Immune Correlates of Resistance and Susceptibility to Tuberculosis

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy
in the Graduate School of The Ohio State University

By

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Abstract

Tuberculosis (TB) has been a prevalent cause of morbidity and mortality in human beings for centuries, and is still a global health emergency today. Although not the headline of news in the United States, TB is rampant in sub-Saharan Africa and southeast Asia, where it is the number one killer of patients with acquired immunodeficiency syndrome (AIDS). Of the estimated 2 billion people infected with *Mycobacterium tuberculosis* (*Mtb*), the etiological agent responsible for TB, only a small percentage progress to active, contagious disease. This is because the immune system of most infected individuals is capable of effectively containing the infection, whereas in a percentage of individuals this containment will eventually fail, leading to reactivation of TB. Reactivation can arise due to numerous factors, including HIV infection, malnutrition, aging, or other immunosuppression. In some individuals, however, reactivation can occur without any overt immune deficiency. In this work, we sought to gain further understanding of the immunological mechanisms behind TB reactivation in susceptible individuals with otherwise intact immune systems.
The differences in susceptibility of human populations are also observed in mice. Inbred strains of mice generally fall into two main categories: those that are relatively resistant to TB reactivation, and those that are relatively susceptible. In this work, we used C57BL/6 mice as a representative of the resistant strains of mice, and CBA/J mice to represent susceptible strains. Previous work by our laboratory has identified a main correlate of reactivation susceptibility in CBA/J mice to be overproduction of the immunosuppressive cytokine, interleukin-10 (IL-10). CBA/J mice genetically deficient in IL-10, were resistant to *Mtb* reactivation and developed human-like TB lesions, a finding that is unprecedented in the field. We also more clearly defined the susceptibility of CBA/J mice to be the result of significant CD8+ T cell dysfunction. Additional studies demonstrated ways to enhance the immune response to *Mtb* by removing the inhibitory receptor, killer cell lectin-like receptor G1 (KLRG1), which we hypothesize could significantly shorten the treatment for TB. Overall, this collective work expands our understanding of how susceptibility to *Mtb* is mediated, and how protective responses may be enhanced to ease the burden of this devastating disease on humanity.
Dedication

This work is dedicated the millions of human lives, past and present, that have been afflicted with tuberculosis. May our discoveries foster new, potent treatments of tuberculosis, and assuage some of the suffering of this world.

_The last enemy that shall be destroyed is death._
Acknowledgements

I would like to extend my most sincere thanks to my advisor, Dr. Joanne Turner, for her dedication in training and molding me into the scientist and philosopher I am today. Most of all I value her constant availability and the freedom of discussion she made possible. I would also like to acknowledge the guidance and support of my dissertation committee members, Dr. Larry Schlesinger, Dr. Virginia Sanders, and Dr. Emilio Flaño. This work would also not have been possible without the assistance of past and present members of the Turner laboratory: Dr. Bridget Carruthers, Dr. Gillian Beamer, Dr. Erin Rottinghaus, Elisha Koivisto, Nandan Gokhale, Rachel Kominsky, and Cynthia Wu.

Dr. Bridget Carruthers’ development of IL-10-deficient CBA/J mice is critical to Chapter 2 of this dissertation, and I would like to extend my sincere thanks to her for all of her work, guidance, and expertise throughout my career.

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Publications


Field of Study

Major Field: Integrated Biomedical Science
Emphasis: Immunology
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Abstract

Introduction

Materials and Methods

Mice

Mtb infection and Colony Forming Unit Enumeration

Lung cell isolation

Cell Purification

ELISpot Assays

Flow cytometry

Immunohistochemistry

Statistics

Results

CD8+ T cells accumulate in the lungs of CBA/J mice as Mtb infection progresses

CBA/J mice develop diffuse, unorganized granulomas after Mtb infection

Accumulated CD8+ T cells from Mtb-infected CBA/J mice express markers of T cell dysfunction

CD8+ T cells from Mtb-infected CBA/J mice are capable of secreting IL-10

CD8+ T cell depletion in CBA/J mice moderately increases pro-inflammatory responses against Mtb

CD8+ T cells from CBA/J mice are clonally expanded

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<td>-/-</td>
<td>Knockout</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ag85</td>
<td>Antigen 85</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>Allophycocyanin-Cy7</td>
</tr>
<tr>
<td>AraLAM</td>
<td>Arabinose Lipoarabinomannan</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine Triphosphatase</td>
</tr>
<tr>
<td>β2M</td>
<td>Beta-2 Microglobulin</td>
</tr>
<tr>
<td>BCG</td>
<td><em>Mycobacterium bovis</em> Bacillus Calmette-Guerin</td>
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<tr>
<td>BMDC</td>
<td>Bone Marrow-derived Dendritic Cell</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine (5-bromo-2-deoxyuridine)</td>
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<tr>
<td>BSL3</td>
<td>Biosafety Level 3</td>
</tr>
<tr>
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<td>Chemokine C-C Ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CIITA</td>
<td>MHC Class II Transactivator</td>
</tr>
<tr>
<td>CFP</td>
<td>Culture Filtrate Protein</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CR</td>
<td>Complement Receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-Lymphocyte Antigen 4</td>
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<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell-Specific ICAM-3 Non-integrin</td>
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<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
</tr>
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<td>DNase</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>DOTS</td>
<td>Directly Observed Treatment, Short-course</td>
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<td>E-cadherin</td>
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<td>ELISA</td>
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<td>Early Secreted Antigenic Target-6</td>
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<td>FcR</td>
<td>Constant fraction receptor</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>Glucocorticoid-Induced Tumor Necrosis Factor Receptor</td>
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<td>GKO</td>
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<td>Granzyme B</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>H&amp;E</td>
<td>Hemotoxylin &amp; Eosin</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
</tr>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<td>Hepatitis C virus</td>
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<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N’-2-Ethenesulfonic Acid</td>
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<td>Human Leukocyte Antigen</td>
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<td>Heme Oxygenase-1</td>
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<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
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<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>IFNγR</td>
<td>IFN-γ Receptor</td>
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<tr>
<td>ICAM</td>
<td>Intracellular Adhesion Molecule</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>INH</td>
<td>Isoniazid</td>
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<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<tr>
<td>ITIM</td>
<td>Immunotyrosine-based Inhibitory Motif</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factors</td>
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<tr>
<td>JAK</td>
<td>Janus Activated Kinase</td>
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<tr>
<td>KLRG1</td>
<td>Killer cell lectin-like receptor subfamily G member 1</td>
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<td>LAM</td>
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<td>Lymphocytic choriomeningitis virus</td>
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<td>MDM</td>
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<td>MDR</td>
<td>Multi Drug-Resistant</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
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<td>MLN</td>
<td>Mediastinal Lymph Node</td>
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<td>MMP9</td>
<td>Matrix Metalloproteinase-9</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose Receptor</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td><em>Mt</em></td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>neg</td>
<td>Negative</td>
</tr>
<tr>
<td>NHP</td>
<td>Nonhuman Primate</td>
</tr>
<tr>
<td>NRAMP</td>
<td>Natural Resistance-associated Macrophage Protein</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor Kappa B</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
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<td>NKT</td>
<td>Natural Killer T cells</td>
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<td>NO</td>
<td>Nitric Oxide</td>
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<td>OADC</td>
<td>Oleic Albumin Dextrose Catalase</td>
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<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature Media</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PD-1L</td>
<td>Programmed death ligand-1</td>
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<td>PDIM</td>
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</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>Phycoerythrin-Cy7</td>
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<td>PerCP-Cy5.5</td>
<td>Peridinin chlorophyll protein-Cy5.5</td>
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<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
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<tr>
<td>PIM</td>
<td>Phosphatidyl myo-inositol mannosides</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear cell</td>
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<tr>
<td>PPD</td>
<td>Purified Protein Derivative</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
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<td>PZA</td>
<td>Pyrazinamide</td>
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<td>RAG</td>
<td>Recombinase-activating Gene</td>
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<td>RD-1</td>
<td>Region of Difference-1</td>
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<td>RIF</td>
<td>Rifampin</td>
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<td>ROI</td>
<td>Reactive Oxygen Intermediates</td>
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<td>RNI</td>
<td>Reactive Nitrogen Intermediates</td>
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<tr>
<td>RPMI</td>
<td>Royal Park Memorial Institute (culture medium)</td>
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<td>S1P</td>
<td>Sphingosine 1-phosphate</td>
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<tr>
<td>SHIP-1</td>
<td>Src homology 2 containing inositol phosphatase-1</td>
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<tr>
<td>SP</td>
<td>Surfactant Protein</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of Cytokine Signaling 3</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell Receptor</td>
</tr>
<tr>
<td>TDM</td>
<td>Trehalose 6,6-dimycolate</td>
</tr>
<tr>
<td>TDR</td>
<td>Totally Drug-Resistant</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>T\text{H}1</td>
<td>T helper Type 1</td>
</tr>
<tr>
<td>T\text{H}17</td>
<td>T helper Type 17</td>
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<tr>
<td>TIM3</td>
<td>T cell immunoglobulin and mucin protein-3</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>V\beta</td>
<td>β chain of the Variable region of the T cell Receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>XDR</td>
<td>Extensively Drug-Resistant</td>
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</table>
Chapter 1: An introduction to the history, significance, and understanding of tuberculosis infection and immunity.

On the etymology of tuberculosis

Tuberculosis or scrofula has been a prevalent cause of morbidity and mortality in human beings and other mammals for millennia. Some authors believe tuberculosis was the first disease known to mankind. It has been known by a variety of different names throughout the centuries, usually determined by the location and description of the affliction and the socioeconomic environment of the age. The most ancient written recordings of the disease come from China, Egypt and India from approximately 4500-1500 BCE. In classical antiquity, it was observed by the Greeks as *phthisis*, literally a wasting away or consumption of the body by pus. The prominence of the disease during this period pervades historical and literary texts, and was well documented by preeminent Greek physicians, including Hippocrates and Galen around 450 BCE. By the 17th century, it also came to be known as White Plague, owing to a massive tuberculosis epidemic across civilized Europe. At the time, sovereign heads of state, like the King of France or England, were also considered the head or caput of the Christian Church and as such had divine and curative abilities. Ceremonies were commonly held where the monarchs
would touch, and thereby cure, people of common afflictions like tuberculosis. This gave rise to the name King’s Evil or Mal du Roi. Over the next centuries, scientific and surgical advances continued to be made and much more was discovered concerning the variation, contagiousness, and etiology of the disease. The identification of *Mycobacterium tuberculosis* is attributed to Robert Koch, whose work in isolation and reinfection of microbes is well documented. This brief etymological history covers over 5000 years, and does not begin to convey the vast number of philosophers, scientists, physicians, writers, clerics, and kings that have dedicated their lives to the study of this disease. Tuberculosis truly has shaped the course of human history, and the amount of lives it has touched or destroyed is staggering.

*An historical account of the origin of Mycobacterium tuberculosis*

From the lessons learned over the centuries, we began to understand the organism responsible for causing tuberculosis, yet the exact origin of the human pathogen is still unclear. The oldest evidence of *Mycobacterium tuberculosis* (*Mtb*) in human remains comes from the Neolithic era, around 9000 years ago, in the settlement of Atlit-Yam in the east Mediterranean, while genetic studies examining mycobacterial tandem repeat sequences have dated the *Mtb* complex (*M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, *M. pinnipedii*, and *M. caprae* species) back 40,000 years, coinciding with the time early humans were thought to be migrating out of eastern Africa. There was some debate over the possible zoonotic origin of *Mtb*, but current genetic studies have shown
that it was likely passed from humans to animals originally. Compared to other ancient bacteria, the *Mtb* complex has an extremely low level of genetic variation over the millennia, with approximately 0.01–0.03% synonymous nucleotide variation. This suggests that our current *Mtb* species is likely a clonal expansion that arose from a single member of a much more diverse progenitor species after an evolutionary bottleneck approximately 20,000 to 35,000 years ago.

*Modern epidemiology and incidence of TB*

The concentration of TB cases grew with human civilization after the Industrial Revolution. From the 17th century onward, TB has been most prevalent in densely populated areas with poor sanitation and public health practices. Today, an estimated 2 billion people are infected with *Mtb*, with the overwhelming majority of cases endemic to sub-Saharan Africa and southeast Asia. The 22 countries listed by the World Health Organization (WHO) as most afflicted with TB are Afghanistan, Bangladesh, Brazil, Cambodia, China, the Democratic Republic of the Congo, Ethiopia, India, Indonesia, Kenya, Mozambique, Myanmar, Nigeria, Pakistan, the Philippines, the Russian Federation, South Africa, Thailand, Uganda, the United Republic of Tanzania, Viet Nam and Zimbabwe. In 2010, global incident cases of TB were approximately 8.8 million, with 1.1 million deaths in human immunodeficiency virus (HIV)-negative individuals, and an additional 0.35 million from HIV-positive patients. Although high, the absolute number of global incident cases has been falling since 2006 and TB mortality has been
declining since 2002. WHO initiatives and task forces have been focused on eliminating TB for decades, and in 1993 TB was declared a world health emergency. Goals were set to reduce the incident cases and mortality of TB by 50% in 2015 compared to 1990. The WHO has divided TB-afflicted countries into larger Regions, some of which have met or are close to meeting the 50% reduction. If current trends continue, all regions except the African Region are expected to reach the goal 18.

**TB treatment, drug resistance, and public health concerns**

Of the approximately 2 billion people infected with *Mtb*, only around 10% will progress to active disease 18. The remaining bulk of infected individuals develop a low-level, latent *Mtb* infection that is controlled by their immune system. Latent TB is characterized by a non-replicating persistence of *Mtb* 19-23. The primary means of diagnosis of TB for over 100 years has been sputum smear microscopy, especially in areas with poor technological availability 24, 25. The standard test is by *Mtb* culture, yet faster molecular tests are being adopted as the endemic area permits technologically 26-29. Vaccination for *Mtb* is practiced in many parts of the world, predominantly in the eastern hemisphere, but except in very rare cases, is not widely used in the United States 30-32. The efficacy of the vaccine strain, *Mycobacterium bovis* Bacille Calmette–Guérin (BCG), is inconsistent, particularly among various age groups, and is not effective against pulmonary TB 33-35. As a result, numerous new vaccines are in clinical trials; ranging from viral vectors expressing *Mtb* antigens (MVA85A) 36, to recombinant BCG (AERAS402) 37.
According to the Center for Disease Control and Prevention\textsuperscript{38}, treatment of latent TB consists of various adaptations of treatment regimens based on the specific case, but each uses the drugs isoniazid (INH) and rifampin (RIF). Depending on the chosen regimen, combinations of these drugs are taken for 6-9 months. Treatment of active TB is attempted with a combination of all first-line anti-TB drugs with the addition of ethambutol (EMB), and (RPT), and pyrazinamide (PZA). These drugs are given in concert to avoid creating resistant strains, which quickly develop after single drug treatment. The primary method of action of each drug is as follows:

- INH: Inhibits synthesis of \textit{Mtb} cell wall mycolic acids\textsuperscript{39,40}
- RIF: Inhibits the action of bacterial DNA-dependent RNA polymerase\textsuperscript{41-43}
- PZA: Inhibits the activity of \textit{Mtb} fatty acid synthase\textsuperscript{44,45}
- EMB: Inhibits arabinosyl transferase necessary for cell wall synthesis\textsuperscript{46,47}
- RPT: A derivate of RIF with similar mechanism of action\textsuperscript{48,49}

Treatment regimens for active TB are usually broken into a 2 month initial phase with all first-line treatments, followed by various options for the continuation phase over the following 4-7 months. One the most crucial factors and deterrents of TB treatment is compliance. Medication must be taken daily and consistently for the entire prescribed length. To help ensure adherence with the exact treatment regimen, directly observed treatment, short-course (DOTS) was established. DOTS is a system put in place by the WHO to ensure TB testing and treatment is carried out with maximum efficiency to boost compliance with prescribed treatment regimens\textsuperscript{50-52}. It ensures that tests are quick and
accurate, treatment is observed, and drugs are always available. Without treatment compliance, *Mtb* can rapidly become resistant to first-line antibiotics. The amount of clinics offering TB drugs in endemic areas are often not equipped to monitor and observe treatments in accordance with DOTS, leading to new, dangerously resistant forms of *Mtb*.

Drug-resistant TB is characterized by resistance to at least one first-line antibiotic. Multi-drug-resistant (MDR) TB is resistant to two first-line treatments which must include INH or RIF. Further resistance is also seen in the form of extensively or extremely drug-resistant TB (XDR) which is resistant to all first-line antibiotics. Treatment of these forms of TB is much more difficult, since each case must be individually examined to determine drug sensitivities. Once the individual’s strain has been determined, treatment with second-line antibiotics including aminoglycosides, fluoroquinolones, and cycloserine (among others) can be started. Recently, cases have been reported as being resistant to all known antibiotics and have been deemed totally drug-resistant (TDR). In such desperate cases, experimental compounds like imipenem, co-amoxiclav, or PA-824 have been used, though their safety and efficacy are unknown.

*Mtb cell wall structure, metabolism, and virulence factors*

*Mtb* drug resistance arises due to the application of selection pressure to a bacterial population which forces only the most fit variations within that population to survive. If treatment is not adhered to properly or completely, this selection pressure is lifted and the
surviving bacteria repopulate the entire population. In *Mtb*, the main determinant of drug resistance is cell wall variation. The cell wall of *Mtb* is thick and complex, comprised of proteins, mycolic acids, polysaccharides (arabinogalactan), peptidoglycan, and various other glycolipids and sugars. The most abundant extractable lipid in the *Mtb* cell wall is trehalose 6,6-dimycolate (TDM), or cord factor. TDM is a main virulence factor of *Mtb*, capable of inhibiting phagosomal acidification and phago-lysosomal fusion. Other key glycolipids of the cell wall are mannose-capped lipoarabinomannan (ManLAM), uncapped LAM (AraLAM), phospho-myoinositol lipoarabinomannan (PI-LAM), phosphatidyl myo-inositol mannosides (PIMs), and phthiocerol dimycocerosates (PDIMs). These are each critical for the virulence and cell wall metabolism of *Mtb*, but studies have shown that many are also capable of immunomodulatory effects.

ManLAM has been shown to direct *Mtb* entry into host macrophages through the mannose receptor rather than other phagocytic receptors like complement receptor 1, 3, or 4 (CR), thereby subverting the generation of a proper pro-inflammatory response.

Although *Mtb* cell wall lipids can be very immunogenic, there are various secreted and cell wall-associated proteins that induce potent changes in the immune response to *Mtb*. It has been identified that virulent *Mtb* possess a specialized ESX-1 protein secretion system that is necessary for its virulence, given that the BCG vaccine strain of *Mtb* lacks nine genes from the ESX-1 locus (known as region of difference (RD1)) and has significantly attenuated growth inside of macrophages and animals. Two of the main proteins secreted by *Mtb* are early secretory antigenic target protein 6 (ESAT6) and
culture filtrate protein 10 (CFP10). Both proteins are encoded by the RD1 genes Rv3874 and Rv3875 \(^{69}\), can interact to form heterodimers \(^{70}\), and have been shown to act primarily on macrophages by inhibiting the production of interleukin-12 (IL-12) and tumor necrosis factor (TNF) \(^{71}\). ESAT6 has also been shown to have pore-forming qualities, and is hypothesized to play a significant role in the escape of *Mtb* from the phagosome \(^{72,73}\).

Another critical component of *Mtb* virulence is the fibronectin-binding protein antigen-85 (Ag85). The Ag85 complex is composed of Ag85A, Ag85B, and Ag85C which are encoded by three different genes at different sites in the mycobacterial genome \(^{74}\). The Ag85 complex functions primarily in *Mtb* cell wall synthesis, acting as a mycolyl transferase, but it appears that the individual subunits may be functionally redundant, as genetic disruption of each component showed no defect in intracellular and *in vitro* growth \(^{75}\). Regardless of the exact role of each component, Ag85 is capable of the induction of potent pro-inflammatory responses in animal models of TB \(^{76-78}\).

*The initial interaction of Mtb with the pulmonary environment*

*Mtb* is primarily a pulmonary pathogen that is transmitted via aerosol droplets and inhaled, passing into the distal airspaces. The lung environment encountered upon initial infection with *Mtb* is very important and can alter the course of infection. Components of pulmonary surfactant, such as surfactant protein (SP)-A, SP-D, and various enzymes can
directly affect the initial interaction of \textit{Mtb} with the immune system. SP-A and SP-D are collectins and both contain C-type (calcium-binding) lectin domains that can recognize carbohydrates domains of \textit{Mtb}. Studies have shown that SP-A can adhere to \textit{Mtb} and enhance phagocytosis by macrophages through the mannose receptor (MR) \textsuperscript{79}. Furthermore, SP-A was found to increase the expression of the mannose receptor (MR) and activate a phosphoinositide 3-kinase (PI3K) and calcium-dependent signaling system in human monocyte-derived macrophages (MDMs) which induced a state of alternative activation \textsuperscript{80}. SP-D has been shown to have very different functions, and may help the host immune response control \textit{Mtb} infection. SP-D was shown to bind and agglutinate \textit{Mtb} subsequently leading to decreased phagocytosis by human MDMs, although the aggregation of \textit{Mtb} by SP-D was shown not to affect bacterial uptake itself \textsuperscript{81}. Further studies have demonstrated that SP-D can increase phago-lysosomal fusion during \textit{Mtb} infection \textsuperscript{82}. Various enzymes are present in pulmonary surfactant that can directly affect the cell wall of \textit{Mtb}. It has been shown that human lung hydrolases can alter the cell wall of \textit{Mtb} such that it dramatically decreases association of \textit{Mtb} with macrophages and generates a protective immune response \textsuperscript{83}. Examination of the effects of human lung hydrolases and surfactant proteins on \textit{Mtb} will continue to expand our understanding of the complex interaction of \textit{Mtb} with the pulmonary environment.

As alluded to previously, the alveolar macrophage is the first cell to encounter \textit{Mtb} in the airway and the phagocytic receptor employed dictates the type of immune response that will be generated. Studies have demonstrated that when \textit{Mtb} is phagocytized by the MR,
a weak and ineffective response is generated. Various factors contribute to which phagocytic receptor is used, but as aforementioned, interaction of SP-A or ManLAM with the macrophage will enhance MR-mediated uptake. This is usually considered to be the preferred method of entry for *Mtb* since it subverts the generation of optimal pro-inflammatory responses. Conversely, another phagocytic receptor used for the uptake of *Mtb* is CR3, which drives a strong pro-inflammatory response. Other receptors, such as Fc receptors (FcR), bind to the Fc region of antibodies during antibody-mediated opsonization of particles and pathogens. FcRs are also capable of recognizing *Mtb*, yet studies have shown it is less preferred than CR.

Pulmonary dendritic cells (DCs) can use different receptors to interact with *Mtb*. Another C-type lectin, DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), is present on human DCs (and certain pulmonary and placental macrophages) and interacts with *Mtb* via ManLAM but not AraLAM. DC-SIGN recognizes dimeric and trimeric mannose residues in ManLAM and requires specific spacing on glycoproteins. Studies have demonstrated that interaction of DC-SIGN with *Mtb* ManLAM prevents maturation of DCs and interferes with toll-like receptor (TLR)-mediated activation. The precise *in vivo* interaction of DC-SIGN with *Mtb* is still being examined, but overall these studies suggest that *Mtb* preferentially binds DC-SIGN as the phagocytic receptor on DCs, allowing for subversion of pro-inflammatory responses.
Macrophage killing mechanisms and intracellular survival of Mtb

*Mtb* is a very successful pathogen, primarily due to its ability to survival within host macrophages. Depending on what phagocytic receptor was used for entry, *Mtb* is capable of subverting the maturation and acidification of the phagosome by preventing the association of a vacuolar ATPase with the phagosomal membrane 

90-93, and impairing phago-lysosomal fusion 

94-96 through the secretion of acid phosphatases 97. Through the secretion of ESAT6, *Mtb* is speculated to escape from the phagosome into the cytosol 98-101, further enhancing its survival. Overall, there are various mechanisms employed by *Mtb* to arrest normal protective macrophage function and impair the generation of pro-inflammatory responses.

It is not until the onset of adaptive immunity, where *Mtb*-specific T cells activate infected macrophages via IFN-γ stimulation that *Mtb* can be killed 102-106. Activated macrophages are better at phagosome acidification and phago-lysosomal fusion 107, 108. Upon successful maturation of the phagolysosome, various compounds and proteins are employed to kill *Mtb*. Lysozyme (muramidase), proteases, and free radicals from reactive oxygen and nitrogen intermediates (ROI, RNI) all attack *Mtb* and attempt to disrupt the cell membrane and lyse the bacteria 109-114. 1,25-dihydroxy vitamin D₃ has also been shown to inhibit the growth of *Mtb* by stimulating the vitamin D receptor on macrophages and enhancing inducible nitric oxide synthase (iNOS) expression 115-118. It is not clear
whether ROI are required to control \textit{Mtb}. Studies have shown that \textit{Mtb} is capable to surviving in the presence of H\textsubscript{2}O\textsubscript{2} \textsuperscript{103}.

\textit{The innate immune response to Mtb}

\textit{Mtb} does not fully activate the innate immune response during its initial interactions with pulmonary phagocytes. Using components of its cell wall, like ManLAM, \textit{Mtb} is capable of entering macrophages and DCs surreptitiously and does not initiate the generation of strong anti-\textit{Mtb} responses \textsuperscript{119, 120}. In addition to phagocytic receptors, antigen-presenting cells (APCs) express numerous pattern recognition receptors (PRRs) to sense foreign particles and pathogens \textsuperscript{121}. Most notable in the context of \textit{Mtb} infection are TLRs, which can be located on the cell surface or intracellularly \textsuperscript{122}. Each TLR has unique pattern recognition. For \textit{Mtb} infection, TLR2, TLR4, and TLR9 seem to be the most important \textsuperscript{123}. TLR2 binds lipid moieties, TLR4 recognizes polysaccharides, and TLR9 (which is intracellular) recognizes prokaryotic methylated DNA sequences. Murine studies have demonstrated that the lack of TLR2 \textsuperscript{124, 125} or TLR4 \textsuperscript{126, 127} impairs protective immunity to \textit{Mtb}. As previously mentioned, interaction of ManLAM with DC-SIGN decreases TLR-mediated protection \textsuperscript{87}. TLR stimulation usually leads to protective cytokine protection and the initiation of pro-inflammatory responses via signaling through NF-\kappaB, activating protein-1 (AP1), or interferon regulatory factors (IRF) \textsuperscript{128}. The pro-inflammatory cytokines IL-12 and TNF have been shown to be produced after TLR ligation by \textit{Mtb}, subsequently recruiting more immune cells to the area and activating T cells during the
initiation of adaptive immunity 129. Studies have demonstrated that mice deficient in TLR2 have significantly decreased production of these pro-inflammatory cytokines 124, 130.

