CIRICULUM VITAE

Patrick W. Kerns
Email: patrick.kerns@som.umaryland.edu
pwkerns@gmail.com

EDUCATION

August, 2007 to Present
University of Maryland School of Medicine
MD/PhD Dual Degree Program
Baltimore, MD

PhD in Molecular Microbiology and Immunology to be conferred August, 2014

Passed Step 1 of the USMLE Boards – July, 2009
Admitted to candidacy for PhD in Molecular Microbiology and Immunology – November, 2010

September, 2002 to May, 2006
Johns Hopkins University, Krieger School of Arts and Sciences
B.S in Molecular and Cellular Biology
Baltimore, MD, GPA = 3.73
Beta Beta Beta Honor Society

June 1998 to June 2002
Montgomery High School
Skillman, NJ

AWARDS/HONORS

2007 to 2008 Academic Year
Nataro Scholar
University of Maryland School of Medicine
MD/PhD Program

EXPERIENCE

August, 2009 – Present
Graduate Research Assistant – Shirtliff Lab
University of Maryland School of Medicine, Baltimore, MD

June, 2009 to August, 2009
Research Lab Rotation – Shirtliff Lab
University of Maryland School of Medicine, Baltimore, MD

June, 2008 to August, 2008
Research Lab Rotation – Nataro Lab
University of Maryland School of Medicine, Baltimore, MD

Working in the laboratory of Dr. James Nataro, I used molecular and cellular biology techniques to improve understanding of the role of the extra-cellular matrix protein fibronectin in the pathogenesis of entero-aggregative *E. coli* (EAEC)

June, 2006 to June, 2007
Research Technologist II
Johns Hopkins School of Medicine, Oncology Department

Investigated the role of gene promoter regulation in the pathogenesis of hematological malignancies using molecular biology techniques to determine promoter methylation levels in patient and cell culture samples. Conducted correlative research on patient samples from a clinical trial of a combined demethylating agent and histone deacetylating agent for treatment of myelodysplastic syndrome (MDS).

May - August, 2005
Neuroscience Internship
Wyeth Research, Princeton, NJ

Preformed a critical role in a drug development team by utilizing a cell-based assay combining a stable cell line and atomic absorption spectroscopy to determine the effect of numerous proprietary compounds on the KCNQ family of ion channels. Additional responsibilities included presenting the data and conclusions at team meetings and planning a schedule for efficient testing and side projects.

September, 2004 -May, 2005
Undergraduate Research Assistant (for academic credit)
Johns Hopkins University, Baltimore, MD

Working in the laboratory of Dr. Joel Schildbach I developed a purification strategy for *E. coli* TrbB protein minus signal peptide, purified samples for use in x-ray crystallographic, mass-spectroscopy, and other biochemical tests to elucidate TrbB's role in the Type IV secretory apparatus and for structural determination.

January - May 2004
Laboratory Assistant,
Johns Hopkins School of Public Health, Baltimore, MD

Working in the laboratory of Dr. Laura Kielkopf I prepared laboratory reagents, preformed established lab protocols including desalting DNA, DNA purification, as well as equipment and supply procurement.

May - August 2003
Molecular Biology Internship,
Wyeth Research, Princeton, NJ

As an intern in the Neuroscience Department I constructed a fusion plasmid for rapid determination of recombinant protein solubility in *E. coli* cells.

PUBLICATIONS

1. Fandy TE, Herman JG, **Kerns P**, *et al.* (2009) Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies. *Blood* **114**: 2764-2773.
2. Harro JM, Peters BM, O'May GA, Archer N, **Kerns P**, Prabhakara R & Shirtliff ME (2010) Vaccine development in *Staphylococcus aureus*: taking the biofilm phenotype into consideration. *FEMS Immunol Med Microbiol* **59**: 306-323.
3. **Kerns PW**, Ackhart DF, Basaraba RJ, Leid JG & Shirtliff ME (2014) *Mycobacterium tuberculosis* pellicles express unique proteins recognized by the host humoral response. *Pathog Dis* **70**: 347-358.

PRESENTATIONS

Poster entitled "*Mycobacterium tuberculosis* biofilms express a distinct set of immunogenic proteins compared to shaking-culture"
Sept. 29 – Oct. 4, 2012
6th Annual ASM Conference on Biofilms, Miami, FL

Poster entitled "*Mycobacterium tuberculosis* Biofilm Exhibits Heterogeneous Transcriptional Activity by Fluorescence in Situ Hybridization"
May 21-24, 2011
111th General Meeting of the American Society for Microbiology
New Orleans, LA

June, 2010
GPILS Program in Molecular Microbiology and Immunology Graduate Student Presentations
University of Maryland School of Medicine, Baltimore, MD
"Identification of Immunogenic *Pseudomonas aeruginosa* Biofilm Proteins for Biofilm Vaccine Creation"

August, 2009
MD/PhD Summer Research Presentations
University of Maryland School of Medicine, Baltimore, MD
"Identification Of *Klebsiella Pneumoniae* Biofilm Immunogenic Proteins For Use in a Recombinant Protein Vaccine"

September, 2008
Medical Student Research Day
University of Maryland School of Medicine, Baltimore, MD
"Progress towards understanding the role of fibronectin in the pathogenesis of Enteroaggregative *E. coli*"

PROFESSIONAL AFFILIATIONS

2007 – Present
Member of the American Medical Students Association (AMSA)

2011 – Present
Member of the American Society for Microbiology (ASM)

ABSTRACTS

Kerns PW, Basaraba RJ, Ackart DF, Ojha A, Nuermberger EL, Shirtliff ME. *Mycobacterium tuberculosis* biofilms express a distinct set of immunogenic proteins compared to shaking-culture. 6th American Society for Microbiology Conference on Biofilms. Miami, FL. Sept. 29 - Oct. 4, 2012.

Kerns PW, Moter A, Peters BM, Leid JG, Basaraba RJ, Ackart DF, Shirtliff ME. *Mycobacterium tuberculosis* Biofilm Exhibits Heterogeneous Transcriptional Activity by Fluorescence in Situ Hybridization. 111th General Meeting of the American Society for Microbiology. New Orleans, LA

ABSTRACT

Title of Dissertation: Development and Testing of a Five-Subunit Biofilm Vaccine for the Prevention of Pulmonary Tuberculosis

Patrick Kerns, Doctor of Philosophy, 2014

Dissertation Directed by: Mark Shirtliff, Ph.D., Associate Professor, Department of Microbial Pathogenesis.

Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis, a disease that kills 1.4 million people and infects 8.7 million people worldwide every year. This bacterium has traditionally been studied using *in vitro* shaking cultures including detergents, which force the bacteria into an artificial style of planktonic growth. We utilized a static model of MTB growth that allows the bacteria to naturally grow as a biofilm pellicle at the air-liquid interface and utilized this model to identify antigens common to both shaking and biofilm cultures through a process of two-dimensional gel electrophoresis combined with western blotting. Having identified proteins that were both immunogenic and produced at three biofilm time points (three, five, and seven weeks) as well as in our control shaking culture, we proceeded to recombinantly express five in their full length forms. Three proteins were purified in their native forms and two proteins first denatured during the purification process and then refolded. We vaccinated female BALB/c and C3HeB/FeJ mice using 25 μg of each of the five antigens combined with either cyclic-di-GMP or DDA/MPL as an adjuvant over the course of three vaccine trials. While all vaccine formulations elicited a strong immune humoral response with high IgG1 and IgG2a titers against the vaccine antigens, there were no significant reductions in the number of CFU/g tissue in spleens or lungs of vaccinated animals

compared to unvaccinated controls. Overall lung burden in BALB/c mice was significantly reduced in BCG vaccinated animals ($p < 0.05$) while there was a trend for bacterial burden reduction in animals vaccinated with the antigen subunits and DDA/MPL adjuvant ($p = 0.055$). In addition, the difference in the immune responses of the difference strains of mice was apparent when the T-helper responses of BALB/c and C3HEB/FeJ mice were evaluated. In the case of testing the DDA/MPL vaccine, BALB/c mice produced a strong antigen-specific IFN-γ response from vaccinated mouse splenocytes compared to the C3HeB/FeJ mice that produced significantly more IL-10 relative to IFN-γ. This indicated the fundamental differences in mouse strain responses to vaccination with BALB/c mice skewing towards a Th1 response relative to C3HeB/FeJ mice that had a more Th2/Treg skewed immune response. In addition, there were fundamental differences in the properties of the adjuvant-dependent immune responses. While DDA/MPL adjuvant showed a trend towards challenge protection, we found that cyclic-di-GMP-treated animals did significantly worse than control animals with an accompanying elevation in lung IFN-γ levels. These results call into question the use of cyclic-di-GMP adjuvant for tuberculosis vaccines, since it may be counterproductive to a protective immune response to MTB. Furthermore, the recombinant proteins 35kdag, CeoB, Mkl, and TB31.7 warrant further investigation as potential diagnostic markers for active or latent tuberculosis.

Development and Testing of a Five-Subunit Biofilm Vaccine for the Prevention of Pulmonary Tuberculosis

by
Patrick W. Kerns

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2014

To my parents Edward H. and Nancy E. Kerns for their tireless work, ceaseless support, and unconditional love. I’m keenly aware that most people live their lives without such fine relations and I consider myself to have been gifted with the most excellent of parents. Also to my superlative mentor Mark E. Shirtliff who encouraged my intellect, taught me the fine points of communication, and made me feel like family.

ACKNOWLEDGEMENTS

Dr. Mark Shirtliff made this work possible by encouraging my desire to work on tuberculosis and by providing the resources to do so. His guidance on this project and constant support are a debt that cannot be repaid. The entire Shirtliff lab over the years were an immense help and joy to work with including Yvonne Acherman, Devon Allison, Nate Archer, Jeffery Freiberg, Jan Harro, Brian Peters, Graeme O'May, Ranj Prabakhara, Megan Prior, Lisa-Marie Schlecht, and Zhenbo Xu.

Dr. Anil Ojha's pioneering work with MTB biofilms was the direct inspiration for this research project and his frequent advice and support as a PhD Committee member are greatly appreciated.

Our initial western blot screens of immunogenic proteins would have been impossible without the infected guinea pig serum from Dr. Randall Basaraba and David F. Ackart. Thanks also go to Dr. Basaraba for his expert evaluation of histological preparations from the vaccine trials.

Dr. Eric Nuermberger and his lab at Johns Hopkins, especially Si-Yang Li, made the testing of this vaccine possible in their ABSL3 facilities. Their expertise in MTB animal infection models was indispensible.

My committee members including doctors Thomas Blanchard, Bob Ernst, Martin Flajnik, Eric Nuermberger, and Anil Ojha were invaluable for their experience and perspective on this research and for numerous helpful suggestions from committee meeting and personal discussions. Committee member emertius Dr. Jeff Lied was also invaluable in the early planning of this research. Their service on my committee is greatly appreciated.

This work would not have been possible without the support of the MD/PhD program including Dr. Terry Rogers, Nancy Malson, Dr. Michael Donnenberg, Dr. Achsah Keegan, and Jane Bacon. Furthermore, the GPILS Graduate Program in Molecular Microbiology and Immunology including Dr. Nicholas Carbonetti and June Green were extraordinarily supportive.

The advice of Dr. C.S. Raman was essential in troubleshooting the cloning, expression, and purification of recombinant proteins for the vaccine. The members of Dr. Bob Ernst's laboratory were valued participants in scientific discussions and were kind enough to lend us regents when we ran out.

My fellow students, in both my medical and graduate school classes, were a constant source of perspective, information, and fun. Thank you all.

Table of Contents

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATION ix

CHAPTER 1: INTRODUCTION 1

Part 1: Biofilm formation in non-tuberculous mycobacteria 4

Part 2: Evidence of biofilm formation in mycobacterium tuberculosis 13

Part 3: Virulence factors of *M. tuberculosis* 17

Part 4: Animal Models of Tuberculosis 21

Part 5: The host immune response to mycobacterium tuberculosis 27

5A. Innate immune response 27

5B. Cell-mediated immune response 34

5C. Humoral immune response 38

Part 6: Conclusions 41

Part 7: Hypothesis and Specific Aims 43

CHAPTER 2: MTB *IN VITRO* BIOFILMS EXPRESS A DISTINCT SET OF IMMUNOGENIC PROTEINS COMPARED TO SHAKING CULTURE 46

Part 1: Background and Significance 46

Part 2: Materials and Methods 49

Part 3: Results 55

Part 4: Discussion 63

CHAPTER 3: PROTECTIVE EFFICACY AND IMMUNE RESPONSE TO A RECOMBINANT, 5 SUBUNIT BIOFILM VACCINE FOR THE PREVENTION OF MTB 69

Part 1: Background and Significance 69

Part 2: Materials and Methods 72

Part 3: Results 83

Part 4: Discussion 99

CHAPTER 4: CONCLUDING REMARKS 105

APPENDIX I: Reagent Recipes 111

REFERENCES 1113

LIST OF TABLES

Table		Page
Table 1.1	Summary of Animal Models of Tuberculosis Infection …...	21
Table 2.1	Comprehensive list of immunogenic proteins identified grouped by expression patterns……………………………...	58
Table 2.2	Sequenced Genomes Used for Conservation Analysis……..	60
Table 2.3	Predicted structure of pellicle-specific proteins. …………...	67
Table 3.1	Primers used in this Research………………………………	74
Table 3.2	Plasmids used in this Research……………………………..	74
Table 3.3	Description of Vaccine Trials………………………………	78
Table 3.4	Human Serum Reactivity to recombinant antigens used in this study ………………………………………………….	84
Table 3.5	Average Bacterial Burdens by Group for All Trials………..	87
Table 3.6	BALB/c to C3HeB/FeJ Mouse IFN-γ Release Ratio by Group and Antigen ………………………………………	95
Table 3.7	C3HeB/FeJ to BALB/c Mouse IL-10 Release Ratio by Group and Antigen ……………………………………...	96

LIST OF FIGURES

Figure		Page
Figure 1.1	World incidences of tuberculosis, 2011………………………...	2
Figure 1.2	*M. marinum* 14-day old biofilm……………………………...	5
Figure 1.3	Extensive *M. ulcerans* infection of the hand of a Nigerian Patient………………………………………………………..	7
Figure 2.1	Static Tuberculosis Biofilm…………………………………….	56
Figure 2.2	Two-dimensional gel electrophoresis gels and matched western Blots……………………………………………………………….	57
Figure 2.3	Pellicle Specific protein conservation…………………………..	62
Figure 2.4	Phylogenetic tree of MTB and related mycobacterium………...	62
Figure 3.0	Diagram of vaccine trial elements and timing of vaccinations, boosts, challenge and harvest………………………………...	79
Figure 3.1	Purified recombinant protein subunits **i**ncluded in the vaccine...	83
Figure 3.2	Challenge-Day Vaccine Immune Response…………………….	85
Figure 3.3	Trial K2 Humoral Response……………………………………	86
Figure 3.4	Trial K2 CFUs/Lungs, Lung Pathology, and Lung Cytokines…	88
Figure 3.5	Trial K3 Humoral Response……………………………………	90
Figure 3.6	Trial K3 Lung and Spleen Bacterial Burden…………………….	91
Figure 3.7	Trial K4 Humoral Response – BALB/c………………………...	92
Figure 3.8	Trial K4 Humoral Response – C3HeB/FeJ……………………..	93
Figure 3.9	IFNy Release Assay……………………………………………	94
Figure 3.10	IL-10 Release Assay……………………………………………	95
Figure 3.11	Rescaled Figures………………………………………………..	96
Figure 3.12	Trial K4 Bacterial Burdens……………………………………..	98
Figure 3.13	Trial K4 right lung lobe weight (BALB/c mice)……………….	99

LIST OF ABBREVIATIONS

35kd_ag: 35 kilodalton antigen
ADCC: Antibody dependent cell-mediate cytotoxicity
Ag85A: Antigen 85A (a.k.a. FbpA or fibronectin binding protein)
AFB: Acid Fast Bacilli (or diagnostic sputum smears stained by this method)
CD#: Cluster of differentiation # (e.g. CD4, CD8, CD1d)
CeoB: Complementation of *E. coli oxyR* B
CMI: Cell-Mediated Immunity
C-di-GMP: Cyclic di-GMP
DDA/MPL: Dimethyldioctadecylammonium liposomes with monophosphoryl lipid-A
DOTS: Direct Observed Therapy, Short course
ELISA: Enzyme-linked Immunosorbant Assay
FcR: Fragment constant receptor
FISH: Fluorescence *in situ* hybridization
GPL: Glycopeptidolipid
H&E: Hematoxylin and Eosin
HbhA: Heparin-binding hemaglutinin A
IFNγ: Interferon gamma
IL-#: Interleukin # (e.g. IL-10, IL-12)
IRF: Interferon regulatory factor
LAM: Lipoarabinomannan
MAC: *Mycobacterium avium* complex (*M. avium* and *intracellulare*)
MBEC: Minimum Biofilm Eradication Concentration assay system
MDR-TB: Multidrug resistant tuberculosis
Mkl: Probable ribonucleotide transport ATP-binding protein Mkl
MTB: *Mycobacterium tuberculosis*
MTBC: *Mycobacterium tuberculosis* complex (*M. tuberculosis, bovis, africanum, microti*, and *canetti)*
MVA-85A: Modified Vaccinia Ankara Antigen 85A vaccine
NK: Natural killer cell
NTM: Non-tubercular mycobacteria
PRR: Pattern recognition receptor
RGM: Rapidly-growing mycobacteria (e.g. *M. smegmatis*)
SGM: Slow growing mycobacteria (e.g. *M. avium*)
STAT1: Signal Transducers and Activators of Transcription 1
TA: Toxin-antitoxin
TB: Tuberculosis (disease)
TB31.7: Tuberculosis 31.7 kilodalton protein
TGF-β: Transforming growth factor beta
TLR#: Toll-like receptor # (e.g. TLR4, TLR9)
TNF-TNF-αR: Tumor necrosis factor alpha/TNF-α receptor
WHO: World Health Organization
XDR-TB: Extensively drug-resistant tuberculosis

CHAPTER 1: INTRODUCTION

Tuberculosis (TB) is a disease that has plagued humans for all of recorded history. Numerous archeological studies have demonstrated evidence of TB in human remains as early as c. 5000 BCE (Suzuki, *et al.*, 2008) with the estimated speciation of the causative organism 15,000 to 20,000 years ago (Sreevatsan, *et al.*, 1997). Tuberculosis in humans is caused by the members of the Mycobacterium *tuberculosis* complex including the obligate human pathogens *Mycobacterium tuberculosis* (MTB), *Mycobacterium africanum*, and *Mycobacterium canetti* as well as *Mycobacterium bovis* which causes disease in humans, cows, and other mammals (Brosch, *et al.*, 2002). While primarily known for the pulmonary infection accompanied by wasting, TB can involve most tissues in the body and in endemic countries is a cause of child death from disseminated tuberculosis, especially tuberculosis meningitis (Principi & Esposito, 2012).

TB arises in about 9 million people worldwide (Fig. 1.1) and is responsible for approximately 1.4 million deaths per year (WHO, 2012). This is down considerably from the 2.5 million deaths per year recorded in 1990 (Dolin, *et al.*, 1994). Much of this decrease is a result of the World Health Organization's (WHO) Stop TB campaign which has cured 36 million people between 1995 and 2008 has prevented an estimated 8 million deaths(World Health Organization, 2013). The Stop TB campaign aims to reduce the global burden of TB substantially by 2015 using techniques such as Direct Observed Therapy, Short Course (DOTS) that is a standardized six-month treatment regime where the patients are monitored by healthcare workers for at least the first two months to help ensure completion of the full course of anti-mycobacterial drugs. The Stop TB program also aims to strengthen primary care resources while monitoring TB-HIV co-infection and the emerging threat of Multidrug and Extensively Drug-Resistant TB (MDR and XDR TB)(World Health Organization, 2006).

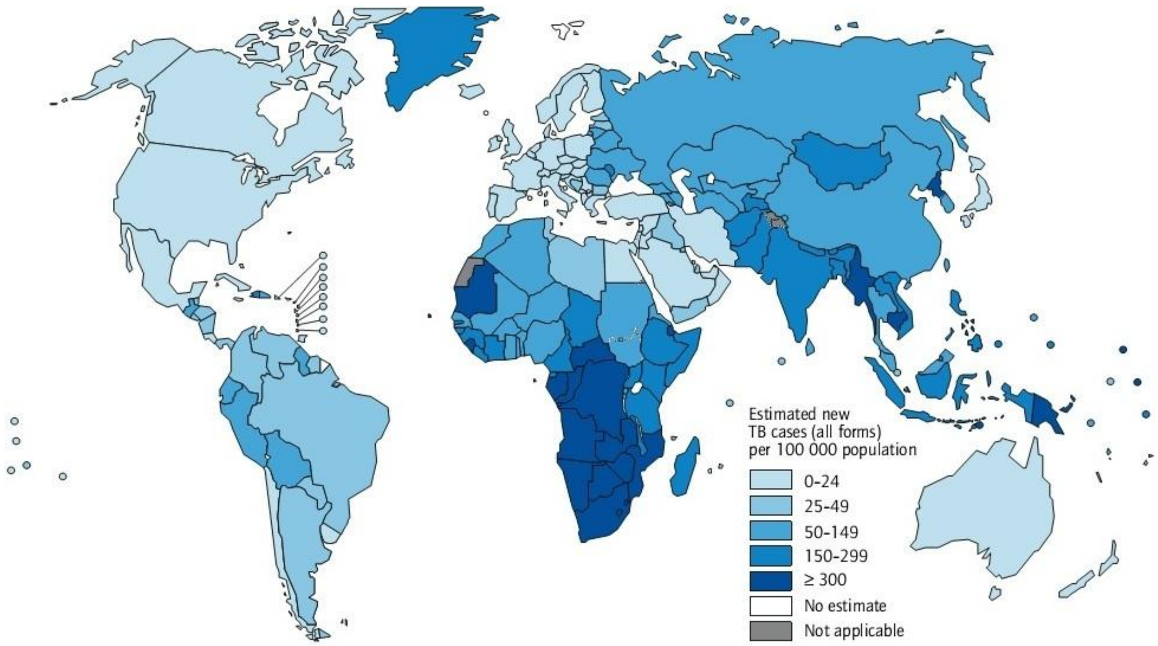


Figure 1.1 World Incidence of Tuberculosis, 2011 (WHO)

Despite this progress against TB using antitubercular chemotherapy, TB remains a global health challenge because of its remarkable ability to persist within both asymptomatic hosts and in patients actively being treated for the disease. When exposed to *Mycobacterium tuberculosis,* (MTB), the causative agent of tuberculosis, ~95% of people will either contain or eliminate the bacteria while 5% will go on to develop active tuberculosis. Of those who contain the disease, approximately 5-10% will experience a reactivation of the disease within their lifetime (Rook, *et al.*, 2005). Risk factors for reactivation include HIV, immunosuppression, diabetes, smoking, and alcohol abuse (Flynn & Chan, 2001, Gupta, *et al.*, 2012). Once the disease has reactivated, it can develop necrotic foci of infection in the lungs, which progress to cavitary lesions when they erode into a bronchus. At this point, the bacterial are freely transmitted as an aerosol that are created as a result of coughing (Helke, *et al.*, 2006).

When active TB patients are treated, modern therapeutic practice requires a minimum of a six-month therapy involving four different antitubercular drugs, with the most common regimen being isoniazid, rifampin, ethambutol, and pyrazinamide (Hopewell, *et al.*, 2006). This lengthy treatment is required because, while the bacterial die rapidly in the lungs during the first two weeks of therapy, a population of phenotypically (not genetically) antibiotic resistant bacteria persist in a viable state despite months of treatment (Jindani, *et al.*, 2003). Stopping treatment before four-to-six months frequently results in relapse and runs the risk of selecting for bacteria resistant to one or more of the prescribed antibiotics (M'Imunya J, *et al.*, 2012).

The abilities of MTB to persist in a discrete focus of infection, demonstrate increased tolerance to antibiotics, and seed other body sites are all qualities of a biofilm infection. Biofilm infections are characterized by four criteria: (1) the bacteria are adhered to a surface, (2) microbes are detectable *in vivo* as aggregates associated with a matrix, (3) the infection is localized with the possibility for dissemination, and (4) antibiotic treatment is either ineffective or the required treatment is lengthy (Parsek & Singh, 2003). These infections are particular pernicious because of this phenotypic, not genetic, tolerance to antibiotics that is gained by bacterial growth in the biofilm. Other biofilm infections, such as bacterial endocarditis or medical implant infections, are often treated by surgical resection or debridement of the infected tissue/implant (Parsek & Singh, 2003). TB has been treated surgically in the past, prior to the development of the six-month four-drug treatment course. Lung resection remains the last resort of recalcitrant cases of XDR-TB (Iddriss, *et al.*, 2012). Beyond the morbidity and mortality associated with biofilm infections, there is a dramatic cost associated with additional surgeries required to treat them.

Part 1 of this chapter will review evidence for biofilm formation and the role it plays in pathogenesis of non-tuberculous mycobacteria (NTM) including *Mycobacterium avium* and *Mycobacterium ulcerans*. Part 2 examines the current evidence that MTB grows in a biofilm, and the implications of this mode of growth. MTB virulence factors will be covered in Part 3. Part 4 will focus on the host innate and adaptive immune response to MTB infection and will underscore the critical nature of the host cell-mediate immune (CMI) response and the role of humoral immunity in immunity to tuberculosis. In Part 5 we will examine the conclusions to be drawn from the current literature and in Part 6 will layout the hypothesis and goals of this research project of the host immune response to TB.

Part 1: Biofilm formation in non-tuberculous mycobacteria

M. tuberculosis complex bacteria (*M. tuberculosis, bovis, canetti, africanum,* and *microti)* are, unfortunately, not the only pathogenic mycobacteria. Non-tuberculous mycobacteria (NTM) are broadly divided into slow growing mycobacteria (SGM), which typically take more than seven days to culture and rapidly-growing mycobacteria (RGM). SGM include *M. marinum,* which predominantly cause soft-tissue infection, *M. ulcerans,* which causes the disfiguring Buruli ulcer, and the *M. avium* complex (MAC, *M. avium* and *intracellulare*), best known for causing lung infection in patients with underlying structural lung disease and disseminated infections in immunosuppressed patients. The

RGM include *M. fortuitum, M. abscessus,* and *M. chelonae* which have caused increasing amounts of human infections recently (Falkinham, 2002, Primm*, et al.*, 2004). An important common link between these NTMs is that they are environmental bacteria causing infections in normal or immunocompromised hosts. In particular, infections due to *M. avium* and *M. intracellulare* are a major concern for HIV/AIDS patients because both organisms are commonly found in hot and cold water lines as well as soil and dust (Guirado*, et al.*, 2012, Zheng & Fanta, 2013). Where these organisms live free in the environment, biofilm formation plays an important role in their survival. This ability to form biofilms also makes them more tenacious pathogens in humans.

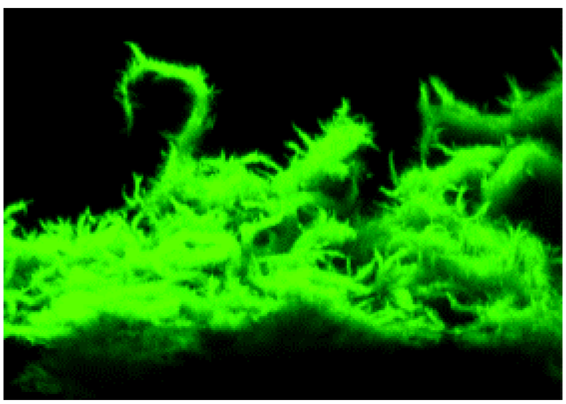


Figure 1.2: *M. marinum* 14 day biofilm (GRP expressing strain GFP-expressing *M. marinum* G13R) (Hall-Stoodley*, et al.*, 2006).

Mycobacterium marinum

Mycobacterium marinum is a SGM that infrequently causes disease in humans, but in fish it is capable of causing a deadly disseminated infection characterized by granuloma formation (Hall-Stoodley, *et al.*, 2006). Human cases of *M. marinum* infection are normally granulomatous skin infections where the bacterium gains access through minor wounds from an environmental source. Because cases of this disease are often seen in those with exposure to aquariums, the disease has been nicknamed "fish-tank granuloma" (Edelstein, 1994).

M. marinum has been demonstrated to form robust biofilms (Fig. 1.2) on solid substrates during *in vitro* growth (Hall-Stoodley, *et al.*, 2006). Studies of the antibiotic susceptibility of *M. marinum* bacteria grown as a biofilm using the Minimum Biofilm Eradication Concentration (MBEC) assay system demonstrate that, unlike the vast majority of biofilms, *M. marinum* did not have any increased antibiotic resistance in biofilm compared to planktonic growth (Bardouniotis, *et al.*, 2003). Mutations in a critical lipooligosaccharide synthesis gene (LOS-II) decreased *M. marinum* survival in macrophages accompanied by decreased sliding motility and biofilm formation (Ren, *et al.*, 2007). Similarly, transposon-inactivation of the gene coding for the cell-wall protein PPE38 caused deficiencies in sliding motility and biofilm formation as well as in cord formation and macrophage phagocytosis. PPE38 appeared to be necessary for stimulating TNFα and IL-6 from infected macrophages, which may promote disease progression through excessive inflammation (Dong, *et al.*, 2012). While not especially deadly to humans, *M. marinum* is economically important because of its impact on fish populations (Knibb, *et al.*, 1993, Burge, *et al.*, 2004). *M. marinum*, however, is an excellent example of the pattern of many mycobacteria pathogens in that it is a robust biofilm former with a heavy environmental reservoir and is usually pathogenic in the setting of host immune system compromise. This compromise can be due to skin barrier compromise, specific mutations, or other host disease processes.

Figure 1.3: Extensive *M. ulcerans* infection of the hand of a Nigerian patient (Chukwuekezie, *et al.*, 2007).

Mycobacterium ulcerans infection – Buruli ulcer

Mycobacterium ulcerans is a SGM responsible for a skin and soft tissue infection known as Buruli ulcer (or Bairnsdale ulcer in Australia) that is common in West and Central Africa (Pidot, *et al.*, 2010). It is the third most common mycobacterial disease after TB and leprosy. *M. ulcerans* recently evolved from *M. marinum* through a process of reductive evolution and acquisition of a key virulence plasmid (Stinear, *et al.*, 2004, Stinear, *et al.*, 2007, Yip, *et al.*, 2007). This virulence plasmid encodes the necrotizing, immunosuppressive cytotoxin mycolactone, which gives *M. ulcerans* the ability to produce widespread tissue destruction (Walsh, *et al.*, 2010). The bacteria are acquired directly from the environment through minor skin trauma, or via an arthropod vector; the disease is not transmissible from person-to-person (Walsh, *et al.*, 2010). Infection begins as a painless, slow-growing nodule that eventually necrotizes, producing an ulcerative lesion with a characteristic undermined edge (van der Werf & Stienstra, 2011). As the disease progresses, underlying tissues can become involved leading to osteomyelitis, joint contractures, or disseminated disease. Treatment for nodules and small ulcers can be with antibiotics alone, but adjunctive surgery to remove dead and diseased tissue is currently standard for large (>5 cm) lesions (Walsh, *et al.*, 2010). The fact that the bacteria grow as a biofilm *in vivo* combined with the necessity of surgery for advanced cases is highly suggestive of a biofilm infection (Parsek & Singh, 2003).

M. ulcerans has been shown to grow in a biofilm both *in vivo* and *in vitro* complete with an extra-cellular matrix containing mycolactone. Additionally the biofilm protects the bacteria against antibiotic treatment and allows for better adhesion in both mammals and insect vectors (Marsollier, *et al.*, 2007). Specifically, the protein *Hsp18* promotes biofilm formation by increasing attachment and aggregation on surfaces (Pidot, *et al.*, 2010). Exposure to algal extracts significantly increased the doubling time of *M. ulcerans in vitro* as well as bolstered biofilm formation and the growth of aquatic plants in areas of endemic Buruli ulcer infection (Marsollier, *et al.*, 2004). A recent study which detected *M. ulcerans* in aquatic plant biofilms also found abundant organisms in the feces of possums suggesting a strong mammalian reservoir (Fyfe, *et al.*, 2010). Taken together, the current evidence suggests that the biofilm mode of growth is involved in the maintenance of an environmental and animal reservoir of *M. ulcerans*. In addition, biofilm growth improves the transmissibility of *M. ulcerans*y via insects to mammalian hosts.

Mycobacterium avium complex

The *Mycobacterium avium* complex (MAC) includes *M. avium* and *M. intracellulare.* MAC disease is the most common NTM infection diagnosed in the United States and poses a significant health threat to immunocompromised individuals (e.g. persons living with HIV with a CD4 count of $<50/\text{mm}^3$) (MacGregor, *et al.*, 2005). These bacteria are commonly isolated from tap water and hospital water systems, and patients can be infected through the gastrointestinal tract or by inhaling the bacteria aerosolized by fixtures such as a showerhead or bathtub inlet (Vaerewijck, *et al.*, 2005, Falkinham, *et al.*, 2008, Nishiuchi, *et al.*, 2009, Beumer, *et al.*, 2010). MAC bacteria can cause a localized lung disease similar to tuberculosis or a disseminated disease, which is less common in the setting of modern highly active antiretroviral therapy for HIV infection (Uchiya, *et al.*, 2013). The treatment for MAC is similar to that for MTB but generally involves fewer drugs administered for longer durations (Griffith, *et al.*, 2007, Brown-Elliott, *et al.*, 2013).

Biofilm formation in *M. avium*, like most NTM, seems to be a strategy for permanent environmental colonization. Growth in the biofilm protects *M. avium* from both chlorine and high-shear conditions found inside water distribution systems (Steed & Falkinham, 2006, Lehtola, *et al.*, 2007, Torvinen, *et al.*, 2007).

Biofilm formation in *M. avium* can be trigged by oxidative stress resulting from signaling with the autoinducer-2 system (Geier, *et al.*, 2008). However, this seems to result from the oxidative stress and doesn't seem to indicate a link between quorum-sensing and biofilm formation in *M. avium*.

Rapidly Growing Mycobacteria – *M. fortuitum*, *M. abscessus*, *M. chelonae*

The rapidly growing mycobacteria group includes *M. abscessus, M. chelonae*, and *M. fortuitum,* which are the agents of a variety of lung, skin and soft tissue, and indwelling medical device infections. In RGM infections, removal of any infected indwelling medical devices is considered necessary for cure (Griffith, *et al.*, 2007). RGM biofilms are resistant to amikacin, ciprofloxacin and clarithromycin (Ortiz-Perez, *et al.*, 2011). This is an important observation because RGM are routinely susceptible to these drugs when cultured in a planktonic state. As a result, these antibiotics have limited success when used to treat RGM infections (Griffith, 2011).

M. abscessus can cause a progressive lung infection in which antibiotics are ineffective and, when it can be safely performed, surgical resection is the optimal treatment (Nessar, *et al.*, 2011). Like *M. smegmatis*, *M. abscessus* is capable of growth with both smooth and rough morphologies in culture. The rough morphotype exhibits less glycopeptidolipid (GPL) synthesis and increased invasion (Howard, *et al.*, 2006). When expressed at highest level during biofilm growth, GPLs make the bacteria less immunogenic by masking cell wall mannosides that could normally induce TNF-α secretion by macrophage stimulation of TLR2 (Rhoades, *et al.*, 2009).

M. chelonae and *M. fortuitum* have been demonstrated to form biofilms *in vitro* on materials common to water distribution systems (Hall-Stoodley, *et al.*, 1998) and are commonly isolated from sewage and tap water (Jin, *et al.*, 1984). *M. chelonae* causes opportunistic infections in a variety of settings including tattooing (Kennedy, *et al.*, 2012), animal bites (Iyengar, *et al.*, 2013), and following surgical procedures (Lee, *et al.*, 2012). Pulmonary infection with *M. chelonae* is rare (Ko, *et al.*, 2013). *M. fortuitum* is also a common environmental isolate and can cause pulmonary disease, skin disease, and osteomyelitis (Kupeli, *et al.*, 2010). There is also a case report of *M. fortuitum* causing endocarditis on a prosthetic heart valve with microscopy demonstrating biofilm adhered to the resected implant (Bosio, *et al.*, 2012).

Biofilm Studies in *M. smegmatis*

M. smegmatis is a RGM that has long been used as a model organism for *M. tuberculosis* because of its non-pathogenicity, genetic and structural similarities to MTB as well as its ability to be easily manipulated genetically. Rarely, *M. smegmatis* can cause disease including skin and soft tissue infections and occasionally, disseminated disease in immunosuppressed patients (Best & Best, 2009). *M. smegmatis* has been a flagship model organism in research into the importance of biofilms in mycobacteria.

