Natural Polymorphism of Mycobacterium tuberculosis and CD8 T Cell Immunity

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NATURAL POLYMORPHISM OF *MYCOBACTERIUM TUBERCULOSIS* AND CD8 T CELL IMMUNITY

A Dissertation Presented
By

RUJAPAK SUTIWISESAK

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

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February 24th, 2020

IMMUNOLOGY AND MICROBIOLOGY PROGRAM
NATURAL POLYMORPHISM OF *Mycobacterium Tuberculosis* AND CD8 T CELL IMMUNITY

A Dissertation Presented

By

RUJAPAK SUTIWISESAK

This work was undertaken in the Graduate School of Biomedical Sciences Program in Immunology and Microbiology

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February 24th, 2020
To my grandparents:

常德 เศรษฐมนูกุล
วิลาวันธ์ สุทธิวิเศษศักดิ์
ชุณห์ สุทธิวิเศษศักดิ์
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Abstract

Coevolution between *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis, and the human host has been documented for thousands of years. Interestingly, while T cell immunity is crucial for host protection and survival, T cell antigens are the most conserved region of the Mtb genome. Hypothetically, Mtb adapts under immune pressure to exploit T cell responses for its benefit from inflammation and tissue destruction for ultimately transmission.

*EsxH*, a gene encoding immunodominant TB10.4 protein, however, contains polymorphic regions corresponding to T cell epitopes. Here, I present two complementary analyses to examine how Mtb modulates TB10.4 for immune evasion. First, I use a naturally occurring *esxH* polymorphic clinical Mtb isolate, 667, to investigate how A10T amino acid exchange in TB10.4 affect T cell immunity. To verify and identify the cause of the immunological differences, I construct isogenic strains expressing EsxH\(^{A10T}\) or EsxH\(^{WT}\). In combination with our recent finding that TB10.4\(_{4,11}\)-specific CD8 T cells do not recognize Mtb-infected macrophages, we hypothesize that TB10.4 is a decoy antigen as it distracts host immunity from inducing other potentially protective responses. I examine whether an elimination of TB10.4\(_{4,11}\)-specific CD8 T cell response leads to a better host protective immunity. The studies of *in vivo* infection and *in vitro* recognition in this dissertation aim to provide a better understanding of the counteraction between immune evasion and protective immunity.
# Table of Contents

Acknowledgements iii

Abstract vi

Table of Contents vii

List of Tables xi

List of Figures xii

List of Abbreviations xiv

CHAPTER I: INTRODUCTION 1

Tuberculosis and Pathogenesis 1
  A. Significance: Global Disease Burden 1
  B. *Mycobacterium tuberculosis* Infection and Pathogenesis 4

Immunity to Tuberculosis 6
  C. Immune Responses to Tuberculosis 6
  D. The underappreciated role of CD8 T cells in TB Immunity 10
  E. Current Vaccine Development and T cell Recognition 12

*Mycobacterium tuberculosis* Evolution and Evasion Mechanisms 14
  F. Evolutions and Transmission of Mtb Clinical Isolates 14
  G. Immune Evasion Mechanisms 16
  H. Evasion of CD8 T cell immunity 19

*esxH* gene, TB10.4 protein, and TB10.4-specific T cells 21

Thesis objectives 25

CHAPTER II. INVESTIGATING RECOGNITION CAPACITIES OF
*Mycobacterium tuberculosis*-SPECIFIC CD8 T CELLS 27

Abstract 28

Introduction 29

Results 32
  Generation of H2-Kb-restricted TB10.44,11-specific TB10RgL, TB10RgR,
  TB10RgP, TB10Rg3 CD8 T cell lines 32
The H2-K\(^b\)-restricted TB10.4\(_{4-11}\)-specific TB10RgL, TB10RgR, TB10RgP and TB10Rg3 CD8 T cells do not recognize infected macrophages nor inhibit intracellular Mtb growth

Generation of TB10.4\(_{20-28}\)-specific CD8 T cells by Trivax-TB10.4\(_{20-28}\) vaccination

The H2-K\(^d\)-restricted TB10.4\(_{20-28}\)-specific CD8 T cells do not recognize infected macrophages

A subdominant H-2K\(^b\)-restricted MTB32a\(_{309-318}\)-specific CD8 T cell does not recognize infected macrophages

Discussion

CHAPTER III. THE EFFECT OF MYCOBACTERIUM TUBERCULOSIS ANTIGENIC POLYMORPHISMS ON HOST T CELL IMMUNE RESPONSES

Abstract

Introduction

Results

*Mycobacterium tuberculosis* clinical isolates reveal highly polymorphic clusters within the known T cell epitopes of the *esxH* gene

Clinical isolate 667 alters hierarchy of immunodominant antigen-specific CD8 T cell responses.

The effect of variations in the TB10.4\(_{4-11}\) epitope on T cell recognition

H-2K\(^b\)/TB10.4\(_{4-11}\)\(^{A10T}\) tetramer verifies the loss of TB10.4\(_{4-11}\)-specific CD8 T cells

The H2-K\(^d\)-restricted TB10.4\(_{20-28}\)-specific CD8 T cell response is unchanged after 667 infection

No cryptic epitope emerged in the absence of immunodominant epitope

667 infection induces less severe disease outcomes

Discussion

CHAPTER IV. THE EFFECTS OF A10T AMINO ACID SUBSTITUTION OF *MYCOBACTERIUM TUBERCULOSIS* ESXH PROTEIN ON HOST CD8 T CELL RESPONSES

Abstract

Introduction

Results

Generation of Isogenic EsxH\(^{WT}\) and EsxH\(^{A10T}\) in Erdman Mtb

Isogenic Erd\(^{WT}\) and Erd\(^{A10T}\) Mtb stimulate different CD8 T cell immunity in C57BL/6
A10T substitution of TB10.4 protein does not affect virulence and bacterial burden.

Discussion

CHAPTER V. DIFFERENTIAL RECOGNITION OF T CELLS GENERATED BY POLYMORPHIC Mycobacterium Tuberculosis INFECTIONS

Abstract

Introduction

Results

Development of Mtb-Infected Macrophage ELISpot and ICS (MIM-E and MIM-ICS) to assess T cell recognition ability.
CD4 and CD8 T cells differ in their ability to recognize Mtb-infected macrophages.
Differential recognition of Erdman- and 667- elicited CD8 T cells.
ErdWT- and ErdA10T-elicited CD8 T cells inefficiently recognize macrophages with different Mtb strain infections.

Discussion

CHAPTER VI: DISCUSSION

CHAPTER VII. MATERIALS AND METHODS

Ethics Statement

Animal

Thioglycolate-elicited peritoneal macrophages (TGPM)

Generation of TCR retrogenic mice

Generation of CD8 and CD4 T cell lines

Peptides

Trivax vaccination and generation of antigen-specific CD8 T cells.

Mycobacterium tuberculosis stains

In vitro infection

Mtbi-Infected Macrophage ELISpot (MIM-E) and Intracellular Cytokine Staining (MIM-ICS)

RMA-S assay

Measurement of cell proliferation.
Mouse infections 154
Peptide library screening 155
Flow cytometry 155
Lung cell purification 156
Barcoded clinical isolate pool infection 156
Generation of isogenic Erd\textsuperscript{WT} and Erd\textsuperscript{A10T} by oligo recombineering 157
RT-PCR of mycobacterial mRNAs 158
Statistical analysis 159

APPENDIX: THE INVESTIGATION OF GRANULYSIN-MEDIATED CD8 T CELL CYTOTOXICITY DURING TUBERCULOSIS 160

Introduction 161
Results 163
  GNLY is expressed in human CD8 T cells 163
  GNLY is expressed in NK cells, but not CD8 T cells, of GNLY-Tg mice 164
  Generation of antigen-specific GNLY-Tg CD8 T cell line 165
  GNLY-Tg CD8 T cells can produce granulysin under limited stimulation conditions 168
  Granulysin-transgenic mice do not exhibit better control of \textit{Mycobacterium tuberculosis} infection 170
  Adoptive transfer of CD8 T cells from GNLY-Tg mice do not exhibit better control of Mtbe infection 173
Discussion 176

References 178
List of Tables

Table 3.1: Identification of esxH nonsynonymous polymorphisms among clinical isolates. 60
Table 3.2: IEDB processing analysis: H-2-Kb Processing prediction results 66
Table 3.3: Sequences of peptide used in this study. 68
List of Figures

Figure 2.1: Generation of H2-Kβ-restricted TB10.4\textsubscript{4-11}-specific TB10RgL, TB10RgR, TB10RgP, TB10Rg3 CD8 T cell lines 35

Figure 2.2: TB10.4\textsubscript{4-11}-specific CD8 T cells do not recognize Mtb-infected macrophages. 39

Figure 2.3: Generation of TB10.4\textsubscript{20-28}-specific CD8 T cells. 42

Figure 2.4: TB10.4\textsubscript{20-28}-specific CD8 T cells do not recognize Mtb-infected macrophages. 44

Figure 2.5: Generation and recognition of MTB32\textsubscript{a309-318}-specific CD8 T cells. 48

Figure 3.1: esxH polymorphisms in clinical isolates are highly clustered within the known T cell epitopes 59

Figure 3.2: 667 infection changes the CD8 T cell immunity hierarchy in C57BL/6. 63

Figure 3.3: Alteration of CD8 T cell immunodominant hierarchy persists during chronic infection in C57BL/6. 64

Figure 3.4: TB10.4\textsubscript{4-11} epitope variants bind and stimulate T cell recognition. 69

Figure 3.5: H-2Kβ/TB10.4\textsubscript{4-11}\textsuperscript{A10T} tetramer verifies the loss of TB10.4\textsubscript{4-11}-specific CD8 T cells 74

Figure 3.6: 667 infection elicited similar CD8 T cell immunity in BALB/c. 76

Figure 3.7: No cryptic epitope in the absence of immunodominant epitope. 79

Figure 3.8: Infection with 667 is less virulent than Erdman. 83

Figure 4.1: Generation of EsxH\textsuperscript{A10T} and EsxH\textsuperscript{WT} isogenic strains 96

Figure 4.2: Infections with isogenic Erd\textsuperscript{WT} and Erd\textsuperscript{A10T} Mtb validate the immunodominant shift of CD8 T cell hierarchy in C57BL/6 mice 99

Figure 4.3: A10T substitution of TB10.4 protein does not change the amount of antigen-specific CD4 and CD8 T cell numbers in BALB/c mice 100
Figure 4.4: Isogenic strain infection does not impact bacterial burden

Figure 4.5: *hsp60*-complemented strains are less virulent than clinical isolate 667 and Erdman Mtb

Figure 4.6: Diagnostic PCR screening of nine streptomycin candidates

Figure 5.1: Measuring T-cell recognition by the Mtb-infected macrophage ELISpot (MIM-E)

Figure 5.2: Similar frequencies of T cells recognizing Mtb-infected macrophages are detected by the MIM-E assay and MIM-ICS.

Figure 5.3: Differences in CD4 and CD8 T-cell recognition of Mtb-infected macrophages.

Figure 5.4: MIM-ICS assay of T cells elicited from Erdman and 667 infection reveals differential recognition capability.

Figure 5.5: Isogenic Erd<sup>WT</sup> and Erd<sup>A10T</sup> infections elicit CD8 T cell responses that differentially recognize infected macrophages early during infection.

Figure 6: Immunodomination is a decoy mechanism Mtb used to evade CD8 T cells

Figure A.1: Granulysin is hypothesized to enhance CD8 T cell cytotoxic functions and provide better protection against Mtb infection

Figure A.2: GNLY is expressed in human CD8 T cells

Figure A.3: GNLY is expressed in GNLY-Tg NK cells, but not CD8 T cells

Figure A.4: Generation of GNLY-Tg antigen-specific CD8 T cell lines

Figure A.5: GNLY-Tg CD8 T cells can produce granulysin under limited stimulation conditions

Figure A.6: GNLY-Tg mice do not exhibit better control of Mtb infection

Figure A.7: Adoptive transfer of CD8 T cells from GNLY-Tg mice do not exhibit better control of Mtb infection
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>β2m</td>
<td>β-2 microglobulin</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>CDR3</td>
<td>Complementarity-determining region 3</td>
</tr>
<tr>
<td>CFP10</td>
<td>10-kDa Culture Filtrate Protein</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II Major Histocompatibility Complex Transactuator</td>
</tr>
<tr>
<td>CPXV</td>
<td>Cowpox Virus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ELISpot</td>
<td>Enzyme-Linked Immunosorbent Spot</td>
</tr>
<tr>
<td>ESAT6</td>
<td>Early secreted antigenic target of 6-kDa protein</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal Sorting Complexes Required for Transport</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>IGRA</td>
<td>Interferon Gamma Release Assay</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KO</td>
<td>Knock Out</td>
</tr>
<tr>
<td>LTBI</td>
<td>Latent Tuberculosis Infection</td>
</tr>
<tr>
<td>LXA4</td>
<td>Lipoxin A4</td>
</tr>
<tr>
<td>MDLN</td>
<td>Mediastinal Lymph Node</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multi-Drug Resistant Tuberculosis</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIM-E</td>
<td>Mtb-Infected Macrophages ELISpot</td>
</tr>
<tr>
<td>MIM-ICS</td>
<td>Mtb-Infected Macrophages Intercellular Cytokine Staining</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>MPEC</td>
<td>Memory Precursor Effector Cell</td>
</tr>
<tr>
<td>Mtbc</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
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<tr>
<td>PPD</td>
<td>Purified Protein Derivative</td>
</tr>
<tr>
<td>RR-TB</td>
<td>Ripampicin-Resistant Tuberculosis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SLEC</td>
<td>short lived effector cell</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporters Associated with Antigen Processing</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGPM</td>
<td>Thioglycolate-elicited Peritoneal Macrophages</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>Tumor Necrosis Factor-(\alpha)</td>
</tr>
<tr>
<td>TST</td>
<td>Tuberculin Skin Test</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensive Drug Resistant Tuberculosis</td>
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</tbody>
</table>
CHAPTER I: INTRODUCTION

Tuberculosis and Pathogenesis

A. Significance: Global Disease Burden

Tuberculosis (TB) is a contagious disease caused by airborne Mycobacterium tuberculosis (Mtb) that transmits via cough. In spite of a 42% decrease in mortality rate between 2000 and 2018, TB still caused 1.5 million deaths in 2018—surpassing human immunodeficiency virus (HIV)—and ranked as the leading cause of death by an infectious agent and one of the top 10 causes of death worldwide (1). Mtb was estimated to infect over 10 million people in 2018 alone, bringing the current total infection number to 1.7 billion people (1). A small proportion (5–10%) of Mtb-infected individuals will develop active TB disease during their lifetime and are able to transmit the mycobacteria to an average of 10 (and up to 200) additional people per year. The probability of progression to active TB, however, increases among people coinfected with HIV, as HIV suppresses the immune system and hinders control of Mtb. Other risk factors include diabetes, malnutrition, smoking and alcohol consumption (2).

Given this immense disease burden, prophylactic and post-exposure treatments are the logical steps toward eradicating TB. Once diagnosed, the current recommended post-exposure treatment for drug-susceptible TB is a 6-month regimen of four first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide; this serves to clear the infection and prevent development of
antibiotic resistance (3-5). Although antibiotic treatments cure approximately 85% of TB patients, an estimated 484,000 people developed drug-resistance in 2018. Of those, there were 187,000 reported cases of either rifampicin-resistant TB (RR-TB) or multidrug-resistant TB (MDR-TB). An estimated 11,600 individuals were also predicted to have extensively drug-resistant TB (XDR-TB), defined by resistance to not only isoniazid and rifampin, but also any fluoroquinolone and injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycine) (6). Treatment for individuals with RR-TB and MDR-TB is longer, more toxic, and more expensive. Compared to an average of forty dollars per person for a 6-month regimen of first-line drugs, the cost to treat antibiotic resistance is over one thousand dollars (1). Combined with other healthcare costs, an average of $110,900 was spent to treat and manage one antibiotic-resistant tuberculosis patient, three times higher than that of an antibiotic-susceptible TB case (7). In addition, it is estimated that only 70% of new cases of active TB were actually reported in 2018, leaving 30% undiagnosed, untreated, and capable of transmitting Mtb or even drug-resistant Mtb (8). The extreme biological, social, and economic burden emphasizes the rising threat from TB and the need for researchers and funders to collaborate efforts in developing higher efficacy antibiotics and a more effective TB vaccine.

Historically, vaccines have been the most cost-effective intervention against infectious diseases. The development of an effective vaccine against TB, however, has been much more challenging. The only licensed prophylactic vaccine for TB
prevention is the Bacillus Calmette–Guérin (BCG, an attenuated strain of *Mycobacterium bovis*) vaccine. The BCG vaccine has been used widely since 1921 and is currently used to prevent severe forms of TB in children, tuberculosis-induced meningitis, and reduce the risk of childhood dissemination (9). However, vaccine performance remains disappointing and inconsistent in adults. Despite controversy, studies reported its inefficiency in high TB burden regions (10-12). In fact, BCG is not recommended for use in the US (13) with the exception of healthcare or humanitarian workers traveling to areas with MDR-TB (14). There is currently no approved prophylactic or post-exposure treatment that is effective in preventing pulmonary TB, the form of the disease that is of public health concern. Many vaccine studies in animal models exhibited encouraging early bacterial burden control but did not affect survival and failed to achieve sterilizing immunity (15-17). Interestingly, individuals successfully treated for active TB are not protected from Mtb reinfection and do not develop sterilizing immunity (18). This raises the question of why immunity elicited by either vaccination or natural infection fail to provide protection. Therefore, it is necessary to understand pathogenesis and what constitutes protective immunity against Mtb in order to ultimately develop a more effective TB vaccine.
B. *Mycobacterium tuberculosis* Infection and Pathogenesis

Dr. Robert Koch discovered an acid-fast, rod-shaped, slow-growing bacillus subsequently named *Mycobacterium tuberculosis* (Mtb) in 1882 (19, 20). The only ecological niche of Mtb is humans, and transmission is from people with active TB to other people via infectious aerosols (i.e., cough). Mtb primarily infects phagocytes, particularly resident alveolar macrophages in the lung, where it survives and replicates to establish a very successful niche within infected hosts (21, 22). Recruitment of other immune cells leads to the aggregation and formation of granulomas, a hallmark of TB. Diverse architectures of granulomas seen in humans (23) and experimental animal models (24, 25) contribute to differential hypotheses as to whether granulomas are beneficial to the Mtb bacterium by spatially restricting the access of protective cells or beneficial to the host by containing Mtb and restricting dissemination. Recent studies suggest that the local spread of actively dividing mycobacteria is facilitated during early granuloma formation (26, 27). Interestingly, individual granulomas within the same infected animal manifested independent fates with respect to bacterial growth (i.e., permissive vs. restrictive environments) (28).

Mtb typically affects the lungs (pulmonary TB) but can also disseminate to other organs (extrapulmonary TB) via the bloodstream and cause meningitis, enteritis, pyelonephritis, and occasionally infiltrates the bone marrow (29). Among individuals infected with Mtb, the majority have latent TB infections (LTBI) and do
not progress to active TB disease. LTBI patients have a 5-20% lifetime risk of reactivation, and the development of active pulmonary tuberculosis is greatest in the first 2 years after infection (30). Interestingly, some household contacts of active TB patients have never converted their Tuberculin Skin Test (TST: type IV hypersensitivity reaction to the tuberculin antigen, indicating prior exposure or infection) (31) or Interferon Gamma Release Assays (IGRA: IFNγ secretion response by Mtb-specific T cells as indication of Mtb infection) (32). This clinical population is termed “resisters” and could represent successful immune responses in preventing or clearing infection. However, additional studies—including more accurate diagnostic tests—are required to understand this interesting subset of individuals.

Overall, it appears that the host immune system is mostly able to contain Mtb infection successfully, with a small percentage of individuals developing disease, which may be a sign of incompetence of host immune responses, an immune evasion strategy, or Mtb-human coevolution. Gaining a better understanding of previous and current global TB prevention efforts (including 13 vaccine candidates and 23 combination drug regimens that are in clinical trials) could transform the future of TB prevalence. Next, I will discuss the immunological pathways and their roles during Mtb infection that influence the disease outcome.
Immunity to Tuberculosis

C. Immune Responses to Tuberculosis

Upon Mtb infection, innate immunity is the first line of defense, recruiting inflammatory cells to the lung before the initiation of adaptive immunity. The main players of innate immunity consist of resident alveolar macrophages, monocyte-derived macrophages, dendritic cells, and neutrophils. There is also growing knowledge of unconventional, innate-like T cells that interact with nonpolymorphic antigen-presenting molecules including CD1a, CD1b, or CD1c-restricted T cells (33), CD1d-restricted invariant natural killer T (iNKT) cells (34), and MR1-restricted mucosal-associated invariant T (MAIT) cells (35), all of which participate in the immune response to Mtb. The recognition of Mtb infection by macrophages results in the optimal production of pro- and anti-inflammatory cytokines to recruit, activate, and differentiate other innate and adaptive cell types. A disruption in the balance of pro- and anti-inflammatory cytokines can lead to detrimental effects on the host (36). Although macrophages can kill Mtb by reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) and efferocytosis (37, 38), they cannot effectively clear the infection. Instead, the initiation of granuloma formation by innate immunity (39) and subsequent recruitment of adaptive immunity players are necessary to control the infection.

The recruited adaptive immune response consists of CD4 and CD8 T cells. CD4 and CD8 T cells are both required for protective immunity against Mtb in the mouse model (40). Importantly, the kinetics of Mtb-specific T cell responses are
delayed in comparison to other viral or bacterial infections. In contrast to detecting the T cell responses within 3-5 days of a viral infection, adaptive immune responses to Mtb infection do not appear at the site of infection until 11 days post-infection in mice (41) and 42 days post-exposure in humans (42). This delay is due in part to the need for live Mtb bacteria to transport from the lungs—the site of infection—through infected dendritic cells (DCs) to mediastinal lymph nodes (MDLN: lung-draining LN) where bacterial antigens are generated (41, 43, 44). CD4 T cells are subsequently primed and activated in MDLN before migrating to the lungs. Interestingly, the migratory Mtb-infected DCs are inefficient at activating CD4 T cells, and the uninfected DCs are the cells that uptake the released proteins to process and activate CD4 T cells (45). Studies in mice with different genetic backgrounds and susceptibilities have helped to elucidate that earlier T cell activation in the MDLN is associated with superior control of Mtb in the lungs (41).

CD4 T cells are required for control of tuberculosis in mice (40), cattle (46), nonhuman primates (47), and humans, as shown in HIV patients who have a reduced number of CD4 T cells (48). In addition, interferon-gamma (IFNγ) production and secretion by CD4 T cells has been repeatedly shown to be critical in limiting bacterial growth and is therefore considered part of the central dogma of how Mtb infection is controlled (43, 49). For example, IFNγ can activate macrophages and their apoptosis to kill Mtb (50). Furthermore, patients with Mendelian susceptibility to mycobacterial diseases (MSMD) harboring mutations in the IFNGR1 or IFNGR2 genes, which encode IFNγ receptors, are more
susceptible to mycobacterial diseases (51). However, IFNγ production does not always correlate with protection against TB. Though causation has not been shown, increased numbers of IFNγ-secreting T cells are more correlated to TB disease burdens than to protection in mice (52). Higher numbers of IFNγ-secreting T cells in humans were also associated with disease progression to active TB (53). When CD4 T cells engineered to produce larger amounts of IFNγ were adoptively transferred, mice displayed worse outcomes (54). These contradictory results with IFNγ challenge the central dogma and suggest that there are other protective mechanisms that contribute to the protection mediated by CD4 T cells in addition to the essentiality of IFNγ.

In addition to IFNγ, tumor necrosis factor α (TNFα) is an essential multifunctional pro-inflammatory cytokine produced by myeloid cells, as well as by CD4 and CD8 T cells, which is essential for control of Mtb infection in animal models and in humans (55-57). LTBI patients have a higher risk of developing active TB when undergoing anti-TNFα treatment for autoimmune diseases. However, similar to IFNγ, active TB patients contain a higher frequency of CD4 T cells that produce TNFα than patients with LTBI (58), suggesting a positive correlation of TNFα with bacterial burden.

The majority of the T cells from diverse host genetic backgrounds are specific to a small subset of peptides among thousands of antigen-derived peptides. This selection results in immunodominant antigens. Several
immunodominant antigens for both class I and class II MHCs have been identified in the murine TB model, including Ag85a, Ag85b, CFP10, ESAT6 and TB10.4 (59). T cell responses to these antigens are also frequently detectable in Mtb-infected people and some are used in TB immunodiagnostic tests (60). Intriguingly, immunodominant antigen-specific CD4 and CD8 T cells do not necessarily represent an effective immune response. In rhesus macaques, induction of IFNγ-producing T cells by an adenovirus vaccine expressing Ag85A along with the immunodominant Mtb antigens Ag85B and TB10.4 did not protect from Mtb infection (61). Similarly, the MVA85A vaccine, which uses the immunodominant antigen Ag85A, induced a high amount of IFNγ secretion (62) and polyfunctional T cells (63) in human subjects, but failed to provide protection (64). Taken together, IFNγ and immunodominant antigen-specific T cells have been used as surrogates for responsive immunity against Mtb infection but may or may not correlate to protection.