Other innate immune cells play important roles in Mtb pathogenesis. Natural killer (NK) cells have been shown to be associated with increased early resistance to intracellular pathogens and can contribute to protective interferon (IFN)-γ secretion 131. Although capable of protection, studies in Mtb infection have shown that NK cells can respond to Mtb but contribute very little to protection 132. Studies using T cell-deficient mice (RAG−/−), show increased IFN-γ production from NK cells after Mtb infection, and increased susceptibility after NK cells removal in RAG−/− mice 133. This suggests that NK cells can contribute to anti-Mtb responses, but may not play an essential role in protection. NK have also been shown to direct the proliferation of another innate immune cell type, γδ T cells 134.

γδ T cells can respond to phosphoantigens from Mtb, such as TUBag4 135, 136. Although γδ T cells can have both innate and adaptive cell functions 137, studies using γδ T cell knockout animals show increased susceptibility only at very high infectious doses 138, 139. Neutrophils or polymorphonuclear (PMN) cells are another cell of the innate immune system that is thought to contribute little to protection against Mtb, yet recent work has shown that PMNs are the most common phagocyte infected by Mtb in the human airway 140. There are many conflicting reports of the role of PMNs in Mtb 141-143, and
interestingly, some studies have demonstrated a detrimental role for PMNs in *Mtb* infection in the mouse model. Overall the innate immune response to *Mtb* can have significant effects on disease progression, but the responsibility and function of individual components cannot clearly be differentiated.

*Generation of the adaptive immune response to Mtb*

The onset of adaptive, T cell-driven immunity to *Mtb* is remarkably delayed after initial infection. In humans, it takes approximately 5-6 weeks after infection to reach a positive tuberculin skin test (purified protein derivative (PPD)), while in mice the earliest T cell responses in the lung have not been detected before day 12 of infection. The underlying cause of this delay is still unclear, but is likely a combination of the slow growth rate of *Mtb* (doubling time is approximately 24 hours), and the fact that although *Mtb* first infects the lungs, *Mtb*-specific CD4+ T cells are not seen in the lung draining lymph node (mediastinal lymph nodes (MLN)) until *Mtb*-containing APCs have migrated to that lymph node. This indicates that the adaptive immune response to *Mtb* is generated in the lung draining lymph nodes and *Mtb*-specific T cells must then migrate back to the lungs before initiating an adaptive immune response. It has been demonstrated that both macrophages and DCs are capable of migrating to the lymph node to prime T cells, yet whether one is used preferentially remains unclear.
Mtb-specific T cells are primed in the MLN and migrate back to the lung by following specific chemokine gradients and by changing the expression of molecules on their surface. Naive T cells express L-selectin (CD62L) which permits entry into the lymph node through afferent lymphatic vessels or the high endothelial venule. Once primed, antigen-experienced T cells downregulate CD62L and upregulate the sphingosine 1-phosphate (S1P) receptor allowing for exit from the lymph node through efferent lymphatics. Once back into the bloodstream, Mtb-specific T cells can follow various chemokine signals to reenter the lungs. The production of TNF and IL-1β by innate immune cells and infected macrophages causes diverse changes to the lung environment and endothelial cells; increasing expression of adhesion molecules like intercellular adhesion molecule 1 (ICAM-1) and chemokines like CCL3, CCL4, CXCL2, CCL2, and CCL5 that facilitate T cell migration.

Humoral immunity in Mtb infection

Since Mtb is an intracellular pathogen, humoral immune responses have been considered less critical for protection against Mtb. Any antibody-mediated effects on immunity to Mtb have historically been discounted. Examination of mice genetically deficient in B cells has produced inconsistent results. Some studies report increased numbers of Mtb, decreased survival, and altered granuloma formation in B cell-deficient mice, while others show little to effect on Mtb control. While antibody-mediated effects do not contribute greatly to Mtb protection, the interaction of B cells with components of
Cell-mediated immunity, like T cells, has recently been shown to be important during TB. Recent murine work has shown that B cells can establish germinal centers and modulate IL-10 levels in \textit{Mtb}-infected lungs \textsuperscript{163}. Overall, the contribution of B cells to \textit{Mtb} immunity is still unclear, but as more studies examine B cells, their importance to \textit{Mtb} infection is becoming more appreciated.

\textit{Cell-mediated immunity to \textit{Mtb} and the contribution of T cell subsets}

The cell-mediated immune response has been shown to be the most critical component of protective immunity to \textit{Mtb}. The arrival and effector function of \textit{Mtb}-specific T cells in the lung takes place after 2-4 weeks of infection in mice \textsuperscript{164, 165}. CD4\textsuperscript{+} T cells are considered the most important cell for combatting \textit{Mtb} infection \textsuperscript{166-168}. This was originally shown by a study demonstrating that adoptive transfer of L3T4\textsuperscript{+} (CD4) or Lyt-2\textsuperscript{+} (CD8) cells from immune competent to immune depleted mice conferred protection against \textit{Mtb} infection \textsuperscript{169}. Another study confirmed this finding, showing that specific depletion of L3T4\textsuperscript{+} or Lyt-2\textsuperscript{+} T cells impaired resistance to \textit{Mtb} infection, and that this was primarily due to depletion of L3T4\textsuperscript{+} cells \textsuperscript{170}.

After discovery of the specific T cell populations responsible for control of \textit{Mtb}, many studies began to identify how protection was established. CD4\textsuperscript{+} T cells can secrete high levels of IFN-\gamma after stimulation. IFN-\gamma knockout mice (GKO) fail to develop RNI and cannot control the growth of \textit{Mtb}. GKO mice develop severe tissue necrosis and succumb
to *Mtb* infection within 20 days \(^{171}\). The protective effect of IFN-\(\gamma\) comes mainly from its signaling through the IFN-\(\gamma\) receptor (IFNGR) on APCs and activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Upon ligation, the IFNGR dimerizes, activating JAK1 and JAK2 which phosphorylate STAT-1 which dimerizes and translocates to the nucleus and binds interferon-gamma activated sequences (GAS) in the promotor regions of target genes \(^{172,173}\). This can lead to the upregulation of major histocompatibility complex (MHC) class II molecules on the APC surface \(^{174}\), increases in ROI and RNI production \(^{175,176}\), and increased ability to produce pro-inflammatory cytokines TNF and IL-1\(\beta\) \(^{177,178}\). This T\(\text{H}1\)-mediated phenotype is essential for control of *Mtb*.

CD4\(^+\) T cells are the predominant source of IFN-\(\gamma\) during infection \(^{168,179}\), but studies have demonstrated that in mice deficient in CD4\(^+\) T cells, IFN-\(\gamma\) returns to wild-type levels by three weeks of *Mtb* infection, indicating that CD8\(^+\) T cells were compensating by producing more IFN-\(\gamma\) \(^{168}\). CD4\(^+\) T cells can have other functions in *Mtb* infection other than IFN-\(\gamma\) production, such as lysis of infection macrophages \(^{180}\), and are critical for protective granuloma formation \(^{181,182}\). CD8\(^+\) T cells were considered less relevant to *Mtb* infection since they recognize cytoplasmic antigens presented in the context of MHC class I, and *Mtb* resides within a cytoplasmic vacuole, but recent work has demonstrated that *Mtb* is capable of escaping the phagosome and can be processed intracellularly \(^{183}\).
In *Mtb* infection, CD8\(^+\) T cells can be either classically restricted to MHC class I, or non-classical MHC molecules, like HLA-E\(^{184}\). Studies using β2 microglobulin deficient mice (and therefore deficient in CD8\(^+\) T cells) showed increased susceptibility to *Mtb* infection\(^{185,186}\). This may have been due to the fact that β2m\(^{-/-}\) mice lose all CD8\(^+\) T cells, not just those classically restricted to MHC class I. β2m\(^{-/-}\) mice developed macrophage-dominated granulomas with delayed lymphocyte accumulation\(^{187}\). More recent studies utilized mice deficient in only MHC class Ia, and therefore lack only classically restricted CD8\(^+\) T cells. These studies showed a similar but less significant increase in *Mtb* susceptibility\(^{188}\). Although not completely understood, it is generally accepted that CD8\(^+\) T cells are protective against *Mtb*, and contribute by lysing target cells and aiding in IFN-γ production.

Historically, IFN-γ and TNF were thought to be the primary cytokines responsible for inducing potent chemokine production from macrophages, and driving protective pro-inflammatory responses. Although these T\(_H\)1 cytokines have long been considered necessary for control of *Mtb* infection, recent work has demonstrated that T cells secreting IL-17 are important during TB. IL-17-producing cells, or T\(_H\)17 cells, can secrete IL-17A\(^{189}\), IL-17F\(^{190}\), IL-21\(^{191}\), and IL-22\(^{192}\), and were first shown to contribute to pro-inflammatory responses by inducing PMN recruitment in *Klebsiella pneumoniae* infection\(^{193}\). However, in *Mtb* infection the role of IL-17 is less well understood. Some studies have shown that IL-17 or IL-23 deficient mice have no defect in control of *Mtb*\(^{194,195}\), while others using high doses of *Mtb* via intratracheal infection have shown a
significant increase in susceptibility without IL-17\textsuperscript{196}. Further studies have identified $\gamma\delta$ T cells as a significant source of IL-17 after high dose $Mtb$ infection\textsuperscript{197}, but whether these findings are representative of human TB is not known. Since the primary mechanism of $T_H^{17}$-mediated pro-inflammation has been shown to be PMN recruitment, it is not surprising that the requirement for IL-17 production in $Mtb$ remains unclear.

*Defining aspects of protective immunity and granuloma formation in Mtb infection*

Even today, researchers have not clearly identified what is necessary for protective immunity to $Mtb$. Much has been learned using genetic knockout models to understand the function and contribution of various aspects of the immune response to $Mtb$, but these studies do not extend to humans. What constitutes a protective anti-$Mtb$ immune response in man? Surely, IFN-$\gamma$ is important, as are CD4$^+$ and CD8$^+$ T cells, but exactly how the immune response assembles to control $Mtb$ infection is still unknown. One of the main hallmarks of proper control of $Mtb$ infection is the formation of mature granulomas or lesions. Since $Mtb$ cannot be eradicated without chemotherapy, the body attempts to contain $Mtb$ within infected macrophages and prevent their dissemination out of the lungs.

Following specific cues from cytokines like TNF, immune cells cluster around infected macrophages and begin to try and contain the infection. Infected macrophages become apoptotic or necrotic and are phagocytized by healthy macrophages, eventually

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developing into heavily vacuolated foamy macrophages and multinucleated giant cells. T cells are intimately involved with the infected macrophages, secreting IFN-γ, and activating and lysing infected cells. Higher levels of cellular organization will dictate how the granuloma matures and how effective it is at containing the infection. Small, concise foci are usually considered protective, while large, diffuse, unorganized lesions are indicative of poor control. The amount of complexity in the spatial and temporal interplay of all the factors involved in adaptive immunity and granuloma formation has made it difficult to accurately model. Work has been done to simulate granuloma formation using mathematical and computer modeling. Although still developing, this work attempts to circumvent the difficulties in observing human granulomas. A major deterrent in the study of Mtb granulomas has been the lack of small animal models capable of making human-like lesions. Human Mtb granulomas mature into concise, organized foci that become fibrotic and necrotic. Mtb granulomas in mice do not develop this same mature, concise, organized structure representative of protective human granulomatous containment, even in relatively resistant mouse strains, like C57BL/6.

Both the physical and computational models of granuloma formation demonstrate that TNF is critical. Early studies in Schistosoma reported that TNF induced ICAM-1, and blockade of TNF completely ablated proper granuloma formation. During Mtb infection, several studies have demonstrated that TNF induces chemokine production necessary for the cellular recruitment involved in granuloma formation and maintenance. TNF also functions to increase the expression of iNOS, thereby enhancing the
production of NO and RNI by macrophages\textsuperscript{209,210}. We have gained much insight into TNF-mediated immune effects in murine infection, but through the use of anti-TNF antibody we are gaining an understanding of TNF’s importance in man. Patients receiving anti-TNF immunotherapy for autoimmune disorders have been shown to have altered \textit{Mtb} granulomas and increased susceptibility of \textit{Mtb} reactivation\textsuperscript{211}. Although shown to be critical for protection, alterations in TNF production can also lead to severe immunopathology\textsuperscript{212,213}, however, TNF is also responsible for limiting inflammation by inducing apoptosis during \textit{Mtb} infection\textsuperscript{214,215}. It is clear that TNF is one of the most important cytokines in \textit{Mtb} infection, and is responsible for maintaining a healthy balance of pro- and anti-inflammatory signals.

\textit{Regulation of protective immune responses}

In addition to generating a strong T\textsubscript{H}1 immunity against \textit{Mtb}, the body must be able to regulate this level of inflammation to avoid autoimmune-related tissue damage\textsuperscript{216,217}. One of the ways this is achieved is through the production of anti-inflammatory cytokines, like IL-10 and transforming growth factor-\textit{β} (TGF\textit{β}). IL-10 is an anti-inflammatory cytokine most readily associated with macrophages, both as a source of IL-10 and as the population most impacted by its action\textsuperscript{218}. Numerous other cells have, however, been shown to secrete IL-10 including DCs, T cells, B cells, neutrophils, eosinophils, and mast cells\textsuperscript{218}. The secretion of IL-10 is mediated by several cytokines including IL-12\textsuperscript{219}, IL-6\textsuperscript{220}, TGF\textit{β}\textsuperscript{221} and IL-27\textsuperscript{222,223} although the exact pathways that
lead to IL-10 secretion are currently unclear. The action of IL-10 on target cells is more clearly described and is mediated by the IL-10 receptor (IL-10R), a dimer consisting of an α and β subunit. Engagement of the IL-10R results in activation of Jak1 and Tyk2 protein tyrosine kinases, and the activation, and DNA binding, of signal transducer and activator of transcription 3 (STAT3), leading to a downstream alteration in biological function of the target cell. The action of IL-10 results in the down-regulation of MHC class II proteins and co-stimulatory molecules such as CD80 and CD86 on the surface of target macrophages. IL-10 also suppresses the production of ROI and RNI in activated macrophages. Therefore, IL-10 diminishes the capacity of innate immune cells to kill pathogens, as well reducing their capacity to generate and maintain responsive antigen specific T cells.

The suppression of effector function after IL-10R engagement is mediated by several key molecules, including heme oxygenase-1 (HO-1) and suppressor of cytokine signaling-3 (SOCS3). Both HO-1 and SOCS3 induce potent changes in JAK/STAT signaling through the MAP kinase system leading to disruption of effector cell function. Although the specifics of IL-10R signaling are still being elucidated it is evident that SOCS3, and potentially SOCS1, are mediators of cellular response including TNF, IFN-γ and nitric oxide production. Furthermore, it appears that SOCS1 and SOCS3 can negatively impact the production of IL-10, indicating an internal SOCS dependent negative feedback loop for the regulation of IL-10. Since IL-10 has such a potent effect on protective immunity, controlling its activity is a vital component of...
immune competence. Several studies report that epigenetic remodeling of the *IL10* locus is the primary regulatory mechanism of IL-10 production \(^{237-240}\). Though chromatin remodeling may be an important first step in IL-10 expression, other molecular factors also regulate IL-10 production. Various transcription factors such as CREB, ATF-1 \(^{241}\), GATA3 \(^{242,243}\) and MAF \(^{244}\) bind to the *il10* promotor but no single factor is sufficient to induce IL-10 expression. Post-transcriptional control of *il10* mRNA is regulated by elements in the 3’ untranslated region (UTR) \(^{245}\) and microRNAs \(^{239,246}\) that lead to *il10* mRNA degradation. Most of these control mechanisms are induced by IL-10 itself in a negative feedback loop \(^{247-249}\). The subtle balance of pro- and anti-inflammatory signals determines the extent of IL-10 expression and production.

Regulation of immune responses is controlled in more ways than with immuno-suppressive cytokines alone. Cell-cell contact via inhibitory receptors as well as B cell-derived antibody production can contribute to the modulation of immune response to *Mtb*. Fcγ receptors can regulate immunity after recognizing antibodies produced by B cells. Recent work has demonstrated that FcγRIIB\(^{-/-}\) C57BL/6 mice have enhanced control of *Mtb* infection, associated with increased numbers of IFN-γ\(^+\)CD4\(^+\) T cells and elevated expression of MHC class II \(^{250}\). Inhibitory signaling receptors, such as programmed death-1 (PD-1), killer cell lectin-like receptor G1(KLRG1), and T cell immunoglobulin and mucin protein-3 (Tim3) have all been shown to inhibit protective immune responses \(^{251-254}\). As with all aspects of immunity disturbing the balance of inflammatory signals can have severe consequences, as demonstrated in PD-1\(^{+-}\) mice infected with *Mtb*. These
mice develop rapid and severe immunopathology with unchecked replication of *Mtb*, and significantly decreased survival after *Mtb* infection compared with control C57BL/6 mice.

The characterization of lymphocytes specifically programmed to control inflammation and maintain homeostasis and self-tolerance is an ongoing area of intense study. Regulatory T cells (T\textsubscript{reg} cells) are a subset of T cells characterized by expression of CD4, CD25 (IL-2R\textsubscript{a}), and forkhead box transcription factor P3 (FoxP3)\textsuperscript{256}. T\textsubscript{reg} cells have been shown to control inflammation primarily through secretion of IL-9, IL-10 and TGF\beta, but are also thought to utilize cell-cell contact inhibition via CTLA-4 and glucocorticoid-induced TNFR-related protein (GITR)\textsuperscript{257-259}. The presence of T\textsubscript{reg} cells in human TB was not discovered until recently\textsuperscript{260, 261}, after which numerous studies have examined their importance to *Mtb* infection\textsuperscript{262-268}. T\textsubscript{reg} cells are generally viewed as negatively restricting protective anti-*Mtb* responses. Adoptive transfer studies have demonstrated that T\textsubscript{reg} cells prevent clearance of *Mtb* by restricting maximal CD4\textsuperscript{+} T cell responses\textsuperscript{269}. T\textsubscript{reg} cells are found at higher concentration in patients with active TB than latent TB, and restrict IFN-\gamma production after ex vivo stimulation with *Mtb* antigens\textsuperscript{270}. Furthermore, studies have demonstrated that T\textsubscript{reg} cells can delay the priming of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell anti-*Mtb* responses in the lymph node\textsuperscript{271, 272}. Overall, the exact contribution of T\textsubscript{reg} cells during *Mtb* control is still unclear, but the majority of work indicates that they negatively impact immunity to *Mtb*.
Animal models of Mtb infection

The susceptibility of different animals to Mtb infection varies greatly. Guinea pigs are inherently susceptible to infection compared to most other animals, such as humans, non-human primates, mice, and rabbits which have varying degrees of susceptibility. Guinea pigs were the most widely used animal model of TB during the time when Robert Koch was examining the etiological agent of TB \(^{273}\). Most researchers chose to use guinea pigs because of their susceptibility to infection with human Mtb \(^{274}\) and because guinea pigs are much closer related to humans physiologically than rodents \(^{275-277}\). Other studies have verified the susceptibility to guinea pigs using aerosol infection with Mtb \(^{278, 279}\). The rabbit model of Mtb infection can demonstrate the formation of cavitary lesions that resemble the granulomas of immunocompetent TB patients \(^{280}\). The liquefaction and cavitation observed during rabbit TB has been well studied \(^{281-283}\). But, as with guinea pigs, the limited availability of reagents and high cost of care makes rabbits a difficult model system of TB.

Concern over limited reagents and animal costs are most prevalent in non-human primate (NHP) model of TB. Cynomolgus macaques and rhesus monkeys are the primary NHPs used for the study of Mtb. The TB disease present in NHPs represents the closest and most accurate model of Mtb infection in humans. Antibody-mediated depletion of T cell subsets is practiced in NHPs but is often incomplete, making any results questionable \(^{284, 285}\). We need to establish clear criteria for what questions each specific animal model is
best equipped to answer. There has been a push to discourage the use of mouse models of TB due to their limited applicability to humans, but it should be acknowledged that murine models are cheap, efficient, and controlled systems to test basic ideas, and the majority of immunology discovered in mice is closely representative of man. Once an idea has been rigorously proven in murine models, it is worth the investment to apply it to higher animal models.

The murine model of *Mtb* has been researched and reviewed extensively. The majority of the seminal work in identifying the critical components of an anti-*Mtb* immune response were done using C57BL/6 mice. As such, the majority of transgenic mice have been created on the C57BL/6 background, which naturally mounts a very strong T$_{H1}$ response to *Mtb*. In this work, we have called into question the applicability and finality of exclusively using C57BL/6 mice. We feel that C57BL/6 mice only represent certain populations of *Mtb*-resistant humans, and that early studies that have discounted the importance of components of the immune response (such as IL-10 and CD8$^+$ T cells) should not be applied further than their own respective models. We believe that use of other, more susceptible mouse strains (like CBA/J mice) will provide insight into how immune system components behave in *Mtb*-susceptible patients.

CBA/J mice have been deemed relatively susceptible to *Mtb* infection compared to C57BL/6 mice after intravenous or aerosol infection. This was determined based on
survival after \textit{Mtb} infection, where C57BL/6 (and BALB/c) mice were able to survive past day 300, while susceptible strains like CBA/J, DBA/2 and C3H/HeJ succumb to \textit{Mtb} infection around day 180 \cite{286}. CBA/J mice carry the Pde6b\textsuperscript{rd1} mutation which causes blindness by wean age, and are natural resistance-associated macrophage protein (NRAMP)-negative, yet studies have shown that NRAMP status does not dictate susceptibility to \textit{Mtb} in 129/sv mice \cite{287}. CBA/J mice also have been reported to have poor granuloma formation \cite{288} and elevated levels of IL-10 \cite{289}. The details of the susceptibility of CBA/J mice to \textit{Mtb} are the primary focus of this collective work, and are explained in detail at various points throughout the text. Overall, CBA/J mice are here examined because they mimic the \textit{Mtb} reactivation of susceptible human populations.

Numerous other inbred mouse strains are used for the study of TB, in addition to C57BL/6 and CBA/J. BALB/c mice have variable susceptibility to \textit{Mtb} depending on the dose and method of infection. Some studies using BALB/c mice deemed them relatively susceptible to \textit{Mtb} infection based on IL-4 secretion \cite{290-292}, while others classify them as resistant particularly based on their survival after intravenous infection \cite{286}. Most work shows that they are more resistant than CBA/J mice, and survive similarly to C57BL/6 after aerosol infection with \textit{Mtb} \cite{69,293}. In contrast, BALB/c mice have been shown to be more susceptible then C57BL/6 mice to \textit{Mycobacterium avium} infection, largely due to increased IL-10 levels \cite{294}. Therefore, BALB/c mice are described as having an intermediate \textit{Mtb}-susceptibility phenotype \cite{295}. Accordingly, susceptibility to \textit{Mtb} infection is not limited to CBA/J mice. DBA/2 \cite{296}, C3H/HeJ \cite{297}, 129/sv \cite{298}, and I/St \cite{299}. 

\cite{27}
strains of inbred mice have all been categorized as susceptible to *Mtb* infection. DBA/2 mice are extremely susceptible to virulent *Mtb* infection, and have been shown to develop widespread dissemination of *Mtb* throughout their lungs, associated with the presence of poorly activated foamy macrophages. Although each of these strains displays susceptibility to *Mtb*, the exact mechanism involved for each has not been fully described.

The factors responsible for the reactivation of latent *Mtb* infection are currently unknown. A large percentage of the work devoted to this issue deals primarily with examining fluctuations in gene expression in *Mtb* during various phases of infection. It has been well established that during latent *Mtb* infection the bacterium undergoes a state of genetic dormancy; where many of its virulence factors are downregulated. Very few studies, however, attempt to examine variations in the immune system of infected individuals that may account for their susceptibility. This is due to the fact that accurate models of latent *Mtb* infection often require the use of non-human primates and other higher order vertebrates. Small animal models are useful tools for examining specific immunological mechanisms, but have not been used extensively for modeling *Mtb* reactivation.

Throughout the body of work described in this text, it was our driving motivation to build on the advances and discoveries of the past, to enhance our understanding of the immune response during *Mtb* infection. The human population is immensely diverse, and as such
no single model system can fully represent how someone will respond to TB. Herein, we explored the central hypothesis that CBA/J mice represent a better model of \textit{Mtb}-susceptible human populations than C57BL/6 mice. We examined the regulation of \textit{Mtb} immunity in both CBA/J and C57BL/6 mice, by infecting them with virulent \textit{Mtb} via aerosol and analyzing the dynamics of pulmonary T cell responses. In doing so, we hoped to gain further understanding of why susceptible individuals progress to active TB, and how the immune response to \textit{Mtb} may be enhanced.
Chapter 2: Interleukin-10 prevents the generation of long-term protective immunity against *Mycobacterium tuberculosis*.

*Abstract:*

The underlying factors that direct the progression from persistent, asymptomatic *Mycobacterium tuberculosis* (*Mtb*) infection to active, contagious disease are currently unknown. Whether this reactivation is induced by *Mtb* or is an inherent defect in the host response is also not clear. Identifying why granulomatous containment of *Mtb* fails in certain individuals is imperative to understanding, and eventually preventing, reactivation. Although *Mtb* susceptibility is notoriously multifactorial, effective pro-inflammatory responses are a main correlate of protection; though the exact dynamics of when and how pro-inflammatory cells and cytokines confer protection are unclear. We have previously shown that high levels of the immunosuppressive cytokine, interleukin-10 (IL-10), dampen protective responses and contribute to the reactivation of *Mtb* infection in CBA/J mice, a mouse strain which fails to develop mature granulomas. We now report that CBA/J mice genetically deficient in IL-10 (IL-10−/−) maintained a reduced and stable *Mtb* bacterial load and had extended survival relative to wild type CBA/J mice. CBA/J IL-10−/− mice had significantly enhanced T\(_H\)1 responses in the lung and draining
lymph nodes, associated with increased T cell activation via higher expression of antigen presentation molecules. Strikingly, CBA/J IL-10\(^{-/-}\) mice developed distinct, mature, fibrotic granulomas as infection progressed.

These findings support the belief that potent pro-inflammatory responses are required for \textit{Mtb} control, but do not address when and how long-term protection against \textit{Mtb} is established. As such, we blocked the action of IL-10 \textit{in vivo} throughout the first 21 days of \textit{Mtb} infection only, in wild-type CBA/J mice. This limited blockade of the IL-10R recapitulated the protective phenotype observed in CBA/J IL-10\(^{-/-}\) mice (reduced CFU, mature granulomas) providing evidence that manipulation of early immune events via the action of IL-10 can have an overt impact on the long-term containment of \textit{Mtb} infection. Taken together, our findings begin to provide a clear understanding of the critical times and constituents required to establish \textit{Mtb} protection.

