of bacterial infection, which helps minimize pathology [92, 285]. In order to test whether the decreased number of these cells in *Nod2*$^{-/-}$ mice had a detrimental effect on bacterial control, we evaluated their function *ex vivo*. To test this, peritoneal macrophages were infected with *M. tuberculosis* and washed after 4h. We then added to these macrophages splenocytes from *Nod2*$^{+/+}$ or *Nod2*$^{-/-}$ mice that had been immunized 4 weeks earlier with BCG. As a control, addition of splenocytes from PBS vaccinated animals provided no measurable control of intracellular bacterial growth when compared with a control group in which no splenocytes were added. This outcome occurred regardless of *Nod2* status. In contrast, splenocytes from BCG vaccinated *Nod2*$^{+/+}$ animals limited *M. tuberculosis* growth; this effect was lost with splenocytes from BCG vaccinated *Nod2*$^{-/-}$ animals (Figure 7).

The cellular composition of spleens includes B-cells, monocytes and T-cells. The control of mycobacteria is facilitated by T-cells, likely due to their production of IFN-γ and ability to generate a Th1 response [284]. In order to test the cellular basis for the difference we observed, we repeated the above experiment using T-cells purified by CD90.2-positive microbeads. These were then added to infected peritoneal macrophages, and cells were cultured with either IFN-γ neutralizing antibodies, or isotype controls. T-cells from vaccinated *Nod2*$^{+/+}$ mice were better able to control mycobacterial growth than those from *Nod2*-deficient animals. This was dependent upon IFN-γ producing cells, as the effect was lost when *Nod2*$^{+/+}$ T-cells were cultured with antibodies that neutralized IFN-γ.

4.7 Survival

Our data showed a discrepancy between the effects of NOD2 in a mouse (where bacterial burden was unaffected) compared to *ex vivo* macrophage studies (where bacterial numbers where reduced), suggesting either biological differences between a simple cell culture model or technical differences in detecting a significant difference *in vivo*. To test whether the early differences in histopathology seen with *Nod2* disruption translated into a deleterious outcome, we evaluated long-term survival after *M. tuberculosis* challenge, in BCG vaccinated mice. At the time of the writing of this manuscript, vaccinated *Nod2*$^{-/-}$ mice had a median survival time of 303 days, whereas the vaccinated *Nod2*$^{+/+}$ had not yet reached a median (Figure 8). This experiment is ongoing and the trend suggests that the loss of NOD2 in vaccinated animals diminishes the protection afforded by the vaccine, as measured by decreased survival.

5. Discussion

Vaccine induced protection requires the integrated responses of T-cells in order to contain bacterial growth and reduce pathology [95]. In particular, the IFN-γ producing Th1, and IL-17 producing Th17 subsets are essential for facilitating this effect [83, 302]. This response is dependent upon host recognition of bacterial ligands through pattern recognition receptors. Previously it has been shown that NOD2 facilitates protection against *M. tuberculosis* through the induction of anti-mycobacterial cytokines, involved in mediating the transition from innate to adaptive immunity [89]. NOD2 recognizes MDP, which in addition to being a component of the BCG cell wall, is also a potent adjuvant [237, 250, 258, 303]. The immunostimulatory action of MDP is required for the induction of cell-mediated immunity [250], and is potentially important for inducing maximum efficacy of BCG vaccination.

We examined established markers of vaccine protection in *Nod2*-deficient mice, such as bacterial burden and histopathology. Here, the loss of *Nod2* does not result in an increase in pulmonary bacterial load, but does increase pathology. Specifically, *Nod2*-deficient mice exhibit larger, more diffuse granulomas compared to their wild-type counterparts. Furthermore, *Nod2* deficiency produced T-cells that were less able to contain intracellular growth *ex vivo*, and had fewer IFN-γ and IL-17 producing cells. At a single cell level, lymphocytes from NOD2 mice appear to facilitate killing of *M. tuberculosis,* but in the lung this effect is absent. This observed discrepancy might be explained by several factors. First, if the differences in bacterial burden are small in an animal model, they may not be detectable due to the resolution of the system. In contrast it is easier to detect small differences in an *ex vivo* culture system. Second, the lung is a

complex environment, with many other cell types becoming involved in responding to infection. Therefore, cross-talk and interference from these cells may affect the ability of T-cells to interact with the macrophage and facilitate bacterial killing.

As a result, we hypothesize that the more salient finding is that NOD2 contributes to adaptive immunity through the generation of mycobacterial specific Th1 and Th17 cells; these are responsible for moderating pathology.

This hypothesis is in agreement with previous investigations examining the role of T-cells, in facilitating granuloma formation [95, 96, 196]. Vaccination studies using CD4-deficient and CD8-deficient mice implicate CD4 T-cells as essential for limiting pathology [95, 96, 304]. It is the contribution of Th1 cells that facilitate this, likely through the expression of IFN-γ [92, 305, 306]. IFN-γ induces production of nitric oxide through NOS2 (NO synthase 2), and *Nod2*-deficient mice have reduced levels of both molecules [89]. Nitric oxide and IFN-γ moderate inflammation, by impeding lymphocyte migration and limiting lymphocyte expansion, through which lesion size during infection is contained [307]. This provides a mechanism for the observed increase in pathology seen with our vaccinated *Nod2*-deficient mice. In addition, the loss of *Nod2* has been shown to restrict the ability of T-cells to differentiate into Th1 type cells during infection with *T. gondii*, due to intrinsic signaling defects. Here, NOD2 is predicted to interact with c-Rel, to induce *Il2* transcription [266]. It is conceivable that a similar effect may be occurring in our experiments.