M. smegmatis readily forms biofilms. *In vitro*, different colony morphologies reflect changes in the bacterial envelope that reflect biofilm-forming abilities. Specifically, the expression of GPLs by the bacteria results in a smooth colony morphology and correlates with both the ability to form biofilms and sliding motility in culture (Recht, *et al.*, 2000, Agusti, *et al.*, 2008). Bacteria deficient in *rpoZ*, the gene encoding the omega subunit of RNA polymerase which controls the stringent response under conditions of nutrient starvation, are unable to properly incorporate GPLs into the cell wall, and are, therefore, also deficient in biofilm formation (Mukherjee & Chatterji, 2008). *M. smegmatis* not expressing GPLs as a result of a mobile insertion sequence can be identified by their rough colony morphology (Kocincova, *et al.*, 2009). The smooth colonies are less likely to be internalized by macrophages and are less invasive in laboratory models but experience a selective advantage in *in vitro* culture (Kocincova*, et al.*, 2009). As GPLs have been shown to be necessary for attachment to polyvinylchloride in models, smooth colony variants may have better attachment on certain environmental surfaces (Recht*, et al.*, 2000).

All mycobacteria encode two versions of the chaperone GroEL, instead of the single version encoded by most bacteria. GroEL2 is essential for growth but GroEL1 is required only for the growth of mature biofilms. GroEL1 interacts with the type II fatty acid synthesis pathway to affect the production of mycolates such as trehalose dimycolate (TDM) which form part of the bacteria's outer cell wall (Ojha*, et al.*, 2005).

Biofilm has also been shown to increase the frequency of DNA transfer by conjugation, suggesting that biofilm can have a significant influence on horizontal gene transfer of important genetic elements such as antibiotic resistance plasmids (Nguyen*, et al.*, 2010). This process is mediated by the expression of the histone-like protein Lsr2 (Gordon*, et al.*, 2008, Nguyen*, et al.*, 2010).

New work studying the role of polyphosphate (polyP) in *M. smegmatis* has demonstrated that this molecule can have a direct effect on biofilm formation (Shi*, et al.*, 2011). PolyP is a polymer of inorganic phosphate containing numerous high-energy bonds that is synthesized from ATP and found in the cells of all fungi, microbes, plants, and animals (Kulaev, 1979). In bacteria, polyP influences the expression of the sigma factor S, critical for stationary phase gene expression; polyP also has pleiotrophic, pro-survival effects in the setting of nutrient limitation, hypoxia, and in promoting the stringent response (Sureka*, et al.*, 2009). Depletion of polyP has been shown to negatively affect biofilm formation in *Pseudomonas aeruginosa*, *Bacillus cereus*, *Burkholderia pseudomallei*, as well as in *M. smegmatis* (Rashid, *et al.*, 2000, Shi, *et al.*, 2004, Tunpiboonsak, *et al.*, 2010, Shi, *et al.*, 2011). Overexpression of an MTB exopolyphosphatase, an enzyme that degrades polyP in *M. smegmatis,* lowers polyP levels resulting in less biofilm formation and alterations to outer membrane lipid structure (Shi, *et al.*, 2011). Down-regulation of the polyP synthesis enzymes leads to reduced survival of MTB in macrophages (Sureka, *et al.*, 2007, Sureka, *et al.*, 2009). This work suggests that biofilm formation is connected to MTB's ability to survive in adverse environments and may be part of MTB's general response to stress.

Part 2: Evidence of biofilm formation in mycobacterium tuberculosis

Investigation into MTB biofilms is relatively recent; prior to 2008 the only mention of MTB biofilms was a paper that recorded small MTB biofilms on spinal implants (Ha, *et al.*, 2005). Later it was demonstrated that MTB biofilms *in vitro* produce a novel species of free methoxy-mycolic acid compared to their planktonic counterparts. The *in vitro* grown biofilms grew best when the culture vessel was sealed for the first three weeks, after which the vessel cap was loosened producing a rich biofilm growth by the fifth week. These biofilms displayed increased survival after treatment with either rifampicin or isoniazid compared to planktonic cultures or a mutant deficient in biofilm formation. Biofilm cultures displayed the biphasic killing curve commonly associated with persister cells, indicating that the biofilm may contain up to a thousand times as many persisters as the mid-log planktonic cultures they were measured against (Ojha, *et al.*, 2008).

Expanding on the previous paper, Ojha et al. used *M. smegmatis* as a model organism to identify the mechanism for the production of the free methoxy-mycolic acids unique to mycobacterial biofilms. They showed that trehalose dimycolate (TDM) is one substrate for the generation of free methoxy-mycolic acids by a novel serine esterase specific for TDM. Deletion of the gene coding for the serine esterase slowed biofilm formation and reduced levels of free mycolic acids. Furthermore, it was demonstrated that TDM hydrolysis is also carried out in MTB (Ojha, *et al.*, 2010, Yang, *et al.*, 2014).

Taken together these two studies demonstrate that biofilm growth in MTB has a unique phenotype that is altered by mutations that have no effect on planktonic growth. MTB forms aggregates in the absence of detergents that protect the organism from the action of antibiotics, most likely by facilitating the development of quiescent persister cells. Persister cell formation in MTB has been show to be associated with expression of toxin-antitoxin (TA) system genes, which are capable of stochastically inhibiting bacterial growth in a small (~1%) portion of the bacterial population. MTB possess ten such TA systems that are activated under experimental conditions indicating a relatively large genetic armamentarium for persister cell formation (Keren, *et al.*, 2011).

Further studies have demonstrated that the pellicle form of biofilm growth, and the antibiotic resistance it confers, is dependent on the product of keto-mycolic acids that make up the bulk of MTB's outer membrane. MTB mutants with deletion of the *mmaA4* gene are unable to produce either methoxy- or keto-mycolic acid species and are highly susceptible to rifampin, a phenotype that could be complemented by co-culture with pellicle-forming wild-type MTB (Sambandan, *et al.*, 2013).

Other bacterial genetic factors that have been implicated in MTB biofilm formation include *Rv3852*, *pks1*, and *Rv3312A*. *Rv3852* encodes a NAP, or nucleoid-associated protein, that when overexpressed in *M. smegmatis*, the nucleoid structure is altered thereby regulating KasA and KasB, vital genes for the biosynthesis of long-chain fatty-acids, and the chaperone GroEL1 (Ghosh, *et al.*, 2013). The gene *pks1* is alone capable of complementing biofilm development in biofilm defective mutants and the gene's significant sequence variability between MTB strains many explain some variability in MTB biofilm formation (Pang, *et al.*, 2012). The last genetic factor associated with MTB biofilms is Rv3312A that is crucial for the production of pili in MTB. These pili play a significant role in biofilm formation as the mutant displays only 32% of the biofilm mass of wild-type MTB (Ramsugit, *et al.*, 2013).

Culture manipulation of MTB indicates that biofilm formation may be an important long-term survival strategy in MTB. Standard cultures containing a low quantity of Tween-80 were able to form microscopic corded structures and experience a rapid CFU decrease as the culture transitioned into stationary phase, but ultimately had greater CFUs at later time points than high detergent cultures that avoided the abrupt onset of stationary phase (Caceres, *et al.*, 2013).

A relatively new model of MTB biofilms has been developed in which the H37Rv MTB strain is grown in tissue culture media supplemented with lysed human peripheral blood leukocytes gaining phenotypic antibiotic resistance. The biofilm's dependence on DNA as a component of its extracellular matrix was demonstrated by the restoration of antibiotic sensitivity following DNaseI treatment (Ackart, *et al.*, 2014). This model was used to demonstrate the ability of two 2-aminoimidazole-based compounds to restore isoniazid susceptibility of established MTB biofilms (Ackart, *et al.*, 2014).

Critics of the application of biofilm principles to MTB infection, and mycobacterial infections in general, make the valid point that a conclusive demonstration that the biofilm mode of growth is important to *in vivo* infections is needed. Perhaps demonstration of attenuation virulence for biofilm-defective mutants would serve to convince some, but the identified biofilm mutant MTB strains have mutations in genes with pleotropic effects (such as fatty acid synthetase II mutants). It may not be possible to separate MTB from biofilm growth, as it is with bacteria with clear biofilm/planktonic switches such as *Staphylococcus aureus*. *In vivo* proteomics studies are another possible strategy to demonstrate biofilm during infection, assuming reliable biofilm protein markers could be identified. Additionally, drug discovery work using *in vitro* biofilms to screen agents that could sensitize MTB to existing antibiotics might be the ultimate proof of principle, potentially reducing treatment time to weeks instead of months.

The most recent research on MTB biofilm formation has shown that the bacteria are capable of forming disinfectant-resistant biofilms on cement, ceramic, and stainless steel substrates (Adetunji, *et al.*, 2014). These data seem to contradict a previous study that found only small amounts of MTB adherent to metal hardware used in orthopedic implants. However, in this study the materials were only cultured with MTB for one week compared to the two to four weeks in the more recent study (Ha, *et al.*, 2005). These studies indicate that MTB has the potential for causing medical-implant associated infections but that the opportunity for such infections is likely minimized by effective control of MTB infection in countries that routinely use medical implants.

Part 3: Virulence factors of *M. tuberculosis*

MTB and the other organisms in the MTB complex do not possess classical virulence factors in the form of secreted toxins, rather they possess a variety of glycolipid, lipoprotein, and proteins factors that manipulate the host immune response or protect the bacteria from numerous stressors (Forrellad, *et al.*, 2013). Following inhalation of a bacterial aerosol, bacterial factors act on macrophages to facilitate rapid phagocytosis of the bacteria. Phagosome-lysosome fusion is then prevented, and the phagosome is maintained in a state suitable for bacterial replication. Eventually, the bacteria will lyse the host cell, and the bacteria can re-infect another macrophage. If the released bacterial cell is not taken up by another macrophage, survival-promoting factors are produced to allow the bacteria to weather lengthy periods of dormancy in the harsh environment of the necrotic granuloma.

One well-characterized group of virulence factors in MTB is the so-called culture-filtrate proteins, some of which are actively secreted. The immunodominant antigens ESAT-6 and CFP-10 are the best-known examples of this group and are secreted into the culture supernatant by the mycobacteria specific *esx-1* secretion system (Samten, *et al.*, 2009). Both proteins are contained in the region of difference 1 (RD1), the only genetic locus that is both present in all virulent MTB and absent in all BCG strains (Brosch, *et al.*, 2002). These proteins have been shown to bind to the surface of macrophage cell lines in 1:1 complexes of ESAT-6:CFP-10 and the ESAT-6 component acts to inhibit T-cell signaling pathways decreasing IFN-γ production (Renshaw, *et al.*, 2005, Samten, *et al.*, 2009).

Superoxide dismutase is an MTB protein critical for protection from reactive oxygen intermediates in the host phagosome. Previously thought to be actively secreted, recent studies show the presence of SodA (as well as GlnA, the glutamine synthetase) in the culture filtrate is a result of very high levels of expression and extracellular stability of this protein following release by autolysis or bacterial leakage (Tullius, *et al.*, 2001). Unfortunately, attempts to make *sodA* deletion mutants in MTB have not been successful (Dussurget, *et al.*, 2001). The protective effect of SodA is such that overproduction of the protein in BCG neutralized the vaccine's normal protective effect in two different animal models (Jain, *et al.*, 2011). In addition to its pathogenic role, it also serves the normal housekeeping function of eliminating superoxide radicals produced as a byproduct of respiration (Smith, 2003).

A number of virulence factors in MTB are in or attached to the complex outer envelope of the bacteria. Lipoarabinomannan (LAM) is a highly branched glycolipid and a major component of the mycobacterial cell wall (Hunter, *et al.*, 1986). It is composed of arabinose and mannan subunits and is critical in preventing phagosome maturation in host macrophages (Mishra, *et al.*, 2011). LAM has also been shown to depress macrophage IFN-γ production and increase TNF-α converting enzyme activity, resulting in lower levels of both critical Th1 cytokines (Smith, 2003, Richmond, *et al.*, 2012). Furthermore, mannose-capped LAM (manLAM) has been shown to prevent IL-12 and TNF-α production by macrophages (Johansson, *et al.*, 2001, Nigou, *et al.*, 2001).

Antigen 85A (Ag85A, FbpA) is a fibronectin binding protein with mycolyltransferase activity is critical for MTB survival in host macrophages. This fibronectin binding ability of Ag85A is believed to facilitate its adherence and phagocytosis *in vivo* (Kuo, *et al.*, 2012). It is found both in the outer envelope of MTB where its mycolyltransferase activity is utilized in the production of the potent glycolipid trehalose dimycolate (Belisle, *et al.*, 1997). Ag85A is strongly immunogenic and has been utilized in experimental TB vaccines to provide a degree of protection (Tanghe, *et al.*, 2000, Tanghe, *et al.*, 2001, Sugawara, *et al.*, 2003, Liang, *et al.*, 2012).

Trehalose dimycolate (TDM) is a glycolipid component of the MTB outer membrane produced in larger amounts by virulent MTB and an incredibly potent toxin. TDM is also known as cord factor because production of it is required for the formation of twisted cords of MTB in liquid culture. This phenotype was identified as a hallmark of virulence in MTB as early as 1947 (Middlebrook, *et al.*, 1947, Noll, *et al.*, 1956). TDM is sufficient to reproduce this cording pattern with TDM-coated glass beads (Hunter, *et al.*, 2006). Pure TDM is capable of forming highly organized crystalline monolayers at phase interfaces or micelles in aqueous environments (Retzinger, *et al.*, 1981, Retzinger, *et al.*, 1982). TDM's incredible toxicity is highly dependent on whether it is present in a monolayer or a micelle. In monolayer form, TDM has a LD_{50} toxicity of 10-30 μg in mice although toxicity is actually a product of surface area of monolayer not dose. This toxin is also a potent trigger of granuloma formation. However, in the micelle form, TDM is less toxic as shown when TDM liposomes were injected into mice with no ill effects with the highest amount tested being 50 mg/mouse (Hunter, *et al.*, 2006). A proposed explanation is that TDM is present in a micelle form on the surface of MTB and in this form is effective at preventing macrophage killing of MTB by assisting in the phagosome maturation arrest (Indrigo, *et al.*, 2003). Formation of caseating granulomas characteristic of pulmonary TB are a direct result of host sensitization to TDM complexed with lipids. As disease progresses to the formation of cavities in the lung TDM is believed to be critical in maintenance of the cavity as well structural organization of the bacterial aggregates lining the cavity (Hunter, *et al.*, 2006).

HbhA is a well-characterized MTB adhesin, which binds heparin and is unusual in that it binds to non-phagocytic cells including airway epithelial cells (Locht, *et al.*, 2006). A knock-out mutant for the gene encoding HbhA displayed decreased dissemination to the spleen in a mouse infection model as did bacteria that were pre-incubated with anti-HbhA antibodies (Pethe, *et al.*, 2001). Similar results showing decreased dissemination of MTB were shown in experimental vaccination of animals with BCG recombinantly expressing HbhA (Kohama, *et al.*, 2008).

Important for long-term survival in the host, the bacterium has a number of factors that are crucial to adaptation to harsh host environments. It has been suggested that one such protein, the chaperone-like protein (Yuan, *et al.*, 1996, Yuan, *et al.*, 1998) is an important regulatory factor in MTB persister formation (Smith, 2003). This has been supported by the discovery that HspX is strongly induced by DosR, a dormancy master regulatory factor, as a consequence of hypoxia, and that the protein is produced in large quantities just prior to the bacterium entering stationary phase (Siddiqui, *et al.*, 2011).

Toxin-Antitoxin (TA) systems were originally identified as mechanisms of plasmid “addiction” in which both a durable protein toxin and a labile protein anti-toxin were expressed off of the same plasmid (Rawlings, 1999). Subsequent loss of the plasmid resulted in degradation of the anti-toxin and bacterial death resulting from the remaining “self” toxin (Wang, *et al.*, 2012). However, the expression of TA systems can also serve to create drug-tolerant persisters cells by interfering with vital cellular processes such as translation and ATP synthesis (Schuster & Bertram, 2013). MTB contains over 65 TA modules, ten of which are overexpressed in MTB persister cells while numerous metabolic pathways were significantly downregulated, including glyoxylate metabolism which supports growth in hypoxic condition (Keren, *et al.*, 2011).

Although not discussed herein, MTB possesses a number of other virulence factors that promote survival in the host. However, the factors described above are representative of the key strategies used in the host and contribute to biofilm formation. A summary of these models is included below in Table 1.1.

Part 4: Animal Models of Tuberculosis

Table 1.1: Summary of Animal Models of Tuberculosis Infection

	Pros	Cons
Mouse	• Many strains, reagents, and tools to study immunology • May be inexpensive • Ease of larger test groups	• Generally, poor replication of human disease histologically
C57BL/6J	• Useful for experiments to determine the genetic basis of susceptibility • Th1 and Th17 biased	• More resistant to MTB infection
BALB/c	• Inexpensive and consistent model of infection • Aerosol dose determines acute vs. chronic course of infection • Th2 biased	• No necrotic granulomas
C3HeB/FeJ (Kramnik)	• Necrotic granulomas are a better pathological representation of human lesions • Strongly Th2 and Treg biased	• Less consistent in terms of bacterial burden and lesion formation compared to BALB/c
Guinea Pig	• Highly susceptible to MTB infection • Classic necrotic	• BCG protection requires explanation • Formation of cavities is

	granulomas • Gold Standard for Vaccine Testing	rare • Susceptibility can lead to rapid fatality • Poor for studying latent TB
Rabbit	• Proven model for cavitary tuberculosis (utilizing *M. bovis*)	• Strains other than MTB *Erdman* are cleared slowly
Non-Human Primate	• NHP infection generates clinical correlates of infection such as blood CBC counts, chest X-ray, PPD skin test, etc. • Highest degree of genetic similarity to humans • Ability to simulate TB/HIV co-infection using SIV • Lung distribution of infection mirrors human • Infection results in either chronic disease or acute cavitary disease in a dose-dependent fashion.	• Very expensive model • Utilized only as last step before human trials • Very few molecular or immunological reagents • No Day 1 sacrifice to determine inoculums amounts • BCG is not as protective in Rhesus macaques

Tuberculosis is most commonly modeled in two animal species: mouse and guinea pig. Each of these models has strong and weak points in terms of how well it replicates the clinical properties of disease in humans. In addition to this, a number of mammalian species can be infected with one member of the MTB complex, *M. bovis*, including deer, badgers, ferrets, feral pigs, and brushtail possums, but these animals are not routinely used experimentally (Biet, *et al.*, 2005). Rabbit infection with *M. bovis* has provento be useful in modeling cavitary TB (Nedeltchev, *et al.*, 2009). Non-human primate models of TB are currently used as a last step prior to any clinical vaccine testing, as in the case of the MVA85 vaccine that recently failed to show protection in humans (White, *et al.*, 2013). Cattle are used in veterinary studies, often with a focus on diagnostic and vaccination research. However, the suggestion has been made that bovine tuberculosis would be an excellent model for human TB with respect to its lung pathology, latency phenotype, and innate and adaptive immune responses. One obvious drawback is the large size of cattle that limits their practical use with virulent TB outside of very specialized ABSL3 facilities (Cassidy, 2006, Van Rhijn, *et al.*, 2008).

The mouse model of tuberculosis has been the primary workhorse for MTB animal research because of the wide variety of strains, ability to achieve larger sample sizes through increased numbers of animals, and the wide availability of immunological reagents (Orme, 2005). C57BL/6 mice are very resistant to MTB infection (Lynch, *et al.*, 1965, Musa, *et al.*, 1987, Medina & North, 1998) and are commonly used in experiments to determine the genetic basis of resistance to TB using both crosses with susceptible strains or knock-out animals on this background (Kramnik, *et al.*, 1998, Scott & Flynn, 2002). BALB/c are commonly used in both vaccination and drug treatment experiments of MTB infection because these animals provide a consistent model of infection. The acute or chronic course of their infection is dependent on the aerosol dosage with approximately 100 CFU/mouse for a chronic infection versus approximately 1000 CFU/mouse for a more acute course (Goodridge, *et al.*, 2014). One strain of mice that has become increasingly available and useful for MTB research in recent years is the C3HeB/FeJ (a.k.a. Kramnik) mouse. These mice are seeing increased use as an experimental model because of their ability to form necrotic granulomas at similar inoculum sizes to BALB/c mice (Kramnik, *et al.*, 1998, Chackerian, *et al.*, 2001, Kamath, *et al.*, 2003, Driver, *et al.*, 2012). While this susceptibility to TB has been shown to be multigenic in origin in C3HeB/FeJ mice (Kramnik, *et al.*, 1998, Sissons, *et al.*, 2009), a mutation in the *sst1* locus appears to be key. The functions of the genes within this complex are unknown but additional analysis indicates that the complex contains the *Ipr1* gene, which encodes an interferon inducible nuclear protein that may limit the multiplication of MTB within macrophages and drive infected macrophages towards apoptosis (Pan, *et al.*, 2005, Kramnik, 2008, Pichugin, *et al.*, 2009). Unfortunately, this model can exhibit greater variability compared to the BALB/c model within experimental groups since animals tend to fall into a higher vs. lower bacterial burden subgroup depending on the formation or lack of formation of large necrotic granulomas (Personal communication with E. L. Nuermberger). Therefore, the standard deviation of CFU/g lung tissue shows a high variance within experimental groups making statistical comparisons between groups challenging.

Guinea pigs are the oldest model organism for MTB infection, having been utilized by Koch in the demonstration that MTB fulfilled his system of postulates demonstrating it as the causative organism of TB (Nobelprize.org, 2013). Guinea pig MTB infection is normally administered via the aerosol route using 20-50 CFU/animal (Baldwin, *et al.*, 1998). This produces classical necrotic granulomas complete with multinucleated giant cells (Turner, *et al.*, 2003). These animals continue to be the most commonly used model for the testing of MTB preventative vaccines (Turner, *et al.*, 2003). Due to their extreme susceptibility to MTB these animals have not classically been suitable for the study of latent TB. However, infection of guinea pigs with a streptomycin-auxotrophic MTB strain (18b) can produce a chronic infection without signs of active disease (Kashino, *et al.*, 2008).

Early models of TB using rabbits resulted in successful infections that cleared after four months except for a small number of animals in which the bacteria persisted

(Lurie, 1928). This pattern of resistance was noted to be similar to humans and recent versions of rabbit trials have utilized corticosteroid injections to increase the percentage of animals that develop a chronic infection that resolves with withdrawal of steroid treatments (Manabe, *et al.*, 2008). The rabbit model of cavitary tuberculosis utilizes *M. bovis* to infect animals that have been pre-sensitized to this bacterial pathogen by administration of heat-killed *M. bovis* with incomplete Freund's Adjuvant (IFA). This model has been used to demonstrate that both the wall and lumen of TB cavities produce a hypoxic environment that causes the infecting bacteria to exploit host lipids via catabolism (Nedeltchev, *et al.*, 2009). A similar model uses the MTB strain HN878 to achieve granulomas and cavities without the pre-sensitization (Subbian, *et al.*, 2011). The HN878 rabbit model has been utilized to demonstrate that poor activation of innate immunity accompanied by delayed T-cell activation are key factors in the formation and persistence of cavities harboring high bacteria burdens for prolonged periods of time (Subbian, *et al.*, 2011). The MTB HN878 rabbit aerosol model has also been utilized to track the course of lung infection using PET-CT imaging that measures the uptake of labeled glucose molecules into individual lung lesions (Via, *et al.*, 2012).

Non-human primate (NHP) models of infection have a large number of benefits in terms of closely modeling both human disease pathology and clinical correlates including CXR, PPD skin tests, and blood CBC. Unfortunately, the drawbacks of NHP models include high cost, inability to sacrifice animals for precise confirmation of CFU deposition following aerosol or intratracheal infection, and an extreme dearth of NHP-specific immunological or molecular reagents (Kaushal, *et al.*, 2012). Like the mouse model of infection, the dosage of MTB bacteria used in the model has a strong effect on the progression of the disease. With small intratracheal inocula of 10-100 CFU/animal cynomolgus macaques develop a latent tuberculosis, but with larger dosages of ~10^3 CFU/animal, the macaques develop a slowly progressive pneumonia. Doses of 10^4 and higher result in a rapidly fatal acute TB (Walsh, *et al.*, 1996). Pathologically, the macaques develop a variety of different granulomas that mimic those seen in clinical cases of TB (Lin, *et al.*, 2009). Animal models in this and other host species can recapitulate the compromise in the host immune system and the resulting relapse of MTB infection by treating animals with corticosteroids or TNF-α inhibitors (Repique, *et al.*, 2002, Manabe, *et al.*, 2008). However, the NHP models are capable of modeling immunodeficiency by TB/HIV co-infection via co-infecting with MTB and the Simian Immunodeficiency Virus (SIV). Rhesus macaques were infected with 25 CFUs of MTB Erdman via the intratracheal route resulting in a latent MTB infection that relapsed following intravenous infection with 10^6 – 10^7 $TCID_{50}$ units of SIV (Diedrich, *et al.*, 2010).

While none of these model systems perfectly reproduces human MTB, it is clear that certain models are particular suited to study particular aspects of TB disease. Mice will continue to be the first choice for experiments, testing drug and vaccine therapies as well as those studying the effects of specific immunological deficits on TB. Guinea pigs, because of their tremendous susceptibility, will likely remain an important intermediate animal model for vaccine research following mouse experiments but proceeding NHP research. Rabbits appear to be the current best model for cavitary disease. NHPs are the all-important experimental subjects that are utilized as a last step prior to human tests of vaccines and will continue to be irreplaceable for the study of TB/HIV co-infection for the foreseeable future.

Part 5: The host immune response to *Mycobacterium tuberculosis*

The host immune response to MTB is complex and incompletely understood. However, the current zeitgeist is that this bacterial pathogen is very adept at manipulating the host immune system, as well as its own metabolism, to ensure survival and promote chronic infection. Following initial contact with alveolar macrophages and pattern-recognition receptors (PRRs) of the host innate immune system in the lungs, MTB manages to conceal its presence and delay the onset of potentially protective adaptive immunity for up to three weeks. Once the host has started to mount an effective immune response the bacteria is able to protect itself from attack by inducing the formation of a host granuloma, an elaborate collection of immune cells that walls off the infection. Years or decades later, the bacteria are capable of escaping this granuloma and rapidly replicating. At this point the host's adaptive immune response is used against itself to destroy host lung tissue giving the bacterium an avenue of escape.

5A. Innate immune response

As the initial obstacle to MTB infection, the innate immune response to MTB is critical in slowing the progress of disease and promoting the adaptive immune response. This section will explore the role of innate immune system components including the central role of macrophages and dendritic cells, pattern-recognition receptors (PRRs) important to innate recognition of MTB, the role of neutrophils and natural killer (NK) cells in infection, and the contribution of the complement system.

Ideally, MTB bacteria arriving in the lungs by the aerosol route would be rapidly recognized via PRRs on macrophages and dendritic cells and then phagocytosed. MTB would then be killed by effector mechanisms including the oxidative burst, acidification of the phagosome, and the release of lysosomal contents into the phagosome through fusion of the two organelles. Bacterial proteins could then be digested and loaded into class II MHC molecules allowing for the efficient presentation of antigen to T-cells resulting in a rapid formation of an adaptive cell-mediated immune response including critical Th1 CD4+ cells. Unfortunately, while the phagocytosis of MTB is efficient, the bacterium can often interfere with the subsequent steps that are required to produce an effective adaptive immune response.

Macrophages are important in innate defense against MTB in the lungs and their initial activity enables the 90% of those infected patients to clear the infection or arrest it in a latent state. Macrophage recognition of MTB is mediated by a number of innate immune receptors. These interactions result in phagocytosis and often lead to MTB killing. However, the interaction between MTB and certain host MTB receptors, such as mincle, can result in decreased killing of the bacteria once phagocytized. This Trojan horse strategy allows the bacterium to enter the phagosome where it halts the natural acidification at pH 7.2 using glycolipid virulence factors such as TDM. MTB also prevents lysosomal fusion with the phagosome and continues to replicate in an environment protected from the host immune system. However, the host is often able to mount a Th1 adaptive immune response to MTB, and macrophages are activated for more efficient killing of MTB through IFN-γ-IFN-γR signaling.

Failing endosome-lysosome fusion, macrophages can still eliminate MTB via the autophagocytic pathway. Autophagy is well-known as a homeostatic mechanism in mammalian cells in which macromolecular complexes and organelles are degraded by fusion of autophagosomes with lysosomes (Ma, *et al.*, 2013). This process has also been demonstrated to be an important immune mechanism for the control of intracellular bacteria both within endosomes, such as MTB, and in the cytoplasm, such as *Listeria* (Gutierrez, *et al.*, 2004, Nakagawa, *et al.*, 2004, Checroun, *et al.*, 2006, Singh, *et al.*, 2006, Py, *et al.*, 2007). While MTB is capable of arresting the fusion of MTB-containing endosomes with lysosomes, autophagy is capable of efficient MTB-killing through the formation of autolysosomes, which contain anti-mycobacterial ubiquitin fragments (Alonso, *et al.*, 2007). This mechanism of MTB killing represents an important point of cross-talk between the innate and adaptive immune systems. Pattern-recognition receptions such as Toll-Like Receptors (TLRs), especially TLR4, can trigger autophagy in response to endosomal and cytoplasmic bacteria (Xu, *et al.*, 2007, Delgado, *et al.*, 2008, Deretic, *et al.*, 2009). The adaptive immune system can also increase autophagy via Th1 cytokines IFN-γ and TNFα, which augment the cytokine's normal effects against intracellular pathogens (Gutierrez, *et al.*, 2004, Djavaheri-Mergny, *et al.*, 2006, Singh, *et al.*, 2006). This Th1-mediated increase in autophagy is mirrored by the autophagy inhibitory effects of Th2 cytokines IL-4 and IL-13 (Harris, *et al.*, 2007).

Apoptosis is another critical mechanism that infected macrophages can utilize to avoid being complicit with MTB by denying the bacteria a protected site for its replication (Lee, *et al.*, 2009). Apoptosis is a carefully controlled process of cell-death in which the cell contents are degraded and encapsulated into small membrane-bound vesicles covered in molecular signals to stimulate phagocytosis. MTB has been shown to activate the extrinsic pathway of apoptosis via paracrine signing by TNF-TNF-αR at a multiplicity of infection of approximately 5:1 (Keane, *et al.*, 1997). The clinically attenuated strain of MTB H37Ra induces apoptosis much better than its virulent relative H37Rv, and this difference appears to be a major cause of H37Ra attenuation (Keane, *et al.*, 2000). Macrophage apoptosis results in the release of considerably lower amounts of viable MTB compared to necrotic cell death (Molloy, *et al.*, 1994, Keane, *et al.*, 2000). Additionally, macrophage ingestion of apoptotic bodies containing bacilli seems to prevent MTB from arresting phagosome development as uninfected macrophages added to cultures containing apoptotic, MTB-infected macrophages appeared to promote phagosome development in uninfected macrophages in a contact dependent manner (Fratazzi, *et al.*, 1997, Lee, *et al.*, 2006).

In addition to the role of TLR4 in promoting autophagy in MTB infected cells, a variety of TLRs including TLR2, TLR4, TLR9 are activated by MTB components (Doherty & Arditi, 2004, Bafica, *et al.*, 2005, Harding & Boom, 2010, Kleinnijenhuis, *et al.*, 2011). TLR2 recognizes a variety of mycobacterial ligands including LAM and TDM as well as several lipoproteins such as LpqH and LprA (Harding & Boom, 2010). TLR4 has been implicated in resistance to MTB infection and shown to recognize MTB's phosphatidylinositol mannosides (Abel, *et al.*, 2002). The importance of these PRRs is reinforced by the effect of knocking out the TLR downstream-signaling protein, Myd88. The results of MTB infection were dendritic cells (DCs) and macrophages with defective TNFα, IL-12, and NO production, and the rapid death of the host (Fremond, *et al.*, 2004).

TLRs protect against MTB through induction of the NF-κB pathway resulting in downstream cytokine production TNFα, IL-6, and IL-12 (Doherty & Arditi, 2004).

C-type lectins (calcium-dependent lectins) including DC-SIGN, dectin-1, mincle, and the mannose receptor are another group of MTB-recognizing PRRs. These receptors bind sugar moieties such as those found on glycosylated proteins (including the 19 and 38 kDa MTB antigens) and glycolipids (such as LAM and TDM) (Tanne & Neyrolles, 2010). DC-SIGN is a C-type lectin expressed on both DCs and macrophages and binds mannose motifs on both LAM and the MTB 19kDa antigen and stimulates phagocytosis of MTB (29, 30, 32). Interestingly, it has been shown that costimulation of TLRs is required for DC-SIGN to robustly effect gene expression since stimulation of DC-SIGN by LAM alone is insufficient (den Dunnen, *et al.*, 2009). C-type lectin Dectin-1 is expressed on macrophages, neutrophils, and DCs, and recognizes MTB antigens, although the specific ligand has yet to be defined. Dectin-1 does not require TLR stimulation to stimulate TNFα and IL-12 secretion (Azad, *et al.*, 2012). Mincle, a macrophage C-type lectin, has been shown to recognize TDM (cord factor) but its downstream signaling through Syk-CARD may serve to promote granuloma formation and MTB survival (Lang, 2013).

DCs are essential to the formation of an adaptive immune response in their role as primary antigen-presenting cells (Banchereau & Steinman, 1998), but are also part of the innate immune system in their ability to recognize and phagocytize MTB in a manner similar to macrophages. DCs require IL-10 specifically (not IFN-γ or TNFα) in order to control the growth of MTB in their phagosomes, but despite this they are still less efficient at killing MTB than macrophages (Fortsch, *et al.*, 2000).

In contrast to the role of macrophages in MTB pathogenesis, the role of neutrophils is significantly less defined despite the fact that they are infected by MTB in greater numbers than macrophages (Eum, *et al.*, 2010). Neutrophils have a clear role at controlling the early response to MTB since experimentally depleting or increasing the numbers of neutrophils present in the lung at the time of challenge with MTB increases or decreases final lung bacterial burden, respectively (Sugawara, *et al.*, 2004, Barrios-Payan, *et al.*, 2006). Neutrophils have been shown to kill MTB, but this ability varies between 50-95% depending on the donor (Neufert, *et al.*, 2001). Virulent MTB appears to be resistant to neutrophil killing by classical reactive oxygen species (ROS) but several mechanisms have been proposed for alternate MTB-killing methods such as anti-microbial peptide release (Miyakawa, *et al.*, 1996, Sharma, *et al.*, 2000, Sharma, *et al.*, 2001, Corleis, *et al.*, 2012). What is less controversial is the ability of neutrophils to recruit monocytes to the site of infection via chemokine/cytokine release (Mantovani, *et al.*, 2011). Furthermore neutrophils may bolster macrophages through the production of neutrophil extracellular traps (NETs) that are DNA and protein-containing structures produced via a process similar to apoptosis (Brinkmann, *et al.*, 2004). These NETs have been shown to be phagocytized by macrophages and this leads to an increase in the production of cytokines IL-1, TNF-α, IL-6, and IL-10 (Braian, *et al.*, 2013). Despite these useful functions early during infection, sustained accumulation of neutrophils correlates with lung pathology. This neutrophil accrual is believed to be either a symptom of an unchecked inflammatory response to infection or a cause of increased pathology through ongoing release of cytotoxic neutrophil contents (Lowe, *et al.*, 2012).

In the light of MTB's ability to infiltrate macrophages and resist killing, it is important to consider the action of NK cells, which are innate lymphocytes capable of identifying and killing infected host cells. NK cells are capable of killing MTB-infected macrophages and monocytes in culture, and this action is dependent on the NK receptors NKp46 and NKG2D, which recognize the ligands vimentin and ULBP-1, respectively (Garg, *et al.*, 2006, Schrama, *et al.*, 2006). NK cells can also promote increases in the number and potency of CD8+ T-cells. In addition, CD1 is expressed on NK cells and is an MHC Class I homolog that binds lipid moieties instead of peptides and presents them to the T-cell receptor (TCR). NK cells interact with lipid or glycolipid antigens presented in the context of the CD1d molecule and react by producing large quantities of IFN-γ, IL4, and GMCSF. The fact that MTB is capable of downregulating CD1 from the cell-surface of infected antigen presenting cells supports the position that these proteins are important for control of MTB infection (Stenger, *et al.*, 1998).

The limited-diversity subset of γδ T-cells, whose antigen-specificity is produced through stereotyped gene rearrangements, have been shown to play a support role in MTB infection, facilitating cross-talk between the innate and adaptive immune systems. γδ T-cells can recognize specific phosphoantigens and then stimulate the maturation of DCs as a response (Ismaili, *et al.*, 2002).

Complement can play a role in the opsonization for MTB for phagocytosis. The majority of this opsonization occurs via complement-receptor 3 (CR3) recognition of bacterial-bound C3B (Schlesinger, *et al.*, 1990). However, due to very low levels of complement in the alveoli where MTB first encounters host macrophages, most phagocytosis results from signaling through non-opsonic receptors such as the TLRs, mannose receptor, and dectin-1 (Kleinnijenhuis, *et al.*, 2011).