\textit{Introduction:}

\textit{Mycobacterium tuberculosis} (\textit{Mtb}), the etiological agent responsible for the disease tuberculosis (TB), remains a severe threat to mankind, infecting nearly one third of the world’s population and causing over 1 million deaths each year \textsuperscript{301}. The majority of individuals that become infected with \textit{Mtb} are capable of asymptomatic containment and control of the infection \textsuperscript{302}. During this asymptomatic phase, \textit{Mtb} persists in balance with the immune system. In some individuals, this control can break down leading to reactivation and contagious TB disease. In man, protective immunity and the
development of latent *Mtb* infection are associated with the formation of small, dense foci, or granulomas, within the lungs that serve to limit *Mtb* infection \(^{303}\). Protective granulomas are characterized by the containment of infection within a fibrotic capsule in which the central core can become necrotic \(^{303,304}\). In this environment, *Mtb* is thought to survive in a non-replicating persistent state until host immunity and granuloma structure break down \(^{19,305}\). Understanding the development and importance of granuloma structure has been challenging due to the lack of animal models that develop structures resembling mature granulomas in humans. Indeed, the greatest limitation of the mouse model, the species most widely used for the study of *Mtb* disease progression, is its failure to generate granuloma structures that are representative of granulomas associated with protection in man \(^{304,306}\).

The factors that determine susceptibility to reactivation TB are not completely understood, but are complex, multifactorial and closely linked to immune competency. Robust T\(_H1\)-driven immune responses with the generation of interferon-\(\gamma\) (IFN-\(\gamma\))-secreting T cells are thought to be essential \(^{171,307,308}\), yet it is unclear how other immune modulators influence the generation of protective immunity and long-term control of *Mtb* infection. The immunosuppressive cytokine interleukin-10 (IL-10) has been implicated in susceptibility to TB in both humans and animal models. Patients with active TB have increased levels of IL-10 in their serum \(^{309,310}\), pleural fluid \(^{311}\), and/or bronchoalveolar lavage fluid \(^{312}\), suggesting a link between elevated IL-10 and TB disease. Whether elevated levels of IL-10 in TB patients are produced to dampen the excessive inflammation associated with active disease, or if IL-10 can itself cause TB reactivation
in man is not known. However, *Mtb* infection of IL-10-expressing transgenic mice indicate that IL-10 production can drive TB reactivation. Other murine studies have confirmed the relationship between IL-10 and TB disease progression by demonstrating that blocking or deleting the action of IL-10 can reduce pulmonary bacterial load, an event highly associated with increased IFN-γ production. The exact mechanism for how IL-10 influences the control of *Mtb* infection remains elusive, but its presence during reactivation TB implicates IL-10 as a significant mediator of TB disease progression.

To determine the role of IL-10 during *Mtb* infection and TB disease progression we developed IL-10 gene-disrupted mice on the CBA/J mouse strain background (CBA/J IL-10−/−). This *Mtb*-susceptible strain succumbs to aerosol *Mtb* infection within 150-200 days, which is associated with reduced T\(^{H1}\)-mediated immunity, poorly organized granulomas, and abundant IL-10 production. Deletion of IL-10 on the CBA/J mouse strain background provided a tractable system to elucidate the role of IL-10 in a model that naturally mimics the elevated levels of IL-10 seen in TB patients. Our findings demonstrate that CBA/J IL-10−/− mice were fully capable of controlling and containing *Mtb* infection which was, as expected, associated with an early and heightened T\(^{H1}\)-mediated immune response. What was particularly striking, however, was that in the absence of IL-10 CBA/J mice formed mature granulomas with *Mtb* encapsulated within a fibrotic capsule that frequently surrounded a necrotic core. Furthermore, the protective phenotype of CBA/J IL-10−/− mice was recapitulated by removing the action of IL-10 throughout the first 21 days of *Mtb* infection in wild-type CBA/J mice. Our data demonstrate that IL-10 has a significant and early role in the generation of immunity that
is closely linked with the capacity to establish mature granulomas and long-term protective immunity against *Mtb* infection. We hypothesize that the early action of IL-10 in humans during an initial encounter with *Mtb* determines the long-term outcome of TB disease.

**Materials and Methods:**

**Mice**

CBA/J mice (Jackson laboratories, Bar Harbor, Maine) were crossed with C57BL/6 IL-10+/− mice (Jackson) for eight generations. At each cross progeny were ear-punched and DNA was screened for the presence of a neomycin cassette at the *il10* gene locus and IL-10+/− mice were selected for further breeding. At the eighth generation, heterozygotes were crossed and IL-10-deficient homozygote CBA/J mice were selected. A homozygous breeder colony of CBA/J IL-10+/− mice was maintained thereafter.

4- to 8-week-old, specific pathogen-free, age/sex-matched CBA/J IL-10+/−, CBA/J wild-type (Jackson or National Cancer Institute, Frederick, MD), C57BL/6 wild-type (Jackson) and C57BL/6 IL-10+/− mice (Jackson) were maintained in ventilated cages inside a biosafety level 3 (BSL3) facility and provided with sterile food and water *ad libitum*. Experiments were unaffected by the recent CBA/J-C3H/HeN genetic contamination at NCI. All protocols were approved by The Ohio State University’s Institutional Laboratory Animal Care and Use Committee.
**Mtb infection and Colony Forming Unit Enumeration**

*Mtb* Erdman (ATCC no. 35801) was obtained from American Type Culture Collection. Stocks were grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and frozen in 1 ml aliquots at –80°C. Mice were infected with *Mtb* Erdman using an inhalation exposure system (Glas-Col) calibrated to deliver 50–100 CFU (for low-dose aerosol) or 500 CFU (high-dose aerosol) to the lungs of each mouse, as previously described.315

At specific time points post *Mtb* infection mice were sacrificed and lung, spleen and mediastinal lymph node aseptically removed into sterile saline. Organs were homogenized and serial dilutions plated onto 7H11 agar supplemented with OADC as previously described.316 Plates were incubated at 37°C for 21 days in order to enumerate bacterial colonies and calculate the bacterial burden.

**Survival Studies**

Groups of CBA/J wild-type and CBA/J IL-10−/− mice were infected with *Mtb* Erdman via aerosol, as previously described, and maintained in a BSL3 facility. Mice were observed daily and euthanized when moribund. Date of euthanasia was recorded. Low-dose infection was performed twice (n= 18 wild-type and 10 IL-10−/−, or 30 wild-type and 25 IL-10−/−). High-dose infection (n=10 wild-type and 10 IL-10−/−).
**Lung cell isolation**

Mice were euthanized by CO\textsubscript{2} asphyxiation and lungs perfused with cold phosphate buffered saline containing 50Units/mL of heparin through the right ventricle. Lungs from individual mice were mechanically disrupted using a GentleMACS dissociator (Miltenyi Biotec) followed by collagenase A (type XI) (0.7 mg/mL, Sigma) and type IV bovine pancreatic DNase (30µg/mL, Sigma) digestion at 37°C for 30 minutes in GentleMACS C-tubes. Lung cell suspensions were passed through a 70 µm nylon cell screen and residual erythrocytes were lysed with Gey’s solution. Viable cells were determined by trypan blue exclusion.

**Cell Purification**

Single lung cell suspensions were adhered to sterile tissue culture dishes for 1hr at 37°C. Non-adherent cells were washed and removed from the plates. CD\textsuperscript{4+} and CD\textsuperscript{8+} T cells were obtained from the non-adherent cell fraction by magnetic cell separation (BD IMAG anti-CD4 particles 551539, anti-CD8 particles 551516) and either placed directly into TRIzol reagent (Invitrogen), homogenized, and frozen at -80°C or used for culture as described below. Purity of CD\textsuperscript{4+} and CD\textsuperscript{8+} T cell populations was determined to be greater than 90% for all experiments by flow cytometry using an LSRII flow cytometer (BD). Adherent cells were scraped off the plates with a sterile razor blade, lysed in TRIzol, homogenized, and immediately frozen at -80°C.
**ELISpot Assays**

Bone marrow-derived dendritic cells (BMDCs) were matured as previously described from female CBA/J IL-10−/− mice. CD4+ and CD8+ T cell populations were purified from age/sex matched infected CBA/J IL-10−/− and CBA/J wild-type mice as described above. 3x10^4 BMDCs were cultured with 2x10^5 CD4+ T cells or CD8+ T cells for 48hr at 37°C in media containing either 10µg/mL *Mtb* culture filtrate protein (CFP), 10µg/mL ovalbumin (OVA) or 10µg/mL anti-CD3 (145-2C11) and 1µg/mL anti-CD28 (37.51). ELISpot reagents were obtained from eBioscience Ready-Set-GO! Spot-forming units (SFU) were enumerated with an ELISpot plate counter (C.T.L.).

**Flow cytometry**

Isolated lung cells or mediastinal lymph nodes (MLN) were suspended in deficient RPMI (Irvine Scientific) supplemented with 0.1% sodium azide (Sigma-Aldrich). Surface targets were detected as previously described (16). Specific Abs and isotype controls were purchased from BD Biosciences: PerCP-Cy5.5 anti-CD3ε (145-2C11), allophycocyanin-Cy7 anti-CD4 (GK1.5), PE-Cy7 anti-CD8 (53-6.7), PerCP-Cy5.5 anti-CD8 (53-6.7), PE-Cy7 anti-IFN-γ (XMG1.2), PE anti-TNF (MP6-XT22), and APC anti-IL-2 (JES6-5H4). Cytokine levels were determined according to the manufacturer’s instructions for intracellular cytokine staining (Cytofix/Cytoperm fixation/permeabilization solution kit with BD GolgiStop, BD Biosciences), following a 4hr incubation with 10µg/mL anti-CD3 (145-2C11) and 1µg/mL anti-CD28 (37.51). Samples were read using a six color BD LSRII flow cytometer and analyzed with FACSDiva software (BD Biosciences) or FlowJo software (Tree Star, Inc).
**Real-time PCR**

RNA was purified using Qiagen RNeasy kit with RNeasy columns and quantified on a NanoDrop spectrophotometer (ThermoSCIENTIFIC). ifng, tnf, ciita, h2a, nos2, tgbf primer/probe sets were obtained from Applied Biosystems.

**Histology**

Caudal lung lobes were taken from infected CBA/J wild-type and IL-10\(^{+/−}\) mice at various timepoints post-infection, inflated and stored in formalin as previously described. 

Tissue sections were prepared and stained with hemotoxylin and eosin, Ziehl-Neelson, Masson’s trichrome, and Von Kossa stains, and were assessed by a board certified veterinary pathologist with no prior knowledge of experimental groups.

**IL-10R Blockade**

CBA/J and C57BL/6 wild-type mice were injected intraperitoneally with 1mg of anti-IL-10R (1B1.3A) or Rat IgG\(_1\) antibody (BioXCell) one day before low-dose aerosol infection with *Mtb* Erdman. On days 6, 13, 20 post-infection 0.2mg of antibody were injected to maintain the blockade. On day 21 post-infection lung and MLN were removed from a subset of mice for T cell purification (lung) or flow cytometry (MLN). Remaining groups of infected mice were sacrificed on day 120 post-infection without any further treatment past day 21. Right caudal lungs were fixed in formalin for sectioning while the remaining lobes were homogenized and plated for CFU enumeration.
Statistics

Statistical analysis performed using GraphPad Prism software for the Students t test per individual time point of each graph. Any comparisons between timepoints of the same experiment utilize a two-way analysis of variance test with Bonferroni post-tests for multiple comparisons. Survival significance was determined by LogRank Mantel-Cox test. * p<0.05, ** p<0.01, *** p<0.001

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I would like to disclose that the breeding of CBA/JIL-10−/− mice, IL-10−/− Mtb growth curves, survival studies, and whole lung cytokine assays/PCR were performed by Dr. Bridget Carruthers. Without her initial work, this study would not exist in its current form. Also, some work examining lymph node responses at early timepoints was performed by Rachel Kominsky. Thank you.
Results:

IL-10 deficiency in CBA/J mice results in long term control of *Mtb* infection

Just as different populations of humans have varying susceptibility to developing active TB disease, inbred mouse strains also have different disease outcomes. Previous studies have categorized the susceptibility of various inbred mouse strains to intravenous or aerosol infection with *Mtb* and demonstrated that inbred mice generally fall into two survival groups: those that lived past day 300 of infection, and those that succumb much earlier (around day 200) \(^{286,319}\). These groups were deemed relatively resistant or relatively susceptible to developing TB disease. C57BL/6 mice have historically been the most well-studied in the context of *Mtb* infection and belong to the relatively resistant survival group. While much insight has been gained into *Mtb* infection dynamics using C57BL/6 mice, they only represent one population. CBA/J mice fall into the relatively susceptible survival group, and we believe accurately represent *Mtb*-susceptible human populations. We used both of these mouse strains to test the relative contribution of IL-10 to *Mtb* disease progression.

Increasing levels of IL-10 in the lung have been linked to TB disease progression in CBA/J mice \(^{289,320}\) and, therefore, we asked whether the complete absence of IL-10 would provide a significant survival advantage for CBA/J mice during *Mtb* infection. CBA/J IL-10\(^{-/-}\) mice were generated and infected with a low-dose aerosol of *Mtb* and the course of infection compared to wild type CBA/J mice. As anticipated, wild-type CBA/J mice displayed escalating colony-forming units (CFU) within the lung and spleen as
infection progressed beyond day 100 (Fig 2.1a, c). In contrast, CBA/J IL-10\(^{+/+}\) mice were capable of reducing the pulmonary \textit{Mtb} burden by day 60 of infection, and subsequently maintained a reduced and stable bacterial load for the entire duration of the study (Fig 2.1a). Splenic analysis displayed an additional reduction in CFU at later time points post-infection (Fig 2.1c), but whether sterilizing immunity could be reached was unclear, as CFU were below the level of detection in some, but not all, animals. Comparison with C57BL/6 wild-type and C57BL/6 IL-10\(^{+/+}\) mice showed that CBA/J IL-10\(^{+/+}\) mice reduced the lung and splenic CFU to levels equivalent to or below wild type C57BL/6 mice (Fig 2.1b, d). Similar to other studies, a moderate reduction in CFU at days 60 and 90 post-infection was observed between C57BL/6 and C57BL/6 IL-10\(^{+/+}\) mice (Fig 2.1b). This effect was transient in the lungs but persisted in the spleen of C57BL/6 IL-10\(^{+/+}\) mice (Fig 2.1d). No mortality was observed in C57BL/6 IL-10\(^{+/+}\) mice within the timeframe studied (not shown) however, CBA/J IL-10\(^{+/+}\) mice survived significantly longer (approximately 20% extension of life-span) after \textit{Mtb} infection compared to wild-type CBA/J mice, with median survival times of 249.5 days (CBA/J) and 296 days (CBA/J IL-10\(^{+/+}\)) (Fig 1e). Interestingly, and despite a significant and sustained reduction in \textit{Mtb} CFU in the lungs of CBA/J IL-10\(^{+/+}\) mice by day 60 post infection, we observed no significant differences in lung or spleen CFU at earlier time points (Fig 2.1f) suggesting that immune responses were not accelerated in the absence of IL-10, a finding that was also verified using early anti-IL-10R treatment of CBA/J mice (not shown). Challenge of CBA/J and CBA/J IL-10\(^{+/+}\) mice with a higher dose of \textit{Mtb} (approximately 500 CFU) showed similar findings for \textit{Mtb} control in the lung (Fig 2.1g) as well as survival (Fig 2.1h).
Figure 2.1 *Mtb* burden and survival of CBA/J and C57BL/6 wild-type and IL-10−/− mice

CBA/J and C57BL/6 wild-type and IL-10−/− mice were infected with a low dose aerosol of *Mtb*. Lungs (a, b) and spleens (c, d) were obtained for analysis and *Mtb* CFU were enumerated on 7H11 plates. CBA/J data representative of three independent experiments with 5 mice per group per timepoint. C57BL/6 data representative of one experiment with 5 mice per timepoint. (e) Mice were maintained inside BSL3 conditions, monitored daily, and euthanized when moribund (n=30 wild-type, 25 IL-10−/−). Data representative of two independent experiments. (f) CFU during early timepoints after *Mtb* infection of wild-type and CBA/J IL-10−/− mice. Data representative of two independent experiments with 5 mice per group per timepoint. (g) High-dose aerosol of wild-type and CBA/J IL-10−/− mice with 10^5 CFU *Mtb* or (h) survival after infection (n=10 mice per group). Data representative of one experiment with 5 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test. Survival significance was determined by LogRank Mantel-Cox test.
Control of *Mtb* infection in CBA/J IL-10^-/- mice is associated with an early and robust Th1 immune response

We examined levels of the Th1 cytokine IFN-γ in total lung homogenates and found both protein (Fig 2.2a) and mRNA (Fig 2.2b) to be significantly elevated in the lungs of CBA/J IL-10^-/- mice at day 21 of infection. IL-12 protein was also significantly elevated in lung homogenates at day 21 post *Mtb* infection (Fig 2.2a). IL-17A mRNA was not significantly altered throughout infection (Fig 2.2b). We observed a concordant increase not only in the number of total lung cells in CBA/J IL-10^-/- mice (Fig 2.2c), but specific increases in CD4^+ (Fig 2.2d) and CD8^+ (Fig 2.2e) T cells. Furthermore, significantly more CD4^+ T cells from IL-10^-/- CBA/J mice expressed the activation marker CD69 (Fig 2.2d) 30 days after *Mtb* infection indicating that T cells in the lung were actively encountering antigen at that time point. Significantly more CD4^+ T cells from CBA/J IL-10^-/- mice were also positive for IFN-γ following *ex vivo* TCR cross-linking (Fig 2.2d). CD8^+ T cells from CBA/J IL-10^-/- mice were increased at day 30 post-infection but did not display significant increases in IFN-γ or CD69 expression (Fig 2.2e).

To determine the capacity of T cells to produce IFN-γ in the absence of *ex vivo* stimulation we isolated CD4^+ and CD8^+ cells from *Mtb*-infected mice and analyzed purified subsets by real-time PCR for IFN-γ message, indicative of *in vivo* capacity. Both CD4^+ and CD8^+ cells from CBA/J IL-10^-/- mice expressed significantly more IFN-γ mRNA than wild-type CBA/J mice after 21 days of infection with *Mtb* (Fig 2.2f) which correlated with the secretion of IFN-γ in lung homogenates (Fig 2.2a) and by T cells
stimulated ex vivo (Fig 2.2d,e). Together, these data demonstrate that IL-10 deficiency enables CBA/J mice to mount a potent yet transient Th1 response in vivo. Despite this limited window of increased IFN-γ production in vivo we observed abundant capacity of CD4+ T cells to secrete Mtb-specific IFN-γ after in vitro culture with Ag85-pulsed bone marrow-derived dendritic cells (Fig 2.2g) indicating that CBA/J IL-10−/− mice generated and maintained a pool of Mtb specific T cells within the lung, but these cells did not appear to be producing abundant IFN-γ in vivo during later timepoints of infection.
Figure 2.2  Cytokine production in wild-type of IL-10−/− CBA/J mice.

Wild-type and CBA/J IL-10−/− mice were infected with a low-dose aerosol of *Mtb*. At various timepoints post-infection, lungs were obtained and processed for analysis. (a) Levels of IFN-γ and IL-12 were obtained by ELISA of whole lung homogenate. (b) mRNA expression of IFN-γ and IL-17a by real-time PCR from whole lung homogenate at indicated times post-infection. (c) Total pulmonary cell numbers, (d) numbers of total pulmonary CD4+, CD4+IFN-γ+, or CD4+CD69+ T cells as determined by flow cytometry. (e) Numbers of total pulmonary CD8+, CD8+IFN-γ+, or CD8+CD69+ T cells as determined by flow cytometry. (f) Real-time PCR analysis of purified pulmonary CD4+ or CD8+ T cells for IFN-γ relative to 18s housekeeping gene. (g) IFN-γ ELISpot of purified pulmonary CD4+ T cells cultured for 48hrs with Ag85-pulsed BMDCs. SFU=spot-forming units. All data sets representative of at least two independent experiments with 5 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s t test.
Figure 2.2
Macrophages from IL-10-deficient CBA/J mice have increased MHC class II and chemokine expression in vivo during initial Mtb infection

MHC class II expression was determined on the surface of pulmonary macrophages (CD11b⁺CD11c⁺ cells) isolated from Mtb-infected wild-type and CBA/J IL-10⁻/⁻ mice by flow cytometry. We found that CBA/J IL-10⁻/⁻ mice had increased numbers of macrophages that expressed MHC class II on their surface (Fig 2.3a) as well as a moderate increase in the proportion of cells that expressed MHC class II with higher mean fluorescence intensity (MFI) (Fig 2.3b). Increased MHC class II expression was verified by real-time PCR where adherent cells from the lungs of Mtb infected CBA/J IL-10⁻/⁻ mice had increased mRNA expression for MHC class II (Fig 2.3c) and the MHC class II transactivator, CIITA (Fig 2.3d). Similar to our findings for T cell responses in CBA/J IL-10⁻/⁻ mice, significant increases in MHC class II expression were only observed at early time points of Mtb infection, after which levels remained stable and low relative to wild type CBA/J mice. We observed no differences throughout infection in nos2 (iNOS) expression (Fig 2.3e), TGFβ (Fig 2.3g), or TLR2 (Fig 2.3h) indicating that macrophages from Mtb infected CBA/J IL-10⁻/⁻ mice were not more bactericidal, compensating for the lack of IL-10 with other immunosuppressive cytokines, or changing expression of TLR2. As anticipated, cells from CBA/J IL-10⁻/⁻ mice expressed significantly more CCL5 and CXCL10 than wild-type mice at day 21 after Mtb infection (Fig 2.3f). Together, these data demonstrate that macrophages from CBA/J IL-10⁻/⁻ mice have enhanced capacity to present Mtb antigens in vivo during early stages of infection.
Wild-type and IL-10⁻/⁻ CBA/J mice were infected with a low-dose aerosol of *Mtb* and lungs were harvested at various times post-infection. Absolute number (a) or mean fluorescence intensity (b) of lung cells positive for CD11c⁺CD11b⁺IA/IE⁺ as determined by flow cytometry. Adherent lung cells were placed in TRIzol and homogenized for real-time PCR analysis; *h2a* (MHC class II) (c), *ciita* (d), *nos2* (e), *ccl3, ccl5, ccr10* (only day 21 post-infection) (f), *tgfb1* (TGFβ) (g), *tlr2* (h). All relative units obtained against 18s housekeeping gene. All data sets are representative of two independent experiments with 5 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test.
Figure 2.3

(a) Figure showing CD1c/CD11c+ Ia/IE expression over days post-infection for CBAU and CBAU IL-10−/−. The y-axis represents the number of cells, and the x-axis represents days post-infection.

(b) Graph showing CD11c/CD11b+ Ia/IE expression with MPL 215 and MPL 206 for CBAU and CBAU IL-10−/−. The y-axis represents the number of cells, and the x-axis represents the number of infected cells.

(c) Graph showing h2a relative units over days post-infection for CBAU and CBAU IL-10−/−. The y-axis represents the relative units, and the x-axis represents days post-infection.

(d) Graph showing delta relative units over days post-infection for CBAU and CBAU IL-10−/−. The y-axis represents the relative units, and the x-axis represents days post-infection.

(e) Graph showing pmd2 relative units over days post-infection for CBAU and CBAU IL-10−/−. The y-axis represents the relative units, and the x-axis represents days post-infection.

(f) Graph showing relative units for Ccl3, Ccl5, and Cxcl10 on day 21 post-infection. The y-axis represents the relative units, and the x-axis represents the day.

(g) Graph showing tgfβ1 relative units over days post-infection for CBAU and CBAU IL-10−/−. The y-axis represents the relative units, and the x-axis represents days post-infection.

(h) Graph showing tgfβ2 relative units over days post-infection for CBAU and CBAU IL-10−/−. The y-axis represents the relative units, and the x-axis represents days post-infection.
CBA/J IL-10−/− mice develop mature granulomas

Analysis of granuloma integrity (Fig 2.4) showed that CBA/J wild type and CBA/J IL-10−/− mice had recruited immune cells into the lung by day 21 of Mtb infection, both forming similar focused aggregates that were associated with the presence of Mtb bacilli (not shown). The cellular makeup of the pulmonary granulomas remained similar at 60 days post infection but diverged significantly thereafter. Morphometric analysis revealed that CBA/J IL-10−/− mice initially had a trend for more granulomas and total granuloma area at day 60 of Mtb infection which subsequently reduced over time (Fig 2.4c, d). In contrast, wild type CBA/J mice continued to develop granulomas and expand granuloma area over the course of infection. CBA/J wild-type mice progressed to develop large macrophage dominated coalescing and disorganized granulomas (Fig 2.4a) that contained numerous acid-fast bacteria (AFB) (Fig 2.4e), as has been previously described 288, 313.

In contrast, by day 90 of Mtb infection CBA/J IL-10−/− mice had developed discrete, organized granulomas (Fig 2.4a) as observed at low magnification. By day 90 post-infection, several granulomas in CBA/J IL-10−/− mice appeared mature, containing a central area of highly vacuolated or foamy macrophages and pyknotic debris surrounded by a rim of lymphocytes that over time contained necrotic centers (day 90 through 120 post infection) (Fig 2.4b). Fewer Mtb bacilli were apparent in the granulomas of CBA/J IL-10−/− mice and the bacteria appeared fragmented or attenuated on AFB staining (Fig 2.4e right panel). Masson’s trichrome staining (Fig 2.4e left, center panel) revealed that CBA/J IL-10−/− mice had formed a fibrous capsule surrounding several of the mature granulomas. Containment of Mtb within mature granulomas, with little accessibility to
immune cells likely accounted for the reduced immune responses observed during that same timeframe. *Mtb* infected CBA/J wild type mice failed to deposit collagen in any organized manner around the foci of inflammation (Fig 2.4e). Von Kossa staining for calcium revealed no significant staining in either group.
Figure 2.4  Pulmonary granuloma formation and maintenance

Wild-type and IL-10−/− CBA/J mice were infected with a low-dose aerosol of *Mtb*. At various timepoints post-infection lungs were removed, fixed in formalin, sectioned, and stained with hematoxylin and eosin (a, b). Total number of granulomas per lung lobe (c) and total area of lung tissue occupied by granulomas (µm²) (d) obtained by Aperio ImageScope. Trichrome stain (e left, center panel) for collagen or Ziehl-Neelsen stain (e right panel) for acid-fast bacilli. Images representative of two independent experiments with 5 mice per group per timepoint. Scale bars 1mm (a), 200µm (e: left panel), 100µm (b, e: center panel), 50µm (e: right panel) * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test.
Figure 2.4

(a) [Images of tissue samples from different conditions and time points (D21, D60, D90, D120, D150).

