IMMUNOLOGIC MECHANISMS AND PREDICTORS OF SUSCEPTIBILITY TO MYCOBACTERIUM 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

It has been estimated that 2 billion people, equivalent to one-third of the world’s population, are currently infected with *Mycobacterium tuberculosis* (*M.tb*), the causative agent of tuberculosis (TB). *M.tb* is typically an aerosol infection that causes disease in the lungs. Most people develop protective immunity that effectively contains *M.tb* bacilli in a clinically silent form (latent TB) that is not contagious. However, in a proportion of individuals, latent TB progresses to contagious active disease (reactivation TB) due to complex environmental, genetic, and immunologic interactions that are incompletely defined. By accurately predicting TB disease outcome, at-risk patients could be specifically targeted for antibiotic and/or immunotherapeutic treatments to prevent active TB. This strategy is important because early aggressive therapies would prevent illness in predisposed individuals, and benefit society by eliminating those patients’ abilities to transmit *M.tb* to others.

This work used comparative murine models to identify immunologic correlates and mechanisms of *M.tb* susceptibility and TB disease progression that may be applicable to humans. Following a low dose *M.tb* aerosol infection of susceptible and relatively resistant mice, we identified three immune mediators that were associated with *M.tb* susceptibility. These mediators, therefore, could predict the outcome and timing of TB disease progression: antigen specific interferon-γ (IFN-γ), interleukin-10 (IL-10), and
chemokine C-C ligand 5 (CCL5). Persistently low antigen specific IFN-γ and low CCL5 were biomarkers of *M.tb* susceptibility and predicted TB disease progression four to five months prior to disease onset in mice. The studies using antigen specific IFN-γ are particularly relevant to man (where access to lung samples is limited) because blood antigen specific IFN-γ accurately predicted lung responses.

In contrast to low IFN-γ and low CCL5, elevated levels of IL-10 in the lungs predicted reactivation TB approximately 4-8 weeks prior to disease onset in *M.tb* susceptible mice. Furthermore, IL-10 was shown to be a single cause of reactivation TB, as disease progression was delayed and protective immunity was enhanced by IL-10 blockade *in vivo*. Overall, these results indicate that single immune mediators may be used as biomarkers of TB disease progression in humans, and furthermore, that appropriately timed immunotherapeutic intervention may prevent the transition from controlled *M.tb* infection to active TB disease.
DEDICATION

This work is dedicated to people affected by tuberculosis. I hope part of this research may translate from animal models to humans, providing insight into immunologic mechanisms that may be targeted for accurate prognosis and for improving disease outcome in TB patients.
ACKNOWLEDGEMENTS

I am grateful for Dr. Joanne Turner’s excellent mentorship and the support of my advisory committee: Dr. Larry Schlesinger, Dr. Paul Stromberg, and Dr. Abhay Satoskar. This work could not have been completed without direct help and advice from current and past members of Dr. Turner’s laboratory, including Dr. Bridget Vesosky, Dr. Barnabe Assogba, Erin Rottinghaus, David Flaherty, Joshua Cyktor, Craig Davis, Rebecca Buttler, and Meredith Motz.

I appreciate the collegial working environments in the Department of Veterinary Biosciences (VBS) and Center for Microbial Interface Biology (CMIB) at the Ohio State University with notable support from the VBS Chairperson, Dr. Michael Lairmore, and the CMIB Director, Dr. Larry Schlesinger, who enthusiastically promoted inter-departmental scientific collaboration.

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VITA

August 12, 1974………………………………………………………………………………Born

1996……………………………………………………..BA Paleobiology, University of Pennsylvania

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PUBLICATIONS


FIELDS OF STUDY

Major Field: Veterinary Biosciences, Anatomic Pathology
Specializations: Immunology
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<tbody>
<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>ATPase</td>
<td>Adenosine Triphosphatase</td>
</tr>
<tr>
<td>β2M</td>
<td>Beta-2 Microglobulin</td>
</tr>
<tr>
<td>BCG</td>
<td>Mycobacterium bovis Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine (5-bromo-2-deoxyuridine)</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine C-C Ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster Designation or 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>
</tr>
<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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<td>Cy5.5</td>
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<td>Cy7</td>
<td>Cyanin dye 7</td>
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<tr>
<td>DAP-12</td>
<td>DNAX activation protein of 12 kDa</td>
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<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell-Specific ICAM-3 Non-integrin</td>
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<td>DD</td>
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</tr>
<tr>
<td>DDA</td>
<td>Dimethyldioctadecyl</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
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<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>ELISPOT</td>
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<td>ELR</td>
<td>Glutamic acid-leucine-arginine</td>
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<td>ERK</td>
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<td>Fas Associated Death Domain</td>
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<tr>
<td>FAS</td>
<td>Apoptosis Stimulating Fragment</td>
</tr>
<tr>
<td>FASL</td>
<td>Apoptosis Stimulating Fragment Ligand</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallizable fraction</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-Induced Tumor Necrosis Factor Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GKO</td>
<td>IFN-γ Knock Out</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N'2-Ethanesulfonic Acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HVEM</td>
<td>Herpesvirus Entry Mediator</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>IFN-γ Receptor</td>
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<td>Jun N-terminal Kinase</td>
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<td>Killer-Cell Immunoglobulin-Like Receptor</td>
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<td>Knock Out</td>
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<td>mAb</td>
<td>Monoclonal Antibody</td>
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<td>mAGP</td>
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<td><em>Mycobacterium tuberculosis</em></td>
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<td>PknG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified Protein Derivative</td>
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<tr>
<td>RANK</td>
<td>Receptor Activator of NF-KappaB</td>
</tr>
<tr>
<td>RD-1</td>
<td>Region of Difference-1</td>
</tr>
<tr>
<td>ROI</td>
<td>Reactive Oxygen Intermediates</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>RNI</td>
<td>Reactive Nitrogen Intermediates</td>
</tr>
<tr>
<td>RPMI</td>
<td>Royal Park Memorial Institute (culture medium)</td>
</tr>
<tr>
<td>SapM</td>
<td>Secreted Acid Phosphatase M</td>
</tr>
<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>
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<tr>
<td>TACE</td>
<td>TNF Converting Enzyme</td>
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<td>T&lt;sub&gt;H2&lt;/sub&gt;</td>
<td>Type 2 T helper</td>
</tr>
<tr>
<td>TIM</td>
<td>TRAF Interacting Motifs</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>TRADD</td>
<td>TNF Receptor-Associated Death Domain</td>
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<td>TRAF</td>
<td>TNF Receptor-Associated Factor</td>
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<td>TRAIL</td>
<td>Tumor Necrosis Factor-Alpha-Related Apoptosis-Inducing Ligand</td>
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<td>TYK</td>
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CHAPTER 1: INTRODUCTION TO TUBERCULOSIS

*Mycobacterium tuberculosis* (*M.tb*), the bacterium that causes human tuberculosis (TB), remains a substantial global health threat. Although *M.tb* can infect many organs, it is typically a lung pathogen transmitted by respiratory secretions from patients with active TB to non-infected individuals. It has been estimated that one-third of the population (2-3 billion people) may be infected with *M.tb* (1). Within the pool of *M.tb*-infected individuals, approximately 9 million people develop active TB each year, and 2 million people die (2). If left untreated, one patient with active pulmonary TB may infect 10-15 other people each year (3), contributing to the estimate that *M.tb* infects one person every second (4). The high *M.tb* infection rate and the substantial global TB disease burden underscore the need for improved TB diagnosis, prognosis, and prevention-oriented control methods.

Although 90% of infected individuals generate protective immunity that successfully controls *M.tb* for their lifetimes, 10% of people develop active TB (4). Within the newly diagnosed active TB cases, approximately 15% occur in immunocompromised individuals (2), while the remaining cases occur in young adults without known immunodeficiencies (5). Therefore, it is unlikely that generalized immunosuppression or age-related immune alterations contribute to the majority of active TB cases. These findings indicate that individuals at risk for active TB can generate
protective *M.tb*-specific immunity but, over time, changes in the immune response allow bacterial growth and TB disease progression. Despite decades of investigation, the complex mechanisms underlying the transition from latent to active TB in immunocompetent people are not fully understood. Biomedical and clinical research identifies many requirements for the initial generation of anti-*M.tb* immunity, but few studies address the immunologic mechanisms responsible for TB disease progression. Whether active TB results from the gradual loss of protective immunity or from the acquisition of detrimental immune responses is addressed in this work. We further attempt to define immune mediators that predict of *M.tb* susceptibility prior to the onset of active TB.

At least three difficulties hamper TB diagnosis and, therefore, global control measures. First, in the absence of clinical signs, non-invasive diagnostic tests cannot differentiate controlled *M.tb* infection from contagious active TB. Second, diagnostic tests cannot predict if or when a person will transition from controlled *M.tb* infection to active TB. Third, vaccination interferes with interpretation of *M.tb* diagnostic tests.

In humans, TB manifests in three clinical forms distinguished by the time post-*M.tb* infection, and the presence of disease symptoms (6). Primary progressive TB typically occurs within 2 years of *M.tb* exposure in children or immunocompromised adults (7, 8). Latent TB is clinically silent, representing strong protective immunity and controlled *M.tb* infection (9). The third disease manifestation, post-primary TB includes reactivation TB that can occur in adults who develop active TB years to decades following initial infection (9). Patients with reactivation TB are of particular interest because they can transmit *M.tb* to others.
The overall goal of this work is to use the murine, low dose, *M.tb* aerosol model to understand immunologic mechanisms of susceptibility to *M.tb*, and to identify molecular targets that may predict TB disease outcomes in humans. Each chapter focuses on a different molecule in the mouse *M.tb* model that may be relevant to man. Chapter 2 shows that low *M.tb* antigen specific interferon-gamma (IFN-γ) from blood samples predicts susceptibility and TB disease progression in mice. Chapter 3 shows that the immunosuppressive cytokine interleukin-10 (IL-10) correlates with, and causes, reactivation TB in mice. Chapter 4 associates low CCL5 with *M.tb* susceptibility in mice, and further demonstrates that CCL5 promotes optimal localization of early protective immune cells to the lungs during *M.tb* infection.

The interface between host cells and *M.tb* bacilli encompass complex events that both trigger and evade immune responses. Before addressing the specific research questions in each data Chapter, this introduction reviews aspects of *M.tb* infection including: 1) bacterial properties, 2) innate immunity, 3) acquired immunity, 4) granuloma formation, 5) vaccination, and 6) murine *M.tb* models.

*Mycobacterium tuberculosis, cell wall components and protein antigens*

*M.tb* is a rod-shaped facultative intracellular pathogen in the order Actinobacteria. *M.tb* organisms produce peptidoglycans but, due to the thickness of their cell walls, stain variably with the Gram stain. *M.tb* bacilli are positive by acid-fast staining methods due to the retention of carbol-fuscin within the thick mycolic acid cell wall layer.
Glycolipids

Mycobacteria, including *M. tb*, have complex cell walls rich in lipids, glycolipids and peptidoglycans, which are believed to protect against environmental stressors and anti-mycobacterial host immune responses (10). Extending from the bacterial plasma membrane to the surface there are sequential layers of peptidoglycans, arabinogalactans, mycolic acids, peripheral lipids, and outer complex polysaccharides: mannosylated lipoarabinomannan (ManLAM), non-capped LAM (araLAM), and phosphatidylinositol mannosides (PIMs) (11, 12). The interactions of *M. tb* cell wall components contribute to immune evasion and bacterial persistence. For example, by interacting with the mannose receptor (MR) on phagocytes, ManLAM fails to trigger pro-inflammatory responses (13, 14). Additionally, ManLAM contributes to poor phagolysosomal fusion, impairs pro-inflammatory cytokine production, and inhibits dendritic cell maturation (10, 15). Some reports are controversial (16). It has been further suggested that LAMs influence Th2-biased immune responses (14), which may not benefit the host.

Immunodominant proteins

*M. tb* has numerous secreted and cell wall-associated proteins that induce immune responses, including early secreted antigenic target-6 (ESAT-6), antigen 85 (Ag85), culture filtrate protein-10 (CFP-10), TB.7.7, heat-shock proteins (Hsp), 16kDa (alpha-crystallin), 38kDa, and others (17, 18). This introduction focuses on the two proteins, ESAT-6 and Ag85, which are used as model antigens in the data chapters.
Early secreted antigenic target-6

Early secreted antigenic target-6 (ESAT-6) is an immunodominant, small protein, abundantly secreted during the logarithmic growth phase of \( M. tb \) broth cultures (19, 20). It is both a mycobacterial virulence factor and a dominant \( M. tb \) antigen (21, 22). Although controversy exists regarding the relative importance of these two properties, the consequences during \( M. tb \) infection \textit{in vivo} may benefit bacterial and host survival. For example, advantages may be conferred to bacterial populations that express ESAT-6 and to host populations that successfully control \( M. tb \) as a result of ESAT-6 recognition.

ESAT-6 and its chaperone protein, CFP-10, are encoded within the region of difference-1 (RD-1) section of the \( M. tb \) genome (23-25). A relatively small number of virulent mycobacteria express ESAT-6, including \( M. tb \), \textit{Mycobacterium bovis}, \textit{Mycobacterium marinum}, and \textit{Mycobacterium kansasii}. ESAT-6 is absent from the vaccine strain, \( M. bovis \) BCG, as well as less pathogenic mycobacteria (26, 27). This relative specificity of ESAT-6 for the human pathogen \( M. tb \) makes ESAT-6 an attractive diagnostic tool, as discussed in Chapter 2.

As a virulence factor, ESAT-6 lyses lipid bilayers (28) and may promote \( M. tb \) escape from phagosomes into the cytoplasm (29), possibly by intercalation into membranes or through pore formation with CFP-10 (30, 31). The ESAT-6:CFP-10 complex also down-regulates the production of anti-mycobacterial reactive oxygen intermediates (ROI) (32). As an individual protein, ESAT-6 contributes to immune evasion by inhibiting Toll-like receptor (TLR) signaling and reducing IL-12p40 production (33). Additionally, ESAT-6 directly inhibits IFN-\( \gamma \) production by CD4 T cells (34).
During *M.tb* infection both humans (35) and mice (36, 37) generate strong ESAT-6 specific proliferation and IFN-γ production. In mice, ESAT-6 specific T cells participate in effector and memory responses against *M.tb* (27, 37). In humans, there is interest in using ESAT-6 specific blood responses to predict TB disease outcomes, an idea we test in Chapter 2 using mice.

**Antigen 85**

Antigen 85 (Ag85) is both a secreted and a cell wall-associated immunodominant antigen (20, 38). Ag85 is a complex of three constituents, Ag85A, Ag85B, and Ag85C that are highly homologous and cross-reactive but are encoded by distinct genes (38). The Ag85 complex contributes to mycobacterial cell wall biosynthesis with its mycolyl acid transferase enzyme activity (39). Ag85A, Ag85B, and Ag85C are functionally redundant, as loss of Ag85A or Ag85B does not alter bacterial growth in cultures or in macrophages (40). One study, however, shows that the lack of Ag85C diminishes cell wall mycolate content, and increases cell wall permeability (41), suggesting that Ag85 could be a drug target.

In the host, Ag85 binds fibronectin (38) and immunoglobulin (42). The consequences of these two findings with regard to TB pathogenesis are uncertain. It has been firmly established that *M.tb* Ag85 induces protective Th1 responses in animal models (43-45), resulting in numerous Ag85 specific IFN-γ secreting T cells, particularly during the *M.tb* growth phase in the lungs (46). These findings may also be true in humans (47, 48). It is anticipated that Ag85-containing vaccines in clinical trials will
boost protective immunity in humans, as discussed below. In Chapter 2, we use Ag85 as a model \( M.tb \) antigen to determine the strength of protective immunity in blood samples, and as a vaccine antigen to show that \( M.tb \) susceptible mice generate Ag85 specific protective immunity.

**Innate immune response to \( M.tb \)**

Resistance to \( M.tb \) requires multiple cell types, cell-to-cell interactions, and many molecular mediators. Protective immunity is imperfect, resulting in cessation of bacterial growth, but not pathogen elimination. Following inhalation, \( M.tb \) bacilli trigger slow innate immune responses from pulmonary alveolar macrophages and/or dendritic cells. The pulmonary alveolar macrophage is widely accepted as the primary cellular host for \( M.tb \), however, recruited blood-derived macrophages and dendritic cells also provide an intracellular niche for \( M.tb \), and participate in host immunity.

**Toll-like receptors in \( M.tb \) recognition and signaling**

Recognition of \( M.tb \) by host phagocytes depends on the interaction of bacterial cell wall components with pattern recognition receptors, including toll-like receptors (TLRs). Of the known TLRs, TLR2, TLR4, and TLR9 may have protective roles during mycobacterial infection, although some controversy remains (49, 50). TLRs may recognize lipoproteins, glycolipoproteins, Hsps, LAM, and other components of \( M.tb \) (51). *In vivo*, the lack of TLR2 (52, 53) or TLR4 (54, 55) impairs resistance to mycobacterial infections in mice. TLR9 may function cooperatively with TLR2 to
promote resistance to *M. tb* (56). Overall, the importance of TLRs in mediating protection against *M. tb* has been shown using mice deficient in the common TLR signaling component, myeloid differentiation primary response gene 88 (MyD88). MyD88 deficiency severely impairs innate responses to *M. tb*, resulting in increased bacterial burden and increased mortality, as compared to wild-type mice (57, 58).

**M. tb** phagocytosis

Simultaneous to recognition of *M. tb* bacilli by lung macrophages and/or lung dendritic cells, bacteria are phagocytosed. This is an important step for host control of *M. tb* and a bacterial evasion mechanism. As discussed below, *M. tb* bacilli enter phagocytic cells following interactions with the MR, complement receptors (CRs), dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN), surfactant protein-A (SP-A), surfactant protein-D (SP-D), Fc receptors (FcRs), and possibly dectin-1. *M. tb* interactions with each molecule are described in more detail below.

**Mannose receptor and complement receptors**

Human monocyte-derived macrophages use the MR and CRs for phagocytosis of virulent *M. tb* strains (59). Interactions of *M. tb* ManLAM with MR promotes *M. tb* survival by limiting phagolysosomal fusion (60), inhibiting protective cytokine production (11), and dampening pro-inflammatory TLR signaling (61). These *in vitro*
results suggest that MR-mediated phagocytosis of *M.tb* directs bacilli to intracellular compartments that favor bacterial persistence.

*M.tb* can also interact with CR1, CR3, and CR4 (62). *M.tb* adherence and/or phagocytosis following interactions with CR3 are the best characterized. Both opsonized (63) and non-opsonized (64) *M.tb* bind to CR3. CR3 interactions may direct *M.tb* bacilli towards intracellular compartments favoring bacterial clearance, but this has not been conclusively demonstrated. *In vivo*, the absence of CR3 has little effect on *M.tb* burden or survival of mice (65), suggesting that *M.tb* engages additional receptors *in vivo* that contribute to protective immunity.

**DC-specific intracellular adhesion molecule-3 grabbing non-integrin**

On dendritic cells, *M.tb* preferentially use DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) to enter host cells, mediated by ManLAM (66, 67). These interactions suppress dendritic cell maturation and TLR signaling (68), suggesting DC-SIGN’s engagement by *M.tb* inhibits protective immune functions. Although lung macrophages from TB patients also express DC-SIGN (69), the contribution of DC-SIGN to *M.tb* susceptibility *in vivo* is unknown, partially because mice do not express DC-SIGN (70-72). Despite this, the murine model of *M.tb* infection provides some understanding of DC-SIGN’s role *in vivo*. Transgenic mice that express human DC-SIGN have improved survival and less lung damage than control mice (73). These results suggest that the overall function of DC-SIGN is to limit *M.tb*-associated immunopathology.
**Pulmonary surfactant proteins**

Surfactant proteins contain calcium-binding (C-type) lectin domains that recognize carbohydrates in *M.tb* LAM, ManLAM (74), and the glycoprotein, Apa (75). Of the four known surfactant proteins, only the hydrophilic members, SP-A and SP-D, directly interact with *M.tb*. SP-A increases *M.tb* phagocytosis by human macrophages (76). Furthermore, SP-A up-regulates MR expression (77), inhibits the production of ROI (78), and inhibits TLR signaling (79). Overall, SP-A contributes to the relative anti-inflammatory state of the lungs and, by these mechanisms, SP-A may promote *M.tb* survival and persistence *in vivo*. Reports of *M.tb* infection in SP-A deficient mice have not been published, however, SP-A modulates pulmonary immune responses and inflammation in other *in vivo* models of respiratory pathogens (80-83).

In contrast to SP-A, the role of SP-D during *M.tb* infection may favor a protective host immune response. *In vitro*, SP-D inhibits *M.tb* uptake and increases phagolysosomal fusion, resulting in decreased *M.tb* survival (84, 85). The exact role of SP-D *in vivo* during *M.tb* infection is currently unknown.

**Dectin-1**

Dectin-1 is another C-type lectin pattern recognition receptor that is expressed on the surface of macrophages (86) and dendritic cells (87). It interacts with β-glucan, a cell wall component of fungi (88). In concert with TLRs, dectin-1 contributes to pro-inflammatory responses against *M.tb* (89, 90). The precise molecular interactions of *M.tb* with dectin-1 have not been determined.
**Fc receptors**

The Fc receptors (FcRs) bind to the Fc portion of antibody-opsonized particulates, including pathogens. FcRs are expressed on the surface of phagocytes, and ligation mediates phagocytosis and signaling that generally results in cell activation. The impact that FcRs have on *M.tb* control is not fully understood. *In vitro*, human monocyte-derived macrophages rely less on FcRs for *M.tb* uptake than CRs (63). *In vivo*, however, FcαR may play a protective role, as delivery of intranasal antibody favored host resistance to infection (91), perhaps by directing *M.tb* towards micobcidal intracellular compartments. Alternatively, FcRs may be associated with immunomodulation, rather than protection, as it has been shown that FcR-bearing lymphocytes, T cells, and B cells suppress responses to mycobacteria (92-94).

Overall, *M.tb* uses a variety of surface receptors for signaling and for entry into host phagocytes. Recognition of *M.tb* by different receptors results in dichotomous outcomes *in vitro*. For example, a protective pro-inflammatory response that limits bacterial growth may be initiated when *M.tb* molecules trigger TLR signaling. In contrast, *M.tb* survival and persistence may be favored following interactions with MR or DC-SIGN. These findings may impact human TB disease outcomes if interactions with different receptors affect intracellular trafficking and *M.tb* survival *in vivo*.
**M.tb intracellular survival**

Following binding and uptake, *M.tb* disrupts phagolysosomal fusion (95, 96) through secreted acid phosphatases (SapM) and protein kinase G (PknG) (15). *M.tb*-mediated phagosomal arrest is associated with retention of the endosomal marker Rab5 and impaired delivery of lysosomal hydrolases, cathepsins, and vacuolar ATPase to the phagolysosome (97, 98). *M.tb* bacilli also evade anti-mycobacterial responses by inhibiting lysosomal acidification, by reducing the production of inducible nitric oxide synthase (iNOS), and by reducing the generation of reactive nitrogen intermediates (RNI) (99-102). Overall, *M.tb* actively resists degradation by host immune cells.

Due to persistence within the phagosome, and *M.tb*’s ability to survive harsh conditions, *M.tb* bacilli have been characterized as non-replicating or persistent. The state of *M.tb* organisms in people with latent TB is difficult to assess, but models provide some insight. The *in vitro* Wayne model of latency demonstrates that *M.tb* genes and proteins associated with persistence in hypoxic environments are expressed (103), as could occur in *M.tb* granulomas. The *in vivo* Cornell model, and related variations, show that intact T<sub>H</sub>1 immune responses are required for long-term suppression of *M.tb* growth in mice (104). Whether or not antibiotic treatment, which is necessary to induce “latency” in the Cornell model, reflects human latent TB is unclear.

**Anti-mycobacterial mediators**

Although *M.tb* can persist within phagocytes, Bhatt *et al* (105) suggest that innate immune responses control and, possibly, eliminate bacilli. Macrophages produce anti-
mycobacterial mediators such as ROI and RNI (106). Murine macrophages (107-110) and human phagocytes (111, 112) inhibit mycobacterial growth in vitro, particularly when activated by IFN-γ, TNF, or vitamin D. Although RNI may be more important than ROI for *M.tb* control in vitro (113), both ROI and RNI contribute to host resistance to *M.tb* infection in vivo. Mice genetically deficient in gp91phox (114) and iNOS (114, 115) develop higher *M.tb* burdens than control mice and exhibit decreased survival. Similar findings may be true in *M.tb*-infected humans, but are difficult to demonstrate in cell culture systems, possibly due to a lack of tetrahydrobiopterin, a cofactor for iNOS (116).

**Protective pro-inflammatory cytokines**

Following *M.tb* infection, both macrophages and dendritic cells from humans and mice are sources of pro-inflammatory cytokines that are necessary for mycobacterial control. More specifically, interactions between interleukin-12 and the IL-12 receptor, tumor necrosis factor and TNF receptors, interleukin-1, and interleukin-6 are crucial for resistance to *M.tb*.

**Interleukin-12, interleukin-23 and interleukin-12 receptor**

Interleukin-12 (IL-12) is a heterodimer, composed of IL-12p40 and IL-12p35 subunits, that contributes to T cell activation (117). IL-12 is critical for resistance to *M.tb* in mice (118), is included in experimental vaccines (119), and is used as an experimental oral immunotherapy (120).
Murine models and *in vitro* experiments with human cells show the importance of IL-12 during *M. tb* infection. *M. tb* infection induces IL-12 from murine and human cells *in vitro* (121-123), with dendritic cells secreting the majority of IL-12 in response to TLR ligation (124). *In vivo*, exogenous IL-12 reduces *M. tb* burden and improves host survival (120, 125-127). Furthermore, the lack of functional IL-12 allows more rapid *M. tb* growth (126) that is associated with reduced pro-inflammatory mediators IFN-γ, iNOS, TNF, and IL-6 (118). All of these results confirm that IL-12 promotes resistance to *M. tb* in mice. Additional studies demonstrate that IL-12p40 (but not IL-12p35) is required for optimal resistance to *M. tb* in mice (128), perhaps by promoting DC migration to lung-draining lymph nodes to initiate antigen specific responses (129).

The IL-12p40 subunit can also form a heterodimer with p19 to form the pro-inflammatory cytokine, IL-23 (130). Although IL-23 is not required for *M. tb* resistance in mice (131), it partially compensates for the lack of IL-12 (132, 133) and it enhances protection when delivered exogenously (134). Together, these results demonstrate that IL-12, IL-12p40, and IL-23 all contribute to *M. tb* protective immunity in mice.

Humans with IL-12 or IL-12R mutations are highly susceptible to mycobacterial infections (135-140), including *M. tb* (141, 142). These reports indicate that intact IL-12/IL-12R signaling pathways are important for *M. tb* resistance in humans. The paucity of human TB cases due to IL-12 or IL-12R mutations is not incompatible with an essential role for IL-12 in *M. tb* resistance but, rather, reflects a general increased susceptibility to intracellular pathogens (137, 143).

As described above, IL-23 is dispensable for *M. tb* resistance in mice. In contrast, IL-23 may be more important in humans. *M. tb* infected human alveolar macrophages up-
regulate IL-23p19 gene expression and produce abundant IL-23 protein, whereas IL-12 is undetectable (144).

**Tumor necrosis factor and tumor necrosis factor receptors**

Tumor necrosis factor (TNF) is a membrane bound, or cleaved, trimer produced by macrophages, dendritic cells, and T cells (145). Both forms of TNF interact with many receptors, including but not limited to, fas (to induce apoptosis), TNFR2 (to induce activation), and decoy-R3 (to modulate immune responses) (146, 147). TNF contributes to *M.tb* protective immunity by three mechanisms: 1) activating antigen presenting cells, 2) promoting granuloma formation/function, and 3) regulating apoptosis.

TNF is required for *M.tb* resistance during acute and chronic infections as shown by using neutralizing antibodies, TNFR knockout (KO) mice, and TNF KO mice (148-152). In all experiments, the loss of TNF significantly reduces survival, and increases *M.tb* burdens. TNF contributes to resistance against *M.tb* by inducing iNOS expression and NO production, and up-regulating RNI production from macrophages (116, 148, 149, 153-156). TNF also up-regulates expression of costimulatory molecules on antigen presenting cells (157), suggesting that TNF promotes T cell priming. However, functional antigen specific T cells accumulate in the lungs of *M.tb* infected, TNF deficient mice (148-152) indicating that T cell priming is not dependent on TNF.

In humans, TNF is also required for *M.tb* control, although the anti-mycobacterial effects of TNF-dependent iNOS and RNI remain controversial (158-160). Growing
evidence, however, suggests that NO is important (116), and is supported by the fact that iNOS and NO from TB patients contributes to \( M.tb \) growth inhibition \textit{in vitro} (161-163).

TNF also promotes protective \( M.tb \) granuloma formation, maintenance, and function in humans (164, 165) and in mice. In mice, loss of TNF delays and disrupts mycobacterial granuloma formation, resulting in disorganized aggregates of poorly activated macrophages, abundant bacilli, and few abnormally located lymphocytes (149, 151, 154, 156, 166, 167). Later studies attribute TNF-dependent granuloma formation specifically to transmembrane TNF (152), possibly through the induction of local chemokines (152, 156, 166, 168) that regulate cell localization to the lungs. Together, these results indicate that TNF is important for T cell migration to macrophages to form functional granulomas.

Under some circumstances, TNF contributes to lung damage (169, 170). A large body of work, however, demonstrates that TNF prevents non-protective inflammation, possibly by inducing apoptosis during mycobacterial infection. Lung inflammation, granuloma necrosis, and morbidity occur during mycobacterial infections when TNF is absent, and are associated with activated T cells, inflammatory cytokines, and apoptosis-associated molecules (148-154, 167). TNF-dependent apoptosis may benefit the host by eliminating non-protective immune cells, and by providing antigenic material that stimulates CD8 T cells (171-178). TNF clearly has multiple protective roles during \( M.tb \) infection in humans and in mice, such as activating macrophages, promoting granuloma formation through chemokine induction, and regulating inflammation. It is likely that all TNF-mediated responses contribute to \( M.tb \) infection and TB disease progression.
**Interleukin-1 cytokine family**

Interleukin-1 (IL-1) is generally considered a pro-inflammatory cytokine, however, four of the eleven IL-1 family members are receptor antagonists or anti-inflammatory (179). IL-1β (pro-inflammatory) and IL-1Ra (receptor antagonist) are the most studied. IL-1β is an inactive cytoplasmic precursor molecule, and requires enzymatic cleavage for secretion and activation. IL-1 is produced by monocytes, macrophages, dendritic cells, B cells, and natural killer (NK) cells (179).

Upon receptor binding, IL-1β induces its own transcription and expression (179, 180). IL-1β also up-regulates anti-mycobacterial mediators that are crucial for *M.tb* control, including iNOS with NO production (116), phagosomal acidification and maturation (180), and other molecules that may play a role in *M.tb* infection such as cyclooxygenase (181) and phospholipase A2 (182). IL-1β also up-regulates adhesion molecules (179), indicating IL-1β may contribute to immune cell recruitment and lung granuloma formation.

A small number of studies in mice demonstrate that IL-1 is required for resistance to *M.tb* or to other mycobacteria. Both IL-1Ra KO mice and IL-1α/IL-1β double KO mice have reduced survival, elevated *M.tb* lung burdens, and variable pulmonary necrosis following *M.tb* infection (183-186). Similar evidence indicates that IL-1 confers *M.tb* resistance in humans (187, 188). Furthermore, reactivation TB has been reported in one patient treated with IL-1Ra (189), suggesting that continual pro-inflammatory effects of IL-1 are important for maintaining control of an established *M.tb* infection.
**Interleukin-6**

Although interleukin-6 (IL-6) is typically considered a pro-inflammatory cytokine it also has pleotropic effects including B cell stimulation, cytotoxic T lymphocyte (CTL) differentiation, macrophage differentiation, and immunoregulation. The differing roles for IL-6 depend upon its interactions with IL-6 receptors and an associated glycoprotein, gp130 (190).