In addition to delaying the initiation of adaptive immune response, Mtb successfully establishes a chronic infection, which suggests continuous Mtb antigen production and immune stimulation. This constant antigen stimulation drives ESAT6-specific CD4 T cells to terminally differentiate and be retained in the pulmonary vasculature, resulting in a decreased capacity to provide protection compared to CD4 T cells in the lung parenchyma (65-67). However, this ESAT6-specific response is a selective occurrence as Ag85B antigen is downregulated by Mtb and Ag85B-specific CD4 T cells do not exhibit dysfunctions (68) (further
demonstrated in section G: Immune Evasion Mechanism). Pulmonary T cells also express cell surface markers consistent with an exhausted phenotype (increased inhibitory receptors TIM3, PD-1, LAG3, 2B4) and function (lowered capacity to produce multiple cytokines) during the chronic infection phase in mice (69).

Chronic infection and dysfunctional CD4 T cell responses may contribute to the inability of the immune responses to clear Mtb infection.

D. The underappreciated role of CD8 T cells in TB Immunity

Different models including antibody depletion in mice and nonhuman primates, along with mouse models with defects in class I MHC presentation (e.g., β2m knockout, TAP knockout, and H-2 K<sup>b</sup>D<sup>b</sup> double knockout) have shown that CD8 T cells are essential for optimal control of Mtb infection (40, 70-73). The appreciation of CD8 T cells’ contribution to protection, however, is masked by the importance of CD4 T cell functions; CD4 T cells help in priming CD8 T cells, recruiting CD8 T cells to the site of infection, and generating recall responses (40, 74-78). Memory CD8 T cells have been shown to be important during TB (79, 80), suggesting that CD8 T cells may provide better protective roles during the chronic phase of infection. This notion is supported by a latent TB study where aerosol-infected mice were antibiotic-treated before permitting bacterial growth and subsequently depleting T cells (81). CD8 T cell depletion during chronic infection
led to a significant increase in bacterial burden, whereas depletion of CD4 T cells did not, indicative of CD8 T cell-mediated killing.

Cytotoxicity and cytokine production are the main effector functions CD8 T cells utilize to clear pathogens. Cytotoxic T lymphocyte (CTL) activities consist of cytotoxic granules such as perforin and granzyme, Fas/FasL (CD95/CD95L) pathway and cytokine secretion. During Mtb infection, CD8 T cells use all three mechanisms of cytotoxicity in vivo to induce apoptosis, the aforementioned efferocytosis, and killing of Mtb infected target cells (38, 82-84). In addition to perforin, which is required to restrict Mtb growth by murine CD8 T cells (83), cytotoxic granules in humans and nonhuman primates also contain granulysin that can directly kill Mtb in vitro (85). Importantly, mice with CD95L and perforin deficiencies are more susceptible to Mtb infection (86, 87), highlighting the importance of cytotoxic pathways for immunity to tuberculosis.

In the absence of CD4 T cells, IFNγ levels in the lungs were unaffected (88), and CD8 T cells were shown to increase their proportion of IFNγ producers in chronic infection (89), suggesting CD8 T cells produce more IFNγ in compensation. While IFNγ secretion by CD8 T cells can activate macrophages and protect mice from Mtb infection in the absence of CD4 T cells (90), it cannot durably substitute CD4 T cells for protection (88). Together, CD8 T cells have been categorized as either IFNγ-secreting cells or CTLs during Mtb infection (91), but which CD8 T cell functions to provide bacterial control is unclear (83, 85, 90, 92). Thus, CD8 T cells,
in concert with other cells of host immune response, collaborate and contribute to the optimal control of TB. A better understanding of the role of CD8 T cells in controlling TB is needed to develop a more effective vaccine.

E. Current Vaccine Development and T cell Recognition

The incompetence of BCG is thought to be a result of many factors including: (a) the genetic diversity of both BCG and Mtb strains prevalent throughout the world; and, (b) the genomic and antigenic differences between BCG and Mtb. Over 20% of known human CD4 and CD8 T cell epitopes in the Mtb genome are deleted from BCG genome including the immunodominant antigens ESAT6 and CFP10 (93). However, antigen loss and sequence variation alone are insufficient to account for the limited efficacy of BCG.

The current strategy to design vaccine candidates aims at eliciting antigen-specific T cell responses using immunodominant antigens as targets. For example, the MVA85A vaccine expresses the Mtb antigen 85A (Ag85A), an abundant secreted protein of Mtb. MVA85A induced high-frequency Ag85A-responsive T cells that produced IFNγ, TNFα, IL-2, and/or IL-17. Using IGRA conversion as an indication of Mtb infection, the researchers reported that MVA85A failed to prevent new infections in both HIV-infected adults and healthy infants who previously received BCG (64, 94). On the other hand, the M72/AS01E vaccine, which employs the same strategy but uses Mtb antigens MTB32A and MTB39A, provides
54% protection against active pulmonary tuberculosis disease to HIV-negative adults with latent Mtb infection in phase IIb clinical trials (95, 96). Together, these studies raise the question of how one could define the protective T cell subsets to better design clinical trials and improve upon the 54% protection.

There are a few confounders I could identify in vaccine studies. First, the measurements of antigen recognition were done with the assumption that antigens, especially immunodominant antigens, are presented by infected cells; hence, all antigen-specific T cells should recognize infected cells. Since CD4 T cells have been shown to require direct contact to recognize and limit intracellular Mtb (97), there is a disconnect in most studies which use peptide or protein as a stimulant, rather than Mtb-infected cells themselves. Secondly, while there is no known immune correlate of protection against tuberculosis, IGRA assays use IFN\(\gamma\) as a readout of responsiveness and a surrogate for recognition of infected cells. IFN\(\gamma\) and other cytokine secretions by CD4 and CD8 T cells can be stimulated by non-cognate (cytokine-driven) or cognate (T cell receptor-dependent) interactions, and indirect recognition through cytokine stimulation may not lead to clearance of infected cells. This is supported by an intravital imaging study that showed not all antigen-specific T cells interact with granulomas (98). Lastly, among hundreds of identified human antigens, the use of two antigens in the M72/AS01E vaccine are encouraging. While this work assumes that either MTB32A or MTB39A or both are presented by infected cells, identification of other antigens presented by infected cells could improve T cell responses and provide full protection to all populations.
Despite the necessary multifunctional effector roles of CD4 and CD8 T cells, immunocompetent animals can only maintain latency, containing but not eradicating Mtb infection. Whether humans can eliminate Mtb, however, is debatable. Recent reports suggest co-evolution of Mtb and humans is the consequence of successful immune evasion mechanisms employed by Mtb. Studying how Mtb subverts and adapts in response to host immunity will further enhance our understanding toward the development of strategies to eliminate Mtb.

*Mycobacterium tuberculosis* Evolution and Evasion Mechanisms

F. Evolutions and Transmission of Mtb Clinical Isolates

The connection between our human ancestors and Mtb is long established. Some studies suggested Mtb coexisted with humans for 6,000 years (99, 100) while one study speculated back as many as 3 million years (101). Given that Mtb is a human obligate pathogen without an environmental or animal reservoirs, all Mtb adaptations are presumably through selections by human immune responses. The long-standing interaction—whereby Mtb infection stimulates inefficient immunity—reflects an evolutionary arms race that needs to allow survival of both the pathogen and its host.

Mtb bacteria that are isolated from sputum or resected lung of tuberculosis patients are referred to as clinical isolates. New technology has facilitated the study of Mtb evolution through the sequencing of clinical isolates. This led to the
categorization of Mtb into lineages based on gene clusters, such as TbD1 deletions (102). Despite the historical belief that Mtb strains are highly uniform because of their slow growing nature and lack of mutation, clinical isolates of virulent Mtb that infect human populations show significant heterogeneity (103). Some Mtb lineages are more globally distributed and contain more variable T cell epitopes, suggestive of interactions with diverse populations and/or evidence of immune escape. Some lineages have strong geographical restriction (104, 105), which could represent the preference in host-adaptation, limited mobility of the infected population or its more recent appearance.

Distinct ethnicity and MHC alleles have previously been reported to impact T cell responses in various infectious diseases including Hepatitis B (106), Hepatitis C (107) and HIV (108). Given that class I MHC is the most polymorphic allele in the human genome (109), this relationship may reflect a host and pathogen co-adaptation, which in turn drives diversification in both MHC and pathogen. Therefore, Mtb genetic diversity and reciprocal host tolerance to mycobacterial infections are important interplaying components, contributing to disease trajectory and transmission patterns.

Unlike many pathogens that spread during an asymptomatic state, Mtb induces human disease, displayed as a cough and fever, and transfers to different hosts. The transmission of Mtb, therefore, depends on pulmonary disease, showing a direct correlation of virulence and transmission. In addition, as opposed to many viruses whose antigenic sequences are variable for immune escape, the
vast majority of antigens encoded in the Mtb genome are conserved (110, 111). This unique antigen conservation has been predicted to benefit the bacteria’s ability to exploit adaptive immune responses, potentially through inflammatory lung and tissue damage. The absence of escape mutations in Mtb genomes could represent a well-adapted, inefficient immunity that Mtb prefers to prevent the clearance of infection.

G. Immune Evasion Mechanisms

Mtb is a remarkably successful pathogen that utilizes multiple mechanisms for evading and subverting immune responses. Given that Mtb infects professional antigen-presenting cells and CD4 T cells are essential to limit Mtb growth, multiple mechanisms to perturb innate immunity (26) and inhibit class II MHC presentation and CD4 T cell recognition (112) are employed by Mtb. Mtb replicates in early phagosomes, inhibits phagosome maturation (113) and lysosomal fusion (114), and evades the immune system. The mycobacterium also induces damaging type I interferon or excessive TNFα secretion (115, 116) to boost its early survival and delay adaptive immunity (117).

Mtb utilizes various strategies to hamper class II MHC presentation such as inhibiting class II MHC molecule intracellular trafficking (118) and blocking IFNγ-mediated upregulation of class II transactivator (CIITA, the master regulator of MHC II genes), which controls genes in the class II MHC pathway in mice and
humans (119-121). (Side note: Mtb cytokine signaling interference may help explain why IFNγ is not correlated to protection in TB). Mtb also diverts its secreted antigens from the class II MHC pathway to the extracellular space through vesicular transport (122, 123). This process, interestingly, enhances CD4 T cell priming and recognition of uninfected cells that take up the exported antigen (45), but evades the direct recognition of infected cells. Blockage of the motor component leads to decreased antigen export, increased antigen presentation of Mtb-infected cells to ESAT6- and Ag85B-specific CD4 T cells, and enhanced bacterial control both in vitro and in vivo (122).

Recently, the Mtb-encoded PE_PGRS47 gene was discovered to play a role in class II MHC inhibition, and its deletion resulted in Mtb attenuation (124). PE_PGRS47 was also reported to disrupt autophagy in infected macrophages, supporting roles of autophagy in class II MHC presentation of mycobacterial antigens and Mtb evasion (125). Another study reported a role of the Mtb-secreted molecule EsxH (or TB10.4) in inhibiting class II MHC presentation (113). The EsxH protein was shown to directly bind Hrs, a component of the endosomal sorting complex required for transport (ESCRT) machinery in human and murine cells, thereby dysregulating phagosomal maturation and antigen processing (126). EsxH overexpression enhanced (while its deletion improved) Mtb-specific CD4 T cell activation. EsxH-deficient mycobacteria were also attenuated in immunocompetent mice, but not in CD4 T cell-deficient mice, indicating EsxH interferes with CD4 T cell activation. In addition, Mtb has also been shown to
regulate its gene expression in vivo to limit T cell responses. Ag85B transcript expression was high during the initiation of Mtb infection in the lungs and decreased later, leading to reduced activation of Ag85B-specific CD4 T cells (127). Conversely, forced expression of Ag85B leads to the attenuation of the Mtb strains because of better antimicrobial activity of Ag85B-specific CD4 T cells in vivo (127).

Last, but not least, Mtb inhibits bacterial clearance by macrophages. Mtb induces infected macrophages to produce lipoxin A4 (LXA4), prevent prostaglandin E2 (PGE2)-dependent plasma membrane repair, and undergo necrosis rather than apoptosis (128). While apoptotic cells can be phagocytosed by uninfected cells (38), necrotic cells allow for the escape of Mtb which can then infect other cells and disseminate the infection (129). The upregulation of LXA4 delays the priming of T cells and therefore promotes the establishment of persistent infection (128). In turn, Alox5 deficient mice (which cannot make LXA4) generated antigen-specific T cells earlier, enhanced effector functions and decreased bacterial burden (130). In parallel, a proapoptotic ΔnuoG Mtb appears earlier in the mediastinal lymph node (131), leading to earlier priming of antigen specific CD4 T cells (132).

These examples—among many other studies—have shown that Mtb manipulates host immune responses at many levels, suggesting that there are additional unknown mechanisms to limit the efficacy and evade host immunity to control TB.
H. **Evasion of CD8 T cell immunity**

Less is known about how CD8 T cells respond during infection and whether Mtb evades CD8 T cell recognition. This could be because: (a) other protective immune components, such as CD4 T cells, mask the influences of CD8 T cells; or, (b) Mtb suppresses CD8 T cell roles. One report showed that mycobacterial antigens localize to phagosomes and segregate from cytosolic class I MHC molecules (133), providing evidence Mtb averts class I MHC antigen presentation for TCR-mediated CD8 T cell recognition. Furthermore, apoptotic vesicles from Mtb-infected macrophages activated CD8 T cells *in vivo*, and vaccination using the vesicles alone was able to provide protection (134). This supports the previous finding that inhibiting apoptosis decreases CD8 T cell activation (133). If, similar to class II MHC, Mtb-infected cells themselves do not present antigen, but rather the bystander uninfected cells do so by acquiring antigens from apoptotic cells (45), this would imply Mtb distracts CD8 T cells from directly recognizing and killing Mtb-infected cells *in vivo*.

Though not reported during Mtb infection, many viruses such as herpesviruses (135, 136) and cowpox viruses (CPXV) (137), evade CTL response by inhibiting class I MHC antigen presentation via retaining class I MHC molecules in the endoplasmic reticulum and preventing peptide loading (138). In addition, the CPXV-specific CTL response in C57BL/6 mice exhibits an immunological phenomenon called immunodominance where a single antigen, B8, dominates
(139), similar to Mtb-specific CD8 T cell response in Mtb-infected C57BL/6 (140, 141). This process was reported to hamper the development of successful vaccines because of lacking efficient effector functions (142). Deleting the immunodominant epitope from the virus gives rise to compensation and domination by subdominant epitopes instead (143, 144), suggesting an inhibition of subdominant epitopes by an immunodominant epitope. This phenomenon is termed immunodomination and also occurs as a mechanism by which memory responses inhibit naïve CD8 T cell responses (145). Interestingly, a recent study indicated that CD8 T cell immunodomination during primary CPXV infections was not affected by viral class I MHC inhibition (137).

Taken together, Mtb utilizes a variety of mechanisms for immune evasion, some of which implicate Mtb’s capability to directly avoid detection, and therefore enhance its survival. Given that CD8 T cells are essential in controlling Mtb infection (71) and there are many reports of CD8 T cell evasion in viral immunity (136, 137), to understand whether the suboptimal contribution of CD8 T cells is due to Mtb modulation of CD8 T cell recognition and functions is of critical value.

We next discuss the immunodominance phenomenon by TB10.4 protein during Mtb infection. As immunodominance has been shown to interfere with the generation of desired immune responses through successful vaccination (142), this emphasizes that evaluating the pre-existing immunity is crucial to predict the outcome of vaccine responses.
**esxH gene, TB10.4 protein, and TB10.4-specific T cells**

Mtb secretion systems are crucial for Mtb pathogenesis and virulence, and their antigenicity are important to understand host and pathogen interactions (146). ESAT6 secretion (ESX, also known as type VII secretion) systems are Mtb secretion systems named after the first identified substrate, the 6 kDa early secretory antigenic target (ESAT6, also known as EsxA). ESX systems and their secretion substrates are the main players of Mtb pathogenicity (147). Of the 5 ESX systems in Mtb (ESX-1 through ESX-5), ESX-1 was the first to be discovered from genomic analysis of the *M. bovis* strain of BCG vaccine where a large deletion of Region of Difference 1 within the *esx1* operon caused attenuation of the strain (148). ESX-1, the most characterized secretion system in Mtb, is essential for microbial resistance and host immune evasion. EsxA or ESAT6, in addition to being described as a substrate for ESX-1 and a cytolytic virulence factor of Mtb (149), is a potent T cell antigen that has been used in many vaccines (150, 151). Similarly, ESX-3 is crucial for Mtb viability and pathogenesis, but much less is known. The ESX-3 secretion system is encoded within the *esx3* operon and plays both iron-dependent and -independent roles in Mtb pathogenesis and survival (152). The iron and zinc acquisition and metal homeostasis roles of ESX-3, however, depend on host genotypes. Since different hosts have varied iron availability, this suggests that Mtb uses ESX-3 to counteract host defense mechanisms in restricting iron nutrients. *esxH*, or *Rv0288* in the *esx3* operon, encodes for a secreted protein EsxH or TB10.4 that is co-expressed with its partner
EsxG from the Rv0287 gene within the same operon (152). Mycobacteria with esxH deficiency exhibit an absence of growth in vivo and require iron supplementation to grow in culture (152). As described earlier (section G: Immune Evasion Mechanisms), the EsxH/TB10.4 protein plays a role in disrupting class II MHC presentation through ESCRT (126) and is the protein of interest.

TB10.4, similar to ESAT6, is an antigenic protein that generates TB10.4-specific T cell responses in human and animal models (153-158) among hundreds of other epitopes that have the potential for recognition by CD8 T cells (59, 159). Axelsson-Robertson et al. have extensively studied human class I MHC, which is the most polymorphic molecule in the human genome, in the context of TB10.4-responsive CD8 T cells using Mtb-infected human PBMCs and immobilized recombinant class I MHCs (153-155). The researchers have studied MHC molecules in Caucasians, Asians and Africans including the human leukocyte antigen (HLA)-A*30 family which is one of the most frequent allele families of African background individuals and found numerous candidate epitopes from TB10.4.

In addition to class I MHC diversity, esxH polymorphisms were reported whereas other antigenic regions were hyperconserved (110). In this study, only a limited number of Mtb clinical isolates were sequenced, so one could argue that the comparison to the H37Rv lab strain as “wild-type” added to the bias in the interpretation of the results. The follow-up study, however, used an unbiased computational approach with a larger set of Mtb genome sequences, and esxH
was again identified as one of the top polymorphic genes (111). The naturally occurring single amino acid exchanges in the TB10.4 protein included A10T, G13D, S27N and A71S (110), all of which are part of previously reported CD8 T cell epitopes (153, 155). This antigenic variation may impact antigen presentation and subsequent T cell recognition.

Following aerosol infections, C57BL/6 mice generate 30-50% of pulmonary CD8 T cells specific for a single epitope, H-2K<sup>b</sup>-restricted TB10.4<sub>4-11</sub> (156, 160-162). H-2K<sup>d</sup>-restricted TB10.4<sub>20-28</sub> CD8 T cells are also elicited at a high frequency in Mtb-infected BALB/c mice, along with I-A<sup>d</sup>-restricted TB10.4<sub>74-88</sub>-specific CD4 T cells (157, 158). Taken together with data from human studies, TB10.4 is an immunodominant antigen, which makes it an attractive target for vaccine development with the assumption that Mtb-infected cells are antigen-presenting cells responsible for these large responses to TB10.4 antigen. As a side note, the secreted immunodominant antigens of Mtb are likely processed through the cross-presentation pathway (133, 163) and blocking bacterial secretion has prevented the priming of Mtb-specific CD8 T cells (162).

Despite the ability to lower bacterial burdens in CD4 and CD8 T cell deficient mice in the adoptive transfer model (160, 161), TB10.4<sub>4-11</sub>-specific CD8 T cell responses elicited by vaccination do not mediate protection in immunocompetent mice (160, 164). In particular, TB10.4-immunized animals generated larger and earlier TB10.4-specific responses, but the vaccinated mice did not exhibit more protective immune responses. Unvaccinated mice were able to catch up and
displayed the same number of TB10.4-specific CD8 T cells by the peak of the infection (160). In addition, the AERAS-402 vaccine, which uses TB10.4 as an antigen, also failed to elicit protective immunity in humans (61, 165).

An explanation for the failure of TB10.4-specific CD8 T memory cells to mediate protection may be due to the failure of TB10.4_{4-11}-specific CD8 T cells to recognize Mtb-infected macrophages as reported by our group (166). We have begun to gain some insights as we were able to detect TB10.4_{4-11}-specific CD8 T cell recognition when using *Listeria monocytogenes* expressing TB10.4 in place of Mtb. We hypothesize that Mtb uses TB10.4, which does not present on Mtb-infected cells, as a decoy antigen to distract host immunity from other protective antigens for immune evasion. Together, the naturally occurring variations in TB10.4 protein could further impact antigen presentation and ultimately CD8 T cell recognition.
Thesis objectives

The majority of Mtb epitopes recognized by human T cells are encoded by regions of the Mtb genome that are hyperconserved, excluding *esxH*. As Mtb-specific T cell responses are required for protective immunity, it is critical to understand the paradoxical notion that Mtb could benefit from T cell immunity.

In this dissertation, I employ immunological and bacterial genetic approaches to investigate how Mtb exploits its antigenic polymorphisms to evade host adaptive immune responses and establish a successful infection. In particular, we hypothesize that polymorphisms in TB10.4 could modulate immunodominant CD8 T cell responses which Mtb may develop as an immune evasion strategy. Chapter II addresses the recognition of Mtb-infected cells by H-2K^d^-restricted TB10.4_{20-28}-specific and H-2K^b^-restricted MTB32a_{309-318}-specific CD8 T cells to determine whether other class I MHC, or only TB10.4_{4-11}, epitopes could act as decoys. Chapter III focuses on antigen-specific T cell responses to polymorphic epitopes and the clinical Mtb isolates containing these polymorphisms. Chapter IV focuses on the cause of antigenic shift described in Chapter III. Finally, Chapter V investigates the ability of polyclonal T cells elicited by Mtb infection *in vivo* to recognize Mtb-infected macrophages. A discussion in Chapter VI of data presented will review the possible areas of improvement and remaining questions for further investigation.
The results and analyses in this dissertation will enhance the understanding of Mtb infections with naturally occurring polymorphic immunodominant antigens and the consequences on antigen-specific T cells responses and disease outcomes. This work directly impacts the strategies for vaccine design and development against tuberculosis and implicates a need for better comprehension of evolution and immune evasion.
CHAPTER II. INVESTIGATING RECOGNITION CAPACITIES OF 
MYCOBACTERIUM TUBERCULOSIS-SPECIFIC CD8 T CELLS

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R.S. and S.B designed all experiments. D.M. provided assistance in maintaining cell lines and repeating experiments in Figure 2.4 and 2.5. R.S. performed all other experiments and statistical analysis. S.B. provided critical feedback.
Abstract

Immunodominant Mtb-specific CD4 and CD8 T cell responses, which signify a selection of few antigens for the generation of T cell immunity, are the primary targets of vaccine development. Despite robust and persistent Mtb-specific CD8 T cell responses during Mtb infection, less is understood regarding why CD8 T cells contribute minimally to protection. In this chapter, I assess the ability of three dominant Mtb-specific CD8 T cells to recognize Mtb-infected macrophages \textit{in vitro} in comparison to Mtb-specific CD4 T cells. The CD8 T cells are specific to: (1) H2-Kb/TB10.4_{4-11}, (2) H2-Kd/TB10.4_{20-28}, and (3) H2-Kb/MTB32a_{309-318} Mtb epitopes. Compared to the capability of Ag85b_{240-254}-specific CD4 T cells in recognizing Mtb-infected macrophages, the Mtb-specific CD8 T cells neither recognized Mtb-infected macrophages nor restricted Mtb growth. We propose that immunodomination could act as a decoy mechanism for Mtb immune evasion; by inducing a large response of CD8 T cells that cannot recognize Mtb-infected cells, Mtb may evade the control of infection by CD8 T cells.
**Introduction**

*Mycobacterium tuberculosis* (Mtb) infected 1.7 billion people globally and caused 1.5 million deaths in 2018 alone (1). While Mtb has demonstrated its capability to subvert host immune responses and establish its niche, CD4 and CD8 T cells have shown their essentiality to control the infections in both human and animal models (40, 167). Yet, the functions of protective immunity are not well-defined. Given that vaccination is the most effective infectious disease intervention, studies have focused on eliciting immunodominant T cell responses, anticipating their ability to mediate protection and prevent disease. This is based on the underlying assumption that immunodominant antigen-specific CD4 and CD8 T cells are primed by infected cells, therefore they should recognize and kill the infected cells.