(b) [Images of tissue samples from different conditions and time points (D90, D120, D150).]
Figure 2.4 (continued)
Early IL-10R blockade of CBA/J mice recapitulates the CBA/J IL-10−/− mouse phenotype during Mtb infection

CBA/J IL-10−/− mice showed a significant and transient alteration in macrophage and T cell phenotype and function, compared to wild-type CBA/J mice, yet altered granuloma formation was not apparent until after 60 days of infection with Mtb. To determine whether the phenotypic changes we observed early in the lymph node and lungs of CBA/J IL-10−/− mice were responsible for the long term control of Mtb infection we treated wild-type CBA/J mice with anti-IL-10R for the first 21 days of infection and assessed T cell function and granuloma formation. Early anti-IL-10R treatment resulted in increased total cell numbers as well as CD4+, CD8+ T cells (Fig 2.5a left) in the MLN at day 21 post Mtb infection in anti-IL-10R treated CBA/J mice, similar to that observed in CBA/J IL-10−/− mice. Additionally, we observed elevated CD69+ T cells (Fig 2.5a center) and moderate increases in IFN-γ+ (Fig 2.5a right) and TNF+ T cells (not shown) in the MLN at day 21 of Mtb infection.

Anti-IL-10R treatment was halted at day 21 and a group of mice were maintained for an additional 100 days to determine the long-term phenotype. CBA/J mice treated with anti-IL-10R for only the first 21 days of Mtb infection exhibited a significantly reduced pulmonary Mtb burden (Fig 2.5b), similar to that observed in CBA/J IL-10−/− mice. Additionally, after anti-IL-10R treatment, pulmonary T cells expressed more IFN-γ mRNA (Fig 5d), while pulmonary antigen-presenting cells expressed significantly more MHC class II and CCL5 mRNA (Fig 5e). Most striking, however, was that at day 120 post Mtb infection anti-IL-10R treated mice had developed mature granulomas that
contained few, small foci (Fig 2.5f: panel a), a fibrotic capsule (Fig 2.5f: panel b) and minimal AFB within the granuloma (Fig 2.5f: panel c), recapitulating the IL-10−/− phenotype. These data indicate that blocking the action of IL-10 in wild type CBA/J mice during the initial stages of *Mtb* infection only was sufficient to enable CBA/J mice to establish organized, mature granulomas that provided long-term effective control of *Mtb*. Furthermore, these data also discount a role for IL-10 in the generation of immune cells during development in CBA/J IL-10−/− mice that could skew T cell subsets or function prior to *Mtb* infection, validating the use of CBA/J IL-10−/− mice in the study of *Mtb* immunity.
**Figure 2.5** IL-10R Blockade of wild-type CBA/J mice

**Panel:** (a) Absolute number of lymph node cells, CD4⁺, and CD8⁺ T cells (left), as determined by flow cytometry at day 21 post-infection. Absolute number of CD69⁺ CD4⁺ or CD8⁺ T cells (center), IFN-γ⁺ CD4⁺ or CD8⁺ T cells (right) as determined by flow cytometry. (b) *Mtb* colony-forming units in the lungs of CBA/J treatment groups at day 120 post-infection. (c) Real-time PCR analysis of purified pulmonary CD4⁺ or CD8⁺ T cells for IFN-γ or (d) pulmonary adherent cells for h2a, ciita, nos2, ccl5 at day 21 post-infection. **Panel e:** H&E staining of anti-IL-10R treated lungs (left), Masson’s Trichrome stain (center), Ziehl-Neelson stain (right) at day 120 post-infection. All data sets represent two independent experiments with 5 mice per group per experiment. Control group = No treatment and Rat IgG1 groups combined for analysis. Scale bars 1mm (a), 100µm (b), 50µm (c) * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s t test.
Early functional T cell properties, but not kinetics, are altered in CBA/J IL-10\(^{-/-}\) mice

Blockade or absence of IL-10 throughout the first 3 weeks of \(Mtb\) infection led to a dramatic change in phenotype and therefore we sought to identify the immunological mechanism for this change. Previous studies have postulated that IL-10 deficiency leads to accelerated and enhanced immune responses in the absence of IL-10. We therefore determined early immune events in MLN and lungs of CBA/J IL-10\(^{-/-}\) mice. We observed no evidence of accelerated immune responses in the absence of IL-10, with lymphocyte composition (Fig 2.6a) and activation of CD4\(^+\) (Fig 2.6b) or CD8\(^+\) (Fig 2.6c) T cells in MLN being equivalent between CBA/J IL-10\(^{-/-}\) and CBA/J mice throughout the first 18 days of \(Mtb\) infection. Significant increases in T cell numbers, and activation of T cells subsets only became evident in the MLN by day 21 of \(Mtb\) infection, indicating no enhanced priming or activation of T cells in the absence of IL-10. \textit{Ex vivo} stimulation of lymph node cells also showed no increased ability for T cells from CBA/J IL-10\(^{-/-}\) mice to secrete IFN-\(\gamma\) (Fig 2.6b,c) or TNF (Fig 2.6b,c) until day 21 of \(Mtb\) infection. At day 21 of infection with \(Mtb\) CBA/J IL-10\(^{-/-}\) mice had significantly more activated CD4 and CD8 T cells that could secrete IFN-\(\gamma\) or TNF indicating that the absence of IL-10 led to enhanced activation of T cells in the MLN. As a measure of protective T\(_{H1}\) effector cells\(^{321}\) we analyzed the number of polyfunctional TNF\(^+\)IFN-\(\gamma^+\)CD4\(^+\) T cells in the MLN and found those to be significantly increased at day 21 post-infection in CBA/J IL-10\(^{-/-}\) mice (Fig 2.6d). We also examined the expression of IFN-\(\gamma\) mRNA in lung T cells from CBA/J IL-10\(^{-/-}\) mice at these same early timepoints during \(Mtb\) infection. Similar to MLN, we observed no alteration in the amount of IFN-\(\gamma\) mRNA detected in CD4\(^+\) or
CD8$^+$ T cells in the lungs of CBA/J IL-10$^{+/}$ mice until day 21 of $Mtb$ infection (Fig 2.6e). Therefore our data indicate that IL-10 can influence the functional properties of CD4$^+$ T cells throughout the first 3 weeks of infection with $Mtb$ but plays no role in accelerating the initial immune response within the MLN or lung.
Figure 2.6   Early lymph node and lung responses to Mtb infection

Wild-type and IL-10−/− CBA/J mice were infected with a low-dose aerosol of Mtb. At various timepoints post-infection, mediastinal lymph nodes and lungs were obtained and processed for analysis. **Panel a:** Total number of lymph node cells determined by trypan blue exclusion, absolute number of lymph node CD4+ or CD8+ T cells as determined by flow cytometry. **Panel b:** Absolute number of lymph node IFN-γ+ CD4+, TNF+ CD4+, CD69+ CD4+ T cells as determined by flow cytometry. **Panel c:** Absolute number of lymph node IFN-γ+ CD8+, TNF+ CD8+, CD69+ CD8+ T cells as determined by flow cytometry. **Panel d:** Absolute number of polyfunctional CD4+ T cells (IFN-γ+TNF+) by flow cytometry and in vivo expression of IFN-γ mRNA by real-time PCR analysis of purified pulmonary CD4+ or CD8+ T cells relative to 18s housekeeping gene. All data representative of two independent experiments with 5 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s t test.
Figure 2.6

(a) [Graph 1]

(b) [Graph 2]

(c) [Graph 3]

(d) [Graph 4]

(e) [Graph 5]
Discussion:

In this study, we have demonstrated that IL-10 affects very early events after *Mtb* infection that contribute to initial granuloma formation. We have shown that without the influence of IL-10, normally susceptible CBA/J mice can effectively and continually control *Mtb* growth, and are resistant to *Mtb* reactivation. This resistance comes largely from an enhancement of pro-inflammatory cytokine production at day 21 post-infection. CBA/J IL-10\(^{-/-}\) macrophages have increased capacity to prime T cells which initiates cellular recruitment and granuloma formation much earlier than in wild-type mice. These granulomas develop into mature systems of distinctly localized infected macrophages and guardian lymphocytes, eventually becoming completely encased in collagen with necrotic cores. The formation of mature, necrotic, human-like granulomas in the murine model of TB is unprecedented. Until now, very little was known about what constitutes protective immunity to *Mtb*. IFN-\(\gamma\) has been shown to be required, but where and when it is most important to anti-*Mtb* immunity was unclear. Here, we have begun to elucidate the details that comprise an effective immune response against *Mtb*.

Historically, studies examining animals genetically deficient in IL-10 have generally reported little to no survival advantage after *Mtb* infection, however, recent studies have demonstrated enhanced and accelerated immunity to *Mtb* in IL-10\(^{-/-}\) mice. Until now, IL-10 transgenic mice only existed on the relatively resistant C57BL/6 and BALB/c backgrounds. We, and others, have shown that removing IL-10 from these resistant mouse strains had only transient and moderate effects on TB disease progression \(^{314,322}\)}
This was not surprising since these mice generate such a robust response originally, and IL-10 may play small part in their TB progression. Although C57BL/6 mice are a reliable model of TB with a multitude of genetic varieties available, they do not represent the vast majority of susceptible human patients that cannot mount an overtly robust response to *Mtb*. IL-10 needed to be removed from a mouse strain that more accurately reflects *Mtb* susceptibility in man. Thus, the effects that we have demonstrated in CBA/J IL-10/− mice provide crucial insight into the role of IL-10 in the population of individuals most at risk for developing and transmitting TB.

The events responsible for the reactivation of latent TB to an active and contagious disease state are still unknown. Various fields of research are devoted to defining the molecular, pathological, or immunological changes that dictate susceptibility to *Mtb* reactivation. The vast majority of findings in this area are correlative and based on associations of individuals with active TB and a deficiency in certain aspects of Th1 immunity, or certain genetic changes in the bacterium. One correlation commonly noted is that individuals with active TB have elevated levels of IL-10 in their serum, pleural fluid, and/or bronchoalveolar lavage fluid. Whether IL-10 is elevated in susceptible individuals with latent TB is unclear in man. However, using animal models of reactivation-susceptibility we know that in CBA/J mice increased IL-10 during the containment phase of infection leads to granulomatous breakdown and *Mtb* reactivation. Blocking the action of IL-10 at this critical late stage of infection prevents reactivation and rescues the survivability of CBA/J mice. Although informative, previous studies have not examined any corrective measures to ensure
optimal T cell responses and granulomas formation are properly established during early infection.

The details of *Mtb* granuloma establishment, maintenance, and structural dynamics are still unclear. Post-mortem analyses and stereo images of human *Mtb* lesions have offered little insight into their cellular makeup and structural fluidity. The largest obstacle in the study of *Mtb* granulomas is the fact that mice do not form human-like granuloma structures, and all of the advanced models and techniques developed in the murine model could only extrapolate hypotheses concerning human lesions. Current advances in positron emission imaging and computer modeling have given TB researchers a better glimpse of the theoretical dynamics of a human granuloma, but still fall short of actual *in vivo* representation. Studies have shown the importance of various types of cells and cytokines to proper murine granuloma formation, but again, whether the same is true of human lesions is unknown. Efforts have made to replicate human granulomas in the mouse model. One study reports that C3HeB/FeJ mice develop hypoxic lesions with moderate necrosis. These lesions are better representations of human granulomas, yet still lack the compact, mature organizational structure observed in latent human infection. It is for these reasons that our model is so important. The *Mtb* lesions that develop in CBA/J IL-10−/− mice have the same compact, mature and organized structure that is seen in human granulomas. Furthermore, CBA/J IL-10−/− granulomas develop central necrosis and become fibrotic. The collagenous, fibrotic capsule surrounding a necrotic core is another hallmark of human *Mtb* lesions.
The exact mechanism IL-10-deficient protection is currently being examined. CBA/J IL-10<sup>−/−</sup> macrophages have the capacity to present more *Mtb* antigens, which can activate more T cells and boost protective immunity at day 21 post-infection. It is possible that the absence of IL-10 inherently changes the receptor repertoire of alveolar macrophages, directing phagocytosis through less *Mtb*-preferred receptors, such as complement receptor 3 (CR3). This would change the intracellular trafficking of *Mtb* and possibly affect phago-lysosomal fusion, acidification, and bactericidal ability of IL-10<sup>−/−</sup> macrophages. After which, priming of T cells and chemokine-mediated recruitment of new cells would be unimpaired. Current studies utilizing confocal microscopy seek to elucidate this trafficking mechanism. Also, it has been demonstrated that IL-10 can arrest phagosomal maturation and acidification<sup>340</sup>. It is likely that *Mtb* is inducing IL-10 from wild-type CBA/J macrophages, which would downregulate MHC class II, alter surface receptor expression and impede the generation of an ideal response. Wild-type CBA/J macrophages may have inherently high expression of IL-10R, making them much more sensitive to *Mtb*-induced IL-10 levels than wild-type C57BL/6 mice, for example. Closer examination of T cell priming in the lymph nodes may reveal exactly how IL-10 prevents the development of long-term protective immunity during this critical stage. We propose that CBA/J IL-10<sup>−/−</sup> mice provide an ideal model system to study the *in vivo* dynamics of human-like granuloma formation and maintenance.

Known consequences of IL-10 deficiency, such as ulcerative colitis and other gut inflammatory disorders, have not been detected in CBA/J IL-10<sup>−/−</sup> mice. These mice approach clearance of *Mtb* from the spleen, and show no other pathological abnormalities.
through the first 200 days post-infection. Yet, despite such stable protection from and containment of *Mtb*, CBA/J IL-10−/− mice succumb to infection after day 300. Necropsy of a subset of animals at these very late stages of infection shows signs of lung damage and immunopathology. The direct cause of death is currently unknown but it is likely that during such long-term infections, age-related deficiencies may start to arise. T cells may reach the end of their lifespan, becoming proliferatively and functionally exhausted; unable to maintain their earlier level of control.

To account for any inherent changes derived from the genetic removal of IL-10, we blocked to IL-10R from day (-1) to day 21 of infection only to determine if altering the initial events of infection would be sufficient to establish control in wild-type CBA/J mice. Early lymph node responses mimicked that of IL-10−/− CBA/J mice and by day 120 post-infection IL-10R treatment had drastically lowered pulmonary *Mtb* burden and mature, organized granulomas were evident. This clearly demonstrates that IL-10 alters the development of protective immunity very early during *Mtb* infection that has long-term consequences. Additionally, this offers new possibilities towards improving the efficacy of *M. bovis BCG* vaccination. By giving anti-IL-10R as an adjuvant with *M. bovis BCG* vaccine, individuals can generate a much stronger response which we hypothesize will lead to a more efficacious vaccine.

Although CBA/J IL-10−/− mice are capable of generating a nearly ideal immune response to *Mtb* for the majority of their normal lifespan, more detailed work could be done to explain why IL-10 is so prevalent and potent in this mouse strain, and if the same
protective phenotype can be achieved in other mouse strains. Given the time, I would first begin by analyzing the expression dynamics of the IL-10R in various cell types, organs, mouse strains, and at times pre- and post-infection. I believe that the published finding of excessive IL-10 production in CBA/J mice is the consequence of a much more fundamental abnormality. If the levels of IL-10R were found to be higher in CBA/J mice than other mouse strains at critical early stages of \textit{Mtb} infection, it would explain why wild-type CBA/J mice fail to mount an effective early response. I would also use two different susceptible mouse strains for these studies, to broaden the application of any findings. It is possible that IL-10-mediated \textit{Mtb} susceptibility is limited to only CBA/J mice, and that they may, like C57BL/6 mice, only represent a part of the total complex human population. Testing other susceptible mouse strains will add power and significance to our findings, if reproducible. It is not feasible to knockout IL-10 in every susceptible inbred mouse strain, but the anti-IL10R treatment during early \textit{Mtb} infection would be sufficient to determine the relevance of our model. If successful, this would validate the hypothesis that \textit{Mtb} is inducing IL-10 expression during the initial phases of immune priming and that only immune systems that express low levels of IL-10R are capable of overcoming this inhibition. The quantities of IL-10 being induced may be minute and difficult to measure, yet still be physiologically critical.

In conclusion, we have demonstrated that the removal of a single cytokine (IL-10) greatly enhances the ability of susceptible mice to prime T cells, form mature granulomas, and continually control \textit{Mtb} infection. This control was mediated by increased expression of MHC Class II on IL-10\textsuperscript{-/-} macrophages which led to enhanced, but not accelerated,
priming of adaptive immunity. The increase in pro-inflammatory cytokines at day 21 helped to recruit the necessary cellular components to construct a rigid and organized granuloma structure, capable of restricting \textit{Mtb} dissemination. We believe that this model accurately reflects the type of immune repertoire present in susceptible human populations; that a genetic tendency towards excessive IL-10 production can be exploited by \textit{Mtb} to dictate a chaotic and unprotected pulmonary environment, ideal for the transmission of \textit{Mtb} to other human beings.
Chapter 3: CD8+ T cells from CBA/J mice are clonally expanded and dysfunctional after *Mycobacterium tuberculosis* infection.

Abstract:

In this study, we show that *Mtb*-susceptible CBA/J mice accumulated CD8+ T cells in their lungs as infection progressed. This accumulation did not correlate with protection as we observed no concordant increase in interferon-γ (IFN-γ)-producing CD8+ T cells during *Mtb* infection. CBA/J CD8+ T cells did not properly localize to the periphery of the granuloma, a phenotype that is thought to contribute to containment of *Mtb*. Comparisons with resistant C57BL/6 mice demonstrated that large proportions of CBA/J CD8+ T cells expressed CD69, programmed cell death-1 (PD-1), and T-cell immunoglobulin domain and mucin domain 3 (Tim3) suggesting a highly activated but dysfunctional phenotype, which we hypothesized was detrimental to protection against *Mtb*. Furthermore, some CBA/J CD8+ T cells coexpressed CD122 and PD-1, identifying them as possible suppressor T cells. Indeed, CBA/J CD8+ T cells were able to secrete the immunosuppressive cytokine interleukin-10 (IL-10) in response to *ex vivo* stimulation compared to C57BL/6 CD8+ T cells.
After *in vivo* CD8⁺ T cell depletion, CBA/J mice had moderately lower levels of *Mtb* in their lungs, but significantly higher numbers of IFN-γ-producing CD4⁺ T cells. This suggested that only a subset of cells may be responsible for the dysfunction. Complete ablation of CD8⁺ T cells likely masks any increase in protection that may be afforded by depleting only the detrimental subset. We therefore examined the diversity of the CD8⁺ T cell repertoire by T cell receptor (TcR) phenotyping. We discovered that CBA/J CD8⁺ T cells are clonally expanded, preferentially expressing TcR Vβ chain 8 (8.2, 8.3) or Vβ 14. Although we saw no significant differences in Th1 surface expression between Vβ 8 and 14, further *ex vivo* analysis demonstrated that Vβ8⁺ CD8⁺ T cells were capable of generating the majority of IL-10 production. However, *in vivo* depletion of Vβ8⁺ cells led to no significant increase in protection against *Mtb*. We also observed the same accumulation and Vβ TcR repertoire in CD8⁺ T cells in CBA/J IL-10⁻/⁻ mice, indicating that IL-10 is not driving the clonal expansions seen in wild-type CBA/J mice. Together, these data demonstrate the extent of *Mtb* susceptibility in CBA/J mice and provide new insight into the mechanisms of *Mtb* reactivation.

*Introduction:*

The exact factors responsible for the reactivation of latent *Mtb* infection are not well understood, and likely involve contributions from both the host and the pathogen. To appreciate the role that the host immune system plays in *Mtb* reactivation, we used relatively resistant (C57BL/6) or susceptible (CBA/J) mice to represent the natural
progression of TB between different human populations. CBA/J mice have been shown to produce low levels of CD4+ T cell-derived IFN-γ during *Mtb* infection\textsuperscript{288, 289, 341-343}, which has been demonstrated to be required for control of *Mtb* infection\textsuperscript{168, 171, 344}. We have previously shown that *Mtb*-susceptibility in CBA/J mice is mediated by elevated pulmonary levels of IL-10\textsuperscript{289}, but we did not address the underlying mechanism behind this phenotype. In addition to decreased CD4+ T cell-derived IFN-γ, CBA/J mice may possess deficient CD8+ T cell responses that may contribute to *Mtb* susceptibility.

The importance of CD8+ T cells during *Mtb* infection is still unclear. Historically, CD8+ T cells have been considered less important than CD4+ T cells in *Mtb* infection, since *Mtb* resides within a cytoplasmic vacuole and is usually presented through MHC class II to CD4+ T cells. Studies attempting to characterize the role of CD8+ T cells in TB by using genetically deficient mice have met with inconsistent results. Some work has demonstrated that CD8+ T cells are dispensable for *Mtb* control\textsuperscript{345}, while others showed increased *Mtb* susceptibility without CD8+ T cells\textsuperscript{186, 187, 346}. The studies demonstrating that CD8+ T cells were required for protection against *Mtb* initially used β2 microglobulin (β2m\textsuperscript{-/-}) mice as an indirect way to prevent the development of CD8+ T cells\textsuperscript{347}. However, the defect in β2m\textsuperscript{-/-} mice extends past CD8+ T cell deficiency and also removes nonclassical MHC class Ib molecules, such as CD1, inhibiting the ability to present *Mtb* lipid antigen\textsuperscript{348}. This issue was addressed by specific genetic disruption of MHC class Ia or CD8 directly. Some of these studies showed a much less significant role for CD8+ T cells during *Mtb* infection\textsuperscript{188, 345}, while others still demonstrated increased susceptibility in CD8+ T cell-deficient mice\textsuperscript{186, 187}. One of the main contributors to CD8+
T cell anti-

\textit{Mtb} responses in humans is the release of the cytotoxic granule, granulysin \textsuperscript{349}, which does not exist in mice \textsuperscript{345, 350}. This may explain why murine studies examining the genetic deletion of CD8\textsuperscript{+} T cells have had inconsistent results \textsuperscript{186, 187, 346}.

The role of CD8\textsuperscript{+} T cells in \textit{Mtb} infection is becoming better understood through recent work. Increasing evidence suggests that \textit{Mtb} can escape from the phagosome into the cytosol leading to intracellular processing and presentation through MHC class I \textsuperscript{183, 351}. The notion of \textit{Mtb} escape into the cytosol has been speculated for years, but these recent studies provided strong evidence that it occurred. Other studies have begun to elucidate how CD8\textsuperscript{+} T cells are able to respond to \textit{Mtb} antigen through MHC class I. These studies demonstrated that apoptotic vesicles from infected macrophages could be cross-presented to CD8\textsuperscript{+} T cells by DCs \textsuperscript{352, 353}. This indicates that apoptosis of infected cells can be beneficial to \textit{Mtb} control by providing activation of CD8\textsuperscript{+} T cells. Additional work has focused on human CD8\textsuperscript{+} T cell responses to \textit{Mtb} to bypass any differences that exist between humans and mice. Overall, these studies reported that human CD8\textsuperscript{+} T cells were capable of IFN-\gamma production and cytotoxic function after \textit{ex vivo} with \textit{Mtb} antigens, and preferentially targeted heavily infected cells for lysis \textsuperscript{354-356}.

Much more is known about the importance of CD8\textsuperscript{+} T cells in viral models, where, although usually protective, CD8\textsuperscript{+} T cells have been shown to become exhausted after chronic antigenic stimulation \textsuperscript{347, 357}. T cell exhaustion is characterized by a lack of functional or proliferative capability, and surface expression of inhibitory molecules, such as programmed cell death-1 (PD-1) and (Tim3). PD-1 has classically been used as
marker of T cell exhaustion in viral infection and in cancer\textsuperscript{358-361}, and mice deficient in PD-1 are extremely susceptible to \textit{Mtb} infection, primarily due to severe immunopathology\textsuperscript{255}. This suggests that in \textit{Mtb} infection, PD-1 not only marks exhausted T cells but actively maintains their suppression. Other studies have found that cells expressing Tim3 are dysfunctional and lack regulation\textsuperscript{362,363}, and coexpression of PD-1 and Tim3 leads to extensive dysfunction of CD8\textsuperscript{+} T cells\textsuperscript{364}. Together, these studies have identified that PD-1 and Tim3 are negative regulators of immunity to \textit{Mtb}, and can have significant effects on the function of CD8\textsuperscript{+} T cells.

In addition to T cell exhaustion, another potent component of immune modulation is the expression of immunosuppressive cytokines, such as IL-10. IL-10 has been studied extensively, and is implicated in driving susceptibility to TB in both humans and animal models\textsuperscript{288,289,309,310,312}. We, and others, have previously demonstrated that \textit{Mtb} susceptibility in CBA/J mice is mediated by excessive pulmonary IL-10 during infection\textsuperscript{289,341-343,365}, although the underlying mechanism remains unclear. Numerous cell types are capable of producing IL-10, but work has shown that IL-10-producing T cells actively suppress the immune response in TB patients\textsuperscript{366}. Furthermore, T cells expressing both PD-1 and CD122 (the β subunit of the IL-2 receptor) have been shown to have suppressive qualities and secrete IL-10\textsuperscript{367}. This indicates that in addition to inefficient CD4\textsuperscript{+} T cell responses, CBA/J mice may possess significant T cell dysfunction that drive \textit{Mtb} susceptibility. We hypothesize that in \textit{Mtb}-infected CBA/J mice, excessive pulmonary IL-10 is the result of dysfunctional CD8\textsuperscript{+} T cells that express markers of immunosuppression.
**Materials and Methods:**

**Mice**

Specific pathogen-free, age/sex-matched CBA/J wild-type (National Cancer Institute, NIH, Frederick, MD), C57BL/6 wild-type (Jackson laboratories, Bar Harbor, Maine) were maintained in ventilated cages inside a biosafety level 3 (BSL3) facility and provided with sterile food and water *ad libitum*. All protocols were approved by The Ohio State University’s Institutional Laboratory Animal Care and Use Committee.

**Mtbc infection and Colony Forming Unit Enumeration**

*Mtb* Erdman (ATCC no. 35801) was obtained from American Type Culture Collection. Stocks were grown according to published methods. Mice were infected with *Mtbc* Erdman using an inhalation exposure system (Glas-Col) calibrated to deliver 50–100 CFU (for low-dose aerosol) to the lungs of each mouse, as previously described

At specific time points post *Mtbc* infection mice were sacrificed and lungs and spleens were aseptically removed into sterile saline. Organs were homogenized and serial dilutions plated onto 7H11 agar supplemented with OADC as previously described. Plates were incubated at 37°C for 21 days in order to enumerate bacterial colonies and calculate the bacterial burden.
**Lung cell isolation**

Mice were euthanized by CO$_2$ asphyxiation and lungs perfused with cold phosphate buffered saline containing 50Units/mL of heparin through the right ventricle. Lungs from individual mice were mechanically disrupted using a GentleMACS dissociator (Miltenyi Biotec) followed by collagenase A (type XI) (0.7 mg/mL, Sigma) and type IV bovine pancreatic DNase (30µg/mL, Sigma) digestion at 37°C for 30 minutes in GentleMACS C-tubes. Lung cell suspensions were passed through a 70 µm nylon cell screen and residual erythrocytes were lysed with Gey’s solution. Viable cells were determined by trypan blue exclusion.