In humans, IL-6 may be a marker of inflammation, or of tissue damage, and possibly contribute to TB disease progression (191-194). In contrast, animal models demonstrate that IL-6 is required for resistance to *M.tb* infection (195, 196), and other mycobacteria (196, 197), by inducing early IFN-γ production (196, 198). As discussed below, IL-6 may contribute to protection against *M.tb* by generating T_{H17} cells, or by inducing IL-17 production.

**Acquired immune response to *M.tb***

In addition to activating innate immune responses following *M.tb* infection, both alveolar macrophages and lung dendritic cells participate in acquired immune responses by antigen processing, presentation, and activation of T cells (199).

**T_{H1} and T_{H2} polarized immune responses**

T_{H1} and T_{H2} CD4 T helper cell subsets are discriminated based on patterns of cytokine secretion (199) that polarize immune responses (200). T_{H1} cells produce interleukin-2 (IL-2), IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-
CSF); while \( T_{H2} \) cells produce IL-3, IL-4, and IL-5 (199). The list of cytokines from \( T_{H1} \) and \( T_{H2} \) cells has since been expanded, however, IL-2 and IFN-\( \gamma \) remain key \( T_{H1} \) cytokines, and IL-4 distinguishes \( T_{H2} \) responses (200).

\( T_{H1} \) cytokines, in particular IFN-\( \gamma \), skew the response towards cell-mediated immunity and protect the host from \( M. tb \) by activating macrophages (127). In contrast, \( T_{H2} \) cytokines, primarily IL-4, promote humoral immunity to extracellular pathogens (201), and play a role in allergies (202). The role for \( T_{H2} \) cytokines in \( M. tb \) infection remains controversial, although increasing evidence suggests that a \( T_{H2} \)-dominated response may impair host resistance to \( M. tb \).

The \( T_{H1}/T_{H2} \) paradigm is a useful, but simplified, model. It fails to account for participation of the antigen presenting cell in T helper cell polarization. For example, if a dendritic cell encounters an intracellular pathogen, resulting in its activation, it may promote \( T_{H1} \) differentiation of naïve T cells through the secretion of IL-12 and expression of costimulation molecules (203). Alternatively, an antigen presentation cell may limit \( T_{H1} \) differentiation when faced with oxidative stress or depletion of reduced glutathione (204, 205).

**Interferon-gamma**

The prototypical \( T_{H1} \) cytokine, interferon-\( \gamma \) (IFN-\( \gamma \)), is required for limiting \( M. tb \) growth and establishing protective immunity. It is produced by a number of cell types, including CD4 T cells, CD8 T cells, \( \gamma \delta \) T cells, NKT cells, NK cells (206, 207), and even antigen presenting cells (208, 209).
In humans, the importance of IFN-γ during *M.tb* infection is evident by individuals with IFN-γ or IFN-γ receptor (IFNGR) mutations who develop severe, difficult-to-treat, mycobacterial infections including *M.tb* (138, 139, 210, 211). IFN-γ enhances anti-mycobacterial functions of human macrophages (212). Furthermore, exogenous IFN-γ improves treatment of TB patients and of patients infected with other mycobacteria (213, 214).

The use of IFN-γ knockout (GKO) mice confirms the requirement of IFN-γ for *M.tb* resistance. Following *M.tb* infection, GKO mice survive less than 50 days. Morbidity is attributed to lung tissue damage, and poor control of *M.tb* growth due to inadequate iNOS expression and low NO production (215-217). IFN-γ stimulates macrophage production of pro-inflammatory cytokines, and up-regulates many surface molecules, including cytokine receptors, major histocompatibility complex (MHC) I, MHCII, costimulation molecules, adhesion molecules, and chemokine receptors (207), all of which may contribute to *M.tb* resistance.

In addition to macrophage activation, IFN-γ also regulates immune cells. IFN-γ promotes T cell loss during mycobacterial infection (218) by up-regulating the mitochondrial pathway for apoptosis, and sensitizing cells to apoptotic signals (219). Additionally, large numbers of activated, antigen specific, CD4 T cells are generated in mycobacterial-infected GKO mice, but those cells fail to undergo apoptosis (220). These studies demonstrate a regulatory role for IFN-γ *in vivo*. Furthermore, when mycobacterial burdens are similar in GKO and wild type mice, the absence of IFN-γ still results in significant accumulation of activated T cells (216). Together, these results indicate the immunomodulatory role of IFN-γ is independent of *M.tb* burden. IFN-γ also regulates
T\textsubscript{H}17 cells. In the absence of IFN-\(\gamma\), IL-17 producing cells are significantly increased in the spleens and the lungs of mycobacterial-infected mice (221). Overall, these findings are important because they indicate that IFN-\(\gamma\) contributes to \textit{M.t}\textsubscript{b} control by activating macrophages, and protects the host from excessive lymphocyte accumulation.

Because IFN-\(\gamma\) is required for \textit{M.t}\textsubscript{b} resistance, it is considered a correlate of protection. Levels of IFN-\(\gamma\) are lower in patients with active TB, when compared to latent TB (222, 223), suggesting that insufficient IFN-\(\gamma\) is associated with TB disease progression. Recent improvements for diagnosing \textit{M.t}\textsubscript{b} infection quantify ESAT-6 specific IFN-\(\gamma\) from blood using IFN-\(\gamma\) Release Assays (IGRAs). Because of the stratification of \textit{M.t}\textsubscript{b} antigen specific IFN-\(\gamma\) responses in human TB patients we, and others (224, 225), hypothesize that IGRAs may also be used to serially monitor patients, or predict TB disease outcomes prior to the onset of clinical signs. This hypothesis is the focus of Chapter 2.

\textit{Interleukin-4, interleukin-5 and interleukin-13}

Interleukin-4 (IL-4), the prototypical T\textsubscript{H}2 cytokine, is produced by CD4 and NKT cells during chronic \textit{M.t}\textsubscript{b} infection (226-228) and stimulates B cells and antibody production (229). IL-5 and IL-13 function similarly as IL-4, but are less well studied in the context of \textit{M.t}\textsubscript{b} infection (230, 231). IL-4 dampens protective T\textsubscript{H}1 immunity by promoting the maturation of alternatively activated macrophages (232, 233), or by inhibiting autophagy (234). Although the lack of IL-4 does not alter \textit{M.t}\textsubscript{b} infection in C57BL/6 mice (235), IL-4 contributes to \textit{M.t}\textsubscript{b} susceptibility in BALB/c mice. Anti-IL-4
significantly reduces *M. tb* burdens when administered during the acute or chronic phases of *M. tb* infection to BALB/c mice (236, 237). In addition, the absence of IL-4 enhances immunotherapy by increasing anti-mycobacterial mediators (NO), pro-inflammatory cytokines (TNF, IL-1β), chemokines (CCL2, CCL3, CCL4, and CCL5) (237), and IFN-γ (238). Overall, these results indicate that the detrimental effect of IL-4 on *M. tb* infection in mice depends on the genetic background.

**T**H2 cytokines may also contribute to *M. tb* susceptibility in humans. In some geographical regions, active TB patients produce abundant IL-4 (239). In contrast, humans with latent TB have increased expression of IL-4δ2 (240, 241), an IL-4 antagonist (242, 243). These data suggest that IL-4 blockade with naturally occurring IL-4δ2 increases *M. tb* resistance in humans. Although IL-4δ2 has been detected in experimental animal models (244, 245), whether IL-4δ2 promotes *M. tb* resistance in animals has not been confirmed.

**T**H17 cells and interleukin-17

**T**H17 CD4 T cells produce IL-17, IL-21, and IL-22. In humans, **T**H17 CD4 T cells also produce IL-26 (246-248). **T**H17 cells are important for resistance to pathogens, mucosal immunity, immunosurveillance, and they contribute to autoimmune diseases (247, 249-253). Generally, **T**H17 cells differentiate and mature in response to pro-inflammatory cytokines such as IL-1, IL-6, and IL-23, however the immunomodulatory cytokine TGF-β also promotes **T**H17 differentiation in mice (246-248, 254, 255). In
addition to T\textsubscript{H}17 CD4 T cells, \(\gamma\delta\) T cells are a source of early and abundant IL-17 during mycobacterial infection (256), including \textit{M.\textit{tb}} (257).

IL-17 is not required for \textit{M.\textit{tb}} resistance in mice (133, 258). Multiple studies, however, demonstrate that T\textsubscript{H}17 cells and IL-17 promote optimal immunity by inducing chemokines that recruit T\textsubscript{H}1 cells (132-134) which produce antigen specific IFN-\(\gamma\), and inhibit \textit{M.\textit{tb}} growth (259). Furthermore, IL-17 deficient mice have impaired mycobacterial granuloma formation (256), confirming that IL-17 is important for cellular trafficking to sites of infection.

IL-17 producing T cells are detected in human TB patients (260) and are induced by \textit{M.\textit{bovis}} BCG vaccination (261). Whether IL-17 producing T cells substantially contribute to protection against \textit{M.\textit{tb}} infection in humans has not been determined. Given the strong evidence in mice, it is likely that T\textsubscript{H}17 cells and IL-17 may also rapidly localize protective cells to the lungs of \textit{M.\textit{tb}} infected humans.

\textit{Immunomodulatory cytokines}

\textit{Interleukin-10}

Interleukin-10 (IL-10) down-regulates acquired and innate immune responses (262, 263) and, in some tissues, maintains homeostasis (264). Multiple immune cell types produce IL-10, including monocytes (265), macrophages (266), dendritic cells (267), CD4 T cells (268), and regulatory CD4 T cells (267, 269, 270) that express \(\text{V}\alpha3.2\) and \(\text{V}\beta14\) TCR (271). Except for synthetic peptide polymers, however, the antigen specificity
for Vα3.2 and Vβ14 TCRs are unknown (271). CD8 T cells are also a source of IL-10 (268, 270, 272).

Functional IL-10 is a homodimer that binds to heterodimeric surface receptors. Following receptor dimerization, the IL-10R1 subunit transduces signals by Janus tyrosine kinase-1 (JAK1) and tyrosine kinase-2 (TYK2) (262). IL-10 ligation and signaling activates signal transducer and activator of transcription-3 (STAT3) (273, 274) and slows the transcriptional rate of inflammatory genes (274). IL-10 also activates STAT1, which inhibits IFN-γ-induced STAT1 phosphorylation (275), perhaps indicating that IL-10 modulates inflammation by competing for intracellular signaling molecules. IL-10 also up-regulates suppressor of cytokine signaling-3 (SOCS3) (276) and inhibits NFκB (277-280), both of which contribute to IL-10’s immunomodulatory effects.

Two exceptions to IL-10-mediated inhibition are its effects on CD8 T cells and B cells. IL-10 promotes proliferation, differentiation, and activation of these cell types. More specifically, exposure of CD8 T cells to IL-10 activates the transcription factors NFκB and AP-1 (281), and increases antigen specific cytolysis in cancer and viral models (282-284). These results suggest that IL-10’s effects on CD8 T cells benefit the host by clearing virally-infected cells and neoplastic cells. In some models, IL-10 is required for the generation and/or maintenance of memory CD8 T cells (285) however, this finding remains controversial (286, 287). B cells increase MHCII expression when cultured with IL-10 (288), possibly indicating an enhanced capacity to interact with T cells. During M.tb infection, the consequences of these alternative roles for IL-10 are unknown.

IL-10 has many biological effects that limit inflammation. IL-10 de-activates monocytes, macrophages, and/or dendritic cells, thereby reducing cytokines, chemokines,
anti-mycobacterial mediators, antigen presentation, costimulation, and inflammatory lipid mediators (262). More specifically, IL-10 inhibits the production of many pro-inflammatory cytokines including IL-1, IL-6, IL-12, IL-18, and IFN-γ (265, 289-294). IL-10 also reduce the production of ROI and RNI (293, 295-299) that are crucial for *M.tbc* control, as discussed previously. IL-10 inhibits immune cell recruitment by reducing pro-inflammatory chemokines including CCL5, CCL4, and CCL3 (300, 301). IL-10 limits antigen presentation by inhibiting endocytic recycling of MHCII peptide-loaded molecules (302) and by inducing membrane-associated ring finger (C3HC4)-1 (MARCH1), an enzyme involved in intracellular trafficking of the MHCII β-chain (303). IL-10 may also induce apoptosis of dendritic cells (262, 304), or prevent maturation of dendritic cells resulting low expression of MHCII and costimulatory molecules, and low IL-12 secretion (305, 306). Overall, IL-10 down-regulates multiple functions of antigen presenting cells, which subsequently diminishes T cell proliferation and cytokine production (307-312).

In addition to indirect inhibition of T cells by macrophage or dendritic cell de-activation, IL-10 directly acts on CD4 and CD8 T cells to inhibit cytokine production (287, 313-315) or to induce anergy (316). Although the *in vivo* significance of these findings are not fully known, the potential for IL-10-mediated T cell anergy could be detrimental to some TB patients (317).

Although IL-10 down-regulates many monocyte and macrophage functions, it does not de-activate all macrophage functions. IL-10 increases FcγR expression on monocytes and promotes macrophage maturation (318, 319). IL-10 also promotes phagocytosis of opsonized organisms (320), but as indicated above, internalized
organisms are not readily killed due to inhibition of RNI and ROI. These functions of IL-10 may be important for maintenance of alternative macrophage activation and for lung homeostasis by promoting particulate removal from alveolar air spaces while minimizing tissue damage due to oxidants.

In many bacterial, fungal, and protozoal infections, IL-10 down-regulates protective immune responses (262). That IL-10 worsens disease has been shown in models of IL-10 excess. For example, IL-10 transgenic mice have reduced survival and increased bacterial replication following *Listeria monocytogenes* infection (321, 322). Similar results are shown with *Leishmania major* infection (321) and with *Trypanosoma cruzi* infection (323). During mycobacterial infection, including *M. tb*, IL-10 transgenic mice have elevated bacterial loads associated with early mortality (324-326). Overall, IL-10 abundance exacerbates several infectious diseases.

When IL-10 is blocked or absent, resistance to infectious diseases improves. IL-10 KO mice (327), or mice treated with anti-IL-10 (328), have enhanced immunity to *L. monocytogenes* (328). Similarly, in the absence of IL-10, immunity and parasite clearance are improved during *L. major* (262, 329) and *T. cruzi* (330-332) infections. During mycobacterial infection, IL-10 blockade or absence improves anti-mycobacterial immunity to low-virulence organisms, *M. avium* (333) and *M. bovis* BCG (334, 335). During virulent *M.tbc* infection, however, the absence of IL-10 does not consistently enhance anti-mycobacterial functions, or promote protective acquired immunity (336-338). Instead, IL-10 may prevent tissue damage during chronic *M.tbc* infection of C57BL/6 mice (339).
In the context of human *M.tb* infection, IL-10 likely functions as both a suppressor of immunity and as a regulatory cytokine that prevents immune-mediated damage. Both functions could have important consequences on reactivation TB, especially within the lung where maintenance of alveolar structure and function are important for gas exchange. The majority of clinical research associates IL-10 abundance with active TB, and disease severity in humans (340-345). Furthermore, T_{H1} responses from cell cultures obtained from TB patients are suppressed by endogenous IL-10 (346), thus defining a mechanism of poor immune responses in humans with active TB. In Chapter 3, we determine the role of IL-10 *in vivo* using mice to model reactivation TB.

*Transforming growth factor-beta*

Transforming growth factor-beta (TGF-β) stimulates growth, apoptosis, tissue repair, angiogenesis, and anti-angiogenesis. It also modulates the immune response by de-activating macrophages and by down-regulating acquired immunity (347-352). TGF-β synergizes with IL-10 to promote immune tolerance and limit pathological inflammation (353-358). TGF-β is secreted by multiple types of immune cells during *M.tb* infection, including monocytes, macrophages, alveolar macrophages, dendritic cells (359, 360) and CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells (270, 361). Similar to IL-10, TGF-β suppresses antigen presenting cell costimulatory molecules (362) and decreases iNOS expression (358). Additionally, TGF-β down-regulates T cell function by inhibiting proliferation, decreasing cytokine production, and reducing T cell responsiveness to IL-12 (363-367).
Animal studies demonstrate that TGF-β can suppress immune responses during mycobacterial infection (112, 368-370). More specifically, in guinea pigs, exogenous TGF-β exacerbates *M.tb* infection by decreasing T\(_H\)1 cytokines and by inhibiting lymphocyte proliferation (368). TGF-β may similarly contribute to *M.tb* infection in humans. TGF-β is detectable in patients with active TB (371-373), and high levels are associated with more severe disease (372). Furthermore, TGF-β inhibitors improve T cell responses and anti-mycobacterial effects *in vitro* (374, 375), and the addition of TGF-β slightly enhanced *M.tb* growth in human cells (376, 377). Together, these studies indicate that TGF-β impairs resistance to *M.tb* in mice and in humans.

*Cell-mediated immunity*

**Classically and alternatively activated macrophages**

Macrophages are broadly categorized into classically and alternatively activated states. Generally, classically activated macrophages require exposure to IFN-\(\gamma\) or bacterial lipopolysaccharide and such activated macrophages promote cell-mediated immunity (232). Classically activated macrophages up-regulate MHCII molecules and costimulatory molecules, produce abundant pro-inflammatory chemokines and cytokines, and up-regulate iNOS expression (232). As described previously, all of these immune mediators contribute to *M.tb* resistance.

In contrast, alternatively activated macrophages contribute to humoral and anti-parasitic immunity, allergies, and organ-specific homeostasis, including the lung (378-381). Alternatively activated macrophages are further subdivided into M2a, M2b, and
M2c populations depending on cytokine exposure and/or surface receptor expression (232). Generally, macrophages in the lungs are maintained in an alternative activation state likely due to SP-A-mediated reduction of NADPH oxidase activity (78), increased MR expression (59), and production of anti-inflammatory cytokines such as IL-1Ra, IL-10, TGF-β (232). This macrophage phenotype likely impairs, or delays, \( M.tb \) protective T\textsubscript{H}1 immunity.

T cells

\textit{αβ CD4 T cells}

αβ CD4 T cells dominate the acquired immune response to \( M.tb \). Successful transfer of cell-mediated mycobacterial resistance is evident in early publications (382, 383). In 1975, live splenocytes from \( M.\text{bovis} \) BCG vaccinated donor mice inhibited \( M.tb \) growth when transferred into syngeneic recipients (384). Over one decade later, two protective T cell subsets that expressed distinct surface markers L3T4 (CD4) and Lyt-2 (CD8) were identified by adoptive transfer (385), and by depletion \textit{in vivo} (386). Together, these results demonstrate that CD4 T cells are the most abundant and efficient cellular mediators of \( M.tb \) protection, especially those CD4 T cells generated during the early phase of \( M.tb \) infection.

CD4 T cell protection manifests in the first 2-4 weeks of \( M.tb \) infection, associated with antigen specific IFN-γ and stabilized bacterial growth (228, 387). \( M.tb \) infection of CD4 KO mice confirms the importance of CD4 T cells for \( M.tb \) resistance. CD4 KO mice have increased \( M.tb \) burdens and reduced survival compared to wild type
mice (388, 389). By 50 days of *M.tb* infection, bacterial loads are significantly higher in CD4 KO mice than controls, and *M.tb* grows until morbidity develops at approximately 150 days (388). Granuloma structure and composition are altered in CD4 KO mice, yet these mice generate antigen specific IFN-γ producing cells (388, 389). Taken together, these results demonstrate that: 1) CD4 cells are essential for protective immunity to *M.tb*; 2) CD4 T cells may contribute to *M.tb* protection by mechanisms independent of IFN-γ; and 3) additional IFN-γ producing cells partially compensate for the absence of CD4+ cells.

Although IFN-γ is believed to be the main protective cytokine produced by CD4 T cells, additional protective mechanisms exist. CD4 T cells lyse *M.tb* infected macrophages (387), suggesting that CD4 T cells promote *M.tb* clearance by releasing bacilli and promoting uptake by another, perhaps more highly activated macrophage. Organized *M.tb* granulomas are required for optimal *M.tb* control (390). In the absence of CD4 T cells, granulomas are large, with abundant neutrophils, and are often necrotic (388, 389). These results indicate that CD4 T cells promote granuloma formation through direct or indirect coordination of cellular recruitment to the lungs.

CD4 T cells also control chronic *M.tb* infection and contribute to protective memory responses. Depletion of CD4 T cells after 6 months of chronic, stable *M.tb* infection results in significant bacterial re-growth, loss of CD4 T cell derived IFN-γ, loss of organized granulomas, and diminished survival (391). In CD4-depleted mice, macrophage function is marginally affected, as measured by expression of TNF and iNOS (391). The fact that CD4 depletion increases *M.tb* burden and reduces survival
without altering known protective macrophage function, indicates that CD4 T cells may have additional, unknown, protective functions during chronic \textit{M.tb} infection.

Immunological memory develops in response to \textit{M.tb} infection (392-394). Furthermore, CD4 T cells mediate antigen specific memory in response to live \textit{M.tb} (395-397). Additional experiments confirm that CD45RB$^{\text{hi}}$, L-selectin$^{\text{lo}}$, CD44$^{\text{hi}}$ memory CD4 T cells produce IFN-$\gamma$ and control \textit{M.tb} (398). Taken together, these results indicate that CD4 T cells participate in long lasting \textit{M.tb} specific immunity and recall responses.

The ability of CD4 T cells to produce IFN-$\gamma$ in response to ESAT-6, CFP-10 and other \textit{M.tb} antigens, provides the foundation for the use of IGRAs to diagnose \textit{M.tb} infection in humans, as discussed in Chapter 2.

\textbf{αβ CD8 T cells}

αβ CD8 T cells promote \textit{M.tb} resistance, as shown by \textit{in vivo} depletion and adoptive cell transfer (385, 386). β2-microglobulin (β2m) KO mice lack MHCI-restricted CD8 T cells and have impaired \textit{M.tb} resistance, but the results are confounded by the lack of other β2m functions (399-403). \textit{M.tb} infection of CD8 KO mice, depletion of CD8$^{+}$ cells, and transfer of CD8$^{+}$ cells confirm that CD8 T cells specifically protect during chronic \textit{M.tb} infection (402, 404), latent \textit{M.tb} infection (405), and during memory/vaccination responses (406, 407). During \textit{M.tb} infection, optimal protective CD8 T cell responses are dependent upon CD4 T cell help (408, 409).
CD8 T cells protect during *M.tb* infection by two mechanisms: 1) antigen specific elimination of heavily *M.tb* infected cells through perforin dependent lysis or Fas/FasL mediated apoptosis (400, 406, 410, 411); and 2) production of pro-inflammatory mediators such as IFN-γ, TNF, or CCL5. Although perforin and fas-fasL are not essential for *M.tb* resistance in mice, these mechanisms contribute to long-term *M.tb* control (404, 411-413). In humans, mycobacterial specific CD8 T cells similarly lyse heavily infected cells (414, 415). CD8 CTL granule contents (granulysin) kill extracellular *M.tb* (416), and expression of granulysin improves *M.tb* resistance in mice (417). In humans and mice, CD8 T cells also produce *M.tb* protective cytokines, IFN-γ and TNF (387, 406, 408), particularly during early *M.tb* infection (418). Furthermore, IFN-γ from CD8 T cells may partially compensate for the lack of IFN-γ from CD4 T cells (388), and contribute to *M.tb* control (419).

CD8 T cells recognize *M.tb* antigens presented in classical (MHCI) and non-classical (CD1) molecules. Respectively, these molecules present peptides (420-422) or lipid-derived antigens (423-427). Both types of antigen presentation may protect against *M.tb*, but the abundance of MHCI restricted CD8 T cells contributes the most to *M.tb* resistance (423, 428-432).

Overall, CD8 T cells contribute to *M.tb* resistance by localizing to granulomas (433), producing macrophage-activating cytokines during early *M.tb* infection, and functioning as CTLs during chronic *M.tb* infection, latent TB, or recall responses.
**γδ T cells**

γδ T cells were first identified in the late 1980s due to expression of distinct TCR genes (434). Although γδ T cells have limited genetic diversity (435), they respond to multiple types of *M. tb* antigens, including peptide antigens (436) and non-protein antigens (206, 437). Furthermore, γδ T cells may participate in resistance to mycobacteria by localizing to granulomas (438), by producing IFN-γ (206), and by producing IL-17 (257, 260). These findings suggest that γδ T cells can promote protective immunity against *M. tb*. However, γδ T cells are rarely observed in human *M. tb* granulomas (439) and only a small fraction of γδ T cells accumulate in response to mycobacterial infection in mice (440, 441). Finally, morbidity increases in γδ KO mice only with high doses of *M. tb* (442, 443). Together these results suggest that γδ T cells do not substantially contribute to *M. tb* resistance *in vivo*, or significantly alter disease outcome.

**Natural killer T cells**

Natural killer T (NKT) cells express both αβ TCR chains and NK markers, such as NK1.1 and Ly-49 (444, 445). Some, but not all, NKT cells display invariant TCRα chain usage (Vα14), which contributes to their glycolipid antigen recognition in CD1 molecules (446-454). NKT cells are present in TB patients (455) and they may contribute to *M. tb* protective immunity by recognizing mycobacterial cell wall components (430, 431, 456, 457). NKT cells rapidly produce cytokines (IFN-γ, TNF and others) that activate macrophages and suppress bacterial growth (430, 453, 458, 459). NKT cells also
lyse regulatory T cells during *M.tb* infection (460), thus eliminating cells that may dampen protective T\(_\text{H}1\) immunity.

NKT cells are a small proportion of T cells in *M.tb* infected mice (458, 461) and the lack of CD1 does not alter survival of *M.tb* infected mice (432). These results show that NKT cells are not required for *M.tb* resistance. However, delivery of the synthetic NKT cell ligand, α-galactosylceramide, improves survival, reduces *M.tb* lung burden, and reduces lung tissue damage in mice (432). Furthermore, in guinea pigs, vaccination with *M.tb* lipids induces equivalent protection as that induced by *M. bovis* BCG vaccination, and reduces lung tissue damage (462) that may have been mediated by NKT cells (463, 464). Overall, these results identify NKT cells as attractive therapeutic and/or vaccination targets, despite their small numbers *in vivo*.

*Natural killer cells*

Similar to innate immune cells, natural killer (NK) cells express non-polymorphic surface receptors. NK cell effector functions, however, resemble conventional T cells, and include cytokine production and target cell lysis (465). Thus, NK cells are frequently considered a bridge between innate and adaptive immunity. NK cell lysis of target cells occurs in response to loss of MHCI expression, as well as complex signal integration from activatory and inhibitory receptors (465, 466).

During *M.tb* infection, NK cells contribute to protective immunity by producing IFN-γ (467), lysing *M.tb* infected cells (467-471), and possibly by directly binding *M.tb* with the NKp44 receptor (472). However, similar to NKT cells and γδ T cells, the small
numbers of NK cells in the lungs during *M.tb* infection suggest that their protective contribution *in vivo* is minimal (461).

**Humoral immunity and B cells**

B cells are bone marrow-derived lymphocytes that mediate humoral immunity by differentiating into short-lived antigen specific, antibody secreting plasma cells or long-lived memory B cells (473). Differentiation, maturation, and antibody class switching depend upon T cell cytokines such as IL-4, IL-5, IL-21 and IFN-\(\gamma\) (473).

The role of B cells during *M.tb* infection may be overshadowed by the dominant role of CD4 T cells and T\(\text{H}1\) mediated protection. However, B cells localize to *M.tb* infected lungs (474), and in experimental models, B cells and purified antibodies can protect against *M.tb* infection. B cell deficient mice have increased numbers of *M.tb* bacilli that is independent of antigen specific IFN-\(\gamma\) (475). Decreased survival and abnormal granuloma formation are also demonstrated in B cell deficient mice (476), although results are not consistent (477, 478). This variation in the response of B cell KO mice to *M.tb* infection suggests a complex role for B cells in *M.tb* infection, possibly involving antibody-mediated protection, and IL-10- or TGF-\(\beta\)-mediated immunomodulation.

Mycobacterial specific antibodies in *M. bovis* BCG infected rabbits and in TB patients (479) may partially confer protection against mycobacterial challenge (480). Additional studies demonstrate that intravenous immunoglobulins (481, 482), intranasal IgG (483, 484), and intranasal IgA (237, 485) reduce *M.tb* lung burden and may prevent
bacterial dissemination. In addition, anti-arabinomannan (IgG3 mAb 9d8) significantly improves survival when administered to \textit{M.tb} infected mice (486). It is uncertain whether IgG3 mAb 9d8, or similarly functioning antibodies, are generated during primary \textit{M.tb} infection or vaccination. These results, however, provide compelling evidence that antibodies can mediate protection against \textit{M.tb}.

\textit{Chemokines and chemokine receptors}

Chemokines are small, secreted cytokines that direct cell migration during embryological development, homeostasis and inflammation (487, 488). Current chemokine nomenclature includes the family name followed by the letter “L’ for ligand, or “R” for receptor, and a number that designates the order of discovery within its family (489, 490). The four chemokine families are defined by the position of conserved, N-terminus cysteine (C) residues, in relationship to intervening amino acid (X) residues: CXC, CC, C, and CX3C. CXC and CC chemokines attract a broad array of leukocytes including neutrophils, monocytes, dendritic cells, lymphocytes, natural killer cells, eosinophils, and basophils (491). In contrast, the C and CX3C chemokine families attract lymphocytes and monocytes (491).

Chemokines within the large CC family recruit cells during inflammatory and infectious disease processes. Specifically, CCL5 may be important in leukocyte recruitment in asthma, experimental allergic encephalitis/multiple sclerosis, herpes viral infection, human immunodeficiency virus (HIV) infection, leishmaniasis, neoplasia,
collagen-induced arthritis/rheumatoid arthritis, schistosomiasis, transplantation rejection, toxoplasmosis, tuberculosis, and vascular disease/atherosclerosis (492).

Chemokine ligand activity is mediated by seven-transmembrane, G-protein coupled receptors. Ligation triggers calcium flux and cytoskeletal rearrangements (487, 488). Chemokine receptors exhibit overlapping ligand specificities. For example, CCR5 binds CCL5, CCL4 and CCL3 (493, 494). There is also promiscuity in the binding of ligands to multiple receptors. For example, CCL5 binds CCR1, CCR5 and others (495, 496). These findings illustrate the redundancy in chemokine and chemokine receptor interactions (493, 494, 497-504), that are summarized below (Tables 1.1 and 1.2). In Chapter 4, we focus on the chemokines and chemokines that are identified below in bold typeface.

### Table 1.1. Chemokines

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Cellular Source of Chemokine</th>
<th>Receptor Interactions</th>
</tr>
</thead>
</table>
| CCL5      | Macrophages, dendritic cells, CD8 T cells/CTLs, endothelial cells, epithelial cells | CCR1
|           |                                                                       | CCR3
|           |                                                                       | CCR4
|           |                                                                       | CCR5
|           |                                                                       | CCR10                 |
| CCL3      | Macrophages, multinucleated giant cells, mesothelial cells             | CCR1
|           |                                                                       | CCR5                 |
| CCL4      | Macrophages                                                            | CCR5                 |

### Table 1.2. Chemokine receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Receptor Expression</th>
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<tbody>
<tr>
<td>CCR1</td>
<td>effector T cells</td>
</tr>
<tr>
<td>CCR3</td>
<td>Th2 cells</td>
</tr>
<tr>
<td>CCR4</td>
<td>semi-naïve T cells and Th2 cells</td>
</tr>
<tr>
<td>CCR5</td>
<td>effector &amp; Th1 T cells, macrophages, dendritic cells</td>
</tr>
<tr>
<td>CCR10</td>
<td>T cells in skin/mucosa</td>
</tr>
</tbody>
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Table 1.2. Chemokine receptors
**M.tb infection, granulomas and chemokines**

The cellular immune response to *M.tb* infection is coordinated in discrete aggregates of inflammatory cells called granulomas. Granulomas restrain mycobacterial growth, limit dissemination, and protect adjacent tissue from detrimental inflammation (505-509), albeit imperfectly (510). The prototypical human *M.tb* granuloma contains central necrosis, mineralization, epithelioid macrophages, and multinucleated giant cells, surrounded by a thin rim of lymphocytes and peripheral fibrosis. This granuloma structure, however, is associated with latent TB (511), and may not represent the wide spectrum of granuloma morphologies associated with active TB or granuloma alterations that may precede active TB (512).