One protein of interest is TB10.4, encoded by *esxH* gene, an immunodominant protein which is a component of the AERAS-402 vaccine (61, 165). Studies of Mtb-infected patients have shown several different epitopes from the TB10.4 protein elicit HLA-restricted CD8 T cells (153-155). In the murine model, 30-50% of CD8 T cells in the lungs of Mtb-infected C57BL/6 mice recognize the TB10.4\textsubscript{4-11} epitope (140, 160, 161), and 10-15% of CD8 T cells are specific for the TB10.4\textsubscript{20-28} epitope in infected BALB/c mice (157, 158). These data indicate that TB10.4 induces immunodominant CD8 T cell responses during Mtb infection. However, whether TB10.4\textsubscript{4-11}-specific CD8 T cells can provide protection is unclear. Adoptive transfer of TB10.4\textsubscript{4-11}-specific CD8 T cells into immunodeficient
mice reduced the bacterial burden (161), but successful vaccination and generation of TB10.44-11-specific CD8 T cells did not provide protection compared to mock-vaccinated control in immunocompetent mice (160). Given the dichotomy of these results, we hypothesize that TB10.44-11-specific CD8 T cells are unable to efficiently recognize Mtb-infected cells. In this chapter, we also investigate the capacity of TB10.420-28-specific CD8 T cells to recognize Mtb-infected cells. These responses will be compared to the ability of CD4 T cells to recognize infected cells by using P25, a CD4 T cell line specific for Ag85B; we have previously demonstrated its capability to recognize infected cells and suppress the growth of intracellular Mtb (166).

Another protein of interest is MTB32A, encoded by Rv0125, which is part of the M72/AS01E vaccine that has had promising results in a phase IIb clinical trial, and is proceeding towards a phase III clinical trial (96). The MTB32A309-318 epitope is recognized by 2-5% of CD8 T cells in the lungs of Mtb-infected C57BL/6 mice (168), indicating it is a major response, although subdominant to the TB10.4 protein. MTB32A309-318-specific CD8 T cells will be evaluated for their capacity to recognize Mtb-infected cells.

IFNγ secretion and the Mtb-Infected Macrophage ELISpot (MIM-E) assay (see (169), and Chapter V for a thorough discussion) will be used to assess T cell recognition of infected macrophages. This assay allows us to compare the recognition of antigens (i.e., in the form of proteins or synthetic peptides) versus Mtb-infected macrophages. Here we determine whether antigen-specific CD8 T
cells, which are elicited by vaccination and can recognize peptide-loaded cells, have the capacity to recognize the same epitope in the context of Mtb infection.
Results

Generation of H2-K<sup>b</sup>-restricted TB10.4<sub>4-11</sub>-specific TB10RgL, TB10RgR, TB10RgP, TB10Rg3 CD8 T cell lines

To determine whether immunodominant TB10.4<sub>4-11</sub>-specific CD8 T cells can recognize Mtb-infected macrophages, I generated TB10.4<sub>4-11</sub>-specific CD8 T cell retrogenic mice named TB10RgL, TB10RgR, TB10RgP and TB10Rg3 (166). We chose to use four different T cells, all specific for the same TB10.4<sub>4-11</sub> epitope to obtain T cell receptors (TCRs) representative of in vivo clonally expanded TB10.4<sub>4-11</sub>-specific CD8 T cells from the lung of infected C57BL/6 mice (161). TB10RgL, TB10RgR, TB10RgP and TB10Rg3 CD8 T cells are all specific to the TB10.4<sub>4-11</sub> epitope but use distinct TCRs that encode related CDR3<sub>α</sub> and CDR3<sub>β</sub> regions (Figure 2.1A). We then derived TB10.4<sub>4-11</sub>-specific CD8 T cell lines from TB10RgL, TB10RgR, TB10RgP and TB10Rg3 retrogenic mice by stimulating purified splenic CD8 T cells with TB10.4<sub>4-11</sub>-pulsed irradiated splenocytes supplemented with IL-2. Stimulated cells were monitored for their proliferation and passaged following expansion. At 4 days post stimulation, TB10RgL, TB10RgR, TB10RgP and TB10Rg3 T cell lines expressed GFP (co-expressed with the TCRs as part of the retroviral vector) and their specific V<sub>α</sub> and V<sub>β</sub> receptors(Figure 2.1B). TB10RgL T cell line was 47% V<sub>β</sub>5<sup>+</sup>GFP<sup>+</sup> and represented the lowest uniform population among four cell lines. At 3 weeks post-stimulation, TB10RgL and all other T cell lines were more than 98% positive for its specific V<sub>α</sub> and V<sub>β</sub> receptors.
(Figure 2.1C). The TB10.44-11-specific CD8 T cell lines were subsequently used in assays at 3-5 weeks post-stimulation and could be maintained in vitro by restimulation with peptide and splenocytes for future use, or frozen down.
Figure 2.1: Generation of H2-K\textsuperscript{b}-restricted TB10.4\textsubscript{4-11}-specific TB10RgL, TB10RgR, TB10RgP, TB10Rg3 CD8 T cell lines

(A) TCR\textalpha and TCR\textbeta family with the sequences of CDR3\textalpha and CDR3\textbeta regions of TB10RgL, TB10RgR, TB10RgP, TB10Rg3 CD8 T cell lines. (B-C) Purified CD8 T cells from TB10RgL, TB10RgR, TB10RgP, TB10Rg3 retrogenic mice were co-cultured with TB10.4\textsubscript{4-11}-pulsed irradiated splenocytes with IL-2 supplement. (B) At four days post \textit{in vitro} stimulation, each T cell line was stained for expression of CD8 and its specific V\textalpha and V\textbeta: TB10RgL and TB10RgR with V\textbeta5 antibody, TB10RgP with V\textbeta4 antibody, and TB10Rg3 with V\textalpha2 antibody. Each T cell line was gated on CD8\textsuperscript{+} and subsequently GFP\textsuperscript{+} and V\textalpha/V\textbeta\textsuperscript{+}. (C) At three weeks post-stimulation, TB10RgL CD8 T cell line was stained for V\textbeta5 expression.
The H2-K^b-restricted TB10.44-11-specific TB10RgL, TB10RgR, TB10RgP and TB10Rg3 CD8 T cells do not recognize infected macrophages nor inhibit intracellular Mtb growth

TB10RgL, TB10RgR, TB10RgP and TB10Rg3 CD8 T cell lines were compared to the Ag85B_{241-256}-specific (P25) CD4 T cell line. To evaluate their ability to recognize Mtb-infected macrophages, thioglycolate-elicited peritoneal macrophages (TGPM) were infected in vitro with H37Rv Mtb, and then co-cultured with each T cell line, with or without its cognate epitope (i.e., synthetic peptide). In the peptide-included condition, all T cell lines sensitively responded by secreting IFN_γ, measured by ELISA, demonstrating the specificity of TB10RgL, TB10RgR, TB10RgP and TB10Rg3 CD8 T cells to the TB10.44-11 epitope and P25 CD4 T cells to the Ag85B_{241-256} epitope (Figure 2.2A). However, only P25 CD4 T cells were able to recognize Mtb-infected macrophages in the absence of cognate peptide. We next asked whether the ability to recognize infected cells, as measured by IFN_γ production, correlated to the ability of each T cell line to restrict bacterial growth. We cultured respective T cell lines with infected macrophages, with or without their cognate antigens, for 96 hours before determining bacterial loads in comparison to the no T cell condition. Similar to their IFN_γ production, only P25 CD4 T cells were able to control bacterial growth without the presence of its cognate antigen (Figure 2.2B).
Using multiple independent T cell lines with the specificity to a single TB10.44-11 epitope, we showed that TB10.44-11-specific CD8 T cells lines are unable to recognize Mtb-infected macrophages.
Figure 2.2: TB10.44-11-specific CD8 T cells do not recognize Mtb-infected macrophages.

C57BL/6 TGPMs were infected with H37Rv Mtb for 2 hours. (A) After 2 hours, bacteria were washed off. TB10.44-11 peptide and Ag85b_{240-254} peptide were added for 1 hour in the peptide-pulsed condition and subsequently washed off. TB10RgL, TB10RgR, TB10RgP, TB10Rg3, or P25 T cells were cocultured with uninfected, Mtb-infected, or peptide-pulsed TGPMs for 3 days, and supernatants were subsequently measured for IFN\_\gamma by ELISA. (B) At one day post H37Rv infection, TB10.44-11 peptide and Ag85b_{240-254} peptide were added in “+” conditions, washed after 1 hour, and TB10.44-11-specific CD8 and P25 CD4 T cells were added respectively. CFUs were determined 96 hours post-coculturing. Results are representative of three experiments. Statistical testing was by one-way ANOVA, using the Dunnett’s posttest compared to No T d5. **** indicated p<0.0001.
Generation of TB10.4<sub>20-28</sub>-specific CD8 T cells by Trivax-TB10.4<sub>20-28</sub> vaccination

We next sought to determine if the failure of TB10.4-specific CD8 T cells to recognize Mtb-infected macrophages was true for other epitopes or genetic backgrounds. As Mtb infection of BALB/c mice elicits an immunodominant CD8 T cell response for the TB10.4<sub>20-28</sub> epitope (157, 158), we determined whether TB10.4<sub>20-28</sub>-specific CD8 T cells could recognize Mtb-infected macrophages. We generated TB10.4<sub>20-28</sub>-specific CD8 T cells by vaccinating BALB/c mice using the Trivax strategy which consisted of anti-CD40 mAb, Poly(I:C) and a short synthetic epitope peptide (i.e., TB10.4<sub>20-28</sub> here) (170, 171). BALB/c mice were injected intravenously with Trivax-TB10.4<sub>20-28</sub> vaccine (160). Vaccinated mice were rested for 3 weeks before subsequent boosting with Trivax-TB10.4<sub>20-28</sub> and monitored for their generation of TB10.4<sub>20-28</sub>-specific CD8 T cells (Figure 2.3A). At one week post-boost, 2.56% of splenic CD8 T cells from Trivax-TB10.4<sub>20-28</sub> mice were specific for H<sub>2K<sup>d</sup></sub>/TB10.4<sub>20-28</sub> tetramer, and we obtained 1.67% tetramer<sup>+</sup> cells after CD8 T cell purification (Figure 2.3B). The H<sub>2K<sup>d</sup></sub>/TB10.4<sub>20-28</sub> tetramer<sup>+</sup> CD8 T cells were of an activated phenotype, determined by high CD44 expression (Figure 2.3B). The purified CD8 T cells were used in a recognition assay and stimulated in vitro to generate TB10.4<sub>20-28</sub>-specific CD8 T cell line as described earlier. Three weeks after stimulation, the TB10.4<sub>20-28</sub>-specific CD8 T cell line contained 70% H<sub>2K<sup>d</sup></sub>/TB10.4<sub>20-28</sub> tetramer-specific T cells with lower CD44 expression, indicating rested phenotype (Figure 2.3C).
Figure 2.3: Generation of TB10.4_{20-28}-specific CD8 T cells.

(A) Schematic representation of vaccination strategy used to generate TB10.4_{20-28}-specific CD8 T cells. (B-C) Flow cytometry representation of H-2K^{d}/TB10.4_{20-28} tetramer staining of (B) ex vivo pre-purified and post-CD8-purified splenic cells at one week post-boost and (C) TB10.4_{20-28}-specific CD8 T cell line at 3 weeks post stimulation. Cells were gated on CD8 T cells and the activated state was determined with CD69 and CD44 antibodies and their respective isotype antibodies.
The H2-Kd-restricted TB10.420-28-specific CD8 T cells do not recognize infected macrophages

We subsequently performed the MIM-E assay [Mtb-Infected Macrophage ELISpot (169)], to determine whether ex vivo H-2Kd-restricted TB10.420-28-specific CD8 T cells and the corresponding in vitro derived CD8 T cell line recognized Mtb-infected macrophages. Here we used TGPM from CB6F1 mice (C57BL/6 x BALB/c F1), so we could compare the recognition by the Kd-restricted CD8 T cells with the I-A^b-restricted P25 CD4 T cell line. Among purified splenic CD8 T cells from Trivax-TB10.420-28 mice, ~0.5% produced IFNγ when stimulated with the TB10.420-28 peptide (Figure 2.4A). Given that 1.67% were H-2Kd/TB10.420-28 tetramer+ (Figure 2.3B), approximately 30% of TB10.420-28-specific CD8 T cells produced IFNγ in response to its cognate epitope. However, IFNγ was not detected in the Mtb-infected macrophage condition (Figure 2.4A). The in vitro derived TB10.420-28-specific CD8 T cell line with 70% H-2Kd/TB10.420-28 tetramer+ (Figure 2.3C) was also able to produce IFNγ in response to its epitope but failed to produce IFNγ when co-cultured with Mtb-infected cells (Figure 2.4B). In addition, only a small population of TB10.420-28-specific CD8 T cells recognized γ-irradiated Mtb. P25 CD4 T cell line recognition of Mtb infected cells indicated that Mtb-infected TGPMs were able to stimulate responses (Figure 2.4C). The data show that, in addition to TB10.44-11-specific CD8 T cells, TB10.420-28-specific CD8 T cells do not recognize Mtb-infected macrophages despite their responsiveness to cognate epitope.
Figure 2.4: TB10.420-28-specific CD8 T cells do not recognize Mtb-infected macrophages.

Thioglycolate-elicited peritoneal macrophages (TGPMs) were obtained from CB6F1 mice for the MIM-E assay. In short, TGPMs were infected overnight with H37Rv and subsequently harvested, washed, and counted along with uninfected cells. Infected and uninfected TGPMs were plated on membrane ELISpot plates with irradiated Mtb, short peptides, or anti-CD3 antibody. (A) ex vivo Trivax-elicited TB10.420-28-specific CD8 T cells, (B) in vitro-derived TB10.420-28-specific CD8 T cell line, or (C) P25 CD4 T cell line were added and co-cultured overnight before following the manufacturer's protocol for ELISpot development and counting. Results are representative of two experiments.
A subdominant H-2K\textsuperscript{b}-restricted MTB32a\textsubscript{309-318}-specific CD8 T cell does not recognize infected macrophages

Since CD8 T cells lines specific for two different TB10.4 epitopes did not recognize Mtb-infected macrophages (Figure 2.2, 2.4), we sought to determine whether a different Mtb epitope recognized by CD8 T cells would recognize Mtb infected macrophages. We chose MTB32a because the protein is a component of the promising M72/AS01E vaccine candidate (96) and MTB32a\textsubscript{309-318} epitope induces a subdominant CD8 T cell response during Mtb infection in C57BL/6 mice (168). We generated MTB32a\textsubscript{309-318}-specific CD8 T cell line by Trivax vaccination as previously described (Figure 2.3). One week post-boost, Trivax-MTB32a\textsubscript{309-318} elicited 18.4\% H-2K\textsuperscript{b}/MTB32a\textsubscript{309-318}-tetramer\textsuperscript{+} among splenic CD8 T cells (Figure 2.5A). Splenic CD8 T cells were purified for MIM-E assay and subsequent generation of MTB32a\textsubscript{309-318}-specific CD8 T cell line. Of note, MTB32a\textsubscript{309-318}-tetramer\textsuperscript{+} population became 6.61\% after CD8 purification, which we considered due to the purification antibody cocktail containing CD11c, a marker that can be expressed on activated T cells. After three weeks, MTB32a\textsubscript{309-318}-specific CD8 T cell line was over 90\% H-2K\textsuperscript{b}/MTB32a\textsubscript{309-318}-tetramer\textsuperscript{+} (Figure 2.5B). Despite their ability to recognize cognate antigen, neither the Trivax-MTB32a\textsubscript{309-318} elicited CD8 T cells nor the MTB32a\textsubscript{309-318}-specific CD8 T cell line recognized Mtb infected cells, as measured by MIM-E or IFN\textgreek{y}-secretion assays, respectively (Figure 2.5C-D). Furthermore, the recognition of \textgreek{y}-irradiated Mtb by \textit{ex vivo} vaccine-elicited
MTB32a$_{309-318}$-specific CD8 T cells was much lower than by the MTB32a$_{309-318}$-specific CD8 T cell line.

Here we showed that, in addition to TB10.4$_{4,11}$-specific CD8 T cells, neither TB10.4$_{20-28}$-specific CD8 T cells nor MTB32a$_{309-318}$-specific CD8 T cells recognized Mtb infected macrophages despite their recognition of their cognate peptide and binding to the peptide-loaded MHC molecule (i.e., as measured by tetramer staining). Thus, I have demonstrated that CD8 T cells specific for three distinct epitopes fail to recognize infected macrophages. We have not yet discovered any antigens that are presented by Mtb-infected cells and recognized by CD8 T cells. These data imply that dominant CD8 T cell responses are elicited by mechanisms other than direct priming by Mtb-infected cells and that Mtb directly suppress CD8 T cell function.
Figure 2.5: Generation and recognition of MTB32a_{309-318}-specific CD8 T cells.

Flow cytometric analysis of H-2K\textsuperscript{b}/MTB32a_{309-318} tetramer staining of (A) ex vivo pre-purified and post-CD8-purified splenic cells at 1 week post-boost and (B) MTB32a_{309-318}-specific CD8 T cell line at 3 weeks post stimulation. Cells were gated on CD8 T cells. (C) Trivax-MTB32a_{309-318} elicited CD8 T cells were used in MIM-E assay as described previously. (D) MTB32a_{309-318}-specific CD8 T cell line was co-cultured with H37Rv-infected or uninfected TGPMs with indicated stimulations for three days. IFN\textsubscript{\gamma} in the supernatants was subsequently measured by ELISA. Results are representative of two experiments.
**Discussion**

T cell immunity is essential during *Mycobacterium tuberculosis* infection in both humans and mice (40, 167). CD4 T cells are vital to provide a protective role against Mtb infection, but less is understood regarding the role of CD8 T cells in protective immunity against tuberculosis. Correlates of protection and what classify an effective T cell response to Mtb are not well-defined and these confounding factors complicate the discovery toward an effective TB vaccine.

In this chapter of my dissertation, I used IFNγ secretion detected by ELISA and ELISpot to investigate the recognition of Mtb-infected macrophages by antigen-specific CD8 T cells. IFNγ production, a common readout for recognition, can be stimulated by TCR-mediated interaction (cognate) and IL-12 and IL-18 cytokines secreted by Mtb-infected cells (non-cognate) (172, 173). Although MHC-mismatched antigen-presenting cells (APC) or MHC-blocking antibodies were not included as controls to verify MHC-restriction, the lack of recognition obviated the need for these controls. In addition, we have used anti-IL12 and anti-IL18 antibodies (to block non-cognate recognition) along with K\(^{b}\)D\(^{b}\) deficient mice (to block cognate recognition) in establishing the MIM-E assay, and did not observe evidence of non-cognate CD8 T cell activation in these assays (169).

Despite the persistence and immunodominance in the lungs of Mtb-infected mice, H-2K\(^{b}\)-restricted TB10.4\(^{11}\), H2-K\(^{d}\)-restricted TB10.4\(^{20-28}\), and H-2K\(^{b}\)-restricted MTB32a\(^{309-318}\)-specific CD8 T cells do not recognize and produce IFNγ.
in response to Mtb-infected macrophages. Interestingly, the recognition of γ-irradiated Mtb ("dead bug") were much lower by ex vivo vaccination-elicited TB10.4_{20-28}-specific and MTB32a_{309-318}-specific CD8 T cells. This finding could present a few issues of some vaccine designs and approaches in using specific epitopes for vaccinations. First, Mtb has been shown to modulate its protein expression (68, 127, 174), and despite viewing a snapshot from γ-irradiated Mtb, antigen abundance could restrict the detection. Secondly, the TCRs generated by vaccination with synthetic peptides could be specific to the short epitopes rather than the bacteria.

Previous reports have evaluated the ability of CD8 T cells to recognize Mtb-infected cells including ex vivo murine CD8 T cell recognition of Mtb-infected macrophages and DCs (175-177) and human CD8 T cell recognition of Mtb-infected DCs (178-180). Given the different abilities of macrophages and DCs to cross-present to CD8 T cells, the interpretation of results will slightly depend on what APCs were used for the infection. Despite lower frequencies than anticipated, these studies agreed that CD8 T cells can recognize and respond to infected cells. Though antigen specificities have not been identified, we have shown that there is a population of polyclonal pulmonary CD8 T cells elicited from Mtb infection that recognize infected macrophages (166). However, our data indicate that not all Mtb-specific CD8 T cells recognize Mtb-infected macrophages. The question of recognition of Mtb-infected macrophages by ex vivo polyclonal CD8 T cells is addressed in Chapter V.
Our results led us to hypothesize that: (1) in contrast to the robust antigen-specific CD8 T cell responses elicited by Mtb infection in vivo, the low recognition ability of these CD8 T cells may be responsible for the disproportionately smaller contribution of CD8 T cells to host defense; and, (2) Mtb employs immunodominant antigens as a decoy to distract the host from generating potentially protective CD8 T cell responses to antigens presented by Mtb-infected macrophages.
CHAPTER III. THE EFFECT OF MYCOBACTERIUM TUBERCULOSIS ANTIGENIC POLYMORPHISMS ON HOST T CELL IMMUNE RESPONSES

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This chapter was included in the manuscript in preparation:

Sutiwisesak R, …, Behar SM. “A natural polymorphism of Mycobacterium tuberculosis in the EsxH gene disrupts immunodomination by the TB10.4-specific CD8 T cell response”

Attributions:

N.H. and S.F. analyzed clinical isolate data in Figure 3.1 and Table 3.1 and guided the performance and analyses of barcoded clinical isolate pool in Figure 3.8. S.B. analyzed and generated Table 3.2. B.S. provided assistance in experiments in Figure 3.7. S.C. performed the survival experiment in Figure 3.8. R.S. and S.B designed all experiments. R.S. performed all other experiments and statistical analysis. S.B. provided critical feedback for interpretation of the data.
Abstract

*EsxH* (*Rv0288*) is an essential gene of Mtb and encodes TB10.4 protein. To overcome the attenuation of Δ*esxH* strain in assessing immunodomination, we used naturally occurring *esxH* polymorphisms from clinical isolates of Mtb. We demonstrated that infection with a clinical isolate expressing polymorphic TB10.4 protein can cause a drastic shift in the CD8 T cell immunodominant hierarchy in C57BL/6 mice. The dominant CD8 T cell response to TB10.4-11 is lost and the MTB32a309-318 response becomes dominant. Our data show that immunodomination occurs during Mtb infection; immunodominant CD8 T cell response toward TB10.4-11 epitope suppresses subdominant CD8 T cell responses specific for MTB32a309-318 and possibly other Mtb antigens.
Introduction

Mtb has coexisted with its human host for thousands of years. The heterogeneity of both clinical Mtb strains and polymorphic MHC alleles reinforces the complexity of host-pathogen interactions, and these interactions could influence the immune outcome of infection. Just as Mtb can be clustered into lineages that correlate with geographic locations (181), human leukocyte antigens (HLA) are also clustered among geographic areas and ethnicities (182). The associations between host genetics and Mtb lineages have been identified as variables that influence Mtb-human co-adaptation and genetic diversification (104, 105).

Recent studies have identified that unlike many other pathogens, which undergo genetic mutation as a strategy for immune escape, Mtb genomes are highly conserved especially in genes that encode proteins recognized by human T cells (110, 111). As Mtb-specific T cell responses are required for protective immunity, it is critical to understand the paradox of why Mtb selects to maintain T cell responses. One notion suggested that Mtb could benefit from the inflammatory environment generated from T cell immunity, which induces disease symptoms and ultimately leads to transmission.

*EsxH* (Rv0288) is an essential gene of Mtb and encodes the TB10.4 protein. TB10.4 is an immunodominant antigen in BALB/c and C57BL/6 mice and stimulates T cell responses in people. The TB10.4 protein has a role in
mycobacterial iron and zinc acquisition (152) and has recently been shown to inhibit class II MHC presentation. Infection with esxH-deficient Mtb strains results in greater CD4 T cell activation and CD4-mediated protection (126). In an evolution study of Mtb encoded antigenic genes with a small group of clinical Mtb isolates, the esxH gene was excluded due to its highly polymorphic nature (110). Importantly, we demonstrated that TB10.44-11-specific CD8 T cells do not recognize Mtb-infected macrophages nor control bacterial growth (Chapter II, and (166)). These findings raise the question of whether Mtb has also adapted to diversify its antigenic genes to subvert host immune pressure.

Given that esxH polymorphisms are prevalent in clinical isolates of Mtb, we sought to determine whether host immune responses are affected by polymorphisms present in clinical Mtb isolates. In this chapter, we investigate how naturally occurring esxH polymorphisms affect immunodominant T cell responses and disease outcomes. We hypothesize that polymorphisms in esxH gene could alter immunodominant TB10.44-11-specific CD8 T cell responses. Here, we also examine the hypothesis that TB10.4 is a decoy antigen as it induces an immunodominant CD8 T cell response that distracts the host from recognizing other class I MHC epitopes that could mediate protective immunity, and will continue to develop this hypothesis in Chapters IV and V.
Results

*Mycobacterium tuberculosis* clinical isolates reveal highly polymorphic clusters within the known T cell epitopes of the *esxH* gene

To characterize the frequency and type of *esxH* polymorphisms present in clinical Mtb isolates, we analyzed whole-genome sequencing data from a total of 3,363 isolates from Holt et al. and Walker et al. spanning lineages 1-4 (183, 184). We identified a total of 86 isolates containing non-synonymous variants in *esxH* (2.56%), the majority of which (77 isolates) contained mutations between amino acids N7 and G13 (Figure 3.1A, Table 3.1). The fraction of isolates with *esxH* variants in lineage 1 (L1) was strikingly different among other lineages. 75 of 594 L1 strains analyzed (12.5%) contained *esxH* polymorphisms, while the rate in L2, L3 and L4 were significantly lower with 4 of 1239 (0.32%) for L2, 0 of 529 (0%) for L3, and 7 of 1001 (0.7%) for L4 (Figure 3.1A, two-sided chi-square: L1 vs L2, $\chi^2 = 147.4$, $p < 0.0001$; L1 vs L3, $\chi^2 = 71.57$, $p < 0.0001$; L1 vs L4, $\chi^2 = 108.7$, $p < 0.0001$).