Nod2-associated vaccine protection also depends on IL-17 producing cells. Deletion studies revealed that IL-17 is involved in granuloma formation during mycobacterial infection [300]. Here, it induces CCL2 and promotes tight cell-to-cell

binding through ICAM-1 and LFA-1, retaining Th1 cells in the early granuloma [300]. IL-17 also stimulates TNF-α production, which supports granuloma formation [308]. Recent evidence has shown that Th17 cells convey protection against *M. tuberculosis*, even in absence of IFN-γ [83]. Khader noted that BCG vaccination produces mycobacterial specific, IL-17 producing $CD4^+$ T-cells that recruit IFN-γ producing cells to the lung [193]. This data demonstrates that interplay exists between cell types and is required for protection. Due to the fact that NOD2 is required for IL-17 production, the inability of vaccinated *Nod2*$^{-/-}$ mice to produce this cytokine could impair granuloma formation at an early time point [264]. Taken together, we propose a model where the loss of IL-17 producing cells leads to early pathology, and that this effect is enhanced by the lack of IFN-γ producing cells later during infection.

The data presented herein reveal a role for NOD2 in vaccine protection against *M. tuberculosis*. Importantly, our results have implications for global vaccination programs, as they suggest that individuals who possess a mutation in *Nod2* may not receive the full benefit of vaccine protection. This might necessitate the design of a vaccine that better stimulates other host pathways. Potential alternatives include Mtb72F (naked DNA), administered as a booster with BCG, which likely acts to stimulate TLR9 [309] or the use of viral vectors to present mycobacterial antigens in a NOD2-independent manner.

6. Acknowledgements

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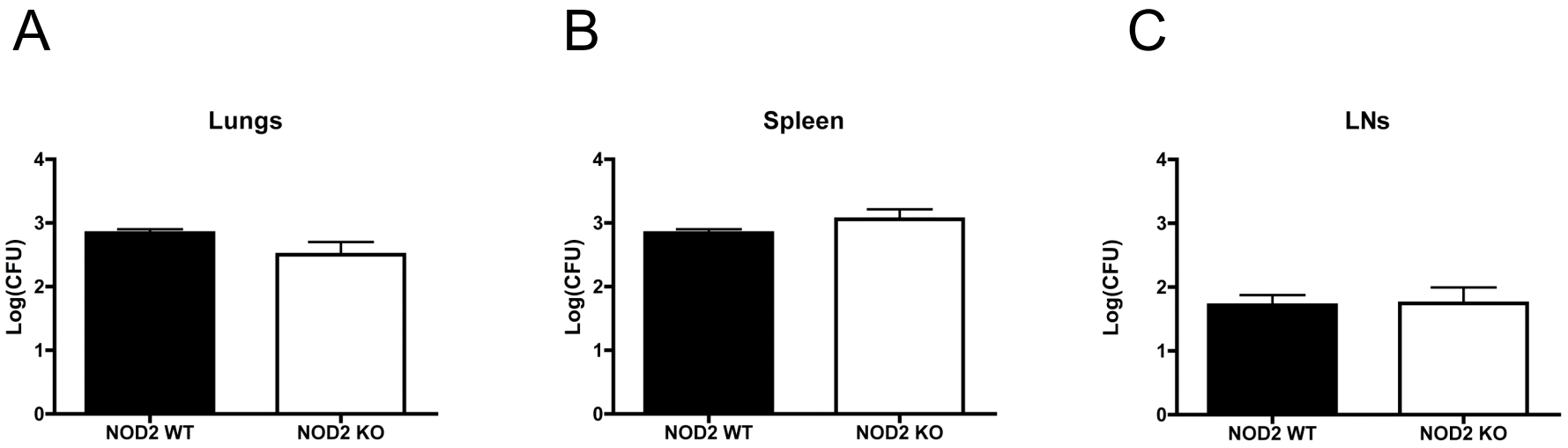
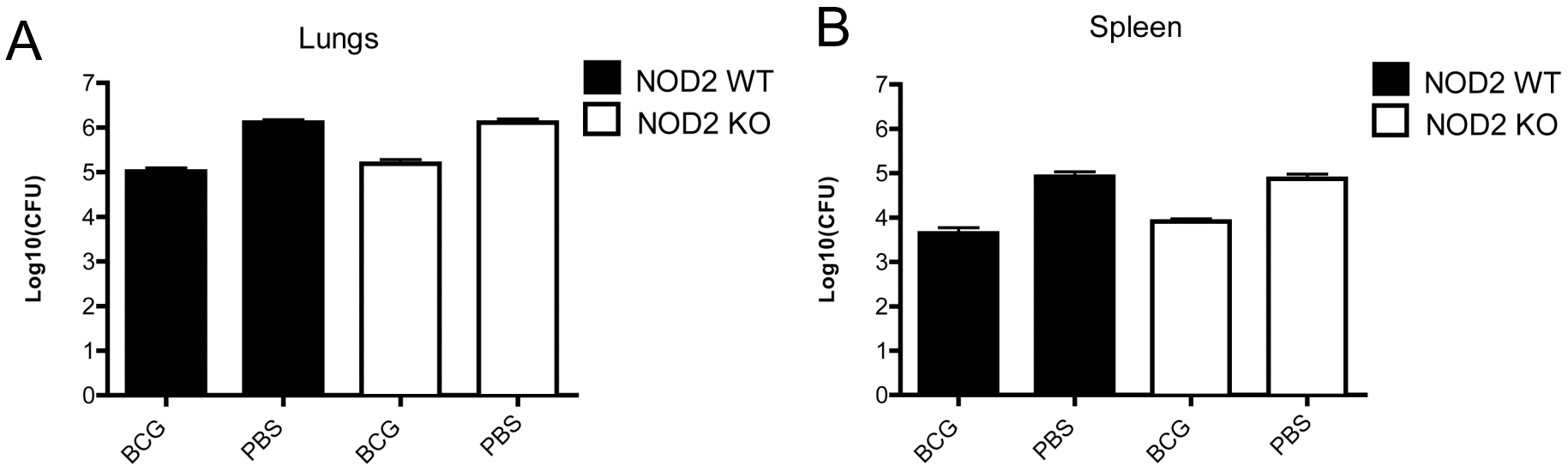
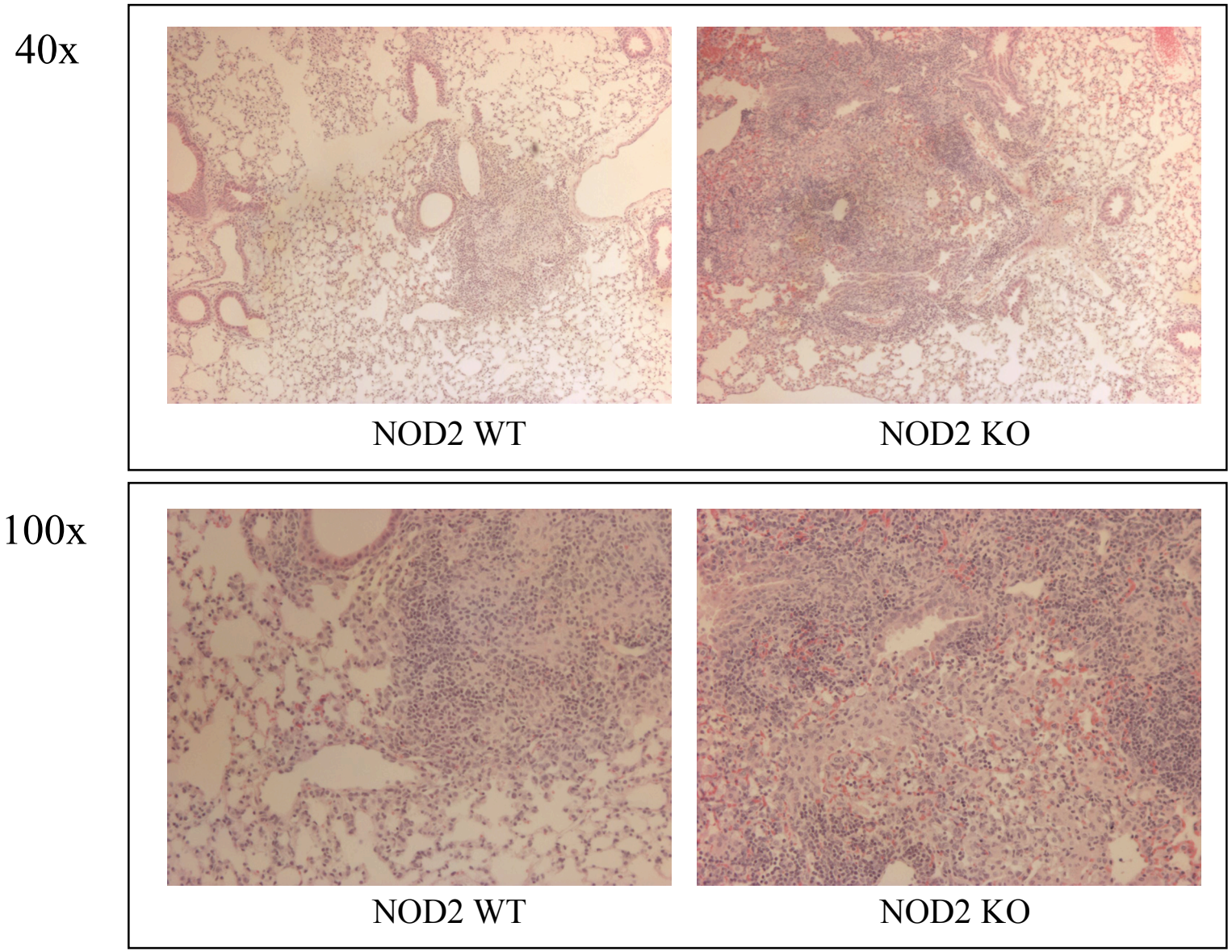
7. Figures and tables