Human and murine *M.tb* granulomas show distinct cellular patterns that are associated with resistance or susceptibility to *M.tb* infection. Human patients with controlled *M.tb* infection (160, 165, 513, 514), and resistant mice (390, 515-520), form compact, lymphocyte-rich granulomas that limit *M.tb* growth for extended periods of time. In contrast, active TB patients (160, 165, 512-514, 521-524) and *M.tb* susceptible mice (149, 390, 515-520) develop large granulomas with abundant foamy macrophages and few lymphocytes, often in association with necrosis and airway erosion.

Because chemokines attract leukocytes, and receptors are differentially expressed by naïve, T\(_H1\), and T\(_H2\) cells, we and others (509) hypothesize that chemokines are essential for T cell recruitment to *M.tb* granulomas. Effector and T\(_H1\) T cells express CCR5 and CCR1, and migrate towards CCL5, CCL4, and CCL3 (493, 494). CCR5 and CCR1 may participate in resistance to *M.tb*. T cells from TB patients migrate when CCR5 interacts with its ligands (525), suggesting that CCR5 contributes to granuloma
formation in vivo. Although less is known about CCR1-mediated migration during M.tb infection, CCR1 is expressed following exposure to M.tb antigens (526). Although additional chemokines and chemokine receptors may contribute to M.tb immune responses (509), Chapter 4 focuses on CCL5 as a correlate of M.tb susceptibility, and defines a role for CCL5 in vivo during early M.tb infection.

Vaccination and TB diagnostic tests

M. bovis bacillus Calmette-Guerin and experimental TB vaccines

M. bovis bacillus Calmette-Guerin (BCG) is the only approved vaccine against M.tb. It is routinely administered to children in countries of high TB prevalence, and it protects against primary, disseminated M.tb infection (527). The efficacy rate of M. bovis BCG, however, varies from 0-80% (527), and immunity wanes after 10-20 years (528-530). Single vaccination with M. bovis BCG, and re-vaccination with M. bovis BCG, afford poor protection against active pulmonary TB in adults, the common and contagious form of TB (531, 532). Furthermore, although widespread negative outcomes are not documented in M. bovis BCG vaccinated humans (533), survival in M.tb challenged guinea pigs significantly decreases following multiple M. bovis BCG vaccinations (534). Vaccine research, therefore, focuses on boosting M. bovis BCG with heterologous antigens, primarily with Ag85.

Most experimental vaccines attempt to increase M.tb specific T_{H1} immunity by improving antigen presentation and increasing the numbers of antigen specific IFN-γ producing cells. In animal models, both successes (535-538) and failures (539) are
reported. Differences may be attributed to antigen or adjuvant choices, method of vaccine delivery, or other technical variations. Nevertheless, nine successful experimental vaccines have progressed to human clinical trials and, of those, seven include Ag85 (540). Results of these trials are not yet available.

Because of the complexity of pre-existing Th2 immune responses in some individuals, there is interest in developing vaccines that actively suppress, prevent, or overcome Th2 responses. Some success has been reported using *M. bovis* BCG that secretes IL-2 (541-543).

There is also interest in identifying immune correlates of protection induced by *M. bovis* BCG, or of protection induced by experimental vaccines. Many studies focus on antigen specific IFN-γ from CD4 or CD8 T cells (535-538), or gene transcription profiling (544). One study in human newborns has identified *M. bovis* BCG vaccinated individuals with divergent immune responses, where blood cell cultures produce either IFN-γ, or IL-10. It is not yet known, however, which individuals will be protected from, or susceptible to, TB disease (545).

**TB diagnostic tests**

The gold standard for diagnosing *M.tb* infection is positive identification of bacilli by culture or sputum smear. False negative results may occur if the precise anatomic site of infection is not accessible, and adjunct tests may support a diagnosis of TB. Additional tests may include radiographs, computed tomography scans, or the Purified Protein Derivative (PPD) skin test. Accurate interpretation of results from these diagnostic
modalities can be difficult. Imaging and PPD skin tests are not always sensitive or specific for \textit{M.tb} infection. Additionally, PPD tests may yield false positive results in BCG vaccinated individuals, or in people exposed to environmental mycobacteria due to shared antigens with \textit{M.tb}. Some limitations with these tests have been overcome by using IGRAs to detect \textit{M.tb} antigen specific IFN-\(\gamma\) from blood cell cultures, or to enumerate the frequency of \textit{M.tb} antigen specific IFN-\(\gamma\) producing cells. IGRAs show enhanced sensitivity and specificity over the PPD skin test, and they are not confounded by \textit{M. bovis} BCG vaccination. IGRAs might be able to discriminate TB disease states, and they may have the potential to predict disease outcome in an \textit{M.tb} infected individual (224, 225, 546). However, it is currently unknown whether IGRAs can indeed be used for risk assessment of reactivation TB. The complexity of interpreting TB diagnostic test results is summarized below, in Table 1.3.

<table>
<thead>
<tr>
<th></th>
<th>Primary TB</th>
<th>Latent TB</th>
<th>Reactivation TB</th>
<th>BCG vaccination</th>
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<td>\textit{M.tb} culture/smear</td>
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<td>Radiographs</td>
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<td>PPD</td>
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<td>IGRAs</td>
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<td>unknown</td>
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Table 1.3. Diagnostic test outcomes associated with TB disease and vaccination status.

\textit{Murine models of M.tb}

We have reviewed the murine model of \textit{M.tb} infection, and favor experimental approaches using multiple inbred mouse strains to identify immunological responses associated with either susceptibility, or resistance, to \textit{M.tb} (547). The majority of experimental research uses the C57BL/6 inbred strain to demonstrate immune mediators
of *M. tb* resistance, including IFN-γ, TNF, CD4, CD8, MHCII, MHCI, iNOS, IL-1, IL-6, IL-12, MyD88, and others. C57BL/6 mice are relatively resistant to *M. tb*, and they survive more than one year following a low dose aerosol of *M. tb*. In contrast, susceptible CBA/J, DBA/2 and C3H/HeJ mice survive approximately 100-200 days following *M. tb* infection. These mouse strains, and others, may model the heterogeneous immune responses that are observed in the genetically diverse human population. Because inbred mouse strains succumb to infection with *M. tb* in variable, but predictable, time periods, researchers may identify immune mechanisms that influence TB disease progression.

This strategy using resistant and susceptible mice can address specific clinical questions, and can generate hypotheses that may be tested *in vivo*. In Chapter 2, we show that susceptible mouse strains produce low levels of *M. tb* antigen specific IFN-γ, that is detectable from blood cells months prior to TB disease progression. These results illustrate that *M. tb* susceptibility has common immunologic outcomes, despite underlying genetic disparities. Furthermore, low *M. tb* antigen specific IFN-γ can be an accurate biomarker predicting heightened risk of active TB disease.

*M. tb* susceptible mice may also be used to identify, and test, immunotherapeutic targets. In Chapter 3, we show that CBA/J mice model reactivation TB in humans. By blocking IL-10 *in vivo*, TB disease outcome is improved. Our results also demonstrate that IL-10 increases shortly before reactivation TB in CBA/J mice. Abundant IL-10, therefore, may be used as a biomarker to predict progression to active TB in humans. In Chapter 4, we identify a third immune mediator, low CCL5, which is also associated with susceptibility to *M. tb* in CBA/J mice. Alone, insufficient CCL5 may not cause susceptibility to *M. tb*, however, low CCL5 may still be a useful biomarker to predict an
increased risk of TB disease. Identification of common immunologic mediators that contribute to \textit{M.tb} susceptibility may translate into useful clinical applications for prognosis of \textit{M.tb} infection and TB disease progression in humans.
CHAPTER 2: BLOOD INTERFERON-γ RELEASE ASSAYS PREDICT MYCOBACTERIUM TUBERCULOSIS SUSCEPTIBILITY AND PROTECTION BY SINGLE PROTEIN VACCINATION.

Abstract:

Current tests for M.tb cannot distinguish active from latent infection, nor can they predict the risk of disease progression in a latently infected person. Interest exists, however, in adapting antigen specific IGRAs to quantify immunological responses that may correlate with increased risk of disease progression, or quantify vaccine induced protective responses. In this study, we used the differential susceptibilities of inbred mouse strains to M.tb infection to evaluate the prognostic capabilities of IGRAs during primary M.tb infection and in M.tb challenge studies following vaccination. Using lung and blood cultures we determined that CBA/J, DBA/2, and C3H/HeJ mice (models of heightened risk of progression to active tuberculosis) produced less antigen specific IFN-γ in response to M.tb CFP, ESAT-6, and Ag85 during primary M.tb infection than the relatively resistant C57BL/6 mouse strain. Reduced IFN-γ secretion in supernatants reflected fewer responding lung and blood cells and additionally, on a single cell level, each individual cell produced less IFN-γ only when stimulated with M.tb antigens.

Our results from vaccination and M.tb challenge studies further showed that vaccination with Ag85 and ESAT-6 in susceptible CBA/J mice clearly induced protective
immunity as determined by reduced \( M.tb \) lung burdens. Furthermore, vaccine induced protective IFN-\( \gamma \) responses were significantly increased in lung and blood IGRAs. Although CBA/J mice had very poor responses to purified dominant antigens during primary \( M.tb \) infection, subcutaneous vaccination induced numerous IFN-\( \gamma \) secreting Ag85 specific and ESAT-6 specific lung cells. Furthermore, in susceptible CBA/J mice, vaccine induced protection was reflected by increased IFN-\( \gamma \) in the blood, reinforcing the concept that blood directly reflects lung responses. These are important findings with direct relevance to man, because our data suggests that TB disease susceptible people may respond favorably to \( M.tb \) single antigen booster strategies currently in human clinical trials.

These studies demonstrate that antigen specific IFN-\( \gamma \) from blood cultures accurately reflect lung responses, indicating that blood can be an appropriate test tissue in humans during primary \( M.tb \) infection, and for determining vaccine induced protection. During primary \( M.tb \) infection in mice, reduced antigen specific IFN-\( \gamma \) production and low frequencies of IFN-\( \gamma \) responding cells from peripheral blood predicted increased risk of TB disease progression across genetically diverse TB disease susceptible mouse strains, suggesting similar results may be true in humans. Furthermore, our results show that IGRAs which detect IFN-\( \gamma \) secretion into cell culture supernatants may lack sensitivity, and therefore may be unable to distinguish \( M.tb \) infected from non-infected individuals. This problem may be addressed by using diagnostic tests with enhanced sensitivity to confirm \( M.tb \) infection status such as those that detect IFN-\( \gamma \) secretion from single cells. Overall, development of efficacious predictive tests for humans could lead to
targeted therapy prior to progression to active TB, thus reducing transmission, incidence, and prevalence rates while maximizing the use of public health resources.

Introduction:

*M. tb*, the causative agent of TB, infects up to one third of the world’s population (548). The majority (90%) of infected adults control *M.tb* in a latent state that is clinically silent and not considered contagious. In contrast, the remaining 10% of *M.tb* infected individuals progress to contagious, active TB and may transmit bacilli to others, resulting in significant global morbidity and mortality each year (549). The mechanisms that contribute to *M.tb* susceptibility and TB disease progression in humans are multifactorial and reflect altered pulmonary immunity due to polygenic interactions, immune status, age and environmental factors (550, 551). These interactions contribute to the common outcome of active TB, associated with increased growth of *M.tb* in the lung and detrimental pulmonary inflammation, which are also common disease characteristics in animal models (516, 519, 552).

Although the predisposing mechanisms underlying progression to active TB are not fully understood, we and others (553-555) propose that the transition from latency to active TB can be detected, quantified and predicted by peripheral immune responses prior to disease onset. Identification of such biomarkers could then be used to quantify risk of TB disease progression, allowing targeted treatment of susceptible individuals. Current diagnostic tests including skin tests and IGRAs cannot accurately distinguish latent *M.tb* infection versus active TB nor can they assess the risk of TB disease progression (553, 554, 556). Research interest does exist, however, in longitudinal assessment of IGRAs for
TB prognostic applications in *M.tb* infected humans. Indeed, some evidence shows that IFN-γ responders stratify into high, middle, and low categories (553), suggesting that IGRA results could also assess risk of developing active TB disease.

Commercial IGRA\s quantify the amount of antigen specific IFN-γ in blood culture supernatants (QuantiFERON-TB Gold™, Cellestis Ltd, Carnegie, Victoria, Australia) or determine the frequency of IFN-γ producing blood leukocytes (T-SPOT.TB™, Oxford Immunotec, Oxford, UK) in response to specific mycobacterial peptides. IGRA peptides are synthetic immunogenic mycobacterial antigens including ESAT-6, CFP-10 and TB7.7. These proteins are almost exclusively expressed by members of the *M.tb* complex and not by environmental, opportunistic mycobacteria, or the vaccine strain *M. bovis* BCG. Immune responses to these peptides are highly sensitive and specific for *M.tb* infection (225), and they stimulate potent T cell responses in mice and man, particularly ESAT-6 (36, 37, 46, 557). It is currently unknown whether the magnitude of *M.tb* antigen specific responses could quantify risk of TB disease progression, or whether antigen specific IFN-γ from the blood accurately reflects responses within the lung. A better understanding of differential responses using IGRA\s in defined animal models of *M.tb* infection may lead to the identification of humans with predicted poor outcomes and holds promise for earlier, targeted therapy.

Using immunocompetent inbred mouse strains (C57BL/6, CBA/J, DBA/2, and C3H/HeJ) with known timelines of TB disease progression (558), we modified IGRA\s to assess antigen specific IFN-γ production from the peripheral blood as an immunological predictor of TB disease outcome. All analyses were performed on mice that had no outward signs of TB disease (i.e. normal body weight, normal hair coat and posture, and
normal socialization, etc) and stable bacterial loads within the lungs. The results, therefore, did not reflect morbidity-associated pulmonary inflammation. Our data demonstrate that mouse strains with reduced survival times following M. tb infection (558) failed to sustain or generate a robust antigen specific IFN-γ response to M. tb CFP, ESAT-6, and Ag85 in both lung and peripheral blood cultures. Most significant was that low IFN-γ production from blood cells was evident throughout the entire infection period in susceptible mice even in the absence of clinical signs, and prior to TB disease progression. Using short term infection studies, low antigen specific IFN-γ production was identified in two additional TB susceptible mouse strains, DBA/2 and C3H/HeJ.

The lungs of M. tb infected CBA/J mice contained few IFN-γ producing Ag85 or ESAT-6 specific CD4 T cells and each individual T cell produced less antigen specific IFN-γ than those from C57BL/6 mice. We therefore hypothesized that CBA/J mice had a limited capacity to generate protective immunity to these single antigens. To test whether CBA/J mice could generate Ag85 specific and ESAT-6 specific protective immune responses, CBA/J mice were vaccinated with purified Ag85 or ESAT-6 and challenged with M. tb. Indeed, the lungs of vaccinated CBA/J mice contained fewer M. tb bacilli during primary infection which was associated with significantly more IFN-γ secreting antigen specific cells. The amount of IFN-γ from blood IGRAs was also significantly increased in vaccinated CBA/J mice compared to controls, extending the relevance of these results to show that M. tb susceptible humans may also respond favorably to single protein vaccination strategies, and that blood protective responses mirror the lungs during M. tb challenge.
Overall, these data indicate that reduced antigen specific responses *ex vivo* can predict an increased susceptibility to TB disease progression in mice. Furthermore, these data demonstrate that the total amount of IFN-γ and the frequency of IFN-γ responding cells in the blood directly reflect immune responses in the lung. These results, therefore, define a simple blood assay as both an indicator of lung *M.tb* specific immunity, vaccine induced protection, and predictor of TB disease outcome.

**Materials and Methods:**

**Mice**

Specific pathogen free eight-week-old female C57BL/6 and CBA/J mice (Charles River Laboratories, Wilmington, MA) and specific pathogen free eight-week-old female C57BL/6J, DBA/2J and C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in ventilated cages within Biosafety Level-3 facilities 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.

**Mycobacterial stocks**

*M.tb* Erdman (ATCC #35801) and *M. bovis* BCG (ATCC #35734) were obtained from American Type Culture Collection (Manassas, VA.). Stocks were grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and frozen in 1ml aliquots at -80°C.
**M.tb infection**

Mice were exposed to an aerosol of *M.tb* Erdman using an Inhalation Exposure System (Glas-col, Terre Haute, IN), calibrated to deliver 50-100 CFU to the lungs of each individual mouse. Confirmation of *M.tb* dose and calculation of *M.tb* burden throughout the experimental infection period were determined by plating serial dilutions of partial lung homogenates onto OADC-supplemented 7H11 agar. *M.tb* CFUs were counted after 3 weeks at 37°C and transformed to a \( \log_{10} \) scale. Mice were weighed weekly. Mice weighing 20% less than the average of age- and sex-matched non-infected control animals with concurrent signs of disease progression (unthrifty hair coats, tachypnea, social isolation) were excluded from the study.

**Vaccination**

Selection of vaccination proteins, preparation and administration methods were modified from Deitrich *et al* (559) and Andersen (560). Briefly, vaccines were freshly prepared as follows: 250µg/ml of synthetic monophosphoryl lipid A (MPL) (Avanti Polar Lipids, Inc., Alabaster, AL) in 0.2% triethylamine in PBS was briefly heated to 70°C and then sonicated for 30 seconds. 2.5mg/ml of dimethyldioctadecyl ammonium bromide (DDA) (Sigma-Aldrich, Co., St. Louis, MO) in PBS was heated at 80°C for 10 minutes and then cooled. *M.tb* antigens at 100 µg/ml (Ag85 or ESAT-6) or equivalent volumes of PBS were added to the cooled DDA solution. Finally, equivalent volumes of the MPL solution and DDA solutions (with or without antigen) were mixed and gently agitated immediately prior to injection. Each CBA/J mouse was injected subcutaneously
interscapularly three times at two week intervals with 200µl (containing 25µg MPL, 250µg DDA, and 10µg of antigen). Unvaccinated control groups included CBA/J mice which received 200µl of PBS or 200µl of adjuvant. As a positive control for vaccination, separate groups of CBA/J mice received one interscapular injection of $2.5 \times 10^5$ *M. bovis* BCG bacilli in 200µl of PBS. Mice challenged with *M.tbc Erdman* by aerosol 10 weeks after the first injection.

**Lung cell isolation**

Single cell suspensions were obtained from the lungs at specific time points post infection (561). Briefly, lungs were perfused through the right cardiac ventricular lumen and pulmonary trunk with 10ml of phosphate buffered saline containing 50U/ml of heparin (Sigma; St. Louis, MO) and placed in Dulbecco’s modification of Eagle’s medium (DMEM, 500ml) (Mediatech; Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals; Fort Collins, CO), 1% HEPES buffer (Sigma), 10ml of 100X non-essential amino acid solution (Sigma), 5ml of penicillin/streptomycin solution (50,000U penicillin, 50mg streptomycin; Sigma) and 0.14% mercaptoethanol (complete DMEM). Single cell suspensions from individual mice were obtained by dicing lung lobes with sterile razor blades, followed by a 30 minute incubation at 37°C with 4ml of complete DMEM containing collagenase XI (0.7mg/ml; Sigma), and bovine pancreatic DNase (30µg/ml; Sigma). 6ml of complete DMEM was subsequently added to dilute enzymatic activity and lung pieces were pressed through sterile 70µm nylon mesh screens (BD Biosciences; San Jose, CA) to obtain a single cell
suspension. Residual red blood cells were lysed using 2ml of ACK lysis buffer (0.15 M \( \text{NH}_4\text{Cl} \), 1mM \( \text{KHCO}_3 \)) for 3 minutes at room temperature followed by washing with complete DMEM. Viable cells were counted using Trypan blue exclusion and re-suspended at working concentrations in complete DMEM.

**Blood collection**

200-300µl of blood was collected from the right cardiac ventricular lumen with a 22 gauge needle immediately following euthanasia and placed into 20U of heparin (Sigma) diluted in 10µl of sterile PBS. Heparinized whole blood was then diluted 1:10 (primary infection) or 1:5 (vaccine studies) in complete DMEM for use in modified antigen specific IGRAs. Blood leukocytes for flow cytometry and ELISpots were obtained by lysing red blood cells with two 4-minute room temperature incubations in ACK lysis buffer, separated by washes in 5-10ml of complete DMEM. Isolated blood leukocytes were counted by Trypan blue exclusion and re-suspended at working concentrations in complete DMEM for ELISPOTS or in FACS buffer (deficient-RPMI (Irvine Scientific; Santa Ana, CA) supplemented with 0.1% sodium azide (Sigma)) for 30 minutes at 4°C to fix cell surface membranes for flow cytometry.

**Bone marrow derived antigen presenting cells**

Bone marrow cells were obtained from the tibiae and femora of age and sex matched non-infected C57BL/6 and CBA/J mice. Cells were differentiated into antigen presenting cells (APCs) using complete DMEM supplemented with 10% conditioned
media derived from GM-EL4 cells, a GM-CSF producing clone kindly provided by Arthur A. Hurwitz (National Cancer Institute, NIH, Frederick, MD). The clone was created by the retroviral transfection described in (562). 1-2x10⁶ bone marrow cells were plated at 37°C in 1ml of GM-EL4 conditioned media in sterile 24-well tissue culture plates. An additional 1ml GM-EL4 conditioned media was added on day 1. GM-EL4 conditioned media was replaced on days 3, 5 and 7. On day 8, differentiated APCs were briefly centrifuged and the GM-EL4 conditioned media removed. Cells were washed and stimulated for 24 hours with 1ml of 50pg/ml endotoxin-free recombinant murine IFN-γ (Peprotech, Rocky Hill, NJ) in complete DMEM. On day 9, APCs were washed in complete DMEM to remove IFN-γ and pulsed for 24 hours with 1ml of the following antigens in complete DMEM: 10µg/ml of ovalbumin (Sigma), 10µg/ml of M.tb CFP, 5µg/ml of M.tb ESAT-6, or 5µg/ml of M.tb Ag85 complex. On day 10, APCs were washed to remove excess antigen, harvested, counted, and re-suspended at working concentrations in complete DMEM. M.tb CFP, M.tb Ag85 and M.tb ESAT-6 proteins were obtained through Colorado State University’s NIH NIAID Contract No. HHSN266200400091C. In some experiments, recombinant M.tb ESAT-6 was expressed and purified from plasmids obtained through Colorado State University’s NIH NIAID Contract No. HHSN266200400091C, as described below.

Expression and purification of recombinant M.tb ESAT-6

Competent BL21 Star (DE3) pLySs E.coli (Invitrogen, Carlsbad, CA) were transformed as per the manufacturer’s instructions with the HIS-tagged esat-6 containing
plasmid pMRLB.7 and plated onto LB agar plates with ampicillin (100µg/ml) plus chloramphenicol (34µg/ml) and grown overnight. 5-10 colonies of positive transformants were inoculated into 40 ml LB broth plus ampicillin (100µg/ml) and chloramphenicol (34µg/ml) and grown overnight at 37 ºC. Seed cultures were used to inoculate 2 L of LB broth plus ampicillin (100µg/ml) and chloramphenicol (34µg/ml) and grown with shaking at 37ºC to an O.D. of approximately 0.5-0.6. Cultures were then rapidly cooled on ice to room temperature, 0.25mM IPTG added to induce esat-6 gene expression, and grown overnight at 25ºC with shaking. Cultures were centrifuged at high speed, the pellet resuspended with protease inhibitors (Roche, Indianapolis, IN), DNase (Sigma-Aldrich) and lysozyme (Sigma-Aldrich) and incubated for 15 minutes at 30ºC prior to lysis by sonication. Lysed bacteria were then centrifuged for 90 minutes at 16,000g and the lysate passed over poly-prep chromatography columns (BioRad, Hercules, CA) with equilibrated His-binding resin (Novagen, San Diego, CA) as per the manufacturer’s instructions. Following washes with 10nM Tris-HCL and application of 0.5% ASB for endotoxin removal and more washes with 10nM Tris-HCL, the purified protein was eluted with 1M imidazole. Residual detergents and salts were removed by dialysis (Slide-A-Lyzer dialysis chambers, Pierce, Rockford, IL) for 48-72 hours in 4 L of 10mM ammonium bicarbonate with complete buffer changes every 6-12 hours. The protein concentration in dialyzed samples was then determined by BCA Assay (Pierce), checked for size using a low molecular weight ladder (Benchmark Pre-Stained Protein Ladder (Invitrogen) on a 16.5% Tris-tricine gel (BioRad) stained with Code Blue (Pierce). Remaining purified ESAT-6 was lyophilized and aliquots tested for endotoxin using the Limulus Amoebocyte Lysate assay as per the manufacturer’s instructions (Endochrome-
K Endosafe Kit, Charles River, Wilmington, MA). Endotoxin levels did not exceed 0.6 ng/mg of protein, substantially less that the quality control standard (10 ng/mg) recommended by Colorado State University. Batches of in-house purified ESAT-6 were then compared to ESAT-6 from Colorado State University in multiple types of cell culture assays and no significant differences were noted (data not shown).

**Lung and blood modified IGRAs by ELISA**

100µl of lung cell suspensions containing 2x10⁵ cells or 100µl aliquots of diluted blood from each individual mouse were cultured in duplicate in 96-well sterile tissue culture plates (BD Falcon Microtest™, Fisher Scientific). 100µl of antigens in complete DMEM were added to cultures, resulting in a final volume of 200µl per well and final antigen concentrations of ovalbumin (10µg/ml; Sigma), *M.tb* CFP (10µg/ml), *M.tb* ESAT-6 (5µg/ml), *M.tb* Ag85 complex (5µg/ml), or concanavalin-A (10µg/ml; Sigma). Cultures were incubated for 72 hours at 37°C with 5% CO₂ and supernatants were stored at -80°C.

IFN-γ was quantified in lung cell and whole blood culture supernatants by ELISA using antibody pairs (BD Biosciences). High-binding 96-well round bottom plates (Nunc Maxisorb Immunoplates, Fisher Scientific) were coated overnight with anti-IFN-γ (R4-6A2), and wells blocked with 10% bovine serum albumin in PBS or complete DMEM containing heat-inactivated 10% fetal calf serum for 2 hours at room temperature. Samples from individual mice or recombinant murine IFN-γ standards (BD Biosciences) were dispensed in duplicate and incubated for 2 hours at room temperature. IFN-γ was
detected with secondary biotinylated anti-IFN-γ (XMG1.2) for 1 hour at room temperature followed by a 30 minute room temperature conjugation with streptavidin horseradish peroxidase (Invitrogen, Carlsbad, CA). Detection was quantified with 3,3’,5,5’-tetramethylbenzidine substrate (DAKO, Carpinteria, CA) and reactions stopped with 0.18M H₂SO₄. Positive samples were quantified by optical density at 450nm wavelength absorbance with 570nm correction and compared to the linear standard curve (ng/ml).

**Lung and blood modified IGRAs by ELISPOT**

At each time point, 6 mice per inbred strain were euthanized and non-adherent lung cells pooled in groups of 2 for CD4 purification using magnetic MACS LS columns and anti-CD4 beads (Miltenyi Biotech, Auburn, CA) as per the manufacturer’s instructions. Prior to labeling with anti-CD4 beads, adherent cells from each individual mouse were removed by incubation in 100mm sterile tissue culture petri dishes for 1 hour at 37°C. CD4 T cell purity was confirmed by flow cytometry (93.5 ± 1.7% of the purified lung cells were CD3⁺CD4⁺). Purified cells from each pair of mice were serially diluted 1:2 in complete DMEM onto pre-coated, blocked, sterile ELISPOT plates as per the manufacturer’s instructions (Ready-Set-Go Mouse IFN-γ ELISPOT kit, eBioscience, San Diego, CA). At least 6 serial dilutions were performed for lung cells. Following serial dilutions, 2.5x10⁴ antigen-pulsed bone marrow derived APCs were added to each well.

Pooled samples from 6 mice per strain were used for ELISPOTs with blood leukocytes. Following red blood cell lysis, leukocytes were counted by Trypan blue exclusion, re-suspended in complete DMEM and serially diluted 1:2 in triplicate onto
pre-coated (Ready-Set-Go Mouse IFN-γ ELISPOT kit, eBioscience), blocked, sterile ELISPOT plates (Millipore, Billerica, MA). At least 3 serial dilutions were performed for blood leukocytes. Following serial dilutions, 2.5x10⁴ antigen-pulsed bone marrow derived APCs were added to each well.

For both lung CD4 and blood leukocyte ELISPOTs, plates were incubated for 36 hours at 37°C. IFN-γ spot forming units were detected and developed as per the manufacturer’s instructions (eBioscience, Ready-Set-Go Mouse IFN-γ ELISPOT kit). Spots were counted and analyzed using the Immunospot Analyzer (C.T.L., Cleveland, OH). The numbers of antigen specific IFN-γ spot forming units were determined by subtracting the number of spots formed in the presence of a negative control antigen (ovalbumin).

**Flow cytometry**

All conjugated and unconjugated antibodies and isotype controls were purchased from BD Biosciences, including Fc Block™ (clone 2.462), PerCP-Cy5.5 anti-CD3ε (145-2C11), APC-Cy7 anti-CD4 (GK1.5), PE-Cy7 anti-CD8 (53-6.7), APC anti-TCR-β chain (H57597), PerCP anti-CD8 (53-6.7), PE-Cy7 anti-IFN-γ (XMG1.2), PE-Cy7 rat IgG1,κ isotype control, FITC anti-I-A/I-E (2G9), and FITC rat IgG2a,κ isotype control. Analyses were performed on lung cells or blood leukocytes from individual mice, except for Figures 3C, 3D, 5B, 5D, and 5F (groups of 6 pooled blood samples) and Figures 5A, 5C, and 5E (3 groups of 2 pooled lung CD4 T cells). For surface staining, aliquots of lung cell suspensions were adjusted to 4x10⁶ cells/ml and placed immediately into 1ml of
FACS buffer, and blood leukocytes were fixed in 500µl of FACS buffer, for 30 minutes at 4°C. For intracellular cytokine staining, separate aliquots of lung cells from individual mice or isolated blood leukocytes from pooled mice were adjusted to 2x10^6 cells/ml in complete DMEM and stimulated with 1µg/ml of purified anti-CD3ε plus 0.1µg/ml of purified anti-CD28, and incubated with GolgiStop® for 4 hours at 37°C as per the manufacturer’s instructions for inhibition of cytokine transport (Cytofix/Cytoperm kit; BD Biosciences). At the conclusion of 4 hours, cells were fixed in FACS buffer for 30 minutes at 4°C.

Following fixation, approximately 5x10^5 to 1x10^6 of lung cells or blood leukocytes were incubated with 0.31µg of Fc Block™ for 10-15 minutes at room temperature, to minimize non-specific antibody binding. Samples were then incubated with 0.31µg of fluorescent antibodies in the dark for 20 minutes at 4°C followed by 3 washes in FACS buffer. Lymphocytes were identified according to characteristic forward and side scatter profiles and 20,000-50,000 events were counted within the lymphocyte gate using a LSRII flow cytometer. CD4 T cells were identified based upon CD3 or TCRβ in combination with CD4 expression. Results were analyzed with FACSDiva software (BD Biosciences). Isotype controls were included for each mouse strain at each time point and used to set gates for analysis.