To understand the evolution of *esxH* variants, we constructed whole-genome single nucleotide polymorphism (SNP)-based phylogenies for each lineage separately (Figure 3.1B). We mapped the *esxH* variations back on the phylogeny to calculate the number of times each SNP has evolved independently. Within L1, each *esxH* variant evolved once except for A10V, which evolved 3 times and could be found in one clade of four isolates and two unrelated isolates (purple
stars, Figure 3.1B). Most esxH variants were relatively rare except P9S and A10T, which were found in 17 and 41 isolates, respectively. Given several of the most frequent esxH variants within L1 represent related isolates rather than independent mutation events, we further calculated whether the acquisition rate of esxH variation in L1 was higher than expected based on the acquisition in L2, L3 and L4. By mapping the genome-wide SNP alignment back to the phylogenetic tree, we could identify 55,703 SNP mutation events within the L1 phylogeny, of which 16 were within esxH. Assuming that each lineage was equally likely to alter esxH by chance as any other, the number of variations in L1 was significantly higher than expected when compared with L3 (0 of 32400 mutations, two-sided chi-square, $\chi^2=9.308$, $p = 0.0023$) and L4 (4 of 57937 mutations, two-sided chi-square, $\chi^2=7.684$, $p = 0.0056$). The trend was consistent compared with L2 (4 of 36353 mutations, $\chi^2=1.783$, $p = 0.075$). Overall, the data suggest that L1 isolates are more likely to acquire esxH polymorphisms compared with the modern L2, L3 and L4 strains. The clustering of polymorphisms within reported T cell epitopes suggests ongoing immune pressure and adaptation between Mtb and its host.
Figure 3.1: *esxH* polymorphisms in clinical isolates are highly clustered within the known T cell epitopes.

Whole genome sequencing data were collected from Holt et al. and Walker et al. (183, 184). (A) Frequency on the Y axis and type of *esxH* polymorphisms on the X axis were graphed in reference to the polymorphic location on the gene. The color of the bars specified the lineages of each clinical isolates: purple for lineage 1 (L1), blue for L2, white for L3, and red for L4. The table indicated the number of total numbers of polymorphisms in each lineage, and the $\chi^2$ and p values were compared to L1. (B) Whole-genome single nucleotide polymorphism (SNP)-based phylogenies represented the polymorphism across all clinical isolates within lineage 1. Each circumference line signified different polymorphism noted on top in the grey box, and each purple dot cluster represented each time the indicated polymorphism evolved independently. Purple stars designated the A10V polymorphism which evolved separately 3 times. The table showed the rate of *esxH* variation acquisition by identifying the genome-wide SNP events within each phylogeny and the number of events which were within *esxH*. 
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</tr>
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<td>4/1239</td>
<td>0/529</td>
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<td>0.3%</td>
<td>0.0%</td>
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</tbody>
</table>

*Also A10V mutant
**Also A10T mutants

Table 3.1: Identification of esxH nonsynonymous polymorphisms among clinical isolates. The number of times each polymorphism was identified among different Mtb lineages is shown, including the number of times each polymorphism arose independently.
Clinical isolate 667 alters hierarchy of immunodominant antigen-specific CD8 T cell responses.

To determine whether \textit{esxH} polymorphisms affect immune responses \textit{in vivo}, we used clinical isolate 667. We selected 667 for study because it has a single nonsynonymous polymorphism in \textit{esxH} that results in the A10T amino acid substitution, the most frequent variant among the clinical isolates (Figure 3.1A). Compared to the well-characterized laboratory reference strain H37Rv, 667 contains over two thousand SNPs, although there are no polymorphisms among the genes encoding other major antigens used to measure T cell responses in the mouse \textit{M. tuberculosis} model (i.e., ESAT6, Ag85B, CFP10, and MTB32a) (185).

Following Erdman aerosol infection in C57BL/6 mice, 26% of lung CD8 T cells were specific for TB10.4-11 while less than 1% were detected after 667 infection (Figure 3.2A-B). Not only did the frequency of TB10.4-specific CD8 T cells differ, but the absolute number of TB10.4-11-specific CD8 T cell was markedly lower after 667 infection, a difference which persisted during the course of infection (Figure 3.2C, Figure 3.3A). We next determined whether the T cell responses to other antigens differed. No differences were detected in the frequency or number of ESAT6-specific CD4 T cells elicited by the two bacterial strains (Figure 3.2B-C). Interestingly, the frequency and number of MTB32a\textsubscript{309-318}-specific CD8 T cells was significantly greater after 667, increasing from 2% in Erdman to 12% in 667 infected C57BL/6 lungs (Figure 3.2B-C). The difference in the number of
MTB32a309-318-specific CD8 T cells also persisted from 5 to 17 weeks post-infection (Figure 3.3B). Independent of the infecting strain, antigen-specific CD8 T cells expressed high levels of KLRG1 and low levels of CD127, consistent with a short-lived effector cell (SLEC) phenotype (Figure 3.3C). Finally, we compared the T cell response elicited by 667 and Erdman infection in 96 mice analyzed individually in 9 independent paired infections. These differences are highly reproducible and show that 667 infection elicits a different hierarchy of Mtb-specific CD8 T cells (Figure 3.2D, E).
Figure 3.2: 667 infection changes the CD8 T cell immunity hierarchy in C57BL/6.

C57BL/6 mice were infected with ~100 aerosolized Erdman or 667 mycobacteria and sacrificed at week 5 post-infection. (A) Representative flow cytometry plots of H-2Kb/MTB32a_{309-318} tetramer and H-2Kb/TB10.4_{4-11} tetramer staining of pulmonary CD8 T cells following Erdman and 667 infection in C57BL/6 mice. (B) Quantification of the percent tetramer staining and (C) the total numbers of pulmonary tetramer-specific cells between Erdman and 667 infections. (D, E) Compiled comparisons of percentages (D) and total numbers (E) of tetramer staining cells in 9 pairs of Erdman and 667 infections. Each point represents the average of each tetramer in individual infection, and each line connects the paired infections. (A-C) Data is representative of nine experiments. Each experiment performed at 4 to 6 weeks post-infection, with 4 to 8 mice per infection. (D, E) Compilation of nine experiments and total of 96 mice are shown. n= 48 Erdman-infected and n=48 667-infected C57BL/6 mice. Statistical testing by a two-tailed, unpaired Student’s T test. **, p<0.01; ***, p<0.005; and ****, p<0.0001.
Figure 3.3: Alteration of CD8 T cell immunodominant hierarchy persists during chronic infection in C57BL/6.

Total number of (A) TB10.4-11 and (B) MTB32a309-318-specific T cells detected by tetramer staining in the lung of infected C57BL/6 mice over the course of infection. (C) Proportion of tetramer-specific and tetramer-nonspecific CD8 T cells that were KLRG1+ or CD127+. Data is representative of three kinetic experiments. Each experiment performed at 5 and 17 weeks post-infection, with 5 mice per group.
The effect of variations in the TB10.44-11 epitope on T cell recognition

We wished to understand why the A10T amino acid exchange in TB10.4 protein abrogated the CD8 T cell response to the TB10.44-11 epitope. As the preponderance of esxH polymorphisms are within the TB10.44-11 epitope, we used the Immune Epitope Database tools to predict differences between the abilities of the variant epitopes to bind class I MHC (186). Only small differences were predicted among the variant epitopes (i.e., P9S, A10T, A10V and M11I) to bind K\(^b\) compared to the most common sequence (i.e., IMYNYPAM, hereafter referred to as “WT”) and all had IC50 values between 5.6 – 9.5 nM (Table 3.2). To experimentally confirm these predictions, we performed an RMA-S K\(^b\) stabilization assay using synthetic WT, P9S, A10T, A10V and M11I peptides (Table 3.3). WT peptide stabilized K\(^b\) expression as well as the SIINFEKL control (Figure 3.4A). Contrary to the prediction algorithm, peptides A10T, M11I, A10V, and P9S were all slightly less effective at stabilizing K\(^b\) than WT peptide. The non-binding control with Y6A/Y8A mutations was designed and predicted to bind K\(^b\) 200-fold less well than the WT epitope, and failed to stabilize K\(^b\) (Table 3.2, Figure 3.4A).
<table>
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<th>tap score</th>
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Table 3.2: IEDB processing analysis: H-2-K\(^b\) Processing prediction results

Predicted processing and binding strength of polymorphic TB10.4\(^4\)-11 epitopes to H2-K\(^b\) MHC-I by [www.iedb.org](http://www.iedb.org) prediction algorithm (186). Each column indicated scores for different processing components and predicted total binding affinity (MHC ic50).

The differences in MHC binding among the variant peptides did not seem large enough to abrogate T cell recognition. However, alterations of the residues that are in contact with the TCR can also affect T cell recognition. To address whether the variations in the TB10.4\(^4\)-11 sequence affect T cell recognition, we used primary TB10.4\(^4\)-11-specific CD8 T cell lines elicited by Erdman Mtb which has the "WT" sequence of the TB10.4\(^4\)-11 epitope (i.e., IMYNYPAM) (Chapter II and (166)). The TB10RgR CD8 T cell line recognized and proliferated in a dose dependent manner to all polymorphic epitopes with the following hierarchy WT ~ M11I > A10T ~ A10V > P9S (Figure 3.4B), an order similar to their ability to stabilize cell surface
expression of K\textsuperscript{b} on RMA-S cells. Thus, we infer that peptide binding to K\textsuperscript{b} was largely driving T cell recognition.

We used additional TB10.4\textsubscript{4-11}-specific CD8 T cell lines (TB10Rg3, TB10RgL and TB10RgP) with distinct TCRs and compared their abilities to recognize the WT and A10T epitopes. All four T cell lines recognized the two TB10 epitopes with a similar hierarchy: TB10RgP ~ TB10Rg3 > TB10RgR > TB10RgL (Figure 3.4C). While TB10RgP, TB10Rg3, and TB10RgR all recognized the WT and A10T epitopes similarly, TB10RgL only recognized the A10T epitope with low avidity (Figure 3.4D). Importantly, the TCRs used by TB10RgR and TB10RgL only differ by two amino acids – one in the CDR3\textsubscript{a} region and one in the CDR3\textsubscript{b} region (Figure 2.1A). As both of these T cells were elicited by the WT epitope, and were clonally expanded \textit{in vivo} (161), these data show the fine specificity of TCRs for their cognate antigens, and reveals how T cells elicited against one Mtb strain (including vaccine strains) may be unable to recognize other Mtb strains. Importantly, these data also show A10T epitope of 667 can both bind to K\textsuperscript{b} and be recognized by T cells. Thus, CD8 T cells should have the capacity to recognize and respond to epitopes with the EsxH\textsuperscript{A10T} polymorphism in 667 infection. The absence of an effective TB10.4\textsubscript{4-11}-specific CD8 T cells response following 667 infection could exemplify the use of escape mutation or dysfunctional APC as another immune evasion strategy.
Table 3.3: Sequences of peptide used in this study.

TB10, A10T, A10V, P9S, M11I, non-binder and positive peptides were used in RMA-S and T cell proliferation assays. The 21-peptide array containing 15- or 16-mers overlapping 11-mer spanning the TB10.4 protein were obtained from BEI resource and used in screening for possible cryptic epitopes.
Figure 3.4: TB10.4-11 epitope variants bind and stimulate T cell recognition.

(A) RMA-S H-2Kb stabilization assay with titrated concentrations of positive control peptide SIINFEKL (brown), WT TB10.4-11 (blue), A10T (red), A10V (green), P9S (orange), M11I (purple), and negative control IMANAPAM (black). H-2Kb surface expressions were presented by the median of fluorescent intensity from antibody staining and flow cytometry. (B) IMYNYPAM-specific TB10RgR T cell proliferation assay was measured by eFlour450 proliferation dye dilution at 48 hours post-stimulation with thioglycolate-elicited peritoneal macrophages pulsed with indicated polymorphic peptides at titrated concentrations. (C, D) TB10RgP, TB10Rg3, TB10RgR, and TB10RgL CD8 T cell lines with different TCR and avidity were used in the T cell proliferation assays described above with WT TB10 peptide (C) or A10T peptide (D). Data is representative of four experiments.
**H-2K^b/TB10.4_{4-11}^{A10T} tetramer verifies the loss of TB10.4_{4-11}-specific CD8 T cells**

Although CD8 T cells elicited by Erdman recognized the polymorphic EsxH^{A10T} epitope, we considered the possibility that CD8 T cells elicited by 667 might not recognize the WT epitope. In other words, could the loss of the TB10.4_{4-11}-specific CD8 response following 667 infection arise because of a failure of the K^b/IMYNYPAM tetramers to bind TCRs specific for IMYNYP^TM? To evaluate this possibility, we used H-2K^b/TB10.4_{4-11}^{WT} tetramer (i.e., loaded with the IMYNYPAM epitope) as well as tetramers loaded with the H-2K^b/TB10.4_{4-11}^{A10T} epitope (i.e., IMYNYP^TM). Following 667 infection, dual tetramer staining showed that CD8 T cells elicited by Erdman infection bound preferentially to H-2K^b/IMYNYPAM tetramers while 667 infection elicited TB10.4-specific CD8 T cells were only detected using H-2K^b/IMYNYP^TM tetramers (Figure 3.5A). When staining the tetramers independently, H-2K^b/IMYNYP^TM tetramers detected more TB10.4-specific CD8 T cells than the H-2K^b/IMYNYPAM tetramers in 667-infected animals, although the number of events was only incrementally greater (Figure 3.5B). Thus, there is a significant reduction of TB10.4_{4-11}-specific CD8 T cells after 667 infection.

We further sought to confirm the specificity of tetramers to the antigen-specific cells following 667 and Erdman infection. To assess, we performed titrated H-2K^b/TB10.4_{4-11}^{WT} and H-2K^b/TB10.4_{4-11}^{A10T} tetramer staining on Mtb infection-elicited pulmonary cells. We observed highly specific CD8 T cells elicited from
Erdman infection to H-2K\textsuperscript{b}/IMYNYPAM tetramer, both frequency and mean fluorescent intensity (MFI) (Figure 3.5C, D, E). The H-2K\textsuperscript{b}/IMYNYPAM tetramer detected a lower frequency of cells from 667 infection, which also had a lower MFI and a lower avidity based on the tetramer-binding dose response (Figure 3.5E). Similarly, the H-2K\textsuperscript{b}/IMYNYPTM tetramer did not bind as well to the Erdman-elicited CD8 T cells (Figure 3.5C, D, F). 667-elicited CD8 T cells, on the other hand, were detected at higher frequencies and higher MFI when stained with H-2K\textsuperscript{b}/IMYNYPTM tetramer, indicating the superior specificity (Figure 3.5C, D, F). Lastly, the detection of H-2K\textsuperscript{b}/MTB32a tetramer was not affected by the presence of either H-2K\textsuperscript{b}/TB10.44.11 tetramers (Figure 3.5 E, F). Together, this result depicted the preferential specificity of Erdman-elicited CD8 T cells to H-2K\textsuperscript{b}/IMYNYPAM tetramer and 667-elicited CD8 T cells to H-2K\textsuperscript{b}/IMYNYPTM tetramer.
Figure 3.5: H-2K\textsuperscript{b}/TB10.4\textsubscript{4-11}\textsuperscript{A10T} tetramer verifies the loss of TB10.4\textsubscript{4-11}-specific CD8 T cells

(A) Representative flow cytometry plot of H-2K\textsuperscript{b}/TB10.4\textsubscript{4-11}\textsuperscript{WT} and H-2K\textsuperscript{b}/TB10.4\textsubscript{4-11}\textsuperscript{A10T} dual tetramer staining of CD8 T cells from the lungs of Erdman- or 667-infected C67BL/6 mice. (B) Total numbers of pulmonary TB10.4-specific CD8 T cells determined by staining with H-2K\textsuperscript{b}/TB10.4\textsubscript{4-11}\textsuperscript{WT} or H-2K\textsuperscript{b}/TB10.4\textsubscript{4-11}\textsuperscript{A10T} tetramers. Each point represents an individual infected mouse from Erdman (filled circle) or 667 (empty circle) infections. n=40 mice from 8 independent infections. (C-F) H-2K\textsuperscript{b}/IMYNYP\textsuperscript{A}M or H-2K\textsuperscript{b}/IMYNYP\textsuperscript{T}M tetramers, together with H-2K\textsuperscript{b}/MTB32\textsubscript{a309-318} tetramer, were serially diluted from 1:100 to 1:25600 to detect antigen-specific cells following Erdman and 667 infections. Cells were gated on CD8 T cell populations. (C) Percentages and (D) mean fluorescent intensity (MFI) of the Erdman-elicited (filled) and 667-elicited (empty) CD8 T cells were graphed for positive H-2K\textsuperscript{b}/IMYNYP\textsuperscript{A}M (black) or H-2K\textsuperscript{b}/IMYNYP\textsuperscript{T}M (red) tetramer staining at different tetramer concentrations. (E-F) Representative flow cytometry plots of (E) H-2K\textsuperscript{b}/IMYNYP\textsuperscript{A}M or (F) H-2K\textsuperscript{b}/IMYNYP\textsuperscript{T}M tetramer staining at indicated dilutions.
The H2-K\textsuperscript{d}-restricted TB10.4\textsubscript{20-28}-specific CD8 T cell response is unchanged after 667 infection

Mtb infection of BALB/c mice elicits H-2K\textsuperscript{d}-restricted CD8 T cells specific for TB10.4\textsubscript{20-28} and I-A\textsuperscript{d}-restricted CD4 T cells specific for TB10.4\textsubscript{74-88} epitope (157, 158). Although the EsxH\textsuperscript{A10T} polymorphism is located outside of the TB10.4\textsubscript{20-28} and TB10.4\textsubscript{74-88} epitopes, we sought to determine whether the EsxH\textsuperscript{A10T} polymorphism affected these T cell responses. Erdman- or 667-infected BALB/c mice were found to generate similar frequencies and numbers of both TB10.4\textsubscript{74-88}-specific CD4 T cell and TB10.4\textsubscript{20-28}-specific CD8 T cell responses (Figure 3.6 A-C). This result suggested that TB10.4 proteins were expressed at similar amounts in 667 bacteria compared to Erdman bacteria during \textit{in vivo} infections. To examine an independent epitope recognized by CD8 T cells, we quantified the response to the EspA\textsubscript{150-158} epitope (157). Both 667 and Erdman elicited similar frequencies of EspA\textsubscript{150-158}-specific CD8 T cells in the lungs of infected mice (Figure 3.6 A-C). The similar CD8 T cell responses following 667 or Erdman infection in BALB/c mice, in contrast to the shift in the CD8 T cell hierarchy observed in C57BL/6 mice, indicates a preferential interaction of specific class I MHC alleles and variant epitopes.
Figure 3.6: 667 infection elicited similar CD8 T cell immunity in BALB/c.

BALB/c mice were infected with ~100 aerosolized Erdman or 667 Mtb and sacrificed at week 5 post-infection. T cell responses were analyzed by tetramer staining and flow cytometry. (A) Representative flow cytometry plot of I-A^d/TB10.4_{74-88}-specific CD4 T cells (top) and H-2K^d/TB10.4_{20-28} or H-2K^d/EspA_{150-158}-specific CD8 T cells (bottom) following Erdman and 667 infection in BALB/c mice. (B) Percentages and (C) total cell numbers of tetramer specific cells from 30 mice from 3 independent timepoints of Erdman (dark purple, dark green, black) or 667 (light purple, light green, grey) infections were graphed. Statistical testing by a two-tailed, unpaired Student’s T test.
No cryptic epitope emerged in the absence of immunodominant epitope

When the immunodominant epitope of ESAT6\textsubscript{1-20} was ablated, new sub-dominant T cell responses to cryptic epitopes of ESAT6 emerged (187). Accordingly, we hypothesized that a loss of the TB10.4\textsubscript{4-11}-specific CD8 T cell responses may lead to the emergence of new TB10.4 epitopes recognized by CD8 T cells following 667 infection in C57BL/6 mice. We used a TB10.4 peptide library consisting of 15mers overlapping by 11 amino acids, supplemented with known peptides corresponding to known epitopes (Table 3.3). These were used to screen the pulmonary and splenic T cells elicited by 667 infection, to identify any possible novel CD8 T cell responses. Consistent with the flow cytometry data using tetramers, we did not detect IFN\textgamma production in response to the immunodominant TB10.4\textsubscript{4-11} epitope in 667 infection, while the MTB32a\textsubscript{309-318} sub-dominant epitope response increased in magnitude (Figure 3.7A). However, no new epitopes from TB10.4 were identified. When screening the peptide library using T cells obtained from the lungs of infected BALB/c mice, we detected IFN\textgamma production that was proportional to the frequencies of antigen-specific T cells based on tetramer staining: similar levels of TB10.4\textsubscript{74-88} and slightly decreased TB10.4\textsubscript{20-28} responses in 667-infected samples (Figure 3.7B). To control for possible genetic variations in antigen presentation between BALB/c and C57BL/6 mice, we infected CB6F1 (C57BL/6 x BALB/c F1) mice, and only responses to the known H2\textsuperscript{b}- and H2\textsuperscript{d}-restricted epitopes were detected (Figure 3.7C). Therefore, unlike ESAT6, no cryptic TB10.4 epitopes emerged after ablation of the immunodominant epitope.
Figure 3.7: No cryptic epitope in the absence of immunodominant epitope.

Single-cell suspensions from the lungs of Erdman- or 667-infected (A) C57BL/6 (B) BALB/c or (C) CB6F1 mice were isolated and incubated with a TB10.4 peptide library (21 peptides of 15mers overlapping by 11 amino acids) and the indicated control peptides. Supernatants were collected at 48 hours post-stimulation and IFNγ secretion was detected by ELISA. Data is representative of at least two experiments. Each experiment performed at 5 weeks post-infection, with 3-5 mice per infection.
667 infection induces less severe disease outcomes

To assess whether the altered CD8 T cell response affected the virulence of the two bacterial strains, we compared the ability of Erdman and 667 to cause disease in C57BL/6 mice after low-dose infection by the aerosol route. Across 13 independent experiments, 667-infected mice had fewer bacteria in their lungs and spleens compared to Erdman-infected mice (Figure 3.8A-B). In two independent experiments, 667-infected mice had prolonged survival compared to Erdman-infected mice, suggesting that 667 is less virulent than Erdman (Figure 3.8C). The ability of mice to resist mycobacterial infection is ultimately determined by both innate and elicited immunity. However, the intrinsic virulence of the bacterial strain could also play a role. For example, while 667 is a clinical isolate, and therefore by definition is a pathogenic strain, Erdman has been passaged through mice to maintain its virulence in the mouse model.

To address this question, C57BL/6 and RAG1 KO mice were infected with a pool of barcoded Mtb clinical isolates, which also contained Erdman and H37Rv, by intravenous route. In the absence of pressure from the adaptive immune response, 667 grew more (ie. fold change from day 1 to week 2 or week 3 post-infection) than Erdman in the spleens of RAG1 KO mice (Figure 3.8D). Both Erdman and 667 strains were similarly controlled by adaptive immunity, as indicated by the similar increase in the relative proportion of the strains between 2 to 3 weeks in the spleens of C57BL/6 mice. The situation differed in the lung;
Erdman had greater overall fitness in the lung, although the relative increase between week 2 to week 3 post-infection in RAG1 KO was similar for both Erdman and 667 (Figure 3.8E), indicating that 667 increased in abundance between week 2 and week 3 post-infection in C57BL/6 mice. Together, these data show that based on its fitness in immunodeficient mice and its ability to compete in the spleen, 667 is not intrinsically attenuated. However, the reduced fitness of 667 in the lung, compared to Erdman, is consistent with the prolonged survival of mice following 667 infection.
Figure 3.8: Infection with 667 is less virulent than Erdman.

The bacterial burden as measured by CFU from lung (A) or spleen (B) homogenates from Erdman (filled) or 667 (open) infected C57BL/6 at different timepoints post-infection. CFU data were compiled from 13 (lung) or 6 (spleen) independent experiments, from 4 to 30 weeks post-infection. (C) The survival of C57BL/6 mice after Erdman (solid) or 667 (dashed) infection, which is one of two independent experiments with similar results. In this experiment, the d1 CFU was 158 (667) or 55 (Erdman). (D-E) A barcoded pool of clinical isolates was administered intravenously to C57BL/6 or RAG1 KO mice, and 5 mice of each strain were harvested, CFU recovered 1, 14, and 21 days post-infection. The relative abundance of the different Mtb strains in the (D) spleen or (E) lung was determined by NextSeq deep sequencing and the BARTI pipeline (188).
Discussion

Specific clinical isolates of *Mycobacterium tuberculosis* have been shown to induce different immune responses, resulting in differential pathogenicity and disease outcomes (189-191). Clinical isolate CDC1551 was identified by a remarkably high rate of TST conversions during an Mtb outbreak but resulted in a low number of active TB cases. It was subsequently shown that CDC1551 elicited earlier and more vigorous Th1 cytokine response in the lung of infected mice compared to Erdman and H37Rv lab strains (190, 192). In contrast, HN878 was associated with a strikingly high proportion of active TB and extrapulmonary disease cases (192); it was later determined that HN878 infection failed to produce an effective Th1 immune response (189). These findings indicate that genetic diversity of Mtb impacts immunological responses and leads to differential disease outcomes (189-191). They also raise the question of what type of immune responses are generated in individuals infected with mixed variant Mtb strains (193). In addition, phenotypical antigen-specific T cell responses have never been examined following infection with Mtb strains bearing polymorphisms in the genes encoding antigens.