**Cell Purification**

Single lung cell suspensions were adhered to sterile tissue culture dishes for 1hr at 37°C. Non-adherent cells were washed and removed from the plates. CD4$^+$ and CD8$^+$ T cells were obtained from the non-adherent cell fraction by magnetic cell separation (BD IMAG anti-CD4 particles 551539, anti-CD8 particles 551516) and used for culture as described below. Purity of CD4$^+$ and CD8$^+$ T cell populations was determined to be greater than 90% for all experiments by flow cytometry using an LSRII flow cytometer (BD).

**ELISpot Assays**

Bone marrow-derived dendritic cells (BMDCs) were obtained from the tibiae and femora of age and sex matched non-infected wild-type or IL-10$^{-/-}$ C57BL/6 or CBA/J mice. Cells were differentiated into dendritic cells using complete DMEM supplemented with 10% conditioned media derived from GM-EL4 cells, a GM-CSF-producing clone kindly
provided by Arthur A. Hurwitz (NCI). 2x10^6 bone marrow cells were plated at 37°C in 1ml of GM-EL4 conditioned media in sterile 24-well tissue culture plates. GM-EL4 conditioned media was replaced on days 2, 4 and 6. 3x10^4 BMDCs were infected overnight with *Mtb* Erdman at an MOI of 1:1 then fixed (for WT DCs) in 2% paraformaldehyde. Infected BMDCs were cultured with 2x10^5 CD8^+ T cells or CD8^- T cells for 72hr at 37°C in media containing either tissue culture media alone or 10µg/mL anti-CD3 (145-2C11) and 1µg/mL anti-CD28 (37.51). ELISpot reagents were obtained from eBioscience Ready-Set-GO! Spot-forming units (SFU) were enumerated with an ELISpot plate counter (C.T.L.).

**Flow cytometry**

Isolated lung cells were suspended in deficient RPMI (Irvine Scientific) supplemented with 0.1% sodium azide (Sigma-Aldrich). Surface targets were detected as previously described. Specific Abs and isotype controls were purchased from BD Biosciences: PerCP-Cy5.5 anti-CD3ε (145-2C11), allophycocyanin-Cy7 anti-CD4 (GK1.5), PE-Cy7 anti-CD8 (53-6.7), PerCP-Cy5.5 anti-CD8 (53-6.7), PE-Cy7 anti-IFN-γ (XMG1.2), PE anti-PD-1 (J43). FITC anti-CD122 (TM-Beta 1). PE anti-Tim3 (HAVCR2) antibody purchased from eBiosciences. Cytokine levels were determined according to the manufacturer’s instructions for intracellular cytokine staining (Cytofix/Cytoperm fixation/permeabilization solution kit with BD GolgiStop, BD Biosciences), following a 4hr incubation with 10µg/mL anti-CD3 (145-2C11) and 1µg/mL anti-CD28 (37.51). Samples were read using an LSRII flow cytometer and analyzed with FACSDiva software (BD Biosciences).
**Immunohistochemistry**

Right cranial lung lobes from individual C57BL/6 or CBA/J mice were inflated with 0.1% optimal cutting temperature (OCT) medium (Tissue-Tek) and flash frozen in OCT media using a Leica CM1850 Cryostat Peltier element. Frozen samples were sectioned at 5µm inside the Leica CM1850 cryostat and affixed to glass slides. Sectioned samples were fixed in acetone and standard immunohistochemical staining for using CD8, CD4, isotypes was performed as previously described \(^{368,369}\).

**Statistics**

Statistical analysis performed using GraphPad Prism software for the Students \(t\) test per individual time point of each graph. Any comparisons between timepoints of the same experiment utilize a two-way analysis of variance test with Bonferroni post-tests for multiple comparisons. * \(p<0.05\), ** \(p<0.01\), *** \(p<0.001\)
Results:

**CD8⁺** T cells accumulate in the lungs of CBA/J mice as *Mtb* infection progresses

CBA/J mice have been classified as relatively susceptible to *Mtb* infection. To examine the immunological basis of this susceptibility, we infected wild-type CBA/J mice and C57BL/6 mice with an aerogenic dose of *Mtb* and examined bacterial growth and immune function throughout the course of infection. As previously described, we observed that CBA/J mice were capable of sufficient control of the infection from day 21-90 of infection, but were unable to prevent *Mtb* reactivation after day 90 (Fig 3.1a). To examine the underlying immunological changes associated with this susceptibility, we used flow cytometry to determine the numbers and phenotype of pulmonary T cells between C57BL/6 and CBA/J mice. Throughout the entire course of *Mtb* infection, CBA/J mice had significantly fewer CD4⁺ T cells in the lungs compared to C57BL/6 mice (Fig 3.1b), which matches previous reports of decreased CD4⁺ T cell-mediated protection in CBA/J mice. CD8⁺ T cells were significantly higher in C57BL/6 mice throughout infection, and maintained relatively constant numbers from day 60-150 of infection (Fig 3.1c). Surprisingly, we observed that in CBA/J mice, CD8⁺ T cells accumulated over time, eventually surpassing the level of C57BL/6 mice at day 150 post-infection (Fig 3.1c). CBA/J mice are able to control *Mtb* growth at early timepoints post-infection, regardless of lower numbers of CD4⁺ T cells, but the reactivation of *Mtb* after day 90 correlates with the increasing numbers of CD8⁺ T cells in CBA/J mice. We therefore examined the ratio of CD4:CD8 T cells in CBA/J mice, and demonstrated that CBA/J mice did not lose CD4⁺ T cells but consistently accumulated CD8⁺ T cells in their...
lungs as *Mtb* infection progressed (**Fig 3.1d**). BrdU staining of CBA/J CD8+ T cells was not significantly higher than C57BL/6 CD8+ T cells throughout infection, confirming that the increased numbers of CBA/J CD8+ T cells was not local proliferation, but likely a recruited accumulation of cells (**Fig 3.1e,f**).

We next sought to determine what was triggering the accumulation of CD8+ T cells in CBA/J mice. CBA/J mice have been shown to have high levels of IL-10 in their lungs as *Mtb* infection progresses. In addition to its immunosuppressive effects, IL-10 has been shown to stimulate the proliferation of CD8+ T cells372. Although we did not observe changes in T cell proliferation (**Fig 3.1e,f**), it did not exclude the possibility that IL-10 may be driving the accumulation of CD8+ T cells in CBA/J mice. We therefore examined the numbers of CD4+ and CD8+ T cells present in the lungs of CBA/J IL-10−/− mice over the course of *Mtb* infection. We did not observe any differences in CD8+ T cell accumulation at late stages of infection between wild-type and IL-10−/− CBA/J mice (**Fig 3.1h**). Rather, CBA/J IL-10−/− mice had increased numbers of CD4+ (**Fig 3.1g**) and CD8+ T cells (**Fig 3.1h**) only at day 30 post-infection compared to wild-type CBA/J mice. This phenomenon is addressed in detail in Chapter 2.
Figure 3.1  Accumulation and characterization of CBA/J CD8+ T cells

C57BL/6, CBA/J, and CBA/J IL-10−/− mice were infected with an aerosolized dose of *Mtb*, and at various times post-infection lungs were removed. (a) C57BL/6 and CBA/J lungs were homogenized and plated on 7H11 plates for CFU enumeration. (b,c) CBA/J and C57BL/6 lung cell were analyzed by flow cytometry for CD4+ and CD8+ T cells. (d) Ration of CBA/J CD4+ to CD8+ T cells. (e,f) 24hr prior to necropsy mice were injected with BrdU, then lung cells were analyzed for expression of BrdU+ CD4+ or CD8+ T cells. (g,h) Absolute numbers of CD4+ or CD8+ T cells in wild-type or IL-10−/− CBA/J mice as determined by flow cytometry. Results representative of at least two independent experiments. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s t test. (c,h) * p<0.05, ++ p<0.01, +++ p<0.001 as obtained by Student’s t test comparing day 90 to day 150 post-infection.
CBA/J mice develop diffuse, unorganized granulomas after *Mtb* infection

Both humans and mice are unable to clear *Mtb* infection without drug therapy. As such, infected individuals and animals attempt to contain *Mtb* infection within arrangements of immune cells called granulomas. T cell subsets are classically thought to localize to distinct areas of the granuloma with CD4$^+$ T cells permeating the center to interact with infected macrophages and CD8$^+$ T cells around the granulomas periphery preventing dissemination of infected cells. CBA/J mice have previously been shown to develop diffuse, unorganized granulomas after *Mtb* infection$^{288}$. Since CBA/J mice have an accumulation of CD8$^+$ T cells, yet are moderately deficient in CD4$^+$ T cell numbers compared to C57BL/6 mice, we hypothesized that CD8$^+$ T cells would more densely populate granulomas in CBA/J mice.

Using immunohistochemical staining for CD4 or CD8, we observed that in *Mtb*-infected CBA/J mice CD8$^+$ T cells did not appear more densely populated, but they did not localize to the granuloma periphery compared to C57BL/6 mice, or permeate into the granuloma center (Fig 3.2a). This was best observed at day 120 in Fig 3.2a (circles), where the large granuloma from a C57BL/6 mouse is not permeated by CD8$^+$ T cells, while the CBA/J lesions at day 120 contain numerous CD8$^+$ T cells in the granuloma center. CD4$^+$ T cell distribution was similar between both CBA/J and C57BL/6 mice at all timepoints recorded (Fig 3.2b), with staining demonstrating CD4$^+$ T cells permeated into the granuloma center. From these histological analyses we have determined that CD8$^+$ T cells from CBA/J mice have altered positioning and are disorganized inside *Mtb* granulomas.
Figure 3.2  Immunohistochemistry of T cell distribution during *Mtb* infection

Wild-type C57BL/6 and CBA/J mice were aerogenically infected with *Mtb* and at various times post-infection lungs were removed, inflated with OCT media, and snap-frozen. Tissue sections were stained with (a) CD8 or (b) CD4 and counterstained with hematoxylin and eosin. Circles represent areas of clear CD8\(^{+}\) T cell arrangement disparity between groups of mice. Representative images from two independent experiments with 4 mice per group per timepoint.
Figure 3.2

(a) CD8+ T cells

(b) CD4+ T cells
Accumulated CD8+ T cells from *Mtb*-infected CBA/J mice express markers of T cell dysfunction

Since CD8+ T cells were accumulating in CBA/J mice after *Mtb* infection, but were not maintaining a border around the granuloma periphery, we hypothesized that CD8+ T cells in CBA/J mice were permeating into the granuloma to contribute to IFN-γ production, in the absence of sufficient CD4+ T cell-derived IFN-γ. We therefore examined the number of T cells that were capable of secreting IFN-γ after *ex vivo* TcR stimulation. As anticipated, we found that significantly fewer CD4+ T cells from CBA/J mice were positive for IFN-γ during the course of *Mtb* infection compared to C57BL/6 mice (Fig 3.3a). The amount of IFN-γ-producing CD4+ T cells was significantly lower in CBA/J mice than C57BL/6 mice, but both groups increased in number concordant with the total number of CD4+ T cells during infection (Fig 3.1b). In contrast, examination of IFN-γ+ CD8+ T cells showed that by day 30 post-infection CBA/J mice have already reached the maximum number of IFN-γ-producing CD8+ T cells present throughout the remainder of *Mtb* infection (Fig 3.3b). Interestingly, this is in stark contrast to the total number of CD8+ T cells in *Mtb*-infected CBA/J mice, which continued to accumulate over time (Fig 3.1c). This suggests that IFN-γneg CD8+ T cells from CBA/J mice may be contributing in another, perhaps dysfunctional, way.

We then further characterized the surface phenotype of CD8+ T cell from *Mtb*-infected CBA/J mice by flow cytometry. Since the number of pulmonary T cells varied significantly over the course of *Mtb* infection between CBA/J and C57BL/6 mice, phenotypic expression was analyzed as the proportion of total CD8+ T cells expressing
each specific marker. We observed that the percentage of CD8+ T cells from CBA/J mice that expressed CD69 continued to increase from day 60-150 post-infection, compared to the proportion of CD69+ CD8+ T cells from C57BL/6 mice that reached their maximum point at day 60 and remained relatively stable (Fig 3.3c). Indeed, the proportions of CD69+CD8+ T cells in CBA/J was significantly higher at day 150 than day 60 (Fig 3.3c). Following the same trend, higher percentages of CD8+ T cells from CBA/J mice expressed the T cell dysfunction marker Tim3 (Fig 3.3d) than C57BL/6 mice throughout Mtb infection. Tim3 was also relatively stable from day 30-150 in C57BL/6 CD8+ T cells, while continually increasing in CBA/J CD8+ T cells. This trend paralleled the levels of bacteria in the lungs of CBA/J mice at late timepoints of Mtb infection, suggesting that their CD8+ T cells are activated and dysfunctional and may be contributing to TB progression.

To further examine this dysfunctional phenotype, we screened for the presence of PD-1 on the surface of CD8+ T cells from CBA/J and C57BL/6 mice. Starting at day 60 post-infection, higher percentages of CBA/J CD8+ T cells expressed PD-1 than C57BL/6 CD8+ T cells (Fig 3.3e). Furthermore, more CD8+ T cells from CBA/J mice coexpressed PD-1 and CD122 (IL-2R β subunit) than C57BL/6 mice for all timepoints except day 150 after Mtb infection (Fig 3.3f). Recent work has demonstrated that coexpression of PD-1 and CD122 marks a subset of CD8+ T cells that have immunosuppressive properties 367. The expression pattern of PD-1+ and PD-1+CD122+ cells did not increase over time as with CD69 and Tim3, and reached its highest point from day 60-90 post-infection, although still significantly higher in CBA/J mice at later timepoints (Fig 3.3e,f). There
was a significant decrease in PD1⁺CD122⁺ CD8⁺ T cells in both C57BL/6 and CBA/J mice at days 120-150 post-infection, which we hypothesize is due to high levels of IL-10 in the lungs that would downregulate expression of the IL-2R. Taken together, these data demonstrate that *Mtb*-infected CBA/J mice accumulate IFN-γ neg CD8⁺ T cells in the lungs, that express known markers of effector T cell exhaustion, dysfunction and immunosuppression.
C57BL/6 and CBA/J mice were infected with an aerosolized dose of Mtb and at various timepoints post-infection lungs were removed and processed for flow cytometry. Absolute numbers of IFN-γ⁺ CD4⁺ (a) or CD8⁺ (b) T cells after 4hr *ex vivo* stimulation with anti-CD3/CD28/GolgiSTOP. Absolute numbers of CD8⁺ T cells expressing CD69 (c), Tim3 (d), or PD-1 (e) after Mtb infection. (f) Absolute number of CD8⁺ T cells expressing both PD-1 and CD122. Data representative of two independent experiments with 4 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test. (c,d) + p<0.05, ++ p<0.01, +++ p<0.001 as obtained by Student’s *t* test comparing only CBA/J mice across all timepoints.
Figure 3.3

(a) RtnR^+CD4^+ T Cells
(b) RtnR^+CD3^+ T Cells
(c) % CD69^+CD8^+ T Cells
(d) % Tim3^+CD8^+ T Cells
(e) % PD1^+CD8^+ T Cells
(f) % CD122^+PD1^+ CD8^+ T Cells

Days Post-Infection
CD8$^+$ T cells from *Mtb*-infected CBA/J mice are capable of secreting IL-10

We have previously reported that a main correlate of susceptibility to *Mtb* reactivation in CBA/J mice is elevated levels of the immunosuppressive cytokine, IL-10, in their lungs as *Mtb* infection progressed. In this study, we have shown that at the same timepoints, CBA/J mice accumulated IFN-γ dysfunctional CD8$^+$ T cells. Although we have demonstrated that IL-10 is not responsible for this accumulation, we hypothesized that CD8$^+$ T cells from *Mtb*-infected CBA/J mice were contributing to IL-10 production, since the numbers of CD8$^+$ T cells parallel the amount of IL-10 produced in CBA/J mice during *Mtb* infection. This disagrees with the normal protective function of CD8$^+$ T cells as being responsible for lysing target cells and contributing to IFN-γ production during *Mtb* infection\(^{179,373-375}\). We purified CD8$^+$ and CD8$^{\text{neg}}$ T cell populations and cultured them for 72 hours with anti-CD3 and anti-CD28 cross-linking antibodies, plus autologous bone marrow-derived dendritic cells that were infected with *Mtb* for 24 hours and measured IL-10 production by ELISpot. DCs were fixed prior to incubation with T cells to prevent changes in DC surface expression or DC-secreted IL-10. We observed that high numbers of CD8$^+$ T cells from CBA/J mice secreted IL-10 compared to C57BL/6 mice throughout *Mtb* infection (Fig 3.4a). CD8$^{\text{neg}}$ T cells were also capable of IL-10 production after TcR stimulation but did not significantly differ between CBA/J and C57BL/6 mice (Fig 3.4b), suggesting that CD4$^+$ T cells from both strains are capable of equivalent IL-10 production when stimulated, but CD8$^+$ T cell IL-10 production is exclusive to CBA/J mice during *Mtb* infection. Additionally, levels of T cell-derived IL-10 were examined by ELISA after 6 day culture with anti-CD3/CD28 which verified the
finding that CBA/J CD8+ T cells produced significantly more IL-10 than CD8+ T cells from C57BL/6 mice (Fig 3.4c).

Numerous studies were undertaken to determine if the IL-10 secretion from CBA/J CD8+ T cells was *Mtb*-specific. Cultures were established as in Fig 3.4a, using only *Mtb*-infected DCs from IL-10−/− C57BL/6 or CBA/J donors. Very few CD8+ (Fig 3.4d) or CD8neg (Fig 3.4e) T cells were capable of producing IL-10 from C57BL/6 or CBA/J mice in response to only *Mtb*-infected DCs. Despite rigorous testing, we were unable to reproduce the same level of IL-10 production without stimulation with anti-CD3/CD28. Although our T cell populations were highly purified, the CD8neg group could contain other lymphocytes besides CD4+ T cells. To determine the IL-10 contribution of other cell populations, we analyzed the expression of IL-10 mRNA by real-time PCR on purified cells expressing the B cell marker, B220. We detected very little IL-10 mRNA in B220+ cell populations, and also observed that B220neg cells had significantly more IL-10 mRNA in C57BL/6 mice than CBA/J mice. This indicates that CD8neg T cell production of IL-10 is not B cell-derived, and is most likely produced by CD4+ T cells. Indeed, further studies using purified CD8+ and CD4+ T cells confirmed this hypothesis after culture with anti-CD3/CD28 (not shown). Overall, our inability to demonstrate *Mtb*-specific IL-10 production from CBA/J CD8+ T cells most likely stems from our experimental design. We attempted numerous experimental variations, including lengthening the period of DC infection, increasing/decreasing the multiplicity of infection (MOI), and including other T cell populations from CBA/J IL-10−/− mice in the culture. After which, we still are not confident that the CD8+ T cells in our assays are seeing *Mtb*
antigen in the correct way, and that further *in vivo* analysis would be a more robust method of testing our hypothesis.
Figure 3.4  IL-10 production by CD8\(^+\) T cells from CBA/J mice
C57BL/6 and CBA/J mice were infected with an aerosolized dose of *Mtb* and at various times post-infection lungs were removed and various cell populations were purified. Spot-forming units (SFU) representing the absolute number of IL-10\(^+\)CD8\(^+\) T cells (a) or CD8\(^{neg}\) T cells (b) per lung after 72hr culture with anti-CD3/CD28. (c) ng/mL of IL-10 produced after 6 day culture of CD8\(^+\) or CD8\(^{neg}\) T cells with anti-CD3/CD28 at day 180 post-infection. SFU per lung of IL-10\(^+\) CD8\(^+\) (d) or CD8\(^{neg}\) (e) T cells cultured with *Mtb*-infected BMDCs for 72hr. (f) IL-10 mRNA expression from purified B220\(^+\) or B220\(^{neg}\) cells at day 125 post-infection. (a,b,d,e) Data representative of two independent experiments with 4 mice per group per timepoint, (c,f) representative of one experiment with 4 mice per group. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test.
CD8+ T cell depletion in CBA/J mice moderately increases pro-inflammatory responses against *Mtb*.

Since CD8+ T cells in CBA/J mice expressed markers of T cell dysfunction and were capable of secreting IL-10 during *Mtb* infection, we hypothesized that they were detrimental to immunity and that antibody-mediated *in vivo* depletion would increase protection against *Mtb*. CD8+ T cells were depleted from wild-type CBA/J mice from day 90-120 after *Mtb* infection via intraperitoneal injection. This time course was used since it represented the period when IL-10 and CD8+ T cells were increasing in the lungs of *Mtb*-infected CBA/J mice\(^ {289}\). At day 120, we observed a trend towards lower bacterial burden (Fig 3.5a) and higher numbers of total (Fig 3.5b) and IFN-γ+ (Fig 3.5c) CD4+ T cells in the lung after CD8+ T cell depletion, though these did not reach statistical significance. We also measured a significant decrease in the total amount of IL-10 in the lungs of CBA/J mice after CD8+ T cell depletion (Fig 3.5d). Depletion of CD4+ T cells led to significantly increased *Mtb* burden and mortality before day 120 (not shown).

Despite the lack of significance, depletion of the entire CD8+ T cell repertoire from CBA/J mice led to moderately decreased levels of pulmonary *Mtb* at day 120 post-infection. When compared to the severe loss of protection after CD4+ T cell depletion, this indicates that CD8+ T cells are not contributing to protection in CBA/J mice, and may be detrimental. Depletion of the entire CD8+ T cell repertoire *in vivo* eradicated both functional and dysfunctional cells, which could have masked any potential increase in protection after CD8+ T cell removal, and it was possible to selectively deplete only the IL-10+ CD8+ T cells. Therefore, we hypothesized that only a subset of CBA/J CD8+ T cells may be detrimental to *Mtb* immunity.
**Figure 3.5  CD8$^+$ T cell depletion in CBA/J mice**

CBA/J mice were infected with an aerosolized dose of *Mtb* and from day 90-120 were treated weekly with anti-CD8 depletion antibody via intraperitoneal injection then sacrificed at day 125 post-infection. (a) Lungs were homogenized and plated on 7H11 plates and CFU enumerated after 21 days. Absolute number of total CD4$^+$ T cells (b) or IFN-γ$^+$CD4$^+$ T cells (c) after CD8$^+$ T cell depletion as determined by flow cytometry. (d) Levels of pulmonary IL-10 after CD8$^+$ T cell depletion as determined by ELISA. Results representative of at least two independent experiments with 5-10 mice per group.

* p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test.
**CD8+ T cells from CBA/J mice are clonally expanded**

We demonstrated that removal of the entire CD8+ T cell population had only moderate effects on *Mtb* burden and CD4+ T cell numbers, yet compared to CD4+ T cell depletion this phenotype was encouraging as CD8+ T cell depletion did not exacerbate infection. Recent studies have demonstrated that CD8+ T cell populations can be clonally expanded during TB \(^{376, 377}\). As such, we hypothesized that a clonal population of detrimental CD8+ T cells arises in CBA/J mice during *Mtb* infection. Therefore, we analyzed the diversity of CD8+ T cell receptor repertoire by flow cytometry after *Mtb* infection. We observed that CD8+ T cells from CBA/J mice primarily expressed only two variable regions of the TcR beta chain (Vβ). Vβ8 (8.2,8.3) and Vβ14 were expressed by approximately 45% of all CD8+ T cells in CBA/J mice at day 120 after *Mtb* infection, compared to more even expression of numerous Vβ TcR in C57BL/6 mice (Fig 3.6a). Expression of Vβ8 and Vβ14 was similar on CBA/J CD4+ T cells but at a much lower frequency (Fig 3.6b).