**Statistics**

Statistical analyses were performed using Prism 4 software (GraphPad Software, San Diego, CA). Multigroup comparisons were analyzed with one-way ANOVA using
Dunnett’s post-test (comparison to non-infected control mice within each strain over time) or Tukey’s post-test (comparison of all groups). Pairwise comparisons between strains within time points used the Student’s \( t \)-test. Statistical significance in all analyses was defined as \(*p<0.05, **p<0.01, ***p<0.001\). Where necessary, one outlier per strain per time point was identified using the Grubb’s outlier test (GraphPad QuickCalcs, [http://www.graphpad.com/quickcalcs](http://www.graphpad.com/quickcalcs)) and removed.

For vaccination studies if no statistically significant differences were detected between PBS and adjuvant control groups, the data were combined and referred to as “Control.”

**Results:**

**CBA/J mice maintain stable \( M.tb \) lung burden with no outward signs of TB disease.**

The ability of inbred mouse strains to survive \( M.tb \) infection segregates into TB disease resistant and susceptible clusters with known survival times (516, 518, 519, 558, 563-566). C57BL/6 mice survive \( M.tb \) infection into their second year of life and are considered relatively resistant (518, 563). In contrast, CBA/J mice are more susceptible to \( M.tb \) infection with significantly decreased survival (558 and Figure 2.1A). Infection in CBA/J mice is associated with a persistently elevated \( M.tb \) bacterial load in the lung with early death due to overwhelming pulmonary disease (516, 519, 564).

In this study, we analyzed immune responses during a period of \( M.tb \) infection when the majority of CBA/J mice showed no outward signs of disease and compared responses to the relatively resistant C57BL/6 mouse strain. Throughout the experimental
time frame (up to 150 days post infection), the majority of \textit{M.tb} infected CBA/J mice exhibited normal weight gain relative to non-infected age- and sex-matched controls (Figure 2.1B). In these experiments, a small proportion of CBA/J mice developed morbidity prior to 150 days of infection and these mice were excluded. All C57BL/6 mice appeared clinically healthy and gained weight at the same rate as non-infected age-and sex-matched control mice over 150 days of \textit{M.tb} infection (data not shown). Despite remaining clinically healthy, CBA/J mice had significantly more \textit{M.tb} CFU recovered from the lungs than C57BL/6 mice at all time points tested (Figure 2.1C).
Figure 2.1. Asymptomatic infection in M.tb susceptible CBA/J mice. C57BL/6 and CBA/J mice were infected with 50-100 CFU of M.tb Erdman by aerosol. Mice were euthanized when signs of morbidity developed (A) and survival results were analyzed by the Logrank test, ***p<0.001. In independent experiments M.tb infected and non-infected age- and sex- matched control CBA/J mice were weighed weekly (B) and results are the average ± SD from 2 independent experiments using 10-52 M.tb infected mice and 4-10 non-infected mice per time point. Weights were analyzed by one-way ANOVA with Tukey's post test. No statistical differences were found. C57BL/6 and CBA/J mice were euthanized at various time points and M.tb CFUs determined in the lungs (C). CFU results are the average ± SD from 2-5 independent experiments each with 4-5 mice per strain per time point, analyzed by one way ANOVA with Tukey's post test, *p<0.05, **p<0.01, ***p<0.001.
A. Percent survival over time post infection (days) for C57BL/6 and CBA/J mice.

B. CBA/J body weight (gm) comparison between non-infected and M.tbc infected mice.

C. Log$_{10}$ M.tbc CFU from lungs over time post infection (days) for C57BL/6 and CBA/J mice.
Low antigen specific IFN-γ secretion from CBA/J lung cells.

Lung cells from infected C57BL/6 and CBA/J mice were cultured with *M. tb* CFP, ESAT-6 or Ag85 to determine antigen specific IFN-γ secretion *ex vivo*. Lung cells from C57BL/6 mice produced abundant IFN-γ in response to CFP (Figure 2.2A) and ESAT-6 (Figure 2.2B) and these responses were sustained throughout the 150 days of *M. tb* infection. When C57BL/6 lung cells were stimulated with Ag85 (Figure 2.2C), however, IFN-γ secretion peaked at 21 days and then declined to levels that were higher than naïve mice but did not achieve statistical significance using Dunnett’s post-test.

In contrast to C57BL/6 mice which had strong sustained responses, lung cells from susceptible CBA/J mice transiently responded to *M. tb* CFP with peak secretion at day 21 post infection (Figure 2.2A). Although this initial protective response was equally potent to C57BL/6 cells and may have stabilized bacterial growth, IFN-γ production declined over time in CBA/J mice. Furthermore, as infection progressed beyond day 60 into the chronic phase of *M. tb* infection, CFP specific IFN-γ secretion from CBA/J lung cells was significantly less than C57BL/6 mice. ESAT-6 induced weak antigen specific IFN-γ from CBA/J lung cells with a small peak at day 60 (Figure 2.2B) and, similar to CFP specific responses, was not sustained. At all time points tested, ESAT-6 specific IFN-γ production was significantly reduced in lung cell cultures from CBA/J mice compared to C57BL/6 mice. In addition, lung cells from CBA/J mice secreted little Ag85 specific IFN-γ, resulting in a small peak at day 21, and at all time points was significantly less than IFN-γ from C57BL/6 lung cells. In fact, when both ESAT-6 and Ag85 IFN-γ secretion from *M. tb* infected mice was compared to naïve CBA/J mice, levels were only marginally elevated.
These data demonstrate that predicted TB disease progression and decreased survival in susceptible CBA/J mice following *M.tb* infection are associated with reduced antigen specific immune responses throughout early and chronic, asymptomatic pulmonary infection. Poor antigen specific IFN-γ, in particular to the dominant *M.tb* antigens ESAT-6 and Ag85 from the primary site of *M.tb* infection can therefore predict accelerated TB disease progression in mice. Because lung cells are not readily available for testing from *M.tb* exposed or infected humans, we subsequently determined whether blood was an appropriate surrogate tissue for lung immune responses.
Figure 2.2. Antigen specific IFN-γ production from lung cells. C57BL/6 and CBA/J mice were infected with 50-100 CFU of M.tb Erdman by aerosol. 2x10^5 lung cells from non-infected (naïve) and M.tb infected mice were cultured with M.tb CFP (A), M.tb ESAT-6 (B) or M.tb Ag85 (C) at the indicated time points. Results are the mean ± SEM of 4 (A, C) or 2 (B) independent experiments with 4-5 mice per strain per time point. Data were analyzed by mouse strain with comparison to non-infected controls by one-way ANOVA with Dunnett’s post test, *p<0.05; **p<0.01, ***p<0.001, ND = no significant difference. Pair wise comparisons between C57BL/6 and CBA/J mice at each time point were analyzed by Student’s t-tests, *p<0.05, ++p<0.01, +++p<0.001. IFN-γ production in response to ovalbumin (negative control) did not exceed 0.12 ± 0.3 ng/ml.
Antigen specific IFN-γ responses from blood are accurate indicators of lung responses.

*M. tb* IGRAs approved for human diagnostics use heparinized whole blood or freshly isolated peripheral blood mononuclear cells co-cultured with *M. tb* antigens. We modified IGRAs for use in *M. tb* infected C57BL/6 and CBA/J mice by stimulating diluted heparinized whole blood with the *M. tb* antigens: CFP, ESAT-6, and Ag85. Blood samples were analyzed in parallel to the lung cultures.

Antigen specific IFN-γ from blood samples generated similar profiles to that observed from isolated lung cells. Blood cell cultures from C57BL/6 mice produced abundant and sustained IFN-γ to *M. tb* CFP (Figure 2.3A) and ESAT-6 (Figure 2.3B) throughout 150 days of infection, while Ag85 (Figure 2.3C) IFN-γ peaked at 21 days of *M. tb* infection. ESAT-6 specific IFN-γ production from blood cell cultures was elevated after 21 days infection in comparison to the lung but was then comparable between 60 and 150 days. Therefore, data indicate that for the relatively resistant C57BL/6 mouse strain, antigen specific IFN-γ production from blood cell cultures directly reflects the immune status of the lung and can be used to predict disease outcome, i.e. long term control of *M. tb*.

Minimal IFN-γ was secreted by blood cell cultures from CBA/J mice in response to *M. tb* CFP (Figure 2.3A), ESAT-6 (Figure 2.3B), and Ag85 (Figure 2.3C). Similar to what was observed for lung cell cultures (Figures 2.2), IFN-γ production from blood cultures of CBA/J mice was significantly less than that observed in C57BL/6 blood cultures. Furthermore, comparison of CBA/J lung and blood profiles showed that antigen specific IFN-γ production from blood was reduced, often to levels that were statistically
equivalent to non-infected (naïve) control animals. Overall, these data show that blood cultures from susceptible CBA/J mice reflect lung specific immunity and due to the substantially reduced responses, the blood may be a better predictor of disease outcome. Furthermore, these results indicate tests with enhanced diagnostic sensitivity may be required to discriminate between *M.tb* infected and non-infected individuals.
Figure 2.3. Antigen specific IFN-γ production from blood. C57BL/6 and CBA/J mice were infected with 50-100 CFU of M.tb Erdman by aerosol and 1:10 diluted whole blood from non-infected (naïve) and infected mice at specific time points post infection were cultured with M.tb CFP (A), M.tb ESAT-6 (B) or M.tb Ag85 (C). Results are the mean ± SEM of 4 (A, C) or 2 (B) independent experiments with 4-5 mice per strain per time point. Data were analyzed by mouse strain with comparison to non-infected controls by one-way ANOVA with Dunnett’s post test, *p<0.05; **p<0.01, ***p<0.001, ND = no significant difference. Pair wise comparisons between C57BL/6 and CBA/J mice at each time point were analyzed by Student’s t-tests, +p<0.05, ++p<0.01, +++p<0.001. IFN-γ production in response to ovalbumin (negative control) did not exceed 0.02 ± 0.07 ng/ml.

It is widely accepted that CD4 T cells are the dominant source of protective antigen specific IFN-γ during M.tb infection (567). We therefore determined whether
poor antigen specific IFN-γ production from the lungs and blood of CBA/J mice was associated with inadequate numbers or function of CD4 T cells.

**Number and function of CD4 T cells in the lungs and blood**

Although primary genetic defects (550), HIV infection (568) and pharmacologic (569) immunosuppression increase the risk of TB disease progression, most active cases are not attributable to any of these immunodeficiencies (550, 570, 571). Furthermore, the majority of TB patients appear to have intact acquired immune responses and produce IFN-γ to *M. tb* antigens and unrelated antigens (317, 572). In conjunction with gene disrupted mice (215, 389), these observations suggest that a critical mass of functional CD4 T cells is optimal for *M. tb* control. To determine whether increased risk of TB disease progression in CBA/J mice was associated with low CD4 T cell numbers in the lungs or blood, CD4 T cell numbers were determined. The proportion of CD4 T cells in the lungs of *M. tb* infected CBA/J mice was significantly less than that of C57BL/6 mice throughout the 150 day study (Figure 2.4A), leading to fewer total CD4 T cells recovered from the lungs (Figure 2.4B) and approximately 25% fewer CD4 T cells in each cell culture well analyzed (data not shown). Although the proportion of CD4 T cells in the blood was increased in CBA/J mice as compared to C57BL/6 mice (Figure 2.4C), the absolute number of CD4 T cells was less (Figure 4D) due to fewer leukocytes recovered from equivalent volumes of blood. These data indicate that reduced numbers of lung and circulating CD4 T cells may contribute to poor TB disease outcome.
Figure 2.4. CD4 T cells in the lung and blood. C57BL/6 and CBA/J mice were infected with 50-100 CFU of *M. tb* Erdman by aerosol. Lung and blood cells were labeled with fluorescent anti-CD3 or anti-TCRβ and anti-CD4 and quantified using flow cytometry. The proportion (A) and absolute numbers (B) of lung CD4 T cells from C57BL/6 and CBA/J mice are shown throughout infection. The proportion (C) and absolute numbers (D) of blood CD4 T cells are shown from day 21 post infection. Results are the mean ± SEM of 4 separate experiments (A, B) each with 4-5 mice per strain per time point. Results shown in C and D are the mean ± SEM from 3 independent experiments using blood from pooled samples. Data were analyzed by pair wise comparisons between strains at each time point using the Student’s *t*-test, *p*<0.05, **p*<0.01, ***p*<0.001.

To determine whether CD4 T cells from CBA/J mice had the potential to respond in an antigen dependent manner during infection, lung and blood cells were stimulated with anti-CD3 and anti-CD28 for 4 hours with GolgiStop™ and accumulated intracellular
IFN-γ quantified by flow cytometry. In lung (Figure 2.5A) and blood (Figure 2.5B) cell cultures the proportion of CD4 T cells from CBA/J mice that produced IFN-γ in response to TCR stimulation was approximately one-half of that from C57BL/6 mice. Therefore, CBA/J mice had fewer CD4 T cells capable responding to TCR mediated stimulation during *M.tb* infection, indicating that CBA/J mice generate or maintain a smaller pool of CD4 T cells that were capable of TCR-mediated IFN-γ secretion.

**Figure 2.5. TCR stimulated IFN-γ producing CD4 T cells from lung and blood.** C57BL/6 and CBA/J mice were infected with 50-100 CFU of *M.tb* Erdman by aerosol. Lung cells (A) or blood leukocytes (B) were stimulated with anti-CD3 and anti-CD28 to cross link and induce TCR signaling, labeled with fluorescent anti-TCRβ and anti-CD4, permeabilized, and labeled with anti-IFN-γ. Intracellular IFN-γ was quantified by flow cytometry for IFN-γ⁺ CD4 T cells. Lung results are the mean ± SEM from 4 independent experiments, each with 4-5 mice per strain per time point, analyzed by strain over time using one-way ANOVA with Dunnett’s post test compared to non-infected mice, *p*<0.05; ***p*<0.001. Direct pair wise comparisons between C57BL/6 and CBA/J mice at each time point were analyzed by Student’s *t*-tests, †*p*<0.05, ‡*p*<0.01, ‡‡*p*<0.001. Blood results are from 2 experiments with 6 mice per strain per time point, analyzed by strain over time using one-way ANOVA with Tukey’s post test. Average isotype control levels for lung CD4 T cells and blood CD4 T cells were 0.38% and 0.46%, respectively.
Because cross-linking with anti-CD3 and anti-CD28 measured the maximal potential for IFN-γ production from T cells triggered by TCR signaling, we next determined whether susceptible CBA/J mice had fewer *M.tb* specific IFN-γ responding cells compared to C57BL/6.

**Decreased frequency of *M.tb* antigen specific T cells in CBA/J mice**

To confirm that the low levels of antigen specific IFN-γ produced by lung and blood cells in culture from CBA/J mice were attributable to reduced numbers of antigen specific cells *in vivo*, we performed ELISpot assays and calculated the frequency of cells capable of secreting IFN-γ in response to *M.tb* CFP, ESAT-6, and Ag85. As anticipated, CBA/J mice had significantly fewer CD4 T cells that could secrete IFN-γ in response to CFP (Figure 2.6A), ESAT-6 (Figure 2.6C), and Ag85 (Figure 2.6E) compared to C57BL/6 mice. Isolated blood leukocytes from CBA/J mice also showed a significantly reduced frequency of IFN-γ responding cells to CFP (Figure 2.6B), ESAT-6 (Figure 2.6D), and Ag85 (Figure 2.6E) compared to blood cells from C57BL/6 mice. ELISpot analysis therefore confirms that CBA/J mice generate or sustain fewer antigen specific CD4 T cells within the lungs and in circulation during *M.tb* infection. To maintain the integrity of the IGRA ELISpot model, CD4 T cells were not purified from blood samples however, depletion of CD4 T cells from *ex vivo* cultures has been shown to nearly abrogate IFN-γ detection (46). These data demonstrate that increased risk of TB disease progression is associated with a reduced precursor frequency of antigen specific CD4 T cells in the lung and the peripheral blood, providing further evidence that antigen specific responses in the blood accurately reflect immune events within the lungs.
Figure 2.6. Frequency of antigen specific IFN-γ producing cells from lungs and blood. C57BL/6 and CBA/J mice were infected with 50-100 CFU of *M.tb* Erdman by aerosol. Purified lung CD4 T cells (A, C, E) and blood leukocytes (B, D, F) were stimulated with *M.tb* CFP (A, B), ESAT-6 (C, D) or Ag85 (E, F) pulsed bone marrow derived antigen presenting cells for 36 hours. IFN-γ spot forming units were counted and the frequency of antigen specific cells was calculated per 100,000 cells by subtracting spots formed in the presence of negative control antigen (ovalbumin). Results shown are the mean ± SEM of 3 (A, B, E, F) or 2 (C, D) independent experiments with 6 mice per strain per time point analyzed by strain over time using one-way ANOVA with Tukey’s post test.
Figure 2.6
Reduced M.tb antigen specific IFN-γ from individual CBA/J lung and blood cells

Our prior results (Figures 2.2 and 2.6) showed significant reductions in total secreted IFN-γ in lung and blood cell culture and significantly fewer M.tb CFP, ESAT-6 and Ag85 specific IFN-γ producing cells from susceptible CBA/J mice compared to C57BL/6 mice. We next addressed single cell production of IFN-γ. This was an important question because the amount of IFN-γ secreted by an individual cell could contribute to macrophage activation within the immunological synapse (573). The ELISPOT IFN-γ spot sizes were measured to determine IFN-γ production per cell. The results show that individual lung CD4 T cells (Figures 7A and 7B) and blood cells (Figures 2.7C and 2.7D) from CBA/J produced less M.tb ESAT-6 and Ag85 specific IFN-γ than cell from C57BL/6 mice, which was statistically significant in all conditions, except day 21 from blood cells. These results confirmed that individual cells from CBA/J mice produced less M.tb antigen specific IFN-γ than C57BL/6 cells and therefore each cell had a diminished capacity to respond protectively when exposed to its antigen.
Figure 2.7. Amount of M.tb antigen specific IFN-γ produced per cell. C57BL/6 and CBA/J mice were infected with 50-100 CFU of M.tb Erdman by aerosol. Purified lung CD4 T cells (A, B) and blood leukocytes (C, D) were stimulated with ESAT-6 (A, C) or Ag85 (B, D) pulsed bone marrow derived antigen presenting cells for 36 hours. The size of IFN-γ spot forming units was measured. Results shown are the mean ± SEM of 3 (B, D) or 2 (A, C) independent experiments with 6 mice per strain per time point analyzed at each time point using Student’s t-tests (*p<0.05, **p<0.01, ***p<0.001).
Thus far, our results show that *M. tb* susceptible CBA/J mice produce less *M. tb* antigen-specific IFN-\(\gamma\) in the lungs and blood by two mechanisms: 1) a decreased frequency of *M. tb* antigen specific IFN-\(\gamma\) producing cells, and 2) each individual responding cell secreted significantly less *M. tb* antigen specific IFN-\(\gamma\) than cells from C57BL/6 mice. Overall, these results show that an increased risk of TB disease progression in CBA/J mice is associated with low IFN-\(\gamma\) production, particularly when initiated by *M. tb* antigen stimulation. In these assays, however, we have not ruled out a general deficiency in IFN-\(\gamma\) production by CBA/J mice or whether TCR stimulation and signaling from individual CBA/J cells respond with abundant IFN-\(\gamma\) secretion.

To determine whether CBA/J mice had a general defect in IFN-\(\gamma\) secretion, lung (Figure 2.8A) and blood (Figure 2.8B) cells from CBA/J and C57BL/6 mice were nonspecifically stimulated with concanavalin-A. The results showed that *M. tb* susceptible CBA/J mice were fully capable of IFN-\(\gamma\) secretion from both lung and blood cells which at some time points was significantly higher than C57BL/6 mice. These data indicate that increased *M. tb* susceptibility in CBA/J mice is not due to a simple inability to produce IFN-\(\gamma\).

Although cells from CBA/J mice did not have general defects in IFN-\(\gamma\) secretion (Figures 2.8), it was possible, however, that CBA/J mice had specific defects in upstream events of IFN-\(\gamma\) secretion, such as the signaling pathways originating from TCR stimulation. To determine whether individual CD4 T cells from CBA/J mice had defects in TCR mediated intracellular signaling leading to IFN-\(\gamma\) production, cell cultures were stimulated with anti-CD3 and anti-CD28 to provide TCR crosslinking and costimulation. This experiment controlled for potential variation in antigen processing and/or
presentation between CBA/J and C57BL/6 mice while providing a maximal TCR specific stimulus. This was important because we have observed that CBA/J CD11c+ lung macrophages expressed significantly less surface MHCII on lung macrophages than C57BL/6 mice during M.tb infection (data not shown). Importantly, the results showed that individual CD4 T cells from CBA/J mice produced equivalent amounts of IFN-γ as C57BL/6 cells (Figure 2.8C), These results indicate, therefore, that TCR mediated signaling pathways leading to IFN-γ production are fully intact in cells from CBA/J mice. Because these results focused on the amount of IFN-γ produced by individual CD4 T cells, they do not contradict the previous analysis (Figure 2.5) which showed a reduced frequency of IFN-γ+ CD4 T cells. Overall, these results confirm that cells from M.tb infected CBA/J mice do not have general or TCR-mediated deficiencies in IFN-γ production on an individual cell level. They furthermore indicate that only M.tb infection antigen specific deficiencies in IFN-γ production are associated with increased risk of TB disease progression in CBA/J mice.
Figure 2.8. General and TCR-specific IFN-γ production from lungs and blood. C57BL/6 and CBA/J mice were infected with 50-100 CFU of M.tb Erdman by aerosol and euthanized at the indicated time points. 2x10^5 lung cells (A) or 1:10 diluted whole blood (B) were stimulated with concanavalin-A and IFN-γ in supernatants was quantified by ELISA. Results are the mean ± SEM of 4 independent experiments each with 4-5 mice per strain per time points. Data were analyzed by one-way ANOVA with Tukey’s post-test (no significant differences) and Student’s t-tests at each time point (*p<0.05). Lung cells were stimulated with anti-CD3 and anti-CD28 plus monensin for four hours to allow accumulation of intracellular IFN-γ. Cells were then labeled with fluorescent anti-TCRβ, anti-CD3, permeabilized and labeled with fluorescent anti-IFN-γ for flow cytometry. Fold change in mean fluorescence intensity (C) was calculated by dividing the average MFI of the CD4 T cell IFN-γ^+ population by the MFI of the negative population.
Overall, we have shown that increased susceptibility to TB disease progression in CBA/J mice was associated with a reduced frequency of protective cells and, at the single cell level, that responding cells produced less *M.tb* specific IFN-γ. Furthermore, the results in the blood mirrored the lung responses, indicating that the immune response in the blood accurately reflects the lung, and therefore blood can be a substitute tissue to test lung specific immunity man. Finally, our results in CBA/J mice showed that *M.tb* susceptibility and increased risk of TB disease progression may be accurately detected immediately following *M.tb* infection and months prior to disease onset.

**Reduced antigen specific IFN-γ production is common amongst *M.tb* susceptible mouse strains.**

To determine whether reduced antigen specific IFN-γ can be used as a common predictor of TB disease outcome, we analyzed two additional *M.tb* susceptible mouse strains (519, 574). Modified IGRAs were performed on lung and blood samples from DBA/2 and C3H/HeJ mice and responses compared to C57BL/6 mice. As previously observed in CBA/J mice, low *M.tb* CFP, ESAT-6, and Ag85 IFN-γ responses accurately predicted an increased risk of TB disease progression from both blood (Figures 2.9A, 2.9C, 2.9E) and lung cultures (Figures 2.9B, 2.9D, 2.8F) during asymptomatic infection. Blood and lung cultures from DBA/2 and C3H/HeJ produced abundant IFN-γ when stimulated non-specifically with concanavalin-A (data not shown), indicating no general defects in IFN-γ production in either mouse strain. These results indicate that a reduced antigen specific IFN-γ production and responses to the known immunodominant antigens
ESAT-6 and Ag85 in particular are common immunological outcomes among susceptible mouse strains during early *M. tb* infection. Overall, these data indicate that blood derived immunological biomarkers may predict both lung responses and TB disease outcome in primary *M. tb* infection across genetically disparate individuals.
Figure 2.9. Antigen specific IFN-γ from blood and lungs. C57BL/6, DBA/2 and C3H/HeJ mice were infected with 50-100 CFU of M. tb Erdman by aerosol. 1:10 diluted whole blood (A, C, E) or 2x10⁵ lung cells (B, D, F) from mice at day 21 post infection were cultured with M. tb CFP (A, B), ESAT-6 (C, D), or Ag85 (E, F) and IFN-γ quantified in the supernatants. Results are the mean ± SEM of 2 independent experiments each with 4-5 mice per strain for blood and 1 experiment with 5 mice per strain for lungs. Data were analyzed by one-way ANOVA with Dunnett’s post-test, *p<0.05; **p<0.01 compared to C57BL/6. Average IFN-γ production to ovalbumin (negative control) was 0.003 ng/ml (blood) and 0.7ng/ml (lung).
Figure 2.9
**Vaccination protects CBA/J mice from M.tb aerosol challenge**

In addition to using IGRAs to predict the risk of TB disease progression in humans, there is also interest in using these assays to quantify BCG specific immune responses (575, 576). We further propose that IGRAs could also quantify vaccine specific protection during *M.tb* challenge, and thus predict whether vaccination was successful in an *M.tb* susceptible individual. Because CBA/J mice are naturally more susceptible to *M.tb* infection than other mouse strains, they are especially suited for vaccine studies which aim to protect *M.tb* susceptible humans. Based on the poor IFN-γ responses of CBA/J mice to Ag85 and ESAT-6 during primary *M.tb* infection, we hypothesized that CBA/J mice would be unable to generate protective responses to these antigens when administered alone. To test this hypothesis, CBA/J mice were vaccinated subcutaneously three times with ESAT-6 or Ag85 in an adjuvant containing DDA and MPL. Negative control groups received PBS or adjuvant. Positive control groups received a single dose of *M. bovis* BCG, as it has previously been shown that *M.tb* susceptible mice responded favorably to *M. bovis* BCG vaccination (564).

Our hypothesis was wrong. CBA/J mice responded favorably to both single proteins when administered as subcutaneous vaccinations. Ag85 (Figure 2.10A) and ESAT-6 (Figure 2.10B) significantly reduced *M.tb* lung burden in comparison to controls (combined PBS and adjuvant groups). They were less protective than *M. bovis* BCG, although ESAT-6 vaccination and *M. bovis* BCG vaccination were not statistically different. Similar results were obtained from the mediastinal lymph nodes (data not shown), suggesting vaccine induced protection was systemic. Overall, these results
indicate that CBA/J mice can generate protective immune responses to the immunodominant \textit{M. tb} antigens Ag85 and ESAT-6.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 2.10. \textit{M. tb} lung burden in vaccinated and challenged CBA/J mice.** CBA/J mice were vaccinated by subcutaneous injection with Ag85, ESAT-6 or \textit{M. bovis} BCG, rested and challenged with 50-100 CFU of \textit{M. tb} Erdman by aerosol. Mice were euthanized at 28 days of \textit{M. tb} infection and bacterial burden in the lungs determined by plating serial dilutions on OADC-supplemented 7H11 agar plates and counting colonies after 3 weeks incubation at 37ºC. Results shown are from two (A) or one (B) independent experiments each with 5-6 mice per group analyzed by one way ANOVA with Tukey’s post test, *p<0.05, **p<0.01, ***p<0.001. Control data was combined from PBS and Adjuvant groups which showed no significant differences.

To determine vaccine induced protective pulmonary mechanisms, isolated lung cells were quantified by flow cytometry. No consistent significant differences were noted in the proportions of T cells, CD4 T cells, activated CD44\textsuperscript{hi} CD4 T cells, CD8 T cells, activated CD44\textsuperscript{hi} CD8 T cells, or CD11c\textsuperscript{+} lung cells (macrophages). However, there were
more total lung cells isolated from the lungs of *M. bovis* BCG vaccinated mice, compared to adjuvant and Ag85 groups (Figure 2.11A), comprised of more CD4 and CD8 T cells, often with an activated CD44<sup>hi</sup> phenotype (Figures 2.11B, 2.11C, 2.11D, 2.11E). ESAT-6 vaccination of CBA/J mice had more profound effects upon the CD4 T cell population, resulting in significantly more activated CD44<sup>hi</sup> CD4 T cells than either control groups or *M. bovis* BCG vaccinated groups (data not shown). Although there were no consistent differences in the numbers of CD11c<sup>+</sup> lung macrophages, a trend for increased activation as determined by MHCII expression was identified (Figure 2.11F). Overall, these results show that *M. bovis* BCG vaccination of CBA/J mice increased the numbers of activated immune cells within the lungs, suggesting that more cells capable of mediating protective immunity were recruited to the lungs. In contrast, following Ag85 vaccination, the numbers and activation of immune cells was comparable to the adjuvant controls and thus suggests that fewer immune cells participated in control of *M.tb* growth. In contrast, ESAT-6 vaccination increased the numbers of total and activated CD4 T cells in the lungs, while CD8 T cells were not affected (data not shown).
Our previous results (Figure 2.11) indicate that *M. bovis* BCG vaccination increased the numbers and activation of lung immune cells following *M. tb* challenge of CBA/J mice. In contrast, Ag85 vaccination did not have the same effect on lung cell recruitment, yet this vaccine was still capable of significant protection. This suggested that the immune cells inducing protective immunity had enhanced function. To more specifically define the function of CBA/J lung immune cells induced by vaccination, the
numbers of antigen specific IFN-γ producing cells were quantified by ELISpot. As expected, vaccination significantly increased the frequency (data not shown) and the absolute number of Ag85 (Figure 2.12A) and ESAT-6 (Figure 2.12B) antigen specific IFN-γ producing lung cells. Although not statistically significant, BCG vaccination slightly increased the numbers of Ag85 specific IFN-γ producing cells above control groups (average number 1837 in control versus average number 2824 IFN-γ SFU per lung in BCG vaccinated) (Figure 2.12A), likely because *M. bovis* BCG expresses Ag85 (38). *M. bovis* BCG lacks the RD1 region, and therefore lacks ESAT-6 (577). As expected vaccination with *M. bovis* BCG did not increase the numbers of ESAT-6 specific cells during *M.tb* challenge. Interestingly, however, vaccination with BCG significantly reduced the numbers of ESAT-6 specific IFN-γ SFU in comparison to control (average number 11340 in control versus 4820 IFN-γ SFU in BCG vaccinated, p<0.01). As expected, vaccination with Ag85 did not alter the numbers of ESAT-6 specific cells nor did vaccination with ESAT-6 alter the numbers of Ag85 specific cells, confirming that single protein vaccination in CBA/J mice generated large numbers of highly specific cells.

Overall, these results indicate that single protein vaccination of *M.tb* susceptible CBA/J mice produces numerous specific protective immune cells but does not alter protective responses to other single protein antigens. In contrast, vaccination with *M. bovis* BCG also induces strong protection in *M.tb* susceptible mice, which likely resulted from more Ag85 specific cells as well as unknown protective antigens shared with *M.tb*. Interestingly, the statistically significant reduction in ESAT-6 specific IFN-γ producing cells in *M. bovis* BCG vaccination as compared to controls may suggest that *M. bovis*
BCG skews the protective immune response away from the typically potent immunodominant *M. tb* antigens encoded in the RD1 region. This conclusion, however, is limited as these experiments were conducted in CBA/J mice which do not normally respond well to the immunodominant antigens ESAT-6 or Ag85 and the effects may be magnified in C57BL/6 mice capable of strong ESAT-6 and Ag85 immune responses.