As most of Mtb-encoded T cell epitopes are more conserved than its essential genes, the lack of antigen-specific T cell studies is partly due to the low sequence variability (110). Comas et al. speculated that some detection of Mtb-infected cells by host immunity might be beneficial for the mycobacteria to establish a latent infection in the granulomas or contribute to transmission (110).
The conservations were confirmed with the unbiased computational study, with the exception of a handful of genes including esxH (111). Here, in addition to the previous study showing that esxH is highly polymorphic, we further reveal the naturally occurring polymorphic hotspots within previously reported human CD8 T cell epitopes of esxH genes that are highly prevalent in lineage 1 of Mtb. The high frequency of amino acid substitutions in TB10.4 in the Indian Ocean of lineage 1 further suggests the co-adaptation with a subset of human leukocyte antigens and ongoing immune evasion.

We determined that the A10T single amino acid exchange in TB10.44-11 epitope in clinical isolate 667 resulted in the frequency of TB10.44-11-specific CD8 T cell responses falling from ~30% (after infection with Erdman or H37Rv) to 1%, possibly reflecting the interference either with antigen presentation or T cell recognition. This is surprising because TB10.44-11-specific CD8 T cells can recognize the polymorphic TB10.44-11 epitopes similarly, and the small reduction in the recognition sensitivity cannot justify for the 30% CD8 T cell loss. In addition, the use of H-2K^b/TB10.44-11^{A10T} helped identified a different TCR repertoire of antigen-specific CD8 T cells in 667 infected mice. This alteration of TCRs in polymorphic epitopes has previously been reported in SIV infections (194) as well as influenza (195).

The Maurer group assessed human CD8 T cell specificity to TB10.44-11^{A10T} by generating human multimers with common MHC allotypes in a South African population. The researchers inferred in their study that most of the esxH
polymorphic substitutions decreased the binding to human class I MHC and, hence, the peptide presentation. However, both the nature of class I MHC molecule and amino acid composition of the epitope determine the affinity of binding affect the priming and outcome of antigen presentation. Human HLAs used in this study are different from H-2Kb in C57BL/6 mice. In addition, while the researchers detected a small population of TB10.44-11A10T-specific CD8 T cells in PBMC from Mtb-infected patients (154), the study did not identify the bacterial strains that subjects were infected with. The undetermined genotypes of Mtb preclude the understanding of whether the multimer-detected CD8 T cells were generated by the TB10.44-11A10T epitope or were instead cross-reactive from the WT epitope.

Interestingly, the typical subdominant response to MTB32a309-318 in Erdman-infected C57BL/6 mice became more dominant during 667 infection. This phenomenon of TB10.44-11 immunodominating MTB32a309-318 was strikingly similar to cowpox viruses (CPXV) where the deletion of the immunodominant epitope from CPXV gave rise to a compensation and domination by subdominant epitopes (137). In the other words, this finding implies that TB10.44-11 may inhibit MTB32a309-318 from inducing antigen-specific CD8 T cell response.

Mice infected with 667 exhibit prolonged survival compared to Erdman-infected mice. During the competitive Mtb pool infection, 667 grows intrinsically slower from day 1 to day 14 post-infection (prior to the initiation of adaptive immunity) compared to Erdman and H37Rv. However, in immunodeficient mice,
the results suggest that 667 is as virulent as Erdman, if not more. Nevertheless, bacterial burdens in the lungs and spleens of 667-infected C57BL/6 are equal or lower than Erdman-infected mice. Together, 667 appears to be subject to clearance by immune responses more efficiently than Erdman Mtb.

This chapter shows that naturally occurring polymorphisms can have drastic effects on host immune responses and is evidence that Mtb could employ immunodomination over subdominant CD8 T cell responses as an evasion strategy. Since TB patients are prone to re-infection (196) and most people in developing countries are BCG-vaccinated, pre-existing immune responses and the genetic diversity of Mtb could impact the outcome of subsequent Mtb infections. A better understanding of T cell immune response to clinical isolates of Mtb will be of value in determining the nature of clinically relevant responses, which in turn could benefit novel TB vaccine designs.
CHAPTER IV. THE EFFECTS OF A10T AMINO ACID SUBSTITUTION OF MYCOBACTERIUM TUBERCULOSIS ESXH PROTEIN ON HOST CD8 T CELL RESPONSES

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Attributions:

K.M., K.P. and C.S. provided guidance in strategies to use for the generation of isogenic Erd⁴⁰⁰ WT and Erd⁴¹⁰ T Mtb. K.M. and K.P. designed and assisted in generating isogenic Erd⁰ WT and Erd⁴¹⁰ T Mtb in Figure 4.1. Shayla B. provided assistance in performing experiments in Figure 4.2 and 4.3. R.S. and S.B designed all other experiments. R.S. performed all experiments and statistical analysis. S.B. provided critical feedback for interpretation of the data.
Abstract

To verify that the change in antigen specificity was not influenced by other genetic polymorphisms in the Mtb clinical isolate, we generated Mtb isogenic strains expressing either EsxH\textsuperscript{WT} or EsxH\textsuperscript{A10T}. Given the essentiality of esxH, the genetic engineering process was achieved by oligo-mediated recombineering to bypass the creation of a genetic deletion strain. Using the isogenic strains, I confirmed that A10T amino acid substitution in TB10.4 caused the drastic shift in immunodominant hierarchy of CD8 T cell responses. The results from infections with isogenic strains validate the immunodomination phenomenon during Mtb infection that could impact the frequency of CD8 T cells that can recognize and contribute to the control of Mtb infection.
Introduction

Chapter III results showed that naturally occurring polymorphisms between pathogenic human isolates of Mtb strains can alter the hierarchy of antigens that CD8 T cells recognize and can affect the outcome of infection. However, there are over two thousand single nucleotide polymorphisms (SNPs) in the Erdman chromosome compared to 667, any of which could affect these processes. To demonstrate that the A10T amino acid substitution of EsxH protein in 667 Mtb is responsible for the altered CD8 T cell response generated by infection, we need precisely altered bacterial strains. Below describes a brief introduction to approaches in *Mycobacterium tuberculosis* genetic engineering.

The precise modification of Mtb genomes has been central for defining the important functions in physiology and pathogenicity of this important pathogen. Previous methods of genetic modification in Mtb have relied on bacterial endogenous recombination systems for the insertion of an inactivated copy of a target gene into the bacterial chromosome using non-replicating vectors (197-199). These methods, however, generated a rare number of desired recombinants because of (1) the low frequencies of plasmids spontaneously integrating into bacterial chromosomes, (2) the inefficient uptake of DNA by electroporation, and (3) illegitimate recombination events, a long-standing problem for mycobacterial gene replacement methods (although useful for random mutagenesis schemes (200, 201). In addition, once the inactivated copy of the target gene was transferred to the chromosome, resolution of the co-integrant required a second crossover
event to complete formation of the allelic exchange recombinant. Since this event occurred at a much lower efficiency, counter-selection approaches such as using the sucrose sensitive marker sacB were adopted (199, 202, 203). The efficiency of the approach has been greatly improved by the use of a specialized transduction scheme, which uses phage to deliver recombination substrates to nearly every cell (204). This method, however, is time-consuming and demands numerous technical steps.

Though these approaches were instrumental in developing gene transfer systems for Mtb, the isogenic EsxH^{A10T}- and EsxH^{WT} expressing Erdman strains described below were established by oligo-mediated recombineering (for recombinogenic engineering). Recombineering was first introduced in Escherichia coli (E. coli) (205, 206) and developed for use in mycobacteria over the last decade to enhance the accuracy and efficiency of genetic modification (207-209). Recombineering in E. coli utilizes the phage lambda Red recombination system for linear double-stranded DNA transformation, while oligo-mediated recombineering requires only expression of the Che9 mycobacteriophage RecT annealase (the lambda Beta protein) to facilitate annealing of SNP-containing oligos to the Mtb DNA replication fork and subsequent integration.

SNPs transferred to Mtb in this fashion allow us to test if the EsxH^{A10T}, in an otherwise identical genetic background, recapitulates the phenotypical change observed in the original clinical isolate 667. Therefore, we genetically engineered isogenic strains that differed only at codon 10 in the EsxH protein to test the
following hypothesis: the A10T amino acid replacement in esxH gene is responsible for the shift in CD8 T cell immunodominance. Since EsxH protein has been described as a virulence factor and its gene knockout led to an attenuation in vivo (152), we also assessed whether the esxH^{A10T} allele affects the virulence of the isogenic strain.
Results

Generation of Isogenic EsxH\textsuperscript{WT} and EsxH\textsuperscript{A10T} in Erdman Mtb

To demonstrate that the EsxH\textsuperscript{A10T} polymorphism is responsible for the altered CD8 T cell response elicited by Mtb, we generated isogenic Erdman strains containing EsxH protein with A10T substitution by oligo recombineering as previously described (207-210) (Figure 4.1A).

The transfer of the original naturally occurring SNP in the clinical isolate 667 strain, to the Erdman strain (which has the “WT” sequence) required the use of an oligo that would create a G/T mismatch at the target site in the replication fork. This mismatch was anticipated to be repaired by the mismatch repair (MMR) system of mycobacteria (K. Murphy, unpublished observations), creating the inefficiency in SNP transfer. To increase the frequency of SNP transfer, we designed a 144 basepair (bp) oligo to create a mismatched “bubble” where 3 out 4 bps of codon 10 and the wobble position of codon 9 were mismatched, yet still encoded only an alanine to threonine substitution in codon 10 in the TB10.4 protein. This design was based on observations from E. coli recombineering where multiple mismatches in a row caused local strand unpairing and caused the 4bp region to become unrecognizable to MMR (211). Below is the 144bp oligo in reverse complement as Okazaki fragment for the lagging strand. The 4bp “bubble” is underlined.
The EsxH<sup>A10T</sup>-conferring oligo was delivered together with an oligo encoding a K43R mutation in the RpsL protein, which confers resistance to streptomycin for selection. As such, streptomycin-resistant colonies are indicative of DNA uptake and successful recombinogenic events, increasing the probability of finding a strain obtaining the EsxH<sup>A10T</sup> modification. Candidate colonies were screened for the A10T exchange by targeted PCR analysis, which was made possible by using the “bubble” sequences in the 3’ end of the primers (see Figure 4.6 in discussion below). The selected colonies were then confirmed by Sanger sequencing.

esxH<sup>A10T</sup> forward primer 5’ TCATGTACAACTACCCACCGACC 3’
esxH<sup>WT</sup> forward primer 5’ TCATGTACAACTACCCACCGGC 3’

The genetic alteration resulted in a single amino acid change in the TB10.4<sub>4.4-11</sub>, changing the epitope from IMYNYP<sub>AM</sub> (i.e., the Erdman epitope) to IMYNYP<sub>TM</sub> (the 667 epitope). Hereafter, we refer to these isogenic strains as Erd<sup>WT</sup> and Erd<sup>A10T</sup>, respectively. The isogenic strains had equivalent expression of transcripts of the genes that encode immunodominant antigens, including esxH (Figure 4.1B).
Figure 4.1: Generation of EsxHA10T and EsxHWT isogenic strains.

(A) Schematic representation of oligo recombineering method to generate isogenic strains, previously described in (210). (B) Relative expression of Mtb (from left to right) Rv0287, Rv0288, Rv0125, Rv3875, Rv3874, Rv1886c and Rv3019c mRNAs in broth culture. Copy numbers of indicated mRNAs were determined by RT-PCR and normalized to constitutively expressed 16S rRNA. (See Materials and Method for primer information)
Isogenic Erd$^{WT}$ and Erd$^{A10T}$ Mtb stimulate different CD8 T cell immunity in C57BL/6

We determined the antigen-specific T cell responses after infection with the isogenic strains Erd$^{WT}$ and Erd$^{A10T}$. The TB10.44-11-specific CD8 T cell response was significantly reduced while the MTB32a$_{309-318}$-specific CD8 T cell response was significantly increased after infection with Erd$^{A10T}$ compared to Erd$^{WT}$ in C57BL/6 mice (Figure 4.2A, B). The magnitude of the CD4 T cell response to ESAT6, Ag85B, and EsxG, elicited by Erd$^{WT}$ and Erd$^{A10T}$ were similar and recapitulated the CD8-specific differences elicited by Erdman versus 667. In addition, similar to what we observed after Erdman and 667 infection, the Mtb-specific CD4 and CD8 T cell responses after Erd$^{WT}$ or Erd$^{A10T}$ did not differ in BALB/c mice (Figure 4.3).

To confirm these results, we compared the antigen-specific T cell responses elicited by Erd$^{WT}$ and Erd$^{A10T}$ in 5 independent paired infections containing a total of 50 C57BL/6 mice. The differences are highly reproducible and show that the EsxH$^{A10T}$ polymorphism in the 667 Mtb strain causes a shift in the hierarchy of CD8 T cell responses observed after infection of C57BL/6 mice (Figure 4.2C, D). Thus, immunodomination by the TB10.44-11 epitope may inhibit the magnitude of the CD8 response to the MTB32a$_{309-318}$ epitope, and prevent it from being a more significant part of the host T cell response to Mtb.
Figure 4.2: Infections with isogenic Erd\textsuperscript{WT} and Erd\textsuperscript{A10T} Mtb validate the immunodominant shift of CD8 T cell hierarchy in C57BL/6 mice.

C57Bl/6 mice were infected by the aerosol route with \textasciitilde100 Erd\textsuperscript{A10T} or Erd\textsuperscript{WT} Mtb and the lung mononuclear cells were analyzed 5 weeks after infection (A) Representative flow cytometry plots of; (top) MTB32a\textsubscript{309-318} tetramer and TB10.4\textsubscript{a11} tetramer binding CD8 T cells; (middle) ESAT6 tetramer binding CD4 T cells; and (C) Ag85B tetramer and EsxG tetramer binding CD4 T cells. (B) Quantification of the frequency of tetramer\textsuperscript{+} T cells (top) and the total tetramer-specific T cell numbers (bottom) elicited by infection with the isogenic Erd\textsuperscript{A10T} and Erd\textsuperscript{WT} Mtb strains. Compiled comparisons of (C) percentages and (D) total numbers of tetramer staining cells in 5 pairs of Erd\textsuperscript{A10T} or Erd\textsuperscript{WT} infections. Each point represents the average frequency or number of tetramer\textsuperscript{+} cells of individual mice, and the lines connect paired infections. (A-B) Data is representative of five experiments. Each experiment performed at 5 to 6 weeks post-infection, with 5 mice per group. (C-D) Compile of five experiments and total of 50 mice is shown. Statistical testing by a two-tailed, unpaired Student's T test. *, p<0.05; **, p<0.01; ***, p<0.005; and ****, p<0.0001.
Figure 4.3: A10T substitution of TB10.4 protein does not change the amount of antigen-specific CD4 and CD8 T cell numbers in BALB/c mice.

(A) Representative flow cytometry plots of (top) CD8 T cells specific for H-2Kd/TB10.420-28 and H-2Kd/EspA150-158 tetramers and (bottom) CD4 T cells specific for I-Ad/TB10.474-88 and I-Ad/Ag85A tetramers at 5 weeks post-infection. Quantification of the (B) frequencies and the (C) numbers of antigen-specific CD4 and CD8 T cell responses determined by tetramer staining after infection with the isogenic ErdA10T and ErdWT Mtb strains in BALB/c. Data is representative of two experiments. Each experiment performed at 5 weeks post-infection, with n=5 mice per group. Statistical testing by a two-tailed, unpaired Student’s T test.
A10T substitution of TB10.4 protein does not affect virulence and bacterial burden

Despite the change in immunodominant antigen-specific CD8 T cell responses, we did not observe differences in pulmonary or splenic bacterial loads after infection with isogenic Erd\textsuperscript{A10T} or Erd\textsuperscript{WT} Mtb strains (Figure 4.4). This result indicated that the EsxH\textsuperscript{A10T} variant did not affect the virulence of the isogenic bacterium.

**Figure 4.4: Isogenic strain infection does not impact bacterial burden.**

Comparisons of pulmonary (left) and splenic (right) colony forming units (CFU) from seven pairs of infections performed with Erd\textsuperscript{A10T} (red) or Erd\textsuperscript{WT} (blue) isogenic Mtb strains. A total of 70 mice were analyzed at 5-, 6- or 19-weeks post-infection as indicated. Each data point represents the bacterial burden from an individual mouse. The different experiments are identified by unique symbols (circle, upward triangle, downward triangle, square, or diamond), and matched symbols at 5- and 19-weeks post-infection indicate the group of mice from the same corresponding infections. Statistical testing by a two-tailed, unpaired Student’s T test.
Discussion

To our knowledge, this is the first demonstration of a single amino acid substitution causing a major antigenic shift in the Mtb-specific CD8 T cell response following infection with virulent Mtb. These results support the hypothesis that immunodomination (as discussed in Chapter III) contributes to the dominance of the CD8 response to TB10.4. These results also give further credence to the importance of pre-existing immunity as a factor to consider when developing vaccination strategies for the prevention of Mtb infections, particularly in individuals vaccinated with BCG or exposed to environmental mycobacteria.

The validation of the immunological outcomes observed after infection with the clinical isolate 667 required the generation of isogenic Erd\textsuperscript{WT} and Erd\textsuperscript{A10T} strains. This was more challenging than originally anticipated. The esx\textit{H} gene is a part of the esx3 operon, which is an essential and a large operon, spanning 11 genes and is almost 15 kB long (152). We used four different strategies to replace the endogenous esx\textit{H} gene with one containing the Esx\textit{H}\textsuperscript{A10T} modification.

(1) In the first scheme, I received a ∆esx\textit{G}-esx\textit{H} knockout H37Rv from Dr. William Jacobs (152), which was marked with a drug-resistant cassette containing the sucrose counter-selection gene, sac\textit{B}. I used dsDNA recombineering to replace this cassette with the esx\textit{H} gene bearing either the Esx\textit{H}\textsuperscript{WT} or Esx\textit{H}\textsuperscript{A10T} alleles, flanked by 500 bp of target homology DNA, and selected for sucrose resistance. However, the gene knockout cassette obtained from the Jacobs lab
contained γδ-resolvase flanking sites, which are typically used to create unmarked gene knockouts. The gene replacement attempts failed, however, because the recombination event between my linear dsDNA substrates and the chromosome was far less efficient than the spontaneous recombination event between the two chromosomally located γδ-resolvase sites. This resulted in the excision of the drug resistance marker without the incorporation of the desired DNA and generation of sucrose-resistant colonies. It is unclear if expression of the Che9c RecET proteins had an effect on the rate of spontaneous recombination between the γδ-resolvase sites.

(2) Next, I used the ORBIT technique, a Che9 RecT-promoted oligo recombineering system that employs an attP site within the oligo, a co-electroporated attB-containing non-replicating plasmid, and the expression of Bxb1 phage Integrase. This system promotes the integration of attP into the target site, followed by integration of the plasmid containing a selectable marker at the nascent attP site (210). I attempted the construction of (a) an A10T substitution in esxH and (b) knockouts of esxH (Rv0288), esxG-esxH (Rv0287-88), and esxG-esxH-espG3 (Rv0287-89). However, even with the iron supplementation to overcome the iron acquisition function of EsxH, we were not able to recover any candidates. We speculated that the disruption of separate parts of this operon was difficult to achieve because of the importance of the relative expression of its individual genes.
(3) Since we received the \( \Delta \text{esxG-esxH} \) strain, we generated complementation strains with an L5-integration plasmid expressing EsxG-EsxH\(^{WT}\) or EsxG-EsxH\(^{A10T}\) from the \( \text{groEL} \) promoter. However, I discovered that despite the normal growth in liquid and agar culture, the \( \text{groEL} \) complemented strains failed to grow during \textit{in vitro} infections and \textit{in vivo} aerosol infections. We later received the \( \Delta \text{esxH} \) KO strain and \( \Delta \text{esxH::esxG-esxH}^{WT} \)-\text{espG3} complementation strain from Dr. Jennifer Philips. The complementation plasmid contained the \( \text{hsp60} \) promoter, a homolog to \( \text{groEL} \) promoter, but with two additional adjacent genes flanking \( \text{esxH} \) in the integration plasmid construct. Through personal communications, we learned that the expression level of \( \text{esxH} \) is critical for mycobacterial virulence; too weak or too strong of expression would result in an attenuation. This could be partly explained by the operon structure, where there are only one or two promoters, and genes expressed later in the operon are likely expressed at lower levels. We later constructed the \( \Delta \text{esxH::esxG-esxH}^{A10T} \)-\text{espG3} complementation strain using \( \text{hsp60} \) promoter. The \( \text{hsp60} \)-complemented strains were able to grow during both \textit{in vitro} infections and \textit{in vivo} aerosol infections. However, the pulmonary bacterial burdens at the peak of infection (4 weeks post-infection) were over 10-fold lower than Erdman and 667 strains (Figure 4.5). The different amounts of bacteria in the lung affected how much T cell immunity was elicited. Therefore, we turned back to engineer the bacterial chromosome to preserve the virulence of the mycobacteria.
Figure 4.5: hsp60-complemented strains are less virulent than clinical isolate 667 and Erdman Mtb.

Comparisons of pulmonary (left) and splenic (right) colony forming units (CFU) after infection with 667 (blue), ΔesxH:esxG-esxH_{A10T}-espG3 (green), ΔesxH:esxG-esxH_{WT}-espG3 (orange) or Erdman (black). C57BL/6 mice were infected by the aerosol route with a dose of ~100 bacteria per mouse (n=5 mice/group).

(4) Finally, I made the recombinant esxH alleles using oligo-mediated RecT-promoted recombineering. Oligo recombineering was at first an unattractive approach due to the need to screen the individual colonies for the successful recombination event. The selection of streptomycin resistance would only indicate a higher likelihood of integration of the target oligo. Nevertheless, since we
employed the “bubble" strategy to avoid MMR, we also took advantage and used the bubble sequences as primers in PCR screening. The process became visually exciting (to observe a band or not) and less expensive (as opposed to sequencing all the candidates). Following the method described in Figure 4.1, we recovered 1 recombinant out of 9 candidates tested (Figure 4.6). Sequencing confirmed the diagnostic PCR results. The successful recombinogenic ErdA10T colony and the ErdWT (ie. failed to incorporate esxH A10T but successful at RpsL) were expanded and used in our subsequent experiments.

Figure 4.6: Diagnostic PCR screening of nine streptomycin candidates.

Comparisons of bacterial PCR of streptomycin resistant colonies using either (A) the esxHWT forward primer; or (B) the esxHA10T forward primer. In both cases the reverse primer for both PCR reactions is 5’ GACGATGCTTAGGCCGACC 3’ from espG3 locus, which generates a 1kb DNA fragment. The sequence of the esxHA10T forward primer is 5’ TCATGTACAACTACCCGACC 3’ and esxHWT forward primer is 5’ TCATGTACAACTACCCCGCG 3’.
Earlier we introduced the decoy hypothesis, namely TB10.4-specific CD8 T cells do not contribute to the recognition of Mtb-infected cells nor to the control of bacterial burden. We were able to assess this hypothesis by determining whether the ablation of the TB10.4\textsubscript{4-11}-specific CD8 T cell response (i.e., after Erd\textsuperscript{A\textsubscript{10T}} infection) improved the outcome of infection. Under the conditions we used, no differences in bacterial burden were observed between the isogenic Erd\textsuperscript{WT} and Erd\textsuperscript{A\textsubscript{10T}} infected mice. However, since the impact of CD8 T cells is generally modest in the presence of CD4 T cells (40), a 30\% loss of TB10.4\textsubscript{4-11}-specific CD8 T cells may not be enough to have an effect on bacterial load \textit{in vivo}. In addition, we learned from Chapter II that MTB32a\textsubscript{309-318}-specific CD8 T cells did not recognize infected macrophages (Figure 2.5). This could also suggest that MTB32a is another decoy antigen. Thus, the compensatory gain of MTB32a\textsubscript{309-318}-specific CD8 T cells may not have been capable of enhancing host resistance to infection. To further evaluate the decoy hypothesis, we next assessed the qualitative differences of T cell responses induced by Erd\textsuperscript{A\textsubscript{10T}} and Erd\textsuperscript{WT} infection in Chapter V.
CHAPTER V. DIFFERENTIAL RECOGNITION OF T CELLS GENERATED BY POLYMORPHIC MYCOBACTERIUM TUBERCULOSIS INFECTIONS

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Attributions:

S.B. provided assistance in all experiments. Y.P. performed the experiment in Figure 5.1 and 5.2. RS performed parts of the experiment in Figure 5.1 and all other experiments. R.S., Y.P. and S.M.B designed the experiments. R.S. and S.M.B performed statistical analysis. S.M.B. provided critical feedback for interpretation of the data.
Abstract

We previously reported that TB10.4\textsubscript{4-11}-specific CD8 T cells do not recognize Mtb-infected macrophages and speculated that TB10.4 acts as a decoy antigen. In this chapter, I investigate whether polyclonal CD8 T cells following infections with clinical isolate Mtb and isogenic strains recognize Mtb-infected macrophages differentially. Specifically, we assess the qualitative functionality of CD8 T cell responses with or without TB10.4\textsubscript{4-11} specificity. Our results demonstrate that immunodomination by TB10.4 potentially impairs the CD8 T cell response to Mtb by diminishing the expansion of other CD8 T cells, some of which might recognize Mtb-infected macrophages and mediate protection.
Introduction

Mtb infection-elicited CD4 and CD8 T cell responses in both humans and animal models are widely appreciated for their role in immunity to control diseases (40, 167). Most studies assess the immunogenicity of vaccine by using crude Mtb fractions, peptides or recombinant proteins as antigens, presuming that most Mtb antigen-specific T cells elicited by natural infection will recognize Mtb-infected cells. However, the cell numbers and cytokine responses by antigen-specific T cells after stimulation with antigens have not correlated with the protection efficacy of vaccines (52, 212).