5B. Cell-mediated immune response

The Th1-polarized, cell-mediated immune response to MTB infection has long been held to be the single most important factor in host defense against MTB. The critical role that Th1 CMI plays in defense against MTB infection has been well documented in a large number of mouse knock-out studies that illustrate the important roles of the cytokines, receptors, and signaling cascade components that undergird the Th1 T-cell response. Data provided by these mouse models of infections are paralleled by studies examining genetic determinants of MTB susceptibility in humans. However, genetic defects in cytokines opposing the Th1 response, especially IL-10, also result in increased disease severity, thereby indicating that an exclusively Th1 cell-mediate immune response, while necessary, is not sufficient for host protection. In addition to the CD4+ T-cells that mediate the Th1 response, CD8+ T-cells also provide a critical element of the anti-MTB immune response by targeting infected host cells. While all of the effector cells are crucial to the control of MTB infection, it has also been shown that much of the late-state disease pathology of tuberculosis is mediated by excesses of Th1 cytokines that initially lead to control of the infection. After considering all these factors, we will also examine how compromise of cell-mediated immunity can provoke reactivation of latent disease.

The optimal response to a MTB aerosol infection results in the rapid phagocytosis of the bacteria by macrophages and dendritic cells, which then move to local lymph nodes to begin the process of signaling CD4 and CD8 T-cells with the appropriate antigen affinity to begin differentiating into Th1 effector cells. Following the expansion of the appropriate clones of T-cell, these clones would begin to migrate to the lung to form a protective granuloma structure around the infection in concert with fibroblasts and B cells. Th1-cells secreting IFN-γ then activates macrophages, improving their ability to kill phagocytized bacteria and present bacterial antigens in MHC class II molecules. This protective granuloma response usually results in the elimination or containment of the bacteria. Unfortunately, this process fails in a small subset of humans, resulting in primary tuberculosis. Even those who contain the infection following initial exposure have a 5-10% chance to experience a reactivation tuberculosis later in life when immune containment of MTB in the granuloma fails and results in the release of the bacilli to produce a secondary tuberculosis.

The necessity of the Th1 CMI response has been strongly supported by examining the effects of mutation or knockout of specific genes in the mouse model of infection. Defects in any of the genes critical for the Th1 response almost invariably lead to dramatically increased bacterial burdens or death of the host mice. This can be seen in the mouse mutants for IFN-γ, the IFN-γ receptor, STAT1, IRF1, IRF8, IL-12, TNFα, and the TNF-α receptor (Flynn, *et al.*, 1993, Kamijo, *et al.*, 1993, Cooper, *et al.*, 1995, Cooper, *et al.*, 1997, Bean, *et al.*, 1999, Jacobs, *et al.*, 2000, Yamada, *et al.*, 2002, Bafica, *et al.*, 2005, Marquis, *et al.*, 2009).

DCs are primarily responsible for stimulating adaptive immunity to MTB because alveolar macrophages are poor APCs with a generally immunosuppressive response (Mihret, 2012). DCs bearing MTB antigens migrate to mediastinal lymph nodes and signal CD4+ T-cells specific for MTB antigens to begin differentiating, primarily into Th1 CD4+ T-cells. The Th1 response is started by secretion of IL-12 and IL-18 from DCs and macrophages, which triggers the release of IFN-γ from CD4+ and CD8+ T-cells as well as NK cells (Altare, *et al.*, 1998). While several different cell types can secrete IFN-γ, IFN-γ secreted by CD4+ T-cells is essential for host survival in the mouse model (Green, *et al.*, 2013). IFN-γR signaling results in further differentiation of Th1 CD4+ T-cells and activates of macrophages for increased killing of intracellular MTB, increases MHC Class II production, autophagy, and cross-presentation of antigen further accelerating the development of the Th1 T-cell response.

In addition to the well-characterized role of CD4+ T-cells in the response to MTB infection, it is known that, unlike NK cells, CD8+ T-cells are required for an optimal response to MTB infection (Woodworth & Behar, 2006). Since the late 1980's it has been appreciated that purified CD8+ T-cells from infected mice could be transferred to irradiated mice and result in protection from MTB infection, although this protection was not as effective as that provided by transferred CD4+ T-cells (Orme, 1987). A large number of MTB antigens have been shown to generate CD8+ T-cell responses including CFP-10, ESAT-6, Ag85A, SodA, and GlnS (Zhu, *et al.*, 1997, Pathan, *et al.*, 2000, Dong, *et al.*, 2004, Shams, *et al.*, 2004). These cells are capable of recognizing MTB proteins presented in the context of MHC class I molecules and killing those cells that harbor intracellular bacteria. The effector mechanisms of these CD8+ T-cells include secretion of IFN-γ and TNF-α as well as the induction of apoptosis in target cells via either Fas/FasL interactions or the granule-exocytosis pathway (Woodworth & Behar, 2006). Fas-ligand-induced apoptosis has been shown to result in decreased viability of released

MTB compared to complement induced cell death (Oddo, *et al.*, 1998). In light of these facts, it is unsurprising that mice with deletion of the Beta-2-microglobulin gene, an essential component of the MHC class I molecule, experience rapid death following infection with MTB (Flynn, *et al.*, 1992).

Despite the necessity of a strong Th1 response, evidence has been discovered that this strong polarization of the immune system can result in injury to the host at later stages of infection. TNF-α can synergize with Oncostatin M secreted from MTB-infected macrophages, allowing unopposed action of the matrix metalloproteases secreted from pulmonary fibroblasts and degradation of lung collagen (O'Kane, *et al.*, 2008). When the important counterbalance on the Th1-axis, IL-10, was deleted, the resulting knockout animals experienced worse long-term outcomes compared to wild-type mice in which the infection was characterized by bacterial regrowth, increased lung pathology, and elevated lung inflammatory cytokine levels (Higgins, *et al.*, 2009).

The reactivation of latent TB is normally associated with some degree of immune impairment. TNFα-blocking antibodies such as etanercept as well as corticosteroid-induced immunosuppression have been shown to cause TB reactivation both in animal models and in humans. Other risk factors for reactivation of tuberculosis include diabetes, smoking, alcoholism, age (Lin & Flynn, 2010).

Like NK cells discussed above, T cells also utilize CD1 molecules, but Type 1 (isoforms a, b, and c) instead of Type 2 (Cd1d)(Montamat-Sicotte, *et al.*, 2011). These CD1-restricted T cells are capable of targeting a number of MTB lipid antigens present in the MTB cell wall (Cohen, *et al.*, 2009).

The process of lung cavitation following reactivation of disease remains poorly understood. Following the reactivation of the disease, a burst of extracellular MTB growth appears to trigger a corresponding inflammatory response causing tissue destruction and cavity enlargement. The main animal model of this phenomenon utilizes rabbits as discussed in Section 4. As the cavity enlarges it erodes airways allowing for transmission of the bacteria to the next host as coughing produces a bacterial aerosol (Canetti, 1950). During this phase the largest number of bacilli are produced and are easily recoverable in sputum samples (Osler, 1892).

5C. Humoral immune response

MTB-infected patients are known to produce a humoral immune response to MTB proteins but the importance of this arm of the immune response in containing or eliminating the infection has long been controversial (Glatman-Freedman & Casadevall, 1998, Abebe & Bjune, 2009). Antibodies are well known for their ability to neutralize adhesins and secreted toxins or to activate complement. In association with effector cells such as macrophages, antibodies enable opsonization and phagocytosis as well as antibody dependent cell-mediate cytotoxicity (ADCC) through Fc receptor signaling. However, the well-documented intracellular growth ability of MTB coupled with conflicting studies as to the usefulness of antibody responses to MTB have brought into question the topic of the humoral response to MTB and its efficacy.

The earliest studies of humoral immune effects on MTB began shortly after the identification of the tubercle bacillus by Koch. One early study showed that serum from dogs immunized with MTB to have some protective effect on rabbits prior to MTB infection (Beretta, 1891). This and other relatively primitive passive immunization experiments were conducted between the 1890's and the 1930's. In these studies, animals or patients treated with a number of MTB-immune serum preparations and then challenged had no predictable effects and many lacked proper experimental controls (Glatman-Freedman & Casadevall, 1998).

The advent of modern immunology and the understanding of antibodies led to a number of experimental studies to evaluate the efficacy of the humoral response to TB in animal models. Intravenous passive transfer of human TB serum to mice already infected with MTB extended the survival of mice compared to control. However, extended survival time was also seen in mice treated with control commercial IgG from non-MTB infected sources (Zitrin & Wasz-Hockert, 1957). Another study utilized the rabbit model where BCG replaces MTB as it is more virulent. In this study, BCG-immune serum in aerosol-infected rabbits was then passively transferred to rabbits one day prior to aerosol challenge with BCG. That study found no significant differences in lung CFUs or lesions between animals receiving BCG-immune serum and those receiving BCG-naïve serum (Reggiardo & Middlebrook, 1974). Other early *in vitro* tests of antibodies on MTB suggested that antibodies may increase phagocyte killing of MTB, support maturation of MTB containing phagosomes, or promote MTB neutralization (Zitrin & Wasz-Hockert, 1957, Armstrong & Hart, 1975, Glatman-Freedman & Casadevall, 1998). However, these studies were carried out between the 1940's and 1975 using relatively crude fractionation of serum and all dealt with polyclonal serum. More recently passive immunization with serum from mice inoculated with MTB extracts delivered in liposomes had a significant protective effect in a SCID mouse model (Guirado, *et al.*, 2006).

Modern knockout mice and monoclonal antibodies have allowed for a more precise and nuanced interrogation of the role of antibody in host defense against MTB infection. In a mouse model of MTB infection, B-cell knockout mice (μMT mice) had a three-to-eight fold increase in CFUs compared to wild-type. This increase was reversed after adoptive transfer of wild-type B cells (Vordermeier, *et al.*, 1996). These mice have also been show to exhibit increased lung immunopathology with an increased influx of neutrophils in the chronic aerosol infection model (Maglione, *et al.*, 2007). Additionally, treatment with monoclonal antibodies against the MTB protein TBA61 reduces bacterial lung loads and reduced lung histological changes in mice relative to monoclonal control (Lopez, *et al.*, 2009). Monoclonal IgG3 against arabinomannan has also improved survival in an endotracheally-infected mouse model and monoclonal IgG1 against the same molecule gave protection in a BALB/c mouse model (Teitelbaum, *et al.*, 1998, Hamasur, *et al.*, 2004).

IgA-based therapies have been attempted based on the rationale that they can provide a number of effector functions without causing additional inflammation. Intranasal treatment of BALB/c mice with IgA anti-α crystalline was shown to dramatically reduce lung CFU counts (10-fold reduction) at early time points (Williams, *et al.*, 2004). An experimental IgA1 molecule has been developed targeting the mycobacterial alpha crystalline antigen, and has been shown to reduce pulmonary infection following intranasal inoculation of the antibody, especially when given in conjunction with IFN-γ. This reduction occurred in an FcαRI-dependent manner and suggests that passive immunotherapy is theoretically possible (Balu, *et al.*, 2011).

In addition to these experiments it has been demonstrated that a number of MTB antigens are capable of eliciting protective immune responses with a significant humoral component. The protein Ag85A elicited a protective effect when used to vaccinate intranasally, along with a strong increase in IgA and IgG2a content of nasal secretions in a mouse model (Giri, *et al.*, 2006). Vaccination with native heparin-binding hemaglutinin A (HbhA) protein from MTB reduced lung CFUs while a yeast-recombinant HbhA only reduced CFUs in the spleen (Parra, *et al.*, 2004, Kohama, *et al.*, 2008).

Antibodies against MTB targets cannot be expected to directly target intracellular bacteria. However, newly inhaled bacilli, bacilli from recently lysed macrophages, and bacilli in cavitary lesions may all be vulnerable to opsonization and neutralization of surface proteins by antibody. Furthermore, uptake of MTB antigens bound to antibody via FcR may be capable of stimulating the adaptive immune response to MTB. Signaling through the FcγR has been shown to be critical for long-term immunity to intracellular pathogens such as Chlamydia (Moore, *et al.*, 2002).

Part 6: Conclusions

We began this chapter by exploring the non-tuberculous mycobacteria and the role that biofilm formation plays in their environmental lifestyle and pathogenesis. The most common pattern for mycobacteria is that of environmental bacteria that can cause opportunistic diseases. *M. ulcerans* is the major exception to this rule. Despite having an environmental reservoir, it has a specific virulence plasmid, and the disease it causes is mediated largely by a single secreted toxin. These lifestyles of related mycobacteria have led many to conclude that MTB, while now an obligate pathogen, evolved from an environmental ancestor. Both MTB and *M. leprae* have evolved into obligate pathogens. However, experiments with MTB complex organism *M. bovis* have also demonstrated long-term viability of these organisms outside of their host, up to six weeks (Tanner & Michel, 1999) showing that pathogenic mycobacteria continue to reap benefits from their origins as free-living mycobacteria.

Biofilm growth has a well-documented effect on bacterial survival both in the wild and *in vivo*. The adherence phenotype of biofilm bacteria allows occupation of unique environmental niches, such as on rocks in flowing streams. Biofilm also creates a robust microbial community with increased resistance to environmental stresses ranging from acid, heat, starvation, and antibiotics.

In vitro MTB grows as a biofilm when not subjected to detergents, and this biofilm has important effects on long-term bacterial survival. As previously mentioned, destroying MTB biofilm with detergent lowers long-term bacteria viability (Caceres, *et al.*, 2013). When treated with the antibiotic rifampin, biofilm-cultured MTB exhibited three logs greater viability following a five day antibiotic treatment relative to detergent-cultured (Ojha, *et al.*, 2008, Sambandan, *et al.*, 2013).

In vivo, TB meets all four accepted criteria of a biofilm infection. AFB smears for MTB routinely identify small clusters of bacteria in sputum. Histological preparations from animal models of cavitary TB clearly show aggregations of bacteria living at the tissue-air interface on the inner-lining of the cavity (Nedeltchev, *et al.*, 2009). In classic pulmonary TB, the bacteria exist solely in the lungs, usually encapsulated inside a granuloma, but have the ability to cause disseminated disease to most tissues in the body. Lastly, the current antibiotic treatment for MTB requires a minimum of six months of treatment with a combination of four antibiotics, and this regimen may still fail to eliminate the pathogen. The only missing piece is the definition and characterization of the extracellular matrix that MTB secretes. It is known that free mycolic-acids are produced by biofilm MTB (Ojha, *et al.*, 2008) but it remains to be seen if this is the only component of the MTB biofilm matrix.

MTB has been studied almost exclusively using detergent-based and intra-macrophage models of growth. Given that the culture environment has a very strong influence on gene expression, this may have significantly biased previous antigen-screen efforts. By proceeding with a detergent-free model of MTB biofilm growth, we may gain insight into the unique proteome of this important human pathogen that may result in novel diagnostic biomarkers or vaccine antigens.

Part 7: Hypotheses and Specific Aims

Hypothesis I: Utilizing an *in vitro* model of MTB biofilm growth will allow for the identification of biofilm-associated proteins and universally expressed proteins with potential use as diagnostic markers and vaccine candidates.

Hypothesis I directly informs Specific Aim 1.

Specific Aim 1: Identify immunogenic MTB proteins expressed at all biofilm time points

A. Grow MTB biofilms to three, five, and seven weeks and control shaking cultures to three weeks of age.
B. Extract membrane proteins and purify by precipitation.
C. Resolve individual proteins by molecular weight and isoelectric point using 2D gel electrophoresis.

D. Select immunogenic proteins by performing 2D Western Blotting using serum from 60-day MTB-infected guinea pigs.
E. Identify immunogenic proteins by peptide-mass fingerprinting.

Hypothesis 2: Production and testing of a recombinant vaccine of biofilm and universally expressed MTB proteins will elicit a strong immune response and protection from tuberculosis in a mouse model of aerosol infection.

Hypothesis 2 directly informs Specific Aims 2 and 3.

Specific Aim 2: Produce and validate a protein-subunit vaccine against biofilm-expressed proteins

A. Select proteins for inclusion in the vaccine on the basis of immunogenicity, pattern of expression, subcellular localization, and published literature.
B. Recombinantly express proteins selected for the vaccine.
C. Purify recombinant proteins and concentrate in PBS frozen stocks.

Specific Aim 3: Vaccinate mice using a recombinant biofilm vaccine and evaluate protection against virulent MTB aerosol challenge

A. Perform two separate, nine-week trials of the five-subunit vaccine comparing vaccinated animals to unvaccinated (PBS) controls and BCG-Pasteur vaccinated (positive control) animals.
B. Examine CFU deposition by aerosol at challenge in deposition group and lung CFUs at five weeks post-challenge in all groups.

C. Identify any impact on lung pathology at five weeks post-challenge using lung histological preparations.

D. Evaluate the immune response to the vaccine by investigating:

    a. Immune response to the vaccine at challenge

    b. Immune response 5 weeks post-challenge by examining antigen-specific IgG isotype titers and lung cytokine profiles

CHAPTER 2: MTB *IN VITRO* BIOFILMS EXPRESS A DISTINCT SET OF IMMUNOGENIC PROTEINS COMPARED TO SHAKING CULTURE

Part 1: Background and Significance

As discussed in Chapter 1, *Mycobacterium tuberculosis* (MTB) causes both acute and chronic infections in humans characterized by tolerance to antibiotics and reactivation to cause secondary tuberculosis. These characteristics have led to renewed interested in the *in vitro* pellicle, or biofilm, mode of growth where the bacteria grow to produce a thick aggregate at the air-liquid interface and display increased phenotypic resistance to antibiotics. We infected guinea pigs with the virulent H37Rv strain of MTB for 60 days and then collected serum. Using this serum we probed membrane protein extracts of MTB H37Ra pellicles grown for 3, 5, or 7 weeks and shaken cultures grown for two weeks, after which they were separated by two-dimensional gel electrophoresis (2DGE) to identify immunogenic proteins. These proteins were then identified using MALDI-TOF/TOF mass spectrometry peptide mass fingerprinting. Immunogenic pellicle proteins were compared across the three pellicle timepoints in order to ensure they were expressed at all timepoints. They were also compared to those membrane proteins harvested and identified from shaken cultures to determine pellicle-specific versus universally expressed proteins. The sequence of immunogenic pellicle-specific proteins were checked for sequence conservation across 15 sequenced MTB clinical isolates, three other members of the MTB complex, as well as *Mycobacterium avium* and *Mycobacterium smegmatis*. The resulting conserved pellicle-specific proteins represent targets for the development of future diagnostic tests and vaccines.

The only vaccine currently used to protect against TB, the Bacille-Calmette Guerin (BCG) vaccine, was first administered in 1921 and has a roughly 80% efficacy at preventing childhood disseminated TB infections such as TB meningitis but is not effective at preventing adult pulmonary TB (Behr, 2002, Liu, *et al.*, 2009). The widespread use of the BCG vaccine in TB endemic countries further complicates TB diagnosis by making the common screening test, the purified protein derivative (PPD or Mantoux Test) useless because of the cross-reactivity to PPD generated by the vaccine (Jin, *et al.*, 2010). The current situation requires that we identify previously overlooked MTB antigens for use in new vaccines and diagnostic tests to diagnose TB in BCG vaccinated individuals.

With the advent of rapid genome sequencing, *in silico* antigen screens have been used to predict MTB antigens for new vaccines and diagnostics (Cole, 2002, Chaitra, *et al.*, 2005, Ewer, *et al.*, 2006, Zvi, *et al.*, 2008, Wang, *et al.*, 2010, Tang, *et al.*, 2011, Sundaramurthi, *et al.*, 2012). Many of these screens have focused on the prediction of CD4 and CD8 T-cell epitopes in an effort to discover antigens capable of stimulating a robust cell-mediated immune response (Wang, *et al.*, 2010, Tang, *et al.*, 2011, Sundaramurthi, *et al.*, 2012). Inevitably these studies either incorporate existing published data demonstrating expression of the protein either *in vitro* or *in vivo* or require additional testing to verify actual protein expression.

In contrast to *in silico* screens, two-dimensional gel electrophoresis (2DGE) is a classic proteomic technique that allows for resolution of individual expressed proteins by each protein's isoelectric point and molecular weight (Kenrick & Margolis, 1970). 2DGE has been used to map mycobacterial proteins and to demonstrate differences between

MTB and BCG. The technique has been applied to MTB in conjunction with mass spectrometry to demonstrate differential protein responses to a variety of stressors such as heat, reactive oxygen/nitrogen species, or hypoxia. Much of the 2DGE proteomics studies on MTB have focused on culture filtrates in order to identify secreted virulence factors or diagnostic targets (Nagai, *et al.*, 1991, Naito, *et al.*, 1998, Malen, *et al.*, 2008). One study by Florczyk et al. examined the difference between the proteome of shaken and standing cultures of BCG Pasteur demonstrating a group of five standing-culture specific proteins (Florczyk, *et al.*, 2001). By western blotting two-dimensional gels using sera from infected hosts, it is further possible to interrogate the humoral immune response to individual proteins (Pheiffer, *et al.*, 2005, Zhang, *et al.*, 2012).

Recent work using the *in vitro* pellicle biofilm model of static MTB growth in minimal media has demonstrated increased survival of bacteria following treatment with antibiotics compared to the frequently used shaken culture of MTB grown in rich media and supplemented with detergents (Tween-80) to prevent MTB's natural clumping phenotype (Ojha, *et al.*, 2008, Sambandan, *et al.*, 2013). Although often existing in an intracellular environment during infection, the bacilli grow at the air-tissue interface in cavitary TB in aggregates microscopically similar to the *in vitro* pellicle mode of growth (Nedeltchev, *et al.*, 2009). Furthermore, in this stage of the disease large numbers of infectious bacteria are produced and dispersed in aerosol form (Osler, 1892). Thus, growing MTB in pellicle form mimics an extracellular stage of MTB infection that has been largely neglected and is critical for transmission (Grosset, 2003).

For diagnostic purposes, identifying new antigens that stimulate a humoral response allows the production of new assays for MTB infection using techniques such as enzyme-linked immunosorbant assay (ELISA), indirect fluorescent antibody assay (IFA), or lateral-flow assay.

Previously, our group used classic immunoproteomic techniques to show *Staphylococcus aureus in vitro* biofilms stably express a distinct set of proteins (Brady, *et al.*, 2006). A group of these biofilm proteins were used to create a multi-subunit vaccine that prevented the development of *S. aureus* osteomyelitis in a mouse model (Brady, *et al.*, 2011). These same antigens were also used as a novel method of immunodetection of *in vitro S. aureus* biofilms (Brady, *et al.*, 2007).

Using the guinea pig model of aerosol infection and *in vitro* pellicle model of MTB growth, we identified a pool of proteins that stimulated a humoral immune response during pulmonary infection. Using 2DGE, western Blotting, and protein identification by Matrix-Assisted Laser Desorption Ionization Time-of-Flight/Time-of-Flight (MALDI-TOF/TOF) mass spectrometry we determined which genes were expressed throughout all three pellicle timepoints. We then separated the data into proteins expressed only in the pellicle timepoints (pellicle-specific proteins) and those expressed in the three pellicle timepoints and the control shaken culture (referred to as universally expressed proteins). We performed additional bioinformatics studies on both pools of proteins to better gauge their usefulness for vaccine and diagnostic purposes.

Part 2: Materials and Methods

Reagents and Growth Media. All reagents and bacterial growth media were obtained from Thermo Fisher Scientific, Waltham, Massachusetts unless noted otherwise.

Strains. The H37Rv strain of *M. tuberculosis* (TMC #102) was obtained from the Trudeau Institute, Saranac Lake, New York. The H37Ra strain of *M. tuberculosis* (ATCC# 25177) was obtained from the American Type Culture Catalog, Manassas, Virginia.

Bacterial Cultures. H37Ra was propagated in 7H9 OADC media with 0.05% Tween-80 and on 7H10 OADC agar. MTB pellicles were grown as previously described (Ojha, *et al.*, 2008). Briefly, 25 ml of Sauton's media in a 250 ml polystyrene bottle was inoculated with 100 µl of a mid-log phase MTB culture grown in 7H9 OADC with 0.05% Tween-80. Biofilms were grown for three, five, and seven weeks in a 37°C incubator. Control shaken cultures were inoculated with 100 µl of the same culture used to inoculate the biofilm cultures into 25 ml of Sauton's media with no Tween-80 added, shaken for two weeks at 180 rpm at 37°C.

Fluorescence In-Situ Hybridization. MTB pellicle was collected, placed on Gold Plus slides and heat fixed on a hotplate for 10 minutes. Slides were then immersed in 95% ethanol for 10 minutes. MTB smears were treated with a mild acid hydrolysis followed by dual mutanolysin (5,000 U/ml) and lysozyme (10 mg/ml) treatment. The MTB smears were then treated with hybridization solution (50% v/v formamide, 10% wt/v dextran sulfate, 0.1% wt/v sodium pyrophosphate, 0.2% wt/v polyvinylpyrrolidone, 0.2% wt/v Ficoll 400, 0.1% v/v Triton X-100, 0.005 M disodium EDTA, 0.01 M NaCl) containing 1 nm/ml of an MTB specific 16s rRNA FISH probe (5' - /Cy5/ GCA TCC CGT GGT CCT – 3') for 90 minutes (Lefmann, *et al.*, 2006). They were then treated with a wash solution (0.005 M Tris, 0.015 M NaCl, 0.1% v/v Triton X-100) for 30 minutes.

The slides were air-dried and mounted using VectaShield mounting solution (Vector Labs, Burlingame, California).

Bacterial Membrane Protein Isolation. H37Ra pellicles were collected at three, five, and seven week timepoints and shaken cultures after two weeks (late-log stage based on growth curves) by the addition of 20% Tween-80 to a final concentration of 0.05% Tween-80, 10% sodium azide to a final concentration of 0.02%, and 25X concentrated Roche complete, EDTA-free protease inhibitor to a final concentration of 1X. Each MTB sample was centrifuged at 4000 × *g* for 15 minutes at 4°C. The pellet was washed twice in PBS with 0.05% Tween-80 and 1X Roche complete, EDTA-free protease inhibitors and resuspended in Mycobacterial Lysis Buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.02% w/v sodium azide) with 1X complete, EDTA-free protease inhibitor. Bacteria were then lysed using 0.1 mm zirconia beads (BioSpec Products Bartlesville, Oklahoma) and a Fastprep FP120 bead beater (Thermo Fisher Scientific, Waltham, Massachusetts) then centrifuged at 15,000 × *g* at 4°C for 10 min and the supernatant was removed. The supernatant was centrifuged again at 23,000 × *g* at 4°C for 30 minutes, then ultracentrifuged at 150,000 × *g* for 1 hour at 4°C to recover the membrane vesicles. Vesicles were resuspended in nanopure water and protein concentration was established using the modified Lowry method.

Two-Dimensional Gel Electrophoresis. Performed as per Brady with modifications (Brady, *et al.*, 2006). An aliquot of 100 µg of membrane protein for each biofilm and shaken culture time point was precipitated using the Perfect Focus protein cleanup kit (G-Biosciences, St. Louis, Missouri) and resolubilized in 250 µl of Urea-thiourea-CHAPS buffer which was used to rehydrate pH 3-10 linear immobilized pH gradient gel strips (GE Healthcare, Pittsburgh, PA). The rehydrated strips were subjected to isoelectric focusing then loaded onto a Criterion precast 12.5% polyacrylamide gel (Biorad Life Science, Hercules, California) covered with an agarose sealing gel, and run at 120 V for and 1.5 h. Proteins were visualized by terminal staining with Sypro Ruby protein stain (Biorad Life Science, Hercules, California) and imaged under UV light. Each time point sample was subjected to the process in triplicate.

Experimental infection with *M. tuberculosis.* Female outbred Hartley guinea pigs [~500 g] were purchased from Charles River Laboratories (North Wilmington, MA) and held in a Biosafety Level III animal laboratory. Culture stocks of *M. tuberculosis* strain H37Rv collected at mid-log phase of growth in Proskauer-Beck liquid medium containing 0.05% Tween-80 and frozen at -80°C. Thawed aliquots were diluted to 1×10^6 CFU/ml in double-distilled sterile water and delivered by low-dose aerosol infection using a Madison chamber aerosol generation device calibrated to deliver approximately 20 bacilli to each animal.

2D Western Blots. Two-dimensional gels were run as described and then equilibrated in semi-dry transfer buffer (48mM Tris, 39 mM Glycine, 0.0375% SDS, 20% methanol) for 10 min. Immobilon P membranes (Millipore, Billerica, Massachusetts) were activated in 100% methanol for 5-10 s, equilibrated in ultrafiltered distilled water for 2 min, and equilibrated in semi-dry transfer buffer for 5-10 min. Semi-dry transfer was conducted using the Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell as per manufacturer's instructions (Biorad Life Science, Hercules, California) for 30 min at 20 V. Following transfer the gel was removed and stained with Sypro Ruby while the membrane was incubated in 5% milk TBS-T buffer overnight at

4°C. The blot was then incubated with a 1:10 dilution of day 60 TB-infected guinea pig serum again overnight at 4°C. The blot was then washed twice and the secondary antibody, a horseradish peroxidase labeled anti-guinea pig IgG (Ref # 61-4620, Life Technologies, Grand Island, New York) was used at a 1:2000 dilution in 5% milk TBST buffer and incubated 1 hour at room-temperature. The blot was then washed and visualized using Super-Signal West Pico-Chemiluminescent Substrate according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, Massachusetts).

Mass Spectrometry. Gel spots of interest were excised and equilibrated in 100 μl of 50 mM ammonium bicarbonate buffer (pH 8.0, 25mM), washed in 100 μl of water, and incubated twice in 100 μl of acetonitrile for 5 min. Acetonitrile was removed and the samples were then placed in a speedvac for 45 min to remove any excess solvent. 20 mg Trypsin (Promega Corp, Fitchburg, Wisconsin) was dissolved in 2 ml of 25 mM, pH 8.0 ammonium bicarbonate and 10 μl were added to each gel spot and incubated at 37°C for 6 h. After digestion, 1 μl of sample solution was spotted directly onto a MALDI target plate and allowed to dry. 1 μl of α-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co.) matrix solution (50:50 acetonitrile/water at 5 mg/ml) was then applied on the sample spot and allowed to dry.

MALDI TOF/TOF mass spectrometry was used to analyze the samples and determine protein identification. Data were acquired with an AB Sciex TOF/TOF™ 5800. AB Sciex software package included TOF/TOF Series Explorer software (v. 4.1.0) with Oracle Database Schema Version (v. 4.0.0), Data Version (4.0.5) to acquire both MS and MS/MS spectral data. The instrument was operated in positive ion reflectron mode, mass range was 850 – 3000 Da, and the focus mass was set at 1700 Da. For MS data, 800 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using a peptide mixture with reference masses 904.468, 1296.685, 1570.677, and 2465.199. Mass spectrometry peak filtering included the following parameters: minimum S/N filter = 5, mass exclusion list tolerance = 0.5 Da, and mass exclusion list for common trypsin and keratin-containing compounds.

Following MALDI MS analysis, MALDI MS/MS was performed on the top 10 most abundant ions from each sample spot. A 1kV positive ion MS/MS method was used to acquire data under post-source decay (PSD) conditions. The instrument precursor average selection window was +/- 3 Da. For MS/MS data, 1000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using reference fragment masses 175.120, 684.347, 813.389, 1056.475, and 1441.634 (from precursor mass 1570.670). For MS/MS peak filtering, the minimum S/N filter = 5.

AB Sciex Protein Pilot™ software (v. 4.0.8085) was used in conjunction with MASCOT to search the respective protein database using both MS and MS/MS spectral data for protein identification. Protein match probabilities were determined using expectation values and/or MASCOT protein scores. For protein identification, the *M. tuberculosis* taxonomy was searched in the NCBI protein database. Other parameters included the following: selecting the enzyme as trypsin; maximum missed cleavages = 2; fixed modifications included carbamidomethyl (C) for 2-D gel analyses only; variable modifications included oxidation (M); precursor tolerance was set at 0.5 Da; MS/MS fragment tolerance was set at 0.5 Da; mass = monoisotopic; and peptide charges were only considered as +1. The significance of a protein match, based on both the peptide mass fingerprint (PMF) in the first MS and the MS/MS data from several precursor ions, is based on expectation values; each protein match is accompanied by an expectation value. The expectation value is the number of matches with equal or better scores that are expected to occur by chance alone. The default significance threshold is $p<0.05$, so an expectation value of 0.05 is considered to be on this threshold. We used a more stringent threshold of 10^{-3} for protein identification.

Bioinformatics. Protein functional information was obtained from TubercuList (http://tuberculist.epfl.ch/). Information on homology was obtained through the use of BLAST or by accessing structural information at the Protein Data Bank (PDB). Additional information was obtained from primary literature. Gene sequences obtained from NCBI and analyzed for conservation using tBLASTn to determine protein identity percentages across 15 sequenced MTB strains (http://blast.ncbi.nlm.nih.gov/). We predicted the protein's secondary structure using Phyre2 (Kelley & Sternberg, 2009).

Part 3: Results

MTB *in vitro* pellicle membrane proteins are immunogenic. MTB H37Ra pellicles from three, five, or seven weeks *in vitro* culture was compared to a control shaken culture of H37Ra grown for two weeks. Pellicles grown in minimal media for 5 weeks demonstrated robust aggregation macroscopically (Fig. 2.1A) and microscopically as examined by confocal microscopy following Fluorescence *in-situ* Hybridization (FISH) staining with an MTB-specific 16S rRNA probe (Fig. 2.1B). Bacteria were mechanically lysed and total membrane protein was obtained by differential centrifugation. Protein samples were assayed and 100 μg of sample was precipitated to remove impurities. Protein in each sample was separated by 2DGE (Fig. 2.2 A, C, E, G) in triplicate and transferred to membranes. Western blotting of the membranes of the separated and transferred three, five, and seven week pellicle protein samples identified a number of immunogenic proteins (Fig. 2.2 B, D, F, H). 35, 37, and 29 distinct immunogenic proteins spots from 3 three, five, and seven week pellicle two-dimensional gels respectively, were analyzed by MALDI-TOF/TOF mass spectrometry to determine protein identities. This yielded a list of 44 distinct immunogenic proteins expressed over the three timepoints, which was also compared to two-dimensional gels of two week shaken culture H37Ra to determine the temporal expression pattern of the proteins (Table 2.1). Of the 44 identified proteins 43 had previously been showed to fractionate into the total membrane fraction of MTB (Lew, *et al.*, 2011).

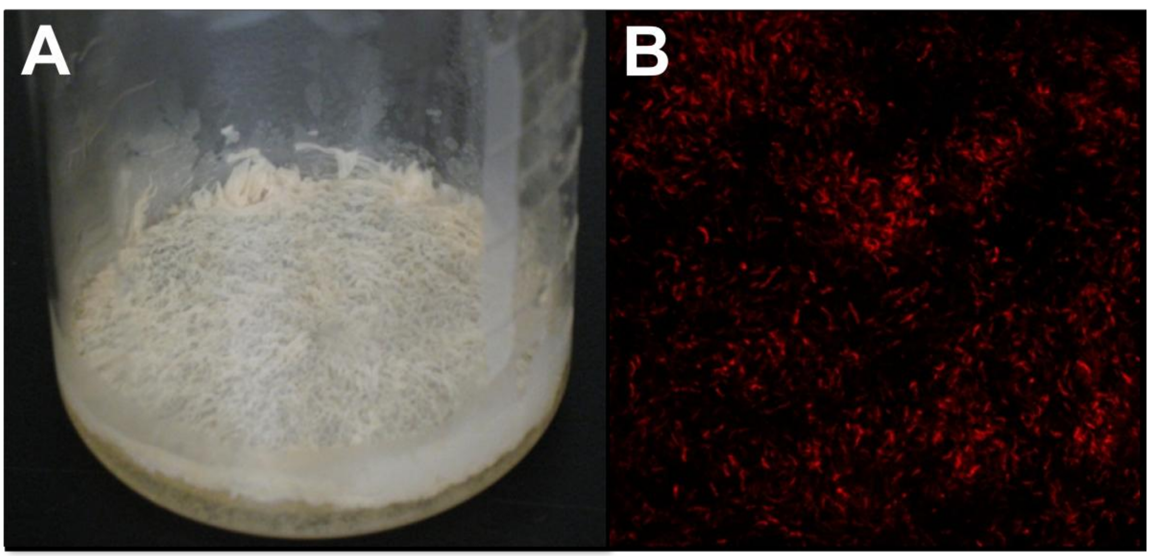


Figure 2.1.Static Tuberculosis Biofilm (A) 5 week-old *Mycobacterium tuberculosis* pellicle grown statically in Sauton's minimal media as described in Ojha et al 2008 and (B) a Fluorescence In-Situ Hybridization stain of the pellicle for MTB 16s rRNA (100x magnification).