**Figure 2.12. Antigen specific IFN-γ producing cells from lungs of vaccinated and *M. tb* challenged CBA/J mice.** CBA/J mice were vaccinated with Ag85, ESAT-6, *M. bovis* BCG or control (results combined from PBS and adjuvant groups), rested, challenged with 50-100 CFU of *M. tb* Erdman by aerosol and euthanized at 28 days of *M. tb* infection. Isolated lung cells were stimulated with Ag85 or ESAT-6. IFN-γ spot forming units were counted and the numbers of antigen specific cells per lung calculated by subtracting spots formed in the presence of negative control antigen (ovalbumin). Results shown are the mean ± SEM of 2 (A) or 1 (B) independent experiments with 5-6 mice per group analyzed by one-way ANOVA with Tukey’s post test, ***p<0.001.
**Vaccination of CBA/J mice increases blood IFN-γ responses during M.tb challenge.**

Because vaccination significantly increased the numbers of lung cells capable of generating a protective immune response, we next determined whether blood responses were also enhanced in vaccinated CBA/J mice using our modified IGRAs. There was more Ag85 specific IFN-γ secreted into whole blood culture supernatants following vaccination with Ag85 (Figure 2.13A) and similar results were achieved with ESAT-6 vaccination (data not shown). Similar to the lung results, there was a trend for increased Ag85 specific IFN-γ secretion following BCG vaccination compared to control, although the changes were not statistically significant.

When CBA/J mice were vaccinated with ESAT-6 (Figure 2.13B), the frequency of responding cells in the blood was increased, although statistical significance was not achieved likely due to variation within the small sample size. Also similar to the lung results, BCG vaccination significantly reduced the numbers of ESAT-6 IFN-γ responding cells in comparison to control, but only when compared by a pair-wise Student’s t-test (not indicated on the graph). Overall, however, these data provide promising results that may be further optimized and translated to humans, indicating that vaccination increases both the amounts of secreted IFN-γ and the frequency of responding cells in the blood. Therefore, similar to our results in primary *M.tb* infection, these vaccination data show that blood IGRAs can predict vaccine induced protection during *M.tb* challenge.
**Figure 2.13. Antigen specific IFN-γ from blood of vaccinated and *M. tb* challenged CBA/J mice.** CBA/J mice were vaccinated with Ag85, ESAT-6, *M. bovis* BCG or control (combined results from PBS and adjuvant groups), rested, challenged with 50-100 CFU of *M.tb* Erdman by aerosol and euthanized at 28 days of *M.tb* infection. Diluted whole blood (A) and isolated PBMCs (B) were stimulated with Ag85 or ESAT-6. IFN-γ secretion into supernatants was measured by ELISA (A) while IFN-γ spot forming units were detected by ELISPOT (B). The frequency of antigen specific cells was calculated by subtracting spots formed in the presence of negative control antigen (ovalbumin). Results shown are the mean ± SEM of 2 (A) or 1 (B) independent experiments with 5-6 mice per group analyzed by one-way ANOVA with Tukey’s post test (*p<0.05; NSD: no significant difference). Samples were pooled for B.

**Discussion:**

These studies show four important conclusions that may have direct relevance to man. First, we have shown using multiple *M.tb* susceptible mouse strains that the low *M.tb* antigen specific IFN-γ at the major site of infection (lung) can be a predictive biomarker of TB disease progression well prior to the onset of clinical signs. Second, we have shown that the protective immune response in the blood directly reflects the immune response in the lungs. This shows that the blood is an appropriate test tissue for
the development of predictive TB tests in humans, which is important for optimization and use of practical diagnostic TB tests in people where lung cells are not readily accessible. Third, we have shown some limitations of the IGRA. In particular, assays which rely on secretion into cell culture supernatant in response to *M. tb* antigens may lack sensitivity and be unable to accurately distinguish *M. tb* infected versus non-infected individuals. Therefore, it may be necessary to use IGRA in combination with additional tests to confirm infection status, or increase the sensitivity of IGRA by additional means. Finally, we have shown that CBA/J mice with poor recognition of immunodominant antigens during primary *M. tb* infection respond very well to single protein vaccination strategies and furthermore that protective responses to vaccination can be detected in the blood. These final results have promising implications for the outcome of Ag85-based vaccines currently in human clinical trials (578), and suggest that *M. tb* susceptible humans may also be protected by single protein antigen vaccines.

Using several different immunocompetent inbred mouse strains we have shown that modified IGRA can successfully identify strains that have shorter survival times in response to infection with *M. tb*. Mouse strains with accelerated TB disease progression (CBA/J, DBA/2, C3H/HeJ) produced suboptimal IFN-γ secretion *ex vivo* from lung and blood cultures in response to *M. tb* antigens CFP, ESAT-6 and Ag85. In CBA/J mice decreased *M. tb* antigen specific IFN-γ secretion was due to low frequencies of antigen specific IFN-γ T cells in the lung and blood, as well as low IFN-γ produced on a single cell level. Importantly, poor *M. tb* antigen specific responses were not related to either general deficiencies in IFN-γ production or in TCR mediated signaling. These results reinforce the concept that the majority of human TB patients are not
immunocompromised, a fact that is reflected across multiple genetically diverse mouse strains. In our modified blood IGRAs, *M. tb* antigen specific responses often provided a more sensitive indicator of risk for TB disease progression than the lung, such that the blood responses were even lower than those in the lung, indicating that very few circulating blood cells produced IFN-γ in response to *M. tb* antigens. These data demonstrate that diagnostic test results based on blood cell culture supernatants such as the (Quantiferon-Gold™) may be very accurate in predicting TB disease outcome but unreliable for use as a first-line screening tool because both non-infected and *M. tb* infected individuals could have equally low *M. tb* antigen specific IFN-γ secretion. We believe, however, that lower sensitivity of the Quantiferon-Gold™-type IGRAs (ELISAs) is a minor limitation that may be easily solved by combining the test with a more sensitive assay. For example, unpublished observations from our laboratory have shown that IFN-γ secretion in supernatants was undetectable, yet the frequency of IFN-γ SFU from equivalent cell densities in parallel cultures was easily detected by T-Spot.TB™-type IGRAs (ELISPOTs). Thus it is possible that a more sensitive assay could distinguish between non-infected and *M. tb* infected individuals based on the frequency of *M. tb* antigen specific IFN-γ responding cells.

Several studies have characterized antigen specific T cell responses in the lungs or spleens of *M. tb* infected mice (326, 579, 580), however, we believe we are the first to study and report the relationship between peripheral blood immune responses with that of lung cells in mice. Using murine models with differential TB susceptibilities we have shown that IFN-γ antigen specific immune responses in the blood are a sensitive indicator of lung specific events. Our studies are relevant to man where the most common source
of antigen specific T cells is the peripheral blood. Few human or animal studies have compared the lung and blood immune responses during long term *M. tb* infection and to our knowledge none have correlated the results with disease outcome. A recent study of human TB patients, however, reported that ESAT-6 specific IFN-γ from normalized cell numbers was elevated in the lungs compared to blood (581) and similar results were obtained from our modified ELISpot IGRAs indicating that in this respect the murine system can accurately model man (Figure 2.6). Due to the small volume of blood obtained from individual mice, blood cell numbers were not normalized in our ELISA IGRAs (Figure 2.5), which may explain why antigen specific IFN-γ secretion was roughly equivalent in the lung and blood samples. Alternatively, it was possible that blood IFN-γ producing cells from mice secreted large quantities of cytokine, yet this was not true when the amount of IFN-γ secreted per cell was calculated (Figure 2.7).

In non-human primates, there is some evidence of individual variation in secreted ESAT-6 specific IFN-γ from blood assays which may be associated with increased risk of TB disease progression. In a study of 4 animals, two had minimal responses to ESAT-6 and of those, one animal had massive infection with hematogenous spread at necropsy (582). Although lung specific responses were not compared to blood, these studies provide additional evidence that low IFN-γ responses to ESAT-6 can be associated with increased susceptibility to TB disease and elevated bacterial burden, similar to our findings in mice. In contrast to our murine models however, both the aforementioned human study and the non-human primate study quantified ESAT-6 specific responses during clinical disease, rather than prior to disease onset.
Our studies also demonstrate that we can successfully predict the risk of different mouse strains to develop TB disease using a simple blood IGRA. The potential to predict TB outcome in man using a simple blood assay could create a powerful tool for the early detection and treatment of susceptible individuals prior to the onset of active TB. Such a strategy would decrease *M. tb* transmission and lead to a reduction in the global burden of tuberculosis. Many clinical research papers (556, 583-585) and reviews (553-555, 586) support the use of IGRAs to monitor or classify stages of TB disease and to predict disease outcome in humans. Additionally, recent analysis (553) of previously published data (240) indicates that healthy contacts of TB patients stratify into low, medium, and high IFN-γ ESAT-6 responders, suggesting that a correlation with TB disease progression might exist in humans. Results from large scale prospective longitudinal studies in humans are not yet available and currently, it is unknown whether low or high responders progress to active TB.

A number of studies have investigated associations between TB disease state and the strength of antigen specific responses. For example, active TB disease has been associated with depressed *M. tb* specific IFN-γ responses (587), whereas latently infected individuals and patients with minimal TB disease had increased numbers of ESAT-6 specific IFN-γ secreting blood cells (588, 589). In contrast, other human studies (556, 590, 591) have associated strong ESAT-6 specific IFN-γ with active TB and/or elevated *M. tb* bacterial load however, subjects were not assessed prior to disease onset and it is unclear whether study participants had ongoing *M. tb* exposure throughout the follow-up periods. These contradictory findings from human IGRA data may be due to small group sizes, differing methodologies or indicate that subpopulations of *M. tb* susceptible
individuals have differential responses to ESAT-6. Larger and longitudinal studies are underway to critically evaluate whether IGRAs can be successfully used to predict TB disease progression in man (553, 555). Using inbred mouse strains with different susceptibilities to \textit{M}. \textit{tb}, our results suggest that TB disease outcome can be predicted by low ESAT-6 antigen specific IFN-\(\gamma\) secretion \textit{ex vivo}.

With the inclusion of highly specific \textit{M}. \textit{tb} antigens, IGRAs are more sensitive and specific for \textit{M}. \textit{tb} infection than the PPD skin test, but cannot yet distinguish latent \textit{M}. \textit{tb} infection versus active TB disease or predict disease outcome. In these experiments, IGRAs were modified to accommodate for the small numbers of lung and blood cells available from mice. ESAT-6 and Ag85 were chosen as model antigens because they are used in the human clinical setting as either diagnostic tests (ESAT-6) (224) or experimental vaccines (Ag85) (578). Complex proteins (CFP) were also included to quantify global CD4 T cell IFN-\(\gamma\) responses to soluble mycobacterial antigens. Incubation times in our assays were 36 and 72 hours which likely resulted in antigen specific IFN-\(\gamma\) production from effector rather than memory T cells (592). Our data clearly indicate that purified ESAT-6 and Ag85 were superior predictors of TB disease susceptibility in our mouse models with better discrimination between the relatively resistant and susceptible strains than seen with CFP. Perhaps most intriguing, however, was our finding that in the blood, ESAT-6 and Ag85 specific IFN-\(\gamma\) production was often unable to distinguish \textit{M}. \textit{tb} infected susceptible CBA/J mice from non-infected mice with ELISA-based IGRAs. Similar patterns have been reported in man where peripheral blood IGRAs failed to detect small proportions of \textit{M}. \textit{tb} infected individuals (593, 594) and TB patients (557). Thus the use of a single IGRA should be used with caution because it could fail to detect
*M.tb* infection in the fraction of individuals most likely to succumb to TB disease. It is important to realize, however, that these limitations do not detract from the significance and potential usefulness of the ability to provide an accurate prognosis. These results show that additional tests may be necessary to confirm *M.tb* infection status, such as the more traditional tests: PPD skin test or microbial culture. Alternatively, it may be possible to increase the test sensitivity by using serial testing in patients, or pursue diagnosis with additional IGRAs based on ELISPOTS, or modify the test to include a less selective pool of *M.tb* antigens which may ensure that all *M.tb* infected individuals are identified. Nonetheless, our results indicate that poor immune responses to complex (CFP) and single antigens (ESAT-6, Ag85) can successfully predict *M.tb* infection outcome in mice.

It is currently unknown how peripheral blood IGRA responses of *M.tb* susceptible inbred mouse strains are affected by vaccination, antibiotic treatment, or immunosuppression in humans. Inconsistent responses from IGRAs have been reported in treated TB patients which include sustained ESAT-6 responses (595), temporally dependent and discordant results (596), and declining frequencies of antigen specific cells (588). The mechanisms underlying these disparities in susceptible humans are currently unknown however, some of these clinically relevant questions may be addressed with the use of multiple *M.tb* inbred mouse strains, such as we describe here.

To expand the relevance of our results using modified IGRAs, we vaccinated CBA/J mice with Ag85, ESAT-6, *M. bovis* BCG as well as PBS and adjuvant vehicle controls and determined that IGRAs can predict protection during *M.tb* challenge. For reporting simplicity, PBS and adjuvant control groups were combined when no
statistically significant differences were detected. This was however, an important finding because it showed that administration of the adjuvant did not significantly alter \textit{M.tuberculosis} infection. Although CBA/J mice had very poor IFN-γ responses to Ag85 and ESAT-6 during primary \textit{M.tuberculosis} infection, vaccination with either single protein antigen provided significant protection and reduced the lung \textit{M.tuberculosis} burden by approximately $\frac{1}{2}$ to 1 Log\textsubscript{10}, which is comparable to that achieved by vaccination of \textit{M.tuberculosis} resistant C57BL/6 mice (559). Although the levels of protection induced by Ag85 or ESAT-6 in CBA/J mice did not reach that achieved with live \textit{M. bovis} BCG, these findings were also typical of experimental vaccines in other mouse strains (559, 564). As expected, vaccination of CBA/J mice increased the frequency and absolute numbers of vaccine antigen specific cells that produced IFN-γ in the lungs. Similar results were found in whole blood cell cultures and PBMC ELISpots, showing that the protective immunity induced by vaccination could also be measured in the blood. Overall these results show that IGRAs may also be used to predict and quantify the protective efficacy of vaccination in \textit{M.tuberculosis} susceptible humans.

In our studies using CBA/J mice and other animal models (calves (597) and C57BL/6 mice (559)), \textit{M. bovis} BCG vaccination decreased ESAT-6 specific IFN-γ blood responses. In all of these studies, it is possible that lower ESAT-6 specific IFN-γ reflected the overall reduced \textit{M.tuberculosis} burden and antigenic load compared to non-vaccinated control animals. Alternatively, these results could suggest that \textit{M. bovis} BCG vaccination skews the immune response away from known immunodominant protective \textit{M.tuberculosis} antigens. This may be a likely scenario as \textit{M. bovis} BCG shares significant homology with \textit{M.tuberculosis} (598, 599) yet BCG-specific protective antigens are poorly described.
An intriguing finding was that CBA/J mice had an initial robust antigen specific immune response to CFP antigens within the lung, which subsequently waned as infection progressed. Loss of acquired immunity occurred, despite an increased bacterial load in the lung at all time points tested. These data suggest that CBA/J mice can initially generate a protective immune response, coinciding with control of *M.tbc* growth within the lung, but for reasons currently unknown fail to maintain a sufficient functional pool of antigenic specific IFN-γ secreting CD4 T cells within the lung. Possibilities for the loss of CFP specific IFN-γ responses could T cell exhaustion or anergy, production of alternate cytokines, such as IL-4, or increased apoptosis of antigen specific cells. An understanding of this mechanism could provide valuable information about why protective immunity cannot be sustained in a subset of individuals with latent *M.tbc* infection, resulting in reactivation of infection and tuberculosis disease.

Increased susceptibility of inbred mouse strains to infection with *M.tbc* has been associated with poor localization of T cells to the lung (519), decreased expression of T cell adhesion molecules (516), delayed *M.tbc* dissemination and subsequent slow T cell priming (519, 574), and increased IL-10 production (326). Poor expression of adhesion molecules may contribute to the reduced number of CD4 T cells localizing to the lungs however, additional mechanisms must contribute as the reduction in IFN-γ and antigen specific cells was not proportional to the reduction in total lung CD4 T cells. Impaired dissemination of *M.tbc* antigens to the regional lymph nodes could also contribute to susceptibility, however, if protective immunity were simply due to slow dissemination and delayed generation of acquired immunity IFN-γ responses should eventually reach levels equivalent to resistant mice, which was not observed. Increased levels of IL-10, an
immunosuppressive cytokine, have been shown within the lungs of CBA/J mice during chronic infection with *M. tb* (326) and the pleotropic effects of IL-10 could certainly contribute to poor antigen specific IFN-γ production. IL-10 has been shown to downregulate macrophage activation by inhibiting MHCII recycling, peptide loading or inhibition of transcription factors (262) all of which could impair the generation or maintenance of antigen specific T cells capable secreting IFN-γ. Furthermore, overexpression of IL-10 on the relatively resistant C57BL/6 background strain led to reduced antigen specific IFN-γ secretion and significantly elevated bacterial burdens (326). The exact mechanism through which IL-10 negatively influences chronic *M. tb* infection is not fully known but in other mycobacterial infections blocking IL-10 promoted macrophage activation, pro-inflammatory cytokine production and production of reactive nitrogen intermediates as well as positively influenced vaccination and therapeutic responses (600, 601).

The use of genetically disparate mouse strains such as CBA/J, DBA/2, and C3H/HeJ mice provide tools to model different susceptibilities to infection with *M. tb*, expand our understanding of immune mechanisms that lead to poor disease outcome, and identify potential biomarkers that can be used to monitor disease progression in man. In this study we have specifically shown that *M. tb* specific IFN-γ predicts both TB disease outcome and protective response to vaccination during *M. tb* challenge. In some instances, IFN-γ production during *M. tb* infection was as low as non-infected mice, and therefore if the test were used alone, it could miss the individuals with the highest risk of TB disease progression. In a clinical setting, this limitation would require additional tests to confirm *M. tb* infection status, such as use of a more sensitive IGRA or a modified test that
included more comprehensive *M.tb* antigens recognized by a broader range of individuals. Furthermore, our experiments in mice reinforce that long-term follow up of *M.tb* exposed individuals using IGRAs are warranted and may yield relevant information that could change the course of TB, not only for individual patients but also for entire populations.

Although our results show that low *M.tb* antigen specific IFN-γ predicts disease outcome across genetically disparate mice, these studies do not fully address the mechanisms underlying poor *M.tb* antigen specific IFN-γ. In this chapter we showed that TCR signaling produced maximal IFN-γ from CBA/J lung cells during *M.tb* infection, yet very few cells responded to *M.tb* antigen by IFN-γ production. Furthermore, vaccination with single protein antigens in CBA/J mice effectively expanded Ag85 and ESAT-6 specific cells, showing that these mice are fully capable of generating and expanding protective *M.tb* specific T cells when administered large doses of a single antigen. These results suggest that susceptible mice have adequate T cell functional capacities, and we could hypothesize that diminished IFN-γ during primary *M.tb* infection is secondary to deficient processing and/or presenting dominant antigens. Future studies should address these issues by quantifying the amount of antigens in the lungs and the peptide availability loaded into MHC molecules. Based on the high *M.tb* lung burden in susceptible CBA/J mice, it is likely that antigenic proteins are abundant in the lungs and should not be limiting factors. It is highly possible, however, that the antigen presenting cells of CBA/J mice express less surface MHC/peptide complexes which in turn would generate fewer IFN-γ producing antigen specific T cells. If this were true, additional
experiments *in vitro* may define differences that could be exploited in antigen processing and presenting pathways between *M.tb* susceptible and *M.tb* resistant mice.

As an alternative hypothesis, it is possible that numerous antigen specific T cells are present in the lungs of susceptible CBA/J mice, but these cells produce other cytokines (such as IL-10, TGF-β, IL-27, IL-4) instead of *M.tb* antigen specific IFN-γ. Future experiments could address this question by using Th1 or Th2 cytokine arrays on purified populations of T cells from the lungs of *M.tb* infected CBA/J mice. Identification of one or more additional antigen specific cytokines could then be manipulated *in vivo* or *in vitro* to define immunological mechanisms that drive *M.tb* susceptibility.

The complex immune response to *M.tb* cannot likely be explained by one underlying mechanism in susceptible mice or humans however, the use of genetically disparate mouse strains can mimic the genetic diversity of the human population. These models, therefore, can be used to identify and validate immunologic mechanisms and biomarkers of TB disease progression in man.

Identification of immunological predictors of TB disease progression and response to vaccination during *M.tb* challenge has the potential to diminish the global TB disease burden by targeted monitoring of at-risk patients and initiation of treatment prior to disease onset, thus limiting *M.tb* transmission. Adaptation of IGRAs, singly or in combination with additional markers such as IL-10 and IL-2 (342, 602), to predict outcomes in humans is an important area of ongoing research. In man, many questions remain, including whether immune responses from peripheral blood truly reflect immune events within the lung and whether the amount or frequency of IFN-γ responding cells correlate with TB disease progression (225, 554, 555).
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CHAPTER 3: INTERLEUKIN-10 PROMOTES *MYCOBACTERIUM TUBERCULOSIS* DISEASE PROGRESSION IN CBA/J MICE.

**Abstract**

Interleukin-10 (IL-10) is a potent immunomodulatory cytokine that affects innate and acquired immune responses. The immunological consequences of IL-10 production during pulmonary TB are currently unknown although IL-10 has been implicated in reactivation TB in man and with *M. tb* susceptibility in mice. Using *Mycobacterium tuberculosis* susceptible CBA/J mice we show that blocking the action of IL-10 *in vivo* during chronic infection stabilized the pulmonary bacterial load and improved survival. Furthermore, this beneficial outcome was highly associated with the recruitment of T cells to the lungs and enhanced T cell IFN-γ production. Our results indicate that IL-10 promotes TB disease progression. These findings have important diagnostic and/or therapeutic implications for the prevention of reactivation TB in man.

**Introduction**

Interleukin-10 (IL-10) is a potent immunomodulatory cytokine that has been shown *in vitro* to directly or indirectly affect multiple cell types including macrophages, monocytes, dendritic cells, B cells, CD4 T cells and CD8 T cells (262). The dominant
function of IL-10 is to de-activate macrophages resulting in diminished Th1 cytokine production (265, 294), decreased production of reactive nitrogen or oxygen species (293) and limited antigen presentation (302), which may have far reaching consequences on both innate and acquired immunity in vivo.

IL-10 has been suggested as a correlate of M.tb susceptibility in both mice and men (235, 337, 342-345) as well as susceptibility to M. avium infection in mice (600). In man, IL-10 can be found in the serum (343, 344) and bronchoalveolar lavage fluid (345) of active TB patients and may be an important clinical biomarker of disease progression (342).

In the murine model, IL-10 deficient C57BL/6 mice may have an early transient resistance to infection with M.tb, but results have been inconsistent with some studies showing no differences in either bacterial burden or growth kinetics over the first few weeks of infection (235, 337, 339). It has been generally accepted that the lack of IL-10 in C57BL/6 mice does not impair the generation of protective immunity as mice controlled M.tb infection at equivalent levels as wild-type controls for at least 100 days with similar kinetics of T cell localization to the lungs (235, 337). A recent publication, however, reported that C57BL/6J IL-10 KO mice survived approximately 190 days following M.tb infection (339), substantially less than similarly infected WT mice (Chapter 2). Although increased M.tb burden was detected, morbidity was attributed primarily to poorly controlled cellular infiltrates and T\textsubscript{H}1 cytokine responses resulting in lung tissue damage (339). These results indicate the complete absence of IL-10 exacerbates tissue damage during chronic M.tb infection. These results show that one role
for IL-10 during chronic *M.tb* infection is to protect the host from immune-mediated tissue damage.

Here and in previous publications (326) it has been shown that C57BL/6 mice do not normally produce abundant IL-10 during *M.tb* infection. Manipulation of IL-10 in this mouse strain therefore, may not fully define the role of IL-10 in driving reactivation TB. Furthermore, IL-10 KO mice have additional defects that may directly or indirectly affect the course of *M.tb* infection such as growth retardation (603), enterocolitis (264, 603), iron deficient anemia (603), and peripheral granulocytosis (603). Although the lungs were reported to be normal, it is unclear whether these potentially confounding variables affected early morbidity (339).

Rather than contradicting our findings, however, the experiments using C57BL/6 IL-10 KO mice complement our CBA/J results. Together, they provide a more thorough understanding of the complexity of IL-10 *in vivo* during *M.tb* infection. Taken together, they indicate that IL-10 has dual roles during *M.tb* infection: when present in small amounts, as detected in C57BL/6 mice during chronic *M.tb* infection, IL-10 limits lung tissue damage by dampening detrimental inflammation. In contrast, IL-10 produced in abundance as detected in CBA/J mice results in *M.tb* growth and reactivation TB.

The CBA/J mouse strain has been defined as relatively susceptible to *M.tb* infection, as it has increased bacterial load within the lungs (516) and earlier mortality than the C57BL/6 strain (558). Furthermore, disease progression in CBA/J mice has been associated with abundant IL-10 within lung macrophages (326). In this chapter we confirmed that CBA/J mice can model reactivation TB disease in man and show many similar clinical signs and lung lesions. We hypothesized that the increased production of
IL-10 during chronic *M. tb* infection in CBA/J mice accelerated TB disease progression. This was supported by the previous findings that C57BL/6 mice engineered to over-express IL-10 had rapid disease progression following *M. tb* infection (326), confirming a clear relationship between the single cytokine IL-10 and poor disease outcome.

The increased amount of IL-10 produced by CBA/J mice during *M. tb* infection provides us with a natural model in which the role of IL-10 can be more accurately defined. In this chapter we used CBA/J mice to determine whether IL-10 caused disease progression during an established chronic *M. tb* infection. Using anti-IL10R1 antibody delivery starting at day 90 post infection, we show that blockade of IL-10 activity led to improved control of *M. tb* bacterial load within the lung and enhanced Th1 immunity without promoting additional lung tissue damage. These data demonstrate that increased IL-10 production exacerbates TB disease and provide supportive evidence that IL-10 can be a therapeutic target for the prevention of reactivation TB.

**Materials and Methods**

**Mice**

Specific pathogen free eight-week-old, female CBA/J and C57BL/6 mice (NCI, Frederick, MD) were maintained in Biosafety Level-3 facilities 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.
M.\textit{tb} infection and quantification of bacterial load

\textit{M.\textit{tb} Erdman} (ATCC \#35801) was obtained from American Type Culture Collection (Manassas, VA.). Stocks were grown and mice infected with \textit{M.\textit{tb} Erdman} using an inhalation exposure system (Glas-col, Terre Haute, IN) calibrated to deliver 50-100 colony forming units (CFU) to the lungs of each mouse as previously described (604). At specific time points post infection, the \textit{M.\textit{tb}} pulmonary load was determined as previously described (18).

Quantification of immune mediators

Lung homogenates were clarified by centrifugation. Cytokines or chemokines were analyzed by ELISA using antibody pairs and standards from BD Biosciences (IFN-\gamma, IL-12p40, IL-10) (San Jose, CA) or R&D Systems (CCL5) (Minneapolis, MN) that were optimized as per the manufacturer’s instructions. Nitrite/nitrates in lung homogenates were quantified using the Greiss reaction by adding Greiss reagent to equal sample volumes. Absorbance was read at 550nm and compared to a sodium nitrite standard curve.

Antibody administration

Anti-IL10R1 mAb (1B1.3A) (Schering-Plough Biopharma, Palo Alto, CA) and whole rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or rat IgG1 isotype control mAb (GL113) (Schering-Plough Biopharma) were diluted to 2mg/ml in sterile PBS and aliquots stored at -80°C. Thawed stocks were maintained at
4°C for up to one month. Antibody administration was modified based on Silva et. al. (601) as follows: at day 90 post infection, 1mg of anti-IL10R1 (1B1.3A) or control antibody was injected into the peritoneal cavity of each mouse, followed by 0.2mg at weekly intervals thereafter until the designated experimental time points. For animals in survival studies, antibody injection was continued weekly until signs of morbidity (weight loss, hunched posture, ruffled hair coat, social isolation) developed when mice were euthanized.

**Lung cell isolation and cell culture**

Mice were euthanized by CO₂ asphyxiation and single cell suspensions obtained from the lung using collagenase/DNase as previously described (604). Viable cells were determined by trypan blue exclusion, counted and re-suspended at 2x10⁶/ml. Lung cells (2.5x10⁵) were cultured in duplicate with 10µg/ml of ovalbumin (Sigma-Aldrich, St. Louis, MO), *M.tb* culture filtrate protein (CFP) (NIH, NIAID Contract No. HHSN266200400091C), or concanavalin-A (Sigma-Aldrich) for 72 hours at 37°C. Supernatants were stored at -80°C until analysis. Cytokine production from cell culture supernatants was quantified by ELISA using antibody pairs and standards from BD Biosciences (San Jose, CA) as described previously (604).

**Flow cytometry**

Isolated lung cells were suspended in deficient-RPMI (Irvine Scientific; Santa Ana, CA) supplemented with 0.1% sodium azide (Sigma-Aldrich). Following 5-10
minutes incubation at room temperature with Fc Block™ (clone 2.462), surface and intracellular targets were detected as previously described (604). Specific antibodies and isotype controls were purchased from BD Biosciences (San Jose, CA): PerCP-Cy5.5 anti-CD3ε (145-2C11), APC-Cy7 anti-CD4 (GK1.5), PeCy7 anti-CD8 (53-6.7), FITC anti-CD11a (2D7), APC anti-CD11c (HL3), APC anti-TCR-β chain (H57597), PerCP anti-CD8 (53-6.7), anti-I-A/I-E (2G9), and PeCy7 anti-IFN-γ (XMG1.2). IFN-γ was determined according to the manufacturer’s instructions for intracellular cytokine staining (CytoFix/CytoPerm Fixation/Permeabilization Solution Kit w/BD GolgiStop™, BD Biosciences), following a 4 hour incubation with anti-CD3 (145-2C11), anti-CD28 (37.51). Samples were read using an LSRII flow cytometer and analyzed with FACSDiva software (BD Biosciences). For some experiments 100µl of BrdU (FITC BrdU Flow Kit, BD Biosciences) was injected into the peritoneal cavity of each mouse 24 hours prior to euthanasia. BrdU incorporation was detected by flow cytometry as per the manufacturer’s instructions (FITC BrdU Flow Kit, BD Biosciences).

Cell purification

Immediately following isolation, lung cells and spleen cells were pooled in groups of three or four for CD11c purification using magnetic MACS LS columns and anti-CD11c beads (Miltenyi Biotech, Auburn, CA) as per the manufacturer’s instructions. Greater than 90% CD11c purity was achieved by passing the positively selected cells over three columns and confirmed by flow cytometry.
**Histology**

The right cranial lung lobes from individual mice were inflated with 10% neutral buffered formalin prior to paraffin embedding, sectioning at 5µm, and staining with hematoxylin and eosin, acid-fast staining by the Ziehl-Neelsen method. Samples were evaluated in a double blind fashion by a board certified veterinary pathologist and graded as previously described (605). For morbidity studies, all lung lobes were collected from individual mice, fixed, sectioned and stained with hematoxylin and eosin as above. Additional sections were stained with Masson’s trichrome and VonKossa stains to detect fibrosis and calcification, respectively with light microscopic analysis performed by the author (GB). All tissue samples were processed, sectioned and stained by the Histotechnology Services within the Department of Veterinary Biosciences at The Ohio State University. Representative slides were digitally scanned and images captures by the Aperio ScanScope (Vista, CA) in the Department of Veterinary Biosciences at The Ohio State University.

**PCR**

Right medial lung lobes were homogenized in 1ml of UltraSpec (Biotecx, Houston, TX) and frozen rapidly at –80°C. Total RNA was isolated by following the manufacturer's instructions, and 1µg was reverse transcribed using an Omniscript RT kit (QIAGEN, Valencia, CA). Real-time PCR was performed with an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using TaqMan Gene Expression Assays for class II transactivator, interferon regulatory factor 1, or the 47KDa GTPase LRG-47
(Applied Biosystems, Foster City, CA). The Delta Delta cycle threshold method was used for relative quantification of mRNA expression; in this analysis 18S was used as an endogenous normalizer.