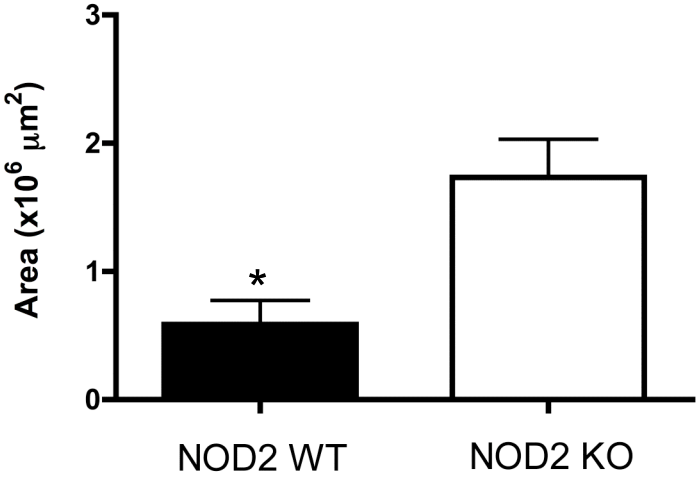
Chapter 4 - Figure 1. *In vivo* persistence of BCG in Nod2+/+ and Nod2-/- mice.
Bacterial burdens in the lungs (A), spleen (B), and lymph nodes (C) at 4 weeks post-vaccination. Nod2+/+ and Nod2-/- mice were subcutaneously vaccinated with BCG Russia. Data represent the ± SEM of 4 mice, where * indicates $p < 0.05$.