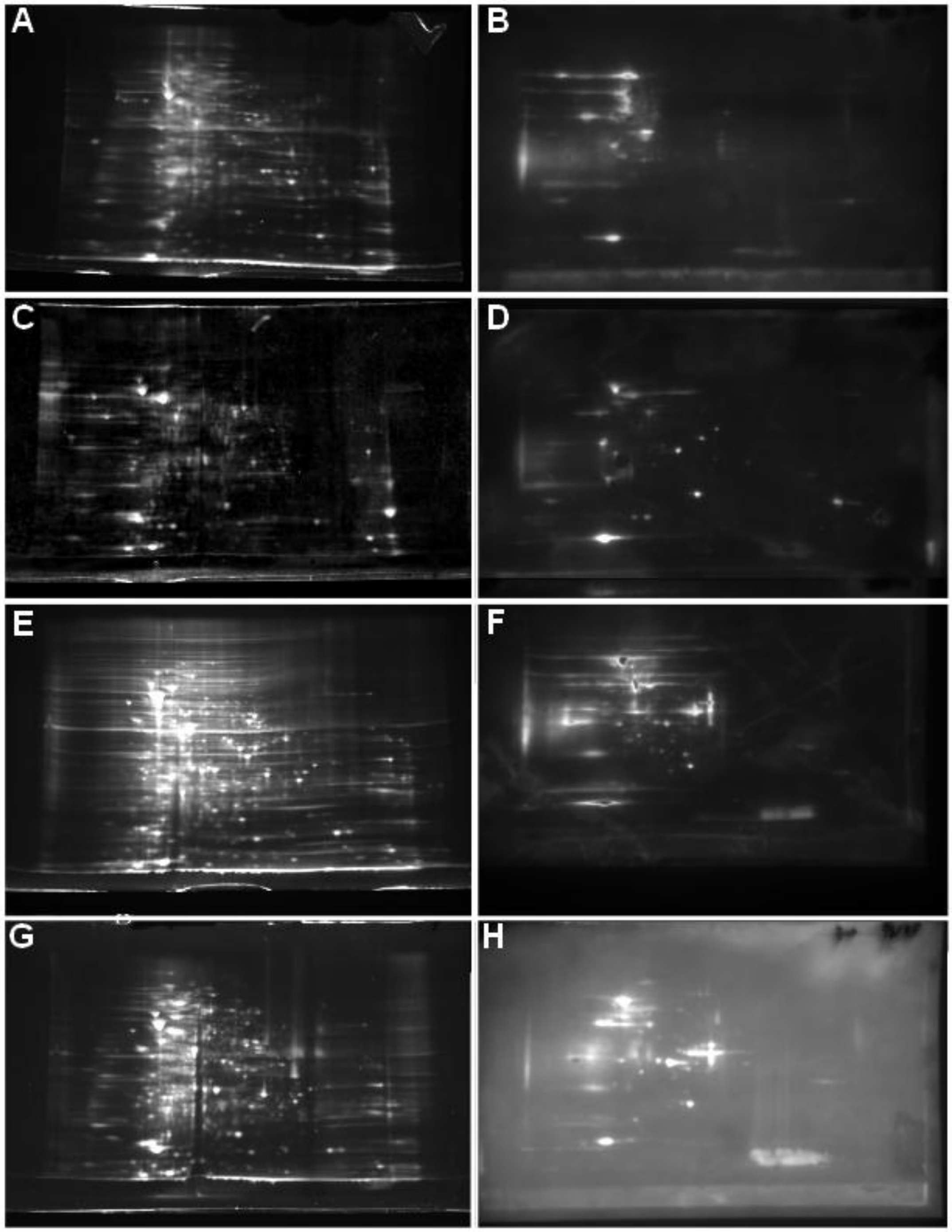


Figure 2.2. Two-dimensional gel electrophoresis gels and matched western blots. 2 week shaken culture (A), 3 week pellicle (C), 5 week pellicle (E), and 7 week pellicle (G) membrane proteins (Sypro Ruby Stain). Two-dimensional Western Blots of 2 week shaken culture (B), 3 week pellicle (D), 5 week pellicle (E), and 7 week pellicle (F) membrane proteins probed with 60-day MTB infected guinea pig serum and visualized with horseradish-peroxidase labeled secondary antibody.

Table 2.1. Comprehensive list of immunogenic proteins identified grouped by expression patterns (Pellicle specific, Universal, or Indeterminate). Protein expression was established for each time point by either mass-spectrometry (MS) or Sypro-Ruby (SR) protein stain of two-dimensional gels. Mass spectrometry identities were determined with an expectation value threshold of 10^{-3} or less, Sypro-Ruby spot identities were representative off at least 3 replicate 2D gels for each time point/sample. Additional protein information including isoelectric point, molecular weight, and functional information obtained from Tuberculist (tuberculist.epfl.ch). Expression at each time point is denoted by + (expressed), - (not expressed), or I (indeterminate).

Abrv	Name	Rv#	pI	MW (kDa)	2W Shaken	3W Pel	5W Pel	7W Pel
Pellicle Specific Proteins								
Rv3237c	Conserved protein	Rv3237c	4.7	17.2	-	+	+	+
CeoB	Complementation of E. coli OxyR Mutant B	Rv2691	6.3	24.2	-	+	+	+
FabG4	3-ketoacyl-acyl carrier protein reductase	Rv0242c	6.4	46.8	-	+	+	+
FhaA	Conserved protein with FHA domain	Rv0020c	4.7	56.9	-	+	+	+
Rv0097	Possible oxidoreductase	Rv0097	6.6	32.6	-	+	+	+
Rv1996	Universal stress protein family protein	Rv1996	7.0	33.9	-	+	+	+
Rv2216	Conserved protein	Rv2216	7.6	31.7	-	+	+	+
Rv2296	Probable haloalkane dehalogenase	Rv2296	7.0	33.4	-	+	+	+
Rv3519	Unknown	Rv3519	10.0	26.0	-	+	+	+
Universally Expressed Proteins								
35kD_ag	35 kDa Antigen	Rv2744c	5.8	29.3	+	+	+	+
AtpD	ATP synthase beta chain	Rv1310	4.6	53.1	+	+	+	+
Eno	Enolase	Rv1023	4.3	44.9	+	+	+	+
FadD2	fatty-acid-CoA synthase	Rv0270	6.8	59.9	+	+	+	+
FixB	Probable electron transfer flavoprotein	Rv3028c	4.4	31.7	+	+	+	+
Frr	Ribosome recycling factor	Rv2882c	5.8	20.8	+	+	+	+
GlnA1	Glutamine synthetase A1	Rv2220	4.8	53.5	+	+	+	+
GlnA2	Glutamine synthetase A2	Rv2222c	5.2	49.6	+	+	+	+
GroEl2	Heat Shock Protein 65	Rv0440	4.6	56.7	+	+	+	+
HbhA	Heparin binding hemagglutinin	Rv0475	9.8	21.5	+	+	+	+
Mkl	Possible ribonucleotide-transport ABC transporter	Rv0655	5.9	39.4	+	+	+	+
MoxR1	Probable transcriptional regulatory protein	Rv1479	6.4	40.8	+	+	+	+
NusA	Probable N utilization substance protein A	Rv2841c	6.9	37.6	+	+	+	+
PepC	Probable aminopeptidase	Rv0800	6.5	46.0	+	+	+	+
PrcA	Proteasome α subunit	Rv2109c	5.2	26.8	+	+	+	+
PyrB	Probable aspartate carbamoyltransferase	Rv1380	7.1	33.8	+	+	+	+
RplE	50S ribosomal protein L5	Rv0716	10.8	21.0	+	+	+	+

RplI	50S ribosomal protein L9	Rv0056	9.7	16.2	+	+	+	+
RplL	50S ribosomal protein L7/L12	Rv0652	4.3	13.4	+	+	+	+
RplM	50S ribosomal protein L13	Rv3443c	10.6	16.3	+	+	+	+
RplR	50S ribosomal protein L18	Rv0720	12.1	13.2	+	+	+	+
RpoA	DNA-directed RNA polymerase α chain	Rv3457c	4.4	37.7	+	+	+	+
RpsE	30S ribosomal protein S5	Rv0721	10.8	22.9	+	+	+	+
Rv1245c	Probable short-chain type dehydrogenase/reductase	Rv1245c	8.0	29.2	+	+	+	+
Rv1771	L-gulono-1,4-lactone dehydrogenase	Rv1771	7.6	48.0	+	+	+	+
SodA	Superoxide dismutase	Rv3846	6.4	23.0	+	+	+	+
Ssb	Single-strand binding protein	Rv0054	4.8	17.3	+	+	+	+
Tb31.7	Universal stress protein family protein	Rv2623	5.6	31.7	+	+	+	+
Tuf	Probable iron-regulated elongation factor TU	Rv0685	5.1	43.6	+	+	+	+
Wag31	DivIVA family protein	Rv2145c	4.5	28.3	+	+	+	+
Indeterminate Proteins								
AtpG	ATP synthase γ chain	Rv1309	5.1	33.9	+	I	+	+
BfrB	Bacterioferritin B	Rv3841	4.5	20.4	I	+	+	-
Fum	Probable fumarase	Rv1098c	5.2	50.1	+	I	+	+
Rv0148	Probable short-chain type dehydrogenase/reductase	Rv0148	5.1	29.8	I	I	+	I
Rv2629	Conserved protein	Rv2629	5.0	40.8	+	I	+	+

A subset of immunogenic proteins is specific to pellicle. In order to identify the subset of immunogenic proteins that were specifically produced in the three pellicle timepoints but not during planktonic shaken growth, we performed comparisons of gels at all timepoints to identify proteins that were expressed during all three pellicle timepoints but not in the shaken control culture as previously described (Brady, *et al.*, 2006). We identified nine proteins that were pellicle-specific in their expression, 30 that were universally expressed, and five that did not fall into either class (Table 2.1). The universally expressed protein class contained a number of housekeeping genes including the glycolysis protein enolase, six ribosomal proteins, and the chaperone GroEl2. Also expressed at all time points was the adhesin heparin-binding hemaglutinin A (HbhA) and the unknown function proteins TB31.7 and 35kD antigen.

Pellicle specific immunogenic proteins are highly conserved. The primary sequence of each of the nine pellicle-specific proteins from H37Ra was compared to 15 sequenced strains of MTB (see Table 2.2) as well as *Mycobacterium bovis* AF2122197, *Mycobacterium africanum* GMO41182, *Mycobacterium canetti* CIPT 140070008, *Mycobacterium smegmatis* mc2 155, and *Mycobacterium avium* 104 using the tBLASTn which uses the Basic Local Alignment Search Tool (BLAST) to compare a protein sequence to a translated genome(Camacho, *et al.*, 2009). The percent identity (Fig. 2.3) demonstrates the extreme conservation of the protein sequences among the members of the *Mycobacterium tuberculosis* complex (MTB, *M. africanum*, *M. bovis*, *M. canetti*) and the lower degree of conservation with the related pathogen *M. avium* or the nonpathogenic *M. smegmatis*. Rv0097 in particular showed high average conservation in the MTB complex (98%) but relatively low sequence conservation in both *M. avium* (25%) and *M. smegmatis* (26%). FabG4 (Rv0242c) displayed a high average degree of conservation among the MTB complex (99%) and with *M. avium* (90%) and *M. smegmatis* (83%). Figure 2.4 shown a phylogenetic tree based on whole-genome sequences is given for comparison with the individual protein sequence conservation shown in Figure 2.3.

Table 2.2. Sequenced Genomes Used for Conservation Analysis

Species	Assembly	Accession	Size (Mb)	GC%
Mycobacterium tuberculosis UT205 chromosome	Mt_UT205	NC_016934.1	4.42	64.9
Mycobacterium tuberculosis RGTB423 chromosome	ASM27710v1	NC_017528.1	4.41	65.6

Mycobacterium tuberculosis RGTB327 chromosome	ASM27708v1	NC_017026.1	4.38	65.6
Mycobacterium tuberculosis KZN1435 chromosome	ASM2362v1	NC_012943.1	4.40	65.6
Mycobacterium tuberculosis KZN 4207 chromosome	ASM15458v2	NC_016768.1	4.39	65.6
Mycobacterium tuberculosis KZN 605 chromosome	ASM15460v2	NC_018078.1	4.40	65.6
Mycobacterium tuberculosis Haarlem/NITR202 chromosome	ASM38990v1	NC_021192.1	4.40	65.6
Mycobacterium tuberculosis H37Rv chromosome	ASM19595v2	NC_000962.3	4.41	65.6
Mycobacterium tuberculosis H37Ra chromosome	ASM1614v1	NC_009525.1	4.42	65.6
Mycobacterium tuberculosis F11 chromosome	ASM1692v1	NC_009565.1	4.42	65.6
Mycobacterium tuberculosis EAI5/NITR206 chromosome	ASM38994v1	NC_021194.1	4.39	65.6
Mycobacterium tuberculosis CTRI-2 chromosome	ASM22443v1	NC_017524.1	4.40	65.6
Mycobacterium tuberculosis CCDC5180 chromosome	ASM27036v1	NC_017522.1	4.41	65.6
Mycobacterium tuberculosis CCDC5079 chromosome	ASM27034v1	NC_017523.1	4.40	65.6
Mycobacterium tuberculosis CAS/NITR204 chromosome	ASM38992v1	NC_021193.1	4.39	65.6
Mycobacterium tuberculosis Beijing/NITR203 chromosome	ASM36482v1	NC_021054.1	4.41	65.6
Mycobacterium tuberculosis ATCC 35801 chromosome	ASM35020v1	NC_020559.1	4.39	65.6
Mycobacterium smegmatis MC2 155	ASM1500v1	NC_008596.1	6.99	67.4
Mycobacterium canetti CIPT 140070008	ASM32880v1	NC_019965.1	4.43	65.6
Mycobacterium bovis AF2122/97	ASM19583v1	NC_002945.3	4.35	65.6
Mycobacterium avium 104	ASM1498v1	NC_008595.1	5.48	69.0
Mycobacterium africanum GMO41182	ASM25335v1	NC_015758.1	4.39	65.6

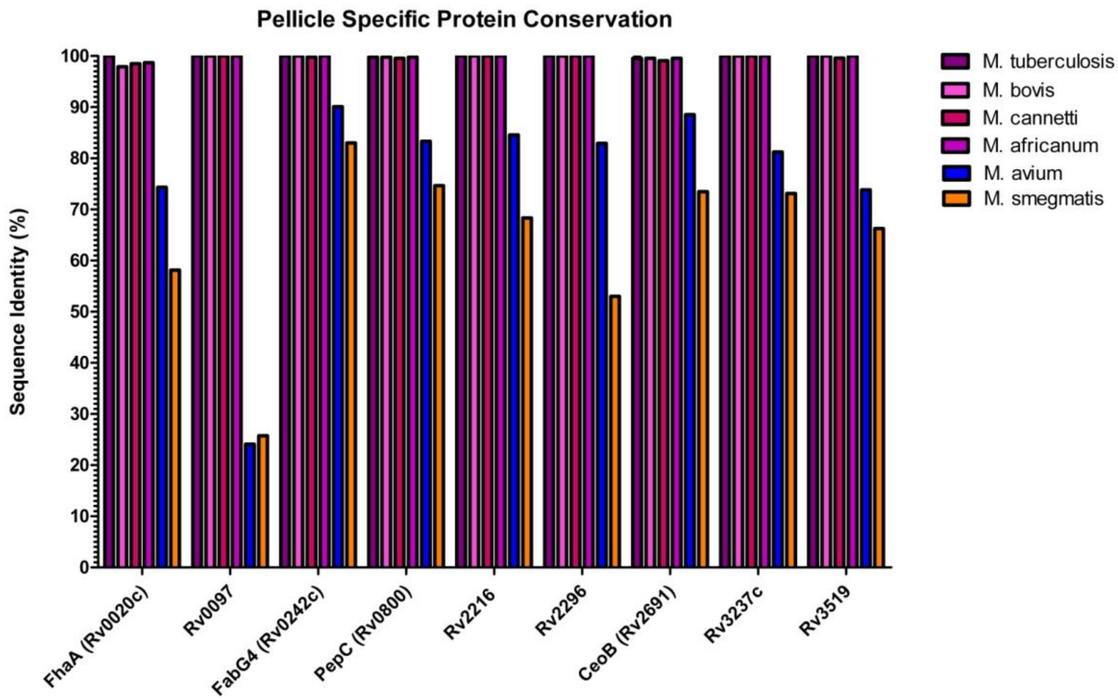


Figure 2.3. Pellicle specific protein conservation. Determined by tBLASTn of protein sequences against translated genomes. *M. tuberculosis* bar represents median conservation of the protein among 15 sequenced MTB strains.

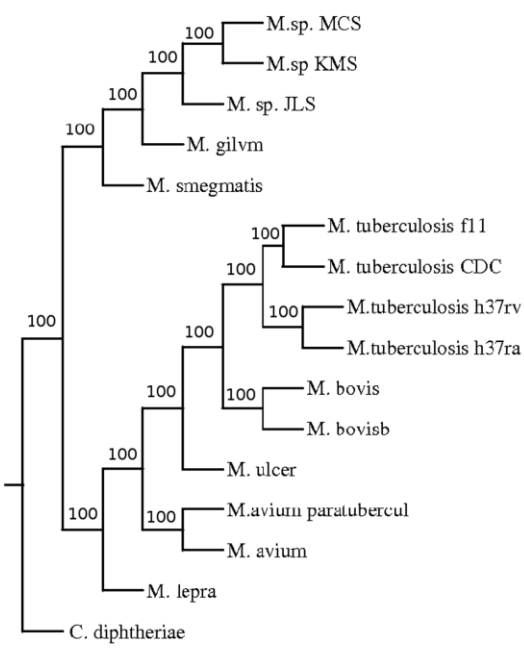


Figure 2.4. Phylogenetic tree of MTB and related mycobacterium (whole genome analysis) for comparison to protein sequence conservation in Fig 2.3 (from Vishnoi et al., 2010).

Part 4: Discussion

A large number of published studies have attempted to identify new TB diagnostic biomarkers in an effort to detect and combat the large worldwide burden of TB. The present work describes a novel approach of we utilizing the host antibody response to detect those antigens unique to the pellicle mode of growth that are expressed and stimulate an immune response *in vivo*. By combining this model of extracellular MTB growth with proven techniques of 2DGE, Western blotting, and MALDI-TOF/TOF protein identification, we were able to identify 44 immunogenic proteins expressed in this model. Out of the 44 immunogenic proteins we identified nine that were specifically expressed in the pellicle mode of growth compared to the control shaken culture. The identification of biomarker antigens for the development of novel diagnostic strategies is advantageous because it allows for a variety of rapid diagnostic tests to be performed on easily isolated serum.

While none of the pellicle-specific proteins identified have well-characterized functions, several existing studies in combination with bioinformatics data shed some light on possible functions for these proteins in MTB pathogenesis. The fork-head domain containing protein FhaA (Rv0020c) has been structurally characterized (Table 2.3) (Roumestand, *et al.*, 2011). Current models of this protein's function describe it as a signal transducer that, when activated by a sensor of peptidoglycan, mediates the repression of more peptidoglycan synthesis (Gee, *et al.*, 2012). This repression would be expected in the nutrient-deprived pellicle and granuloma since macromolecular synthesis is inhibited.

Another pellicle-specific protein, Rv0097, was expressed preferentially in the three pellicle timepoints compared to shaking culture and was highly (98%) conserved within the MTB complex. However, only 25% amino acid sequence similarity was found when compared to *M. avium* and *M. smegmatis*. Disruption of Rv0097 in *M. bovis* (Mb0100) has been shown to abolish production of phthiocerol dimycocerosates (PDIM), a lipid virulence factor that protects MTB against nitric oxide-dependent killing in macrophages (Rousseau, *et al.*, 2004, Hotter, *et al.*, 2005, Astarie-Dequeker, *et al.*, 2009). Considering the large amounts of nitric oxide produced by granuloma macrophages in mycobacterial infections, increased expression of Rv0097 might allow for more PDIM production to protect bacteria from this host response (Ehlers, *et al.*, 1999).

CeoB (Rv2691) is a TrkA homolog; the *trk* potassium uptake system is a medium-affinity system for potassium uptake (Bossemeyer, *et al.*, 1989). CeoB was noted for its ability to complement the *E. coli oxyR* mutant, which is susceptible to the antitubercular drug isoniazid unlike wild type *E. coli,* which is resistant. When CeoB and its homolog CeoC were transformed into the mutant, it regained its natural resistance (Chen & Bishai, 1998). The most common mutations leading to isoniazid residence in clinical isolates of MTB are those of the catalase-peroxidase enzyme KatG, which activates the isoniazid pro-drug (Cade, *et al.*, 2010). Other gene mutations have been identified including *kasA, ndh, ahpC,* and *inhA* (Rindi, *et al.*, 2005). Besides low metabolic rates, it is possible that increased expression of CeoB could be responsible for part of the phenotypic resistance to isoniazid displayed by MTB biofilms (Ojha, *et al.*, 2008).

Rv3519 contains homology with known acetoacetate decarboxylase proteins, which produce CO_2 and acetone from acetoacetate (May, *et al.*, 2013). Acetoacetate is produced in the human liver from acetyl-CoA and secreted into the blood under conditions of starvation or diabetes, metabolized in the brain to CO_2 and acetone, both of which are excreted by the pulmonary route as gas (Miekisch, *et al.*, 2001). Acetone is utilized by *M. smegmatis* as both a carbon source and an energy source, suggesting MTB could utilize Rv3519 to obtain acetone from acetoacetate (Lukins & Foster, 1963). Diabetes is a known risk factor for TB (Dooley & Chaisson, 2009). One possible mechanism for this could be that acetone excreted into the lungs in diabetic patients provides an additional carbon source for MTB. Furthermore, an acetone responsive transcriptional activator, *mimR* has been found in *M. smegmatis* (Kotani, *et al.*, 2007, Furuya, *et al.*, 2011). Given acetone's high permeability across biological membranes there is a possibility that it could act as the, currently uncharacterized, diffusible signaling molecule hypothesized to play a critical role in mycobacterial biofilm development (Ojha, *et al.*, 2008). Recent metabolomic studies have shown that both acetoacetate and acetone are increased in serum concentration during TB infection confirming that MTB would have access to these carbon sources (Zhou, 2013).

FabG4 (Rv0242c) is one of five FabG proteins in the MTB genome. As part of the critical fatty acid synthetase II system (FASII) the protein InhA is transcribed with one FabG protein which uses NADPH to reduce β-oxoacyl-ACP to β-hydroxyacyl-ACP (Dutta, *et al.*, 2013). FabG4 in particular has been shown to be expressed in several minimal media models of growth (Beste, *et al.*, 2009, Sharma, *et al.*, 2010).

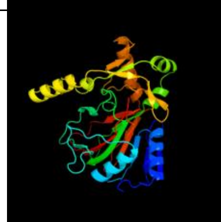
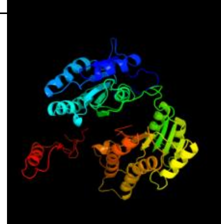
PepC (Rv0800) has been annotated as a probable aminopeptidase but nothing is known about its specific cellular function (Ribeiro-Guimaraes & Pessolani, 2007). Its production during long-term pellicle growth could reflect a need to break down or cannibalize specific mycobacterial proteins. Studies in *E. coli* have demonstrated that certain bacterial proteases are induced strongly in late stationary phase and that mutants for the *clpP* and *clpX* genes had reduced viability after stationary phase demonstrating that a subset of bacterial proteases are critical to surviving low-nutrient conditions (Weichart, *et al.*, 2003).

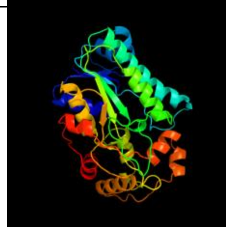
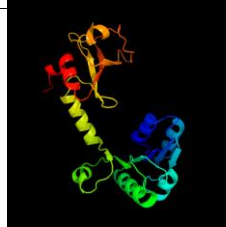
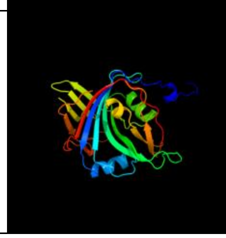
Rv2216 is predicted to be a nucleoside diphosphate sugar epimerase, a class of enzymes that converts sugars (linked to nucleoside diphosphates such as UDP) between different epimers. These enzymes play a crucial role in metabolism and have been shown to be essential virulence factors required for colonization. They are also critical to the formation of important carbohydrate molecules such as the backbone of bacterial lipopolysaccharide (LPS) (Allard, *et al.*, 2001). In mycobacteria it is more likely that Rv2216 might play a role in the synthesis of complex carbohydrate molecules like arabinogalactan similar to the function of UDP-Gal*p* epimerase, believed to be encoded by Rv3634, which converts UDP-Glucose to UDP-Gal*p* during the synthesis of galactan (Kaur, et al., 2009).

This study represents an original set of results with the discovery of novel biomarkers through examining the immunoproteome of tuberculosis that can be studied for rapid diagnostic technologies in subsequent research. A recent high-throughput study examining sera from over 500 tuberculosis patients detected 484 immunogenic proteins of which 5 were also detected in this study (35kd_ag, GroEl2, HbhA, RplE, and RplL) (Kunnath-Velayudhan, *et al.*, 2010). Utilizing these proteins in a diagnostic capacity will require further testing using human serum to recognize recombinant or native-purified, pellicle-specific protein to determine whether MTB infection in humans produces sufficient antibody titers to recognize these antigens. Furthermore, testing of latent vs.

active tuberculosis patient serum against these proteins may be useful to identify whether any of these antigens would be suitable for diagnosis of latent TB. Lastly, the utilization of these proteins in a potential multivalent vaccine that provides protection against MTB challenge is a necessary subsequent step that will be explored in the following chapter.

Table 2.3. Predicted structure of pellicle-specific proteins.

Gene	Phyre2 Structure Prediction	Conser-vation	Coverage	ID %	Template
FhaA (Rv0020c) Actual Structure (Roumestand, *et al.*, 2011)		n/a	n/a	n/a	Actual structure
Rv0097		100	94	23	Fe(ii)/α-ketoglutarate dependent taurine2 dioxygenase from *Pseudomonas putida* kt2440
Rv3237c		99.8	66	26	Putative Potassium Channel Related Protein *Pyrococcus horikoshii OT3*
FabG4 (Rv0242c)		100	89	99	FabG1 from M. tuberculosis H37Ra
PepC (Rv0800)		100	98	36	Human aspartyl aminopeptidase (dnpep)

Rv2296		100	99	30	*Haloalkane dehalogenase* from *Xanthobacter autotrophicus*
Rv2216		100	97	32	human epimerase family protein sdr39u12 (isoform2) with nadph
CeoB (Rv2691)		100	97	22	potassium transporter peripheral membrane component (trka) from vibrio2 vulnificus
Rv3519		100%	91%		Acetoacetate Decarboxylase (*Clostridium acetobutylicum*)

CHAPTER 3: PROTECTIVE EFFICACY AND IMMUNE RESPONSE TO A RECOMBINANT, 5 SUBUNIT BIOFILM VACCINE FOR THE PREVENTION OF MTB

Part 1: Background and Significance

In Chapter 2 we demonstrated that MTB *in vitro* pellicles produce a set of distinct immunogenic proteins compared to shaken culture. Additionally, there was a group of 30 immunogenic proteins that was expressed in both the shaking and biofilm models. Using this information, we developed a five-subunit biofilm vaccine by selecting four of these universally-expressed proteins (35kD_ag, HbhA, Mkl, and TB31.7) with the addition of the biofilm-expressed protein CeoB. We tested this vaccine using two mouse strains, two adjuvants, two vaccination strategies, and performed detailed analysis of both the adaptive and humoral responses to the vaccine as well as its ability to protect vaccinated animals from aerosol challenge with virulent MTB.

New experimental vaccines for tuberculosis are needed because the current vaccine, Bacille Calmette-Guerin (BCG), developed in 1919, is ineffective at preventing pulmonary TB. BCG was originally derived by serially sub-culturing *Mycobacterium bovis* in media containing ox-bile for 13 years, a process resulting in a clinically attenuated strain that was subsequently considered as a vaccine (Luca & Mihaescu, 2013). Initially administered orally, the vaccine later began to be administered by cutaneous injection. Following World War II most European countries adopted the vaccine for routine vaccination of children, but the United States chose to refrain to preserve the utility of the tuberculin test (precursor of the purified protein derivative or Mantoux test).

The efficacy of the BCG vaccine is a debated topic. What is clear is that the vaccine is effective at preventing early childhood tuberculosis especially TB meningitis, which it reduces by ~80% (Lanckriet, *et al.*, 1995). This makes BCG a valuable tool in TB-endemic countries. Its efficacy in preventing adult pulmonary TB, however, has been found to be between 0 – 80% effective in different studies (Liu, *et al.*, 2009). One possible explanation for this variable efficacy is after decades of continuous culture, there are now several regional variants of the BCG vaccine, the mose widely used being BCG-Pasteur, BCG-Copenhagen, and BCG-Tice (Behr, 2002). As a result, each of these regional variants has undergone antigenic drift with resulting variability in protective efficacy. Another possible explanation for the widely varying efficacies reported is the regional variation seen in environmental mycobacteria. When local populations are exposed to local mycobacterial strains that have antigens that are homologous to those that provide protection in the BCG vaccine against MTB, the BCG vaccine could act as a booster, thereby improving efficacy. Alternatively, early exposure of populations to the antigens from some local mycobacterial strains could interfere with the creation of a protective immune response via original antigenic sin, in which a previous exposure to similar bacteria impeding the ability to form an optimal immune response, which has been demonstrated to occur between MTB and *M. leprae* (Bothamley, et al., 1991).

Numerous vaccines have attempted to improve upon the effectiveness of BCG. Advocates of this approach cite the safety profile of BCG, its low expense, single-dose activity, and easy storage as compelling reasons to improve BCG instead of developing an entirely new vaccine (Shaban, *et al.*, 2013). One promising approach is creation of recombinant BCG strains that express one or more antigens from virulent MTB such as a recent BCG strain that expresses MTB proteins ESAT-6, Ag85B, and Rv3620c (Yang, *et al.*, 2014). Several of these vaccines such as rBCG-30 and rBCG85C have shown promise in reducing animal CFU burdens by 0.5 to 1.87 logs compared to parental BCG strains (Horwitz, *et al.*, 2000, Jain, *et al.*, 2008). Other strategies have utilized BCG as part of a prime-boost strategy either with recombinant protein, DNA vaccination, or liposomes (Ferraz, *et al.*, 2004, Guerrero & Locht, 2011, Lu, *et al.*, 2011).

Other vaccine projects have sought to exploit other biological vectors for use in an MTB vaccine. Modified Vaccinia Ankara (MVA) is an avirulent Vaccinia vector that was adapted to produce the immunodominant MTB antigen Ag85A, thereby yielding the MVA-85A vaccine. It was intended that this vaccine would be used as a boosting mechanism in the setting of previous BCG vaccination. MVA-85A showed promising results in animal models, where lung CFUs were reduced by 2.5 logs in mice and 1 log in rhesus macaques, and mortality was prevent in a guinea pig-survival model (Goonetilleke, *et al.*, 2003, Williams, *et al.*, 2005, Verreck, *et al.*, 2009). The vaccine has also been shown to produce a long-lasting Th1 immune response to MTB of up to 6 years (Tameris, *et al.*, 2014). Unfortunately, the vaccine yielded no significant protection in humans when tested in 1399 infants previously vaccinated with BCG in a TB endemic area (Nicol & Grobler, 2010, Tameris, *et al.*, 2013).

Recombinant protein vaccination approaches have also been attempted utilizing a number of different antigens including ESAT-6/CFP-10, and Ag 85A (Brandt, *et al.*, 2000, Hu, *et al.*, 2013) combined with a variety of adjuvants and administration strategies including alum, liposomes, and covalent bonding to polyethylene glycol (Doherty, *et al.*, 2007). One of the benefits of a pure recombinant protein vaccination strategy is safety since no chance of secondary infections or systemic spread in immunocompromised patients would result. Also, the defined composition, ability to alter the immune response to vaccine by altering adjuvant formulation, and the ability preserve the PPD skin test for screening of tuberculosis infection in vaccinated individuals are all benefits of a recombinant vaccine.

While a large number of these vaccines continue to be tested in both animals and humans, the world remains without a reliable, effective TB vaccine. Previous experience in our lab has demonstrated the effectiveness of a multi-subunit vaccine targeted against biofilm and constitutively expressed proteins in the prevention of *Staphylococcus aureus* biofilm infections in animal models. This four-subunit vaccine was formulated using four recombinantly-produced proteins that were identified using two-dimensional gel electrophoresis and western blotting combined with expression profile through RNA microarrays (Brady, *et al.*, 2006, Brady, *et al.*, 2011). We utilized a similar approach to formulate a biofilm vaccine for prevention of MTB infections.

We prioritized antigens discovered during the immunoproteomic screens in Chapter 2 and then recombinantly-expressed five MTB antigens in *E. coli* and *M. smegmatis*. We purified these antigens and used them to perform three vaccination and aerosol MTB challenge trials with several variations of adjuvant, vaccination protocol, mouse strain, and challenge dosage.

Part 2: Materials and Methods

Bacterial Strains. Arctic Express *E. coli* (BL21 lineage) were obtained from Agilent Technologies (Santa Clara, CA, USA) and are BL21 *E. coli* cells modified by the replacement of some endogenous chaperones with cold-temperature optimized chaperones. *Mycobacterium smegmatis* strain mc^2 155 was obtained from the American Type Culture Catalog (Manassas, VA, USA).

Vaccine Protein Selection. Antigens were selected from the list of antigens generated in Chapter 2 for inclusion in the multivalent vaccine on the basis of 1) presence in the membrane fraction of MTB, 2) immunogenicity by 2D western blot, 3) presence in three, five, and seven-week biofilm cultures, 4) literature or predictive models indicating outer membrane protein localization, and 5) literature documentation of a specific pathogenic role for the protein.

Cloning. *Rv2691, Rv2623, Rv2744c*, *Rv0655* DNA sequences were retrieved from NCBI database for *M. tuberculosis* H37Rv, had *Bsa*I restriction endonuclease recognition sites added flanking the MTB gene, and were codon optimized for expression in *E. coli* by Genescript and provided on pUC57 vectors (Piscataway, NJ, USA). *Rv2744c* and *Rv0655* (encoded 35kD_ag and Mkl respectively) were amplified by PCR (primers in Table 3.1). PCR products were fused to the pBAD thio/topo plasmid using the pBAD/TOPO ThioFusion Expression Kit (Invitrogen) per manufacturer instructions. *Rv2691* and *Rv2623* (CeoB and TB31.7, respectively) were excised from their pUC57 vectors by restriction digest using the restriction endonuclease BsaI (New England Biolabs, Ipswich, MA) to generate asymmetric sticky ends. The vector pASK-IBA14 was also digested by BsaI and the insert was ligated into the cut vector using T4 DNA Ligase (New England Biolabs, Ipswich, MA) for 15 minutes at room temperature. The ligation mixture was transformed into BL21 *E. coli* cells by heat shock, incubated at 37ºC with SOC media for one hour, and selected on LB agar with 50 µg/ml ampicillin. All constructs (Table 3.2) were confirmed by restriction digest to liberate the insert followed by running the digest on an agarose gel to confirm insert and vector size, as well as by sequencing from the vector into the insert from both the 5' and 3' ends of the insert. Sequencing results were compared to reference sequences using the UGENE software platform.

Table 3.1: Primers used in this research

Name	Sequence	Purpose
35kd_ag forward	5' - ATG GCG AAC CCG TTT GTG AAA – 3'	Cloning 35kD_ag into pBAD-thio/topo plasmid
35kd_ag reverse	5' - TTG ACC GTA CGG CTG TTC TG – 3'	Cloning 35kD_ag into pBAD-thio/topo plasmid
Mkl forward	5' –GTC CGC TAC TCT GAT TCC TAC-3'	Cloning Mkl into pBAD-thio/topo plasmid
Mkl reverse	5' –TTA CTG GCC AAT TTC GTG CAC C-3'	Cloning Mkl into pBAD-thio/topo plasmid

Table 3.2: Plasmids used in this research

Plasmid	Backbone	Gene Insert	Resistance	Induction Agent	Affinity Tag	Reference
pASK-CeoB	pASK-IBA14	*Rv2691*	Ampicillin	Anhydro-tetracycline	Streptag	This work
pASK-TB31.7	pASK-IBA14	*Rv2623*	Ampicillin	Anhydro-tetracycline	Streptag	This work
pBAD-35kD_ag	pBAD-thio/topo	*Rv2744c*	Ampicillin	Arabinose	6x HIS	This work
pBAD-Mkl	pBAD-thio/topo	*Rv0655*	Ampicillin	Arabinose	6x HIS	This work
PMV3-38	pMV206	*Rv0475*	Hygromycin	Constitutive	6x HIS	(Delogu, *et al.*, 2004)

Recombinant Antigen Expression and Purification:

35kD_ag Expression and Purification. Chemically competent BL21 *E. coli* were transformed by heat shock with pBAD 35kD_ag and plated on LB agar with 100 µg/ml ampicillin. Large scale cultures were inoculated with 1:100 dilution of overnight culture into LB media and grown at 37ºC to an OD600 ~ 0.5 before being induced with 1 ml of 20% arabinose. After three hours at 37ºC cultures were collected by centrifugation at 6,000 × g for 10 minutes. Cells were lysed at 10,000 psi for three passes on a French

Press and centrifuged at 15,000 × *g* for 15 minutes to sediment cell debris and insoluble protein. Pellets were denatured in Denaturing Equilibration Buffer (6 M Guanidine Hydrochloride, 20 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole) for >30 minutes then centrifuged at 15,000 × *g* for 15 minutes to sediment debris. The supernatant was applied to a Hispur Ni-NTA agarose resin column (Thermo) bound, washed, and eluted according to manufacturer instructions. Elution fractions were precipitated using Chloroform Methanol and the pellets resuspended in 1X SDS-PAGE Sample Buffer before being run on a 12.5% Acyramide Tris-HCL Criterion Gel at 130 volts for 90 minutes. Elution fractions containing protein were desalted on a PD-10 column (GE Healthcare) using PBS buffer and stored at -20ºC for use in vaccination, ELISA, or Antigen Specific Recall experiments.