**Statistics**

Pairwise comparisons used the Student’s $t$-test; significance was defined as *$p<0.05$, **$p<0.01$, or ***$p<0.001$. Multigroup comparisons used one-way ANOVA with Tukey’s post-test; significance was defined as *+$p<0.05$, ++$p<0.01$, or +++$p<0.001$. Logrank analysis was used for survival studies.

**Results**

**CBA/J mouse model of reactivation TB**

Following a low dose aerosol of *M.tbc, CBA/J* mice survived approximately 200 days with at least 10-fold increased *M.tbc* lung burdens as compared to C57BL/6 mice (Chapter 2). It was next necessary to establish the kinetics of TB disease progression in the CBA/J model of reactivation TB. This was important to replicate previous results that were performed within different BSL-3 facilities (326) and to define a time frame which experimental blockade of IL-10 would have a measurable effect. The *M.tbc* lung burdens from non-symptomatic CBA/J mice (Chapter 2) were re-analyzed. Multivariate analysis showed a significant increase in *M.tbc* lung load between days 90 and 120 of infection which stabilized at day 150 of infection in the surviving mice (Figure 3.1A). It is
important to emphasize that these mice did not have outward signs of TB disease, yet the bacterial loads increased 10-fold. Therefore re-growth from approximately 1 to 10 million bacteria was well tolerated by the mice, preceded TB disease and indicated that experimental manipulation aimed at preventing \textit{M.tbc} re-growth and disease progression should start at day 90 of infection.

To further establish CBA/J mice as a model of reactivation TB, we confirmed that mice with TB disease had clinical signs, bacterial growth, and microscopic lung lesions similar to human patients with active TB. Following \textit{M.tb} infection, morbid (hunched posture, ruffled hair coat, or social isolation) CBA/J mice weighed approximately 25% less that the non-symptomatic \textit{M.tbc} infected mice and non-infected controls (Figure 3.1B). Weight loss is also a clinical symptom of active TB disease in humans (606). To confirm that TB disease in CBA/J mice was associated with bacterial growth, and not a result of purely host-driven inflammatory responses, the \textit{M.tbc} burden in morbid mice was determined (Figure 3.1C). \textit{M.tb} continued to grow another 10-fold as TB disease developed from 7 to 8 Log\textsubscript{10}. Although the total numbers of \textit{M.tb} bacilli in the lungs of human TB patients cannot be accurately quantified, bacterial growth is believed to be a cause of TB disease progression and increased severity of TB disease (9).
Figure 3.1. Chronic non-symptomatic *M.tb* infection and TB disease in CBA/J mice. CBA/J mice were infected by aerosol with 50-100 CFU of *M.tb* Erdman. Non-symptomatic mice were euthanized at the indicated time points and lung *M.tb* burdens determined by counting colonies from serially diluted homogenized lung tissue (A). Noninfected age and sex-matched control mice and *M.tb* infected mice were weighed weekly between days 90 and 150 of infection (B). From separate experiments, *M.tb* lung burdens from *M.tb* infected non-symptomatic mice and mice with TB disease (weight loss) were determined by counting colonies from serially diluted homogenized lung tissue during chronic *M.tb* infection between 90 and 150 days (C). Results shown (A) are the average ± SEM from 3-5 independent experiments each with 4-5 mice per time point, analyzed by one way ANOVA with Tukey’s post test, ***p<0.001. Results shown (B) are the combined average ± SEM from three independent experiments with 25 noninfected mouse-weights, 347 infection mouse-weights, and 27 morbidity mouse-weights, analyzed by one way ANOVA with Tukey’s post test, ***p<0.001. Results shown (C) are the combined average ± SEM of 30 non-symptomatic *M.tb* infected mice and 14 mice with TB disease (weight loss) analyzed by the Student’s *t*-test, ***p<0.001.
In addition to harboring more than 8 Log$_{10}$ $M.tb$ CFU, microscopic examination of the lungs of CBA/J mice with TB disease showed similar pathological features as
human TB patients, such as cavitation, fibrosis, erosion and ulceration of airway epithelium, extracellular *M. tb* in necrotic debris and intracellular in phagocytes (607). There was extensive lung damage due to cellular infiltration with focal cavitation, necrosis and bronchi/bronchioles filled with cellular debris (Figure 3.2A, Table 3.1). Additionally, there was a cavitated *M. tb* pyogranuloma that communicated with adjacent conducting airways (Figure 3.2B, Table 3.1), a characteristic of contagious humans able to transmit *M. tb* (512, 607). Panel 3.2C shows necrotic cellular debris within an airway lumen and epithelial changes due to chronic inflammation (hyperplasia, dysplasia, leukocyte transmigration), surrounded by pyogranulomatous pneumonia. Panel 3.2D shows acid-fast (red) *M. tb* bacilli within the airway luminal cellular debris, within transmigrating phagocytes, and within adjacent pyogranulomatous foci. These are all features of active contagious TB disease in humans which have been recapitulated in morbid CBA/J mice.

Additional stains of the lung sections from CBA/J mice with TB disease highlight peripheral fibrosis (blue) surrounding granulomas by Masson’s Trichrome staining (Figure 3.3A, 3.3B, Table 3.1). Extensive regions of alveolar septal fibrosis were also noted (data not shown). Within necrotic granulomas, small dark brown to black calcified foci were detected by Von Kossa staining (Figures 3.3C, 3.3D, Table 3.1) but were visualized only at high magnification (Figure 3.3D). Although it has been accepted that mice lack caseous necrosis, fibrosis and calcification, these generalizations rely heavily on examination of lesions from *M. tb* resistant C57BL/6 mice with well controlled asymptomatic *M. tb* infection (512). In contrast, morbid CBA/J mice share similar lesions of reactivation TB as seen in humans, and therefore may better model reactivation TB.
Figure 3.2. Lung lesions of TB disease in CBA/J mice. CBA/J mice were infected by aerosol with 50-100 CFU of *M.tb* Erdman. When signs of morbidity developed (weight loss, hunched posture, ruffled hair coat, social isolation) mice were euthanized, lungs fixed in 10% neutral buffered formalin, paraffin embedded and sectioned for hematoxylin and eosin staining (A, B, C) or acid-fast staining (D). Slides were scanned by AperioScan scope and digital images captured. Panel A shows extensive lung damage due to cellular infiltration with focal cavitation, necrosis and bronchi/bronchioles filled with cellular debris; bar = 500µm. Panel B shows communication of a cavitated lesion with adjacent conducting airways; bar = 200 µm. Panel C shows necrotic cellular debris within the airway lumen, epithelial alterations (hyperplasia, dysplasia, leukocyte transmigration; bar = 50µm. Panel D shows acid-fast (red) *M.tb* bacilli within the luminal cellular debris, transmigrating phagocytes, and phagocytes within adjacent pyogranulomatous foci; bar = 50µm.
Figure 3.3. Fibrosis and calcification in lungs of CBA/J mice with TB disease. CBA/J mice were infected by aerosol with 50-100 CFU of \textit{M. tb} Erdman. When signs of morbidity developed (weight loss, hunched posture, ruffled hair coat, social isolation) mice were euthanized, lungs fixed in 10% neutral buffered formalin, paraffin embedded and sectioned for Masson’s trichrome (A, B) and Von Kossa (C, D) staining to demonstrate peripheral fibrosis (dark blue regions) and calcification within necrotic granulomas (dark brown to black foci), respectively. Bars A, C = 500µm; Bars B, D = 50µm.
Table 3.1. Microscopic lung lesions of TB disease in CBA/J mice. CBA/J mice were infected by aerosol with 50-100 CFU of M.tb Erdman. When signs of morbidity developed (weight loss, hunched posture, ruffled hair coat, social isolation) mice were euthanized, lungs fixed in 10% neutral buffered formalin, paraffin embedded and sectioned for hematoxylin and eosin, acid-fast, Masson’s trichrome, and Von Kossa staining.

<table>
<thead>
<tr>
<th>Estimated area of affected lungs</th>
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<tr>
<td>Necrosis</td>
<td>6/6</td>
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<tr>
<td>Cavitation</td>
<td>+ 1/6; +/- in 2/6</td>
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<tr>
<td>Fibrosis</td>
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<tr>
<td>Calcification</td>
<td>4/5</td>
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<tr>
<td>Airway involvement*</td>
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* Epithelial hyperplasia and/or erosion with bronchial, bronchiolar, and alveolar intraluminal neutrophils, macrophages, and cellular debris containing intracellular or extracellular M.tb bacilli

IL-10 and IL-10R expression in M.tb infected mice.

It has previously been published that CBA/J mice produced abundant IL-10 during chronic M.tb and had disease progression similar to genetically manipulated IL-10 transgenic mice (326). To extend and confirm these results under new laboratory conditions, IL-10 mRNA, IL-10 protein and IL-10 receptor (IL-10R) expression were quantified. After day 90, during chronic M.tb infection, IL-10 mRNA increased over time in CBA/J mice (Figure 3.4A) but not in C57BL/6 mice (data not shown). Furthermore, in comparison to the relatively resistant C57BL/6 mouse strain, IL-10 protein in CBA/J lungs was significantly higher at days 60, 120, and 150 days post M.tb infection (Figure 3.4B, Barnabe Assogba). IL-10 continued to increase substantially over time during late infection (120 and 150 days) in CBA/J mice, while the levels stabilized in C57BL/6 mice (Figure 3.4B).
In addition to expressing higher levels of IL-10 in the lungs during chronic *M. tb* infection, CBA/J mice had significantly more CD11c<sup>+</sup> lung cells (macrophages) that expressed the IL-10R than C57BL/6 mice (Figure 3.4C and 3.4D), indicating that CBA/J mice had a population of cells that were more sensitive to IL-10 blockade than C57BL/6 mice. We also evaluated additional cell populations for IL-10R expression but neither CD4 T cells nor CD8 T cells consistently expressed abundant IL-10R (data not shown). These data indicate that lung CD11c<sup>+</sup> cells (macrophages) are likely the target cells for IL-10 mediated immunosuppression during *M. tb* infection, consistent with literature reviews (262).
**Figure 3.4. IL-10 and IL10R in mice.** CBA/J and C57BL/6 mice were infected with 50-100 CFU of *M.tb* Erdman, and euthanized at the indicated time points. Total RNA was isolated and relative IL-10 mRNA abundance was determined by the delta-delta CT method of quantification using 18S mRNA as an endogenous normalizer (A). Data are from one experiment with 4-5 mice per time point. IL-10 protein was detected by ELISA in clarified lung homogenates (B). Data are the mean ± SD of one experiment with 4 mice per strain per time point analyzed by Student’s *t*-tests (**p<0.01, ***p<0.001) at each time point and by one-way ANOVA with Tukey’s post test for multigroup comparisons over time (‘’p<0.05, ‘’’p<0.001). Lung cells were isolated from C57BL/6 and CBA/J mice, stained with fluorescent anti-CD11c and anti-IL10R, and quantified by flow cytometry (C). Data are combined from days 115 and 150 of chronic *M.tb* infection from one experiment with 8-10 mice per strain per time point analyzed by the Student’s *t*-test, *p<0.05. One representative histogram of IL-10R^+^ CD11c^+^ cells (top) and identically gated isotype control (bottom) are shown in (D).
Figure 3.4

The phenotype of lung macrophages is complex (608, 609) and we recognize these cells cannot be solely defined by CD11c expression. However, we focused on the
CD11c\(^+\) population because this marker included \textit{M.tb} infected lung cells. During chronic \textit{M.tb} infection in both C57BL/6 and CBA/J mice approximately 91% of the \textit{M.tb} CFU was recovered from CD11c\(^+\) lung cells, while only 8.8% was recovered from CD11c\(^-\) cells (Figure 3.5A). In contrast within the spleen, only 5% of the recovered \textit{M.tb} was in the CD11c\(^+\) population while 95% of the \textit{M.tb} was in the CD11c\(^-\) population (Figure 3.5B).

We have confirmed that the immunocompetent inbred CBA/J mouse strain produced abundant IL-10 during \textit{M.tb} infection (326), allowing for the \textit{in vivo} assessment of IL-10 function in a natural model of heightened \textit{M.tb} susceptibility. CBA/J mice also possessed more CD11c\(^+\) lung cells capable of responding to IL-10 than C57BL/6 mice, providing additional evidence that CBA/J mice may be more sensitive to IL-10 mediated immunosuppressive effects that other mice. Overall, CBA/J mice provide an ideal model for dissecting the role of IL-10 in TB disease progression.

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Figure 3.5. *M. tb* resides in CD11c⁺ cells in the lung but not spleen. C57BL/6 and CBA/J mice were infected by aerosol with 50-100 CFU of *M. tb* Erdman. CD11c⁺ cells were purified by magnetic bead separation and passed over multiple columns to achieve purity of > 90% from the lungs and spleens of mice during chronic *M. tb* infection (day 200 for C57BL/6 mice and day 100 from CBA/J mice). Pooled samples from three C57BL/6 or pooled samples from four CBA/J mice were plated in duplicates onto OADC-supplemented 7H11 plates. Colonies were counted after 21 days incubation at 37°C, the total number of CFU normalized to 100% and the fractional recovery from CD11c⁺ and CD11c⁻ cell populations from the lungs (A) and spleens (B) was calculated. Results shown are the average ± SEM, analyzed by the Student’s *t*-test.

**Anti-IL10R1 treatment stabilized *M. tb* growth and improved survival in CBA/J mice.**

To determine whether IL-10 directly influenced TB disease progression, we blocked the signaling chain of the IL-10 receptor by administering anti-IL10R1 (262, 601) during chronic *M. tb* infection in CBA/J mice. *M. tb* infected CBA/J mice were administered anti-IL10R1 starting at day 90 post infection. Day 90 was specifically chosen as the start point because this occurs before the substantial increase in lung IL-10 (Figure 3.4) and prior to *M. tb* re-growth associated with increased morbidity in CBA/J
mice (Figure 3.1). Anti-IL10R1 treatment was continued weekly throughout the experimental infection. The results showed a significant and predictable increase in bacterial burden in the lungs (326) of CBA/J mice that received control antibody (Figure 3.6A), which approached \(8 \log_{10} M.\text{tb} \text{ CFU}\), a level at which CBA/J mice rarely survive (Figure 1). In contrast, treatment with anti-IL10R1 stabilized the pulmonary bacterial load for up to 150 days (Figure 3.6A). A separate group of mice was assigned to survival studies (Figure 3.2B). Weekly antibody treatment was continued throughout survival experiments and mice were euthanized according to established morbidity criteria (516). Mice treated with anti-IL10R1 survived approximately 60 days longer than mice receiving control antibody (median survival: 192 days for anti-IL10R1 treated versus 133 days for control antibody), and the results were close to statistical significance (\(p=0.075\)). Overall, these data indicate that blocking the activity of IL-10 in vivo in the CBA/J mouse strain during chronic infection with \(M.\text{tb}\) lead to control of bacterial growth within the lung and extended survival.

As expected, IL-10 blockade in the relatively resistant C57BL/6 mouse strain did not stabilize the \(M.\text{tb}\) lung load during the time frame studied (Figure 3.6C). This finding is consistent with results suggesting that IL-10 does not contribute to disease progression due to bacterial re-growth C57BL/6 mice (10, 11) (339). An unexpected finding in C57BL/6 mice was that the \(M.\text{tb}\) burden was significantly lower at day 115 compared to day 150, and was slightly lower that the control group at day 115. The \(M.\text{tb}\) CFU recovered from the day 115 was less than typical (average 4.8 versus 5.7 \(\log_{10}\)) during stable infection in C57BL/6 mice (Chapter 2) It was possible that anti-IL10R1 treatment worsened \(M.\text{tb}\) infection in this mouse strain. Alternatively, IL-10 blockade could have
had a beneficial effect on *M.tb* load in C57BL/6 mice prior to day 115 that was not sustained. Further experiments would have to be performed to confirm or refute these results.
Figure 3.6. IL-10 blockade improves disease outcome in CBA/J mice. CBA/J and C57BL/6 mice were aerogenically infected with 50-100 CFU of *M. tb* Erdman and injected weekly with anti-IL10R1 or control antibody starting at day 90 post infection. Mice were euthanized at days 115 and 150, lungs homogenized, plated onto 7H11 agar, and *M. tb* CFU determined after 21 days incubation at 37°C (A). Data (A, B) are the mean ± SEM of 5 independent experiments each with 3-6 mice per treatment group per time point, analyzed pairwise by Student’s t-tests (*p<0.05) and by one-way ANOVA with Tukey’s post-test for multigroup comparisons (†p<0.05). For survival, separate groups of anti-IL10R1 treated mice and controls were euthanized when signs of morbidity developed (B) and statistical significance determined by Log rank analysis. Survival data are combined from 3 independent experiments with total numbers. Results from C57BL/6 mice (C) are the average ± SD from one experiment with 2-3 mice per treatment group per time point analyzed pairwise by time point by the Student’s *t*-test, **p<0.01.
**Anti-IL10R1 treated CBA/J mice have more lung T cells.**

As CD4 and CD8 T cells are important for generating and maintaining protective *M.tuberculosis* immune responses, we next determined whether improved control of infection was associated with increased T cell numbers within the lung. After 115 days no significant differences in total numbers of viable cells, CD4 T cells, or CD8 T cells were observed (Figures 3.7A, 3.7B, 3.7C). In contrast, at 150 days post infection the total number of lung cells, CD4 T cells, and CD8 T cells were increased in mice that received anti-IL10R1, although statistical significance was not reached. In contrast to the T cell populations, the numbers of lung CD11c^+^ cells were not increased due to anti-IL10R1 treatment (Figure 3.7D). These results indicate that IL-10 blockade primarily affected T cells, although this cannot be stated with complete certainty as not all types of immune cells were evaluated (e.g. neutrophils).

We next sought to determine whether increased T cell numbers resulted from increased T cell proliferation, inhibition of apoptosis, or cellular recruitment into the lungs. To test proliferation *in vivo* in the lungs, mice were injected with BrdU 24 hours prior to euthanasia and intranuclear BrdU incorporation determined by flow cytometry. Results of three independent experiments failed to identify a consistent increase in the number of proliferating CD4 or CD8 T cells in the lung (data not shown). To determine whether anti-IL10R1 treatment inhibited apoptosis, lung T cells were labeled for flow cytometric analysis of surface FAS ligand (FASL), a transmembrane protein in the tumor necrosis factor family which initiates the extrinsic pathway of apoptosis (610). Results of one experiment did not detect significant differences in the numbers of FASL^+^ CD4 T cells or FASL^+^ CD8 T cells that could be attributed to anti-IL10R1 treatment (data not...
shown). Taken together, these results show that IL-10 blockade did not significantly affect either T cell proliferation or T cell apoptosis \textit{in vivo} during chronic \textit{M.tb} infection and these mechanisms do not explain increased T cell numbers in anti-IL10R1 treated mice.
Figure 3.7. Anti-IL10R1 treatment increased lung cellularity during *M.tb* infection. CBA/J mice were aerogenically infected with 50-100 CFU of *M.tb* Erdman, injected weekly with anti-IL10R1 or control antibody starting at day 90 post infection, and euthanized at days 115 and 150. Isolated lung cells were counted, fixed, and labeled with anti-CD3, -CD4, -CD8 and -CD11c for flow cytometric analysis. Total number of lung cells isolated (A), number of CD4 T cells (B), number of CD8 T cells (C) and numbers of CD11c+ cells (D) are the mean ± SEM of 4-5 independent experiments, each with 3-6 mice per group per time point, analyzed by Student’s *t*-tests for pairwise comparisons and one-way ANOVA with Tukey’s post test for multigroup comparisons.
T cell recruitment into *M. tb* infected lung tissue could have contributed to increased numbers in the absence of functional IL-10. To determine whether cell recruitment contributed to more T cells in anti-IL10R1 treated mice, T cells from the lung were analyzed for CD11a<sup>bright</sup> expression. At day 150 post infection, the numbers of CD4 and CD8 T cells expressing CD11a<sup>bright</sup> (Figure 3.8A, 3.8B) were significantly increased in mice treated with anti-IL10R1. In contrast, mice treated with control antibody showed moderate increases in CD11a<sup>bright</sup> T cells. Importantly, within both CD4 and CD8 T cell populations, multivariate analysis showed a significant increase in CD11a<sup>bright</sup> expression across all groups and time points as a result of anti-IL10R1 treatment. These data show that IL-10 blockade promotes T cell recruitment into the lungs. Furthermore, a similar role for IL-10 down-modulating CD11a expression has been shown *in vitro* using cells from human TB patients (611).
We have shown above that IL-10 blockade in CBA/J mice stabilized *M. tb* growth, increased survival, and increased T cell recruitment into the lungs. Given that TB disease outcome was improved as a result of anti-IL10R1 treatment, we hypothesized that granulomas would show more lymphocytic infiltrates and less tissue damage (caseation necrosis) than control antibody. Histopathological analysis was performed on formalin-fixed paraffin embedded lung lobes from anti-IL10R1 and control treated mice (Figure 3.9A). No consistent differences were detected in the amounts or location of lymphocytic infiltrates by light microscopy of hematoxylin and eosin stained sections. In both control
and anti-IL10R1 treated mice, lymphocytes were present within granulomas, at the periphery of granulomas, and formed perivascular cuffs (data not shown). These results suggest that IL-10 blockade had minimal effects on lymphocytes in *M.tb* infected lungs; however, we acknowledge that visual pattern recognition can be subjective and further quantitative analysis may yield more accurate results.

In the same tissue sections, granulomas in control and anti-IL10R1 treated mice were evaluated for evidence associated with lung tissue damage (caseating necrosis) and TB disease progression (inflammatory cells and *M.tb* bacilli in airways) (Figure 3.9A). Caseating granulomas were scored by a board certified veterinary pathologist without knowledge of the treatment groups or time points. Mice treated with anti-IL-10R1 had less variation in the granuloma scores, and a trend for lesions of less severity than control mice. Therefore lung lesions were not substantially altered by IL-10 blockade, providing additional evidence that increased lung cellularity was not detrimental to anti-IL10R1 treated mice.
Figure 3.9. Granuloma scores in CBA/J mice treated with anti-IL10R1. CBA/J mice were infected by aerosol with 50-100 CFU of \textit{M.tbc} Erdman. Lung lobes from individual mice were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned for hematoxylin and eosin staining, and graded by a board certified veterinary pathologist without knowledge of treatment groups or time points. Results are combined from 3 individual experiments with 3-6 mice per treatment group per time point, shown as the average ± SEM. Anti-IL10R1 treated mice at day 150 of \textit{M.tbc} infection had uniform granuloma scores and therefore error bars were not generated.

\textit{IFN-\gamma} production from lungs of anti-IL10R1 treated CBA/J mice.

Our results indicate that IL-10 blockade had a beneficial effect in the CBA/J reactivation TB model, likely mediated through increased CD4 and CD8 T cell protective functions that directly or indirectly prevented \textit{M.tbc} re-growth. Importantly, the additional cells within the lungs of anti-IL10R1 treated mice did not contribute to detrimental inflammation as assessed by granuloma scoring.
Since IL-10 inhibits antigen specific responses from T cells (262), multiple methods were used to assess functional effects of IL-10 blockade on lung cells during chronic M.tb infection. First, lung cell suspensions were cultured for four hours with anti-CD3 and anti-CD28 to stimulate TCR mediated cross-linking and signaling in the presence of GolgiStop\textsuperscript{TM} to allow for intracellular accumulation of IFN-\(\gamma\). The proportion (\%) of IFN-\(\gamma^+\) producing CD4 and CD8 T cells within the lungs of mice treated with anti-IL10R1 was not consistently statistically increased (data not shown). When the absolute numbers of IFN-\(\gamma^+\) T cells were calculated at day 115, there were no significant differences between control and anti-IL10R1 treated mice (Figures 3.10A, 3.10B). In contrast, after 150 days infection there was a substantial increase in the number of IFN-\(\gamma^+\) T cells in the lungs of anti-IL10R1 treated CBA/J mice. Statistical significance between anti-IL10R1 treated and control antibody treated mice was observed in the CD8 T cell population (Figure 3.10B) and a trend for more IFN-\(\gamma^+\) cells was observed within the CD4 T cells population (Figure 10A, p=0.08). These data indicate that anti-IL10R1 treatment during chronic M.tb lung infection increased the number of CD4 and CD8 T cell within the lung that could produce IFN-\(\gamma\) when stimulated through the TCR. This method of cross-linking eliminates the need for lung antigen presenting cells. The results, therefore, show the maximum TCR mediated IFN-\(\gamma\) responses but may not reflect true M.tb antigen specific responses.

To evaluate M.tb antigen specific responses, equivalent numbers of lung cells from control and anti-IL10R1 were cultured with M.tb culture filtrate protein (CFP) and IFN-\(\gamma\) secretion into cell culture supernatants was quantified by ELISA (Figure 3.10C). As anticipated (326), M.tb CFP specific IFN-\(\gamma\) production was low (less than 1ng/ml)
from controls. In contrast, at day 115 post infection lung cell cultures from anti-IL10R1 treated mice had significantly increased IFN-\(\gamma\) production relative to the control group suggesting that IL-10R1 treatment enhanced the function of either antigen presenting cells, or antigen specific T cells or both during the first 25 days of anti-IL10R1 therapy. Interestingly, IFN-\(\gamma\) production in anti-IL10R1 treated mice declined to levels similar to the mice treated with isotype controls by day 150 to similar levels as untreated mice (Chapter 2). Why long-term antigen specific IFN-\(\gamma\) secretion was not maintained during IL-10 blockade is uncertain and will be addressed in the discussion. It could reflect the stabilized \textit{M. tb} load in treated mice, a concept which has been proposed in treated TB patients (590, 591).

As a third method of IFN-\(\gamma\) quantification, we directly measured IFN-\(\gamma\) mRNA and protein levels in lung tissue. Because these methods do not rely on external stimulation, results could be the best indicator of \textit{in vivo} functional effects of IL-10 blockade. Consistent changes, however, were not detected in lung levels of IFN-\(\gamma\) mRNA due to anti-IL10R1 treatment (not shown). This suggests that blocking IL-10 does not directly affect IFN-\(\gamma\) gene transcription at the tissue level. When protein was quantified \textit{in vivo}, mice that received control antibody showed stable IFN-\(\gamma\) protein levels (Figure 10D). In contrast, mice receiving anti-IL10R1 treatment had significantly less IFN-\(\gamma\) protein within the lung after 115 days infection relative to control treated mice; however these levels increased significantly by 150 days (Figure 3.10D).

Taken together, these data indicate that short term IL-10 blockade enhanced antigen specific responses, while long term IL-10 blockade allowed accumulation of IFN-\(\gamma\) within the lung and promoted cellular recruitment of T cells with the potential to secrete
TCR mediated IFN-\( \gamma \). This pool of T cells with the potential to respond could be an important result of anti-IL10R1 treatment by increasing a cell population that could immediately respond protectively when their antigens were encountered, as could happen during periods of bacterial re-growth.
Figure 3.10. Anti-IL10R1 treatment increased IFN-γ during chronic \textit{M.\textit{tb}} infection. CBA/J mice were aerogenically infected with 50-100 CFU of \textit{M.\textit{tb}} Erdman, injected weekly with anti-IL10R1 or control antibody starting at day 90 post infection, and euthanized at days 115 and 150. Isolated lung cells were stimulated with anti-CD3 and anti-CD28 for 4 hours, fixed, labeled with anti-TCRβ, -CD4, -CD8 permeabilized, and labeled with anti-IFN-γ for intracellular flow cytometry. Numbers of IFN-γ⁺ CD4 T cells (A) and IFN-γ⁺ CD8 T cells (B) are the mean ± SEM of 4 independent experiments, each with 3-6 mice per group per time point with pairwise analyses using Student’s \textit{t}-tests (*p<0.05) and multigroup comparisons using one-way ANOVA (†p<0.05, NSD = no significant difference). Isolated lung cells were stimulated \textit{ex vivo} with \textit{M.\textit{tb}} culture filtrate protein (CFP) and secreted antigen specific IFN-γ in supernatants was quantified by ELISA (C). Data are the mean ± SEM of 2 independent experiments each with 4-5 mice per treatment group per time point with pairwise analyses by Student’s \textit{t}-tests (***p<0.001) and multigroup comparisons using one-way ANOVA (†p<0.05). IFN-γ in homogenized lung tissue was quantified by ELISA (D). Data shown are the mean ± SEM of three independent experiments each with 3-6 mice per treatment group per time point, and analyzed in pairs by Student’s \textit{t}-tests (*p<0.05).
Figure 3.10
**Immune mediators in the lungs during IL-10 blockade**

The main function of IL-10 is to down-regulate macrophage activation (262) and many of these same functions are enhanced by IFN-γ (207). We therefore expected that known protective functions of macrophages and secreted immune mediators would increase when released from IL-10 mediated inhibition. Because reactive nitrogen species rapidly degrade, the stable end-products, nitrites and nitrates, were quantified in clarified lung homogenates (Figure 3.11A). Contrary to our hypothesis, lungs from control mice had nearly 1nMol more nitrates and nitrates than IL-10R1 treated mice and in both groups, nitrites and nitrates increased slightly over time although statistical differences were not achieved. These results suggest that increased production of reactive nitrogen species did not stabilize *M.tb* growth in anti-IL10R1 treated mice. No consistent differences were found in IL-12p40 (Figure 3.11B) or TNF (data not shown) attributable to anti-IL10R1 treatment, suggesting that these cytokines did not contribute to improved *M.tb* control and survival when IL-10 was blocked.

We previously showed that T cell recruitment was enhanced in the lungs of anti-IL10R1 treated mice. To determine whether IL-10 blockade increased chemokine production in the lungs, as a possible mechanism to explain increased T cell migration, the amount of CCL5 (RANTES) was quantified in the lungs by ELISA (Figure 3.11C). The data show a trend for increased CCL5 in anti-IL10R1 treated mice suggesting that CCL5 contributed to lung cell migration when IL-10 was blocked. This finding also suggests that IL-10 limits chemokine expression during chronic *M.tb* infection and prevents migration of protective immune cells.
Many of the known protective mediators were not significantly increased at the lung tissue level by IL-10 blockade, or were not increased above controls (Figures 3.11A, 3.11B). It was possible that disruption of unknown regulatory feedback loop resulted in elevated IL-10 in mice treated with anti-IL10R1. To determine whether anti-IL10R1 treatment resulted in IL-10 which could have overwhelmed the receptor blockade, IL-10 protein was measured in lung tissue (Figure 3.11D). Anti-IL10R1 treated mice did not have elevated IL-10 in the lungs and the levels remained relatively stable. In contrast, IL-10 protein levels within the lungs of mice receiving control antibody significantly increased between 115 and 150 days (Figure 3.11D), as expected in the normal course of *M.tb* infection in CBA/J mice (326). Therefore, IL-10 blockade did not excessively increase IL-10 and antibody treatments did not alter the kinetics of IL-10 in CBA/J lungs.
Figure 3.11. Lung immune mediators following anti-IL10R1 treatment. CBA/J mice were aerogenically infected with 50-100 CFU of *M. tb* Erdman, and injected weekly with anti-IL10R1 or control antibody starting at day 90 post infection. Mice were euthanized at days 115 and 150, lungs homogenized and nitrites/nitrates (A), IL-12p40 (B), CCL5 (C), and IL-10 (D) quantified by ELISA in clarified supernatants. Data shown are the mean ± SEM of two (A), four (B), one (C) or three (D) independent experiments each with 3-6 mice per treatment group per time point, and analyzed in pairs by Student’s *t*-tests (*p*<0.05).
**Downstream effectors of IFN-γ signaling during IL10 blockade**

In our model anti-IL10R1 treatment had significant effects on IFN-γ production, from both CD4 T cells and CD8 T cells. To assess possible mechanisms by which abundant IFN-γ stabilized *M.tb* growth and enhanced survival, we quantified additional effectors up-regulated by IFN-γ signaling that could have improved antigen presentation and enhanced protective immunity. It has been shown that IFN-γ increased the expression of the class II transactivator (CIITA), the factor responsible for MHCII transcription (612). As shown in Figure 3.12A (Barnabe Assogba), statistically significant differences were not detected in the gene expression of CIITA between controls and anti-IL10R1 treated mice, although there was a slight trend for increased CIITA mRNA during IL-10 blockade. IL-10 has also been shown to slow the endocytic recycling of MHCII molecules (302). It was possible therefore, that inhibition of IL-10 could increase surface MHCII expression on antigen presenting cells in the absence of transcriptional changes.