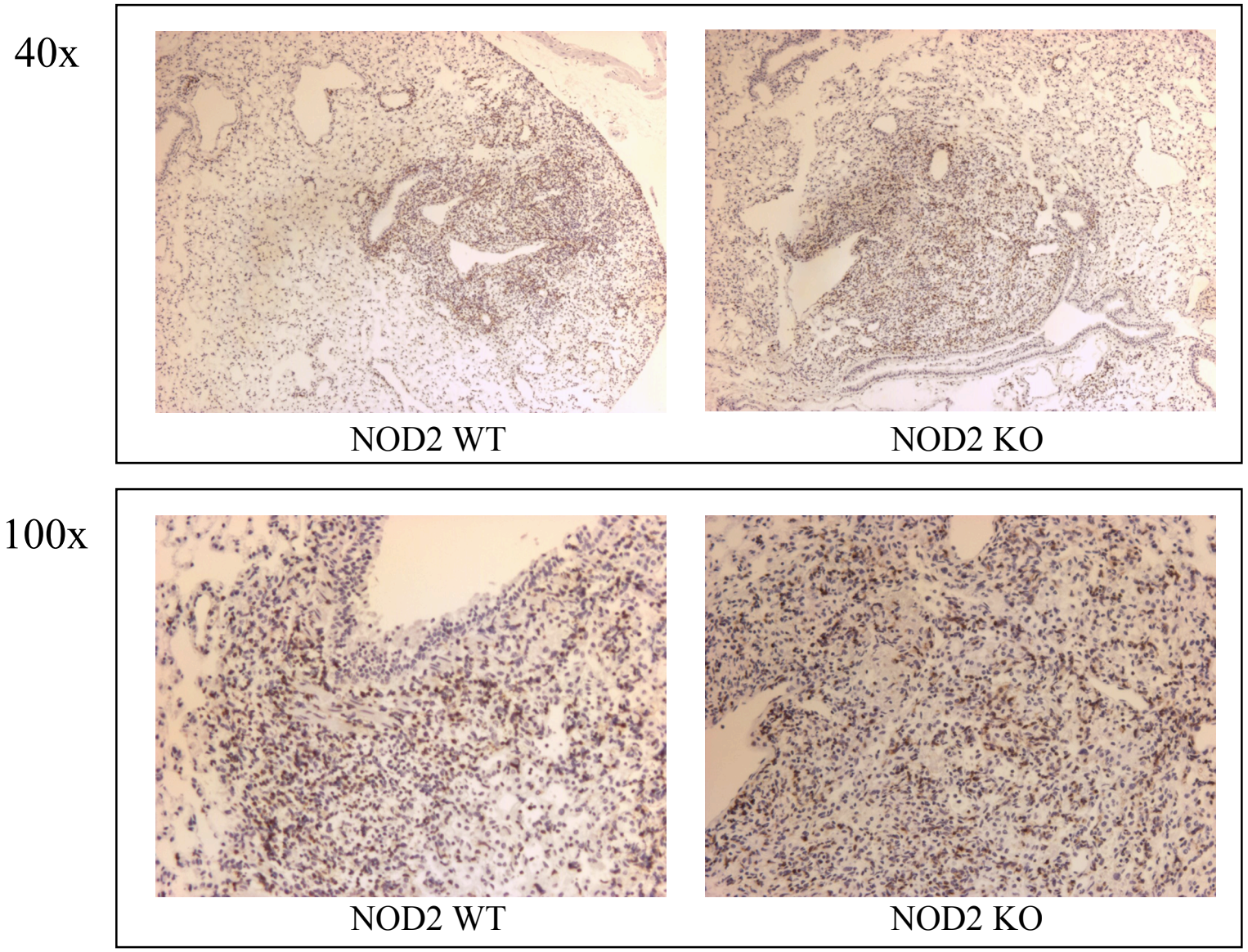
Chapter 4 - Figure 2. Pulmonary and splenic bacterial burden in vaccinated mice.
Bacterial growth in the (A) lungs and (B) spleens Nod2+/+ and Nod2-/- mice were vaccinated with either BCG or PBS 4 weeks prior to infection with ~ 200 CFU of M. tuberculosis. At 4 weeks post-infection bacterial growth was determined in the lungs (A) and spleen (B). Duplicate experiments were performed, and values are from one expermient. Data represent the ± SEM of 13 mice, where * indicates $p < 0.05$.



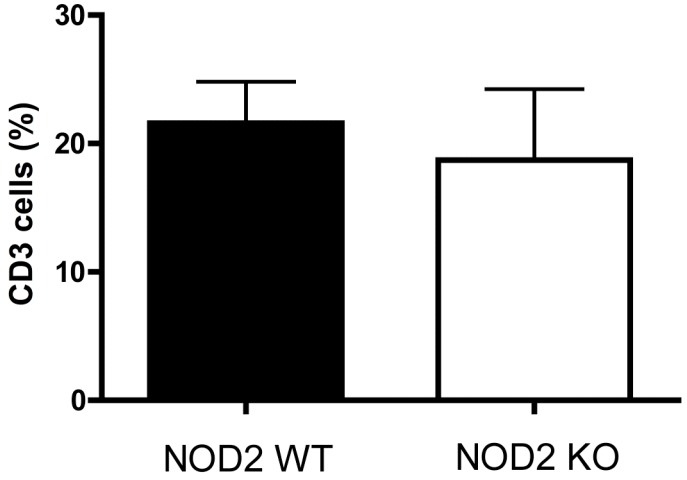
Chapter 4 - Figure 3A. *In vivo* pathology in vaccinated mice.
Lesions from BCG vaccinated *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ mice at 4 weeks post-infection. The experiment was performed in duplicated, and 5 mice per group were used, and lungs were stained with H & E. Micrographs (magnification, 40X and 100X) shown are representative lung sections from each group.