More detailed examination over the course of *Mtb* infection showed that percentages of CD8+ T cells expressing Vβ8 or Vβ14 were significantly higher throughout *Mtb* infection in CBA/J mice, being most significant at late timepoints (Fig 3.6c,d).
Figure 3.6  

Vβ TcR expression in CBA/J and C57BL/6 mice

C57BL/6 and CBA/J mice were infected with an aerosol dose of *Mtb* and at various timepoints post-infection lungs were removed and processed for flow cytometry. Percentages of CD8+ (a) or CD4+ (b) T cells expressing specific Vβ TcRs at day 120 post-infection. Percentages of CD8+ T cells expressing Vβ8 (c) or Vβ14 (d) over the course of *Mtb* infection. Data representative of at least two independent experiments with 4 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test.
Figure 3.6

(a) Vβ Chain

(b) Vβ Chain

(c) Vβ Chain

(d) Vβ Chain
Since the highest proportion of cells expressed Vβ8 and Vβ14, we analyzed these subsets further by flow cytometry to determine if the clonal phenotype of the lungs was present in other organs. We observed relatively the same distribution of Vβ TcR on CD8+ T cells in the mediastinal lymph nodes (MLN) of CBA/J mice, but very few cells expressing any Vβ TcR in the spleen and peripheral blood. This is illustrated by the percentages of CD8+ T cells expressing Vβ8 (Fig 3.7a) or Vβ14 (Fig 3.7b) in the lung, MLN, spleen, and blood. No other Vβ chain had increased expression in any of the secondary organs (not shown). We observed that Vβ expression was highest in the lungs and MLN, so we focused further examination on these organs. Closer investigation of the surface expression of Vβ8+ or Vβ14+ CD8+ T cells revealed that higher percentages of Vβ8+ CD8+ T cells expressed CD69 in the lungs (Fig 3.7c) at day 60 and in the MLN at days 60, 120, 150 (Fig 3.7d) compared to Vβ 14+CD8 + T cells in Mtb-infected CBA/J mice. Evaluation of PD-1 expression showed that more Vβ8+CD8+ T cells expressed PD-1 only in the lung (Fig 3.7e). Expression of PD-1 by any Vβ TcR CD8+ T cell was very low in the MLN (Fig 3.7f), and other organs (not shown). Together, these data show that Vβ8+CD8+ T cells from CBA/J mice are the predominant clonal population in the lungs and MLN, and that their expression of CD69 and PD-1 parallels the expression of total CD8+ T cells. We hypothesize that Vβ8+CD8+ T cells are the detrimental population of CD8+ T cells in CBA/J mice and that specific depletion of only this subset will increase protection against Mtb.
C57BL/6 and CBA/J mice were infected with an aerosol dose of *Mtb* and at various timepoints post-infection lungs, spleens, mediastinal lymph nodes, and peripheral blood were obtained and processed for flow cytometry. Percentages of CD8+ T cells expressing Vβ8 (a) or Vβ14 (b) in multiple organs over the course of *Mtb* infection. Percentages of Vβ8+CD8+ T cells (c) or Vβ14+CD8+ T cells (d) expressing CD69, or Vβ8+CD8+ T cells (e) or Vβ14+CD8+ T cells (f) expressing PD-1 throughout *Mtb* infection. Data representative of at three independent experiments with 4 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test.
Figure 3.7

(a) 

(b) 

(c) 

(d) 

(e) 

(f)
Therefore, we purified the population of Vβ8+CD8+ T cells from CBA/J mice or C57BL/6 mice (control) and cultured them with CBA/J or C57BL/6 IL-10−/− BMDCs stimulated with anti-CD3/CD28 for 72 hours. Significantly more Vβ8+ T cells were capable of producing IL-10 than Vβ8neg T cells from Mtb-infected CBA/J mice (Fig 3.8a). Residual supernatants from this experiment were then assayed for the level of IFN-γ produced by ELISA. We observed no difference between Vβ8+ or Vβ8neg T cells ability to secrete IFN-γ (Fig 3.8b). This supported our hypothesis that only the Vβ8+ CD8+ T cells were detrimental via their secretion of IL-10, and that depletion of only this specific subset of CD8+ T cells in CBA/J mice would lead to significant enhancement of Mtb control. We obtained Vβ8 depletion antibody and treated CBA/J mice from day 90-120 during Mtb infection, as we previously performed with total CD8+ T cells. We did not observe a significant decrease in Mtb burden (Fig 3.8c) or a significant enhancement of total CD4+ (Fig 3.8d) or IFN-γ+ CD4+ (Fig 3.8e) T cells after Vβ8 depletion. We did observe a moderate decrease in the number of CD4 and CD8+ T cells capable of secreting IL-10 (Fig 3.8f). Though this did not reach statistical significance, it supports our finding that Vβ8+CD8+ T cells are the main subset producing IL-10.
Figure 3.8  In vivo depletion of Vβ8+ T cells in CBA/J mice
C57BL/6 and CBA/J mice were aerogenically infected with Mtb and at day 150 lungs were removed and processed into a single cell suspension. (a) Vβ8+ and Vβ8neg T cells were cultured for 72hr with anti-CD3/CD28 and IL-10 SFU determined by ELISpot. (b) Supernatants from (a) were analyzed for IFN-γ levels by ELISA. CBA/J mice were treated with anti-Vβ8 depletion antibody or isotype control antibody from day 90-120 then sacrificed at day 125 post-infection. (c) Lungs were homogenized and plated onto 7H11 plates and CFU enumerated. Absolute number of total T cells (d) or IFN-γ+ T cells (e), or IL-10+ T cells (f) after Vβ8+ cell depletion as determined by flow cytometry. Results representative of at two independent experiments with 5 mice per group. Control group = no treatment and isotype control. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s t test.
Discussion:

In this study, we have demonstrated that CD8\(^+\) T cells from CBA/J mice accumulate over the course of \textit{Mtb} infection. This accumulation does not yield an equivalent increase in IFN-\(\gamma\)-CD8\(^+\) T cells, and CBA/J CD8\(^+\) T cells do not establish their position around the periphery of pulmonary granulomas. Upon further phenotypic examination, we discovered that CD8\(^+\) T cells from CBA/J mice express the T cell dysfunction markers PD-1 and Tim3, as well as coexpression of PD-1\(^+\)CD122\(^+\) suggesting possible immunosuppressive activity. After \textit{ex vivo} purification and culture, CD8\(^+\) T cells from CBA/J mice were shown to be capable of secreting IL-10 after TcR stimulation compared to nearly a complete lack of IL-10 production from C57BL/6 CD8\(^+\) T cells. Depletion of the entire CD8\(^+\) T cell population in \textit{Mtb}-infected CBA/J mice led to a significant reduction in pulmonary IL-10 levels, but only moderate enhancement of protective CD4\(^+\) T cell numbers and bacterial control. Further investigation revealed severely limited TcR diversity in CBA/J mice, with approximately 45\% of all CD8\(^+\) T cells expressing either V\(\beta\)8 or V\(\beta\)14. We identified that V\(\beta\)8\(^+\) T cells were capable of IL-10 production and more expressed CD69 or PD-1 compared to V\(\beta\)8\(^{neg}\) T cells from \textit{Mtb}-infected CBA/J mice, although antibody-mediated depletion of V\(\beta\)8\(^+\) cells did not significantly enhance protection against \textit{Mtb}.

CBA/J susceptibility to \textit{Mtb} infection has been well documented, yet the exact mechanism of this susceptibility is still not entirely understood. We have previously demonstrated that CBA/J mice produce high levels of IL-10 in their lungs during...
infection with *Mtb*. Blockade of the IL-10R from day 90-120 of *Mtb* infection results in significantly reduced *Mtb* burden and enhanced T\(_{H1}\) immunity. Although this study clearly demonstrated a major component of CBA/J susceptibility, it did not address where the excess IL-10 is coming from or why CBA/J mice have increased IL-10. Here, we have demonstrated that an unlikely source, CD8\(^+\) T cells, may be responsible for some of the IL-10 produced during *Mtb* infection in CBA/J mice, although we observed that CD4\(^+\) T cell were also capable of IL-10 production. It is important to note that although CD4\(^+\) T cells could produce IL-10, it was not significant different between C57BL/6 and CBA/J mice. This indicates that CD4\(^+\) T cells are ordinarily capable of self-regulation by IL-10 in both strains, but that IL-10 secreted from CD8\(^+\) T cells was unique to CBA/J mice, suggesting a detrimental role.

The surface phenotype of CBA/J CD8\(^+\) T cells supports this data. High percentages of CD8\(^+\) T cells from CBA/J mice expressed PD-1, which has been demonstrated in *Mtb* infection to mark T cells that have become exhausted and unable to secrete pro-inflammatory cytokines. Studies have demonstrated that in chronic murine *Mtb* infection PD-1\(^+\) T cells are capable of proliferation but not secretion of IFN-\(\gamma\) and that this inhibition can be overcome by direct TcR stimulation\(^{378,379}\). Although we saw no evidence of increased proliferation, this matches our data that CD8\(^+\) T cells are accumulating in the lungs of CBA/J mice, but the majority are not producing IFN-\(\gamma\) *in vivo* (although they are capable after TcR stimulation). We did not see significant increases in proliferative responses, but this may be due to the fact that PD-1\(^+\)CD8\(^+\) T cells have not been extensively studied in *Mtb* infection and may not behave identically
to PD-1+CD4+ T cells. The accumulation (and not proliferation) of CBA/J CD8+ T cells suggests that levels of chemokines may be significantly altered in CBA/J mice. Previous work has addressed this hypothesis and found that CCL5 levels were significantly lower in CBA/J mice than C57BL/6 mice at early timepoints of *Mtb* infection. This study did not, however, examine the levels of chemokines at late timepoints of *Mtb* infection that would correlate to the times when CD8+ T cells were accumulating in CBA/J mice. It is likely that either CCL5, CXCL10 or CCL12 are altered from day 90-150 of *Mtb* infection in CBA/J mice and that this is responsible for the accumulation of CD8+ T cells at these times. It has been previously reported that CCL5 is significantly lower in CBA/J mice during early timepoints of *Mtb* infection leading to reduced ability to properly recruit and organize cells in the lung (unpublished observations). The accumulation of CD8+ T cells later in infection is potentially the result of overcompensation of this early chemokine deficiency by the overproduction of chemokines thereafter, or purposeful recruitment due to increasing *Mtb* burden.

It has also been previously observed that CBA/J mice fail to develop organized granulomas after *Mtb* infection. CBA/J granulomas are usually large, diffuse and lack the characteristic organization found in C57BL/6 granulomas, where CD4+ T cells are located throughout the lesion where they can interact directly with infected macrophages. CD8+ T cells in *Mtb*-infected C57BL/6 mice tend to remain around the periphery of the granuloma and are thought to lyse any infected cells before they can disseminate. It is possible that the increased numbers of CD8+ T cells in CBA/J mice are also not cytotoxic, and do not contribute to the containment of *Mtb* within the granuloma. Studies
of murine granuloma formation have not yet been able to effectively represent human TB lesions since they do not develop mature, concise and necrotic granulomas. As such, the inferences that we can make about CBA/J susceptibility based on their granulomas are limited, though CBA/J IL-10−/− mice (discussed in Chapter 2) could be used as a model of human-like granulomas.

Even compared to C57BL/6 mice it is difficult to base any hypotheses, since neither are accurate representation of human granuloma formation and maturation. The C57BL/6 mouse strain has been used extensively to study the immune response to Mtb, yet it mounts an overtly powerful T_{H1} response to intracellular pathogens. It could be argued that using C57BL/6 mice as a means of comparison with CBA/J mice will not give an accurate representation of Mtb susceptibility. Here, we use C57BL/6 mice as a measure of an optimal T_{H1} response to Mtb, and base our inferences on the dynamics of CBA/J T cell activity rather than manipulation of the already potent C57BL/6 response. It is likely that the inability to form organized granulomas stems from improper cytokine and chemokine signals during critical times after Mtb infection.

CBA/J mice fail to develop organized granulomas and accumulate dysfunctional CD8+ T cells as Mtb infection progresses. We have further classified the extent of this dysfunction to include coexpression of PD-1 and CD122, which has recently been shown to mark IL-10-producing suppressor CD8+ T cells. Furthermore, purified CD8+ T cells can secrete IL-10 after ex vivo TcR stimulation. Unfortunately, stimulation of purified CD8+ T cells from CBA/J mice with Mtb antigens did not lead to IL-10 production. We attempted a
variety of approaches to correct this issue. First it was thought that additional DC stimulation was required for proper CD8$^+$ T cells activation and IL-10 production, and that using fixed DCs was restricting this interaction. We therefore used C57BL/6 IL-10$^{-/-}$ and recently created CBA/J IL-10$^{-/-}$ mice (or CBA/J-C57BL/6 F1 progeny) as the source for the DCs. This permitted us to use unfixed DCs in our culture without concern of false-positive IL-10 production from DCs, yet it did not yield $Mtb$-specific IL-10 production from CBA/J CD8$^+$ T cells. Next, it was thought that exogenous $Mtb$ antigens would not be presented through MHC class I to CD8$^+$ T cells, and that CD4$^+$ T cell help would be necessary for cross-presentation. This was tested using whole wild-type CBA/J T cells incubated with CBA/J IL-10$^{-/-}$ DCs, yet still had no $Mtb$-specific IL-10 production. Various infection and culture lengths were tested with no success. Other methods of measuring IL-10 were sought out, such as intracellular flow cytometry and real-time PCR, but neither were capable of consistently detecting IL-10.

It is likely that the main issue preventing $Mtb$-specific IL-10 from being induced by CBA/J CD8$^+$ T cells is that the T cells are not recognizing the $Mtb$ antigens properly in vitro. Further studies could be attempted to demonstrate that CD8$^+$ T cell-derived IL-10 in CBA/J is $Mtb$ specific. Exogenous $Mtb$ will phagocytized and presented through MHC class II to CD4$^+$ T cells unless they are directed otherwise. We could add a chemical such as streptolysin to the infected DCs cultures to disrupt the DC cell membranes and direct the presentation of intracellular antigens through MHC class I. Additionally, studies have shown that in order for APCs to properly process and cross-present $Mtb$ antigens to CD8$^+$ T cells, CD4$^+$ T cell help is required. Our newly generated CBA/J IL-10$^{-/-}$ mice would be
ideal for testing this hypothesis. We hypothesize that *ex vivo* culture of DCs with CBA/J IL-10<sup>−/−</sup> CD4<sup>+</sup> T cells during *Mtb* infection will lead to proper antigen processing and presentation. Purified CD8<sup>+</sup> T cells from wild-type CBA/J mice could then be added to the culture, and assayed for IL-10 production.

To bypass the entire antigen-presentation issue, a more elegant experiment could be completed again using CBA/J IL-10<sup>−/−</sup> mice. We could adoptively transfer total CD8<sup>+</sup> T cells or Vβ8<sup>+</sup> CD8<sup>+</sup> T cells purified from wild-type CBA/J mice at day 150 post-infection (the peak of IL-10 production capacity) into IL-10<sup>−/−</sup> CBA/J mice at various times post-infection. If CBA/J CD8<sup>+</sup> T cells are secreting IL-10 *in vivo*, the addition of dysfunctional wild-type CD8<sup>+</sup> T cells to CBA/J IL-10<sup>−/−</sup> mice will negatively impact protection against *Mtb*. This would demonstrate not only if CBA/J CD8<sup>+</sup> T cells are detrimental, but at what time post-infection they exert the strongest effect. CD8<sup>+</sup> T cells are not the only source of IL-10 in CBA/J mice during *Mtb* infection, but since they express markers of dysfunction, we hypothesize that they are producing IL-10 inappropriately and do not contribute to protection against *Mtb*.

In addition to IL-10 production, we also observed that CD8<sup>+</sup> T cells from CBA/J mice have severely restricted diversity of their TcR repertoire. This significantly limits the amount of antigens that CBA/J CD8<sup>+</sup> T cells are capable of recognizing. CBA/J mice have an endogenous virus, mouse mammary tumor virus (MTV-6), that selectively deletes various TcR Vβ chains such as Vβ8.1, and Vβ 17a<sup>381, 382</sup>. This alone is not sufficient to explain the limited TcR diversity in CBA/J mice. It is possible that *Mtb* is
also deleting certain subsets of CD8+ T cells and driving clonal expansion of less protective cells (Vβ8, Vβ14). Although depletion of specific Vβ population did not significantly enhance protective responses, we did observe differential capacity to secrete IL-10. This suggests that Vβ subsets of CD8+ T cells have different functional capacity, and that this could be exploited by Mtb. We also observed a decrease in the numbers of IL-10*CD4+ T cells after Vβ8 depletion. This is primarily due to the fact that CBA/J mice do not tolerate intraperitoneal injection well, and that isotype control groups had increased numbers of total lung cells after treatment, thus skewing the absolute number of cells expressing IFN-γ and IL-10 (though neither were significantly higher). Percentages of T cells expressing IFN-γ or IL-10 after antibody treatment were not significantly different (not shown).

To gain more insight into the details of CBA/J Vβ expression, we could first spectratype the Vβ8 and Vβ14 CD8+ T cell populations to determine whether they are true clonal expansion or merely share the same common Vβ chain. We could then perform more rigorous Vβ purification, depletion, and adoptive transfer studies to determine if other Vβ+ TcR T cells were either protective, detrimental, or anergic. Eventually, we could obtain blood samples from Mtb infected individuals and screen for the presence of clonally expanded CD8+ T cell populations. Clonal expansions are common in the elderly, in childhood TB and in many viral models, yet their importance in Mtb infection is still unclear. Drawing comparisons between Mtb-susceptible human and murine population based on preferential expression of clonally expanded CD8+ T cells would be a quick and convenient means to screen for Mtb-susceptibility.
In this study, we have demonstrated that a key component of CBA/J *Mtb*-susceptibility is CD8$^+$ T cells. Although they are ordinarily considered protective against *Mtb*, we have shown that they are dysfunctional, secrete IL-10 at late stages of *Mtb* infection, and do not follow an organized pattern of granuloma arrangement. Although total or V$\beta$-specific CD8$^+$ T cell depletion did not significantly enhance immune responses to *Mtb*, a noticeable trend towards increased protection was observed; which is in contrast to the severe *Mtb*-susceptibility after CD4$^+$ T cell depletion. During each depletion experiment, the course of treatment was only from day 90-120. Extending the length of treatment is not feasible, since mice begin to produce antibodies against the depletion antibody, but if the onset of treatment was varied we may see significant changes in *Mtb* immunity. CD8$^+$ T cells in CBA/J mice may be more detrimental at earlier timepoints of infection, although their maximum capability to secrete IL-10 suggested late stages of infection, and always examining the response at day 120 may be missing the most critical time of their *in vivo* relevance. Overall, we have demonstrated that rather than contributing to protective T$_{H1}$ responses, CD8$^+$ T cells from CBA/J mice have a dysfunctional and possibly detrimental effect on the immune response to *Mtb*.

This is of importance because of new vaccine studies that seek to enhance the CD8$^+$ T cell response in TB patients. Boosting the numbers and activity of CD8$^+$ T cells may be beneficial for the fraction of human patients that react similarly to C57BL/6 mice, but if susceptible individuals have this altered CD8$^+$ T cell phenotype, CD8$^+$ T cell vaccination would have the complete opposite effect. It is for this reason that the dynamics of the CD8$^+$ T cell response need to be fully examined in patients with active TB. Merely
showing low CD8$^+$ T cells numbers and functional activity is not enough, we must verify that their CD8$^+$ T cells do not match our CBA/J phenotype. Upon further clarification of our findings, it may be possible to screen these patients for the presence of clonally expanded CD8$^+$ T cells as a marker of their dysfunction, thereby preventing the unintentional exacerbation of TB disease.
Chapter 4: Killer cell lectin-like receptor G1 negatively regulates protective immunity to *Mycobacterium tuberculosis*.

Abstract:

In this study, we analyzed the expression of killer cell lectin-like receptor G1 (KLRG1) on T cells between relatively resistant (C57BL/6) and susceptible (CBA/J) mice after *Mycobacterium tuberculosis (Mtb)* infection. We demonstrated that KLRG1 is associated with submaximal T cell responses in C57BL/6 mice during chronic *Mtb* infection, and that genetic removal of KLRG1 leads to significantly increased protection against *Mtb*. KLRG1−/− C57BL/6 mice were capable of maintaining a significantly lower level of *Mtb* in their lungs than wild-type C57BL/6 mice. This was primarily associated with increased numbers of CD4+ T cells expressing the activation marker CD69 and capable of secreting gamma interferon (IFN-γ) and tumor necrosis factor (TNF). Furthermore, KLRG1−/− T cells were capable of significantly more cytotoxic release at day 90 post-infection, which appeared to be a critical time for either control or regrowth of *Mtb*. As anticipated, this enhanced immunity to *Mtb* led to significantly extended survival after *Mtb* infection in KLRG1−/− mice. Interestingly, significant differences in *Mtb* burden and T cell responses were only apparent after day 90 of *Mtb* infection. This suggests that KLRG1 does not
impact the early course of disease in C57BL/6 mice but is involved in the maintenance and regulation of T cell responses during chronic Mtb infection. Also, we did not see significant increases in proliferative capability of KLRG1−/− T cells, indicating that protection against Mtb was not mediated by renewed proliferative capacity, but by an enhancement of protective cytokine production and cytotoxic ability. In this study, we have demonstrated that removal of KLRG1 significantly boosted immunity to Mtb in already resistant C57BL/6 mice.

Introduction:

The dynamics of T cell responses during chronic Mycobacterium tuberculosis (Mtb) infection are not completely understood. CD4+ and CD8+ T cells must work in conjunction for optimal control of Mtb to be established. Viral models have extensively demonstrated that chronic antigenic stimulation can lead to the decline of functional and proliferative responses from both CD4+ and CD8+ T cells. In some ways, the chronic nature of TB can resemble a chronic viral infection, with persistent levels of an intracellular pathogen in constant contact with the immune system. This chronic exposure to antigen can eventually lead to T cell dysfunction, or exhaustion characterized by T cells being functionally and/or proliferatively nonresponsive. Since control of Mtb infection relies so heavily on protective T cell responses, T cell exhaustion could exacerbate TB disease.
Another property of T cell exhaustion occurs when T cell populations become terminally differentiated, or senescent, and are no longer able to proliferate. Studies have demonstrated that T cell senescence can be identified by the expression of certain phenotypic markers on T cell subsets. Killer cell lectin-like receptor G1 (KLRG1), programmed death-1 (PD-1), and T-cell immunoglobulin domain and mucin domain 3 (Tim3) all represent cell-intrinsic negative regulatory pathways that mark T cell subsets as functionally or proliferatively exhausted. Exhaustion usually results from either cell intrinsic signals, such as expression of inhibitory molecules like PD-1, or extrinsic signals from immunosuppressive cytokines. This negative regulation develops over the course of chronic infection and eventually serves to create a new differentiated state of T cells; where normal pro-inflammatory signals no longer trigger proliferation or effector function. One of the first effector functions to be lost after exhausted is interleukin (IL)-2 secretion, followed by TNF, however IFN-γ secretion appears to be more resilient. Although T cell exhaustion was first described in CD8+ T cells, several studies indicate that CD4+ T cells can also become dysfunctional. Whether their dysfunction arises from the same molecular differences is not known. The majority of this work used chronic viral models of lymphocytic choriomeningitis virus (LCMV), human immunodeficiency virus (HIV), hepatitis C virus (HCV), or hepatitis B virus (HBV).

Little is known about the in vivo function of KLRG1, especially in Mtb infection. KLRG1 is used as a marker of terminally differentiated T cells in the mouse model, while CD57
is used in humans. Unlike most natural killer (NK) inhibitory receptors which bind classical major histocompatibility complexes (MHC), KLRG1 binds to members of the cadherin family (E-, N-, R-cadherin). In vitro studies have suggested that when KLRG1 on the surface of a T cell binds e-cadherin, this inhibits antigen-induced proliferation via the T cell receptor (TcR). Another study demonstrated that antibody-mediated activation KLRG1 in vitro suppressed the cytolytic function and IFN-γ production of NK cells. The structure of KLRG1 has been solved, and contains an immunoreceptor tyrosine-based inhibition motif (ITIM) that when phosphorylated recruits Src homology 2 containing inositol phosphatase-1 (SHIP-1) and a protein tyrosine phosphatase SHP-2 which negatively regulate PI3K and lead to suboptimal TcR signaling. Recent work suggests that KLRG1+ cells have defective Akt phosphorylation after TcR activation, explaining the proliferative dysfunction of terminally differentiated cells. KLRG1 is postulated to mark T cells that have reached a state of terminal differentiation, being able to secrete protective cytokines but unable to proliferate. This phenotype has been confirmed in Mtb infection using adoptive transfer of either KLRG1+ or PD-1+ T cells and examining their in vivo fate. PD-1+ cells were capable of differentiating into KLRG1+ cytokine-producing cells, but KLRG1+ cells were unable to differentiate into proliferating PD-1+ cells. Other studies have identified a protective role for KLRG1+ CD8+ T cells in γ-herpesvirus infection. From these studies it is clear that KLRG1 plays a significant role in infection, but its affect can vary and appears to be infection-specific.
In this study, we demonstrated that KLRG1<sup>−/−</sup> C57BL/6 mice had a significant survival advantage after infection with <i>Mtb</i>. This was associated with reduced <i>Mtb</i> burden after day 60 of infection, and increased pulmonary CD4<sup>+</sup> T cell production of IFN-γ and TNF. Although viral models suggest CD8<sup>+</sup> T cells are most influenced by KLRG1, we show that increased protection against <i>Mtb</i> in KLRG1<sup>−/−</sup> C57BL/6 mice appears to be mediated by CD4<sup>+</sup> T cells. Until this point, researchers have only speculated at the <i>in vivo</i> importance of KLRG1. It is usually identified only as a marker of senescent or exhausted T cell populations in viral models, and any putative functions that have been hypothesized are based solely on <i>in vitro</i> data. We have demonstrated that removal of KLRG1 from C57BL/6 mice increases their resistance to and survival after <i>Mtb</i> infection.
Materials and Methods:

Mice

Specific pathogen-free, age/sex-matched CBA/J wild-type (NCI), C57BL/6 wild-type (Jackson) and C57BL/6 KLRG1\(^{-/-}\) mice were maintained in ventilated cages inside a biosafety level 3 (BSL3) facility and provided with sterile food and water \textit{ad libitum}. KLRG1\(^{-/-}\) animals were kindly provided by Dr. Hanspeter Pircher, Freiburg University, Germany. All protocols were approved by The Ohio State University’s Institutional Laboratory Animal Care and Use Committee.

\textit{Mtb} infection and Colony Forming Unit Enumeration

\textit{Mtb} Erdman (ATCC no. 35801) was obtained from American Type Culture Collection. Stocks were grown according to published methods. Mice were infected with \textit{Mtb} Erdman using an inhalation exposure system (Glas-Col) calibrated to deliver 50–100 CFU (for low-dose aerosol) or 500 CFU (high-dose aerosol) to the lungs of each mouse, as previously described\(^{315}\).

At specific time points post \textit{Mtb} infection mice were sacrificed and lungs and spleens were aseptically removed into sterile saline. Organs were homogenized and serial dilutions plated onto 7H11 agar supplemented with OADC as previously described\(^{316}\). Plates were incubated at 37°C for 21 days in order to enumerate bacterial colonies and calculate the bacterial burden.
Survival Studies

Groups of wild-type and KLRG1\(^{-/-}\) C57BL/6 mice were infected with \(Mtb\) Erdman via aerosol, as previously described, and maintained in a BSL3 facility. Mice were observed daily and euthanized when moribund. Date of euthanasia was recorded. Low-dose infection was performed twice (\(n= 25\) wild-type and 25 KLRG1\(^{-/-}\), or 30 wild-type and 25 KLRG1\(^{+-}\)).

Lung cell isolation

Mice were euthanized by CO\(_2\) asphyxiation and lungs perfused with cold phosphate buffered saline containing 50Units/mL of heparin through the right ventricle. Lungs from individual mice were mechanically disrupted using a GentleMACS dissociator (Miltenyi Biotec) followed by collagenase A (type XI) (0.7 mg/mL, Sigma) and type IV bovine pancreatic DNAse (30\(\mu\)g/mL, Sigma) digestion at 37\(^\circ\)C for 30 minutes in GentleMACS C-tubes. Lung cell suspensions were passed through a 70 \(\mu\)m nylon cell screen and residual erythrocytes were lysed with Gey’s solution. Viable cells were determined by trypan blue exclusion.

KLRG1 blockade

Wild-type C57BL/6 mice were administered 250\(\mu\)g anti-KLRG1 blocking antibody or Rat IgG isotype control every other day for 20 days by intraperitoneal injection. On day 21 after treatment onset, mice were sacrificed and lungs harvested for CFU determination. This study was conducted at three separate times post-infection: day 20-
40, day 60-80, day 130-150. The results of each study representative of one experiment with 5 mice per group.

**Cell Purification**

Single lung cell suspensions were adhered to sterile tissue culture dishes for 1hr at 37°C. Non-adherent cells were washed and removed from the plates. CD4+ and CD8+ T cells were obtained from the non-adherent cell fraction by magnetic cell separation (BD IMAG anti-CD4 particles 551539, anti-CD8 particles 551516) and either placed directly into TRIzol reagent, homogenized, and frozen at -80°C or used for culture as described below. Purity of CD4+ and CD8+ T cell populations was determined to be greater than 90% for all experiments by flow cytometry using an LSRII flow cytometer (BD).