We have demonstrated that immunodominant TB10.4-11-specific CD8 T cells do not recognize Mtb-infected macrophages (Chapter II and (166)). Other studies have shown that CD4 T cells specific for Ag85b_{240-254}, another immunodominant antigen, responded poorly in granulomas due to constriction of antigen locations (98, 127). Importantly, direct recognition of infected cells by CD4 T cells is required for optimal control of Mtb infection (97).

Following aerosol infection, T cells are primed by dendritic cells in lung draining MDLN before trafficking back to the lung (41, 43). We consider that the antigens presented (or cross-presented) by uninfected DCs in the lymph nodes perhaps differ from the antigens presented by infected macrophages in the lung. Thus, MDLN-primed T cells may not recognize Mtb-infected cells in the lung. This
could also explain why the number of antigen-specific T cells may not correlate with vaccine-induced protection.

To qualitatively evaluate the differences of T cells elicited from Erdman, 667, and isogenic Erd\textsuperscript{WT} and Erd\textsuperscript{A10T} infections, we developed modified IFN\textsubscript{γ} ELISpot and intracellular cytokine staining (ICS) assays, using a low multiplicity of infection (MOI) to assess T cell recognition. By specifically measuring the frequency of T cells that recognize Mtb-infected macrophages, this assay could provide another criterion to help elucidate the differences in T cell immunity elicited by polymorphic mycobacteria and clinical isolates.
Results

Development of Mtb-Infected Macrophage ELISpot and ICS (MIM-E and MIM-ICS) to assess T cell recognition ability.

We modified our established *in vitro* macrophage infection model (166), and aimed to maximize the proportion of infected macrophages, preserve macrophage viability, and achieve physiologically-relevant low numbers of bacteria per macrophage (213) for the Mtb-Infected Macrophage ELISpot (MIM-E) assay. At a multiplicity of infection (MOI) of 4, we found that 70% of macrophages were infected with 93% viability after 18–24 h (Figure 5.1A, B). In contrast, fewer than 35% of macrophages were infected at a MOI of 1 (Figure 5.1C). Although 90% of the macrophages were infected at a MOI of 20, macrophage viability decreased drastically (Figure 5.1C). The median actual MOI was 5 bacteria per macrophage and ranged from 2–13 (Figure 5.1D). Thus, a MOI of 4 maximized the percentage of infected macrophages while maintaining cell viability.

To first test the sensitivity of the assay, we diluted ESAT6-specific CD4 T cells (hereafter, C7 T cells) into an excess of naive splenic T cells from uninfected C57BL/6 mice, and performed an IFN\(_\gamma\) enzyme-linked immunospot (ELISpot) assay to measure the frequency of T cells that recognize Mtb-infected macrophages. Minimal cytokine production of C7 T cells occurred when culturing with uninfected macrophages (Figure 5.1E). The naive C57BL/6 T cells produced undetectable amount of IFN\(_\gamma\) when cultured with infected macrophages or ESAT63.
peptide-pulsed uninfected macrophages (data not shown). When the C7 and naive T cell mixture was co-cultured with Mtb-infected macrophages, we observed IFN\(\gamma\) spots at \(~40\text{−}50\%\) yield of the input C7 cells, and the ELISpot assay sensitively detected as low as 0.04\% of C7 present in the total T cells (Figure 5.1E). When ESAT6\(_{3-17}\) cognate peptide was supplemented, \(~150\) spots per 200 C7 T cells produced IFN\(\gamma\) (i.e., 70 \text{−}80\% of input C7 T cells in Figure 5.1E). Application of the assay to Ag85b-specific CD4 T cells (hereafter, P25 T cells) showed that over 50\% of the P25 T cells that recognized Ag85b\(_{240-254}\) cognate epitope could recognize Mtb-infected macrophages (Figure 5.1F).

We next adapted the MIM-E assay for a flow cytometry-based technique. Pulmonary T cells from infected mice were cultured with Mtb-infected macrophages and analyzed by both ELISpot (MIM-E) and intracellular cytokine staining (MIM-ICS) in parallel. Both assays resulted in similar frequencies of CD4 and CD8 T cells that produced IFN\(\gamma\) in response to Mtb-infected macrophages (Figure 5.2A-C).

Based on our results, the Mtb-Infected Macrophage ELISpot (MIM-E) and ICS (MIM-ICS) represent two sensitive and specific approaches to identify T cells that recognize Mtb-infected macrophages.
Figure 5.1: Measuring T-cell recognition by the Mtb-infected macrophage ELISpot (MIM-E)

(A-B) After infection with H37Rv or Rv.YFP at an MOI of 4 for 18 h, CD11b^+ purified thioglycolate-elicited peritoneal macrophages (TGPM) were measured for the percentage of cells that were (A) infected and (B) viable. (C) TGPM were infected with Rv.YFP at MOI of 1, 4 or 20, for 18–24 h and the percentage of infected macrophages and cell viability were assessed. (D) The actual MOI was determined by plating CFU. (E) The Mtb-infected macrophage ELISPOT (MIME) assay was performed by infecting macrophages with H37Rv at an MOI of 4, for 18–24 h. A titrated number of C7 T cells were mixed with polyclonal T cells (10^5/well) from uninfected mice and added to Mtb-infected macrophages (10^5/well). The ESAT-6_{3-17} peptide was added to the wells, indicated in blue. (F) MIME assay was performed with P25 T cell line. UI indicated uninfected macrophages, Mtb for Mtb-infected macrophages, pep for Ag85b_{240-254} peptide, and αCD3 for soluble anti-CD3 mAb. Two independent experiments are shown. Each experiment was normalized by subtracting the background and defining the αCD3 response as the maximal (i.e., 100%) response. Data are representative of two independent experiments with three technical replicates per experiment that yielded similar results (A, B, C, E, F), or 12 independent experiments with three technical replicates (D)
Figure 5.2: Similar frequencies of T cells recognizing Mtb-infected macrophages are detected by the MIM-E assay and MIM-ICS.

Purified pulmonary CD4 or CD8 T cells were cultured with Mtb-infected macrophages, subjected to intracellular cytokine staining (ICS) and analyzed by flow cytometry. (A) Representative flow plots showed the frequency of pulmonary CD4 (left column) or CD8 (right column) T cells expressing CD69 and producing IFNγ after 6-hour culture with macrophages with (top row) or without (bottom row) Mtb infection. (B-C) The MIM-E or the Mtb-infected macrophage-ICS (MIM-ICS) assay were used to calculate the frequency of CD4 (B) or CD8 (C) pulmonary T cells that recognized Mtb-infected macrophages. U indicated uninfected macrophages, Mtb indicated Mtb-infected macrophages, and n.s. signified not significant (t test).
**CD4 and CD8 T cells differ in their ability to recognize Mtb-infected macrophages**

Total of T cell recognition of individual Mtb antigens should hypothetically be similar to that of Mtb-infected macrophages. To assess this assumption, we took advantage of the megapool of 300 peptides (p300) representing 90 antigens frequently recognized by human CD4 T cells from healthy IGRA+ individuals (214). The p300 peptide pool contains epitopes that are also recognized by murine CD4 and CD8 T cells (based on data available in the IEDB). The advantages of the megapool over other crude Mtb antigens such as PPD, culture filtrate proteins or Mtb lysate are the equimolar concentrations among all epitopes, and that the epitopes are in the form of peptides rather than proteins, allowing efficient presentation by class I MHC. We compared IFNγ production by pulmonary T cells from C57BL/6 mice of 4-week post Erdman Mtb infection with H37Rv-infected macrophages or the p300 megapool. The CD4 T cell response was higher toward Mtb-infected macrophages than to the p300 megapool (Figure 5.3A), indicating that the p300 megapool does not represent all epitopes presented by Mtb infection of murine macrophages. In contrast, CD8 T cells responded better to p300 megapool stimulation (Figure 5.3A). The skewed CD8 T cell response suggested the mismatched repertoire of antigens used to prime CD8 T cells (such as TB10.41–15 of p300) compared to the antigens presented by Mtb-infected cells. In addition, the proportion of IFNγ producing CD4 T cells was higher than that of CD8...
T cells, indicating that polyclonal CD4 T cells recognize Mtb-infected cells better than polyclonal CD8 T cells at the peak of infection (Figure 5.3A).

The number of intracellular Mtb could impact how much CD4 and CD8 T cells can recognize the infected macrophages. A study by Lewinsohn et al. showed that a human CFP10-specific CD8 T cell clone only recognized DCs with high numbers of infecting bacteria (179). To determine whether the bacterial dosage affects polyclonal T cell recognition, we performed MIM-ICS assay of purified CD4 and CD8 T cells from the lungs of infected C57BL/6 mice with infected macrophages using a range of MOIs. Polyclonal CD4 T cells readily recognized infected macrophages and produced IFNγ at a low MOI of 0.2, while the recognition increased at a MOI of 1.2 and plateaued at a MOI of 5.8 (Figure 5.3B). In contrast, polyclonal CD8 T cells did not significantly recognize Mtb-infected cells at low MOI of 0.2 and 1.2, but required a higher MOI for recognition and IFNγ production. (Figure 5.3B). This result indicated that pulmonary CD4 T cells recognize Mtb-infected macrophages with greater sensitivity than CD8 T cells.
Figure 5.3: Differences in CD4 and CD8 T-cell recognition of Mtb-infected macrophages.

(A) CD4 (left) and CD8 (right) T cells obtained from Mtb-infected C57BL/6 mice were assessed for their recognition of Mtb-infected macrophages or the 300 peptide megapool (p300) by MIM-ICS. Data was shown from two independent experiments (signified by open or closed symbols), each with five mice/group, analyzed 4 wpi. (B) The frequency of pulmonary CD4 and CD8 T cells that produced IFNγ upon stimulation with Mtb-infected macrophages were determined by ICS. The target MOIs were indicated and the actual MOIs were shown in parentheses. Data are representative of two independent experiments using T cells from five individual mice at 4 or 22 weeks post-infection. Each line represents an individual mouse. The statistical test was a two-way ANOVA with Tukey’s post-test; actual p values are shown.
Differential recognition of Erdman- and 667-elicited CD8 T cells

We previously showed that TB10.4\textsubscript{4-11}-specific CD8 T cells do not recognize infected macrophages (Chapter II and (166)). Given the dominance of the TB10.4\textsubscript{4-11}-specific CD8 T cell response in the lung, we hypothesized that TB10.4 may be acting as a decoy antigen. Our finding that following 667 infection—few TB10.4\textsubscript{4-11}-specific CD8 T cells were detected and instead, a greater expansion of MTB32a-specific CD8 T cells were elicited—supported this idea (Figure 3.2). Therefore, we asked whether the abrogation of the immunodominant TB10.4\textsubscript{4-11} epitope resulted in an expansion of CD8 T cells that recognized Mtb-infected macrophages. To test this hypothesis, we quantified T cell recognition of Mtb-infected macrophages by the MIM-ICS assay. CD4 T cells from Erdman- or 667-infected C57BL/6 mice recognized H37Rv-infected and 667-infected macrophages similarly, and in a dose-dependent manner (Figure 5.4A, B). Thus, these two bacterial strains elicit CD4 T cell responses that had a similar capacity to recognize infected macrophages; both in vitro infections were successful, and the antigen presentation of class II MHC was not different between 667- and H37Rv-infected macrophages. There was little or no recognition of infected macrophages by CD8 T cells at a low MOI, as previously shown (Figure 5.3). Contrary to our prediction, Erdman-elicited CD8 T cells, but not 667-elicited CD8 T cells, recognized H37Rv-infected macrophages at a high MOI (Figure 5.4C). When the reciprocal experiment of CD8 T cells from Erdman- or 667-infected mice to recognize 667-infected macrophages was measured, neither Erdman nor 667-elicited CD8 T cells
significantly recognized 667-infected macrophages (Figure 5.4D). While these experiments do not support the decoy hypothesis, the results of lower IFNγ responses of 667-elicited CD8 T cells and lower stimulation of 667-infected macrophages show how the mycobacterial strain, or possibly lineage, can profoundly affect antigen presentation and the CD8 T cell response.
Figure 5.4: MIM-ICS assay of T cells elicited from Erdman and 667 infection reveals differential recognition capability.

CD4 (A, B) or CD8 T cells (C, D) purified from the lungs of Erdman- or 667-infected mice were cultured with H37Rv-infected (A, C) or 667-infected (B, D) macrophages and the MIM-ICS assay was performed. The X axis is the actual multiplicity of infection (MOI) as determined by CFU plating. Results from two different experiments are pooled and plotted on each graph. Each box and whisker plot represent results from 5 individual mice, with 2 experimental replicates shown. Statistical significance was determined by multiple t testing, and p values <0.05 are shown above the bars.
Erd WT- and Erd A10T-elicited CD8 T cells inefficiently recognize macrophages with different Mtb strain infections

667-elicited CD8 T cells recognized H37Rv infection less than their Erdman-elicited counterparts, but by using 667-infected macrophages, we considered that there may be a defect in class I MHC presentation and T cell priming during in vivo 667 infection (Figure 5.4). To investigate this possibility, we sought to determine the recognition capabilities of pulmonary T cells between the isogenic Erd WT and Erd A10T infections using MIM-ICS assay. Similar to the observation of ex vivo Erdman- and 667-elicited CD4 T cells, we detected the comparable capability of polyclonal CD4 T cells from Erd WT and Erd A10T infections in recognizing infected macrophages regardless of the bacterial strain used for macrophage infections (Figure 5.5A). This observation persisted throughout the course of infection as the ex vivo polyclonal CD4 T cells from mice at 19 weeks post-infection recognized Mtb-infected macrophages equivalently (Figure 5.5E, F). When considering CD8 T cells, however, the recognition capability of early-induced ex vivo CD8 T cells was different from that of the CD8 T cells isolated later in infection. At 6 weeks post-infection, both CD8 T cells recognized Erd WT-infected macrophages similarly. In contrast, Erd A10T-elicited CD8 T cells produced IFNγ in response to Erd A10T-infected macrophages significantly better than Erd WT-elicited CD8 T cells (Figure 5.5B-D). At 19 weeks post-infection, despite the similar trend observed, Erd WT- and Erd A10T-elicited CD8 T cells did not recognize infected macrophages differentially (Figure 5.5G-H).
Since we detected slight preferential responses of Erd\textsuperscript{WT}-elicited CD8 T cells to Erd\textsuperscript{WT}-infected macrophages and Erd\textsuperscript{A10T}-elicited CD8 T cells to Erd\textsuperscript{A10T}-infected macrophages, we wished to confirm our previous observation that infected macrophages did not present the TB10.4 epitope (166). We used ΔesxH Mtb strain for macrophage infection and observed the MIM-ICS\textsuperscript{+} proportions to be similar between Erd\textsuperscript{WT-} and Erd\textsuperscript{A10T-}elicited CD4 and CD8 T cells (Figure 5.6I-J), indicating that the T cells that recognize infected macrophages were not TB10.4-specific.

Together, these findings show that polymorphic Mtb can induce different T cell immune responses that lead to differential recognition ability. CD8 T cells that contribute to recognition are not TB10.4-specific, supporting the hypothesis that TB10.4 is a decoy antigen. Yet, how much CD8 T cells can recognize and contribute to the outcome of disease depends on the context of infection.
Figure 5.5: Isogenic Erd\textsuperscript{WT} and Erd\textsuperscript{A10T} infections elicit CD8 T cell responses that differentially recognize infected macrophages early during infection.

C57BL/6 were aerosol infected with Erd\textsuperscript{A10T} or Erd\textsuperscript{WT} Mtb and analyzed by MIM-ICS assay for recognition capabilities as described earlier. Percent IFN\textsubscript{γ}+ populations of CD4 (A, E, F, I) and CD8 (B, C, D, G, H, J) T cells at 6 weeks (A-D) and 19 weeks (E-J) post isogenic \emph{in vivo} infection were presented with actual macrophage \emph{in vitro} infection doses on x axes. Macrophages were infected with; H37Rv (A, B); Erd\textsuperscript{WT} (C, E, G); Erd\textsuperscript{A10T} (D, F, H); and \textDelta\text{esxH} (I, J). Data are representative of two experiments. Each experiment was performed with 5 mice per group. Statistical testing by a two-tailed, unpaired Student’s T test with Holm-Sidak post-test; actual p values are shown.
Discussion

We developed the MIM-E and MIM-ICS assays to quantify T cells that recognize Mtb-infected macrophages. To mimic the physiological conditions of 1–5 bacteria per infected cell in the lungs (213), we achieved a median MOI of 5. Under these conditions, 70% of macrophages are infected with 93% viability. The proportion of infected cells is crucial because bystander or uninfected cells have been shown to present antigens released from Mtb-infected cells or from dying cells (122, 130, 133, 134). Therefore, the maximized infection rate allowed us to study the direct recognition of Mtb-infected cells.

In Chapter II of this dissertation, we reported that TB10.44-11-specific CD8 T cells do not recognize infected macrophages [Figure 2.2 and (166)]. Together with a discrepancy between the fraction of T cells that recognize peptide epitopes versus Mtb-infected macrophages (Figure 5.3), we inferred that: (1) the ~10% of total CD8 T cells that recognize Mtb-infected macrophages in C57BL/6 are specific for unknown antigens; and, (2) CD8 T cell epitopes may not be efficiently presented by Mtb-infected macrophages (but are overrepresented in the p300 peptide pool). Although the p300 peptide pool was generated from known human class II MHC-restricted epitopes and species differences could impact the results, we predict that if a C57BL/6-specific peptide pool was available, a larger difference between the recognition of antigens versus the Mtb-infected macrophages would be observed. In addition, we find that CD4 T cells detect Mtb-infected macrophages more efficiently at a lower MOI than CD8 T cells which required more
heavily infected cells. This finding agrees with a previous report showing human CD8 T cell clones preferentially recognize heavily infected DCs (179).

To decipher the decoy hypothesis and understand whether a CD8 T cell response without TB10.4_{4-11} specificity would enhance the recognition, we evaluated the IFNγ production of T cells elicited by Erdman infection in comparison to 667 infection, and ErdWT to ErdA10T infections. Regardless of the bacterial strain used during in vivo and in vitro infections, polyclonal CD4 T cells all recognized infected macrophages similarly. To our surprise, 667-elicited and ErdA10T-elicited CD8 T cells behaved differently in a manner that was opposite from what we would have predicted based on the decoy hypothesis. Fewer 667-elicited CD8 T cells recognized H37Rv-infected macrophages than Erdman-elicited CD8 T cells. However, we gained insight from the 667-infected macrophages stimulation condition which poorly induced CD8 T cell responses, suggesting an overall defect in class I MHC antigen presentation by 667-infected cells. In support of the decoy hypothesis, CD8 T cells elicited from ErdA10T infection recognized ErdA10T-infected cells better than ErdWT-elicited CD8 T cells. However, their ability to recognize is context dependent: no differences were observed with ErdWT-infected macrophages or when T cells were obtained from chronically infected mice. Furthermore, as we determine that MTB32a_{309-318}-specific CD8 T cells do not recognize Mtb-infected macrophages (Figure 2.5), the compensatory increase in the frequency of MTB32a_{309-318}-specific CD8 T cells observed after 667 or ErdA10T
infection is unlikely to lead to better recognition of Mtb-infected cells. Rather, Mtb could exploit immunodomination as the decoy mechanism.

As the isogenic-infected C57BL/6 mice exhibited similar bacterial burdens in the lungs and comparable dissemination to the spleens (Figure 4.4), we cannot correlate MIM-ICS+ CD8 T cells with in vivo bacterial loads. Since direct CD4 T cell recognition of infected cells is required for bacterial control in vivo (97), it is likely that MIM-ICS+ CD4 T cells recognize Mtb-infected cells and provide protection in vivo. Interestingly, we observed a modest increase in the frequency of MIM-ICS+ T cells during the chronic phase of infection (Figure 5.5B versus C), even though these T cells may become dysfunctional (69) or suboptimally interact with infected APCs because they fail to enter the lung parenchyma (215, 216). Therefore, following the initial activation and subsequent contraction of T cells without antigen stimulation, the efficiently recognizing cells could be enhanced in their fraction and representation in the MIM-ICS+ population.

A limitation of this first version of MIM-E and MIM-ICS is its focus on IFNγ. Despite being the central dogma of protective immunity against TB, IFNγ does not always correlate with protection (52-54) and there are currently no correlates of protection in tuberculosis. Other cytokines such as IL-2, IL-17, and TNFα are produced by T cells after TCR activation; therefore, both the MIM-E and MIM-ICS assays can be adapted to include more cytokines. Another limitation is the duration of the assays, which are limited to the first 48 hours of in vitro infection. For
example, the expression of Ag85B varies during the course of infection, but importantly, is downregulated after three weeks post-infection (68, 127). As we are only measuring the antigens presented by macrophages within the first 48 hours of in vitro infection, we may fail to detect T cells that recognize antigens that are only expressed in the lung or late during infection. Lastly, the use of thioglycolate-elicited macrophages could lead to bias. Antigen-presentation by macrophages and DCs have some important differences, including the rate of protein degradation in the phagosomes. Macrophage phagosomes are more degradative than DC phagosomes, potentiating the differences in antigen presentation (217, 218). Since macrophages play an important role in Mtb biology and disease progression (219-221), we opted to assess T cell recognition of Mtb-infected macrophages. Many human and murine studies use Mtb-infected DCs and report very low frequencies of lymph node and lung T cells that recognized Mtb-infected DCs (89).

Other studies have shown that after in vitro expansion, human and murine CD8 T cell lines recognize and kill Mtb-infected DCs or macrophages (179, 222-225). These studies have been useful for characterizing antigen specificity and T cell functions but cannot deduce the ex vivo frequency of T cells that recognize infected macrophages. Further information is limited concerning the capacity of ex vivo T cells to recognize Mtb-infected cells (79, 89, 175, 179, 222-225). Barriers to these experiments include the low frequency of antigen-specific T cells in human blood and technical difficulties using live Mtb-infected cells. Possible confounders
also include the use of total lung cells instead of purified T cells and the
dependence on blocking antibodies against class II and class I MHC to estimate
the frequencies of CD4 and CD8 T cells that recognize Mtb-infected DCs. However, using class II MHC blocking antibodies does not exclude the noncognate
activation of CD4 T cells (169).

The differential recognition of Mtb-infected macrophages by CD8 T cells
generated from in vivo infections with different bacterial strains show that bacterial
polymorphisms can have important effects on immunogenicity. The unexpected
results of 667-elicited and ErdA10T-elicited CD8 T cells argue against the decoy
hypothesis that focuses particularly on the TB10.4 protein. We, therefore, propose
that immunodomination could be a decoy mechanism for immune evasion, by
which Mtb elicits large antigen-specific responses that do not contribute to
recognition of Mtb infection, but inhibit the generation of CD8 T cell responses to
other mycobacterial antigens. A more comprehensive study to elucidate the
epitopes presented by Mtb-infected cells, using a variety of bacterial strains, will
be critical to better understanding the extent of this hypothetical evasion strategy,
but also to identify Mtb antigens presented by infected cells. Finally, as we believe
that recognition of Mtb-infected macrophages by vaccine-elicited T cells will be a
prerequisite to protection, we expect that the MIM-E and MIM-ICS assays will
complement other approaches to assess T-cell-based vaccine candidates.
CHAPTER VI: DISCUSSION

“To know the history of science is to recognize the mortality of any claim to universal truth,” said Evelyn Fox Keller, a physicist (and feminist) at Massachusetts Institute of Technology. Previous findings that have led to new development and scientific discovery are extensively indispensable. Yet, flexibility in understanding is crucial to allow new and old ideas to be questioned, contradicted and accepted. In this dissertation, Mtb-specific and infection-elicited polyclonal CD4 T cells have been confirmed for their importance during tuberculosis. We observed a higher proportion of infection-driven CD4 T cells in recognizing Mtb-infected macrophages than that of CD8 T cells throughout the experiments in Chapter V. However, the highly responsive CD4 T cells only led to the stabilization of bacterial burden and did not further reduce bacterial burden. We, therefore, challenge the dogma that CD4 T cells are the critical component of T cell immunity against Mycobacterium tuberculosis infections, and propose—based on our finding—that Mtb evades CD8 T cell immunity and CD8 T cells would have the potential to eliminate infection if their functions were not subverted.

Immunodomination, an immunological phenomenon described as the suppression of subdominant responses by an immunodominant antigen-specific CD8 T cell response, has been observed in many viral models (136-139). However, immunodomination has never been reported during bacterial infection. Even though many robust immunodominant Mtb-specific CD8 T cell responses have been reported in humans and mice, CD8 T cell contribution to protective immunity
against Mtb infection has been largely inefficient. Thus, we hypothesize that immunodomination could act as the decoy mechanism used by Mtb to diminish the generation of CD8 T cells that are specific to other Mtb antigens, some of which can provide protection.