To assess whether *in vivo* IL-10 blockade increased MHCII expression on lung CD11c$^+$ cells, the specific mean fluorescent intensity of surface MHCII was determined (Figure 3.12B). IL-10 blockade did not significantly affect MHCII expression on CD11c$^+$ lung cells although there was a slight trend for more MHCII in mice treated with anti-IL10R1. It is possible that these small differences enhanced antigen presentation to CD4 T cells despite the lack of statistical significance.

We previously showed that the numbers of lung CD8 T cells capable of TCR mediated IFN-γ was significantly increased by anti-IL10R1 treatment (Figure 3.10). To determine whether IL-10 blockade increased MHCI expression, we quantified lung gene expression of interferon regulatory factor 1 (*Irf1*) (Figure 3.12C, Barnabe Assogba), a
target of IFN-γ signaling that contributes to MCHI expression (207). Again, *Irf1* gene expression in the lungs was not significantly affected by anti-IL10R1 treatment, but there was a trend for increased expression in mice where IL-10 was blocked, suggesting that antigen presentation to CD8 T cells was also enhanced.

As a third downstream mediator of IFN-γ signaling that could affect *M.tb* growth, we quantified gene expression of the 47kDa GTPase LRG-47, a protein important for phagolysosomal acidification and control of intracellular microbial pathogens (613). As shown in Figure 3.12C (Barnabe Assogba), anti-IL10R1 treatment did not significantly increase lung mRNA levels of LRG-47 compared to controls. In both groups, however there were trends for increased LRG-47 mRNA over time. These results show that gene transcription of the LRG-47 in lung tissue was independent of IL-10.

Overall, these data indicate that IL-10 blockade resulted in small increases in the expression of molecules that could enhance antigen presentation to CD4 and CD8 T cells (Figure 3.12), although statistical significance was not achieved. In conjunction with the changes in lung immune mediators (Figure 3.11), these data suggest that certain macrophage functions were enhanced by IL-10 blockade (antigen presentation and chemokine production). Other macrophage functions, however, such as those associated with bacterial killing were not affected (nitrates/nitrates and phagosomal acidification). It is possible, therefore, that the cumulative effects of these small changes during anti-IL10R1 treatment contributed to a beneficial outcome that could not be detected by a single immunological assay.
Figure 3.12. Downstream effectors of IFN-γ following anti-IL10R1 treatment. CBA/J mice were aerogenically infected with 50-100 CFU of M. tb Erdman, and injected weekly with anti-IL10R1 or control antibody starting at day 90 post infection. Mice were euthanized at days 115 and 150, lung tissue homogenized in Ultraspec™, total RNA isolated, reverse transcribed, and relative gene expression quantified for CIITA (A), irf1 (C), and lrg-47 (D) using ribosomal RNA as an endogenous control. Results shown (A, C, D) are the average ± SEM of one experiment with 3-4 mice per time point per treatment group, analyzed pairwise by Student’s t-test and one-way ANOVA. Lung cells were isolated from separate groups and labeled fluorescently with anti-CD11c and anti-MHCII (B) for flow cytometric analysis. The mean fluorescence intensity (MFI) of MHCII on CD11c⁺ cells was calculated by subtracting the fluorescence intensity of the isotype controls for each timepoint, which did not exceed 1960. Data are the combined average ± SEM from four independent experiments with 3-6 mice per treatment group per time point, analyzed pairwise by Student’s t-test and one-way ANOVA.
Discussion

We used \( M.\text{tb} \) susceptible CBA/J mice to model reactivation TB with bacterial re-growth and disease progression following a long period of stable bacterial control. We further showed that step-wise increases in \( M.\text{tb} \) burden in mice was associated with disease progression which may also occur in man. Like humans, there was individual variability in the time to TB disease onset and morbid CBA/J mice developed clinical signs, overwhelming \( M.\text{tb} \) burdens and microscopic lung lesions that resembled reactivation TB.

Previous publications strongly associated IL-10 abundance during chronic \( M.\text{tb} \) infection with progressive TB disease in mice \((326)\) and man \((342, 572)\). We confirmed the kinetics of IL-10 production in the lungs of CBA/J mice repeated across BSL-3 facilities \((326)\), which was an important foundation for this work. Ongoing and future research in our laboratory has focused on identifying the cellular source of IL-10 and specific conditions which stimulate IL-10 secretion during \( M.\text{tb} \) infection. It would also be important to identify the mechanisms leading to elevated IL-10 in CBA/J mice, although evidence has not yet been published. Possibilities include inhibition of all-trans retinoic acid \((267)\), decreased degradation of IL-10, increased binding of IL-10 promoting transcription factors. Identifying the cellular sources and molecular mechanisms resulting in increased IL-10 could provide additional targets to block IL-10 \textit{in vivo}, confirming that IL-10 truly drives TB disease. We used anti-IL-10R1 because it has been shown to be effective in other intracellular pathogen models \((329, 601)\) and did not trigger unwanted intracellular signaling or activation (personal communication Rene de Waal Malefyt). Furthermore, we showed that CBA/J mice had more IL-10R\(^+\) CD11c\(^+\)
lung cells, indicating there were more cellular targets for anti-IL10R1 treatment than C57BL/6 mice.

Our data provide evidence that IL-10 production during chronic *M.tb* infection promoted bacterial growth and exacerbated disease. This was shown by blocking the biological action of IL-10 *in vivo* using anti-IL10R1 antibodies which were delivered during specific time points of *M.tb* infection. Anti-IL10R1 treatment stabilized lung *M.tb* burden, increased median survival time, and an increased number of cells within the lung by day 150 of infection. Increased cellularity within the lung was associated with augmentation of CD8 and CD4 T cells that expressed CD11a<sup>bright</sup>, indicating that IL-10 blockade promoted T cell recruitment, while proliferation and apoptosis were not affected. In addition, more T cells were capable of secreting IFN-γ when functional IL-10 was absent, suggesting that IL-10 inhibits the recruitment of *M.tb* protective cells, a similar finding may be true in human TB patients (611). Overall, these data indicate that IL-10 production during *M.tb* infection leads to poor T cell responses and impaired bacterial control. Furthermore, these deficiencies can be overcome by blocking the action of IL-10 *in vivo*.

Short term anti-IL10R1 treatment significantly increased *M.tb* CFP specific IFN-γ secretion *ex vivo* from lung cells of anti-IL10R1 treated mice compared to lung cells from control mice. We can hypothesize that this early availability (day 115) of antigen specific T cells contributed to the stable *M.tb* load detected at day 150. Why we saw a decrease in *ex vivo* antigen specific IFN-γ secretion during the later (day 150) time point is unclear. This finding, however, was consistent across multiple experiments. In some models, the amount of IFN-γ was proportional to the *M.tb* burden (591) and it is possible that a
stabilized bacterial load in anti-IL10R1 treated mice led to fewer actively-secreting antigen specific cells at day 150. Alternatively, by using *M.tb* CFP in this assay we may have inadvertently selected for CD4 T cell responses because the antigens would be primarily processed through exogenous endocytic pathways and presented to CD4 T cells. The decline in antigen specific IFN-γ, therefore could have reflected changes in the relative importance of T cell populations for *M.tb* protection as infection progressed, suggesting that CD8 T cells were the dominant source of IFN-γ at day 150.

Long term treatment with anti-IL10R1 led to more CD4 and CD8 T cells within the lung that were capable of TCR mediated IFN-γ secretion which for CD8 T cells was statistically significant. These results indicate that IL-10 blockade increased a population of cells capable of immediately mounting a protective response when their antigens were encountered. Analysis of IFN-γ within the whole lung homogenate supported these findings, with a significant increase in IFN-γ between day 115 and 150 of infection in anti-IL10R1 treated mice. Why control treated mice had increased IFN-γ in whole lung homogenates at day 115 is uncertain; it is possible that IFN-γ was increased as a result of subtle differences in lung bacterial growth or turnover that were not detected by the CFU assay.

In addition to dampening protective immunity, IL-10 can also down-regulate an overly exuberant immune response and potentially prevent tissue damage (262), as has been recently shown during *M.tb* infection in another mouse strain and model (339). Indeed, the increased cellularity within the lungs of anti-IL10R1 treated CBA/J mice alludes to a role for IL-10 in preventing detrimental pulmonary inflammation. Anti-IL10R1 treatment did not, however, lead to any overt histopathological changes in the
lungs and therefore we consider these increases to be reflective of enhanced protective immunity rather than a failure to dampen inflammatory responses. Furthermore, the lung cell numbers recovered from anti-IL10R1 treated CBA/J mice were comparable to M.tb resistant C57BL/6 mice (data not shown). In fact, we have previously shown that C57BL/6 mice have more CD11abright CD4 T cells in the lungs during M. tuberculosis infection than CBA/J mice (516) and again, the increased number of CD11abright T cells in anti-IL10R1 treated CBA/J mice suggests acquisition of a protective immune response. Furthermore, Ely et. al. have implied that CD11abright T cells localize to lung tissue during an antigen specific immune response (614). In these experiments, in addition to enhanced T cell adhesion molecule expression we also showed that chemokines, such as CCL5 could play a role in cell migration when IL-10 was blocked. Overall, these results indicate that IL-10 inhibits T cell recruitment and migration in the lungs of CBA/J mice and suggests that reactivation TB in man may result in part from defective localization of protective immune cells.

The failure to reach statistical significance in some of our data is likely multifactorial reflecting the complexity of M.tb susceptibility and TB disease progression in CBA/J mice (558); the choice of only two selected time points for analysis which cannot account for the wax and wane of immunity that has been described by others (615); the pleotropic effects of IL-10; and technical limitations that were focused on establishing in vivo relevance. The increased survival in anti-IL10R1 treated mice compared to controls was close to statistical significance, but was not extended to survival seen in M.tb resistant mouse strains in our laboratory (Chapter 2). Therefore, it is unlikely that M.tb susceptibility in CBA/J mice is controlled by IL-10 alone. Other
immune mediators (e.g. IFN-γ (Chapter 2) and CCL5 (Chapter 4)) could contribute to susceptibility and the underlying mechanisms may not be reversed by anti-IL10R1 treatment. Furthermore, although anti-IL10R1 treatment increased survival compared to controls (Figure 3.6), the median survival was not longer than seen in untreated CBA/J mice (Chapter 2). These results indicate that antibody treatment complicated TB disease progression and alternative methods of IL-10 blockade should be pursued for therapeutic considerations.

It is also possible that the extensive antibody treatments may have stimulated anti-rat antibody production and a loss of functional activity of the anti-IL10R1 antibody, reducing the long term biological impact of the antibody treatment. In preliminary studies some mice generated anti-rat antibodies after multiple injections (data not shown), although it is uncertain whether the antibodies interfered with the epitope recognition and function of anti-IL10R1. Further studies could address the biological activity of anti-IL10R1 present, which would be an important consideration should IL-10 blockade be considered as an adjunct immunotherapy in human TB. Alternative strategies to study the role of IL-10 during chronic *M.tb* infection, such as IL-10 deficient CBA/J mice are currently underway and may provide valuable tools to confirm the role of IL-10 in reactivation TB.

It is important to acknowledge that we are manipulating a complex *in vivo* system of *M.tb* infection and IL-10. Our readout of a protective response was based upon known immunity during *M.tb* infection and IL-10 function. Molecules such as IFN-γ, IL-12, TNF, nitric oxide, and MHCII are essential for control of *M.tb* infection (118, 217) and are down-regulated by IL-10 (262). Consequently these were the parameters that we
measured, primarily focused on the cellular responses and lung tissue level to assess consequences of treatment in vivo. We found few statistically significant differences in the expression of these known protective molecules when we manipulated the action of IL-10. However, this may not be surprising as IL-10 itself has pleotropic effects on multiple types of immune cells and many mechanisms contribute to IL-10 mediated immunomodulation (262). More sensitive assays or experiments may have been necessary to achieve statistical differences between control and anti-IL10R1 groups. For example, the functional consequences of IL-10 blockade could have been better evaluated by purifying T cell subsets or, possibly, separating M.tb infected and non-infected macrophages from the lungs.

We believe that numerous small changes in immune parameters prevented M.tb re-growth and improved survival in anti-IL10R1 treated mice. The stabilization of M.tb burden, rather than reduced bacterial load, may have resulted from enhanced antigen specific immunity (CIITA, MHCII, Irf1, CD4 and CD8 T cell derived IFN-γ), and cellular recruitment (CD11abright, CCL5) which were not able to substantially boost microbicidal functions (nitrates/nitrates, lrg-47). The overall importance of this study, however, is that despite failing to find significant differences in some immunological parameters, we could modify the course of infection by blocking the action of IL-10. The possibility therefore exists that enhanced protection is associated with alternate cells/molecules that have yet to be clearly defined.

In summary, we have shown that modulation of IL-10 in vivo using a mouse strain that naturally produces abundant IL-10 during infection with M.tb leads to stabilized M.tb load, extended survival, and enhanced T cell responses within the lung. It
is important to recognize that the detection of an improved phenotype following the *in vivo* manipulation of one single immunological mediator is a highly significant finding. To date, *in vivo* removal of cytokines during *M.tbc* infection have rarely shown a beneficial effect (236, 616) and the depletion or absence of a single cytokine typically impairs resistance (118, 149, 217) or promotes inflammation and tissue damage (339, 616). The complete absence of IL-10 shortened survival in C57BL/6 mice with morbidity attributed to pathological inflammation (339). These results suggest that a small amount of IL-10 may be beneficial to the host during *M.tbc* infection by protecting delicate lung tissues from immune mediated damage. In contrast, our results identify IL-10 abundance as a detriment, causing reactivation TB by inhibiting recruitment of protective T cells and dampening multiple facets of antigen specific immunity. Furthermore, that IL-10 blockade altered the CBA/J susceptibility phenotype has particular relevance to studies in man where a spectrum of susceptibility and IL-10 responses can be observed. From these results, it would be intriguing to speculate that IL-10 could be also targeted as an immunotherapy in humans.
CHAPTER 4: LOW CCL5 CORRELATES WITH, BUT DOES NOT CAUSE, SUSCEPTIBILITY TO MYCOBACTERIUM TUBERCULOSIS.

Abstract:

Control of Mycobacterium tuberculosis (M.tb), the causative agent of tuberculosis, requires localization of immune cells to form protective granulomas. The mechanisms governing cell recruitment to granulomas, however, are not fully understood. In vitro, the chemotactic cytokine CCL5 is secreted by M.tb infected macrophages and induces T cell migration. In this chapter, we again used M.tb susceptible and resistant mouse strains and have identified CCL5 as potentially important for T cell recruitment to M.tb granulomas in vivo. In M.tb susceptible CBA/J mice, low T cell numbers and lymphocyte-poor granulomas observed during early M.tb infection were associated with significantly reduced CCL5 mRNA and protein in lung tissue as compared to C57BL/6 mice. We further showed that CBA/J mice had fewer CCR5+ T cells in the lungs suggesting a mechanism by which reduced CCL5 affected cell migration. As infection progressed, however, lung T cells in CBA/J mice eventually reached equivalent numbers as in C57BL/6, indicating that additional CCR5 ligands or CCL5 receptors may have compensated for diminished CCL5 production. These experiments identified mouse strain dependent differences in chemokine secretion during M.tb infection and logically indicated that low CCL5 could be a correlate of M.tb
susceptibility. However, these two mouse strains are genetically heterogeneous and other immunological differences could account for variation in *M. tb* susceptibility. To more accurately define the role of CCL5 during *M. tb* infection, additional studies were conducted in CCL5 deficient mice on the C57BL/6 background. CCL5 KO mice had delayed T cell recruitment and fewer cells capable of antigen specific IFN-γ resulting in impaired control of *M. tb* growth. In CCL5 KO mice, lung T cells and antigen specific cells eventually reached or exceeded the levels of WT mice, likely mediated by CCR5-CCL4 interactions which partially compensate for the lack of CCL5.

Overall, these results illustrate that rapid and abundant CCL5 production specifically contributed to early control of *M. tb* growth through protective T cell recruitment to *M. tb* lung granulomas *in vivo*. These studies indicated that low CCL5 may be an early biomarker of *M. tb* susceptibility, but that suboptimal CCL5 or even the complete absence of CCL5 are unlikely to be a single cause of TB disease progression.

**Introduction:**

Following an infection with *M. tb*, 90-95% of infected individuals contain the bacilli for extended periods of time within granulomas (latent TB). In Chapter 4 we determined that slow T cell recruitment to the lungs, lymphocyte poor granulomas and poor control of *M. tb* growth in *M. tb* susceptible CBA/J mice was associated with low lung CCL5 mRNA and protein levels. This striking mouse-strain dependent difference identified another possible correlate of *M. tb* susceptibility and justified further
experiments to determine the role of CCL5 \textit{in vivo} under genetically controlled conditions.

In both humans and in mice, \textit{M.\textit{tb}} granulomas are composed of multiple immune cell types that orchestrate T_{H1} protective immunity, including IL-12 (126, 567), TNF (149, 160), and IFN-\textit{\gamma} (215, 617). The organization and cellular composition of granulomas varies by time, host species and pathogen, but all granulomas control pathogens, protect adjacent tissue (390, 618-623) and provide niches for pathogen persistence (624, 625). In humans, primates, and mice, recruitment of immune cells to \textit{M.\textit{tb}} infected lungs and granulomas is similar. Early granulomatous foci consist of neutrophils and macrophages, and after a few weeks neutrophils decline while activated macrophages and lymphocytes increase (521, 626). During later stages of \textit{M.\textit{tb}} infection and in particular in active TB disease, human patients show a spectrum of pathological changes including central caseation necrosis, mineralization, cavitation and peripheral fibrosis, (516, 520, 521, 618, 627) which CBA/J mice can model (Chapter 3).

As discussed in Chapters 2 and 3, the ability of mice to control \textit{M.\textit{tb}} infection depends on the inbred strain background (516, 518, 519, 558, 563-565, 628) with complex underlying causes (519, 558, 564, 565, 629-635). Furthermore, \textit{M.\textit{tb}} susceptibility in humans and in mice is associated with granuloma composition. Patients with controlled or latent TB (160, 165, 513, 514) and resistant mice (390, 515-520) form compact lymphocyte-rich granulomas that limit \textit{M.\textit{tb}} growth for extended periods, and in mice localize many lung antigen specific IFN-\textit{\gamma} \textit{CD4} T cells (Chapter 2). In contrast, active TB patients (160, 165, 513, 514, 521-524) and \textit{M.\textit{tb}} susceptible mice (149, 390, 515-520) develop large macrophage dominated granulomas with few lymphocytes.
Furthermore, it has been shown that \textit{M.tb} susceptible mice do not localize as many effector T cells to the lungs following \textit{M.tb} infection (516, 636), indicating that lymphocyte poor granulomas do not provide optimal protective immunity. All of these observations suggest that lung T cell migration may be important determinants of disease outcome by promoting lymphocyte-rich granuloma formation.

We hypothesized in Chapter 4 that specific chemokines produced during \textit{M.tb} infection recruit activated T cells to the lungs for protective granuloma formation. We focused on CCL5 and its receptors because both have been detected in humans and mice infected with \textit{M.tb} (637, 638) and promoted lymphocyte migration \textit{in vitro} (499). Furthermore, CCL5 blockade decreased immune cells recruited to lung mycobacterial granulomas in other model systems (639). These observations strongly supported our hypothesis that CCL5 directs T cell migration \textit{in vivo} promoting lymphocyte-rich granulomas and controlled \textit{M.tb} growth. In this Chapter, we studied the consequences of CCL5 expression on granuloma formation, protective antigen specific responses and \textit{M.tb} susceptibility. First we identified low CCL5 as a correlate of \textit{M.tb} susceptibility in CBA/J mice, detected very early during \textit{M.tb} infection. Second we exploited this phenotypic difference to justify experiments in CCL5 gene disrupted mice. Overall, our data showed that CCL5 recruited early IFN-\(\gamma\) producing antigen specific T cells to the lungs and promoted rapid lymphocyte-rich granulomas which controlled \textit{M.tb} growth. We furthermore confirmed that reduced and absent CCL5 were not associated with T cell priming defects, indicating specifically that CCL5 mediated T cell migration to \textit{M.tb} granulomas in the lungs.
Because chemokines display promiscuity in ligand receptor binding we investigated CCL5-related chemokines, CCL4 and CCL3, as well as the receptors CCR1 and CCR5 (495, 496). Our results indicated that lung T cells used CCR5 and not CCR1 for lung migration following \( M.\text{tb} \) infection. We also showed that CCL4 but not CCL3 partially compensated for the absence of CCL5 during early \( M.\text{tb} \) infection. Furthermore, we suggest that the temporal availability of chemokines produced at the sites of \( M.\text{tb} \) infection may have a greater influence on granuloma formation \textit{in vivo} than the \textit{in vitro} functional redundancies.

\textbf{Materials and Methods:}

\textbf{Mice}

Specific pathogen free eight-week-old female C57BL/6 and CBA/J mice (Charles River Laboratories, Wilmington, MA) were maintained in ventilated cages within Biosafety Level-3 facilities and provided with sterile food and water \textit{ad libitum}. All protocols were approved by The Ohio State University’s Institutional Laboratory Animal Care and Use Committee.

\textbf{\( M.\text{tb} \) stocks}

\( M.\text{tb} \) Erdman (ATCC #35801) was obtained from American Type Culture Collection (Manassas, VA.). Stocks were grown in Proskauer-Beck liquid medium containing 0.05\% Tween 80 to mid-log phase and frozen in 1ml aliquots at -80°C.
**M. tb infection**

Mice were exposed to an aerosol of *M. tb* Erdman using an Inhalation Exposure System (Glas-col, Terre Haute, IN) that was calibrated to deliver 50-100 CFU to the lungs of each individual mouse. Confirmation of *M. tb* dose and calculation of *M. tb* burden throughout the experimental infection period were determined by plating serial dilutions of partial lung homogenates onto OADC-supplemented 7H11 agar. *M. tb* CFUs were counted after 3 weeks at 37°C. For experiments lasting longer than 90 days, mice were weighed weekly. Mice weighing 20% less than the average of age- and sex-matched non-infected control animals with concurrent signs of disease progression (unthrifty hair coats, tachypnea, social isolation) were euthanized and excluded from the experiments.

**Real time PCR**

Right medial lung lobes were homogenized in 1 ml of UltraSpec (Biotecx, Houston, TX) and frozen rapidly at –80°C. Total RNA was isolated by following the manufacturer's instructions, and 1µg was reverse transcribed using an Omniscript RT kit (QIAGEN, Valencia, CA). Real-time PCR was performed with an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using TaqMan Gene Expression Assays for CCL5 (Applied Biosystems, Foster City, CA). The Delta Delta cycle threshold method was used for relative quantification of mRNA expression; in this analysis 18S was used as an endogenous normalizer.
Cytokine and chemokine protein levels

Lung homogenates were briefly centrifuged and clarified supernatants assayed for CCL5, CCL4, CCL3, CXCL10, and CCL12 using ELISA antibody pairs and standards, or ELISA DuoSet kits from R&D Systems (Minneapolis, MN) and optimized following the manufacturer’s instructions. TNF in clarified lung homogenates, and IL-2 and IFN-γ from lymph node cell culture supernatants were assayed using ELISA antibodies and standards from BD Biosciences (San Jose, CA) that were optimized following the manufacturer’s instructions.

Lung histology

The right cranial lung lobes from individual mice were inflated with 10% neutral buffered formalin prior to paraffin embedding, sectioning at 5µm, and staining with hematoxylin and eosin or Ziehl-Neelsen’s acid-fast modified stain. Slides were evaluated by a board certified veterinary pathologist (Dr. Paul Stromberg, Department of Veterinary Biosciences, The Ohio State University, Columbus, OH) or scanned and granulomas counted and measured using an AperioScan Scope (Vista, CA) in the Department of Veterinary Biosciences, The Ohio State University, Columbus, OH by the author (GB).
Lung cell isolation

Single cell suspensions were obtained from the lungs at specific time points post infection as described (561). Briefly, lungs were perfused through the right cardiac ventricular lumen and pulmonary trunk with 10ml of phosphate buffered saline containing 50U/ml of heparin (Sigma; St. Louis, MO) and placed in Dulbecco’s modification of Eagle’s medium (DMEM, 500ml) (Mediatech; Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals; Fort Collins, CO), 1% HEPES buffer (Sigma), 10ml of 100X non-essential amino acid solution (Sigma), 5ml of penicillin/streptomycin solution (50,000U penicillin, 50mg streptomycin; Sigma) and 0.14% mercaptoethanol (complete DMEM). Single cell suspensions from individual mice were obtained by dicing lung lobes with sterile razor blades, followed by a 30 minute incubation at 37°C with 4ml of complete DMEM containing collagenase XI (0.7mg/ml; Sigma), and bovine pancreatic DNase (30µg/ml; Sigma). 6ml of complete DMEM was subsequently added to dilute enzymatic activity and lung pieces were pressed through sterile 70µm nylon mesh screens (BD Biosciences; San Jose, CA) to obtain a single cell suspension. Residual red blood cells were lysed using 2ml of ACK lysis buffer (0.15 M NH₄Cl, 1mM KHCO₃) for 3 minutes at room temperature followed by washing with complete DMEM. Viable cells were counted using Trypan blue exclusion and re-suspended at working concentrations in complete DMEM.
**Lung cell culture for IFN-γ ELISPOTS**

2.5 x 10^5 lung cells from individual mice were serially diluted 1:2 in complete DMEM for at least 4 dilutions onto pre-coated and blocked ELISPOT plates (Millipore) as per the manufacturer’s instructions (Ready-Set-Go Mouse IFN-γ ELISPOT kit, eBioscience, San Diego, CA). Lung cells were incubated with 10µg/ml of ovalbumin (negative control), 10µg/ml of *M.tb* culture filtrate protein (CFP), 5µg/ml *M.tb* early secreted antigenic target-6 (ESAT-6) or 1µg/ml anti-CD3 (BD Biosciences) plus 0.1µg/ml of anti-CD28 (BD Biosciences) (positive control) at 37°C and 5% CO₂ for 24 hours. After 24 hours, supernatants were removed and spots were developed as per the manufacturer’s instructions (eBioscience). Spot forming units (SFU) were counted and analyzed using the Immunospot Analyzer (C.T.L., Cleveland, OH). Antigen specific IFN-γ spot forming units were determined by subtracting the number of spots formed in the presence of a negative control (media alone or ovalbumin).

**Lymph node harvest and cell culture**

The lung-draining mediastinal lymph nodes from individual mice were harvested with small curved forceps and pressed gently through sterile 70µm nylon mesh screens (BD Biosciences; San Jose, CA) to obtain a single cell suspension. Residual red blood cells were lysed as described above. Viable cells were counted using Trypan blue exclusion and re-suspended in complete DMEM. 1 x 10^5 lymph node cells from individual mice were cultured in duplicate wells at 37°C 5% CO₂ for 72 hours in the absence (for IL-2 secretion) or presence (for antigen specific IFN-γ) of 5µg/ml of Ag85.
After incubation, the supernatants were stored at -80°C until the experiment had ended and all time points could be assayed by ELISA as described above.

Flow cytometry

The following conjugated and unconjugated antibodies and isotype controls were purchased from BD Biosciences: Fc Block™ (clone 2.462), PerCP-Cy5.5 anti-CD3ε (145-2C11), APC-Cy7 anti-CD4 (GK1.5), PE-Cy7 anti-CD8 (53-6.7), APC anti-TCR-β chain (H57597), PerCP anti-CD8 (53-6.7), FITC I-A/I-E (2G9), FITC rat IgG2a (2G9), PE anti-CD195 (C34-3448), PE rat IgG2c (C34-3448). In some experiments, PE anti-CD195 (HM-CCR5) and PE hamster IgG isotype control were obtained from Biolegend (San Diego, CA). Polyclonal anti-CCR1 (Abcam, Cambridge, MA), was conjugated with an APC as per the manufacturer’s instructions (Prozyme, San Leandro, CA) and concentration optimized for use.

Aliquots of 5x10^5 to 2.5x10^6 lung or lymph node cells from individual mice were fixed in FACS buffer (deficient-RPMI (Irvine Scientific; Santa Ana, CA) supplemented with 0.1% sodium azide (Sigma)) for 30 minutes at 4°C. Cells were then incubated with 0.31µg of Fc Block™ for 10-15 minutes at room temperature, to minimize non-specific antibody binding. For surface staining, samples were incubated with 0.31µg of fluorescent antibodies in the dark for 20 minutes at 4°C followed by at least 3 washes in FACS buffer. For in vivo proliferation, mice were injected with BrdU 24 hours prior to euthanasia. Cells were surface labeled with fluorescent antibodies, permeabilized and labeled with anti-BrdU, anti-CCR5, anti-CCR1 or isotype controls as per the
manufacturer’s instructions (BrdU flow cytometry kit; BD Biosciences). Lymphocytes were identified according to characteristic forward and side scatter profiles and 20,000-50,000 events were counted within that gate using a LSRII flow cytometer. Results from individual mice were analyzed with FACSDiva software (BD Biosciences). Isotype controls were used to set gates for analysis.

Statistics

Statistical analyses were performed using Prism 4 software (GraphPad Software, San Diego, CA). Multigroup comparisons were analyzed with one-way ANOVA using Dunnett’s post-test (comparison to non-infected control mice within each strain over time) or Tukey’s post-test (comparison of time points within strains). Pairwise comparisons at the same time points used the Student’s $t$-test. Statistical significance in all analyses was defined as *p<0.05, **p<0.01, ***p<0.001. Where necessary, one outlier per strain per time point was identified using the Grubb’s outlier test (GraphPad QuickCalcs, http://www.graphpad.com/quickcalcs) and removed.

Results

*M. tb* susceptibility is associated with delayed T cell recruitment to the lungs

Our results in Chapter 2 and those of others showed that stabilized *M. tb* growth was associated with an influx of lymphocytes and detection of protective IFN-γ approximately 3 weeks after *M. tb* infection (574, 580, 615, 640). Furthermore, in Chapter
2 we showed equivalent progressive *M.tb* growth during the first 14 days of infection in CBA/J and C57BL/6 mice, a time frame associated with innate immune responses. Between 14 and 21 days, CBA/J mice were less able to control *M.tb* growth as compared to C57BL/6 mice, leading to sustained high bacterial burdens by 21 days.

The kinetics of *M.tb* growth indicated that CBA/J mice had early impaired *M.tb* specific protective immunity. To detect early lung immune responses to *M.tb*, that were not apparent in Chapter 2, we evaluated days 7, 14, 21, and 35 and quantified total cellularity, CD4 T cells, CD8 T cells and CD11c$^+$ cells in *M.tb* infected CBA/J and C57BL/6 mice. No significant differences in total lung cell numbers or in immune cell subsets were detected between non-infected mice, or on day 7 of *M.tb* infection (data not shown). At days 14, 21, and 35, however, the lungs of CBA/J mice contained significantly fewer total cells (Figure 4.1A), CD4 T cells (Figure 4.1B) and CD8 T cells (Figure 4.1C) compared to C57BL/6 mice. In contrast, the numbers of isolated CD11c$^+$ cells (Figure 4.1D) from the lungs were similar in both mouse strains. Therefore, reduced cellularity in CBA/J lungs resulted from poor T cell localization while macrophages were recruited in similar numbers as in *M.tb* resistant C57BL/6 mice. These results indicate that T cells in *M.tb* infected CBA/J lungs may not migrate efficiently, but do not eliminate the possibilities of diminished antigen specific proliferation in the lungs or impaired T cell priming with a secondary delay in lung recruitment.
Figure 4.1. Cellularity in the lungs of *M. tb* infected mice. C57BL/6 and CBA/J mice were infected with 50-100 *M. tb* Erdman CFU by aerosol and euthanized at the indicated time points. Total viable isolated lung cells were counted (A) and fluorescently labeled with anti-CD3, anti-CD4 (B), anti-CD8 (C), and anti-CD11c (D) for analysis by flow cytometry. Results are the combined mean and SEM of 3 (A, B, C) or 2 (D) independent experiments with 4-5 mice per strain per time point with statistical analysis by Student’s *t*-tests at each time point (*p*<0.05, **p*<0.01, ***p*<0.001). No significant differences were detected between non-infected mice or at day 7 post infection (data not shown).