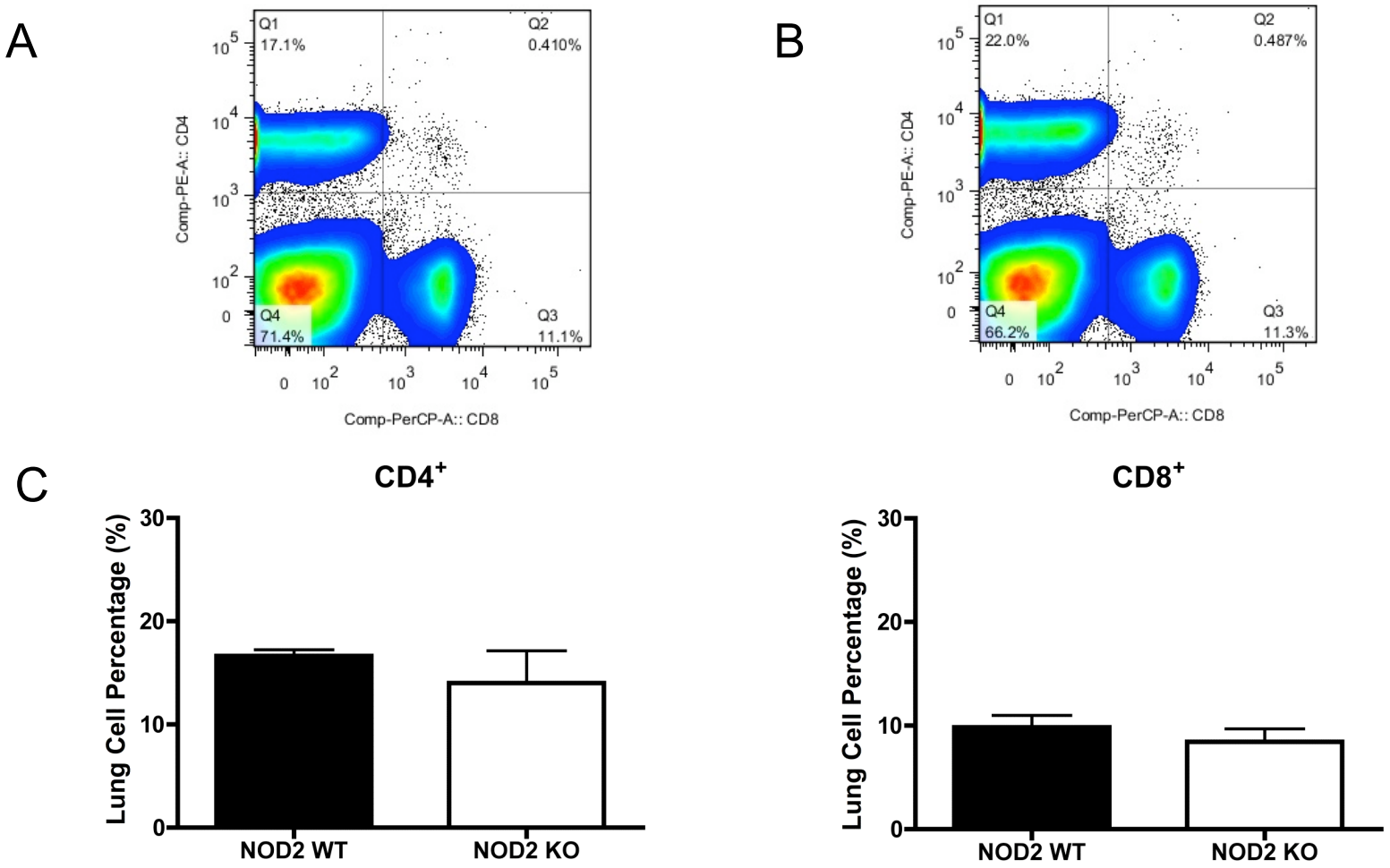
Chapter 4 - Figure 3B. Calculations of *in vivo* pathology in vaccinated mice.
Representative granulomas were selected, and the area of lesions was calculated. Data represent the ± SEM of 4 mice, where * indicates $p < 0.05$.



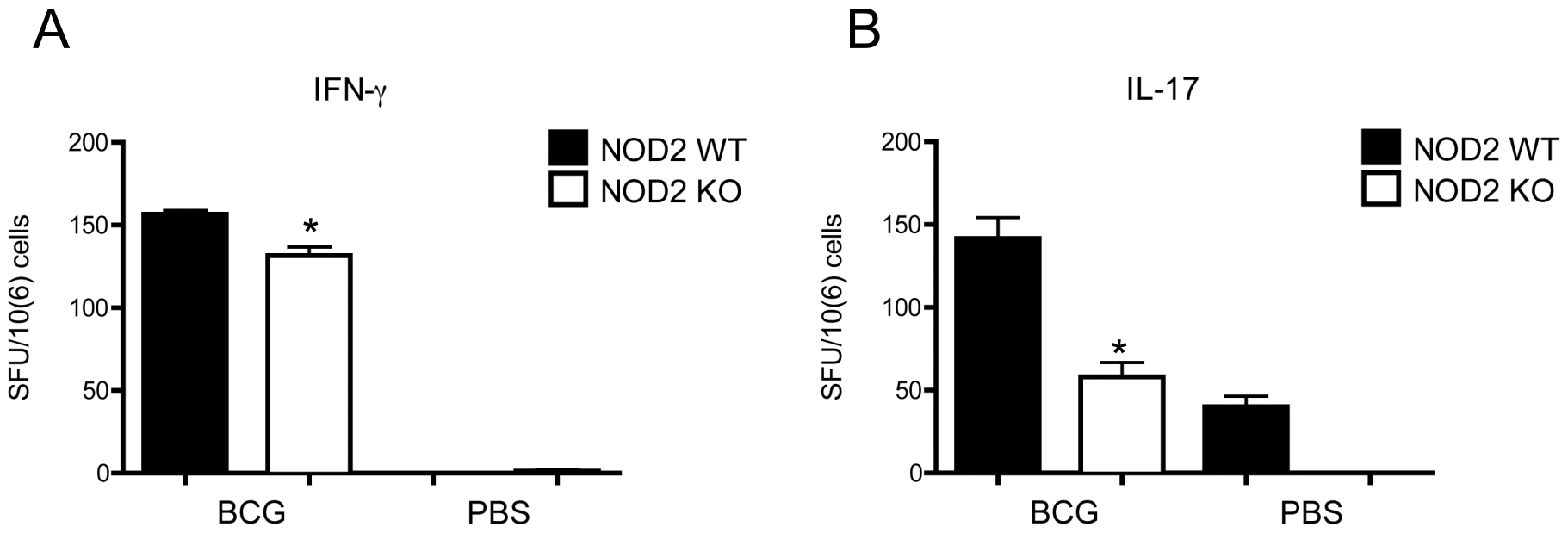
Chapter 4 - Figure 4A. Immunohistochemical staining for CD3-positive cells.
Lung sections from BCG vaccinated *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ mice at 4 weeks post-infection. CD3-positive cells are stained brown. 5 mice per group were used. Micrographs (magnification, 40X and 100X) shown are representative lung sections from each group.