CeoB Expression and Purification. Arctic Express *E. coli* containing the pASK-CeoB plasmid were subcultured in LB media containing 100 µg/ml ampicillin and diluted 1:100 into 2 L LB media and grown to OD600~0.5 at 37ºC, protein production was then induced by addition of 100 µl of 2 mg/ml anhydrotetracycline per 1 L of culture and incubated at 37ºC for an additional 3 hours. Bacteria were collected by centrifugation at 6,000 × *g* for 10 min and resuspended in PBS. Bacteria were lysed in a French Press at >10,000 psi with three passes and centrifuged at 15,000 × *g* for 15 min to sediment inclusion bodies. Pellets were denatured in Denaturing Equilbration Buffer (recipe) and centrifuged to remove debris (15,000 × *g* for 10 min). Denatured protein solution was diluted in Strep-Tag Buffer W 1:20 and centrifuged to remove insoluble protein. The supernatant was purified using IBA Strep-Tactin resin by manufacturer's protocol. All elution fractions were pool and concentrated on an Amicon Ultra 10,000 MWCO filter (Millipore) and buffer exchanged with three washes of PBS.

HbhA Expression and Purification. *M. smegmatis* strain mc^2 155 was electroporated with the plasmid pMV3-38 obtained from the Delogu Lab (Universitá Cattolica del Sacro Cuore, Rome, Italy) as previously described (Goude & Parish, 2008). The plasmid encodes for a N-terminal 6xHis tag fused to the HbhA protein under the constitutive expression of the mycobacterial *hsp60* promoter. Bacteria were subcultured on Middlebrook 7H11 media (Becton Dickinson, Franklin Lakes, NJ) under selection of 50 µg/ml hygromycin. Small-scale liquid cultures (10 ml) were made using Middlebrook 7H9 media with 50 µg/ml hygromycin and were grown for 3 days at 37ºC. Large-scale (1-2 L) cultures were made using a 1:100 dilution of overnight culture into Sauton's minimal media (See Appendix I) and grown at 37ºC for 4 days before being harvest by centrifugation. Cell pellet was resuspended in Ni-NTA native equilibration buffer with the addition of 25 µl of 20% Triton X-100 per and 100 µl of 10 mg/ml lysozyme per 100 ml of buffer. After lysozyme addition, incubate bacterial suspend on ice for 15 min. Bacteria were lysed by sonication on ice, with 5 cycles of 3 min (50% duty cycle, setting 5 of 10) with 3 min of rest in between each cycle. Debris was removed by centrifugation (15,000 × *g* for 10 min) and sample was bound to a Thermo Ni-NTA column as per manufacturer's instructions, with the modification of adding one 100 mM imidazole wash before eluting. Protein was concentrated and buffer exchanged on a Millipore Amicon Centrifugal Filter MWCO 10,000 (Millipore) and stored at -20ºC.

Mkl Expression and Purification. Arctic Express *E. coli* containing the pBAD-Mkl plasmid were subcultured in LB media containing 100 µg/ml ampicillin and diluted 1:100

into 4 L M9 Minimal Media and grown to OD600~0.5 at 37ºC, lowered to 10ºC for 15 min before being induced with 1 ml/L of 20% arabinose. Cultures were incubated at 10ºC for an additional 3 days before bacteria was harvested by centrifugation. Cells were resuspended in Ni-NTA Native Equilibration Buffer and lysed in a French Press at >10,000 psi with three passes and centrifuged at 15,000 × g for 15 min to remove debris. Supernatant was purified on Thermo Ni-NTA resin as per manufacturer's protocol. Protein was concentrated and buffer exchanged on a Millipore Amicon Centrifugal Filter MWCO 10,000 (Millipore) and stored at -20ºC.

TB31.7 Expression and Purification. Arctic Express *E. coli* containing the pASK-TB31.7 plasmid were subcultured in LB media containing 100 µg/ml ampicillin and diluted 1:100 into 4 L M9 minimal media and grown for eight hours at 37ºC before the temperature was lowered to 10ºC for 15 min. Protein production was then induced by addition of 100 µl of 2 mg/ml anhydrotetracycline per 1 L of culture and cultures were incubated at 10ºC for an additional 3-4 days. Bacteria were collected by centrifugation at 6,000 × *g* for 10 min and resuspended in PBS. Bacteria were lysed in a French Press at >10,000 psi with three passes and centrifuged at 15,000× *g* for 15 min to sediment remove debris. The supernatant was purified using IBA Strep-Tactin resin by manufacturer's protocol. Pure elution fractions were pool and concentrated on an Amicon Ultra 10,000 MWCO filter (Millipore) and buffer exchanged with three washes of PBS.

Animal Care and Use Protocol. All described mouse experiments were performed in the ABSL3 (Animal Biosaftey Level 3) facility in the basement of Johns Hopkins University School of Medicine Building CRBII under the animal care and use protocol

M013M33 expiration date 2/09/2014. All experiments used female BALB/c or C3HeB/FeJ mice between five and seven weeks of age at the beginning of the experiment.

Vaccination. Vaccinated mice were injected with a 200 µl of PBS containing 25 µg of each protein (35kD_ag, CeoB, HbhA, Mkl, TB31.7) and 140 µg of cyclic-di-GMP (Invivogen, San Diego, CA) or 250 µg/25 µg of DDA/MPL subcutaneously in their right flank. The priming dose was administered four weeks prior to MTB-aerosol challenge and the boosting dose was administered two weeks prior to challenge. Positive-control animals receiving the BCG vaccine were injected subcutaneously with 100 µl of BCG Pasteur from a frozen stock (~1 $\times 10^5$ CFU/animal) four weeks prior to aerosol challenge and received no boosting dose. Adjuvant only control animals were injected with 200 µl of PBS containing 140 µg of c-di-GMP. Individual vaccine trials are described in Table 3.3 and Figure 3.0

Table 3.3: Description of Vaccine Trials

Trial	**Adjuvant**	**Subunits**	**Mouse Strains**	**Groups**
K2	C-di-GMP	35kD_ag, CeoB, HbhA, Mkl, TB31.7	BALB/c	PBS, BCG, Vaccine,
K3	C-di-GMP		BALB/c	PBS, Adjuvant, BCG, Vaccine
K4	DDA/MPL		BALB/c, C3HeB/FeJ	BALB/c: PBS, BCG, Vaccine, BCG/Vaccine C3HeB/FeJ: PBS, Adjuvant, BCG, Vaccine, BCG/Vaccine

Note: Trial K1 is omitted because it no vaccine was tested – it was conducted for training purposes and also resulted in the collection of naïve BALB/c serum used for the baseline in antibody titer ELISAs.

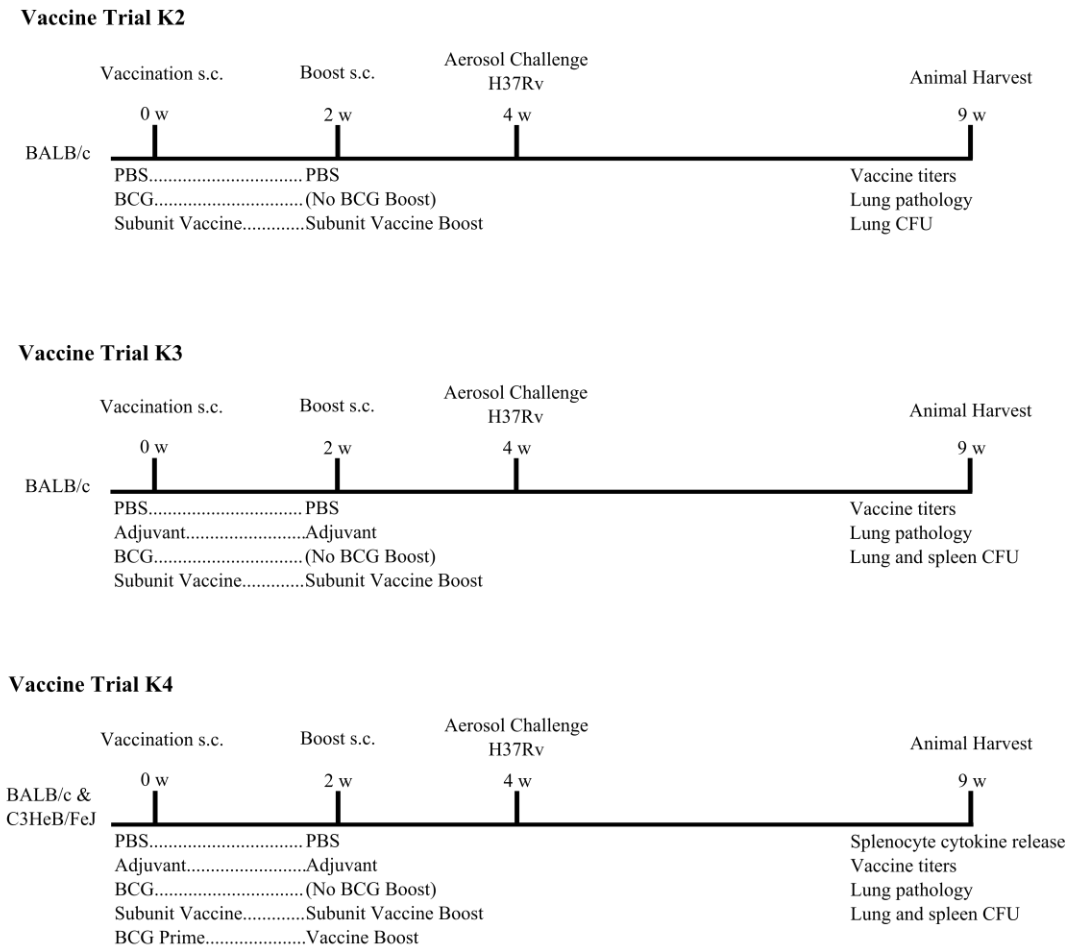


Figure 3.0. Diagram of vaccine trial elements and timing of vaccinations, boosts, challenge and harvest.

Aerosol Challenge. Infection with *M. tuberculosis* was acomplished by placing the mice in the Inhalation Exposure System (Glas-Col, Terre Haute, IN, USA). The mice were exposed to an infectious aerosol for 30 min and were in the chamber for a total of 90 minutes while the instrument purges bacilli with multiple air exchanges through HEPA-filtered intake and exhaust. The animals are free to move about within this chamber and experience no pain, distress or discomfort. Deposition of MTB CFUs by aerosol was verified for each experiment by including a group of five mice in the aersol challenge and sacrificing them within 24 after exposure. Their lungs were homogenized, serially diluted, and plated to determine CFU counts for each animal. Vaccine trial K2 saw an average deposition of ~20 CFU/animal while vaccine trial K3 saw an average deposition of ~480 CFU/animal. Trial K4 saw deposition of ~500 CFU/animal.

Sample Collection. Animals were euthanized by inhaled overdose of Isoflurane followed by cardiac puncture, cervical dislocation, and necropsy with removal of vital organs. Blood from cardiac puncture was separated in serum and coagulated RBC pellets by centrifugation at 2,500 rpm for 30 minutes. Lungs were removed whole and homogenized for determination of CFU counts or preserved in neutral buffered formalin for histological preparation. For BALB/c mice in Trial K3 the lungs were separated into left and right halves with the right being homogenized for CFU counts and left being preserved for histological examination. Harvested spleens were divided in half with half being homogenized and plated for CFU determination. The remaining half spleen was processed to isolate splenocytes (described in Antigen Specific Recall section below).

CFU Determination. Lungs (or spleens) were homogenized manually in 2.5 ml of PBS using a sterile Tenbroeck Tissue Grinder and serially diluted in PBS. Two, 0.5 ml aliquots of each dilution were plated on 7H11 Selective Agar (Becton Dickinson, Franklin Lakes, NJ), sealed in polyethylene bags (Ziploc), and incubated at 37ºC for three weeks before counting colonies.

Lung Homogenate Cytokines. One ml of lung homogenates was set aside immediately after homogenization and mixed with complete, EDTA-free protease inhibitor (Roche) before being frozen at -80°C. Homogenates were freeze-thaw cycled between dry ice and 37°C water bath three times before being centrifuged at 15,000 × *g* for five minutes. 19 samples (5 each from Trial 2 vaccinated, unvaccinated, and BCG groups and four from the adjuvant only group) were analyzed using 175 µl of the cell supernatant in an 8-plex Luminex Multianalyte System specific for IFN-γ, IL-1beta, IL-4, IL-6, IL-10, IL-12p70, TNF-α, TGF-beta.

Histology. Lungs from MTB infected animals were placed in 10% neutral-buffered formalin (Sigma) for fixation before being embedded in paraffin blocks and sectioned. Histological evaluation of sections was performed on hematoxylin and eosin (H&E) stained preparations and lung bacterial burden was assessed using Ziehl-Neelsen (ZN) stained adjacent sections. Lungs were evaluated by a trained veterinary pathologist for lung pathology grade (H&E stained sections), bacterial burden (ZN stained sections), and imaged.

Antigen Specific Antibody Titers. Indirect ELISAs were performed using 0.5 µg of recombinant antigen suspended in PBS bound to Nunc Maxisorp ELISA plates (Nunc) overnight at 4ºC. Plates were washed three times with 300 µl of PBST (PBS + 0.05% Tween-20) and blocked with 1% BSA in PBS for 1 hour at 37ºC. Serum samples were added at a starting concentration of 1:500 dilution for PBS and BCG samples and either 1:4,000 or 1:32,000 for vaccinated animal serum, then diluted 1:2 on the ELISA plate (100 µl starting volume, 50 µl removed and mixed with 50 µl of PBS+1% BSA in each well). Plates were incubated with serum dilution for 1 hour at 37C before being washed three times as before. Then a 1:1000 or 1:2000 dilution of secondary antibody bound to HRP was added to the plate and incubated for 1 hour 37C. After an additional three washes 100 µl of Opteia TMB substrate was added to each well and incubated for 10 min before being neutralized by the addition of 50 µl of 2 N Sulfuric Acid. Absorbance at 450 nm was measured in individual wells. Antibody titers were called as the reciprocal of the last dilution with an OD higher than the threshold of naïve serum wells plus two standard deviations.

Splenocyte Stimulation Protocol. Splenocytes were isolated from spleens by mechanical disruption in Hanks Balanced Salt Solution, and then strained through a 40 micron cell strainer (Becton-Dickenson) before being centrifuged at 500 × *g* for five min. Cells were resuspended in 1X RBC Lysis Buffer (155 mM Ammonium Chloride, 12 mM Sodium Bicarbonate, 0.1 mM EDTA) for five min, then 10 ml of PBS was added and the cells were sedimented by centrifugation at 500 × *g* for five min. Cells were resuspended in two ml of RPMI 1640 + 10% FBS, cell concentration was counted on a hemocytometer and normalized to 5×10^6 cells/ml. 100 µl of cells were aliquoted per well of a 96-well plate and 100 µl of RPMI-1640 + 10% FBS containing 20 µg/ml of stimulating antigen. Cells were stimulated for two days before harvesting supernatants by 96-well plate filtration. Supernatants were assayed using R&D Systems ELISA Duoset reagents (mouse IL-10 and IFN-γ) to perform cytokine ELISA by manufacturer's instruction.

Human Serum Indirect ELISA. Human serum samples were analyzed for their ability to recognize vaccine recombinant antigens using indirect ELISA. Antigens were plated as for Antigen Specific Antibody Titer experiments (0.5 µg/well in PBS, overnight). Plates were washed three times with 200 µl PBS-Tween 20 (0.05%) and blocked with 1% BSA in PBS for one hour at 37ºC. Buffer was removed from wells and 100 µl human serum diluted in PBS + 1% BSA (1:10 or 1:100 dilution of serum**)** was applied and incubated for one hour at 37ºC. Plates were removed from the incubator and washed as before. 100 µl of mouse anti-human IgG1 (clone Hp6070) horseradish peroxidase labeled antibody

(Invitrogen, Cat. No. MH1715) diluted 1:1000 in PBS + 1% BSA was added and incubated for an hour at 37ºC**.** Plates were washed a final time as previously. BD Opteia TMB Substrate was mixed as per manufacturer's instructions and 100 µl was added to each well and incubated for 10 mins before the addition of 50 µl 2N sulfuric acid to stop the reaction. Plates were read for absorption at 450 nm. Threshold values for antigen recognition were set at the average of ten TB-negative patient OD450 values plus two standard deviations; all values above this were recorded as positives.

Part 3: Results

Vaccine Antigens Expressed Recombinantly and Purified. All proteins selected for this vaccine were identified in the membrane fraction of MTB H37Ra during initial screens and have large hydrophobic sequences. As expected, early expression attempts resulted in little-to-no soluble protein during conventional 37ºC bacterial growth. This barrier was overcome in different ways for different proteins. 35kD_ag and CeoB were both expressed at 37ºC and then denatured before being refolded into a soluble form. HbhA was expressed in *M. smegmatis* as previously described using the pMV3-38 plasmid from the Delogu Lab (Delogu, *et al.*, 2004). The proteins Mkl and TB31.7 were expressed at 10ºC for 3-5 days in large volumes allowing the purification of usable

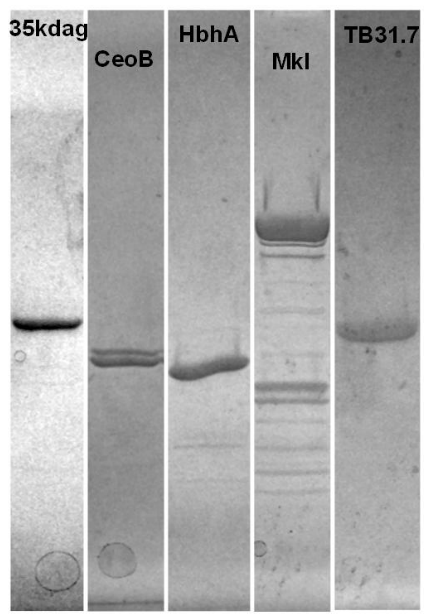


Figure 3.1. Purified recombinant protein subunits included in the vaccine. All recombinant proteins' identities were confirmed by molecular weight and peptide mass fingerprinting.

quantities of soluble recombinant protein. These proteins can be seen in Fig. 3.1. CeoB is visible in two forms, not uncommon for expression with pASK-IBA14, with the smaller species having the OmpA signal sequence cleaved off.

Human TB positive patient serum recognizes recombinant HbhA, Mkl, and TB31.7 antigens. Serum from twenty confirmed TB-positive and ten confirmed TB-negative human patients obtained through the World Health Organization (WHO) via FIND Diagnostics was used to probe recombinant antigens used in vaccination studies K2, K3, and K4 by indirect ELISA assay. Serum samples were tested at both 1:10 and 1:100 dilutions and antibody recognizing the antigen was identified by using a secondary antibody binding human IgG1. Serum samples that recognized antigen with OD450 values exceeding the TB-negative samples plus two standard deviations were recorded as positives. HbhA and Mkl (Table 3.4) were both recognized by a minority of human serum in both the 1:10 and the 1:100 dilutions, while TB31.7 was only recognized in the 1:10. 35kd_ag and CeoB antigens were not recognized by TB-positive patient serum at higher rates than TB-negative serum.

Antigen	Positives (1:10 dilution)	Positives (1:100 dilution)
35kd_ag	0/20	0/20
CeoB	0/20	0/20
HbhA	4/20	2/20
Mkl	2/20	3/20
TB31.7	3/20	0/20

Table 3.4: Human Serum Reactivity to recombinant antigens used in this study, expressed as number of 20 TB positive patients whose serum recognized the antigens at levels exceeding the average of 10 TB negative patients serum plus two standard deviations.

Five Subunit Vaccine with Cyclic-di-GMP Stimulates High Antibody Titers. Two vaccine trials, K2 and K3 (see Table 3.3 and Fig. 3.0), were conducted using the five recombinant subunits (35kD_ag, CeoB, HbhA, Mkl, and TB31.7) and the bacterial second messenger molecule c-di-GMP as an adjuvant. These trials were conducted in BALB/c mice which, as discussed in Chapter 1, are a standard first-line testing strain for both MTB vaccines and pharmaceuticals. A subgroup of animals vaccinated as part of Trial K2 was sacrificed prior to challenge and serum was used to determine antigen-specific, isotype-specific antibody titers (Fig. 3.2). Titers were high for all antigens tested and ranged between 10^5 and 10^7 GMT for isotypes IgG1, IgG2A, and IgG2B demonstrating a robust humoral immune response to the vaccine.

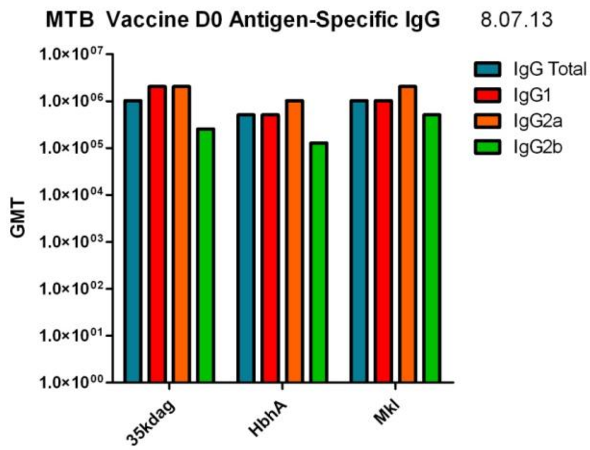


Figure 3.2. Challenge-Day Vaccine Immune Response. Vaccination with the five-subunit vaccine produces a strong IgG, IgG1, and IgG2a response with a lesser IgG2b response specific vaccine antigens 35kdag,

Trial K2 Vaccinated Animals Develop Strong Antibody Titers Compared to Unvaccinated and BCG Vaccinated Animals. Animals that had been vaccinated in K2 with adjuvant C-di-GMP, as well as the five vaccine subunits, had serum collected at sacrifice five weeks following challenge with virulent MTB H37Rv. All vaccinated animals exhibited high antibody titers specific to vaccine components with titers $>10^4$ across all isotypes (Fig. 3.3). Unvaccinated animals had uniformly low-to-undetectable titers while BCG vaccinated animals exhibited strong recognition of HbhA protein.

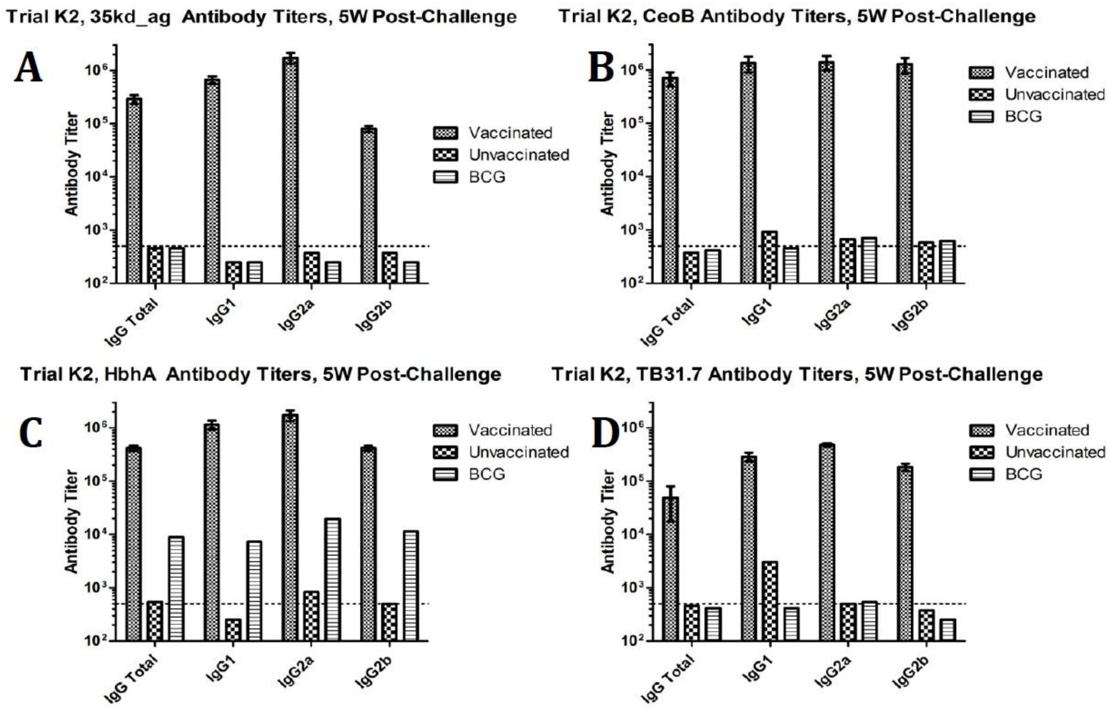


Figure 3.3. Trial K2 Humoral Response (A) Trial K2 35kD_ag antibody titers 5 weeks post-challenge (B) Trial K2 CeoB antibody titers 5 weeks post-challenge (C) Trial K2 HbhA antibody titers 5 weeks post-challenge (D) Trial K2 TB31.7 antibody titers 5 weeks post-challenge. Dashed line denotes limit of detection (1:500 dilution).

Trial K2 Vaccinated BALB/c mice are not Protected Against Aerosol Challenge Compared to Unvaccinated Mice. Despite the robust response to the vaccine antigens seen in Fig. 3.3, whole-lung CFU counts at five weeks post aerosol challenge (~20 CFU/animal deposition) showed similar bacterial burdens in both vaccine-treated mice and unvaccinated (PBS control) animals. BCG animals showed a typical level of protection with a 1.16 log fewer CFU compared to unvaccinated animals (See Table 3.5). A pilot lung histology study on one animal per group suggests that lung pathology may have been increased in both BCG and test vaccine groups (Fig. 3.4B). An assay of lung homogenate cytokines found a pattern that echoed the lung CFU results, showing lower

IFN-γ, IL-1b, TNF-α, and TGFβ for the BCG group relative to PBS or test vaccine (See Fig. 3.4C).

Table 3.5: Average MTB Burdens by Group for All Trials.

Trial	Group	N	Mouse Strain	Average Lung CFU +/- SD (Log10)	Average Spleen CFU +/- SD (Log10)
K2 (C-di-GMP)	Unvaccinated (PBS)	6	BALB/c	5.98±0.72	N/A
	Vaccine	8	BALB/c	5.94±0.53	N/A
	BCG	6	BALB/c	4.82±0.38	N/A
K3 (C-di-GMP)	Unvaccinated (PBS)	4	BALB/c	7.09±0.09	5.06±0.22
	Adjuvant (C-di-GMP)	4	BALB/c	7.48±0.07	5.37±0.23
	Vaccinated	7	BALB/c	7.31±0.08	5.09±0.14
	BCG	5	BALB/c	6.99±0.28	4.24±0.32
K4 (DDA/MPL)	Unvaccinated (PBS)	5	BALB/c	5.68±0.27	N/A
	Vaccine	5	BALB/c	4.97±0.12	N/A
	BCG/Vaccine	5	BALB/c	4.22±0.31	N/A
	BCG	5	BALB/c	3.81±0.19	N/A
	Unvaccinated (PBS)	4	C3HeB/FeJ	5.67±0.09	4.35±0.13
	Adjuvant (DDA/MPL)	4	C3HeB/FeJ	7.02±0.46	4.60±0.29
	Vaccine	4	C3HeB/FeJ	7.14±0.45	4.46±0.34
	BCG/Vaccine	4	C3HeB/FeJ	5.17±0.17	4.05±0.20
	BCG	4	C3HeB/FeJ	5.31±0.40	4.12±0.28

Lung CFU is based on the CFU/Whole Lungs measurement for K2 and K3, for CFU/Half-Lung for K4 BALB/c mice, and CFU/Whole Lungs for K4 C3HeB/FeJ mice. Spleen numbers are based on CFU/half-spleen for all measurements.

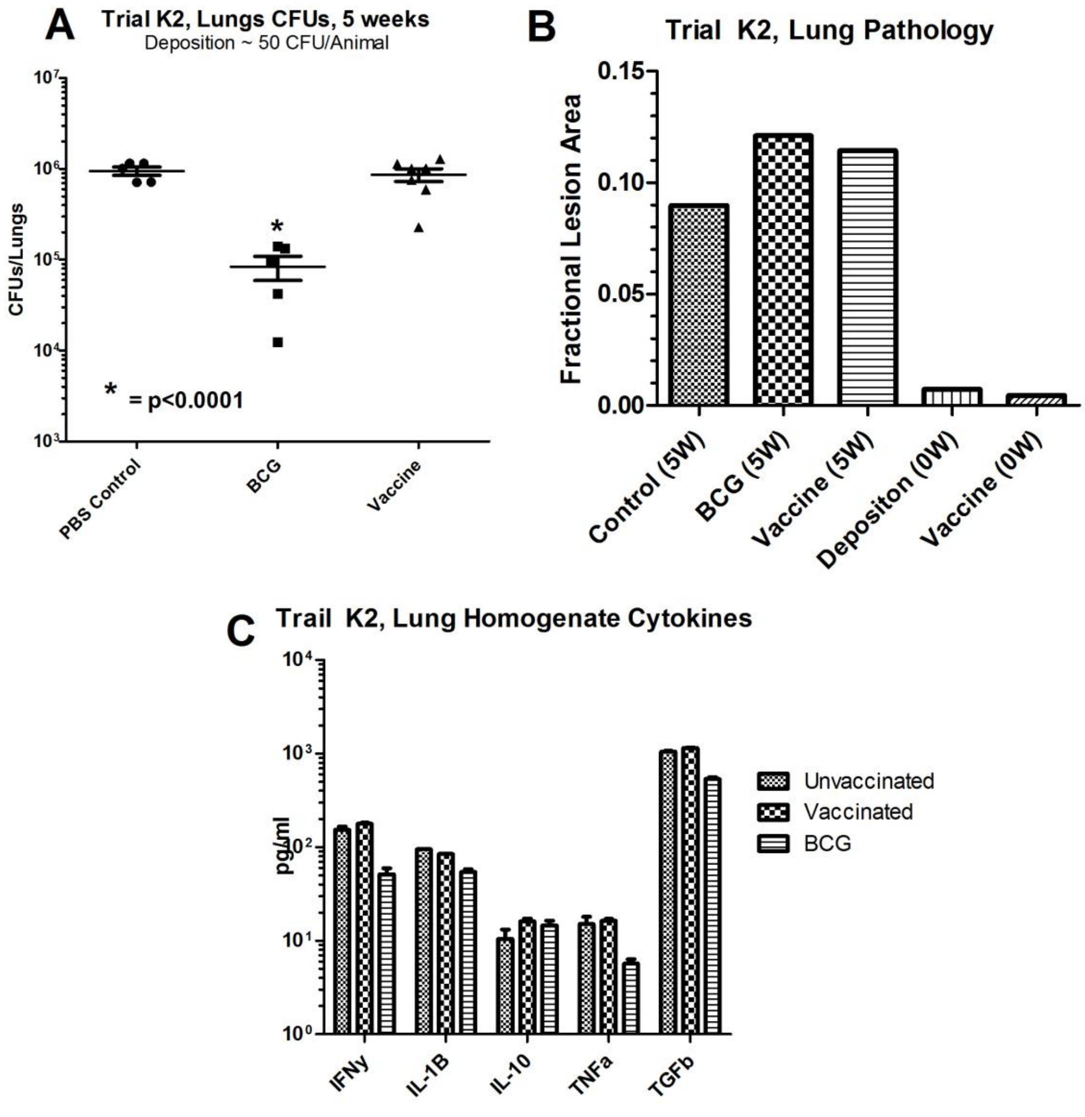


Figure 3.4. Trial K2 CFUs/Lungs, Lung Pathology, and Lung Cytokines (A) Trial K2 CFU/lungs 5 weeks after challenge with H37Rv MTB. (B) Trial K2 lung histological determination of lesion area. (C) Multiplex ELISA of lung homogenate cytokines, 5 weeks post-challenge.

Trial K3 Adjuvant Treated Animals Develop Higher Lung and Spleen Bacterial Burdens than PBS Control. BALB/c mice in vaccine study K3 (C-di-GMP adjuvant) formed humoral immune responses very similar to that of K2 after being treated with the same vaccine formulation (Fig 3.5). On aerosol challenge CFU deposition was ~480 CFU/animals compared to the lower dose of ~20 CFU/animal in Trial K2. Following this higher inoculum, mice treated solely with C-di-GMP developed significantly higher spleen and lung bacterial burdens compared to PBS control (Fig. 3.6 A, B). Animals receiving the test vaccine exhibited a lung burden intermediate to both PBS control and C-di-GMP alone that was significantly different from both groups. BCG vaccinated mice did not have significantly lower lung CFU counts compared to PBS control, but did have a significantly lower spleen CFU counts compared to PBS control (Fig. 3.6B).

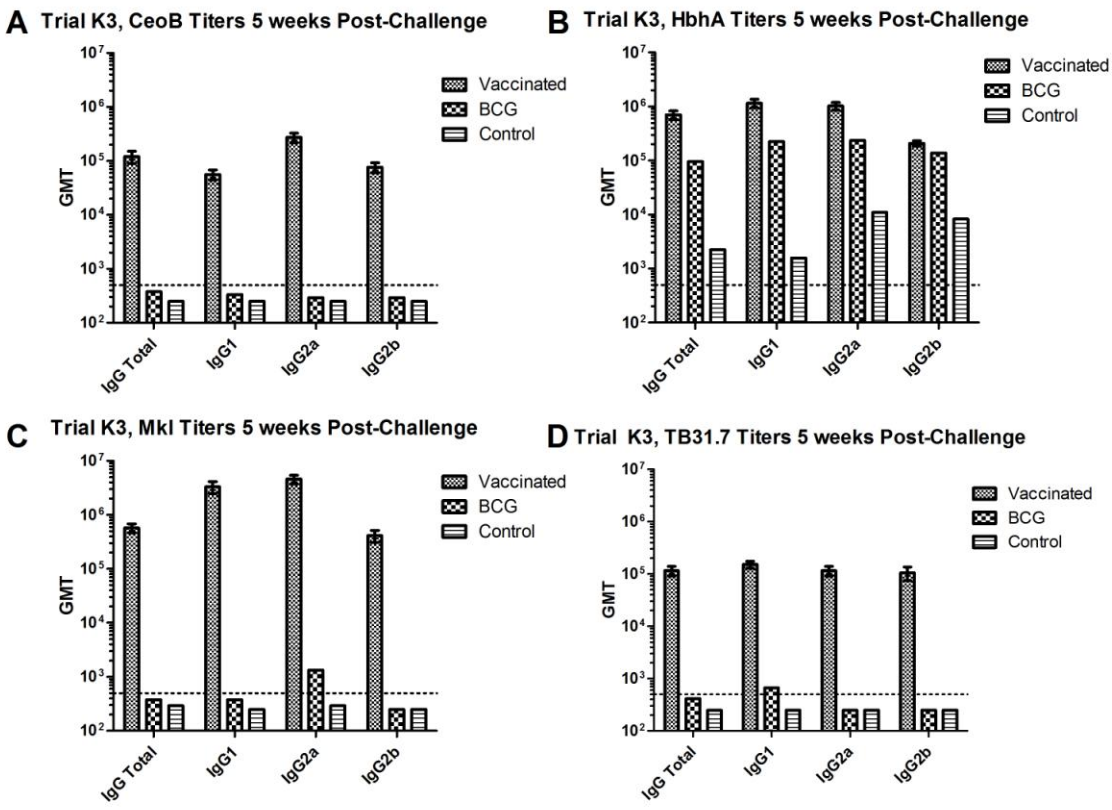


Figure 3.5. Trial K3 Humoral Response (A) Trial K3 CeoB antibody titers 5 weeks post-challenge (B) Trial K3 HbhA antibody titers 5 weeks post-challenge (C) Trial K3 Mkl antibody titers 5 weeks post-challenge (D) Trial K3 TB31.7 antibody titers 5 weeks post-challenge. Dashed line denotes limit of detection (1:500 dilution).