Figure 6: Immunodomination is a decoy mechanism Mtb used to evade CD8 T cells

Mtb (red rods)-infected cells transfer diverse class I MHC epitopes to bystander uninfected cells. The bystander cells subsequently induce high proportions of CD8 T cells that can only recognize uninfected cells. Consequently, a small proportion of CD8 T cells, elicited by infected cells, can efficiently recognize and kill Mtb-infected cells.
We speculate that the decoy mechanism may be the consequence of Mtb in infected cells actively inhibiting class I MHC antigen presentation. In turn, only uninfected cells can present Mtb antigens. Since the definition of an effective immune response includes not only the containment but also the clearance of Mtb infection, supporting evidence of the decoy hypothesis would comprise limited abilities of immunodominant CD8 T cells to both recognize and kill Mtb-infected cells. Polyclonal CD8 T cells without specificity for immunodominant epitopes are also expected to have greater capabilities in recognizing and killing Mtb-infected cells. Vice versa, if the decoy hypothesis does not occur, we anticipate the recognition ability of CD8 T cells without specificity to immunodominant epitopes could either worsen or remain unaffected.

To assess the hypothesis, we first evaluated the immunodominant CD8 T cell responses in Mtb-infected C57BL/6 mice, whereby 30% of CD8 T cells are specific for a single TB10.44-11 epitope. Supporting the decoy hypothesis, TB10.44-11-specific CD8 T cells did not recognize Mtb-infected macrophages nor control Mtb growth [Figure 2.2 and (166)]. Using mice with different genetic backgrounds and MHC haplotypes that present different Mtb epitopes, we investigated the recognition capability of TB10.420-28-specific CD8 T cells that are generated during Mtb infection in BALB/c mice (157). We showed that TB10.420-28-specific CD8 T cells, elicited by vaccination, also did not recognize Mtb-infected macrophages (Figure 2.4).
Our lab has reported the phenomenon we termed immunofocusing during Mtb infection in BALB/c mice (157). In this study, we highlighted the immunodominant CD8 T cell response against the TB10.4<sub>20-28</sub> epitope and a subdominant CD8 T cell response against the EspA<sub>150-158</sub> epitope during the natural course of Mtb infection in BALB/c mice. When a subdominant EspA<sub>150-158</sub>-specific CD8 T cell response was primed by vaccination using recombinant vaccinia expressing the EspA gene, a memory CD8 response to EspA was elicited and transiently boosted following challenge with Mtb. However, by four weeks after infection, the hierarchy of dominant TB10.4<sub>20-28</sub>-specific CD8 T cell response was restored and persisted throughout the remainder of the infection. This also resulted in unchanged bacterial burden. In retrospect, considering the requirement of direct engagement and recognition of the infected cells for killing (97), the inability of TB10.4<sub>20-28</sub>-specific CD8 T cells to directly recognize infected macrophages (Figure 2.4) potentially explains why TB10.4<sub>20-28</sub>-specific memory CD8 T cells failed to mediate protection.

We sought to identify CD8 T cells with known antigen specificity that can recognize Mtb-infected cells in order to study the protective functions and effects. We chose MTB32a<sub>309-318</sub>, a different CD8 T cell epitope from an independent protein. This epitope was selected because MTB32a is a component of the promising M72/AS01 vaccine and we can detect a subdominant response of MTB32a<sub>309-318</sub>-specific CD8 T cells in Mtb-infected C57BL/6 mice. However, we
have shown that MTB32a\textsubscript{309-318}-specific CD8 T cells, elicited by vaccination, also do not recognize infected macrophages (Figure 2.5)

The failure of two immunodominant and one subdominant CD8 T cell responses from two different mouse strains to recognize Mtb infected cells raises the possibility that this is a general feature of CD8 T cell responses and not unique to a particular epitope or host genetic background. Remarkably, C57BL/6 and BALB/c mice are more resistant to Mtb infection than C3H mice (226, 227), but the majority of CD8 T cells in Mtb-resistant mice do not recognize Mtb-infected cells [Chapter II, V, and (166)]. In addition, it has been shown that the intrinsic susceptibility of C3H mice is due to a delay in the CD8 T cell response (41); induction of an earlier CFP10-specific CD8 T cell response through vaccination lowers their susceptibility (228). This further supports the idea that Mtb could dampen the contribution of CD8 T cells in protection. The remaining questions are whether CFP10-specific CD8 T cells and what proportion of polyclonal CD8 T cells from Mtb-susceptible C3H mice recognize Mtb-infected cells. The frequency of CD8 T cells that recognize infected macrophages could also be examined in the newly identified Mtb-susceptible and Mtb-resistant collaborative cross (CC) diverse inbred mouse strains (25) to gain more insights into the contribution of CD8 T cells in protective T cell responses.

Therefore, chapter II has demonstrated that during Mtb infection, infection-driven immunodomination may be responsible for the failure of vaccination to
promote better control of infection. We have now observed this phenomenon after vaccination with four distinct CD8 T cell epitopes [(157, 160) and unpublished].

The most direct approach to study immunodomination is to eliminate the immunodominant response. However, the gene encoding TB10.4 protein, \textit{esxH}, is an essential gene, and Mtb with \textit{esxH} deficiency are attenuated \textit{in vivo}. Generally, pathogens evolutionarily conserve their essential genes for survival, but \textit{esxH} is surprisingly highly polymorphic. As Mtb is an obligate human pathogen, the antigenic variation suggests an ongoing adaptation to the host immune pressure. This is particularly exciting because the \textit{esxH} hyperpolymorphism is focused only in lineage 1 of Mtb. Together with the geographical clustering of both Mtb lineage and class I MHC—the most polymorphic allele in human genome (229)—we may be observing evidence of ongoing immune evasion during Mtb-human co-evolution.

To overcome the attenuation of Mtb with \textit{esxH} deficiency and broaden the understanding of \textit{esxH} polymorphisms, we employed a clinical isolate with a naturally occurring \textit{esxH} polymorphism to study the impact of immunodomination of TB10.4-specific CD8 T cells responses in C57BL/6 mice. We demonstrated a switch in the immunodominant antigens through infections with the clinical isolate 667 containing a single A10T amino acid substitution in the TB10.4 protein. I subsequently generated isogenic Mtb strains expressing EsxH\textsuperscript{A10T} or EsxH\textsuperscript{WT} and revealed the immunodomination phenomenon during Mtb infection through EsxH\textsuperscript{A10T}. MTB32a\textsubscript{309-318}-specific CD8 T cells became the immunodominant
response in the absence of TB10.4_{4-11}-specific CD8 T cells during 667 and Erd^{A10T} infection (Figure 3.2 and 4.2). When examining the cause further using in vitro assays, we observed the preserved recognition ability of TB10.4_{4-11}-specific CD8 T cells to the cognate “WT” and polymorphic A10T epitopes, and the A10T epitope was able to bind and present on H-2K^b (Figure 3.4). Through a collaboration with Dr. Sylvie Le Gall, an A10T long peptide was shown to be digested faster and finer than the wild-type counterpart. Thus, we hypothesize that the loss of TB10.4_{4-11}-specific CD8 T cell response is due to a decreased amount of the epitope presented. Despite yet-to-be-identified specific mechanisms, the EsxH^{A10T} variant has been shown to change the hierarchy of antigen-specific immune responses.

The finding of increased MTB32a_{309-318}-specific CD8 T cells was serendipitous because, aside from H-2K^b/TB10.4_{4-11} tetramer, H-2K^b/MTB32a_{309-318} tetramer was the only Mtb-specific tetramer available for H2^b restriction. We, therefore, speculate that there are potentially more changes in other Mtb-specific epitopes and that the loss of TB10.4_{4-11}-specific response may allow the space for other antigen-specific cells to expand.

To evaluate whether TB10.4 acted as a decoy antigen to distract host immunity from generating protective responses, we utilized 667 and Erd^{A10T} Mtb to assess whether host immunity without TB10.4_{4-11}-specific CD8 T cells could recognize infected macrophages better in Chapter V. Contrary to the decoy hypothesis, we did not observe different bacterial burdens following Erd^{WT} and Erd^{A10T} infections. However, given that Mtb may suppress CD8 T cell functions, a
30% change of CD8 T cell antigen specificity might not display in protection. In addition, we have determined that MTB32a$_{309-318}$-specific CD8 T cells do not recognize infected macrophages (Figure 2.5), suggesting that MTB32a could also act as another decoy antigen. Taken together, the correction of immunodominant CD8 T cell responses might not lead to the control of Mtb infection.

This highlights our finding that not all T cell responses elicited by Mtb provide benefits to the host [Chapter V, (166, 169)]. Even though TB10.4 is highly variable, and its polymorphism results in a change in hierarchy of the antigen-specific CD8 T cell responses, the compensatory response of a different decoy immunodominant epitope could still result in a benefit toward Mtb rather than the host. The decoy hypothesis supports the notion that T cell responses may provide a survival advantage for the bacterium (110, 111); by generating T cells that lack recognition and clearance abilities, T cell responses may accompany inflammation and pathology that create an environment to permit bacterial growth and transmission. Thus, Mtb focuses the CD8 T cell response on the immune response that may not contribute to the bacterial control, successfully evading T cell immunity and enabling it to establish a persistent infection.

Studies of the TB10.4 protein are complicated because of its role in impairing the ESCRT pathway and affecting class II MHC presentation (113, 126). On the one hand, we do not suspect the A10T substitution of TB10.4 protein affects its essentiality and reported function, but the polymorphisms reveal regions that
can be modified instead. We found that H37Rv lab strain and Erd\textsuperscript{WT} Mtb did not interfere with class I or II MHC presentations in infected macrophages as the polyclonal CD8 and CD4 T cells could produce IFN\textsubscript{\gamma} in response. In addition, we observed that polyclonal CD4 and CD8 T cells produced IFN\textsubscript{\gamma} in response to ΔesxH Mtb-infected macrophages. When using complemented ΔesxH::esxH\textsuperscript{WT} or ΔesxH::esxH\textsuperscript{A10T} strains for macrophage infections, we did not observe a decline of recognition by polyclonal CD4 or CD8 T cells. On the other hand, at similar infecting doses to H37Rv and Erd\textsuperscript{WT} Mtb, stimulations with clinical isolate 667- and isogenic Erd\textsuperscript{A10T}-infected macrophages exhibited a reduction in the proportion of responsive CD8 T cells, suggesting impaired class I MHC presentation. Thus, our data do not agree with the idea that TB10.4 function is detrimental to class II MHC antigen presentation. Rather, the EsxH\textsuperscript{A10T} polymorphism has more impact on class I MHC. However, the differences between the studies include 1) infection protocols: both the length of infection and the length of co-culture, and 2) the macrophages used in the studies: TGPMs vs. BMDMs. Furthermore, ΔesxH Mtb has defects in intracellular growth, so the finding that CD4 T cells mediate better bacterial reduction perhaps have been partially helped by the enhanced killing by innate macrophage antimicrobial mechanisms (126).

The experimental setup to assess the recognition capabilities of polyclonal CD8 T cells from 667, Erd\textsuperscript{WT} and Erd\textsuperscript{A10T} \textit{in vivo} infections were complex and, therefore, made interpreting the results difficult. Although a few conclusions can be inferred, results may be interpreted differently depending on further \textit{in vivo}
characterization of bacterial burden and kinetics. Therefore, to fully understand the qualitative differences of CD8 T cell responses elicited by polymorphic Mtb infections, further studies are needed to include more phenotypic markers. To identify the polyclonal CD8 T cell responses that contribute to the recognition of infected cells, one could sort IFNγ-secreting cells using a Miltenyi recombinant antibody that is bi-specific to CD45 and IFNγ. The sorted cells could subsequently be TCR sequenced, examined for antigen specificity, and derived into a polyclonal cell line that can recognize Mtb infection. In addition, the purified CD8 T cells could also be analyzed by single cell RNASeq to compare molecular functional differences of elicited CD8 T cells and to investigate other cell subsets. In parallel, immunopeptidomic analysis to identify epitopes presented on Mtb-infected and uninfected macrophages will enhance the possibility of distinguishing antigens for future studies. Identification of presented epitopes and the responsive T cells could lead to the generation of protective immunity and overcome the decoyed immune responses.

We used IFNγ production to study the recognition ability of elicited polyclonal CD8 T cells in Chapter V. However, the effector functions and the ability to kill Mtb-infected cells were not determined. To further evaluate the bacterial control functions of differentially elicited CD8 T cells, one could perform an adoptive transfer experiment of isogenic-elicited CD8 T cells into Mtb-infected recipients and determine the bacterial burden. The depletion of CD8 T cells in isogenic-infected mice may also help determine survival differences based on the
contribution of a different repertoire of CD8 T cell specificities. To test the protective impact of cross-recognition of CD8 T cells from Erd\textsuperscript{A10T} to Erd\textsuperscript{WT}-infected macrophages (and the inability of CD8 T cells from Erd\textsuperscript{WT} to recognize Erd\textsuperscript{A10T}-infected macrophages), one could infect mice with Erd\textsuperscript{A10T} or Erd\textsuperscript{WT} Mtb, treat with antibiotics to clear the infections, and challenge with the reciprocal strain to determine the antigen-specific CD8 T cell responses and bacterial burden.

We recognize that the C57BL/6 murine model cannot represent what happens during human infection, and the wide polymorphisms of class I MHC in humans contributes to the possibility that this phenomenon might not occur in people. Despite no differences in bacterial burden following infection with isogenic strains, a pathology study could be used to determine if inflammation differs. One could use the clinical isolate or isogenic Mtb strain to infect a different animal model, such as monkey, to induce more distinct granuloma phenotypes and compare the pathological outcomes.

Nevertheless, Chapter III and IV showed that a single amino acid substitution can alter host immune response drastically. With the heterogeneity of clinical Mtb isolates in the human population, the apprehension that Mtb-induced immunodominance will supersede a vaccine-driven response poses a question about the current vaccine strategies in development. In addition, since people with a history of Mtb infection and antibiotic treatment are able to get reinfected with Mtb, Mtb-elicited immune responses may be inefficient due to Mtb’s arsenal of
immune evasion strategies. As immunological space is conceptually limited (230, 231), considering whether there are cross-reactivities among existing immune responses from BCG vaccinated, previously infected and subsequently cured, and currently infected individuals will be critical for the development of efficacious new treatments.

What constitutes protective immunity against tuberculosis is not fully understood and is reflected in the lack of immune correlates of protection. This knowledge gap has hampered the development of an effective vaccine. Since direct engagement and recognition of TCRs to the infected cells are required for killing of infected cells (97), successful vaccines should be able to induce CD4 and CD8 T cells that can recognize infected, rather than uninfected, cells. A recent study using immunopeptidomic analysis to identify epitopes presented on BCG-infected macrophages showed that only a small subset of peptides consistently presented in 4 different replicates (232). Since BCG, but not Mtb, infections are generally subjected to clearance by CD4 and CD8 T cell immunity, epitopes presented on the Mtb-infected macrophages may be even more diverse to escape T cell recognition. Successful vaccines should also be able to induce a broad repertoire of CD4 and CD8 T cells that can recognize (possibly diverse) epitopes presented on infected cells.

Numerous microbial and host factors determine whether a protein from an intracellular bacterium elicits a T cell response. These include the bacilli’s
intracellular niche, the protein’s abundance, and whether it is secreted. Many T cell-based vaccine studies assume that Mtb-specific T cells elicited by natural infection will recognize infected cells. Consequently, such T cells elicited by vaccination should mediate protection against Mtb. The failure of TB10.4_{4-11} and TB10.4_{20-28}-specific CD8 T cells to recognize Mtb-infected macrophages demonstrated that not all Mtb-specific T cells recognized Mtb-infected macrophages (98, 122, 127, 166). Consistently, the AERAS-402 vaccine expressing Mtb antigens elicited CD8 T cells that failed to recognize or only modestly recognized Mtb-infected DCs (165). This further emphasizes the possibility that some Mtb antigens, even those that induce immunodominant responses, may not be presented by Mtb-infected cells (166). A strategy to enumerate T cell recognition of Mtb infection could broaden our understanding of host–pathogen interactions and assist in the development of new vaccines.

Vaccination with immunodominant antigens such as Ag85b and ESAT6 induces CD4 T cell responses that provide limited protection (233, 234); thus perhaps these antigens are not the best stimulators of protective immunity. In addition, the recruitment of memory T cell responses specific for the vaccinated immunodominant antigens is only transiently augmented (160, 235). Our findings identify a population of polyclonal CD8 T cells that recognize Mtb-infected macrophages, supporting a previous study that showed cytotoxic functions of polyclonal CD8 T cells from infected mice in killed Mtb-infected cells (223). For efficient vaccine development, it may be useful to screen for (and eliminate from
consideration) Mtb antigens that resemble TB10.4, in that they induce robust T cell responses but fail to recognize and kill infected macrophages. Thus, future vaccine developments can benefit by identifying antigen targets based on their ability of being presented by infected cells rather than on their immunogenicity alone.

The finding that a large proportion of CD8 T cells are specific for TB10.4, yet the epitope is not able to be efficiently recognized on infected macrophages, is perplexing. Previous studies have identified that dendritic cells can pick up apoptotic vesicles containing live Mtb or parts of bacteria and prime naïve T cells in the LN (133, 134). While they portrayed this process in the context of T cell priming in the draining LN, this process could occur in the infected lung, where an uninfected bystander macrophage engulfs a dying Mtb-infected macrophage. Subsequently, peptides generated from dead bacteria or its proteins could be presented by uninfected APC (122). In parallel, if live Mtb actively inhibits antigen presentation (236), this could lead to MHC-restricted and TCR-dependent recognition biases towards uninfected cells rather than infected cells. Together, the persistence of the T cell response to uninfected cells would lead to inflammation unaccompanied by bacterial clearance. It is generally held that T cell-dependent lung inflammation creates the necessary environment to promote a cough and person-to-person transmission. Thus, in agreement with the rationale by Comas et al. (110), hyperconserved T cell epitopes in the Mtb genome could drive T cell-mediated inflammation. In addition, our insights suggest that if the inflammation is mediated by immunodominant CD8 responses, inflammation may
occur in the absence of negative consequences to the bacterium as the T cell responses are unable to recognize infected cells.

We propose that the epitopes presented by dendritic cells in LN, which prime T cell responses, were mismatched from those presented by macrophages in the lung at the site of infection during the effector function phase. We speculate that TB10.4, which is a secreted protein, may be taken up and presented by uninfected dendritic cells to prime T cell responses, but infected cells themselves do not present TB10.4 epitopes. The actual mechanisms of naïve T cell priming and recognition of bacteria-infected cells are important to facilitate development of strategies to enhance recognition of infected cells—either through blocking the antigen presentation by uninfected cells in the lung or by augmentation of antigen presentation by infected cells. One could employ immunopeptidomic analysis similar to the recent study that identified epitopes presented on BCG-infected macrophages (232). However, the main limitation of the study was the low rate of infection, with only 30% of cells being infected. The proportion of infected cells is crucial because it is unknown whether epitopes were presented on Mtb-infected DCs, or by uninfected DCs that acquire antigen through uptake of apoptotic blebs containing Mtb proteins (133, 134), or by the transfer of antigens from cell to cell (45). Sorting for infected and uninfected cells prior to analysis would increase the information regarding which antigens were presented by each subset, but the high number of cells needed for such an analysis (over $5 \times 10^7$ cells) may impede the sorting attempt. The immunopeptidomic study may help facilitate development of
a vaccine by considering which epitopes should be included in vaccine candidates to target infected cells, in order to overcome the decoy immune evasion.

Last, but not least, there has been a growing body of literature supporting the role of antibodies against Mtb infections. Despite the correlation of higher antibody titers with disease severity (237) and inconsistent protections from antibody transfer (238, 239), mice without B cells or without ability to secrete antibodies are more susceptible to infection (240, 241). Depletion of B cells in non-human primates also increased bacterial burden and lesions (242). Furthermore, elevated antibody titers have been identified in “resister” populations, emphasizing the evidence for a possible role for antibodies in Mtb protective immunity. Therefore, induction of antibody responses in addition to protective T cell responses could lead to the ultimate effective vaccine against tuberculosis.

Finally, I have demonstrated that a single nucleotide polymorphism in the genome of Mtb can lead to the generation of a vastly different immunodominant CD8 T cell hierarchy. Therefore, the large genetic heterogeneity of existing clinical Mtb isolates in TB patients would be expected to drive diverse T cell responses. In addition, this dissertation showed that the majority of CD8 T cells induced in natural Mtb infections are inefficiently responsive to Mtb-infected cells. This may occur because the immunodominant CD8 T cells only “see” uninfected cells as targets and not the actual infected cells. In fact, the low overall frequency of T cells that recognize infected cells is consistent with Mtb using multiple strategies to evade
detection by the immune system. Characterizing the CD4 and CD8 T cells that can recognize low-MOI-infected macrophages, to mimic the physiological conditions of infected cells in the lungs (213), is crucial as this may identify antigens that are more likely to be presented during natural infection or post-vaccine challenge.

To conclude, there are knowledge gaps in the current understanding of Mtb pathogenesis, its immune evasion strategies, and the immune responses generated. Given the long-standing interaction between Mtb and human populations, it is not surprising that immune pressures have shaped the evolution of Mtb as an invasive pathogen. The inability of host immunity to eliminate Mtb could be a consequence of the exploitation of host immunity by Mtb to aid in its transmission and avoid elimination. This dissertation presents a detailed example of an ongoing immune evasion strategy used by Mtb to diminish the contribution of CD8 T cells to host defenses. This work provides a foundation to understand and direct future studies to identify and generate protective CD8 T cell responses.
CHAPTER VII. MATERIALS AND METHODS

Ethics Statement

Studies involving animals were conducted following relevant guidelines and regulations, and the studies were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School (Animal Welfare A3306-01), using the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Office of Laboratory Animal Welfare.

Animal

C57BL/6J, BALB/c and CB6F1 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions at University of Massachusetts Medical School (UMMS) animal facilities. Mice were 8 to 9 weeks old at the start of all experiments. C57BL/6J, BALB/c and CB6F1 mice were used for in vivo infection and as donor for isolating thioglycolate-elicited peritoneal macrophages. Infected mice were housed in biosafety level 3 facilities under specific pathogen-free conditions at UMMS.

Thioglycolate-elicited peritoneal macrophages (TGPM)

Thioglycolate-elicited peritoneal macrophages were obtained 4-5 days after intraperitoneal injection of donor mice with 3% thioglycolate solution, as described (34). CD11b microbeads (Miltenyi, Biotec, Germany) were used to purify for
macrophages population. 1x10^5 or 1x10^6 macrophages were plated per well of 96-well plate or Nunc Up-Cell 12-well plate. Macrophages were maintained in culture with RPMI 1640 media (Invitrogen Life Technologies, ThermoFisher, Waltham, MA) supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine (all from Invitrogen Life Technologies) and 10% heat-inactivated fetal bovine serum (HyClone, GE Healthcare Life Sciences, Pittsburgh, PA), referred as supplemented complete media.

**Generation of TCR retrogenic mice**

TCR retroviral constructs and retrogenic mice were generated using protocols developed by the Vignali lab as described (243, 244). Details of the TCRs and cloning strategies were discussed in (161, 166). Retroviral-mediated stem cell gene transfer was performed using congeneric marked bone marrow and transferred into opposite-marked recipients that were lethally-irradiated at 1200 rads administered using a GammaCell 40 Cs^{137} Irradiator (Theratronics, Ottawa, ON, Canada). Reconstitution was measured 6-8 weeks later.

**Generation of CD8 and CD4 T cell lines**

Retrogenic mice expressing TB10RgL, TB10RgR, TB10RgP and TB10Rg3 TCR specific for the TB10.44-11 epitope were generated as previously described (161, 166). In short, CD8 T cells were isolated from these mice by negative isolation with MojoSort (Biolegend), stimulated *in vitro* with irradiated splenocytes pulsed with the peptide TB10.44-11 in complete media containing IL-2. After the initial
stimulation, these T cells were split every two days for 3-4 divisions and rested for two to three weeks. After the initial stimulation, the cells were cultured in complete media containing IL-2 and IL-7.

**Peptides**

Polymorphic peptides A10T, A10V, P9S, and M11I were purchased from Genscript (Piscataway, NJ). WT (IMYNYPAM), negative control (IMANAPAM), Mtb32a₃₀₉-₃₁₈ (GAPINSATAM) and ESAT6₁₋₁₅ (MTEQQWNFAGIEAAA) peptides were purchased from New England Peptides (Gardner, MA). TB10.4 peptide library was obtained from BEI Resource.