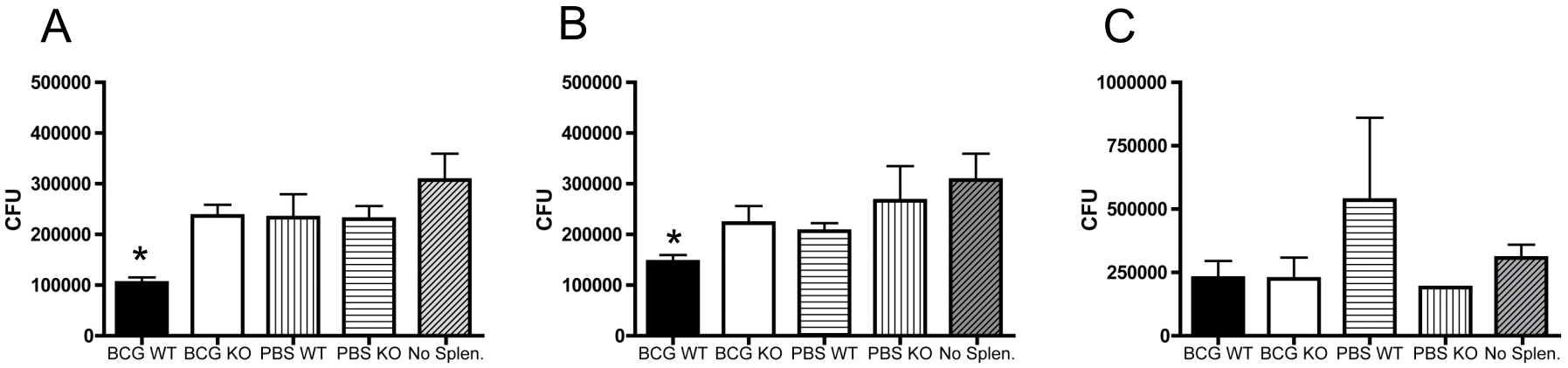
Chapter 4 - Figure 4B. Calculation of CD3-positive cells present in granulomas.
Percentages of CD3-postive cells present in representative granuloma was calculated. Data represent the ± SEM of 4 mice, where * indicates $p < 0.05$.



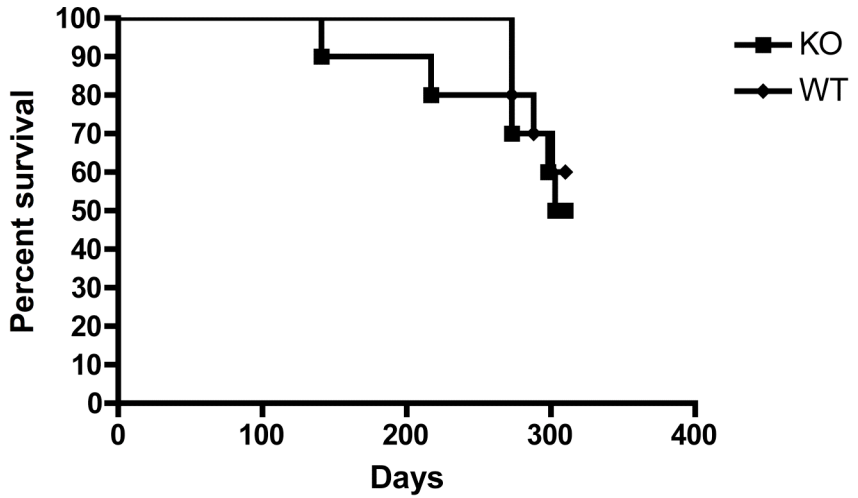
Chapter 4 - Figure 5. Lung T-cell Infiltration.
Infiltration of $CD4^+$ and $CD8^+$ T-cells was evaluated in the lungs of vaccinated NOD2 wild-type (A) and NOD2 knock-out (B) mice infected with *M. tuberculosis*, 4 weeks post-infection. (C) The percentage representing the average relative size of each T-cell population are quantified. Data represent the ± SEM of 4 mice, where * indicates $p < 0.05$.



Chapter 4 - Figure 6. Antigen specific T-cell response to *M. tuberculosis.*
Splenocytes isolated from BCG or PBS vaccinated mice after 4 weeks were cultured in the presence of MTB CF. ELISpot was used to determine the frequency of IFN-γ (A) and IL-17 (B) producing cells after 24h. Data represent the ± SEM of 5 mice, where * indicates $p < 0.05$.



Chapter 4 - Figure 7. T-cell mediated control of intracellular bacteria.
(A) Splenocytes from NOD2 WT or NOD2 KO, BCG vaccinated or PBS vaccinated mice after 4 weeks were added to peritoneal macrophages infected with *M. tuberculosis* (MOI = 5). CD90.2-positive cells from vaccinated mice were purified and were added to infected macrophages with (B) or without (C) anti-IFN-γ antibodies. Bacterial CFUs were determined after 96h. Experiment was performed in duplicated using Data represent the mean ± SEM of 8 mice per group from 2 separate experiments, where * indicates $p < 0.05$.



Chapter 4 - Figure 8. Loss of *Nod2* reduces survival times in vaccinated mice.
Nod2$^{+/+}$ and *Nod2*$^{-/-}$ mice were vaccinated with either BCG 4 weeks prior to infection with ~ 400 CFU of *M. tuberculosis*. NOD2-deficient mice show a trend towards decreased survival times. (Experiment ongoing).

Chapter 5: Discussion and opportunities for research

Discussion and opportunities for future research

This thesis describes studies examining host-pathogen interactions of the BCG vaccine. The first and second sections investigated the pathogen, specifically the role of RD2. The third section discusses host susceptibility, by examining the role of the NOD2 receptor in vaccination. Generation of protective immunity to a particular disease requires a vaccine attenuated enough to stimulate the host, coupled with proper recognition and activation of host pathways to generate a response. Therefore an improved understanding of the BCG vaccine requires the study of bacterial virulence factors and how the host recognizes and responds to these factors. This section will be divided into three themes: 1) the role of RD2 in mycobacterial virulence; 2) the role of RD2 in vaccination; and 3) the role of NOD2 in vaccination.