**Cytokines Assays**

5x10^5 purified CD4+ (>90% purity) or CD4+ pulmonary T cells were cultured with *Mtb* culture-filtrate protein (CFP) for 48hrs at +37°C. After incubation, plates were frozen at -80°C until all timepoints were completed. IFN-γ ELISA antibodies and standards obtained from BD Biosciences and processed as previously described. Colormetric reactions were read on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). The level of TNF in cell supernatants was determined using a mouse TNF ELISA Ready-SET-Go! kit from Ebioscience (San Diego, CA) according to manufacturer's instructions.
Flow cytometry

Isolated lung cells were suspended in deficient RPMI (Irvine Scientific) supplemented with 0.1% sodium azide (Sigma-Aldrich). Surface targets were detected as previously described. Specific Abs and isotype controls were purchased from BD Biosciences: PerCP-Cy5.5 anti-CD3ε (145-2C11), APC-Cy7 anti-CD4 (GK1.5), PE-Cy7 anti-CD8 (53-6.7), PerCP-Cy5.5 anti-CD8 (53-6.7), PE-Cy7 anti-IFN-γ (XMG1.2), PE anti-TNF (MP6-XT22), PE anti-CD69 (H1.2F3). PE or APC anti-KLRG1(MAFA), PE anti-Tim3 (RMT3-23), PE-Cy7 anti-PD1 (J43) antibodies purchased from eBiosciences. Cytokine levels were determined according to the manufacturer’s instructions for intracellular cytokine staining (Cytofix/Cytoperm fixation/permeabilization solution kit with BD GolgiStop, BD Biosciences), following a 4hr incubation with 10µg/mL anti-CD3 (145-2C11) and 1µg/mL anti-CD28 (37.51). Mice were injected with BrdU 24 hours prior to necropsy, and intracellular BrdU was quantified using BD FITC BrdU kit (BD 559619) as described. Samples were read using an LSRII flow cytometer and analyzed with FACSDiva software (BD Biosciences).

Histology

Caudal lung lobes were taken from infected wild-type and KLRG1-/- C57BL/6 mice at various timepoints post-infection, inflated and stored in formalin as previously described. Tissue sections were prepared and stained with hemotoxylin and eosin, or Ziehl-Neelsen stains, and were assessed by a board certified veterinary pathologist with no prior knowledge of experimental groups.
Statistics

Statistical analysis performed using GraphPad Prism software for the Students $t$ test per individual time point of each graph. Any comparisons between timepoints of the same experiment utilize a two-way analysis of variance test with Bonferonni post-tests for multiple comparisons. Survival significance was determined by LogRank Mantel-Cox test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Results:

C57BL/6 mice have increased numbers and proportions of KLRG1+ T cells compared to CBA/J mice after Mtb infection.

The majority of the seminal work examining Mtb pathogenesis and immunity used C57BL/6 mice as the model system \(^{166, 167, 171, 185}\). Previous studies have classified C57BL/6 mice as relatively resistant to TB, based on survival after intravenous or aerogenic infection with Mtb \(^{286, 288, 289}\). The inbred mouse strains used in the study fell into two main groups: those that survived past approximately day 300 of intravenous infection, and those that succumbed to infection around day 150. The mouse strains in these groups were predominantly differentiated by their status as natural resistance-associated macrophage protein (NRAMP) positive or negative, although manipulations of NRAMP in mice have not significantly affected Mtb susceptibility in 129/sv, DBA/2, or BALB/c mice \(^{287, 425}\). CBA/J mice were deemed relatively susceptible to Mtb in the aforementioned study, and mount a significantly weaker pro-inflammatory response to Mtb than relatively resistant C57BL/6 mice. As a result, CBA/J mice have significantly higher pulmonary and systemic levels of Mtb colony-forming units (CFU) throughout Mtb infection than C57BL/6 mice.

CBA/J mice accumulate CD8+ T cells in their lungs as Mtb infection progresses (Chapter 3). Since KLRG1 is important for controlling proliferative responses in the presence of chronic antigen stimulations, we hypothesized that CD8+ T cells from CBA/J mice would have low levels of KLRG1. Therefore, we infected wild-type C57BL/6 and CBA/J mice
with an aerosolized dose of *Mtb* and analyzed their T cell repertoire for expression of
KLRG1 by flow cytometry. CBA/J mice had significantly fewer numbers of CD4⁺ (Fig
4.1a) and CD8⁺ (Fig 4.1c) T cells expressing KLRG1 throughout *Mtb* infection compared
to C57BL/6 mice. To address the fact the C57BL/6 mice generally have higher numbers
of T cells than CBA/J mice during *Mtb* infection, we also examined the proportions of T
cells expressing KLRG1 and saw the same highly significant phenotype (Fig 4.1b, d).
Since CBA/J are relatively susceptible to *Mtb* infection, we hypothesized that the
expression of KLRG1 on T cells may be necessary for optimal T_H1 function.
Figure 4.1  KLRG1$^+$ T cells in Mtb-infected C57BL/6 and CBA/J mice.
CBA/J and C57BL/6 mice were infected with a low dose aerosol of Mtb. At various timepoints post-infection, lungs were obtained and processed for analysis. Cells were analyzed directly ex vivo without stimulation by flow cytometry. Lymphocytes were gated upon, then subgated onto CD3$^+$CD8$^+$ T cells or CD3$^+$CD4$^+$ T cells. Absolute number (a) or proportion (b) of CD3$^+$CD8$^+$ T cells expressing KLRG1. Absolute number (c) or proportion (d) of CD3$^+$CD4$^+$ T cells expressing KLRG1. Data representative of two independent experiments with 4 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s $t$ test.
Higher numbers and proportions of KLRG1\textsuperscript{neg} CD4\textsuperscript{+} T cells express CD69 in C57BL/6 mice at day 90 of \textit{Mtb} infection.

To characterize the phenotype of KLRG1 positive or negative T cells, we infected C57BL/6 and CBA/J mice with an aerosolized dose of \textit{Mtb} and measured the expression of the early activation marker CD69 on KLRG1\textsuperscript{+} or KLRG1\textsuperscript{neg} T cells by flow cytometry. Interestingly, in C57BL/6 mice both the proportions and absolute number of CD4\textsuperscript{+} T cells expressing CD69 was significantly increased in the KLRG1\textsuperscript{neg} population at day 90 of \textit{Mtb} infection (Fig 4.2a). Proportions of KLRG1\textsuperscript{+} CD4\textsuperscript{+} T cells expressing CD69 were increased from day 120-150 of infection (Fig 4.2a, right), which we hypothesize is a consequence of increasing \textit{Mtb} burden at late stages of infection. We observed no significant change in the amount of CD8\textsuperscript{+} T cells expressing CD69 in either the KLRG1\textsuperscript{+} or KLRG1\textsuperscript{neg} populations (Fig 4.2b).

In \textit{Mtb}-susceptible CBA/J mice, we also observed a small increase in the proportions of CD4\textsuperscript{+} T cells expressing CD69 in KLRG1\textsuperscript{neg} population at day 60 and day 120 of infection, but this was not reproduced in absolute numbers (Fig 4.2c). Furthermore, in CBA/J mice we did not observe any change in CD8\textsuperscript{+} T cells expressing CD69 in KLRG1\textsuperscript{+} or KLRG1\textsuperscript{neg} populations (Fig 4.2d). From these data, it is clear that only CD4\textsuperscript{+} T cell activation is affected by the presence of KLRG1 in our model. Although this only describes T cell activation, it is an indication that our previous hypothesis was incorrect. Here, we show that in C57BL/6 mice KLRG1\textsuperscript{neg} CD4\textsuperscript{+} T cell subsets are more activated.
than KLRG1⁺ subsets, and that although few CBA/J T cells express KLRG1, their activation was not equally enhanced. It is possible that any potential increase in T cell activation in CBA/J mice is masked by the presence of high levels of IL-10, as previously shown. We therefore hypothesized that in C57BL/6 mice, KLRG1neg CD4⁺ T cell subsets are more protective against *Mtb* infection, and that removal of KLRG1 will boost Th1 immunity.
Figure 4.2 KLRG1$^+$ or KLRG1$^{neg}$ expression of CD69 on T cells in *Mtb*-infected C57BL/6 and CBA/J mice.

CBA/J and C57BL/6 mice were infected with a low dose aerosol of *Mtb*. At various timepoints post-infection, lungs were obtained and processed for analysis. Cells were analyzed directly *ex vivo* without stimulation by flow cytometry. Lymphocytes were gated upon, then subgated onto CD3$^+$CD4$^+$ T cells or CD3$^+$CD8$^+$ T cells. Cells were further subgated into KLRG1$^+$ or KLRG1$^{neg}$ populations. (a) Absolute number or proportion of C57BL/6 KLRG1$^{+/neg}$ CD3$^+$CD8$^+$ T cells expressing CD69. (b) Absolute number or proportion of C57BL/6 KLRG1$^{+/neg}$ CD3$^+$CD4$^+$ T cells expressing CD69. (c) Absolute number or proportion of CBA/J KLRG1$^{+/neg}$ CD3$^+$CD8$^+$ T cells expressing CD69. (d) Absolute number or proportion of CBA/J KLRG1$^{+/neg}$ CD3$^+$CD4$^+$ T cells expressing CD69. Data representative of two independent experiments with 4 mice per group per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s t test.
Figure 4.2

(a) Graph showing the number of CD8^+ T-cells over days post-infection for different experimental groups.

(b) Graph showing the percentage of CD8^+ T-cells over days post-infection for different experimental groups.

(c) Graph showing the number of CD8^+ T-cells over days post-infection for different experimental groups.

(d) Graph showing the percentage of CD8^+ T-cells over days post-infection for different experimental groups.
KLRG1 deficiency leads to increased protection against *Mtb*.

To test our hypothesis that removal of KLRG1 from relatively resistant C57BL/6 mice would significantly enhance immune function, we treated C57BL/6 and CBA/J mice with antibodies against KLRG1. We treated *Mtb*-infected C57BL/6 mice and CBA/J mice (not shown) with anti-KLRG1 antibody by intraperitoneal injection every other day for 20 days at three times post-infection: one group from day 20-40, the next from day 60-80, and the final group from day 130-150 post-infection. CFU was determined at day 40, 80, and 150 post-infection. None of the treatment courses led to a significant change in *Mtb* CFU (Fig 4.3a). The efficacy and mechanism of action of our anti-KLRG1 antibody was not fully established, and therefore impeded our ability to make definitive statements based on these results.

To reliably determine the importance of KLRG1 to *Mtb* infection, we obtained C57BL/6 mice genetically deficient in KLRG1 (KLRG1\(^{-/-}\)) and infected them with a low-dose aerosol of *Mtb*. Following normal *Mtb* growth kinetics during the first 60 days of infection, after day 90 KLRG1\(^{-/-}\) mice maintained a level of pulmonary *Mtb* burden of approximately 5 log (Fig 4.3b) until the last timepoint measured (day 150). Wild-type C57BL/6 mice follow a similar trend until day 90, when levels of pulmonary *Mtb* continue to increase to over 1 log above KLRG1\(^{-/-}\) mice until day 150 (Fig 4.2b). Splenic changes in CFU between wild-type and KLRG1\(^{-/-}\) mice were much more moderate and wild-type C57BL/6 CFU were only significantly higher at day 150 post-infection (Fig 4.2c). KLRG1 is also expressed on natural killer (NK) cells, which have been shown to
contribute to the initial innate immune response to *Mtb* \(^{131-133}\). To examine the effect of KLRG1 removal on NK cells, we analyzed pulmonary *Mtb* CFU at very early times post-infection and saw no significant differences between C57BL/6 wild-type or KLRG1\(^{-/-}\) mice (Fig 4.2d). In addition to lower *Mtb* burden, KLRG1\(^{-/-}\) mice had significantly extended survival after *Mtb* infection compared to wild-type C57BL/6 mice (Fig 4.2e), whose survival was within the normal range for C57BL/6 mice after *Mtb* infection \(^{286}\). The normal lifespan of a C57BL/6 mouse ranges from 600-800 days under normal circumstances \(^{426-428}\). At day 365 of infection a subset of KLRG1\(^{-/-}\) mice from the survival study were euthanized and *Mtb* burden was found to have increased to only 6 log (Fig 4.3f). This does demonstrate TB disease progression, and reflects why KLRG1\(^{-/-}\) mice eventually succumb to infection. Wild-type mice were nearly all deceased at day 365 and could not be euthanized without affecting the survival curve. These data confirm our original hypothesis that the lack of KLRG1 on pulmonary T cells will boost protective immunity to *Mtb*.
Figure 4.3  *Mtb* burden and survival of wild-type or KLRG1−/− C57BL/6 mice

C57BL/6 wild-type and KLRG1−/− mice were infected with a low dose aerosol of *Mtb*. Wild-type C57BL/6 mice were administered 250µg anti-KLRG1 or Rat IgG isotype control antibody via intraperitoneal injection every other day for 20 days for three separate treatment courses (day 20-40, day 60-80, day 130-150). At day 40, 80, 150 post-infection treated mice were sacrificed and lungs obtained for CFU determination on 7H11 plates (a). Data representative of one experiment with 5 mice per group per treatment course. *Mtb*-infected wild-type and KLRG1−/− C57BL/6 mice were sacrificed at various timepoints post-infection and lungs (b,d,f) and spleens (c) removed for CFU enumeration on 7H11 plates. Survival mice were maintained inside BSL3 conditions, monitored daily, and euthanized when moribund (n=25 wild-type, 25 KLRG1−/−) (e). Data representative of two independent experiments. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s *t* test. Survival significance was determined by LogRank Mantel-Cox test.
Figure 4.3

(a) Log₁₀ Mt. Lung CFU vs. Days Post-Infection for wildtype and anti-KLRG1.

(b) Log₁₀ Mt. Lung CFU vs. Days Post-Infection for C57BL/6 and KLRG1⁻⁻⁻.

(c) Log₁₀ Mt. Spleen CFU vs. Days Post-Infection for C57BL/6 and KLRG1⁻⁻⁻.

(d) Log₁₀ Mt. Lung CFU vs. Days Post-Infection for C57BL/6 and KLRG1⁻⁻⁻.

(e) % Survival vs. Days Post-Infection for C57BL/6 and KLRG1⁻⁻⁻.

(f) Log₁₀ Mt. Lung CFU vs. Days Post-Infection for C57BL/6 (no survivors) and KLRG1⁻⁻⁻.
KLRG1−/− mice have significantly enhanced CD4+ T cell responses to Mtb

Although studies using viral models suggest KLRG1 most affects CD8+ T cells, the numbers of CD69+KLRG1−/− CD8+ T cells from wild-type C57BL/6 mice were not significantly different than KLRG1+CD8+ T cells. Since KLRG1−/− mice were capable of maintaining control of Mtb infection better than wild-type C57BL/6, we hypothesized that, as with KLRG1−/− wild-type CD4+ T cells, KLRG1−/− CD4+ T cells would be highly activated. After low-dose aerosol with Mtb, lungs were harvested and pulmonary cells analyzed by flow cytometry. KLRG1−/− mice maintained a high level of total CD4+ T cells (Fig 4.4a) and CD69+ CD4+ T cells (Fig 4.4c) compared to wild-type mice from days 90-120 of infection. Similar to our findings with KLRG1+ and KLRG1− subset, CD8+ T cell numbers and activation were not altered in KLRG1−/− mice (Fig 4.4b, d). Since more activated KLRG1−/− CD4+ T cells were present in the lung at days 90-120, but the level of Mtb was unchanged, we hypothesized that KLRG1−/− CD4+ T cells were also capable of secreting IFN-γ. Indeed, we observed more KLRG1−/− CD4+ T cells were capable of continually high IFN-γ production upon ex vivo T cell receptor stimulation (Fig 4.4e) also from day 90-120 post-infection. CD8+ T cell IFN-γ expression was not significantly enhanced in KLRG1−/− mice (Fig 4.4f). We observed no significant difference in immune responses before day 90 post-infection. This indicates that there is no appreciable change in early innate immune function, although the absolute numbers and proportions of NK1.1+ cells (NK cells) were moderately increased at day 90 post-infection in KLRG1−/− mice (Fig 4.4g,h).
Of particular note is the observation that protective CD4+ T cell responses were not significantly increased in KLRG1−/− mice from day 60-90, but rather the CD4+ T cell responses of C57BL/6 mice were significantly decreased (Fig 4.4a,c,e). This same phenomenon was not observed in CD8+ T cells (Fig 4.4b,d,f). Since KLRG1 has been shown to inhibit antigen-induced proliferation, we hypothesized that KLRG1−/− CD4+ T cells were capable of more proliferation at day 90 compared to wild-type C57BL/6 mice, and that this would explain why C57BL/6 CD4+ T cell numbers drop significantly at this time.
Figure 4.4  Pulmonary T cell composition and protective phenotype of KLRG1^−/− mice

Wild-type and KLRG1^−/− C57BL/6 mice infected with an aerosolized dose of *Mtb*. At various timepoints post-infection groups of mice were sacrificed and lungs removed for processing by flow cytometry. Absolute numbers of CD4^+^ T cells (a), CD8^+^ T cells (b), CD69^+^CD4^+^ T cells (c), CD69^+^CD8^+^ T cells (d). Absolute numbers of IFN-γ^+^CD4^+^ T cells (e) or IFN-γ^+^CD8^+^ T cells (f) after 4hr incubation with anti-CD3, anti-CD28, and GolgiSTOP export blocker. Absolute numbers (g) or percentages (h) of NK1.1^+^ cells as determined by flow cytometry. Data representative of two independent experiments with 5 mice per group, per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s t test.
KLRG1−/− T cells do not have increased proliferation but have enhanced cytotoxic capability

Since C57BL/6 mice had a significant decrease in CD4+ T cell numbers compared to KLRG1−/− mice at day 90 after Mtb infection, we hypothesized that proliferation would be altered in KLRG1−/− mice. However, we observed no significant difference in BrdU staining in CD4+ T cells (Fig 4.5a) or CD8+ T cells (Fig 4.5b) from KLRG1−/− or wild-type C57BL/6 mice during this important time after Mtb infection. Since proliferation was unaltered in KLRG1−/− mice, it is possible that KLRG1−/− T cells would have better cytotoxic function, preventing the increase in Mtb burden observed in wild-type mice at day 90. As a measure of the cytotoxic ability and degranulation of T cells, we analyzed the percent loss of perforin or granzyme B from the intracellular compartment of KLRG1−/− or wild-type C57BL/6 mice. KLRG1−/− CD4+ T cells (Fig 4.5c), but predominantly CD8+ T cells (Fig 4.5d) had significantly enhanced release of perforin at only day 90 post-infection. Additionally, CD8+ T cells but not CD4+ T cells from KLRG1−/− mice had significantly increased release of granzyme B at day 90 post-infection (Fig 4.5e,f). Together, these data demonstrate that it is an enhancement of cytotoxic ability at day 90 that prevents KLRG1−/− CD4+ T cell activity from decreasing.
Figure 4.5  Proliferation and cytotoxic ability of pulmonary T cells from wild-type or KLRG1−/− mice
Wild-type and KLRG1−/− C57BL/6 mice were infected with an aerosolized dose of Mtb. At various timepoints post-infection lungs were removed and processed for flow cytometry. 24hrs prior to necropsy, mice were injected with BrdU. Absolute number of BrdU+CD4+ (a) or CD8+ (b) T cells as determined by flow cytometry. Data representative of one experiment with 5 mice per group, per timepoint. Percent loss of perforin in CD4+ (c) or CD8+ (d) T cells, or GZB in CD4+ (e) or CD8+ (f) T cells. Degranulation represented as percent loss of perforin and granzyme B (GZB) determined by subtracted remaining perforin/GZB expression from 100. Data representative of two independent experiments with 5 mice per group, per timepoint. * p<0.05, ** p<0.01, *** p<0.001 as obtained by Student’s t test.
Figure 4.5
KLRG1<sup>−/−</sup> CD4<sup>+</sup> T cells are capable of more <i>Mtb</i>-specific IFN-γ and TNF production

Day 90 after <i>Mtb</i> infection appears to be a critical time for either control or regrowth of <i>Mtb</i>, and during this time, lung granulomas are attempting to contain the infection. Our previous data demonstrated that more KLRG1<sup>−/−</sup> CD4<sup>+</sup> T cells were activated and capable of secreting IFN-γ after ex vivo TcR stimulation, but we have not shown that this enhanced CD4<sup>+</sup> T cell response is <i>Mtb</i>-specific. Since CD4<sup>+</sup> T cells are critical for protective granuloma formation and maintenance, we also analyzed the production of TNF by pulmonary T cells in response to <i>Mtb</i>. TNF, along with IFN-γ, has been shown to be required for granuloma formation.

Therefore, we examined the ability of KLRG1<sup>−/−</sup> CD4<sup>+</sup> or CD4<sup>neg</sup> T cells to produce IFN-γ and TNF in response to ex vivo culture with <i>Mtb</i> antigens. Purified pulmonary CD4<sup>+</sup> or CD4<sup>+</sup> T cells were cultured with antigen-presenting cells pulsed with <i>Mtb</i> culture filtrate protein (CFP) for 48hrs. KLRG1<sup>−/−</sup> CD4<sup>+</sup> T cells produced significantly more <i>Mtb</i>-specific IFN-γ (<strong>Fig 4.6a</strong>) and TNF (<strong>Fig 4.6c</strong>) than wild-type cells from day 90-150 of infection. Interestingly, CD4<sup>+</sup> T cells from both groups of mice were capable of very similar IFN-γ and TNF production from day 30-60 of infection, after which KLRG1<sup>−/−</sup> CD4<sup>+</sup> T cells cytokine production greatly increases (<strong>Fig 4.6a,c</strong>). Importantly, <i>Mtb</i>-specific IFN-γ production from wild-type C57BL/6 CD4<sup>+</sup> T cells remained relatively stable from day 30-150, demonstrating that CD4<sup>+</sup> T cells at day 90 were equally as protective, just reduced in number (<strong>Fig 4.6a</strong>). Since these results are normalized to cell number it suggests that, together with our previous intracellular staining (<strong>Fig 4.4e</strong>),
KLRG1\(^{-/}\) mice have high numbers of CD4\(^{+}\) T cells that can secrete more IFN-\(\gamma\) at day 90 post-infection.

Since *Mtb* antigen was added exogenously to the culture, presentation to CD8\(^{+}\) T cells will be limited. Despite this, KLRG1\(^{-/}\) CD4\(^{-}\) T cells produced significantly more IFN-\(\gamma\) (*Fig 4.6b*) than wild-type mice at day 30, 120, and 150 post-infection. CD4\(^{-}\) T cells from KLRG1\(^{-/}\) mice were only capable of more TNF production at day 150 post-infection (*Fig 4.6d*), suggesting a small, but significant enhancement in cytokine-mediated protection by CD8\(^{+}\) T cells. These results present a duplicitous role for KLRG1 on T cells during *Mtb* infection. Removal of KLRG1 does not renew the proliferative capability of C57BL/6 CD4\(^{+}\) T cells, but fundamentally enhances their ability to secrete pro-inflammatory cytokines and resist deletion at day 90 post-infection. Although not directly tested, we hypothesize that the ability of KLRG1\(^{-/}\) mice to maintain their level of CD4\(^{+}\) T cells comes from a burst of cytotoxic activity from pulmonary CD8\(^{+}\) T cells, subsequently leading to enhanced cytokine production and sustained control of *Mtb* infection. Together, we hypothesize that these enhanced T cell responses help maintain containment of *Mtb*. 
Figure 4.6  Levels of *Mtb*-specific IFN-γ and TNF in wild-type and KLRG1<sup>−/−</sup> C57BL/6 mice.

Wild-type and KLRG1<sup>−/−</sup> C57BL/6 mice were infected with *Mtb* via aerosol and at various timepoints post-infection lungs were removed and processed into a single cell suspension. Using magnetic beads, CD4<sup>+</sup> T cells were purified and cultured with *Mtb* CFP for 48hr. The production of IFN-γ (a,b) or TNF (c,d) from CD4<sup>+</sup> or CD4<sup>neg</sup> T cells was analyzed by ELISA.
**KLRG1^- T cells have no enhanced ability to recognize *Mtb* antigens**

Since the removal of KLRG1 leads to significant enhancement of protective immunity to *Mtb*, we (and others) have hypothesized that KLRG1^- T cells may be better at responding to smaller quantities of peptide antigen than wild-type mice. We therefore cultured purified CD4^+ T cells with bone marrow-derived dendritic cells in the presence of a serial dilution of *Mtb* Ag85 peptide for 48 hours. We observed no difference in the capacity of KLRG1^- T cells to produce IFN-γ in response to differing concentrations of *Mtb* Ag85 peptide (**Fig 4.7a**). Additionally, we observed no difference when culturing with whole *Mtb* CFP antigen, or various concentrations of TcR stimulus (**Fig 4.7b**). Intracellular flow cytometry for IFN-γ, IL-2, or TNF showed little to no cytokine production (not shown).