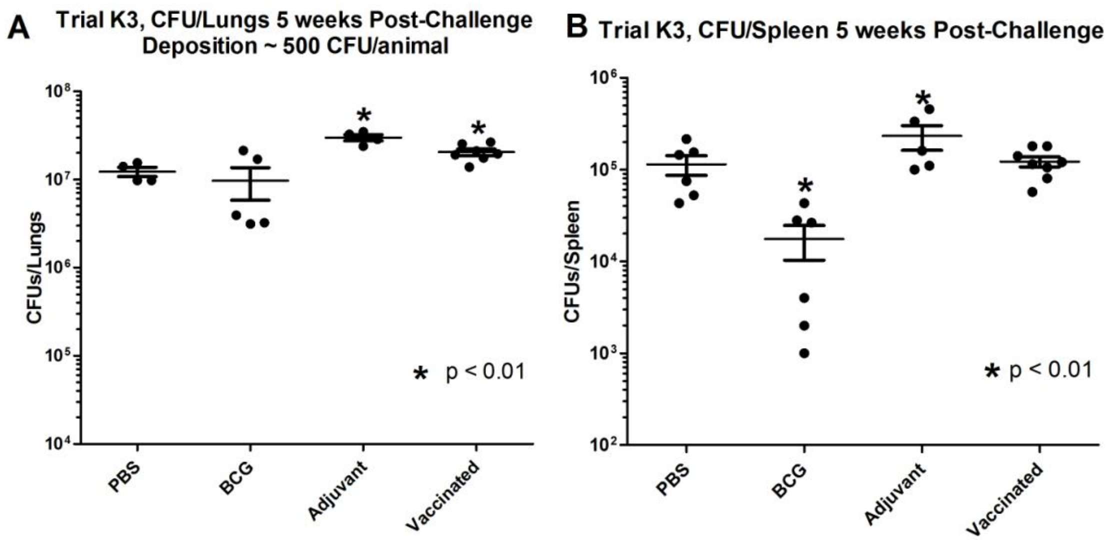


Figure 3.6. Trial K3 Lung and Spleen Bacterial Burden (A) Trial K3 lung bacterial burden (B) Trial K3 bacterial burden by half-spleen.

Vaccination using the Five Subunits with DDA/MPL produces a robust humoral response in both BALB/c and C3HeB/FeJ mice. With the sample dosage of vaccine antigen as in Trials K2 and K3, the adjuvant DDA/MPL produced antigen-specific antibody titers greater than 10^4 GMT for all proteins in both BALB/c and C3HeB/FeJ mouse strains (Fig. 3.7, Fig. 3.8). The subunit vaccine (prime and boost) and the BCG prime followed by the subunit vaccine-boost achieved comparable levels of humoral immunity in BALB/c mice with the exception of Mkl-specific IgG1 and IgG2B titers in which the BCG prime followed by the recombinant vaccine boost strategy achieved significantly lower antibody titers. As seen in Trials K2 and K3, the BCG vaccine was able to produce measurable titers against HbhA significantly above PBS baseline, but still lower that the titers induced by recombinant multivalent vaccine.

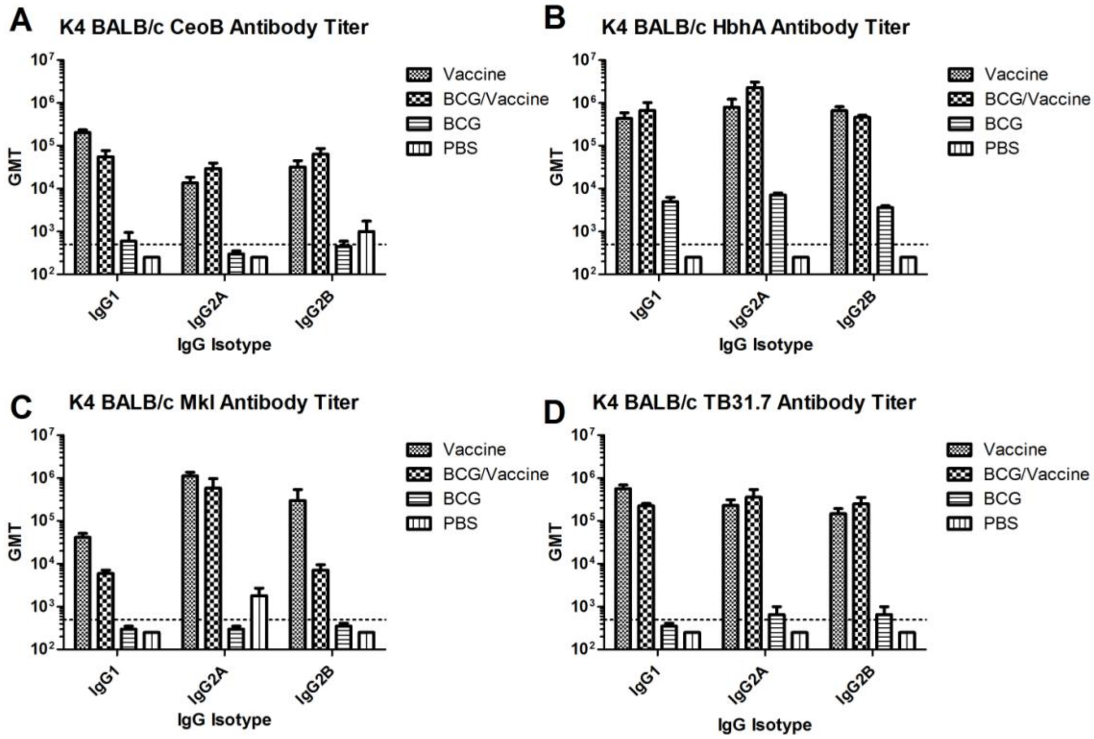


Figure 3.7. Trial K4 Humoral Response – BALB/c (A) Trial K4 BALB/c CeoB antibody titers 5 weeks post-challenge (B) Trial K4 BALB/c HbhA antibody titers 5 weeks post-challenge (C) Trial K4 BALB/c Mkl antibody titers 5 weeks post-challenge (D) Trial K4 BALB/c TB31.7 antibody titers 5 weeks post-challenge. Dashed line denotes limit of detection (1:500 dilution).

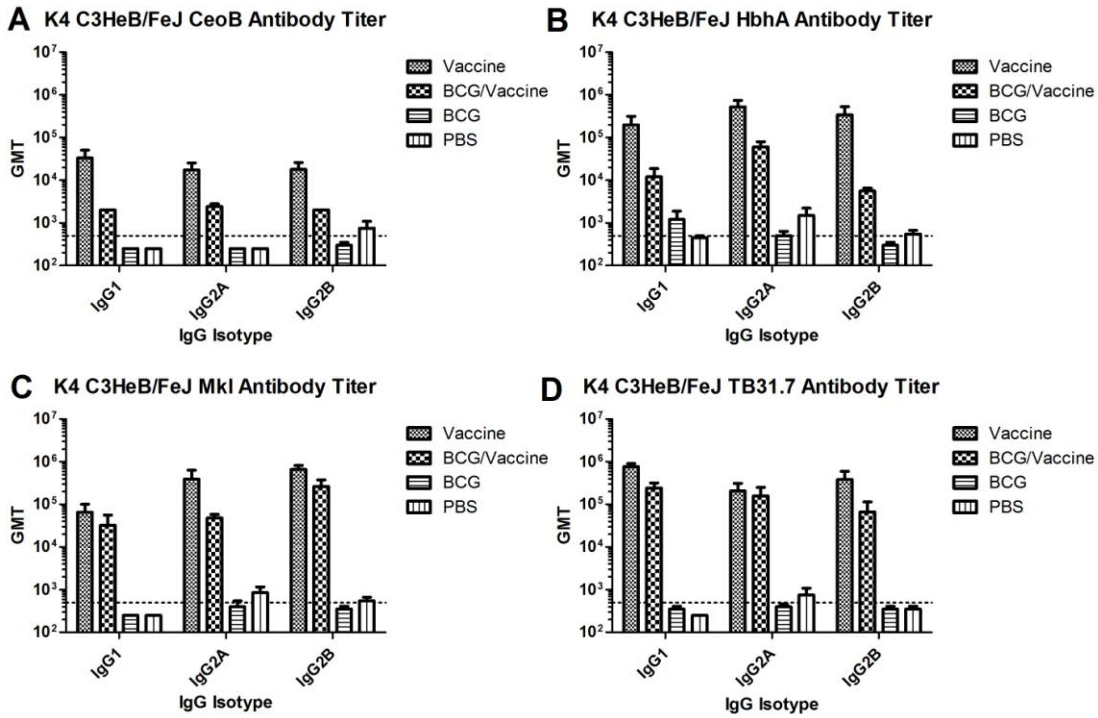


Figure 3.8. Trial K4 Humoral Response – C3HeB/FeJ (A) Trial K4 C3HeB/FeJ CeoB antibody titers 5 weeks post-challenge (B) Trial K4 C3HeB/FeJ HbhA antibody titers 5 weeks post-challenge (C) Trial K4 C3HeB/FeJ Mkl antibody titers 5 weeks post-challenge (D) Trial K4 C3HeB/FeJ TB31.7 antibody titers 5 weeks post-challenge. Dashed line denotes limit of detection (1:500 dilution).

Trial K4: BALB/c mice display a greater IFN-gamma cytokine response in comparison to C3HeB/FeJ mice display a greater IL-10 response. Following mouse sacrifice at five weeks post-challenge, splenocytes were isolated and stimulated with vaccine antigens for two days at 37ºC and 5% CO_2. BALB/c IFN-γ levels in the splenocyte supernatant were higher than comparable levels from C3HeB/FeJ mouse splenocytes for all trial treatment groups (Fig. 3.9, Table 3.6) with BALB/c to C3HeB/FeJ IFN-γ release ratios ranging from 7.3 to 67 for PBS control animals and between 4 – 8.5 for test vaccine animals. The reverse was true for IL-10 where the C3HeB/FeJ mouse splenocytes yielded 6 to 32 times more IL-10 compared to matched groups of BALB/c mouse splenocytes (Fig. 3.10, Table 3.7). For IFN-γ and IL-10, higher quantities of cytokines were released in animals that had been primed with the test subunit vaccine or with the BCG vaccine boosted with the subunit vaccine. Figure 3.11A and 3.11B contain rescaled versions of 3.10A and 3.9B, respectively, for clarity.

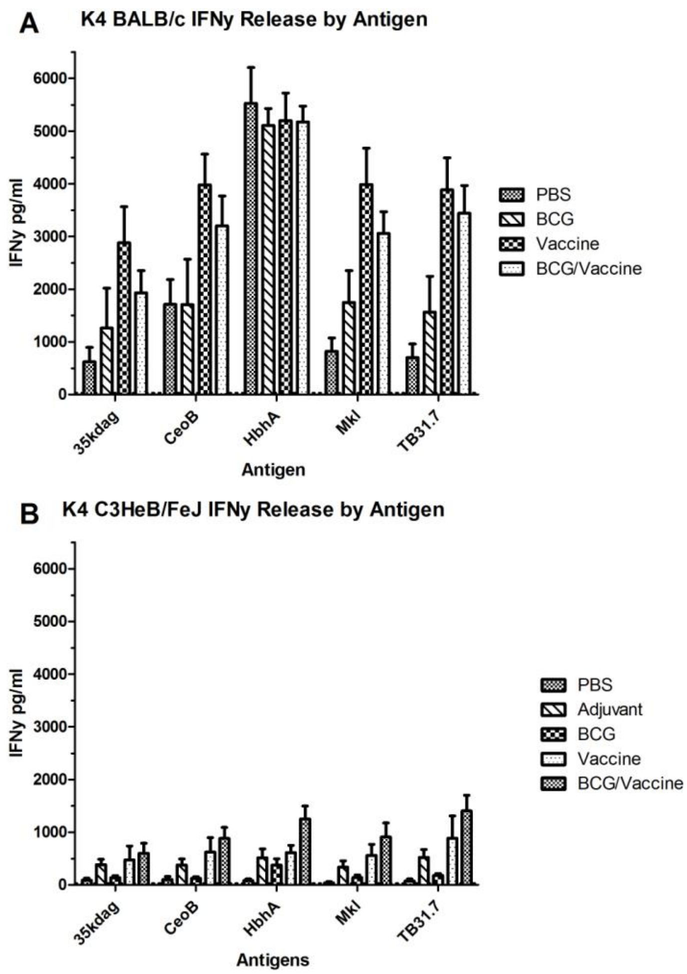


Figure 3.9. IFNy Release Assay (A) Trial K4 BALB/c mouse IFN-γ splenocyte stimulation assay, 5 weeks post-challenge, (B) Trial K4 C3HeB/FeJ mouse IFN-γ splenocyte stimulation assay, 5 weeks post-challenge. Axes equal for comparison. See Fig. 3.11 for rescaled version of (B).

Table 3.6: BALB/c to C3HeB/FeJ Mouse IFN-γ Release Ratio by Group and Antigen

	PBS	BCG	Vaccine	BCG/Vaccine
35kD_ag	7.3	9.3	6.1	3.2
CeoB	16.4	13.7	6.4	3.6
HbhA	67.4	13.7	8.5	4.1
Mkl	21.2	12.6	7.1	3.4
TB31.7	9.4	8.7	4.4	2.5

Average IFN-γ release for each antigen/treatment group was found and the average for BALB/c was divided by the average for C3HeB/FeJ mouse matched group to provide an indication of fold difference in INFy release.

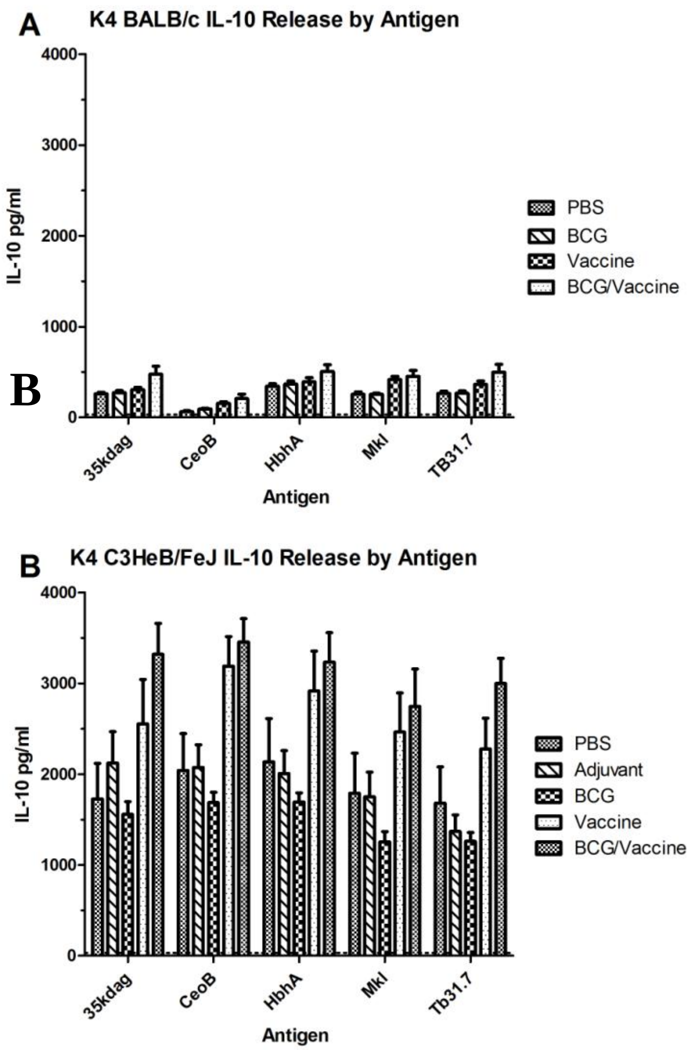


Figure 3.10. IL-10 Release Assay (A) Trial K4 BALB/c mouse IL-10 splenocyte stimulation assay, 5 weeks post-challenge, (B) Trial K4 C3HeB/FeJ mouse IL-10 splenocyte stimulation assay, 5 weeks post-challenge. Axes equal for comparison. See Fig. 3.11 for rescaled version of (A).

Table 3.7: C3HeB/FeJ to BALB/c Mouse IL-10 Release Ratio by Group and Antigen

	PBS	BCG	Vaccine	BCG/Vaccine
35kD_ag	6.7	5.7	8.4	7.0
CeoB	32.5	18.4	20.6	16.7
HbhA	6.2	4.6	7.5	6.4
Mkl	7.0	5.0	5.9	6.1
TB31.7	6.4	4.7	6.3	6.0

Average IL-10 release for each antigen/treatment group was found and the average for C3HeB/FeJ was divided by the average for BALB/c mouse matched group to provide an indication of fold difference in IL-10 release.

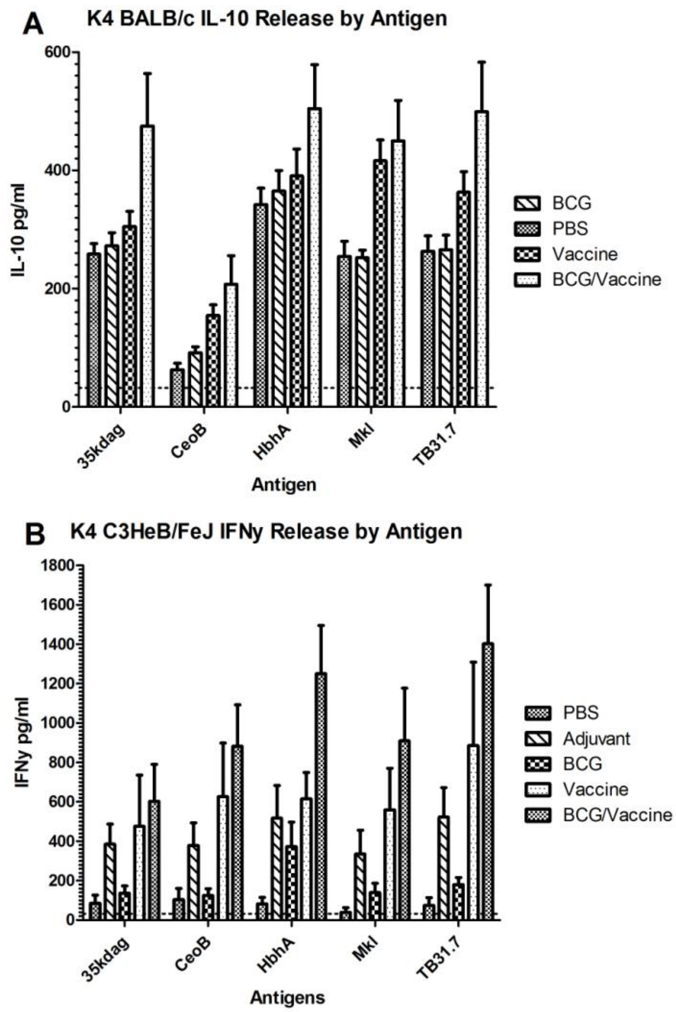


Figure 3.11. Rescaled Figures (A) Trial K4 BALB/c mouse IL-10 splenocyte stimulation assay, 5 weeks post-challenge, (B) Trial K4 C3HeB/FeJ mouse IFN-γ splenocyte stimulation assay, 5 weeks post-challenge.

Trial K4: Vaccine results in lower, but not significant, lung bacteria burden compared to PBS control in BALB/c mice. BALB/c mice receiving the subunit vaccine had lower bacterial burdens per lung compared to PBS controls (p=0.055) (Fig. 3.12A). The difference between these groups was equalized when CFU were normalized against lung tissue weight as a result of the general increased size of PBS mouse lungs compared to that of vaccinated, or BCG primed, vaccine boosted animals (Fig. 3.12B, Fig. 3.13). BCG-primed, subunit vaccine-boosted BALB/c mice had significantly reduced lung mass compared to both PBS and BCG vaccinated mice indicating a possible reduction in lung pathology in the BCG primed, vaccine-boosted group.

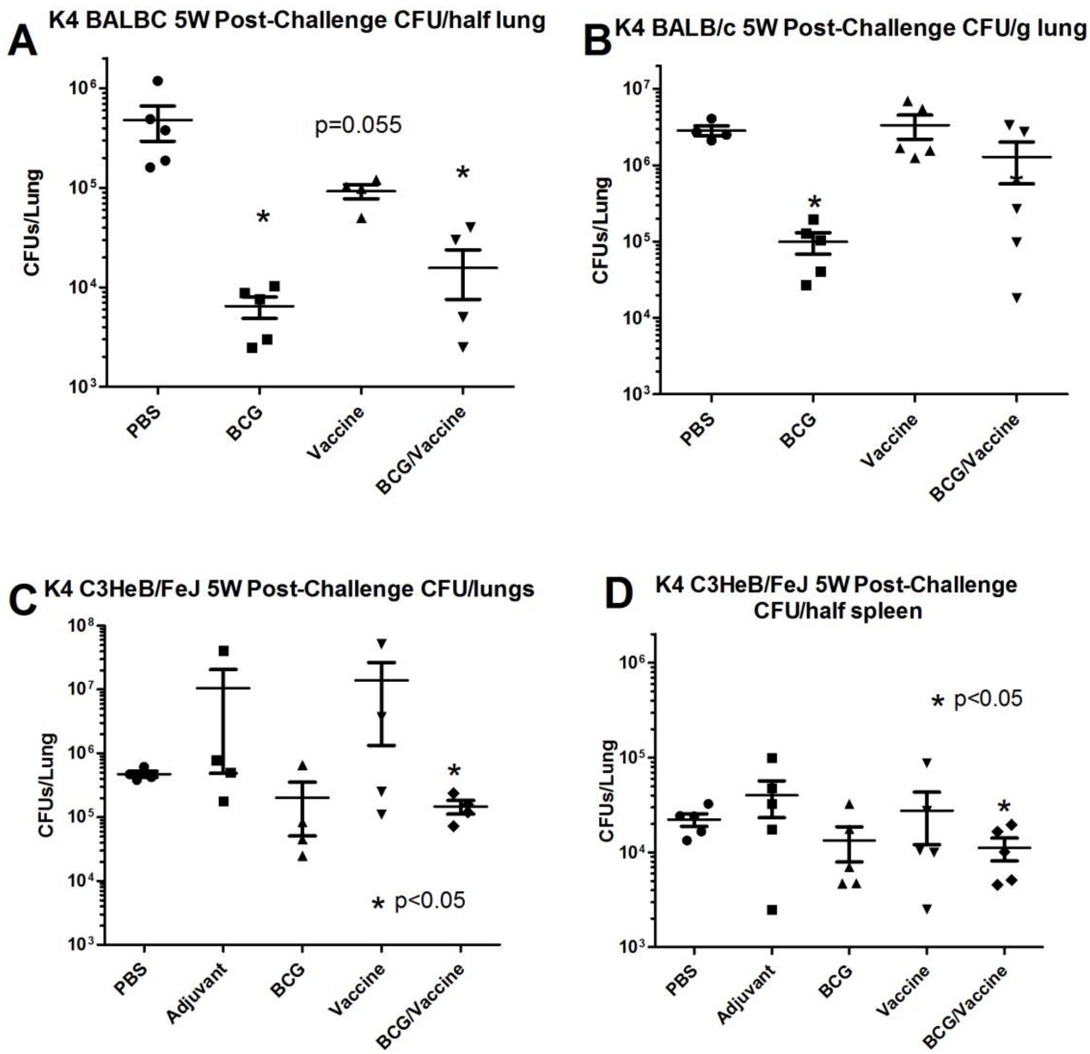


Figure 3.12.Trial K4 Bacterial Burdens (A) Trial K4 BALB/c lung bactrial burden by half-lung (B) Trial K4 BALB/c lung bacterial burden by g lung (C) Trial K4 C3HeB/FeJ lung bacterial burden by whole lungs (D) Trial K4 BALB/c spleen bacterial burden by half-spleen. * is $p < 0.05$.

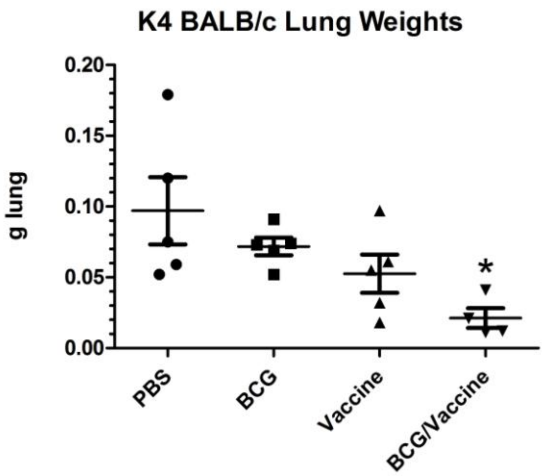


Figure 3.13. Trial K4 right lung lobe weight (BALB/c mice).

Trial K4: BCG-prime, vaccine-boost resulted in lower bacterial burdens compared to PBS controls in C3HeB/FeJ Mice. Lung and spleen bacterial burdens were significantly reduced in BCG-primed, vaccine-boosted C3HeB/FeJ mice compared to PBS control (Fig 3.12 C, D). BCG-vaccinated mice did not exhibit a statistically significant decrease in CFU/organ compared to PBS. However, BCG and BCG prime, subunit vaccine-boosted groups were not statistically significantly different from each other and the lack of statistically significant reduction in bacterial burden in BCG-vaccinated mice compared to PBS controls may have been a result of a large standard deviation rather than a real additional benefit of combining the BCG and subunit vaccines in a prime-boost strategy.

Part 4: Discussion

Both vaccine formulations (C-di-GMP, DDA/MPL) and the BCG-prime, vaccine-boost strategy generated high antibody titers specific for vaccine proteins. These high antibody titers, however, failed to significantly impact lung or spleen bacterial burden.

Although Trial K4 gave the indication that BCG vaccination boosted with the recombinant vaccine had significantly reduced lung CFUs in C3HeB/FeJ mice, this experimental group could not be statistically separated from BCG vaccination alone. This presents the possibility that any benefits associated with BCG-prime, vaccine-boost are solely due to BCG, despite the fact that the BCG group's large standard deviation precluded finding it significantly lower than PBS. We are unable to reject the null hypothesis that high antibody titers recognizing MTB biofilm-expressed vaccine proteins 35kD_ag, CeoB, HbhA, Mkl, and TB31.7 are not effective at reducing lung bacterial burdens.

Testing of vaccine antigens against human serum resulted in positive recognition of proteins HbhA, Mkl, and TB31.7 at rates to be expected of non-dominant MTB antigens (Kunnath-Velayudhan, 2010). These antigens may have a future role in diagnostic tests for MTB infection, most likely as part of an array of MTB proteins. It is also possible that expression of the proteins in a different host (other than *E. coli)* might be required for certain epitopes recognized by infected humans. The lack of response to 35kd_ag or CeoB is best explained by those proteins denaturation during their purification process.

In Trial K4, DDA/MPL-vaccinated and BCG-prime, vaccine-boosted BALB/c mice all formed a robust IFN-γ response specific to each antigen. This response was also present, but extremely diminished, in C3HeB/FeJ mice. While the BCG-prime, vaccine-boosted BALB/c animals demonstrated significant protective reductions in MTB concentrations in the lungs of challenged animals, it could not be discerned if this was due to protection provided by the BCG vaccine or due to the multivalent recombinant vaccine. In addition, the multivalent, vaccinated BALB/c mice with DDA/MPL adjuvant failed to show protection following challenge, although these animals showed a trend towards reduced MTB burden in the lungs of challenged mice ($p=0.055$). Considering the near significant trend towards protection, the discovered immunogenic antigens in this study are worthy of further study.

One particular antigen, HbhA, was confirmed as the antigen that was immunologically significant in MTB, capable of eliciting major immune responses during infection in BALB/c through antibody titers and IFN-γ release assays. However, we failed to find a statistically significant protective effect of vaccinating with HbhA containing vaccine for preventing MTB dissemination to the spleen as has been found in other studies where HbhA was used as a single agent (Guerrero & Locht, 2011). This could be a result of *M. smegmatis*-produced HbhA being insufficiently close to native HbhA to achieve a protective effect, or could be a result of different route of administration of the vaccine (subcutaneous compared to intranasal).

In our challenge experiments we utilized H37Rv bacteria grown to mid-log phase in shaking culture for aerosol infections of vaccinated animals. The guinea pig serum used to identify the antigens comprising the subunit vaccine was harvested following infection with pellicle-grown H37Rv. Thus detected antigens were likely skewed towards the proteomic profile of MTB pellicle compared to mid-log growth. It is possible that our challenge experiments testing the subunit vaccine if conducted with biofilm-grown H37Rv would yield different results despite its greater virulence compared to mid-log grown H37Rv. Also, we used protein extracts from pellicle-grown H37Ra, the clinical attenuated relative of H37Rv, for the initial antigen screen. While H37Ra is highly conserved relative to the parental H37Rv strain it has undergone 73 insertion/deletion events relative to H37Rv (Zheng, 2008). Grossly, the two strains form identical pellicles in culture. However, it is possible that our screen missed superior antigens that may have been present in H37Rv but not H37Ra.

Bacterial burden results were obtained following sacrifice at five weeks post-challenge. While this is sufficient time for BCG to demonstrate a protective effect in most cases, it is not uncommon for MTB vaccine studies to determine vaccine effects at 8, 10, or 20 weeks post-challenge in order to better distinguish protective effects of candidate vaccines compared to BCG-vaccinated controls. Extending the post-challenge period prior to harvest might have increased the statistical significance of observed reductions in CFU in the vaccine group in Trial K3.

Cyclic di-GMP appears to have a direct immune modulating effect on the host, resulting in increased bacterial burden following administration of it alone, as a single adjuvant. This was evident in Trial K3, but not obvious in Trial K2, possibly as a result of the higher initial inoculums in Trial K3 (roughly one order of magnitude greater). The size of the initial dose is known to determine whether the animal model resembles an acute versus a chronic course. Both dosages are within the range appropriate for a chronic model of infection, but the greater inoculums size in Trial K3 gave the mice less time to mount an immune response before reaching a similar bacterial burden as that of Trial K2. C-di-GMP is known to signal through the STING receptor, resulting in the production of Type I interferon (Burdette, *et al.*, 2011, Yin, *et al.*, 2012). This class of interferon is known to negatively impact the function of IFN-γ, which could result in impaired Th1 response to MTB explaining the increase in lung CFU counts seen in Trial K3(Stanley, *et* *al.*, 2007, Teles, *et al.*, 2013). On the basis of these results it seems unlikely the cyclic-di-GMP has a place in either immunomodulatory treatment of MTB or as an adjuvant in any potential vaccine despite its ability to produce high vaccine-antigen-specific antibody titers.

The cytokine-release assays results performed on mouse splenocytes from Trial K4 were striking for the extreme differences between the BALB/c and C3HeB/FeJ mice in terms of the cell-mediated immune response. BALB/c mice skewed heavily towards the Th1 axis by producing dramatically larger amounts of IFN-γ relative to C3HeB/FeJ, mice which produced high levels of the anti-inflammatory cytokine IL-10. IL-10 directly opposes the action of IFN-γ in activating macrophages and the production of large amounts of the cytokine could play a significant role in the genetic susceptibility of the C3HeB/FeJ mouse strain. The ability of IL-10 to interfere in the classic Th1 response by opposing the production of IL-12 and TNFa would be expected to materially affect the IgG1 vs. IgG2A titers, skewing it towards IgG2A in the C3HeB/FeJ mice. This Th2 bias in the humoral response in C3HeB/FeJ mice was not observed. This could indicate that the kinetics of the IL-10 producing cells stimulated in the splenocyte stimulation assay lagged behind the development of the humoral response by a significant margin. To make that determination however, antibody titers and splenocyte stimulation assays would need to be performed at several timepoints from vaccination through challenge to the final harvest timepoint to describe the kinetics of the humoral and adaptive immune response.

The C3HeB/FeJ model proved less consistent than the BALB/c model in terms of lung bacterial burdens, with some mice in the same treatment group displaying lung CFU burdens that were different by as much as two orders of magnitude. This tendency towards a bimodal distribution of CFUs might be dealt with by use of larger experiment groups which could undergo a stratified analysis grouping high-burden mice together for analysis and doing likewise for normal-burden animals.

CHAPTER 4: CONCLUDING REMARKS

While there is an appreciation of the biofilm-forming properties of environmental mycobacteria, the role of biofilm formation in the pathogenesis of mycobacterial diseases is only beginning to be understood. Recently published research indicates that MTB grows in a biofilm mode of growth both *in vitro* as an air/surface pellicle and *in vivo* inside hypoxic lung cavities where they are closely apposed to the interior surface of the cavity. Greater understanding of how this mode of growth contributes to the pathogenesis and especially the persistence of MTB in infected patients will yield fruit for new tuberculosis diagnostics, vaccines, and drugs.

We found that MTB biofilms produce distinct proteomic signatures as compared to forced planktonic (Tween-80) growth. Using the *in vitro* model of MTB biofilm growth, we established a signature of 44 immunogenic proteins associated with the MTB biofilm (relative to shaking culture) of which 30 were expressed at all examined time points. We also found nine proteins that were specific to the biofilm stages of growth and could give further insight into MTB adaptations to the biofilm mode of growth.

The biofilm-specific protein FhaA (Rv0020c) is considered to be an element of signal transduction to reduce bacterial peptidoglycan synthesis. MTB is known to modify its peptidoglycan during dormancy and resuscitation, and promoting factors are known to hydrolyze peptidoglycan (Kana, *et al.*, 2008, Lavollay, *et al.*, 2008). Together these facts suggest that a greater understanding of the mechanisms of peptidoglycan signaling and regulation during dormancy and reactivation might be applied to new drugs designed to reactivate MTB persisters for killing by drug therapy.

Another biofilm-specific protein of interest is Rv3519, an acetoacetate decarboxylase protein that might play a role in increasing access to carbon sources acetone and acetoacetate, both of which are present in serum in elevated concentration during MTB infection. Considering both the poor prognosis of MTB in diabetic individuals, further research into this protein as a potential metabolic virulence factor is warranted. Likewise, better understanding of the biofilm specific protein Rv0097, critical for phthiocerol dimycolate (PDIM) production, might lead to adjunct therapies that could interfere with PDIM production, potentiating the killing of MTB by host-produced nitric-oxide.

The biofilm-specific proteins FabG4 and CeoB both have relevance to antibiotic therapy. CeoB itself can complement *E. coli oxyR* mutant to restore resistance to isoniazid indicating that the potassium flux mediated by CeoB and other TrkA system proteins have some bearing on the effectiveness of the most important antitubercular drug. The target of isoniazid, InhA, is always produced with a FabG protein as a partner suggesting that an alternate way to target the critical FASII system in MTB is to employ FabG inhibitors as antibiotics, an approach which has been utilized in other bacteria (Sohn, *et al.*, 2008, Cukier, *et al.*, 2013). Specifically targeting FabG4 might give a degree of specificity against biofilm-growing bacteria.

Aside from these biofilm-specific proteins as drug targets, both they and the 30 universally-expressed proteins represent potential diagnostic and vaccine candidates. From our group of 30 universally expressed proteins studies have previously identified 35kD_ag, TB31.7, Rv2629, and Tuf as proteins with high vaccine potential based on both published literature and on bioinformatics prediction (Zvi, *et al.*, 2008). Our screen also concurred with a high-throughput study of tuberculosis patient serum on five immunogenic proteins (35kd_ag, GroEl2, HbhA, RplE, and RplL) (Kunnath-Velayudhan, *et al.*, 2010)

The two different formulations of the vaccine, both with C-di-GMP and DDA/MPL as adjuvants, as well as the BCG-prime, vaccine-boost approach elicited strong humoral and cell-mediated immune responses specific to the antigens. However, these responses did not materially alter CFU burdens in the lungs or spleen, with the exception of a trend for reduction (p=0.055) seen in BALB/c mice vaccinated with the multivalent recombinant vaccine with DDA/MPL adjuvant. It is possible that with repetition of this experiment either with increased group size or an extended post-infection period this trend would have reached statistical significance. However, it would most likely still be inferior to BCG in its protective effect suggesting that future effort would be best expended refining the vaccine composition prior to additional animal tests.

Cyclic di-GMP decreases the host's ability to defend against MTB infection. This detrimental effect seemed to be partially mitigated by the inclusion of the five biofilm-expressed subunits in the full formulation of the vaccine However, this does not imply any actual effectiveness of the protein subunits for the prevention of active MTB disease. Alteration of the vaccine by substituting the DDA/MPL adjuvant for c-di-GMP resulted in a trend towards reduced lung burdens in vaccinated BALB/c. A prime-boost strategy using the BCG vaccine for the initial vaccination followed two weeks later by a boosting dose of five-subunit vaccine resulted in a significant reduction in bacterial populations. However, it could not be determined if this was a significant reduction below BCG vaccination alone.