1. The role of RD2 in mycobacterial virulence

The first study contained within this thesis examines the role of RD2 in virulence of *M. tuberculosis*. The results presented clearly demonstrate that RD2 is required to achieve maximum virulence. Infection with H37Rv:ΔRD2 resulted in decreased bacterial burden and histopathology, and increased survival times in *Rag*$^{-/-}$ mice. Macrophage studies revealed that the genes *Rv1979c-Rv1982c* moderate host innate defenses to facilitate bacterial survival. Our method to investigate the role of these genes began with examining their role in an animal model and then proposing a virulence mechanism in the host cell. This methodology has also been applied to the study of other hypothesized virulence factors. As described in Chapter 1, this approach demonstrated that the loss of RD1 was responsible for attenuating BCG [135]. Subsequent work has determined

specific functions of RD1 genes in host-pathogen interactions [132]. In the work presented in Chapter 2 we have investigated host-pathogen interactions involving RD2 genes, and this suggests several possible future directions.

a) How does MPT64 moderate apoptosis?

As shown in Chapter 2 it is likely that MPT64 inhibits apoptosis, which is supported by clinical data [60, 61]. However, data to directly demonstrate the mechanism by which MPT64 inhibits apoptosis is an avenue for future research. To formally examine the above hypothesis the macrophage model system could be used. Preliminary experiments would include infection with *M. tuberculosis* strains where *mpt64* is deleted or over-expressed, coupled with the measurement of apoptotic markers. It has recently been determined that necrosis is advantageous to the bacterium. Whether MPT64 only inhibits apoptosis or pushes the host towards necrosis could also be examined [63]. Microarray studies of the host cell may provide insight into signaling pathways that are affected. This approach could be combined with experiments using 5-lipoxygenase or prostaglandin E synthase deficient mice, which result in increased apoptosis or increased necrosis, respectively, during a mycobacterial infection. Therefore these mice would be model systems to determine whether *M. tuberculosis* over-expressing MPT64 alter these host-processes. A hypothesized outcome would be that MPT64 over-expressing strains have greater bacterial burden and histopathology than wild-type *M. tuberculosis*, although it remains possible that expression of MPT64 needs to be at an 'ideal' level, such that both under-expression and over-expression of this bacterial protein negatively impact on the outcome of infection.

b) What is the function of Rv1985c?

As noted in Chapter 2, complementation of the RD2 knock-out strain with *Rv1985c-Rv1986* partially restored lung bacterial burden, implicating a role for these genes in virulence. It has been shown previously that *Rv1985c* is upregulated in response to hypoxic conditions meant to mimic latency [143]. Determining the genes regulated by *Rv1985c* could provide insight into mycobacterial latency and persistence. *M. tuberculosis* strains where *Rv1985c* has been deleted could be used to infect C57BL/6 mice to investigate long-term persistence or alternatively could be introduced into the Cornell model of latency. Long-term infection experiments revealed RD1 deficient H37Rv to be more virulent than BCG despite comparable phenotypes at early time points [133]. In our studies (described in detail in Chapter 2) time points beyond 6 weeks were not investigated. This would be a logical next experiment to determine whether *Rv1985c* affects long-term persistence.

2. The role of RD2 in vaccination

Having demonstrated that RD2 is required for virulence in *M. tuberculosis*, the second report of this thesis describes its role in vaccine protection. It has been hypothesized that the ongoing *in vitro* evolution of the BCG vaccine favored the deletion of virulence-associated genes. Consequently it is believed that the vaccine has become over-attenuated [108]. As described in Chapter 1, early BCG strains are predicted to be more protective than late strains [120]. The results presented in Chapter 3 demonstrate that the presence of RD2 in BCG increases both the ability of the vaccine to disseminate and its immunogenicity. Additionally, RD2 containing vaccines protected better against

disseminated TB, but did not convey any increase in pulmonary protection in a murine model, despite increased antigenic repertoire. This supports the hypothesis that increased immunogenicity does not necessarily result in better protective efficacy [197]. Our findings have several implications for vaccine design.

a) Can RD2 antigens stimulate better markers of protection?

First, traditional immunological markers such as IFN-γ are not the only indicators of protection, thus additional and novel markers are still needed. A potential candidate to investigate is IL-2. This cytokine has been associated with central memory T-cells, whereas IFN-γ is associated with effector memory T-cells [310]. It has been hypothesized that central memory T-cells are important for protection, and that improving the induction of this subset is would enhance the effectiveness of BCG [103]. As noted in Chapter 1, a number of proteins encoded by RD2-genes are highly immunogenic in different animals ranging from mice to cattle [148, 278, 287, 288]. Determining if any of these antigens induce IL-2 production and central memory T-cells could help optimize vaccine design. Additionally, these antigens may have potential as boosters to stimulate pre-existing immunity.

b) Can RD2 antigens protect against other forms of disease?

Expanding the antigenic repertoire in BCG will only increase protection if those antigens are unique to *M. tuberculosis,* or provide protection against different forms of the disease (i.e. latency). Recent evidence has shown that Rv1986 is an antigen associated with latent tuberculosis (Gideon et al – in submission). This discovery, coupled with a

hypothesized role for *Rv1985c* during hypoxic conditions, makes these two genes interesting candidates for study. Complementation of BCG Pasteur with *Rv1985c*, *Rv1986* or this dyad could reveal the conditions required for their regulation, and whether each requires the other for optimal function. Also of interest would be to determine whether BCG complemented with *Rv1985c-Rv1986* would provide superior long-term protection in a mouse compared to conventional BCG.