**Trivax vaccination and generation of antigen-specific CD8 T cells.**

“Amphi-TB1₀₂₀-₂₈” (MFVMFVQGYAGTLQSL) and Mtb32a₃₀₉-₃₁₈ (GAPINSATAM) peptides were purchased from Genscript (Piscataway, NJ) and reconstituted in DMSO. High molecular weight polyinosinic:polycytidylic acid [poly(I:C)] was obtained from InvivoGen (San Diego, CA). Anti-CD40 mAb (clone FGK4.5) was purchased from BioXCell (West Lebanon, NH). Trivax vaccines were prepared by mixing 100 μM of peptide, 50 μg poly(I:C), and 50 μg aCD40 mAb, in a total volume of 200 μL sterile PBS and administered intravenously. Mice were boosted with the same vaccine 3 weeks later. CD8 T cells were isolated from spleen by negative isolation with MojoSort (Biolegend)
**Mycobacterium tuberculosis stains**

Unless indicated, Erdman strain was used for *in vivo* infection, and H37Rv strain was used for *in vitro* infection. Erdmann WT and Erdmann A10T were generated from Erdman background (as described below and in Chapter IV) and used for both *in vivo* and *in vitro* infections. ΔesxH strain was gifted from Dr. Jennifer Philips (Washington University at St. Louis) and used for *in vitro* infection. Barcoded clinical isolate pool was gifted from Dr. Sarah Fortune (Harvard University), and used in competitive infection.

**In vitro infection**

Indicated Mtb of each experiment was thawed, grown in 7H9 media until reaching log-phase with OD600 of 0.6-1, washed, and opsonized with TB coat (RPMI 1640, 1% heat-inactivated FBS, 2% human serum, 0.05% Tween-80) before filtering through 5uM filter to remove clumps. The bacteria were counted in Petroff-Hausser chamber before adjusting the volume to respective multiplicity of infection (MOI) of interest. Actual MOI were determined by lysing bacterial culture with 10% Triton X-100 in water, serial diluting the lysates and plating in on Middlebrook 7H10 or 7H11 plates. Colony forming unit (CFU) was enumerated after culture for 21 days at 37°C and 5% CO2.
**Mtb-Infected Macrophage ELISpot (MIM-E) and Intracellular Cytokine Staining (MIM-ICS)**

MIM-E and MIM-ICS were previously described (169). Briefly, TGPMs were isolated and infected *in vitro* at indicated MOI overnight. Infected and uninfected TGPMs were harvested the next day and plated 1x10⁵ cells per well on ELISpot plate that had been coated with the IFNγ capture antibody and blocked with complete media as per the manufacturer protocol, or on 96-well plates. Where indicated, 10μM single peptides or 2 μg/ml peptide megapool 300 were added to uninfected macrophage prior to the addition of T cells. Indicated T cells were isolated and co-incubated with TGPMs at 37C in non-shaking 5% CO2 incubator 18-24 hours and follow manufacturer protocol for ELISpot development (BD Bioscience), or 5 hours and follow with standard ICS staining.

**RMA-S assay**

RMA-S H-2Kb cell line was gifted from Dr. Lawrence Stern laboratory (University of Massachusetts Medical School, Worcester MA) and maintained in completed RPMI. RMA-S cells were plated 5x10⁴ per well in 96-well plates. WT and polymorphic peptides were titrated starting at 100uM down to 1pM and incubated onto RMA-S cells overnight at 27C in 5% CO2 incubator. RMA-S cells were subsequently shifted to 37C in 5%CO2 incubator for 1-2 hours before washing and staining for surface expression of H2Kb. Stained cells were then analyzed by flow cytometry.
Measurement of cell proliferation.

Analysis TB10Rg3 cell proliferation was measured in vitro after stimulation by labeling purified T cells with 5 μM of the cell proliferation dye eFluor 450 (eBiosciences). Proliferation, as measured by dye dilution, was measured by flow cytometry 72 hours after co-culture with APCs coated with a serial dilution of peptides

Mouse infections

Eight- to nine-week old female C57BL/6, BALB/c, or CB6F1 mice were infected via the aerosol route with indicated bacterial strains in each experiment. Frozen bacterial stocks were thawed, diluted in 0.9% NaCl - 0.02% Tween 80), and sonicated before loading into a nebulizer for Glas-col aerosol chamber (Terre Haute, IN) to deliver approximately 100 CFU of bacteria to the lungs of each mouse. The Mtb infecting dose from the lung were evaluated 24 hour after aerosol infection by plating lung homogenates on 7H11 agar plates from Hardy Diagnosis. Animal experimentation was performed under authorization from IACUC. At different times post-infection, mice were euthanized, lungs were removed after perfusion of pulmonary arteries with 10mL of cold RPMI1640. Lung cell suspensions were prepared by coarse dissociation using the GentleMACS tissue dissociator (Miltenyi Biotec, Germany). Tissue was digested for 30 min at 37°C with 250 U/mL collagenase (Sigma) in complete RPMI1640 followed by homogenization in the GentleMACS dissociator and sequential straining through
70 μm and 40 μm nylon cell strainers (Falcon). Lungs and spleens were also aseptically removed, individually homogenized, and plated to determine viable bacteria.

**Peptide library screening**

At indicated timepoint post infection, mice were euthanized by CO2 inhalation and cervical dislocation. Lungs and spleens were harvested and processed as described above. Total lung cells were enumerated and 1x10⁵ cells were plated in 96-well plates. Each peptide in peptide library and control peptides were diluted and added to the plate in triplicates. The cells were incubated at 37°C 5%CO2 for 48 hours before supernatants were filtered through 0.2μM plate filter and subsequently determined the amount of secreted IFNγ by ELISA (Biolegend, CA)

**Flow cytometry**

The following cell surface antigens were detected by flow cytometry using Zombie Violet or Aqua Fixable viability dye and the following antibodies: mouse CD4 (clone GK1.5), CD8 (clone 53-6.7), CD3ε (clone 145-2C11), CD19 (clone 6D5), CD44 (clone IM7), CD62L (clone MEL-14), CD127 (clone A7R34), KLRG1 (clone 2F1/KLRG1), CD69 (clone H1.2F3), IFNg (clone XMG1.2), F4/80 (clone BM8) and H-2Kb (clone AF6-88.5) (all from Biolegend). BV421-conjugated TB10.44-11-loaded, BV421-conjugated IMYNPTYM-loaded, PE-conjugated Mtb32a309-318-loaded H-2Kb tetramers, APC-conjugated ESAT6-loaded I-Ab tetramer, PE-conjugated TB10.420-28-loaded H-2Kd tetramer and BV421-conjugated TB10.473-88-
loaded I-A<sup>d</sup> tetramer were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). (Samples were fixed with 1% paraformaldehyde/PBS for 1 hour before being analyzed by a MACSQuant flow cytometer (Miltenyi Biotec). FlowJo Software (Tree Star, Portland, OR) was used to analyze the collected data. Single lymphocytes were gated by forward scatter versus height and side scatter for size and granularity, and dead cells were excluded.

**Lung cell purification**

Following infections, mice were euthanized, lungs were aseptically removed, individually homogenized for cell isolation. Pulmonary cells were purified by positive selection using anti-CD90.2 microbeads on AutoMACS (Miltenyi Biotec, Germany). The isolated cells were counted on a hemocytometer and plated in supplemented complete RPMI 1640 media.

**Barcoded clinical isolate pool infection**

Barcoded Mtb strain generation, infection, and analysis were previously described (188). Briefly, selected clinical isolates were individually tagged with unique 8 basepair sequence, grown to log phase, pooled, and used for intravenous infection at 1x10<sup>6</sup> Mtb per mouse. At indicated time post infection, lungs and spleens were harvested, homogenized and plated on 7H10 supplemented with oleic albumin dextrose catalase (OADC) and 20 ng/ml kanamycin. After 3 weeks of incubation,
the plates were counted for CFU, and colonies were scraped for genomic DNA extraction and sequencing by NextSeq and analysis using Python.

**Generation of isogenic Erd\textsuperscript{WT} and Erd\textsuperscript{A10T} by oligo recombineering**

Oligo recombineering with long oligo containing desired base pair change were generated as described previously (208, 209, 245). Shortly, Erdman was grown in 7H9 broth into a log phase before following standard protocol to electroporate pKM444 plasmid containing RecT annealase. Electroporated strains was selected on 7H10 agar plates containing 20 ng/ml kanamycin, picked at 3 weeks post plated, and PCR verify the presence of pKM444 plasmid. An Erdman strain containing the Che9 phage RecT producing plasmid was grown in 7H9 broth to an O.D. of 0.5. Anhydrotetracycline (Atc) was added to a final concentration of 500 ng/ml to induce expression of RecT from the P\textsubscript{Tet} promoter; the cells were grown overnight and prepared for electroporation with esxH\textsuperscript{A10T}-conferring oligo (1 ug) and an oligo targeting the \textit{rpsL} gene (0.1 ug) designed to generate a K43R mutation in the RpsL protein, which confers resistance to streptomycin. Following outgrowth, the culture was plated on 7H10 agar plates containing 20 ng/ml streptomycin. Selection of streptomycin screens for cells that pick up the DNA and are recombinogenic, increasing the frequency of finding the desired SNP. Candidate colonies were picked and screened for the targeted change by PCR analysis.
RT-PCR of mycobacterial mRNAs

To quantitate expression of Mtb genes, indicated bacteria were grown to OD600 of 0.8-1 before centrifugation to pellet intact bacterial cells. Bacterial pellets were resuspending in TRIzol (Invitrogen) and quickly homogenizing with silica beads and Bead beater homogenizer. RNA was extracted using Direct-zol RNA Miniprep Plus Kits (Zymo Research, Irvine CA). RT-PCR was carried out as previously described (127) with mRNA copy number normalized to the constitutively expressed 16S rRNA. The following RT-PCR primers were used in this study.

16S rRNA:  Fw 5’-AGCTCGTAGGTGGTTTGTCG-3’
            Rv 5’-CCACCGCTACACCAGGAATT-3’;
Rv0288:    Fw 5’-AGCTTGGGTGCGGAGAT-3’
            Rv 5’-TGCTGGACATCGCATGATAG-3’;
Rv0287:    Fw 5’-GCTCAGGCGTTTCACCA-3’
            Rv 5’-CCAACAAGGTGTTGACTTTGG-3’;
Rv3875:    Fw 5’-GTCCATTCCATTCCCTCCTTGAC-3’
            Rv 5’-TTGCTGGACACCCCTGGTA-3’;
Rv1886c:   Fw 5’-ACAACTCACCTGCACGTTAT-3’
            Rv 5’-GACAGTCCCCAGCTGGTAGTA-3’;
Rv0125:    Fw 5’-AAGAGACATTGAACGGGTTGAT-3’
            Rv 5’-CTGGGACAGCTGGAAGTTATC-3’;
Rv3874:    Fw 5’-ATCTCCGGCGACCTGAA-3’
            Rv 5’-CGAGTTCTGCTTCTGCTTAT-3’;
Rv3019c: Fw 5’-GTCAACTATCCGCGATGAT-3’
Rv 5’-TATCACCTGCCAAGCAC-3’;

Statistical analysis

Data are represented as mean ± standard error of the mean (SEM). For comparing two groups, a two-tailed, unpaired student’s t-test was used. For more than two groups, the data were analyzed using a one-way ANOVA. A p value < 0.05 was considered to be statistically significant. Analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA)
APPENDIX: THE INVESTIGATION OF GRANULYSIN-MEDIATED CD8 T CELL CYTOTOXICITY DURING TUBERCULOSIS

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Attributions:

R.S. and S.B designed all experiments. R.S. performed all experiments and statistical analysis. S.B. provided critical feedback for interpretation of the data.
**Introduction**

CD8 T cells, also known as cytotoxic T lymphocytes (CTL), recognize and eliminate infected cells by their effector molecules, which fall into two broad categories: cytotoxins, which are stored in specialized cytolytic granules, and cytokines such as IFNγ and other related membrane-associated proteins, which are synthesized *de novo* (246). CD8 T cells also mediate killing through the Fas-Fas ligand (FasL) pathway, which is upregulated upon target cell stimulation and induce downstream caspase ultimately leading to apoptosis of the target cell. Although CTL have been shown to use IFNγ-, FasL- and cytotoxic-mediated killing during tuberculosis in murine models (82, 83, 86, 90, 91, 223), the contribution of cytolysis by human cytolytic granules are not well-understood.

Murine cytolytic granules contain granzymes (Gzm) and perforin (PFN). Perforin is a pore-forming molecule that is necessary for the delivery of granzymes, which induces apoptosis. Perforin and granzymes are released early after CD8 T cell activation into the immunological synapse between the CD8 T cell and a target cell (247). The process of cytotoxic degranulation is class I MHC-restricted and proposed to play an important role in control of *in vivo* Mtb infection (83, 223). However, these components are unable to kill intracellular bacteria. A major cytotoxic granule present in human and non-human primates, but not in mice, is a saponin-like pore forming protein called granulysin (GNLY) (248). Granulysin alters the membrane integrity on bacteria and mediates the antimicrobial activity
of CTL against Mtb in human, which leads to the direct killing of intracellular mycobacteria \textit{in vitro} (85).

\textbf{Figure A.1:} Granulysin is hypothesized to enhance CD8 T cell cytotoxic functions and provide better protection against Mtb infection

Since murine model lacks granulysin in cytotoxic granule components, \textit{in vivo} studies to elucidate the roles of granulysin during Mtb infection had not been feasible. After the generation of granulysin-transgenic (GNLY-Tg) mice (249), GNLY-Tg mice have been shown to be protected and survive from intracellular \textit{Toxoplasma gondii} and \textit{Trypanosoma cruzi} infections while they are lethal to WT mice (250). Given that human CD8 T cells recognize Mtb infected macrophages and directly kill through granulysin \textit{in vitro}, in this appendix of dissertation, I explore the roles of granulysin during \textit{Mycobacteria tuberculosis} (Mtb) infection \textit{in vivo}. We hypothesize that granulysin enhances the cytotoxic functionalities of CD8 T cells and contributes to protection against Mtb infection (Figure A.1).
Results

GNLY is expressed in human CD8 T cells

To set up the system of granulysin detection, I tested the conditions to stimulate GNLY expressions and detection with the human GNLY antibody for intracellular staining. Consistent with literature, IL-2 (not shown) and IL-15 are the strongest stimulators for the expression of GNLY in human PBMCs (Figure A.2).

![Figure A.2: GNLY is expressed in human CD8 T cells](image)

Human PBMCs were isolated from peripheral blood and stimulated \textit{in vitro} with anti-human CD3 and anti-human CD28 antibodies, with or without recombinant IL-15 cytokines. At 8 days post stimulations, cells were stained for granulysin and granzyme B expression.
GNLY is expressed in NK cells, but not CD8 T cells, of GNLY-Tg mice

We subsequently sought to verify the granulysin expressions of GNLY-Tg mice using exact protocol from the research group who generated the transgenic mice (249). Even though we were able to detect the GNLY expression only in NK cells, to our surprise, GNLY was not detectable in CD8 T cells in any stimulation conditions (Figure A.3).

![Figure A.3: GNLY is expressed in GNLY-Tg NK cells, but not CD8 T cells](image)

Spleens from C57BL/6 littermate control and GNLY-Tg mice were isolated and stimulated in vitro with anti-CD3 and anti-CD28 antibodies, with indicated cytokines. At 12 days post stimulations, cells were stained for granulysin and granzyme expression. Cells were gated by NK1.1 and CD8 antibodies, followed by isotype controls from GzmB and GNLY.
Generation of antigen-specific GNLY-Tg CD8 T cell line

We considered the possibility that granulysin expression needs high amount of stimulation to be expressed, and short-term stimulation might not suffice. We therefore vaccinate GNLY-Tg and C57BL/6 littermate control mice with Trivax-B8R vaccine to generate B8R-specific CD8 T cells lines (Figure A.4A). We elected for B8R epitope because B8R has been shown to be a strong stimulator during vaccination (160) and H-2Kb/B8R tetramer is available to use for detection. At 2 weeks post boost, we isolated splenocytes and stimulated in vitro with IL-2 or IL-15. However, we could not detect granulysin expression on CD8 T cells at 5 days post stimulations (Figure A.4B). We therefore derived CD8 T cells from Trivax-B8R vaccinated GNLY-Tg and C57BL/6 littermate control into primary B8R-specific CD8 T cell lines. At 3 weeks post stimulation and derivation, CD8 T cell lines from both GNLY-Tg and C57BL/6 littermate control continuously expressed high level of granzyme B, but did not produce detectable granulysin (Figure A.4C).
A. 

- **Prime**: 
  - GNLY⁺/⁻ Tg or C57Bl/6
  - Triavax B8R
  - Anti-CD40 mab
  - Poly(I:C)
  - B8R peptide

- **Boost**: Every 3 weeks as needed

- **1 week post boost**: Bleed - Tetramer stain check
  - Purify splenic CD8 T cells

B. 

- **C57Bl/6 Littermate**
  - NK
  - CD8
  - Granulysin
  - Granzyme B
  - IL-15
  - IL-2

- **GNLY⁺/⁻**
  - NK
  - CD8
  - Granulysin
  - Granzyme B

C. 

- **Isotype**
  - GNLY Tg
  - C57Bl/6 littermate

- Granulysin

- Granzyme B
Figure A.4: Generation of GNLY-Tg antigen-specific CD8 T cell lines

(A) C57BL/6 littermate control and GNLY-Tg mice were vaccinated with TrivaxB8R (anti-CD40, Poly(I:C) and B8R peptide) and boosted 3 weeks afterward. (B) Spleens were isolated at 2 weeks post vaccination and stimulated in vitro with indicated cytokines. At 5 days post stimulations, cells were stained for granulysin and granzyme expression. Cells were gated by NK1.1 and CD8 antibodies, followed by isotype controls from GzmB and GNLY. (C) Spleens were isolated, and CD8 T cells were purified with untouched negative selection kit. Purified CD8 T cells were subsequently stimulated in vitro with B8R-pulsed irradiated splenocytes with IL-2 supplement. At 3 weeks post stimulations, cells were stained for granulysin and granzyme expression. Cells were gated on CD8, followed by isotype controls from GzmB and GNLY.
GNLY-Tg CD8 T cells can produce granulysin under limited stimulation conditions

To further determine whether there are conditions that GNLY would express in CD8 T cells, we subsequently restimulated B8R CD8 T cell line with B8R-pulsed splenocytes supplementing with various combinations of IL-2, IL-7, IL-12, IL-15, and IL-21 cytokines. For the first time, we were able to induce and detect granulysin production by CD8 T cells from GNLY-Tg mice at day 8 post stimulation (Figure A.5). Similar to human CD8 T cells, IL-2 and IL-15 were the strongest stimulators for GNLY production. Importantly, subsequent stimulations of the cell line appeared to lower the threshold for GNLY production, allowing granulysin to be induced and detected easier (RS, unpublished observation).
Figure A.5: GNLY-Tg CD8 T cells can produce granulysin under limited stimulation conditions

B8R-specific C57BL/6 littermate control and GNLY-Tg CD8 T cell lines were stimulated in vitro with B8R-pulsed irradiated splenocytes and indicated cytokines. At indicated days post stimulations, cells were stained for granulysin and granzyme expression. Cells were gated by isotype controls from GzmB and GNLY.
Granulysin-transgenic mice do not exhibit better control of *Mycobacterium tuberculosis* infection

Given that granulysin can be expressed on CD8 T cells, but heavy stimulations were needed, we decided to infect C57BL/6 littermate control and GNLY-Tg mice and determined the contribution of granulysin at the chronic phase of infection. At 16 weeks post infection, despite ability to detect granulysin in NK cells in pulmonary compartment, both antigen-specific and non-specific CD8 T cells did not produce granulysin (Figure A.6A). There were no differences in the total numbers of NK cells, antigen-specific and non-specific CD8 T cells, and the MPEC and SLEC phenotypes of CD8 T cells were not different between GNLY-Tg mice and littermate controls (Figure A.6B-E). Interestingly, more than half of granzymeB-expressing NK cells were expressing granulysin (Figure A.6F). Finally, there was no differences in pulmonary bacterial burdens. Together, this data shows that granulysin-expressing NK cells do not provide protection during Mtb infection. However, the role of granulysin on CD8 T cells remains unknown as the transgenic CD8 T cells do not express granulysin.
Figure A.6: GNLY-Tg mice do not exhibit better control of Mtb infection

Age-matched C57BL/6 littermate control and GNLY-Tg mice were infected with Erdman Mtb at ~100 CFU. At 16 weeks post infected, infected mice were sacrificed for flow cytometry and bacterial control analysis. (A) Representative flow cytometry plots of granulysin and granzyme staining on indicated tetramer positive and tetramer negative CD8 T cells or NK cells. Cells were gated by isotype controls from GzmB and GNLY. (B) Proportion of CD8 T cells and NK cells in the lung of infected mice. (C) Proportion of tetramer positive cells in pulmonary CD8 T cells of infected mice. (D) Frequencies of activated CD8 T cell, granzyme B-expressing CD8 T cell, short-lived effector CD8 T cells, and memory precursor CD8 T cells in the lung of infected mice. (E) Total numbers of pulmonary CD8 T cells, antigen-specific CD8 T cells, and NK cells. (F) Proportion of NK cells that express both GzmB and GNLY or only GzmB. (G) Pulmonary bacterial loads of C57BL/6 littermate control and GNLY-Tg mice. Statistical testing by a two-tailed, unpaired Student’s T test.
Adoptive transfer of CD8 T cells from GNLY-Tg mice do not exhibit better control of Mtb infection

We considered the observation that transgenic CD8 T cells needed to be heavily stimulated to express granulysin. To remove the other crucial immunological players during tuberculosis and focus CD8 T cells to be the sole player, I adoptively transferred purified naïve CD8 T cells from GNLY-Tg or C57BL/6 littermate control to TCRα KO recipients that lacks T cells. The adoptive transfer animals were infected via aerosol route with Mtb at ~100 CFU one day after. Despite successful transferring of CD8 T cells which yielded the same numbers and similar phenotypes of CD8 T cells in the lungs and spleens of infected mice (Figure A.7A-B), we were not able to detect any granulysin productions from CD8 T cells (data not shown). Although CD8 T cells from both GNLY-Tg and C57BL/6 mice helped lower the number of bacteria significantly compared to the non-transferred animals, there was no differences in pulmonary and splenic bacterial burdens between the groups (Figure A.7C-D). In addition, we did not observe a prolong survival when extending the study further (Figure A.7E). Together, we did not detect granulysin nor its contribution on CD8 T cells to immunity against Mtb infection.
Figure A.7: Adoptive transfer of CD8 T cells from GNLY-Tg mice do not exhibit better control of Mtb infection

Age-matched C57BL/6 littermate control and GNLY-Tg mice were used as donors to purify CD8 T cells for adoptively transferring to TCRα KO recipients 24 hours prior to aerosol infection. At 5 weeks post infected, infected mice were sacrificed for flow cytometry and bacterial control analysis. (A) Total numbers of pulmonary and splenic CD8 T cells following infection. (B) Frequencies of memory precursor CD8 T cells, short-lived effector CD8 T cells, and activated CD8 T cell in the lung of infected mice. (C-D) Bacterial burden in the (C) lungs and (D) spleens of TCRα KO adoptively transferred with CD8 T cells from C57BL/6 littermate control or GNLY-Tg mice. (E) Survival study of Mtb-infected adoptively transferred TCRα KO mice. Statistical testing was by one-way ANOVA, using the Dunnett posttest compared to No Tx. ***, p<0.005; ****, p<0.0001.
**Discussion**

Consistent with previous reports, IL-2 and IL-15 were the strongest stimulators for the expression of GNLY in human PBMCs (251), mouse CD8 T cells and mouse NK cells (249). However, while GNLY expression in NK cells were detected following *in vivo* Mtb infection, no expression was detected in CD8 T cells. In addition, there was no difference in bacteria control between transgenic and wild-type mice. Our lab has previously identified the low amount of IL-2 detected *in vivo* following Mtb infection (Miye Jacques, unpublished observation). This could partly contribute to the situation where there were not enough cytokines to stimulate GNLY expression on the GNLY-Tg CD8 T cells *in vivo*. In fact, poor production of IL-2 following Mtb infection potentially impairs immunity to TB. In particular, this could result in suboptimal activation of CD8 T cells. One could address this issue by giving the GNLY-tg or control mice with exogenous IL-2 and IL-15, and determining whether their ability to control bacterial growth *in vitro* is enhanced.

However, based on our observations, we postulated that the GNLY-Tg mice did not have the appropriate expression on CD8 T cells similarly to human CD8 T cells as we expected. Despite the effort to use specific and strong promoter, the granulysin transgenic mice were generated using bacteria artificial chromosome system where the transgene was randomly integrated (249). The researchers performed *in situ* hybridization to identify the location of the transgene, and subsequently showed the RNA and protein expressions of granulysin. Yet, subsequent studies utilizing the GNLY-Tg mice did not display the detection of...
GNLY protein on CD8 T cells (250). The incorrect GNLY expression, therefore, impeded our effort to study multiple aspects of granulysin including (1) granulysin contribution to resistance against in vivo Mtb infection and (2) whether the resistance was mediated by CD8 T cells.

Given that granulysin has shown its cytotoxicity in vitro with human CD8 T cells (85), studying granulysin during in vivo Mtb infection would enhance the understanding of granulysin role for bacterial clearance. In human, granulysin consists of three different isoforms without known distinct functions (252). We have recently cloned human granulysin isoform 2 and 3 into retroviral vectors and expressed into stable cell lines, along with generating granulysin isoform 1 lentiviral vector. We aim to transduce the granulysin-expressing virus into CD8 T cells for in vitro Mtb infection study, and generate retrogenic mice expressing granulysin to further elucidate the role of granulysin in vivo.

This work will recognize T cell effector functions that are crucial for the control of Mtb infection and the potential targeted mechanisms to improve the design and development of vaccines against Mycobacterium tuberculosis.
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