Utilization of the Kramnik mouse (C3HeB/FeJ) model to test the vaccine resulted in a better understanding of the immunological response of these mice to vaccination. As communicated privately by Dr. Nuermberger, the mice exhibited a tendency to develop a biophasic distribution of CFU/organ which complicated statistical analysis of bacterial burdens. However, the pathological value of these animals indicates that modifications to the model for vaccine testing in the C3HeB/FeJ mice might be made in order to exploit the strain for better research. Specifically, it may be necessary to use larger numbers of animals, expecting that each treatment group will in fact represent two groups (high and normal bacterial burden) as well as stratifying the analysis of the two subgroups. This mouse model may better reflect the potential effectiveness of the vaccine in non-human and human primates because of the C3HeB/FeJ model's greater histological similarity to lung disease in humans with the formation of frank caseating lesions.

BALB/c mice were used for the bulk of the vaccine trials in this work because of their consistent reaction to aerosol MTB infection. Whereas C57BL/6 mice, with their stronger Th1 bias, are highly resistant to MTB infection, BALB/c mice develop either an acute or chronic infection in a dose-dependent fashion with increasing aerosol inoculum. However, the results of the splenocyte stimulation assays paint the BALB/c mice as strongly Th1 in comparison to C3Heb/FeJ mice suggesting that BALB/c mice occupy the middle of a spectrum between C57BL/6 and C3Heb/FeJ mice in terms of both Th1 response and resistance to infection.

Possible future experiments that could be performed to expand and clarify this research include:

- Individual protein subunit recombinant expression in BCG followed by utilization of these clones to vaccinate mice for a MTB challenge model. This would allow analysis of the value of each protein in a proven MTB vaccination protocol, produced in as close to MTB native conformation as possible.
- Development of polyclonal antibodies to these five protein subunits for utilization in both western blots of cavitary lesions recovered from animal models or human patients, as well as immunofluorescence assays to examine histological preparations of MTB infected tissues for evidence of biofilm-expressed protein production. This would lend greater credence to a role for biofilm in the pathogenesis of MTB infection.
- Western blots to examine MTB-infected sputum samples for biofilm-expressed protein production.

In summary, this work has identified MTB proteins expressed in the biofilm mode of growth that may be employed in the development of new diagnostics and vaccines as well as suggesting a number of new drug target for MTB chemotherapy. Several of these identified proteins also suggest starting points for new research projects into the basic bacteriology of MTB, especially regarding biofilm formation, persister formation, and antibiotic resistance. We successfully expressed five recombinant MTB membrane proteins, purified them, and used them to carry out three major nine-week vaccination and challenge trials. While no statistically significant reduction of bacterial burdens was observed, we identified a trend (p=0.055) towards reduction in lung MTB burden in those animals vaccinated with the multivalent recombinant vaccine coupled with the DDA/MPL adjuvant. Therefore, alternative or additional antigens may be added to this vaccine in order to provide for a more robust protection against MTB challenge. In addition, we identified the harmful nature of C-di-GMP as an adjuvant on MTB-infected mice and have elucidated the differences in the immunological responses of the C3HeB/FeJ mouse model in relation to the gold-standard BALB/c mouse model. As the number of publications acknowledging the ability of MTB to form biofilms grows, it is to be hoped that this new paradigm of MTB biofilms will further inform investigations into the basic bacteriology of MTB as well as the development of new drugs, vaccines, and diagnostics for tuberculosis.

APPENDIX I: Reagent Recipes

Oleic Acid-Dextrose-Catalase (OADC) Culture Supplement

1. Aliquot 950 ml of dH20.
2. Add to it:
   - 8.5 g NaCl
   - 50 g Bovine Serum Albumin Fraction V
   - 20 g D-dextrose
   - 40 mg catalase
   - 0.5 g Oleic acid
3. Stir until dissolved and filter sterilize.

M9 Minimal Media

1. Make M9 salts
2. To make M9 Salts aliquot 800ml H_2O and add
   - 64g Na_2HPO_4-$7H_2O$
   - 15g KH_2PO_4
   - 2.5g NaCl
   - 5.0g NH_4Cl
   - Stir until dissolved
   - Adjust to 1000ml with distilled H_2O
   - Sterilize by autoclaving
3. Measure ~700ml of distilled H_2O (sterile)
4. Add 200 ml of M9 salts
5. Add 2 ml of 1M $MgSO_4$ (sterile)
6. Add 20 ml of 20% glucose (or other carbon source)
7. Add 100 µl of 1M $CaCl_2$ (sterile)
8. Adjust to 1000 ml with distilled H_2O

Middlebrook 7H9 liquid media

1. To 900 ml dH20 add:
   - 4.7 g Middlebrook 7H9 powder (Difco, cat. 0713-01-7)
   - 100 ml Oleic Acid-Dextrose-Catalase (OADC)
   - 10 ml 50% glycerol
   - 2.5 ml 20% Tween-80
2. Dissolve Middlebrook 7H9 powder in dH20 then add ADS (or OADC), glycerol, and Tween-80. Sterilize by filtration through 0.22 micron pore membrane. Store at 4°C for 3 months.

Middlebrook 7H10 solid media

1. Aliquot 900 ml dH20.
3. Add 19 g Middlebrook 7H10 powder (Difco, cat. 0627-17-4)
4. Autoclave at 15 lb/in^2 for 20 min. Let cool on bench with stirring for 30 min.

5. Add 100 ml Albumin-Dextrose-Saline (ADS) or Oleic Acid-Albumin-Dextrose-Catalase (OADC), and 10 ml 50% glycerol.

Middlebrook 7H11 Solid Media
As Middlebrook 7H10 Solid Media with the addition of 1 g/L tryptone (pancreatic digest of casein).

Sauton's Minimal Media
1. To ~800 ml of distilled H20 add:
   - 0.5 g KH2PO4
   - 0.5 g MgSO4,
   - 4.0 g L-asparagine
   - 60 ml glycerol
   - 0.05 g Ferric ammonium citrate
   - 2.0 g citric acid
   - ml 1% ZnSO4
2. Adjust total volume to 900 ml with dH20.
3. Adjust pH to 7 using 33% ammonium hydroxide solution.
4. Adjust final volume to 1000 ml with dH20.
5. Autoclave, cool.
6. (optional for non-biofilm cultures) Add 2.5 ml sterile filtered 20% Tween-80.

REFERENCES

(2006) The Stop TB Strategy. World Health Organization.

(2013) Guidelines for the Prevention and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents. Vol. 2013 National Institutes of Health.

Abebe F & Bjune G (2009) The protective role of antibody responses during *Mycobacterium tuberculosis* infection. *Clin Exp Immunol* **157**: 235-243.

Abel B, Thieblemont N, Quesniaux VJ, *et al.* (2002) Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. *J Immunol* **169**: 3155-3162.

Ackart DF, Lindsey EA, Podell BK, Melander RJ, Basaraba RJ & Melander C (2014) Reversal of *Mycobacterium tuberculosis* phenotypic drug resistance by 2-aminoimidazole-based small molecules. *Pathog Dis*.

Ackart DF, Hascall-Dove L, Caceres SM, *et al.* (2014) Expression of antimicrobial drug tolerance by attached communities of *Mycobacterium tuberculosis*. *Pathog Dis*.

Adetunji V, Kehinde A, Bolatito O & Chen J (2014) Biofilms formed by *Mycobacterium tuberculosis* on cement, ceramic, and stainless steel surfaces and their controls. *J Food Prot* **77**: 599-604.

Agusti G, Astola O, Rodriguez-Guell E, Julian E & Luquin M (2008) Surface spreading motility shown by a group of phylogenetically related, rapidly growing pigmented mycobacteria suggests that motility is a common property of mycobacterial species but is restricted to smooth colonies. *J Bacteriol* **190**: 6894-6902.

Allard ST, Giraud MF & Naismith JH (2001) Epimerases: structure, function and mechanism. *Cell Mol Life Sci* **58**: 1650-1665.

Alonso S, Pethe K, Russell DG & Purdy GE (2007) Lysosomal killing of *Mycobacterium* mediated by ubiquitin-derived peptides is enhanced by autophagy. *Proc Natl Acad Sci U S A* **104**: 6031-6036.

Altare F, Durandy A, Lammas D, *et al.* (1998) Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* **280**: 1432-1435.

Armstrong JA & Hart PD (1975) Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. *J Exp Med* **142**: 1-16.

Astarie-Dequeker C, Le Guyader L, Malaga W, Seaphanh FK, Chalut C, Lopez A & Guilhot C (2009) Phthiocerol dimycocerosates of *M. tuberculosis* participate in macrophage invasion by inducing changes in the organization of plasma membrane lipids. *PLoS Pathog* **5**: e1000289.

Azad AK, Sadee W & Schlesinger LS (2012) Innate immune gene polymorphisms in tuberculosis. *Infect Immun* **80**: 3343-3359.

Bafica A, Scanga CA, Feng CG, Leifer C, Cheever A & Sher A (2005) TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*. *J Exp Med* **202**: 1715-1724.

Bafica A, Scanga CA, Serhan C, Machado F, White S, Sher A & Aliberti J (2005) Host control of *Mycobacterium tuberculosis* is regulated by 5-lipoxygenase-dependent lipoxin production. *J Clin Invest* **115**: 1601-1606.

Baldwin SL, D'Souza C, Roberts AD, *et al.* (1998) Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. *Infect Immun* **66**: 2951-2959.

Balu S, Reljic R, Lewis MJ, *et al.* (2011) A novel human IgA monoclonal antibody protects against tuberculosis. *J Immunol* **186**: 3113-3119.

Banchereau J & Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* **392**: 245-252.

Bardouniotis E, Ceri H & Olson ME (2003) Biofilm formation and biocide susceptibility testing of *Mycobacterium fortuitum* and *Mycobacterium marinum*. *Curr Microbiol* **46**: 28-32.

Barrios-Payan J, Aguilar-Leon D, Lascurain-Ledezma R & Hernandez-Pando R (2006) [Neutrophil participation in early control and immune activation during experimental pulmonary tuberculosis]. *Gac Med Mex* **142**: 273-281.

Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD & Britton WJ (1999) Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. *J Immunol* **162**: 3504-3511.

Behr MA (2002) BCG--different strains, different vaccines? *Lancet Infect Dis* **2**: 86-92.

Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ & Besra GS (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* **276**: 1420-1422.

Beretta C (1891) On the use of dog's serum in the treatment of tuberculosis. *Br Med J* **2**: 1041-1043.

Best CA & Best TJ (2009) *Mycobacterium smegmatis* infection of the hand. *Hand (N Y)* **4**: 165-166.

Beste DJ, Espasa M, Bonde B, Kierzek AM, Stewart GR & McFadden J (2009) The genetic requirements for fast and slow growth in mycobacteria. *PLoS One* **4**: e5349.

Beumer A, King D, Donohue M, Mistry J, Covert T & Pfaller S (2010) Detection of *Mycobacterium avium* subsp. paratuberculosis in drinking water and biofilms by quantitative PCR. *Appl Environ Microbiol* **76**: 7367-7370.

Biet F, Boschiroli ML, Thorel MF & Guilloteau LA (2005) Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). *Vet Res* **36**: 411-436.

Bosio S, Leekha S, Gamb SI, Wright AJ, Terrell CL & Miller DV (2012) *Mycobacterium fortuitum* prosthetic valve endocarditis: a case for the pathogenetic role of biofilms. *Cardiovasc Pathol* **21**: 361-364.

Bossemeyer D, Borchard A, Dosch DC, Helmer GC, Epstein W, Booth IR & Bakker EP (1989) K+-transport protein TrkA of *Escherichia coli* is a peripheral membrane protein that requires other trk gene products for attachment to the cytoplasmic membrane. *J Biol Chem* **264**: 16403-16410.

Bothamley G, Beck JS, Britton W, Elsaghier A & Ivanyi J (1991) Antibodies to *Mycobacterium tuberculosis*-specific epitopes in lepromatous leprosy. *Clin Exp Immunol* **86**: 426-432.

Brady RA, Leid JG, Camper AK, Costerton JW & Shirtliff ME (2006) Identification of *Staphylococcus aureus* proteins recognized by the antibody-mediated immune response to a biofilm infection. *Infect Immun* **74**: 3415-3426.

Brady RA, Leid JG, Kofonow J, Costerton JW & Shirtliff ME (2007) Immunoglobulins to surface-associated biofilm immunogens provide a novel means of visualization of methicillin-resistant *Staphylococcus aureus* biofilms. *Appl Environ Microbiol* **73**: 6612-6619.

Brady RA, O'May GA, Leid JG, Prior ML, Costerton JW & Shirtliff ME (2011) Resolution of *Staphylococcus aureus* biofilm infection using vaccination and antibiotic treatment. *Infect Immun* **79**: 1797-1803.

Braian C, Hogea V & Stendahl O (2013) *Mycobacterium tuberculosis*- Induced neutrophil extracellular traps activate human macrophages. *J Innate Immun* **5**: 591-602.

Brandt L, Elhay M, Rosenkrands I, Lindblad EB & Andersen P (2000) ESAT-6 subunit vaccination against *Mycobacterium tuberculosis*. *Infect Immun* **68**: 791-795.

Brinkmann V, Reichard U, Goosmann C, *et al.* (2004) Neutrophil extracellular traps kill bacteria. *Science* **303**: 1532-1535.

Brosch R, Gordon SV, Marmiesse M, *et al.* (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proc Natl Acad Sci U S A* **99**: 3684-3689.

Brown-Elliott BA, Iakhiaeva E, Griffith DE, *et al.* (2013) *In vitro* activity of amikacin against isolates of *Mycobacterium avium* complex with proposed MIC breakpoints and finding of a 16S rRNA gene mutation in treated isolates. *J Clin Microbiol* **51**: 3389-3394.

Burdette DL, Monroe KM, Sotelo-Troha K, *et al.* (2011) STING is a direct innate immune sensor of cyclic di-GMP. *Nature* **478**: 515-518.

Burge EJ, Gauthier DT & Van Veld PA (2004) In vitro response of the striped bass natural resistance-associated macrophage protein, Nramp, to LPS and *Mycobacterium marinum* exposure. *Comp Biochem Physiol C Toxicol Pharmacol* **138**: 391-400.

Caceres N, Vilaplana C, Prats C, *et al.* (2013) Evolution and role of corded cell aggregation in *Mycobacterium tuberculosis* cultures. *Tuberculosis (Edinb)*.

Cade CE, Dlouhy AC, Medzihradszky KF, Salas-Castillo SP & Ghiladi RA (2010) Isoniazid-resistance conferring mutations in *Mycobacterium tuberculosis* KatG: catalase, peroxidase, and INH-NADH adduct formation activities. *Protein Sci* **19**: 458-474.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K & Madden TL (2009) BLAST+: architecture and applications. *BMC Bioinformatics* **10**: 421.

Canetti G (1950) Exogenous reinfection and pulmonary tuberculosis a study of the pathology. *Tubercle* **31**: 224-233.

Cassidy JP (2006) The pathogenesis and pathology of bovine tuberculosis with insights from studies of tuberculosis in humans and laboratory animal models. *Vet Microbiol* **112**: 151-161.

Chackerian AA, Perera TV & Behar SM (2001) Gamma interferon-producing CD4+ T lymphocytes in the lung correlate with resistance to infection with *Mycobacterium tuberculosis*. *Infect Immun* **69**: 2666-2674.

Chaitra MG, Hariharaputran S, Chandra NR, Shaila MS & Nayak R (2005) Defining putative T cell epitopes from PE and PPE families of proteins of *Mycobacterium tuberculosis* with vaccine potential. *Vaccine* **23**: 1265-1272.

Checroun C, Wehrly TD, Fischer ER, Hayes SF & Celli J (2006) Autophagy-mediated reentry of *Francisella tularensis* into the endocytic compartment after cytoplasmic replication. *Proc Natl Acad Sci U S A* **103**: 14578-14583.

Chen P & Bishai WR (1998) Novel selection for isoniazid (INH) resistance genes supports a role for NAD+-binding proteins in mycobacterial INH resistance. *Infect Immun* **66**: 5099-5106.

Chukwuekezie O, Ampadu E, Sopoh G, *et al.* (2007) Buruli ulcer, Nigeria. *Emerg Infect Dis* **13**: 782-783.

Cohen NR, Garg S & Brenner MB (2009) Antigen presentation by CD1 Lipids, T cells, and NKT cells in microbial immunity. *Adv Immunol* **102**: 1-94.

Cole ST (2002) Comparative mycobacterial genomics as a tool for drug target and antigen discovery. *Eur Respir J Suppl* **36**: 78s-86s.

Cooper AM, Magram J, Ferrante J & Orme IM (1997) Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. *J Exp Med* **186**: 39-45.

Cooper AM, Roberts AD, Rhoades ER, Callahan JE, Getzy DM & Orme IM (1995) The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. *Immunology* **84**: 423-432.

Corleis B, Korbel D, Wilson R, Bylund J, Chee R & Schaible UE (2012) Escape of *Mycobacterium tuberculosis* from oxidative killing by neutrophils. *Cell Microbiol* **14**: 1109-1121.

Cukier CD, Hope AG, Elamin AA, *et al.* (2013) Discovery of an allosteric inhibitor binding site in 3-Oxo-acyl-ACP reductase from *Pseudomonas aeruginosa*. *ACS Chem Biol* **8**: 2518-2527.

Delgado MA, Elmaoued RA, Davis AS, Kyei G & Deretic V (2008) Toll-like receptors control autophagy. *EMBO J* **27**: 1110-1121.

Delogu G, Bua A, Pusceddu C, Parra M, Fadda G, Brennan MJ & Zanetti S (2004) Expression and purification of recombinant methylated HBHA in *Mycobacterium smegmatis*. *FEMS Microbiol Lett* **239**: 33-39.

den Dunnen J, Gringhuis SI & Geijtenbeek TB (2009) Innate signaling by the C-type lectin DC-SIGN dictates immune responses. *Cancer Immunol Immunother* **58**: 1149-1157.

Deretic V, Delgado M, Vergne I, Master S, De Haro S, Ponpuak M & Singh S (2009) Autophagy in immunity against *Mycobacterium tuberculosis*: a model system to dissect immunological roles of autophagy. *Curr Top Microbiol Immunol* **335**: 169-188.

Diedrich CR, Mattila JT, Klein E, *et al.* (2010) Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T cell depletion and not virus load. *PLoS One* **5**: e9611.

Djavaheri-Mergny M, Amelotti M, Mathieu J, *et al.* (2006) NF-kappaB activation represses tumor necrosis factor-alpha-induced autophagy. *J Biol Chem* **281**: 30373-30382.

Doherty TM & Arditi M (2004) TB, or not TB: that is the question -- does TLR signaling hold the answer? *J Clin Invest* **114**: 1699-1703.

Doherty TM, Dietrich J & Billeskov R (2007) Tuberculosis subunit vaccines: from basic science to clinical testing. *Expert Opin Biol Ther* **7**: 1539-1549.

Dolin PJ, Raviglione MC & Kochi A (1994) Global tuberculosis incidence and mortality during 1990-2000. *Bull World Health Organ* **72**: 213-220.

Dong D, Wang D, Li M, *et al.* (2012) PPE38 modulates the innate immune response and is required for *Mycobacterium marinum* virulence. *Infect Immun* **80**: 43-54.

Dong Y, Demaria S, Sun X, *et al.* (2004) HLA-A2-restricted CD8+-cytotoxic-T-cell responses to novel epitopes in *Mycobacterium tuberculosis* superoxide dismutase, alanine dehydrogenase, and glutamine synthetase. *Infect Immun* **72**: 2412-2415.

Dooley KE & Chaisson RE (2009) Tuberculosis and diabetes mellitus: convergence of two epidemics. *Lancet Infect Dis* **9**: 737-746.

Driver ER, Ryan GJ, Hoff DR, Irwin SM, Basaraba RJ, Kramnik I & Lenaerts AJ (2012) Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **56**: 3181-3195.

Dussurget O, Stewart G, Neyrolles O, Pescher P, Young D & Marchal G (2001) Role of *Mycobacterium tuberculosis* copper-zinc superoxide dismutase. *Infect Immun* **69**: 529-533.

Dutta D, Bhattacharyya S, Roychowdhury A, Biswas R & Das AK (2013) Crystal structure of hexanoyl-CoA bound to beta-ketoacyl reductase FabG4 of *Mycobacterium tuberculosis*. *Biochem J* **450**: 127-139.

Edelstein H (1994) *Mycobacterium marinum* skin infections. Report of 31 cases and review of the literature. *Arch Intern Med* **154**: 1359-1364.

Ehlers S, Kutsch S, Benini J, *et al.* (1999) NOS2-derived nitric oxide regulates the size, quantity and quality of granuloma formation in *Mycobacterium avium*-infected mice without affecting bacterial loads. *Immunology* **98**: 313-323.

Eum SY, Kong JH, Hong MS, *et al.* (2010) Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest* **137**: 122-128.

Ewer K, Cockle P, Gordon S, *et al.* (2006) Antigen mining with iterative genome screens identifies novel diagnostics for the *Mycobacterium tuberculosis* complex. *Clin Vaccine Immunol* **13**: 90-97.

Falkinham JO, 3rd (2002) Nontuberculous mycobacteria in the environment. *Clin Chest Med* **23**: 529-551.

Falkinham JO, 3rd, Iseman MD, de Haas P & van Soolingen D (2008) *Mycobacterium avium* in a shower linked to pulmonary disease. *J Water Health* **6**: 209-213.

Ferraz JC, Stavropoulos E, Yang M, *et al.* (2004) A heterologous DNA priming-*Mycobacterium bovis* BCG boosting immunization strategy using mycobacterial Hsp70, Hsp65, and Apa antigens improves protection against tuberculosis in mice. *Infect Immun* **72**: 6945-6950.

Florczyk MA, McCue LA, Stack RF, Hauer CR & McDonough KA (2001) Identification and characterization of mycobacterial proteins differentially expressed under standing and shaking culture conditions, including Rv2623 from a novel class of putative ATP-binding proteins. *Infect Immun* **69**: 5777-5785.

Flynn JL & Chan J (2001) Tuberculosis: latency and reactivation. *Infect Immun* **69**: 4195-4201.

Flynn JL, Goldstein MM, Triebold KJ, Koller B & Bloom BR (1992) Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci U S A* **89**: 12013-12017.

Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA & Bloom BR (1993) An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* **178**: 2249-2254.

Forrellad MA, Klepp LI, Gioffre A, *et al.* (2013) Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence* **4**: 3-66.

Fortsch D, Rollinghoff M & Stenger S (2000) IL-10 converts human dendritic cells into macrophage-like cells with increased antibacterial activity against virulent *Mycobacterium tuberculosis*. *J Immunol* **165**: 978-987.

Fratazzi C, Arbeit RD, Carini C & Remold HG (1997) Programmed cell death of *Mycobacterium avium* serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. *J Immunol* **158**: 4320-4327.

Fremond CM, Yeremeev V, Nicolle DM, Jacobs M, Quesniaux VF & Ryffel B (2004) Fatal *Mycobacterium tuberculosis* infection despite adaptive immune response in the absence of MyD88. *J Clin Invest* **114**: 1790-1799.

Furuya T, Hirose S, Semba H & Kino K (2011) Identification of the regulator gene responsible for the acetone-responsive expression of the binuclear iron monooxygenase gene cluster in mycobacteria. *J Bacteriol* **193**: 5817-5823.

Fyfe JA, Lavender CJ, Handasyde KA, *et al.* (2010) A major role for mammals in the ecology of *Mycobacterium ulcerans*. *PLoS Negl Trop Dis* **4**: e791.

Garg A, Barnes PF, Porgador A, *et al.* (2006) Vimentin expressed on *Mycobacterium tuberculosis*-infected human monocytes is involved in binding to the NKp46 receptor. *J Immunol* **177**: 6192-6198.

Gee CL, Papavinasasundaram KG, Blair SR, *et al.* (2012) A phosphorylated pseudokinase complex controls cell wall synthesis in mycobacteria. *Sci Signal* **5**: ra7.

Geier H, Mostowy S, Cangelosi GA, Behr MA & Ford TE (2008) Autoinducer-2 triggers the oxidative stress response in *Mycobacterium avium*, leading to biofilm formation. *Appl Environ Microbiol* **74**: 1798-1804.

Ghosh S, Indi SS & Nagaraja V (2013) Regulation of lipid biosynthesis, sliding motility, and biofilm formation by a membrane-anchored nucleoid-associated protein of *Mycobacterium tuberculosis*. *J Bacteriol* **195**: 1769-1778.

Giri PK, Verma I & Khuller GK (2006) Enhanced immunoprotective potential of *Mycobacterium tuberculosis* Ag85 complex protein based vaccine against airway *Mycobacterium tuberculosis* challenge following intranasal administration. *FEMS Immunol Med Microbiol* **47**: 233-241.

Glatman-Freedman A & Casadevall A (1998) Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against *Mycobacterium tuberculosis*. *Clin Microbiol Rev* **11**: 514-532.

Goodridge A, Zhang T, Miyata T, Lu S & Riley LW (2014) Antiphospholipid IgM antibody response in acute and chronic *Mycobacterium tuberculosis* mouse infection model. *Clin Respir J* **8**: 137-144.

Goonetilleke NP, McShane H, Hannan CM, Anderson RJ, Brookes RH & Hill AV (2003) Enhanced immunogenicity and protective efficacy against *Mycobacterium tuberculosis* of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J Immunol* **171**: 1602-1609.

Gordon BR, Imperial R, Wang L, Navarre WW & Liu J (2008) Lsr2 of *Mycobacterium* represents a novel class of H-NS-like proteins. *J Bacteriol* **190**: 7052-7059.

Goude R & Parish T (2008) Electroporation of mycobacteria. *J Vis Exp*.

Green AM, Difazio R & Flynn JL (2013) IFN-gamma from CD4 T cells is essential for host survival and enhances CD8 T cell function during *Mycobacterium tuberculosis* infection. *J Immunol* **190**: 270-277.

Griffith DE, Aksamit T, Brown-Elliott BA, *et al.* (2007) An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* **175**: 367-416.

Griffith DE, Aksamit T, Brown-Elliott BA, *et al.* (2007) An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* **175**: 367-416.

Griffith DEW, R.J. (2011) Rapidly growing mycobacterial infections in the HIV-negative patient.

Grosset J (2003) *Mycobacterium tuberculosis* in the extracellular compartment: an underestimated adversary. *Antimicrob Agents Chemother* **47**: 833-836.

Guerrero GG & Locht C (2011) Recombinant HBHA boosting effect on BCG-induced immunity against *Mycobacterium tuberculosis* infection. *Clin Dev Immunol* **2011**: 730702.

Guirado E, Amat I, Gil O, *et al.* (2006) Passive serum therapy with polyclonal antibodies against *Mycobacterium tuberculosis* protects against post-chemotherapy relapse of tuberculosis infection in SCID mice. *Microbes Infect* **8**: 1252-1259.

Guirado E, Arcos J, Knaup R, *et al.* (2012) Characterization of clinical and environmental *Mycobacterium avium* spp. isolates and their interaction with human macrophages. *PLoS One* **7**: e45411.

Gupta A, Kaul A, Tsolaki AG, Kishore U & Bhakta S (2012) *Mycobacterium tuberculosis*: immune evasion, latency and reactivation. *Immunobiology* **217**: 363-374.

Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI & Deretic V (2004) Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* **119**: 753-766.

Ha KY, Chung YG & Ryoo SJ (2005) Adherence and biofilm formation of *Staphylococcus epidermidis* and *Mycobacterium tuberculosis* on various spinal implants. *Spine (Phila Pa 1976)* **30**: 38-43.

Hall-Stoodley L, Keevil CW & Lappin-Scott HM (1998) *Mycobacterium fortuitum* and *Mycobacterium chelonae* biofilm formation under high and low nutrient conditions. *J Appl Microbiol* **85 Suppl 1**: 60S-69S.

Hall-Stoodley L, Brun OS, Polshyna G & Barker LP (2006) *Mycobacterium marinum* biofilm formation reveals cording morphology. *FEMS Microbiol Lett* **257**: 43-49.

Hamasur B, Haile M, Pawlowski A, Schroder U, Kallenius G & Svenson SB (2004) A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab') fragment prolong survival of mice infected with *Mycobacterium tuberculosis*. *Clin Exp Immunol* **138**: 30-38.

Harding CV & Boom WH (2010) Regulation of antigen presentation by Mycobacterium tuberculosis: a role for Toll-like receptors. *Nat Rev Microbiol* **8**: 296-307.

Harris J, De Haro SA, Master SS, Keane J, Roberts EA, Delgado M & Deretic V (2007) T helper 2 cytokines inhibit autophagic control of intracellular *Mycobacterium tuberculosis*. *Immunity* **27**: 505-517.

Helke KL, Mankowski JL & Manabe YC (2006) Animal models of cavitation in pulmonary tuberculosis. *Tuberculosis (Edinb)* **86**: 337-348.

Higgins DM, Sanchez-Campillo J, Rosas-Taraco AG, Lee EJ, Orme IM & Gonzalez-Juarrero M (2009) Lack of IL-10 alters inflammatory and immune responses during pulmonary *Mycobacterium tuberculosis* infection. *Tuberculosis (Edinb)* **89**: 149-157.

Hopewell PC, Pai M, Maher D, Uplekar M & Raviglione MC (2006) International standards for tuberculosis care. *Lancet Infect Dis* **6**: 710-725.

Horwitz MA, Harth G, Dillon BJ & Maslesa-Galic S (2000) Recombinant bacillus calmette-guerin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc Natl Acad Sci U S A* **97**: 13853-13858.

Hotter GS, Wards BJ, Mouat P*, et al.* (2005) Transposon mutagenesis of Mb0100 at the ppe1-nrp locus in *Mycobacterium bovis* disrupts phthiocerol dimycocerosate (PDIM) and glycosylphenol-PDIM biosynthesis, producing an avirulent strain with vaccine properties at least equal to those of M. bovis BCG. *J Bacteriol* **187**: 2267-2277.

Howard ST, Rhoades E, Recht J*, et al.* (2006) Spontaneous reversion of *Mycobacterium abscessus* from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. *Microbiology* **152**: 1581-1590.

Hu S, Chen H, Ma J*, et al.* (2013) CpG7909 adjuvant enhanced immunogenicity efficacy in mice immunized with ESAT6-Ag85A fusion protein, but does not confer significant protection against *Mycobacterium tuberculosis* infection. *J Appl Microbiol* **115**: 1203-1211.

Hunter RL, Olsen MR, Jagannath C & Actor JK (2006) Multiple roles of cord factor in the pathogenesis of primary, secondary, and cavitary tuberculosis, including a revised description of the pathology of secondary disease. *Ann Clin Lab Sci* **36**: 371-386.

Hunter SW, Gaylord H & Brennan PJ (1986) Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. *J Biol Chem* **261**: 12345-12351.

Iddriss A, Padayatchi N, Reddy D & Reddi A (2012) Pulmonary resection for extensively drug resistant tuberculosis in Kwazulu-Natal, South Africa. *Ann Thorac Surg* **94**: 381-386.

Indrigo J, Hunter RL, Jr. & Actor JK (2003) Cord factor trehalose 6,6'-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. *Microbiology* **149**: 2049-2059.

Ismaili J, Olislagers V, Poupot R, Fournie JJ & Goldman M (2002) Human gamma delta T cells induce dendritic cell maturation. *Clin Immunol* **103**: 296-302.

Iyengar KP, Nadkarni JB, Gupta R, Beeching NJ, Ullah I & Loh WY (2013) *Mycobacterium chelonae* hand infection following ferret bite. *Infection* **41**: 237-241.

Jacobs M, Brown N, Allie N, Chetty K & Ryffel B (2000) Tumor necrosis factor receptor 2 plays a minor role for mycobacterial immunity. *Pathobiology* **68**: 68-75.

Jain R, Dey B, Khera A*, et al.* (2011) Over-expression of superoxide dismutase obliterates the protective effect of BCG against tuberculosis by modulating innate and adaptive immune responses. *Vaccine* **29**: 8118-8125.

Jain R, Dey B, Dhar N*, et al.* (2008) Enhanced and enduring protection against tuberculosis by recombinant BCG-Ag85C and its association with modulation of cytokine profile in lung. *PLoS One* **3**: e3869.

Jin BW, Saito H & Yoshii Z (1984) Environmental mycobacteria in Korea. I. Distribution of the organisms. *Microbiol Immunol* **28**: 667-677.

Jin L, Wang G, Zhao X, *et al.* (2010) Characterization and immune effect of the hepatitis B-BCG combined vaccine for using a needle innoculation. *Vaccine* **28**: 6041-6051.

Jindani A, Dore CJ & Mitchison DA (2003) Bactericidal and sterilizing activities of antituberculosis drugs during the first 14 days. *Am J Respir Crit Care Med* **167**: 1348-1354.

Johansson U, Ivanyi J & Londei M (2001) Inhibition of IL-12 production in human dendritic cells matured in the presence of Bacillus Calmette-Guerin or lipoarabinomannan. *Immunol Lett* **77**: 63-66.

Kamath AB, Alt J, Debbabi H & Behar SM (2003) Toll-like receptor 4-defective C3H/HeJ mice are not more susceptible than other C3H substrains to infection with *Mycobacterium tuberculosis*. *Infect Immun* **71**: 4112-4118.

Kamijo R, Le J, Shapiro D, *et al.* (1993) Mice that lack the interferon-gamma receptor have profoundly altered responses to infection with Bacillus Calmette-Guerin and subsequent challenge with lipopolysaccharide. *J Exp Med* **178**: 1435-1440.

Kana BD, Gordhan BG, Downing KJ, *et al.* (2008) The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth in vitro. *Mol Microbiol* **67**: 672-684.

Kashino SS, Napolitano DR, Skobe Z & Campos-Neto A (2008) Guinea pig model of *Mycobacterium tuberculosis* latent/dormant infection. *Microbes Infect* **10**: 1469-1476.

Kaur D, Guerin ME, Skovierova H, Brennan PJ & Jackson M (2009) Chapter 2: Biogenesis of the cell wall and other glycoconjugates of *Mycobacterium tuberculosis*. *Adv Appl Microbiol* **69**: 23-78.

Kaushal D, Mehra S, Didier PJ & Lackner AA (2012) The non-human primate model of tuberculosis. *J Med Primatol* **41**: 191-201.

Keane J, Remold HG & Kornfeld H (2000) Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *J Immunol* **164**: 2016-2020.

Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ & Kornfeld H (1997) Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. *Infect Immun* **65**: 298-304.

Kelley LA & Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* **4**: 363-371.

Kennedy BS, Bedard B, Younge M, *et al.* (2012) Outbreak of *Mycobacterium chelonae* infection associated with tattoo ink. *N Engl J Med* **367**: 1020-1024.

Kenrick KG & Margolis J (1970) Isoelectric focusing and gradient gel electrophoresis: a two-dimensional technique. *Anal Biochem* **33**: 204-207.

Keren I, Minami S, Rubin E & Lewis K (2011) Characterization and transcriptome analysis of *Mycobacterium tuberculosis* persisters. *MBio* **2**.

Kleinnijenhuis J, Oosting M, Joosten LA, Netea MG & Van Crevel R (2011) Innate immune recognition of *Mycobacterium tuberculosis*. *Clin Dev Immunol* **2011**: 405310.

Knibb W, Colorni A, Ankaoua M, Lindell D, Diamant A & Gordin H (1993) Detection and identification of a pathogenic marine Mycobacterium from the European seabass Dicentrarchus labrax using polymerase chain reaction and direct sequencing of 16S rDNA sequences. *Mol Mar Biol Biotechnol* **2**: 225-232.

Ko Y, Kim W, Shin BS*, et al.* (2013) Nontuberculous mycobacterial lung disease caused by *Mycobacterium chelonae*: a case report. *Tuberc Respir Dis (Seoul)* **74**: 191-194.

Kocincova D, Winter N, Euphrasie D, Daffe M, Reyrat JM & Etienne G (2009) The cell surface-exposed glycopeptidolipids confer a selective advantage to the smooth variants of *Mycobacterium smegmatis* in vitro. *FEMS Microbiol Lett* **290**: 39-44.

Kohama H, Umemura M, Okamoto Y*, et al.* (2008) Mucosal immunization with recombinant heparin-binding haemagglutinin adhesin suppresses extrapulmonary dissemination of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) in infected mice. *Vaccine* **26**: 924-932.

Kotani T, Yurimoto H, Kato N & Sakai Y (2007) Novel acetone metabolism in a propane-utilizing bacterium, *Gordonia* sp. strain TY-5. *J Bacteriol* **189**: 886-893.

Kramnik I (2008) Genetic dissection of host resistance to *Mycobacterium tuberculosis*: the sst1 locus and the Ipr1 gene. *Curr Top Microbiol Immunol* **321**: 123-148.