Studies using collected human samples will likely be the only approach to determine whether Rv1986 enhances the BCG vaccine. The aforementioned studies revealed that Rv1986 is a latency-associated T-cell antigen, and further characterization of these T-cells is required. As an initial experiment, Rv1986-specific T-cells could be isolated and their antimycobacterial properties examined through *in vitro* and *ex vivo* methods. If these cells are more adept at controlling mycobacterial infection, a BCG strain engineered to over-express Rv1986 could be considered for clinical trials. It has been demonstrated that BCG efficacy is improved when the vaccine is supplemented with antigens from *M. tuberculosis* [127, 309, 311]. Therefore BCG over-expressing one of these antigens in addition to Rv1986 may provide enhanced protection against acute infection and latent disease.

3. The role of NOD2 in vaccination

Chapter 4 was focused on the stimulation of host receptors by the bacterium, and how this affected the generation of immunity. Specifically, we examined the role of *Nod2* in vaccine efficacy. The results demonstrated that *Nod2* is required for maximal vaccine protection, as *Nod2*$^{-/-}$ mice are not fully-immunodeficient (as compared to say SCID

mice), but do show increased pulmonary pathology. Our data suggest that this protective effect is mediated through the production of key cytokines: IFN-γ and IL-17, by T-cells. Both have known roles in facilitating granuloma formation, and recent studies have highlighted how they interact with each other. Specifically, in vaccinated mice, IL-17 producing T-cells can recruit IFN-γ producing T-cells to the lungs following *M. tuberculosis* infection [193]. Therefore, we proposed a model to explain this *Nod2*$^{-/-}$ associated phenotype. Here the loss of IL-17 producing cells leads to early pathology, and this effect is exacerbated by the lack of IFN-γ producing cells later during infection. Our findings suggest several future research questions, which may have implications for vaccine design.

a) Does the loss of Nod2 affect memory T-cells in vaccination?

Our findings indicate that the loss of *Nod2* affects T-cells in vaccinated mice. Several other reports have also demonstrated a role for NOD2 in T-cell activation and differentiation during infection or ligand stimulation [89, 264, 266]. Therefore, investigation into whether *Nod2* affects the generation of effector memory or central memory T-cells following vaccination could be pursued.

b) Are the proportion of MDP moieties in BCG and *M. tuberculosis* different?

MDP is the predicted ligand for NOD2, and the active component in Freund's adjuvant that is required for the generation of cell-mediated immunity [261]. In mycobacteria there exist chemical variants of MDP, specifically *N*-glycolyl MDP and N-acetyl MDP [312]. It has been recently demonstrated that the *N*-glycolyl MDP variant is

better recognized by NOD2, and consequently is more immunogenic [250]. However, whether BCG has similar proportions of glycolated MDP as *M. tuberculosis* is not known. It is possible that BCG does not elicit full protective immunity because it has less of the immunogenic variant of MDP than *M. tuberculosis*. Mass spectrometry could provide data comparing ratios under *in vitro* conditions. However, even if *in vitro* ratios are similar, the proportion of these MDP molecules may be different under *in vivo* conditions. To investigate this, bacterial mRNA could be harvested from macrophages infected with either BCG or *M. tuberculosis*, and expression levels of the *namH* gene (or its homologue) could be determined. This gene encodes the bacterial hydrolase that converts *N*-acetyl MDP to *N*-glycolyl MDP [312]. Therefore its expression provides an indirect measurement of the amount of *N*-glycolyl MDP. The *namH* homologue present in *M. tuberculosis*, *Rv3818*, has been shown to be part of a gene cluster upregulated during macrophage infection. Therefore *N*-glycolyl MDP levels may change inside the phagosome for this bacterium [313].

c) Does increased stimulation of NOD2 increase the protective immune response?

The finding that *N*-glycolyl MDP is more immunogenic [250], suggests that vaccine protection could be enhanced by increased stimulation of NOD2. This could be accomplished by engineering a BCG strain to over-express its *namH*-homologue, *BCG3880*. First Vaccination-challenge experiments in mice, where the protective efficacy of *namH* over-expressing BCG strains is compared to wild-type BCG, should be performed. As a control, one could also generate a BCG strain that fails to produce *N*-glycolyl entirely, through the targeted disruption of *namH*. If it is demonstrated that

BCG over-expressing *namH* increases protection, it will be necessary to fully characterize the immune response generated by the vaccine. Particular attention should be focused on whether this engineered vaccine enhances the number of antigen-specific T-cells. It has been shown that NOD2 triggers autophagy in dendritic cells, which is a process that is important for antigen presentation by BCG [256, 314]. Accordingly BCG over-expressing the ligand for NOD2 would have increased antigen presentation. If this phenomenon were demonstrated, it would provide a method for priming the host with any number of *M. tuberculosis* antigens. A possibility would be to create a BCG strain that over-expresses *namH*, Rv1986 (a latency antigen), and an *M. tuberculosis* associated antigen (such as ESAT-6). Theoretically, this vaccine would allow the host to develop T-cells that would protect against different forms of disease.

Perspectives

"I feel as if I were really beginning to work now," said Martin. "This new quinine stuff may prove pretty good. We'll plug along on it for two or three years and maybe we'll get something permanent – and probably we'll fail."

Arrowsmith, Sinclair Lewis

The method of finding candidate virulence genes, determining their mechanism of action in the host cell, and evaluating their role in vaccination remains the best approach for improving the vaccine. A prevailing opinion in vaccinology proposes that the evolution of BCG has created a vaccine that is over-attenuated to the point of impotence [108]. If true, the replacement of missing genes should improve the vaccine [315]. This work demonstrates that finding candidate virulence genes, determining their mechanism

of action in the host cell, and evaluating their role in vaccination remains a promising approach. Yet, the overall process is more complex and nuanced, with potential dissociation between desirable and undesirable attributed of a vaccine. As shown here, genes required for immunogenicity and virulence do not always enhance vaccine protection. However, the study of these genes enhances our understanding of the host-pathogen interactions responsible for disease.

Improving the BCG cannot simply involve the addition of factors to the bacterium, unless those enhancements also lead to better recognition by the host. Additionally further study in host immune pathways allows us to determine alternative ways to activate them in deficient individuals. In conclusion, an understanding of what constitutes a protective immune response against TB will only be achieved through study of the interplay between the BCG vaccine and the host.

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