It was next thought that whole lung lymphocytes (CD4^+ T cells, CD8^+ T cells) with B cells as endogenous antigen-presenting cells would better address the question of peptide recognition. This also did not yield any difference in IFN-γ production after stimulation with a battery of peptides and controls (**Fig 4.7c,d**). From this, we were unable to demonstrate any enhancement of KLRG1^- T cells to recognize lower concentrations of *Mtb* peptides, yet more testing needs to be done to determine if this contributes to the mechanism of KLRG1-mediated immune suppression.
Wild-type and KLRG1−/− C57BL/6 mice infected with an aerosolized dose of *Mtb*, and at day 180 and day 200 lungs were removed and processed into a single cell suspension. Using magnetic beads, CD4+ T cells were purified and cultured with wild-type BMDCs in the presence of a serial dilution of *Mtb* Ag85 peptide (a), or controls (b) (tissue culture media, whole *Mtb* CFP antigen, anti-CD3/CD28) for 48hr and IFN-γ production was measured by ELISA. Data representative of one experiment with 5 mice per group. Whole lung cells were adhered to plates and non-adherent lymphocytes were stimulated as above. Amount of IFN-γ produced after stimulation with Ag85 peptide (c) or control (d) was determined by ELISA. Data representative of one experiment with 5 mice per group.
KLRG1<sup>−/−</sup> mice do not have altered granuloma structure during <i>Mtb</i> infection

Protection against <i>Mtb</i> is closely linked to organized granulomatous containment. Since protective responses were enhanced in KLRG1<sup>−/−</sup> mice during <i>Mtb</i> infection, we hypothesized that pulmonary granulomas in KLRG1<sup>−/−</sup> mice would be more mature, therefore enabling the control of <i>Mtb</i> growth demonstrated in Fig 4.3. However, after histological analysis we observed no differences in the ability of either group to develop mature granulomas from day 90-220 post-infection (not shown). Both wild-type and KLRG1<sup>−/−</sup> C57BL/6 mice displayed mild, multifocal, unorganized granulomas and increased inflammation from day 90-120. Lymphocyte aggregates were seen around airway vessels with few neutrophils across timepoints and groups of mice. There was no evidence of necrosis or caseation, or a difference in the amount of epithelioid and foamy macrophages between wild-type or KLRG1<sup>−/−</sup> C57BL/6 mice. Acid-fast staining revealed normal bacilli primarily within macrophages across all timepoints in both groups of mice. From this analysis, we have concluded that KLRG1-mediated inhibition does not significantly affect granuloma structure and organization.
Discussion:

In this study, we have demonstrated that KLRG1 has \textit{in vivo} functional consequences during chronic \textit{Mtb} infection. The majority of viral models examining KLRG1 have shown that it is either dispensable for control \cite{424}, or simply marks senescent or nonreplicative T cells \cite{429-432}. We believe KLRG1 is acting in two distinct ways to suppress the full protective capability of T cells during \textit{Mtb} infection. Primarily, KLRG1\textsuperscript{−/−} mice had increased numbers of CD4\textsuperscript{+} T cells that expressed the early activation marker CD69, suggesting that more CD4\textsuperscript{+} were encountering antigen \textit{in vivo}. This led to more KLRG1\textsuperscript{−/−} CD4\textsuperscript{+} T cells being able to secrete IFN-γ after \textit{ex vivo} TcR stimulation. Furthermore, upon culture with \textit{Mtb} antigens KLRG1\textsuperscript{−/−} CD4\textsuperscript{+} T cells secreted significantly more IFN-γ and TNF from day 90-150 of \textit{Mtb} infection. Secondly, we observed that KLRG1\textsuperscript{−/−} T cells were capable of significantly enhanced cytotoxic responses at day 90 post-infection.

Day 90 appears to be critical in our model as the time when wild-type C57BL/6 mice have a sharp decrease in CD4\textsuperscript{+} T cells and an increase in \textit{Mtb} burden. We hypothesize that the cytotoxic burst, primarily from CD8\textsuperscript{+} T cells, restricts this spread of pulmonary \textit{Mtb} at day 90 in KLRG1\textsuperscript{−/−} mice. It is possible that increased cytotoxic lysis of infected cells yields an acute, local increase of \textit{Mtb} antigen that activates and recruits more CD4\textsuperscript{+} T cells to secrete more IFN-γ at day 90 in KLRG1\textsuperscript{−/−} mice. It is not clear why wild-type CD4\textsuperscript{+} T cells are decreased in C57BL/6 mice at day 90, yet it correlates with an increase in \textit{Mtb} burden in the lungs at that time. It is possible that at day 90, \textit{Mtb} exerts a robust
burst of regrowth and tissue damage. This would expose more cell junctions and increase the availability of the ligand of KLRG1, E-cadherin. In animals with intact KLRG1 signaling, this increase in KLRG1:E-cadherin interaction would restrict T cell responses and afford Mtb the ability to spread.

Since we observed no significant differences between wild-type and KLRG1\(^{-/-}\) mice before day 90, it indicates that KLRG1 is most important during late stages of chronic infection, as previously described. Our work attempting to block KLRG1 in vivo did not significantly affect Mtb CFU at any timepoint studied, however, we did not confirm that our treatment regimen led to complete in vivo blockade of KLRG1. If antibody treatment successfully ablated KLRG1 signaling, it is possible that the timepoints chosen were inappropriate. KLRG1 signaling may need to be removed for several more weeks before any alteration in Mtb CFU is observed. The majority of referenced work dealt primarily with categorizing T cell populations based on the expression of KLRG1\(^{252, 394, 403, 408, 413, 422}\), while our work is the first demonstration of an in vivo consequence of KLRG1 removal during infection.

Our work demonstrates that removal of KLRG1 allows granulomas to maintain a high level of containment and organization beyond the time when wild-type granuloma structure begins to deteriorate. We have shown a very clear phenotype of increased protection and containment of Mtb, but we have not directly identified the mechanism of action. We have attempted several studies to elucidate the underlying mechanism of KLRG1 removed but have met with inconsistent results. First, we attempted to activate
KLRG1 *in vitro* using activating antibodies. If we could activate KLRG1 on infected cells we could learn what is being inhibited in the context of *Mtb* infection. This study, however, proved ineffective and the activating antibody was soon taken off the market. We hypothesize that KLRG1 prevents maximal T cell activation (as a safety precaution), and that its removal allows for higher PI3K activity and Akt phosphorylation. We do not believe that KLRG1−/− T cells are less terminally differentiated, since we saw no significant difference in proliferative capacity. At day 90 post-infection, KLRG1−/− mice could secrete more IFN-γ and TNF which led to more recruitment of peripheral T cells, thus increasing total T cell numbers and subsequent replenishment of terminal granuloma cells. This T cell recruitment and activation, combined with the burst of cytotoxic ability at day 90, maintains granuloma integrity and containment of *Mtb*.

KLRG1 is thought to be present on the surface of NK cells, and certain antigen-experienced effector memory populations of T cells (T_{EM}). Studies have demonstrated that fewer KLRG1_{neg} T_{EM} cells secrete IFN-γ after treatment with PMA/ionomycin than KLRG1_{+} cells. As with all components of *Mtb* immunity, a single aspect of the immune response is never sufficient to confer protection. In addition to early effector responses, the memory response to *Mtb* has been studied extensively. Various types of memory cells have been investigated and deemed important during murine and human TB. Some human studies focus of T_{EM} cells which are delineated as being CD4+CD45RO+CD45RA−CD27−CD28−CCR7−, and are very fast responders to *Mtb* antigens. These cells have shown to be induced by latent *Mtb* infection,
and can secrete IFN-γ despite simultaneous expression of PD-1. Similarly, we observed significant increases in the number of KLRG1−/− CD4+ T cells expressing PD-1 (not shown). This contradicts previous work that identified PD-1+ CD4+ cells as proliferative but unable to produce protective cytokines. It may be possible that the effect of PD-1 is highly dependent on the availability of PD-1L, and that in the absence of PD-1L, T_{EM} cells can produce abundant cytokines. The memory phenotype of KLRG1−/− CD4+ T cells was not analyzed, but we hypothesize that maintained control of Mtb infection in KLRG1−/− mice would permit the development of more T_{EM} cells. CD8+ T cells also constitute populations of memory cells. Of particular note is the recent classification of T stem cell memory (T_{SCM}) cells that are comprised of antigen-experienced CD8+ T cells which are CD44^{low}CD62L^{high}CD122^{high}sca-1+. T_{SCM} cells are capable of self-renewing their population while simultaneously giving rise to both T central memory (T_{CM}) and T_{EM} cell subsets. We have attempted to classify the numbers of T_{SCM} cells in wild-type and KLRG1−/− mice but could not detect cells based on the phenotypic criteria. It is likely that removal of KLRG1 impacts both effector and memory populations of T cells, by removing any restriction for maximal T cell activation throughout Mtb infection.

A legitimate concern when removing the natural balances of the immune system is the development of destructive autoimmune responses. Uninhibited TcR stimulation may be beneficial for controlling Mtb growth and dissemination, but this has the potential to lead to tissue damage and lymphoproliferative disorders such as lymphoma and leukemia in other uninfected organs. KLRG1−/− mice have survived up to 500 days after infection with
MTB, and we have not observed hepatosplenomegaly or inflammatory bowel diseases upon gross dissection. Recent work has demonstrated that PD-1−/− mice acutely develop severe immunopathology and exacerbation of TB disease. It is likely that the inhibition provided by KLRG1 is significantly less potent than other forms of negative regulation from immunosuppressive cytokines. This would allow for increased TCR signaling in KLRG1−/− mice, but would not bypass inhibition by IL-10 or TGFβ, thus maintaining the balance of other pro- and anti-inflammatory signals in the body.

CBA/J mice have extremely low proportions of cells that express KLRG1, yet they are relatively susceptible to MTB reactivation. We did observe a small but significant increase in the proportions of activated KLRG1− CD4+ T cells from CBA/J mice, indicating that KLRG1 is functioning in the same way as in C57BL/6 mice, but this phenotype is not as influential. KLRG1 was not blocked or altered in CBA/J mice during this study, but we would hypothesize that even if it were it would not confer any increase in protective response to MTB. The reason for this is that the susceptibility of CBA/J mice is not dictated merely by KLRG1-mediated inhibition of TcR signaling. The MTB susceptibility of CBA/J mice has been documented previously as being driven primarily by excessive production of IL-10 during key stages of the immune response to MTB (Chapter 2, 3). The anti-inflammatory signal of IL-10 ligation would likely overwhelm any increase in T cell response afforded by low expression of KLRG1 in CBA/J mice. We first examined the level of KLRG1 in CBA/J mice as a possible explanation for the accumulation of CD8+ T cells that develops in CBA/J mice as MTB infection progresses (Chapter 3).
may be that in CBA/J mice, KLRG1 has a stronger effect on proliferation than T cell activation and cytotoxicity, as the literature supports 421,424, and if KLRG1 was removed in CBA/J mice it may only exacerbate their phenotype.

Here, we have demonstrated that protective responses to Mtb can be enhanced by removing the action of KLRG1. In CBA/J mice, low KLRG1 expression alone cannot serve to repair Mtb susceptibility driven by other immunosuppressive factors. We believe that removal of KLRG1 may be used in conjunction with existing Mtb therapies to boost their efficacy. Simply recruiting or transferring more T cells to the infection site is not sufficient, we must ensure those cells are capable of maximal TcR signaling for the duration of their lifespan. This will maintain and extend the anti-Mtb protective response long enough to effectively restrict its growth and dissemination until further chemotherapy can eradicate the infection. We believe the addition of anti-KLRG1 antibody to current treatment courses could significantly shorten the amount of time required to cure Mtb infection.
Chapter 5: Final Summary and Significance of Findings

In this work, we have demonstrated the formation of human-like granulomas in the mouse model of \textit{Mtb} infection by removing the action of IL-10 during critical early immune events. Secondly, we have more thoroughly explained the susceptibility of CBA/J mice based on significant CD8$^+$ T cell dysfunction. And thirdly, we have demonstrated that protective T cell responses can be enhanced by removing receptor-mediated inhibitory signals, leading to prolonged control of \textit{Mtb} infection. Together, these findings contribute to our understanding of protective immunity to \textit{Mtb}, and enhance our knowledge of immune correlates that may predict \textit{Mtb} susceptibility.

Although mouse models of \textit{Mtb} infection are useful, certain aspects of murine TB do not manifest in the same way as human TB. Primarily, murine \textit{Mtb} granulomas do not possess the same maturity in organization and structure that is characteristic of protective human lesions. Human \textit{Mtb} granulomas have been described as highly stratified and often exhibit a thick fibrotic capsule surrounding infected macrophages. Eventually these granulomas develop neovascularization and exhibit necrosis, leading to caseation and cavitation during later stages of maturity $^{304,445-449}$. Murine granulomas do not display the
same level of organization, and typically do not develop necrosis or fibrosis. This has significantly hindered the study of granuloma formation and maintenance. Researchers have resorted to *in vitro* systems and computational models in order to properly study human granulomas, since mouse lesions were not representative. In Chapter 2, we addressed this problem, and put forth a new murine model of TB granuloma formation, capable of closely representing human lesions. CBA/J IL-10−/− mice and wild-type CBA/J mice treated with anti-IL10R blocking antibody (from day -1 to day 21) developed mature, concise, stratified granulomas after *Mtb* infection. These lesions were enclosed in a thick fibrotic layer, and exhibited central necrosis. Accordingly, treated and IL-10−/− CBA/J mice maintained control of *Mtb* growth, survived significantly longer than control groups, and had enhanced T_{H}1 responses. C57BL/6 IL-10−/− mice have a transient increase in *Mtb* control, but do not form mature granulomas (unpublished observations). Therefore, IL-10 is thought to play small role in the natural protective response to *Mtb* in C57BL/6 mice, although overexpression of IL-10 can lead to *Mtb* susceptibility in C57BL/6 mice. We believe the CBA/J IL-10−/− model is ideal for understanding of how protective containment of *Mtb* is established. This is the most natural occurrence of human-like *Mtb* granulomas in mice, and could be invaluable for the investigation of granuloma formation and maintenance in man.

Although highly significant, the results described in Chapter 2 could be enhanced to further explain *Mtb*-susceptibility. Specifically, the formation of the human-like granulomas could be more closely examined to discover the exact mechanism behind the
IL-10 mediated suppression. Human granuloma formation is associated with elevated levels of the matrix metalloproteinase MMP-9, and vascular endothelial growth factor (VEGF), both of which are important for neovascularization. This suggests that tissue remodeling is critical to proper granuloma formation. Since both MMP-9 and VEGF are downregulated after treatment of cells with IL-10, it is likely that wild-type CBA/J mice and patients with active TB (both of which have elevated levels of IL-10) are unable to generate the remodeling processes necessary for mature granuloma formation. CBA/J IL-10<sup>−/−</sup> mice do not develop granulomas faster than wild-type mice, their lesions simply exhibit a significantly higher level of cellular organization and <i>Mtb</i> containment. This suggests that IL-10 is not significantly delaying the onset of granuloma formation, but is restricting their complexity and maturation. Critical to our understanding of this process is our finding that blockade of the IL-10R during the first three weeks of infection led to human-like granuloma formation by day 120 post-infection. This indicates that whatever change is required to develop mature granulomas takes place early after infection.

We hypothesize that removing the effect of IL-10 during the first 21 days of infection enables critical tissue remodeling events to be initiated, mediated by increased expression of MMP9 and VEGF. Although both MMP9 and VEGF can be important during late stage granuloma maturation, we believe they may play a significant role in the proper establishment of human-like <i>Mtb</i> lesion in CBA/J IL-10<sup>−/−</sup> mice. This hypothesis could be tested if we examined the expression of MMP9 and VEGF in lung samples from CBA/J
wild-type of IL-10$^{-/-}$ mice and found them increased in the IL-10$^{-/-}$ mice. We could then begin a series of experiments to block the action of MMP9 and/or VEGF in CBA/J IL-10$^{-/-}$ mice or anti-IL-10R-treated wild-type CBA/J mice. If blockade of MMP9 or VEGF restores the susceptibility of CBA/J mice, we will have identified the specific mechanism by which IL-10 inhibits mature granuloma formation.

Another intriguing observation was that *Mtb* bacilli inside CBA/J IL-10$^{-/-}$ macrophages appeared to be less healthy than those in wild-type macrophages after Ziehl-Neelson staining. *Mtb* bacilli in CBA/J IL-10$^{-/-}$ granulomas appeared attenuated and individualized, compared to the robust clumps of *Mtb* in wild-type CBA/J granulomas. As such, we hypothesized that *Mtb* inside CBA/J IL-10$^{-/-}$ granulomas were not as viable as those in wild-type mice. We therefore attempted to excise only the granulomas from CBA/J wild-type of IL-10$^{-/-}$ mice and enumerate granuloma-specific CFU, but did not observe a significant difference in *Mtb* growth. This work needs refined and replicated before we can exclude the possibility that *Mtb* inside CBA/J IL-10$^{-/-}$ granulomas are less viable than in wild-type granulomas. It is likely that CBA/J IL-10$^{-/-}$ granulomas are more hypoxic (another hallmark of human lesions) and that this stress disrupts the *Mtb* cell wall, affecting Ziehl-Neelson staining. Mice could be treated with Hypoxyprobe-1 and lung sectioned stained to determine the level of hypoxia between CBA/J wild-type or IL-10$^{-/-}$ mice. Together, our findings in CBA/J IL-10$^{-/-}$ mice provide the groundwork for
testing or confirming aspects of granuloma formation that, until now, could only be speculated upon.

In Chapter 3, we looked more closely at the susceptibility of CBA/J mice to Mtb reactivation. Our previous work demonstrated that IL-10 was critical to this susceptibility, but we did not fully characterize the extent and details of its immunosuppression in CBA/J mice. We subsequently discovered that CD8+ T cells from CBA/J mice accumulated over time, expressed markers of T cell dysfunction, and were capable of secreting IL-10 at a much higher level than C57BL/6 CD8+ T cells. Furthermore, CBA/J CD8+ T cells were clonally expanded, preferentially expressing Vβ8, which was capable of more IL-10 production that other Vβ TcR cells.

These data are of particular importance to Mtb infection because of new Mtb vaccines designed to boost the numbers of activity of CD8+ T cells. These approaches assume that enhancing the activity of CD8+ T cells will be universally beneficial; more cytotoxic responses and IFN-γ production to stimulate more efficacy in CD4+ T cells responses. This is ordinarily true, CD8+ T cells contribute to protective immune responses, but we have demonstrated that, in susceptible populations, CD8+ T cells exhibit considerable dysfunction. Increasing the numbers and activity of dysfunction cells during Mtb would be very detrimental to immunity. CBA/J CD8+ T cells are clonally expanded and can secrete IL-10. This not only increases the risk of IL-10-mediated
immunosuppression, but significantly restricts the range of antigens able to be recognized by CD8\(^+\) T cells. This work is critical to ensure that future treatments/vaccines designed to boost immunity to \textit{Mtb} are equally efficacious across both resistant and susceptible populations.

These data add more complexity to the interaction of \textit{Mtb} and the immune system. An over-simplified view is that \textit{Mtb} is harmful and the immune system is protective, and that only individuals with weakened immune systems will be susceptible to disease. It is becoming increasingly clear that \textit{Mtb} can induce immunosuppression through various component of the immune system, such as T\(_{\text{reg}}\) cells. \textit{Mtb} has been shown to induce T\(_{\text{reg}}\) activity, delaying T cell priming in the lymph node \textsuperscript{271}. Older studies (plus a current resurgence) describe the presence of a subset of CD8\(^+\) T cells with immunosuppressive function \textsuperscript{461,462}, similar to CD4\(^+\) T\(_{\text{reg}}\) cells. These suppressor CD8\(^+\) T cells were found to be predominantly restricted to the nonclassical MHC class Ib molecule, HLA-E (or Qa-1 in mice) \textsuperscript{462-464}, and suppress CD4\(^+\) T cell responses based on their peptide:MHC binding avidity \textsuperscript{465}. An important consideration is that unlike CD4\(^+\) T\(_{\text{reg}}\) cells, CD8\(^+\) suppressor cells can distinguish self from non-self \textsuperscript{184}.

Recent studies have identified a phenotypically distinct subset of regulatory CD8\(^+\) T cells capable of secreting IL-10 \textsuperscript{466}. It is possible that, in susceptible populations, \textit{Mtb} triggers the activity of suppressor CD8\(^+\) T cells, as uninfected CBA/J CD8\(^+\) T cells did not secrete
IL-10 after TcR stimulation in our studies. The expression of Vβ TcRs in CBA/J mice was similar between infected and noninfected groups, though absolute numbers increased accordingly with CD8+ T cell accumulation. This indicates that although important, the clonal nature of CBA/J T cells is not induced by infection. Another important finding is that the regulatory effect of suppressor CD8+ T cells required cell-cell contact through inhibitory receptors. We hypothesize that CBA/J mice possess increased levels of suppressor CD8+ T cells, which may explain why our attempts to stimulate IL-10 production with *Mtb* ex vivo were not successful, since we lacked the proper stimulation. This also provides an explanation for the increase in PD-1 and Tim3 we observed, since both molecules are indicated to mark suppressor CD8+ T cells. To determine the presence of suppressor CD8+ T cells in CBA/J, we could purify PD-1+CD8+ T cells and measure IL-10 production by ELISpot, as with CD8+ T cells in Chapter 3. If more PD-1+CD8+ T cells produce IL-10 than PD-1negCD8+ T cells, this would provide evidence that they are marking suppressor T cells in CBA/J mice. Furthermore, we could block the action of PD-1 in vivo during the period of elevated IL-10 production in CBA/J mice (day 90-150), and assay for the level of pulmonary IL-10 after necropsy. These experiments would demonstrate that it is not different Vβ TcR populations, but PD-1+ or PD-1neg CD8+ T cells that represent the detrimental cell subset in CBA/J mice. Overall, this work stresses that in susceptible individuals, enhancing total CD8+ T cell responses may not be the most beneficial treatment strategy.
In Chapter 4, we examined immunosuppression in another context. C57BL/6 mice mount a strong immune response to *Mtb* capable of controlling the infection. However, we determined that the inhibitory receptor, KLRG1, restricts maximal T cell responses in C57BL/6 mice during *Mtb* infection. Using flow cytometry, we analyzed the specific phenotype of T cells positive or negative for KLRG1, and found that more KLRG1$^\text{neg}$ CD4$^+$ T cells expressed the early activation marker CD69 that KLRG1$^+$ CD4$^+$ T cells. This indicated that KLRG1 was restricting the activation of certain T cells, and we hypothesized that removing KLRG1 would enhance activation of CD4$^+$ T cells leading to more IFN-$\gamma$ production. We obtained KLRG1$^{-/-}$ C57BL/6 mice and observed that they are capable of enhanced control of *Mtb* infection compared to wild-type C57BL/6 mice. This was primarily associated with enhanced numbers of activated CD4$^+$ T cells producing high levels of IFN-$\gamma$ in response to stimulation with *Mtb* antigen, and increased release of cytotoxic granules at day 90 post-infection. Interestingly, we observed that immune responses were not enhanced across all timepoints in KLRG1$^{-/-}$ mice, but rather wild-type mice lose numbers of activated CD4$^+$ T cells only at day 90 post-infection. Since KLRG1$^{-/-}$ mice do not display a similar reduction in activated cells, it suggests that, at day 90, KLRG1 inhibits the proliferation or recruitment of CD4$^+$ T cells, or that it mediates the deletion of certain cells.

Our work with KLRG1 is the first to show an *in vivo* consequence of KLRG1-deficiency. Until this point, studies examining KLRG1 have been predominantly viral models, and use KLRG1 expression as an indicator of T cell exhaustion. One of the only viral studies
to demonstrate a consequence of KLRG1 activity in mice overexpressed KLRG1 in NK cells and, after activating-antibody treatment, observed lower production of TNF and IFN-\(\gamma\). Here, we showed that the absence of KLRG1 enables resistant C57BL/6 mice to maintain optimal control of \(Mtb\) infection and significantly extends survival.

This study sheds light on a phenomenon that has been observed in our studies for years; that at day 90 post-infection with \(Mtb\) there is a significant decrease in immune function (unpublished observations). One hypothesis is that since we always use mice of approximately the same age (4-8 weeks old) for our \(Mtb\) studies, that at this time in their lives they undergo some physiological change, independent of infection. We have observed this phenomenon is male and female mice which excludes any gender-related explanations. Another explanation is that this may reflect a significant turnover of immune cells at day 90 post-infection. Whether the immune response to \(Mtb\) is a fluid system with constant cell death and replacement, or whether protective cells maintain activity for months then reach the end of their lifespan is unclear. This could be tested by transferring GFP-expressing cells into a GFP\(^{\text{neg}}\) mouse and infecting with \(Mtb\). This would enable the tracking of how long the donor cells survived after \(Mtb\) infection, and determine whether or not day 90 is a critical time for cell turnover. The better we understand the intricacies of the murine immune system, we can more accurately explain the experimental results obtained from them.
Through our work with KLRG1, we were able to significantly enhance control of \textit{Mtb} in an already resistant mouse strain. Very few studies have been able to show an enhancement in immune function of C57BL/6 mice by deleting component of their immune system. We believe that our findings will enable physicians to significantly shorten the length of time required to cure TB. Over the past decades, treatment of TB has remained largely unchanged and generally requires 6-9 months before a patient is cured of infection. None of the first-line antibiotics directly enhance the immune response against \textit{Mtb}, and rely on weakening the bacteria enough that the body can eradicate it. It has certainly been shown that blocking regulatory component of the immune system can boost protective function, but this can be dangerous and potentially lead to autoimmune disorders. We hypothesize that the negative regulation of KLRG1 is mild, and that blocking KLRG1 throughout the course of \textit{Mtb} treatment will not trigger autoimmunity. By blocking KLRG1 in conjunction with normal \textit{Mtb} treatment, we could ensure that pulmonary T cell responses are enhanced and \textit{Mtb} is weakened leading to more robust pro-inflammatory responses. We believe that this will significantly shorten the amount of time needed to cure \textit{Mtb}, while avoiding autoimmune complications.

Each of these chapters define novel mechanisms for increasing the resistance of mice to \textit{Mtb} infection, and provide insight into how current therapies may be enhanced. We believe that anti-IL-10R antibody could be used as an adjuvant with current BCG vaccination. Since we have shown that short-term blockade of IL-10 is sufficient to afford control of \textit{Mtb} infection, anti-IL-10R adjuvant would reliably generate protective
T cell responses after BCG vaccination. Furthermore, our studies with detrimental populations of CD8+ T cells have cautioned that universal activation of all T cells is not conducive to protective immunity. New DNA vaccines must be specific in the types of CD8+ T cells they induce. By selectively targeting only protective CD8+ T cells (PD-1
\textsuperscript{neg}), new vaccines could safely boost protective immunity to both viral and \textit{Mtb} infections without deleterious effects from detrimental subsets of cells. Finally, our studies on KLRG1 removal have significant implications for TB treatment. We have shown that KLRG1 is a subtle negative regulator of certain subsets of T cells, and that removing this inhibition safely enhances T cell responses. As in highly active antiretroviral therapy (HAART) and first-line antibiotics against \textit{Mtb}, combination therapies have been shown to the most successful treatments of these subversive diseases. Therefore, we submit that our work provides novel adjustments to current treatment strategies and vaccination against TB that could be quickly implemented and enhance the efficacy of the immune response to \textit{Mtb}.

Throughout this collective work, we have examined aspects of the immune response to \textit{Mtb} responsible for immunomodulation, and have shown that removal of these restrictions leads to enhanced outcome of TB disease. We have characterized the extent of \textit{Mtb} susceptibility in CBA/J mice, and demonstrated their immune dysfunction. Our work with CBA/J mice was dedicated to examining susceptible population of humans; populations that may not be accurately represented by using C57BL/6 mice alone. Our findings with KLRG1 serve to further our understanding of the complexities of immune
regulation, and offer significant therapeutic potential. The development of the CBA/J IL-10−/− granuloma model is the most significant finding herein, and offers a rare opportunity to study the dynamics of human-like immune responses to *Mtb*. Taken together, our work furthers the understanding of how various components of the immune response vary among different populations, and how natural regulatory function may be manipulated to alter the course of TB. Whether by enhancing current treatment, or repairing dysfunctional granuloma formation, it is our sincere hope that this work will serve as the groundwork for new studies and foster the development of new treatment strategies for *Mtb*.
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