Kramnik I, Demant P & Bloom BB (1998) Susceptibility to tuberculosis as a complex genetic trait: analysis using recombinant congenic strains of mice. *Novartis Found Symp* **217**: 120-131; discussion 132-127.

Kulaev IS (1979) *The biochemistry of inorganic phosphates*. Wiley, New York.

Kunnath-Velayudhan S, Salamon H, Wang HY*, et al.* (2010) Dynamic antibody responses to the *Mycobacterium tuberculosis* proteome. *Proc Natl Acad Sci U S A* **107**: 14703-14708.

Kuo CJ, Bell H, Hsieh CL, Ptak CP & Chang YF (2012) Novel mycobacteria antigen 85 complex binding motif on fibronectin. *J Biol Chem* **287**: 1892-1902.

Kupeli E, Bozkurt E, Azap O & Eyuboglu FO (2010) *Mycobacterium fortuitum* infection presenting as community-acquired pneumonia in an immunocompetent host. *J Bronchology Interv Pulmonol* **17**: 356-358.

Lanckriet C, Levy-Bruhl D, Bingono E, Siopathis RM & Guerin N (1995) Efficacy of BCG vaccination of the newborn: evaluation by a follow-up study of contacts in Bangui. *Int J Epidemiol* **24**: 1042-1049.

Lang R (2013) Recognition of the mycobacterial cord factor by Mincle: relevance for granuloma formation and resistance to tuberculosis. *Front Immunol* **4**: 5.

Lavollay M, Arthur M, Fourgeaud M, *et al.* (2008) The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J Bacteriol* **190**: 4360-4366.

Lee J, Hartman M & Kornfeld H (2009) Macrophage apoptosis in tuberculosis. *Yonsei Med J* **50**: 1-11.

Lee J, Remold HG, Ieong MH & Kornfeld H (2006) Macrophage apoptosis in response to high intracellular burden of *Mycobacterium tuberculosis* is mediated by a novel caspase-independent pathway. *J Immunol* **176**: 4267-4274.

Lee RP, Cheung KW, Chiu KH & Tsang ML (2012) *Mycobacterium chelonae* infection after total knee arthroplasty: a case report. *J Orthop Surg (Hong Kong)* **20**: 134-136.

Lefmann M, Schweickert B, Buchholz P, *et al.* (2006) Evaluation of peptide nucleic acid-fluorescence in situ hybridization for identification of clinically relevant mycobacteria in clinical specimens and tissue sections. *J Clin Microbiol* **44**: 3760-3767.

Lehtola MJ, Torvinen E, Kusnetsov J, *et al.* (2007) Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli*, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Appl Environ Microbiol* **73**: 2854-2859.

Lew JM, Kapopoulou A, Jones LM & Cole ST (2011) TubercuList--10 years after. *Tuberculosis (Edinb)* **91**: 1-7.

Liang Y, Wu X, Zhang J, *et al.* (2012) Immunogenicity and therapeutic effects of Ag85A/B chimeric DNA vaccine in mice infected with *Mycobacterium tuberculosis*. *FEMS Immunol Med Microbiol* **66**: 419-426.

Lin PL & Flynn JL (2010) Understanding latent tuberculosis: a moving target. *J Immunol* **185**: 15-22.

Lin PL, Rodgers M, Smith L, *et al.* (2009) Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infect Immun* **77**: 4631-4642.

Liu J, Tran V, Leung AS, Alexander DC & Zhu B (2009) BCG vaccines: their mechanisms of attenuation and impact on safety and protective efficacy. *Hum Vaccin* **5**: 70-78.

Locht C, Hougardy JM, Rouanet C, Place S & Mascart F (2006) Heparin-binding hemagglutinin, from an extrapulmonary dissemination factor to a powerful diagnostic and protective antigen against tuberculosis. *Tuberculosis (Edinb)* **86**: 303-309.

Lopez Y, Yero D, Falero-Diaz G, *et al.* (2009) Induction of a protective response with an IgA monoclonal antibody against *Mycobacterium tuberculosis* 16kDa protein in a model of progressive pulmonary infection. *Int J Med Microbiol* **299**: 447-452.

Lowe DM, Redford PS, Wilkinson RJ, O'Garra A & Martineau AR (2012) Neutrophils in tuberculosis: friend or foe? *Trends Immunol* **33**: 14-25.

Lu J, Wang C, Zhou Z, *et al.* (2011) Immunogenicity and protective efficacy against murine tuberculosis of a prime-boost regimen with BCG and a DNA vaccine expressing ESAT-6 and Ag85A fusion protein. *Clin Dev Immunol* **2011**: 617892.

Luca S & Mihaescu T (2013) History of BCG Vaccine. *Maedica (Buchar)* **8**: 53-58.

Lukins HB & Foster JW (1963) Methyl ketone metabolism in hydrocarbon-utilizing Mycobacteria. *J Bacteriol* **85**: 1074-1087.

Lurie MB (1928) The fate of human and bovine tubercle bacilli in various organs of the rabbit. *J Exp Med* **48**: 155-182.

Lynch CJ, Pierce-Chase CH & Dubos R (1965) A genetic study of susceptibility to experimental tuberculosis in mice infected with mammalian tubercle bacilli. *J Exp Med* **121**: 1051-1070.

M'Imunya J M, Kredo T & Volmink J (2012) Patient education and counselling for promoting adherence to treatment for tuberculosis. *Cochrane Database Syst Rev* **5**: CD006591.

Ma Y, Galluzzi L, Zitvogel L & Kroemer G (2013) Autophagy and cellular immune responses. *Immunity* **39**: 211-227.

MacGregor RR, Hafner R, Wu JW, *et al.* (2005) Clinical, microbiological, and immunological characteristics in HIV-infected subjects at risk for disseminated *Mycobacterium avium* complex disease: an AACTG study. *AIDS Res Hum Retroviruses* **21**: 689-695.

Maglione PJ, Xu J & Chan J (2007) B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with *Mycobacterium tuberculosis*. *J Immunol* **178**: 7222-7234.

Malen H, Softeland T & Wiker HG (2008) Antigen analysis of *Mycobacterium tuberculosis* H37Rv culture filtrate proteins. *Scand J Immunol* **67**: 245-252.

Manabe YC, Kesavan AK, Lopez-Molina J, *et al.* (2008) The aerosol rabbit model of TB latency, reactivation and immune reconstitution inflammatory syndrome. *Tuberculosis (Edinb)* **88**: 187-196.

Mantovani A, Cassatella MA, Costantini C & Jaillon S (2011) Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* **11**: 519-531.

Marquis JF, LaCourse R, Ryan L, North RJ & Gros P (2009) Disseminated and rapidly fatal tuberculosis in mice bearing a defective allele at IFN regulatory factor 8. *J Immunol* **182**: 3008-3015.

Marsollier L, Stinear T, Aubry J, *et al.* (2004) Aquatic plants stimulate the growth of and biofilm formation by *Mycobacterium ulcerans* in axenic culture and harbor these bacteria in the environment. *Appl Environ Microbiol* **70**: 1097-1103.

Marsollier L, Brodin P, Jackson M, *et al.* (2007) Impact of *Mycobacterium ulcerans* biofilm on transmissibility to ecological niches and Buruli ulcer pathogenesis. *PLoS Pathog* **3**: e62.

May A, Fischer RJ, Maria Thum S, Schaffer S, Verseck S, Durre P & Bahl H (2013) A modified pathway for the production of acetone in *Escherichia coli*. *Metab Eng* **15**: 218-225.

Medina E & North RJ (1998) Resistance ranking of some common inbred mouse strains to *Mycobacterium tuberculosis* and relationship to major histocompatibility complex haplotype and Nramp1 genotype. *Immunology* **93**: 270-274.

Middlebrook G, Dubos RJ & Pierce C (1947) Virulence and morphological characteristics of mammalian tubercle bacilli. *J Exp Med* **86**: 175-184.

Miekisch W, Schubert JK, Vagts DA & Geiger K (2001) Analysis of volatile disease markers in blood. *Clin Chem* **47**: 1053-1060.

Mihret A (2012) The role of dendritic cells in *Mycobacterium tuberculosis* infection. *Virulence* **3**: 654-659.

Mishra AK, Driessen NN, Appelmelk BJ & Besra GS (2011) Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction. *FEMS Microbiol Rev* **35**: 1126-1157.

Miyakawa Y, Ratnakar P, Rao AG, Costello ML, Mathieu-Costello O, Lehrer RI & Catanzaro A (1996) In vitro activity of the antimicrobial peptides human and rabbit defensins and porcine leukocyte protegrin against *Mycobacterium tuberculosis*. *Infect Immun* **64**: 926-932.

Molloy A, Laochumroonvorapong P & Kaplan G (1994) Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. *J Exp Med* **180**: 1499-1509.

Montamat-Sicotte DJ, Millington KA, Willcox CR, *et al.* (2011) A mycolic acid-specific CD1-restricted T cell population contributes to acute and memory immune responses in human tuberculosis infection. *J Clin Invest* **121**: 2493-2503.

Moore T, Ananaba GA, Bolier J, Bowers S, Belay T, Eko FO & Igietseme JU (2002) Fc receptor regulation of protective immunity against *Chlamydia trachomatis*. *Immunology* **105**: 213-221.

Mukherjee R & Chatterji D (2008) Proteomics and mass spectrometric studies reveal planktonic growth of *Mycobacterium smegmatis* in biofilm cultures in the absence of rpoZ. *J Chromatogr B Analyt Technol Biomed Life Sci* **861**: 196-202.

Musa SA, Kim Y, Hashim R, Wang GZ, Dimmer C & Smith DW (1987) Response of inbred mice to aerosol challenge with *Mycobacterium tuberculosis*. *Infect Immun* **55**: 1862-1866.

Nagai S, Wiker HG, Harboe M & Kinomoto M (1991) Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*. *Infect Immun* **59**: 372-382.

Naito M, Izumi S & Yamada T (1998) Two-dimensional electrophoretic analysis of humoral responses to culture filtrate of *Mycobacterium bovis* BCG in patients with leprosy and tuberculosis. *Int J Lepr Other Mycobact Dis* **66**: 208-213.

Nakagawa I, Amano A, Mizushima N, *et al.* (2004) Autophagy defends cells against invading group A Streptococcus. *Science* **306**: 1037-1040.

Nedeltchev GG, Raghunand TR, Jassal MS, Lun S, Cheng QJ & Bishai WR (2009) Extrapulmonary dissemination of *Mycobacterium bovis* but not Mycobacterium tuberculosis in a bronchoscopic rabbit model of cavitary tuberculosis. *Infect Immun* **77**: 598-603.

Nessar R, Reyrat JM, Davidson LB & Byrd TF (2011) Deletion of the mmpL4b gene in the *Mycobacterium abscessus* glycopeptidolipid biosynthetic pathway results in loss of surface colonization capability, but enhanced ability to replicate in human macrophages and stimulate their innate immune response. *Microbiology*.

Neufert C, Pai RK, Noss EH, Berger M, Boom WH & Harding CV (2001) *Mycobacterium tuberculosis* 19-kDa lipoprotein promotes neutrophil activation. *J Immunol* **167**: 1542-1549.

Nguyen KT, Piastro K, Gray TA & Derbyshire KM (2010) Mycobacterial biofilms facilitate horizontal DNA transfer between strains of *Mycobacterium smegmatis*. *J Bacteriol* **192**: 5134-5142.

Nicol MP & Grobler LA (2010) MVA-85A, a novel candidate booster vaccine for the prevention of tuberculosis in children and adults. *Curr Opin Mol Ther* **12**: 124-134.

Nigou J, Zelle-Rieser C, Gilleron M, Thurnher M & Puzo G (2001) Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J Immunol* **166**: 7477-7485.

Nishiuchi Y, Tamura A, Kitada S, *et al.* (2009) *Mycobacterium avium* complex organisms predominantly colonize in the bathtub inlets of patients' bathrooms. *Jpn J Infect Dis* **62**: 182-186.

Nobelprize.org (2013) Robert Koch and Tuberculosis. Vol. 2014

Noll H, Bloch H, Asselineau J & Lederer E (1956) The chemical structure of the cord factor of *Mycobacterium tuberculosis*. *Biochim Biophys Acta* **20**: 299-309.

O'Kane CM, Elkington PT & Friedland JS (2008) Monocyte-dependent oncostatin M and TNF-alpha synergize to stimulate unopposed matrix metalloproteinase-1/3 secretion from human lung fibroblasts in tuberculosis. *Eur J Immunol* **38**: 1321-1330.

Oddo M, Renno T, Attinger A, Bakker T, MacDonald HR & Meylan PR (1998) Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. *J Immunol* **160**: 5448-5454.

Ojha A, Anand M, Bhatt A, Kremer L, Jacobs WR, Jr. & Hatfull GF (2005) GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. *Cell* **123**: 861-873.

Ojha AK, Trivelli X, Guerardel Y, Kremer L & Hatfull GF (2010) Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. *J Biol Chem* **285**: 17380-17389.

Ojha AK, Baughn AD, Sambandan D, *et al.* (2008) Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol Microbiol* **69**: 164-174.

Orme IM (1987) The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis*. *J Immunol* **138**: 293-298.

Orme IM (2005) The use of animal models to guide rational vaccine design. *Microbes Infect* **7**: 905-910.

Ortiz-Perez A, Martin-de-Hijas N, Alonso-Rodriguez N, Molina-Manso D, Fernandez-Roblas R & Esteban J (2011) Importance of antibiotic penetration in the antimicrobial resistance of biofilm formed by non-pigmented rapidly growing mycobacteria against amikacin, ciprofloxacin and clarithromycin. *Enferm Infecc Microbiol Clin* **29**: 79-84.

Osler W (1892) *The Principles and Practices of Medicine*. New York.

Palanisamy GS, Smith EE, Shanley CA, Ordway DJ, Orme IM & Basaraba RJ (2008) Disseminated disease severity as a measure of virulence of *Mycobacterium tuberculosis* in the guinea pig model. *Tuberculosis (Edinb)* **88**: 295-306.

Pan H, Yan BS, Rojas M, *et al.* (2005) Ipr1 gene mediates innate immunity to tuberculosis. *Nature* **434**: 767-772.

Pang JM, Layre E, Sweet L, Sherrid A, Moody DB, Ojha A & Sherman DR (2012) The polyketide Pks1 contributes to biofilm formation in *Mycobacterium tuberculosis*. *J Bacteriol* **194**: 715-721.

Parra M, Pickett T, Delogu G, Dheenadhayalan V, Debrie AS, Locht C & Brennan MJ (2004) The mycobacterial heparin-binding hemagglutinin is a protective antigen in the mouse aerosol challenge model of tuberculosis. *Infect Immun* **72**: 6799-6805.

Parsek MR & Singh PK (2003) Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol* **57**: 677-701.

Pathan AA, Wilkinson KA, Wilkinson RJ, *et al.* (2000) High frequencies of circulating IFN-gamma-secreting CD8 cytotoxic T cells specific for a novel MHC class I-restricted *Mycobacterium tuberculosis* epitope in *M. tuberculosis*-infected subjects without disease. *Eur J Immunol* **30**: 2713-2721.

Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, Locht C & Menozzi FD (2001) The heparin-binding haemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. *Nature* **412**: 190-194.

Pheiffer C, Betts JC, Flynn HR, Lukey PT & van Helden P (2005) Protein expression by a Beijing strain differs from that of another clinical isolate and *Mycobacterium tuberculosis* H37Rv. *Microbiology* **151**: 1139-1150.

Pichugin AV, Yan BS, Sloutsky A, Kobzik L & Kramnik I (2009) Dominant role of the sst1 locus in pathogenesis of necrotizing lung granulomas during chronic tuberculosis infection and reactivation in genetically resistant hosts. *Am J Pathol* **174**: 2190-2201.

Pidot SJ, Porter JL, Tobias NJ, *et al.* (2010) Regulation of the 18 kDa heat shock protein in *Mycobacterium ulcerans*: an alpha-crystallin orthologue that promotes biofilm formation. *Mol Microbiol* **78**: 1216-1231.

Primm TP, Lucero CA & Falkinham JO, 3rd (2004) Health impacts of environmental mycobacteria. *Clin Microbiol Rev* **17**: 98-106.

Principi N & Esposito S (2012) Diagnosis and therapy of tuberculous meningitis in children. *Tuberculosis (Edinb)* **92**: 377-383.

Py BF, Lipinski MM & Yuan J (2007) Autophagy limits *Listeria monocytogenes* intracellular growth in the early phase of primary infection. *Autophagy* **3**: 117-125.

Ramsugit S, Guma S, Pillay B, Jain P, Larsen MH, Danaviah S & Pillay M (2013) Pili contribute to biofilm formation in vitro in *Mycobacterium tuberculosis*. *Antonie Van Leeuwenhoek* **104**: 725-735.

Rashid MH, Rumbaugh K, Passador L, Davies DG, Hamood AN, Iglewski BH & Kornberg A (2000) Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **97**: 9636-9641.

Rawlings DE (1999) Proteic toxin-antitoxin, bacterial plasmid addiction systems and their evolution with special reference to the pas system of pTF-FC2. *FEMS Microbiol Lett* **176**: 269-277.

Recht J, Martinez A, Torello S & Kolter R (2000) Genetic analysis of sliding motility in *Mycobacterium smegmatis*. *J Bacteriol* **182**: 4348-4351.

Reggiardo Z & Middlebrook G (1974) Failure of passive serum transfer of immunity against aerogenic tuberculosis in rabbits. *Proc Soc Exp Biol Med* **145**: 173-175.

Ren H, Dover LG, Islam ST, Alexander DC, Chen JM, Besra GS & Liu J (2007) Identification of the lipooligosaccharide biosynthetic gene cluster from *Mycobacterium marinum*. *Mol Microbiol* **63**: 1345-1359.

Renshaw PS, Lightbody KL, Veverka V, *et al.* (2005) Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. *EMBO J* **24**: 2491-2498.

Repique CJ, Li A, Collins FM & Morris SL (2002) DNA immunization in a mouse model of latent tuberculosis: effect of DNA vaccination on reactivation of disease and on reinfection with a secondary challenge. *Infect Immun* **70**: 3318-3323.

Retzinger GS, Meredith SC, Takayama K, Hunter RL & Kezdy FJ (1981) The role of surface in the biological activities of trehalose 6,6'-dimycolate. Surface properties and development of a model system. *J Biol Chem* **256**: 8208-8216.

Retzinger GS, Meredith SC, Hunter RL, Takayama K & Kezdy FJ (1982) Identification of the physiologically active state of the mycobacterial glycolipid trehalose 6,6'-dimycolate and the role of fibrinogen in the biologic activities of trehalose 6,6'-dimycolate monolayers. *J Immunol* **129**: 735-744.

Rhoades ER, Archambault AS, Greendyke R, Hsu FF, Streeter C & Byrd TF (2009) *Mycobacterium abscessus* glycopeptidolipids mask underlying cell wall phosphatidyl-myo-inositol mannosides blocking induction of human macrophage TNF-alpha by preventing interaction with TLR2. *J Immunol* **183**: 1997-2007.

Ribeiro-Guimaraes ML & Pessolani MC (2007) Comparative genomics of mycobacterial proteases. *Microb Pathog* **43**: 173-178.

Richmond JM, Duffy ER, Lee J, *et al.* (2012) Mannose-capped Lipoarabinomannan from *Mycobacterium tuberculosis* induces soluble tumor necrosis factor receptor production through tumor necrosis factor alpha-converting enzyme activation. *Infect Immun* **80**: 3858-3868.

Rindi L, Bianchi L, Tortoli E, Lari N, Bonanni D & Garzelli C (2005) Mutations responsible for *Mycobacterium tuberculosis* isoniazid resistance in Italy. *Int J Tuberc Lung Dis* **9**: 94-97.

Rook GA, Dheda K & Zumla A (2005) Immune responses to tuberculosis in developing countries: implications for new vaccines. *Nat Rev Immunol* **5**: 661-667.

Roumestand C, Leiba J, Galophe N, *et al.* (2011) Structural insight into the *Mycobacterium tuberculosis* Rv0020c protein and its interaction with the PknB kinase. *Structure* **19**: 1525-1534.

Rousseau C, Winter N, Pivert E, *et al.* (2004) Production of phthiocerol dimycocerosates protects *Mycobacterium tuberculosis* from the cidal activity of reactive nitrogen intermediates produced by macrophages and modulates the early immune response to infection. *Cell Microbiol* **6**: 277-287.

Sambandan D, Dao DN, Weinrick BC, *et al.* (2013) Keto-mycolic acid-dependent pellicle formation confers tolerance to drug-sensitive *Mycobacterium tuberculosis*. *MBio* **4**.

Sambandan D, Dao DN, Weinrick BC, *et al.* (2013) Keto-mycolic acid-dependent pellicle formation confers tolerance to drug-sensitive *Mycobacterium tuberculosis*. *MBio* **4**: e00222-00213.

Samten B, Wang X & Barnes PF (2009) *Mycobacterium tuberculosis* ESX-1 system-secreted protein ESAT-6 but not CFP10 inhibits human T-cell immune responses. *Tuberculosis (Edinb)* **89 Suppl 1**: S74-76.

Schlesinger LS, Bellinger-Kawahara CG, Payne NR & Horwitz MA (1990) Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J Immunol* **144**: 2771-2780.

Schrama D, Terheyden P, Otto K, *et al.* (2006) Expression of the NKG2D ligand UL16 binding protein-1 (ULBP-1) on dendritic cells. *Eur J Immunol* **36**: 65-72.

Schuster CF & Bertram R (2013) Toxin-antitoxin systems are ubiquitous and versatile modulators of prokaryotic cell fate. *FEMS Microbiol Lett* **340**: 73-85.

Scott HM & Flynn JL (2002) *Mycobacterium tuberculosis* in chemokine receptor 2-deficient mice: influence of dose on disease progression. *Infect Immun* **70**: 5946-5954.

Shaban K, Amoudy HA & Mustafa AS (2013) Cellular immune responses to recombinant *Mycobacterium bovis* BCG constructs expressing major antigens of region of difference 1 of *Mycobacterium tuberculosis*. *Clin Vaccine Immunol* **20**: 1230-1237.

Shams H, Klucar P, Weis SE, *et al.* (2004) Characterization of a *Mycobacterium tuberculosis* peptide that is recognized by human CD4+ and CD8+ T cells in the context of multiple HLA alleles. *J Immunol* **173**: 1966-1977.

Sharma P, Kumar B, Singhal N, Katoch VM, Venkatesan K, Chauhan DS & Bisht D (2010) Streptomycin induced protein expression analysis in *Mycobacterium tuberculosis* by two-dimensional gel electrophoresis & mass spectrometry. *Indian J Med Res* **132**: 400-408.

Sharma S, Verma I & Khuller GK (2000) Antibacterial activity of human neutrophil peptide-1 against *Mycobacterium tuberculosis* H37Rv: in vitro and ex vivo study. *Eur Respir J* **16**: 112-117.

Sharma S, Verma I & Khuller GK (2001) Therapeutic potential of human neutrophil peptide 1 against experimental tuberculosis. *Antimicrob Agents Chemother* **45**: 639-640.

Shi T, Fu T & Xie J (2011) Polyphosphate deficiency affects the sliding motility and biofilm formation of *Mycobacterium smegmatis*. *Curr Microbiol*.

Shi X, Rao NN & Kornberg A (2004) Inorganic polyphosphate in *Bacillus cereus*: motility, biofilm formation, and sporulation. *Proc Natl Acad Sci U S A* **101**: 17061-17065.

Siddiqui KF, Amir M & Agrewala JN (2011) Understanding the biology of 16 kDa antigen of *Mycobacterium tuberculosis*: scope in diagnosis, vaccine design and therapy. *Crit Rev Microbiol* **37**: 349-357.

Singh SB, Davis AS, Taylor GA & Deretic V (2006) Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* **313**: 1438-1441.

Sissons J, Yan BS, Pichugin AV, Kirby A, Daly MJ & Kramnik I (2009) Multigenic control of tuberculosis resistance: analysis of a QTL on mouse chromosome 7 and its synergism with sst1. *Genes Immun* **10**: 37-46.

Skerry C, Pokkali S, Pinn M, Be NA, Harper J, Karakousis PC & Jain SK (2013) Vaccination with recombinant *Mycobacterium tuberculosis* PknD attenuates bacterial dissemination to the brain in guinea pigs. *PLoS One* **8**: e66310.

Smith I (2003) *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin Microbiol Rev* **16**: 463-496.

Sohn MJ, Zheng CJ & Kim WG (2008) Macrolactin S, a new antibacterial agent with FabG-inhibitory activity from *Bacillus* sp. AT28. *J Antibiot (Tokyo)* **61**: 687-691.

Sreevatsan S, Pan X, Stockbauer KE, Connell ND, Kreiswirth BN, Whittam TS & Musser JM (1997) Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc Natl Acad Sci U S A* **94**: 9869-9874.

Stanley SA, Johndrow JE, Manzanillo P & Cox JS (2007) The Type I IFN response to infection with *Mycobacterium tuberculosis* requires ESX-1-mediated secretion and contributes to pathogenesis. *J Immunol* **178**: 3143-3152.

Steed KA & Falkinham JO, 3rd (2006) Effect of growth in biofilms on chlorine susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare*. *Appl Environ Microbiol* **72**: 4007-4011.

Stenger S, Niazi KR & Modlin RL (1998) Down-regulation of CD1 on antigen-presenting cells by infection with *Mycobacterium tuberculosis*. *J Immunol* **161**: 3582-3588.

Stinear TP, Mve-Obiang A, Small PL*, et al.* (2004) Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. *Proc Natl Acad Sci U S A* **101**: 1345-1349.

Stinear TP, Seemann T, Pidot S*, et al.* (2007) Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. *Genome Res* **17**: 192-200.

Subbian S, Tsenova L, Yang G, *et al.* (2011) Chronic pulmonary cavitary tuberculosis in rabbits: a failed host immune response. *Open Biol* **1**: 110016.

Subbian S, Tsenova L, O'Brien P, *et al.* (2011) Phosphodiesterase-4 inhibition combined with isoniazid treatment of rabbits with pulmonary tuberculosis reduces macrophage activation and lung pathology. *Am J Pathol* **179**: 289-301.

Sugawara I, Udagawa T & Yamada H (2004) Rat neutrophils prevent the development of tuberculosis. *Infect Immun* **72**: 1804-1806.

Sugawara I, Yamada H, Udagawa T & Huygen K (2003) Vaccination of guinea pigs with DNA encoding Ag85A by gene gun bombardment. *Tuberculosis (Edinb)* **83**: 331-337.

Sundaramurthi JC, Brindha S, Shobitha SR, Swathi A, Ramanandan P & Hanna LE (2012) In silico identification of potential antigenic proteins and promiscuous CTL epitopes in *Mycobacterium tuberculosis*. *Infect Genet Evol* **12**: 1312-1318.

Sureka K, Sanyal S, Basu J & Kundu M (2009) Polyphosphate kinase 2: a modulator of nucleoside diphosphate kinase activity in mycobacteria. *Mol Microbiol* **74**: 1187-1197.

Sureka K, Dey S, Datta P, *et al.* (2007) Polyphosphate kinase is involved in stress-induced mprAB-sigE-rel signalling in mycobacteria. *Mol Microbiol* **65**: 261-276.

Suzuki T, Fujita H & Choi JG (2008) Brief communication: new evidence of tuberculosis from prehistoric Korea-Population movement and early evidence of tuberculosis in far East Asia. *Am J Phys Anthropol* **136**: 357-360.

Tameris M, Geldenhuys H, Luabeya AK, *et al.* (2014) The candidate TB vaccine, MVA85A, induces highly durable Th1 responses. *PLoS One* **9**: e87340.

Tameris MD, Hatherill M, Landry BS, *et al.* (2013) Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* **381**: 1021-1028.

Tang ST, van Meijgaarden KE, Caccamo N, *et al.* (2011) Genome-based in silico identification of new *Mycobacterium tuberculosis* antigens activating polyfunctional CD8+ T cells in human tuberculosis. *J Immunol* **186**: 1068-1080.

Tanghe A, Denis O, Lambrecht B, Motte V, van den Berg T & Huygen K (2000) Tuberculosis DNA vaccine encoding Ag85A is immunogenic and protective when administered by intramuscular needle injection but not by epidermal gene gun bombardment. *Infect Immun* **68**: 3854-3860.

Tanghe A, D'Souza S, Rosseels V, *et al.* (2001) Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. *Infect Immun* **69**: 3041-3047.

Tanne A & Neyrolles O (2010) C-type lectins in immune defense against pathogens: the murine DC-SIGN homologue SIGNR3 confers early protection against *Mycobacterium tuberculosis* infection. *Virulence* **1**: 285-290.

Tanner M & Michel AL (1999) Investigation of the viability of *M. bovis* under different environmental conditions in the Kruger National Park. *Onderstepoort J Vet Res* **66**: 185-190.

Teitelbaum R, Glatman-Freedman A, Chen B, Robbins JB, Unanue E, Casadevall A & Bloom BR (1998) A mAb recognizing a surface antigen of *Mycobacterium tuberculosis* enhances host survival. *Proc Natl Acad Sci U S A* **95**: 15688-15693.

Teles RM, Graeber TG, Krutzik SR, *et al.* (2013) Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. *Science* **339**: 1448-1453.

Torvinen E, Lehtola MJ, Martikainen PJ & Miettinen IT (2007) Survival of *Mycobacterium avium* in drinking water biofilms as affected by water flow velocity, availability of phosphorus, and temperature. *Appl Environ Microbiol* **73**: 6201-6207.

Tuli SM (2007) Tuberculosis of the spine: a historical review. *Clin Orthop Relat Res* **460**: 29-38.

Tullius MV, Harth G & Horwitz MA (2001) High extracellular levels of *Mycobacterium tuberculosis* glutamine synthetase and superoxide dismutase in actively growing cultures are due to high expression and extracellular stability rather than to a protein-specific export mechanism. *Infect Immun* **69**: 6348-6363.

Tunpiboonsak S, Mongkolrob R, Kitudomsub K, Thanwatanaying P, Kiettipirodom W, Tungboontina Y & Tungpradabkul S (2010) Role of a *Burkholderia pseudomallei* polyphosphate kinase in an oxidative stress response, motilities, and biofilm formation. *J Microbiol* **48**: 63-70.

Turner OC, Basaraba RJ & Orme IM (2003) Immunopathogenesis of pulmonary granulomas in the guinea pig after infection with *Mycobacterium tuberculosis*. *Infect Immun* **71**: 864-871.

Uchiya K, Takahashi H, Yagi T, *et al.* (2013) Comparative genome analysis of *Mycobacterium avium* revealed genetic diversity in strains that cause pulmonary and disseminated disease. *PLoS One* **8**: e71831.

Vaerewijck MJ, Huys G, Palomino JC, Swings J & Portaels F (2005) Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol Rev* **29**: 911-934.

van der Werf TS & Stienstra Y (2011) Buruli ulcer (*Mycobacterium ulcerans* infection).

Van Rhijn I, Godfroid J, Michel A & Rutten V (2008) Bovine tuberculosis as a model for human tuberculosis: advantages over small animal models. *Microbes Infect* **10**: 711-715.

Verreck FA, Vervenne RA, Kondova I, *et al.* (2009) MVA.85A boosting of BCG and an attenuated, phoP deficient M. tuberculosis vaccine both show protective efficacy against tuberculosis in rhesus macaques. *PLoS One* **4**: e5264.

Via LE, Schimel D, Weiner DM, *et al.* (2012) Infection dynamics and response to chemotherapy in a rabbit model of tuberculosis using [(1)(8)F]2-fluoro-deoxy-D-glucose positron emission tomography and computed tomography. *Antimicrob Agents Chemother* **56**: 4391-4402.

Vishnoi A, Roy R, Prasad HK, Bhattacharya A (2010) Anchor-based whole genome phylogeny (ABWGP): a tool for inferring evolutionary relationships among closely related microorganisms. PLoS One **5**(11):e14159.

Vordermeier HM, Venkataprasad N, Harris DP & Ivanyi J (1996) Increase of tuberculous infection in the organs of B cell-deficient mice. *Clin Exp Immunol* **106**: 312-316.

Walsh DS, Portaels F & Meyers WM (2010) Recent advances in leprosy and Buruli ulcer (*Mycobacterium ulcerans* infection). *Curr Opin Infect Dis* **23**: 445-455.

Walsh GP, Tan EV, dela Cruz EC, *et al.* (1996) The Philippine cynomolgus monkey (Macaca fasicularis) provides a new nonhuman primate model of tuberculosis that resembles human disease. *Nat Med* **2**: 430-436.

Wang J, Zhang H & Wang H (2010) Analysis of predicted CD8+ T cell epitopes from proteins encoded by the specific RD regions of *Mycobacterium tuberculosis* for vaccine development and specific diagnosis. *Mol Biol Rep* **37**: 1793-1799.

Wang X, Lord DM, Cheng HY, *et al.* (2012) A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nat Chem Biol* **8**: 855-861.

Weichart D, Querfurth N, Dreger M & Hengge-Aronis R (2003) Global role for ClpP-containing proteases in stationary-phase adaptation of *Escherichia coli*. *J Bacteriol* **185**: 115-125.

White AD, Sibley L, Dennis MJ, *et al.* (2013) Evaluation of the safety and immunogenicity of a candidate tuberculosis vaccine, MVA85A, delivered by aerosol to the lungs of macaques. *Clin Vaccine Immunol* **20**: 663-672.

WHO (2012) Global tuberculosis report 2012. World Heath Organization.

Williams A, Goonetilleke NP, McShane H, Clark SO, Hatch G, Gilbert SC & Hill AV (2005) Boosting with poxviruses enhances *Mycobacterium bovis* BCG efficacy against tuberculosis in guinea pigs. *Infect Immun* **73**: 3814-3816.

Williams A, Reljic R, Naylor I, *et al.* (2004) Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs. *Immunology* **111**: 328-333.

Woodworth JS & Behar SM (2006) *Mycobacterium tuberculosis*-specific CD8+ T cells and their role in immunity. *Crit Rev Immunol* **26**: 317-352.

Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE & Eissa NT (2007) Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* **27**: 135-144.

Yamada H, Mizuno S & Sugawara I (2002) Interferon regulatory factor 1 in mycobacterial infection. *Microbiol Immunol* **46**: 751-760.

Yang E, Lu Y, Xu Y, Liang Q, Wang C, Wang H & Shen H (2014) Recombinant BCG coexpressing Ag85B, ESAT-6 and Rv3620c elicits specific Th1 immune responses in C57BL/6 mice. *Microb Pathog* **69-70**: 53-59.

Yang Y, Kukla K, Montelaro R, Reinhart T, Sissons J, Aderem A, OjhA A (2014) A hydrolase of trehalose dimycolate induces nutrient influx and stress sensitivity to balance intracellular growth of *Mycobacterium tuberculosis*. *Cell Host and Microbe* **15**(2): 153–163

Yin Q, Tian Y, Kabaleeswaran V, *et al.* (2012) Cyclic di-GMP sensing via the innate immune signaling protein STING. *Mol Cell* **46**: 735-745.

Yip MJ, Porter JL, Fyfe JA, *et al.* (2007) Evolution of *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria from a common *Mycobacterium marinum* progenitor. *J Bacteriol* **189**: 2021-2029.

Yuan Y, Crane DD & Barry CE, 3rd (1996) Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. *J Bacteriol* **178**: 4484-4492.

Yuan Y, Crane DD, Simpson RM, Zhu YQ, Hickey MJ, Sherman DR & Barry CE, 3rd (1998) The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. *Proc Natl Acad Sci U S A* **95**: 9578-9583.

Zhang L, Wang Q, Wang W, *et al.* (2012) Identification of putative biomarkers for the serodiagnosis of drug-resistant *Mycobacterium tuberculosis*. *Proteome Sci* **10**: 12.

Zheng C & Fanta CH (2013) Non-tuberculous mycobacterial pulmonary infection in the immunocompetent host. *QJM* **106**: 307-315.

Zhou A, Ni J, Xu Z, Wang Y, Lu S, Sha W, Karakousis PC, Yao Y (2013) Application of the 1H NMR spectroscopy-based metabolomics to sera of tuberculosis patients. *J Proteome Res* **12**: 10. 4642-4649

Zhu X, Stauss HJ, Ivanyi J & Vordermeier HM (1997) Specificity of CD8+ T cells from subunit-vaccinated and infected H-2b mice recognizing the 38 kDa antigen of *Mycobacterium tuberculosis*. *Int Immunol* **9**: 1669-1676.

Zitrin CM & Wasz-Hockert O (1957) Preliminary experiments on passive transfer of protective humoral antibodies in tuberculosis. *Am Rev Tuberc* **76**: 256-262.

Zvi A, Ariel N, Fulkerson J, Sadoff JC & Shafferman A (2008) Whole genome identification of *Mycobacterium tuberculosis* vaccine candidates by comprehensive data mining and bioinformatic analyses. *BMC Med Genomics* **1**: 18.