T cell proliferation *in vivo* was evaluated in CBA/J and C57BL/6 mice by BrdU intranuclear incorporation. C57BL/6 lung BrdU$^+$ CD4 T cells and BrdU$^+$ CD8 T cells increased substantially by three weeks of *M. tb* infection compared to non-infected mice and the numbers were sustained (Figures 4.2A, 4.2B). In contrast, the numbers of BrdU$^+$ CD4 T cells from CBA/J lungs increased slowly and remained lower than in C57BL/6
mice (Figure 4.2A). Similarly, CBA/J lung BrdU+ CD8 T cells were fewer than in C57BL/6 lungs (Figure 4.2B). In both T cell subsets, statistically differences were achieved at 21 days of \textit{M.tb} infection. Overall, these results show that CBA/J lungs contained fewer proliferating T cells in response to \textit{M.tb} infection compared to C57BL/6, which suggested delayed and/or defective antigen specific proliferation. However, a limitation of this \textit{in vivo} assay is that it cannot determine whether the BrdU was incorporated in the lymph node during T cell priming or in the lungs during a second encounter with \textit{M.tb} antigens. It is possible, therefore, that CBA/J lungs contained fewer BrdU+ CD4 and CD8 T cells because the cells which recently proliferated in the lymph node were less able to migrate to lungs to form \textit{M.tb} granulomas.
Figure 4.2. Lung CD4 and CD8 T cell BrdU incorporation during *M. tb* infection. C57BL/6 and CBA/J mice were infected with 50-100 CFU of *M. tb* Erdman by aerosol and euthanized at the indicated time points. 24 hours prior to euthanasia, BrdU was administered to individual mice by intraperitoneal injection. Single cell suspensions from the lungs were fixed and labeled with fluorescent anti-CD3, anti-CD4, anti-CD8, permeabilized, and labeled with anti-BrdU for flow cytometry. The number of BrdU⁺ CD4 T cells (A) and BrdU⁺ CD8 T cells (B) are the combined average ± SEM from at least two independent experiments each with 4-5 mice per strain per time point analyzed by strain over time with one-way ANOVA and Dunnett’s post test, *p<0.05, **p<0.01 or analyzed pairwise at each time point by Student’s *t*-test, ++p<0.01.

Similar T cell priming in *M. tb* infected C57BL/6 and CBA/J mice

Lower numbers of lung T cells in CBA/J mice could have resulted from inadequate T cell priming. To evaluate *in vivo* T cell priming, CD4 T cells and subsets of activated CD4 T cells were enumerated in the lung-draining mediastinal lymph nodes. Lymph nodes from *M. tb* infected C57BL/6 and CBA/J mice contained similar numbers
of cells for 35 days (Figure 4.3A). There were fewer cells in CBA/J lymph nodes at day 21, however the difference was not statistically significant. CD4 T cell numbers were equivalent, except at day 35 when more cells were isolated from CBA/J lymph nodes than C57BL/6 (Figure 4.3B). For three weeks, CBA/J and C57BL/6 lymph nodes contained similar numbers of activated CD44\(^{hi}\) CD4 T cells (Figure 4.3C) and CD4 T cells that co-expressed CD44\(^{hi}\) CD45RB\(^{lo}\) (Figure 4.3D). By five weeks of \(M\.tb\) infection, CBA/J mediastinal lymph nodes had more CD44\(^{hi}\) CD4 T cells, but fewer CD44\(^{hi}\) CD4 T cells that co-expressed CD45RB\(^{lo}\), a marker which is down regulated as T cells transition from naïve to memory effector cells (641). The significance of this finding is uncertain, but it could indicate that as infection progressed CBA/J CD4 T cells became aberrantly activated but were not maturing into effector cells. For the focus of this Chapter, however, it was important to show that early CD4 T cell priming appears intact in CBA/J lymph nodes, and activation was similar to C57BL/6 mice. These results suggest that reduced lung T cell numbers were not due to defective priming in CBA/J mice. At five weeks of infection, differences in T cell numbers and activation may have been associated with late T cell priming events or reflect the fact that lymph nodes become active sites of \(M\.tb\) infection (data not shown) and granuloma formation (data not shown).

When lymph node cell function was analyzed by cytokine secretion, IL-2 (Figure 4.3E) and \(M\.tb\) Ag85 specific IFN-\(\gamma\) (Figure 4.3F) from CBA/J mice were higher than C57BL/6, although marked variation between individual mice was evident. Nevertheless, these results indicated that T cell priming and cytokine production in CBA/J mice was
efficient, and occurred at a similar rate across mouse strains. Neither cytokine was detected at day 14 of *M.tb* infection, likely as a result of ELISA insensitivity.

Overall, *M.tb* susceptible CBA/J mice were capable of T activation and cytokine secretion in the lymph nodes at equivalent or higher levels than C57BL/6 mice, indicating that reduced lung T cell numbers were not secondary to inadequate T cell priming. These results therefore, further supported our hypothesis that lung T cell migration was impaired in CBA/J mice, and suggested that fewer lung BrdU* T cells (Figure 4.2) were due to deficient efferent trafficking from the lymph node.
Figure 4.3. Evidence of T cell priming in *M. tb* infected mice. C57BL/6 and CBA/J mice were infected with 50-100 *M. tb* Erdman by aerosol and euthanized at the indicated time points. Single cell suspensions from the lung-draining mediastinal lymph nodes were fixed and labeled with fluorescent anti-CD3, anti-CD4, anti-CD44 and anti-CD45RB for flow cytometry. The total number of lymph node cells (A), number of CD4 T cells (B), number of activated CD44^{hi} CD4 T cells (C) and percent of co-expressing CD44^{hi} CD45RB^{lo} CD4 T cells (D) are shown. Results are the combined averages ± SEMs for three independent experiments (A, B), two independent experiments (C), or one experiment (D) with 4-5 mice per strain per time point analyzed by Student’s *t*-tests at each time point, * p < 0.05. No significant differences were detected in the numbers of lymph node cells from non-infected mice (data not shown). 1x10^5 lymph node cells were cultured for 72 hours at 37ºC without an *M. tb* antigen for IL-2 secretion (E) or with *M. tb* Ag85 for antigen specific IFN-γ secretion (F). Results are the average ± SEM from one experiment with 4-5 mice per strain per time point, analyzed by Student’s *t*-tests, ND = none detected.
Figure 4.3
CBA/J mice form lymphocyte poor *M.tb* granulomas.

To determine whether low T cell numbers between two and five weeks of *M.tb* infection affected granuloma size and composition in CBA/J mice, lung tissue from both mouse strains was evaluated by light microscopy. In *M.tb* susceptible CBA/J mice, no granulomatous foci were detected at 14 days of *M.tb* infection while in C57BL/6 mice, small nascent granulomas (neutrophils and macrophages) were occasionally identified (Figure 4.4 and Table 4.1). These results indicated that granuloma formation was delayed in *M.tb* susceptible CBA/J mice as compared to C57BL/6 mice. Because there were fewer T cells isolated from the lungs of CBA/J mice (Figure 4.1B, 4.1C), but similar numbers of CD11c+ cells (Figure 4.1D), we anticipated that CBA/J mice would form smaller granulomas than C57BL/6 mice. After 21 and 35 days of *M.tb* infection, however, CBA/J granulomas were typically larger than C57BL/6 granulomas, contained many activated and epithelioid macrophages, and had developed necrotic foci. Similar to established *M.tb* granulomas (326, 516), early CBA/J granulomas were dominated by macrophages that appeared more numerous than lymphocytes (Table 4.1). In general, granuloma cellular composition in C57BL/6 and CBA/J lungs were similar to the flow cytometry calculation (Figure 4.1). One exception appeared to be the lung macrophages in CBA/J mice, which visually appeared more numerous in the lung tissue sections than lymphocytes, a subjective finding that contrasts with the numbers of CD11c+ cells calculated from single cell suspensions. It was possible lung macrophages down-regulated CD11c+ expression during isolation into single cell suspensions, but this was considered unlikely because nearly 70% of lung macrophages similarly obtained expressed CD11c (Erin Rottinghaus, unpublished observations.) It is more likely that
CD11c+ lung macrophages were lost as a result of adherence to sterile plasticware necessary for processing.

Figure 4.4. *M. tb* lung granulomas in mice during early infection. C57BL/6 and CBA/J mice were infected by aerosol with 50-100 *M. tb* Erdman CFU and euthanized at the time points indicated. Lung lobes from individual mice were inflated with 10% neutral buffered formalin, fixed for 3 weeks at room temperature, sectioned at 5µm, and stained with hematoxylin and eosin. Slides were scanned and images captured by Aperio ScanScope. Day 14, bar = 50µm; Day 21, bar = 50µm; Day 35, bar = 250µm.

<table>
<thead>
<tr>
<th>Table 4.1</th>
<th>Granuloma diameter, range (mm)</th>
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<tr>
<td>Day 35</td>
<td>0.2-1</td>
<td>0.2-2.5</td>
</tr>
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</table>

* L = lymphocytes, M = macrophages, N = neutrophils

Table 4.1. *M. tb* lung granulomas in mice during early infection. C57BL/6 and CBA/J mice were infected by aerosol with 50-100 *M. tb* Erdman CFU and euthanized at the time points indicated. Lung lobes from individual mice were inflated with 10% neutral buffered formalin, fixed for 3 weeks at room temperature, sectioned at 5µm, and stained with hematoxylin and eosin. Slides were scanned and images captured by Aperio ScanScope. Day 14, bar = 50µm; Day 21, bar = 50µm; Day 35, bar = 250µm.
CBA/J lung macrophages produce low CCL5 during *M.tb* infection

CCL5 directs migration of activated lymphocytes *in vitro* (498, 638, 642) and CCL5 has been detected *in vivo* in TB patients and in animal models (499, 643-645). To demonstrate that fewer T cells in susceptible CBA/J mice was associated with reduced chemokines, CCL5 gene expression and protein levels were quantified in the lungs during *M.tb* infection. The results showed that CCL5 gene expression (Figure 4.5A) and protein levels (Figure 4.5B) were significantly decreased in the lungs of *M.tb* susceptible CBA/J mice as compared to C57BL/6 mice. Assays using homogenized lung tissue could not ascribe CCL5 production to a particular cell source, so we used adherent lung cells to confirm that macrophages from *M.tb* infected CBA/J mice secreted significantly less CCL5 than C57BL/6 lung macrophages (Figure 4.5C). These results could not exclude CCL5 production from other cell types however, they provide evidence that *M.tb* susceptibility is associated with low levels of macrophage derived CCL5. Furthermore, our data provide a mechanism (CCL5 mediated chemotaxis) by which T cells may migrate to granulomas to localize T$_{H1}$ immunity.

We identified mouse strain dependent differences in T cell numbers and lung CCL5 levels. However, it was possible that *M.tb* susceptible CBA/J mice had global deficiencies in T cell responsive chemokines that could decrease T cell migration to granulomas. To address that possibility, CXCL10 (Figure 4.5D) and CCL12 (Figure 4.5E) in lung tissue were quantified. Both of these chemokines attracted T cells *in vitro* in response to mycobacterial antigens and have been detected *in vivo* during *M.tb* infection (627, 645-648). No strain dependent differences were detected in either CXCL10 or...
CCL12 levels suggesting that these chemokines do not influence T cell recruitment or granuloma formation in *M.tb* susceptible CBA/J mice.

The levels of TNF (Figure 4.5F) were also measured in the lungs because TNF is necessary for resistance to *M.tb* (149) and more importantly for this Chapter, for the formation and maintenance of mycobacterial granulomas by inducing chemokine production (166, 649). Our results showed a trend for decreased TNF in the lungs of *M.tb* susceptible CBA/J mice, but the differences were not statistically significant. Low TNF may have contributed to the susceptibility of *M.tb* infection in CBA/J mice *M.tb*, but we believe this to be unlikely as the levels were similar to C57BL/6 lungs. Furthermore, the optimal amount of TNF for resistance to mycobacterial infections is unknown as both TNF absence and TNF abundance caused disease (170).
Figure 4.5. Chemokines and TNF in the lungs of mice. C57BL/6 and CBA/J mice were infected with 50-100 M.tb Erdman CFU by aerosol. Lung tissue was analyzed for CCL5 exon specific mRNA (A). CCL5 protein in lungs (B) and secreted from adherent lung cells (C), as well as TNF (D), CXCL10 (E), and CCL12 (F) from lung homogenates were quantified by ELISA. Gene expression was normalized against non-infected mice (not shown) using ribosomal RNA as an endogenous control. For CCL5 gene expression, results are the average ± SEM from one experiment with 4-5 mice per strain per time point, analyzed by one-way ANOVA with Tukey’s post test: *p<0.05, ***p<0.001. Results shown are the combined average ± SEM from three (B) or two (D, E, F) individual experiments each with 4-5 mice per strain per time point analyzed at each time point by Student’s t-tests: *p<0.05, **p<0.01, ***p<0.001; N.S. = no significant difference. For CCL5 production by adherent lung cells (C), results are the average ± SEM from one experiment with 2 mice per strain cultured in duplicate and analyzed by the Student’s t-test, **p<0.01.
Delayed recruitment of CCR5\(^+\) T cells to CBA/J lungs

CCR5 is preferentially expressed by effector and T\(_{H}1\) T cells (650), directs chemotaxis towards \(M.tb\) infected cells (646, 651), and has been detected in TB patients (642, 652), suggesting that CCR5 participates in cellular migration to \(M.tb\) lung granulomas \textit{in vivo}. To investigate a relationship between CCL5 and CCR5 during \(M.tb\) infection, the proportion and absolute numbers of CCR5\(^+\) T cells were determined. CCR5\(^+\) CD4 T cells and CCR5\(^+\) CD8 T cells were equivalent in non-infected mouse lungs and at day 7 of \(M.tb\) infection in C57BL/6 and CBA/J mice (data not shown). At 14 and 21 days of \(M.tb\) infection, however, significantly fewer CCR5\(^+\) CD4 T cells were recovered from the lungs of susceptible CBA/J mice than C57BL/6 mice (Figure 4.6A). By multivariate analysis, C57BL/6 mice recruited numerous CCR5\(^+\) CD4 T cells to the lungs at a faster rate by three weeks of \(M.tb\) infection while CBA/J mice required five weeks to achieve comparable numbers. Therefore, the mouse strain dependent difference in numbers of CCR5\(^+\) CD4 T cells was lost by day 35 post infection. Because CCL5 remained low at these time points, additional chemokines promoted CCR5\(^+\) CD4 T cell recruitment into CBA/J lungs. Alternatively, CCL5 may have reached lung threshold levels that induced T cell migration as suggested by Figure 4.5B.

The numbers of lung CCR5\(^+\) CD8 T cells were similar from non-infected mice and day 7 of \(M.tb\) infection in both mouse strains (data not shown). In C57BL/6 mice, CCR5\(^+\) CD8 T cells increased moderately over five weeks of \(M.tb\) infection (Figure 4.6B). In CBA/J mice, CCR5\(^+\) CD8 T cells were recruited slowly during the first three weeks with a significant increased at five weeks. Together, these results associated CCL5 abundance in C57BL/6 mice with steady recruitment of CCR5\(^+\) CD8 T cells, while low
CCL5 in CBA/J mice resulted in slightly fewer CCR5+ CD8 T cells early, followed by a sharp increase at five weeks. Overall, this suggested that additional CCR5 ligands produced by five weeks of *M.tb* infection compensated for low CCL5 levels to promote CD8 T cell lung migration.

Taken together, these data suggest that CCR5 mediated early CD4 T cell recruitment to the lungs during *M.tb* infection in response to abundant CCL5 as modeled by *M.tb* resistant C57BL/6 mice. In contrast, early recruitment of CD8 T cells may be less dependent on CCR5 as the numbers of CCR5+ CD8 T cells were less than CCR5+ CD4 T cells. In the *M.tb* susceptible CBA/J model, we associated diminished CCL5 levels with low T cell recruitment during *M.tb* infection and poor granuloma formation. We furthermore showed that CCR5+ T cells in CBA/J lungs reached equivalent or higher numbers than C57BL/6, implicating additional chemokines compensate for low CCL5. Overall, these results suggest that CCL5-CCR5 interactions on T cells may be especially critical for early *M.tb* control, a hypothesis which we address below.
Figure 5.6. CCR5⁺ CD4 and CD8 T cells in the lungs of mice. C57BL/6 and CBA/J mice were infected with 50-100 *M. tb* Erdman CFU by aerosol. Isolated lung cells were counted, fixed, and fluorescently labeled with anti-CD3, anti-CD4, anti-CD8, and anti-CCR5 for flow cytometry. Nonspecific binding by isotype control antibodies was less than 1% within each parent population. Results from CD4 T cells (A) and CD8 T cells (B) are the combined average ± SEM from 2 independent experiments each with 4-5 mice per strain time point. Statistical analyses were performed at each time point by the Student’s *t*-tests ** *p* ≤ 0.01; *** *p*<0.001 and within mouse strains over time by one-way ANOVA with Tukey’s post-tests, +*p*<0.05; ++*p*<0.01; N.S. = no significant difference.

CCL5 recruits T cells, macrophages, and antigen specific IFN-γ secreting cells to *M. tb* infected lungs.

Comparative analyses exploiting immunological differences between *M. tb* infected C57BL/6 and CBA/J mice provided the foundation for establishing CCL5 as a correlate of *M. tb* susceptibility. Limitations of this approach, however, were the genetic heterogeneity that influenced *M. tb* protective immunity including the haplotype (558), function of antigen specific CD4 T cells (Chapter 2), production of IL-10 (Chapter 3), or
other immunological differences (e.g. TNF levels, Figure 6) that may have been biologically relevant but were not statistically significant. To eliminate confounding effects due to genetic background differences, we used Ccl5 gene deleted (CCL5 KO) C57BL/6 mice to determine that CCL5 mediated lung T cell recruitment and M.tb granuloma formation.

Based on the results in C57BL/6 and CBA/J mice, we hypothesized that lack of CCL5 would delay CD4 T and CD8 T cell recruitment but have minimal effects on CD11c+ lung macrophages. As expected, the numbers of CD4 T cells (Figure 4.7A) and CD8 T cells (Figure 4.7B) in CCL5 KO lungs were significantly lower than WT controls at day 14 of M.tb infection. At 21 and 35 days, CD4 and CD8 T cells had reached near-equivalent levels regardless of CCL5 status. These results therefore, confirmed that CCL5 abundance was required for early CD4 and CD8 T cell lungs migration.

Because macrophages can express CCR5 and migrate in response to CCL5 (653), we hypothesized that CCL5 also promoted lung macrophage migration. We focused on CD11c+ lung cells because approximately 70% of lung macrophages expressed this marker (Erin Rottinghaus, unpublished observations) and M.tb bacilli were contained within this population (Chapter 3). CCL5 KO lungs contained fewer CD11c+ cells than WT controls, which reached statistical significance at days 14 and 21 of M.tb infection (Figure 4.7C). CCL5, therefore, was also required for optimal lung macrophage migration during M.tb infection. As seen with T cells, a fraction of CD11c+ cells entered the lungs in the absence of CCL5 illustrating that additional chemokines contributed.

In our previous experiments using M.tb susceptible CBA/J mice, CCL5 dependent migration of CD11c+ cells was not evident as the numbers of CD11c+ cells were
equivalent as in C57BL/6 lungs (Figure 4.1D). However, it had been reported that CCL5 KO mice recruited fewer monocytes/macrophages to sites of dermal inflammation (654), similar to the lungs of \textit{M.tb} infected CCL5 KO mice. Together these results illustrate that CCL5 promoted maximal CD11c\(^+\) migration as seen in CCL5 KO mice and furthermore that CD11c\(^+\) cells required small amounts of CCL5 for effective migration as evident in CBA/J mice.

Overall, our results indicate that CCL5 was required for recruiting T cells and CD11c\(^+\) macrophages to the lungs during very early \textit{M.tb} infection (between two and three weeks post infection). Since the numbers of cells may not correspond to protective immune responses, we next determined whether the absence of CCL5 caused diminished localization of antigen specific IFN-\(\gamma\) producing cells by ELISPOT in response to a broad array of protein antigens (\textit{M.tb} CFP) and to the single immunodominant antigen (\textit{M.tb} ESAT-6). As expected, the results showed that the numbers of \textit{M.tb} CFP specific IFN-\(\gamma\) (Figure 4.7D) and the numbers of \textit{M.tb} ESAT-6 specific IFN-\(\gamma\) (Figure 7E) producing cells in the lungs were significantly reduced in CCL5 KO mice at day 14 of \textit{M.tb} infection. At days 21 and 35 of \textit{M.tb} infection the numbers of antigen specific IFN-\(\gamma\) producing lung cells were increased in CCL5 KO mice. Overall these results show that the absence of CCL5 delays but does not prevent the generation of \textit{M.tb} protective immunity.
Figure 4.7. Lung immune cells and antigen specific responses during *M. tb* infection.

CCL5 knockout (KO) mice and C57BL/6 wild-type (WT) controls were aerogenically infected with *M. tb* Erdman, euthanized at the indicated time points and lung cells labeled with anti-CD3, anti-CD4 (A), anti-CD8 (B), and anti-CD11c (C) for flow cytometry. The numbers of *M. tb* culture filtrate protein (CFP) specific (D) and early secreted antigenic target-6 (ESAT-6) specific (E) IFN-γ producing lung cells were determined by ELISPOT. Antigen specificity was calculated by subtracting the SFU formed in the presence of an irrelevant antigen (ovalbumin). Data are the mean ± SEM of 2 independent experiments each with 4-5 mice per group per time point, analyzed pairwise by Student’s *t*-tests (*p*<0.05) at each time point. No differences were noted in either the numbers of lung cells or IFN-γ producing cells from non-infected CCL5 KO and WT control mice (not shown).

These flow cytometry and *ex vivo* antigen stimulation assays demonstrate that CCL5 was required for the early localization of T cells and IFN-γ producing antigen specific cells to the lungs. In CCL5 KO mice, numerous antigen specific IFN-γ producing cells were in the lungs at three and five weeks of *M. tb* infection (Figures 4.7D, 4.7E),
showing that the cells could function when antigen was encountered. It was possible however, that IFN-γ producing cells in vivo did not migrate to M.tb infected macrophages and would be dispersed throughout the lungs. This was an important point to address because granulomas provide necessary cell-to-cell contact and their structure reflects functional integrity including the ability to limit pathogen growth as well as minimize lung tissue damage.

**CCL5 promotes rapid formation of M.tb lung granulomas.**

Our previous results showed that CCL5 was necessary for the early recruitment of T cells, macrophages, antigen specific IFN-γ producing cells to the lungs. Since we hypothesized that CCL5 mediated T cell migration to M.tb granulomas, it was necessary to directly visualize lung granulomas. When tissue sections were evaluated by a board-certified veterinary pathologist without knowledge of the groups, the results indicated that the absence of CCL5 delayed granuloma formation. This was evident by very small inflammatory foci at day 14 and the widespread interstitial inflammation at day 21 of M.tb infection in CCL5 KO mice (Table 4.1). In contrast, at those same time points WT mice had formed discrete granulomas and interstitial inflammation was not prominent (Table 4.1). These results indicated that CCL5 was important for early M.tb granulomas and the absence of CCL5 resulted in interstitial inflammation with delayed granuloma formation. Therefore when CCL5 was absent, the protective cells may not have encountered M.tb infected macrophages in vivo leading to inadequate M.tb control despite high numbers of antigen specific IFN-γ producing cells (Figures 4.7D, 4.7E).
Future microscopy studies using frozen sections and immunohistochemistry will identify the locations of CD4 and CD8 T cells, i.e. within granulomas or the lung interstitium and determine the numbers of IFN-γ producing cells in \( M.\text{tb} \) granulomas.

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<td>M, N, L</td>
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<tr>
<td></td>
<td></td>
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<td>35</td>
<td>WT</td>
<td>M, L</td>
<td>Granulomas</td>
</tr>
<tr>
<td></td>
<td>CCL5 KO</td>
<td>M, L, N</td>
<td>Granulomas</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Perivascular</td>
</tr>
</tbody>
</table>

* \( L = \) lymphocytes, \( M = \) macrophages, \( N = \) neutrophils

Table 4.1. \( M.\text{tb} \) lung granulomas in CCL5 KO & WT mice. CCL5 KO and C57BL/6 WT mice were infected by aerosol with 50-100 \( M.\text{tb} \) Erdman CFU and euthanized at the time points indicated. Lung lobes from individual mice were inflated with 10% neutral buffered formalin, fixed for 3 weeks at room temperature and sectioned at 5µm and stained with hematoxylin and eosin. Results have been summarized from one experiment with 4-5 mice per group per time point. There were no differences in lung sections from CCL5 and WT non-infected mice, nor were granulomas present (not shown).

Overall, these data provide strong evidence that CCL5 is indeed important for early lung granuloma formation. For this experiment, multiple mice were evaluated per group per time point, but only one lung section per mouse was examined. Therefore,
further quantitative analyses using serial tissue sections will be performed to confirm these results.

**Accelerated *M.tb* growth in CCL5 KO mice**

Because T cells, granulomas, and antigen specific IFN-γ cells were delayed in the absence of CCL5, we hypothesized that CCL5 KO mice would have impaired control of *M.tb* growth. By light microscopy, similar numbers of acid-fast *M.tb* were present in CCL5 KO and WT control lungs (data not shown), but this method lacks sensitivity and is not accurate for determining *M.tb* burden (655).

When CFUs were recovered, the lungs of CCL5 KO and WT mice contained similar *M.tb* burdens at 14 days showing that CCL5 was not important for the purely innate pulmonary immune responses. As expected at time points associated with acquired immunity, days 21 and 35, CCL5 KO mice lungs contained significantly more *M.tb* CFU than WT (Figure 4.8). Finally, *M.tb* load declined to equivalent levels in CCL5 KO and WT by day 100 of infection, showing that CCL5 was not required for bacterial stabilization during chronic infection.

Taken together, these data indicate that CCL5 recruits IFN-γ producing protective CD4 and CD8 T cells to the lungs and to granulomas between two and three weeks of *M.tb* infection, controlling *M.tb* growth. In the absence of CCL5, recruitment of protective immune cells was delayed, allowing *M.tb* growth to double. Furthermore, protective immunity in the absence of CCL5 eventually controlled and stabilized *M.tb*, likely through the production of other chemokines.
Figure 4.8. *M. tb* growth in CCL5 KO and C57BL/6 WT mice. CCL5 KO and C57BL/6 WT mice were infected with 50-100 CFU of *M. tb* Erdman by aerosol, euthanized at the indicated time points and *M. tb* burden determined in lungs. Data are the combined average ± SEM of 2 independent experiments analyzed pairwise by Student’s *t*-tests (*p<0.05) at each time point. No differences were noted in *M. tb* uptake in the lungs at day 0 of infection (not shown).

**T cells use CCR5 and not CCR1 to enter *M. tb* infected lungs.**

Using KO mice, we confirmed that CCL5 promoted recruitment of protective cells and granuloma formation in the lungs between two and three weeks of *M. tb* infection. We next determined the T cell chemokine receptor requirements for CCL5 dependent lung migration. We focused on CCR5 and CCR1 because they are expressed on effector and T<sub>H</sub>1 cells and interact with CCL5 (656) and may directly activate T cells...
In vitro, CCR5-CCL5 interactions are high affinity, so we further hypothesized that T cells would preferentially use CCR5.

To test our hypothesis we focused on day 14 of *M.tb* infection because this time point consistently showed that low CCL5 (CBA/J mice) or absent CCL5 (KO mice) significantly reduced the numbers of lung T cells. Significantly fewer CCR5⁺ CD4 T cells and CCR5⁺ CD8 T cells (Figure 4.9A) were recovered from CCL5 KO lung than WT C57BL/6, reflecting that 2.5-10% of T cells were CCR5⁺. Surface receptor expression, however, may not reflect functional migration since chemokine receptors are endocytosed following ligand binding. Therefore, internalized CCR5 may better indicate recent migration in response to its ligands. We permeabilized lung cell membranes and labeled for CCR5 and CCR1 to quantify recent functional interactions. The absolute numbers of CCR5⁺ CD4 T cells and CCR5⁺ CD8 T cells increased nearly 5-fold when cells were permeabilized (Figures 4.9A, 4.9B). This reflected that approximately 60% of permeabilized T cells were CCR5⁺ (Figures 4.9D, 4.9E), which suggested that most T cells used CCR5 to migrate within *M.tb* infected lungs. In contrast, the absolute number (Figure 4.9C) and the numbers of permeabilized CCR1⁺ T cells (Figure 4.9E) was small indicating that CCR1 was a minor contributor to lung T cell migration.

As expected due to the absence of CCL5, the lungs of CCL5 KO mice contained significantly fewer CD4 and CD8 T cells that internalized either CCR5 or CCR1 (Figures 4.9B, 4.9C). Therefore, both receptors had recently bound CCL4 and/or CCL3 indicating that these chemokines also mediated lung T cell migration. Importantly, the numbers of
migrated T cells in CCL5 KO lungs were significantly less than WT lungs, showing that these ligands could not fully compensate for the lack of CCL5.

Overall these results indicate that T cells preferentially used CCR5-ligand interactions to migrate in *M. tb* infected lungs. Furthermore, CCR5-CCL5 interactions account for half of the migrated CCR5⁺ T cells, while CCR5-CCL3 and CCR5-CCL4 recruited fewer T cells. Finally, our results show that few T cells internalized CCR1, illustrating that CCR1 played a relatively minor role in CD4 and CD8 T cell lung migration during early *M. tb* infection.
Figure 4.9. T cell chemokine receptor expression in *M.tb* infected lungs. CCL5 KO and C57BL/6 WT mice were infected with 50-100 CFU of *M.tb* Erdman by aerosol, euthanized at day 14 of infection and lung cells fixed and labeled with anti-CD3, anti-CD4, anti-CD8, anti-CCR5 (A) for flow cytometry. In separate experiments, lung cells were labeled with anti-CD3, anti-CD4, anti-CD8, permeabilized and labeled with anti-CCR5 (B) and anti-CCR1 (C). Data are the combined average ± SEM of 3 independent experiments (A), two independent experiments (B), or from one experiment (C) each with 4-5 mice per group analyzed pairwise by Student’s *t*-tests (*p<0.05). Representative histogram plots gated on permeabilized CD4 T cells (D, F) or permeabilized CD8 T cells (E) show detection of CCR5 (D, E) or CCR1 (F) with corresponding isotype controls below.
CCL4 compensates for the lack of CCL5 during early *M.tb* infection.

The results using CCL5 KO mice established that CCL5 was important for early protective *M.tb* specific immunity and a large proportion of T cells used CCR5. It was apparent that additional CCR5 ligands such as CCL3 and CCL4 also participated in T cell lung migration and granuloma formation as CCL5 KO mice eventually localized antigen specific cells to the lungs, formed granulomas, and limited *M.tb* growth. To determine which ligand contributed to CCR5 mediated CD4 and CD8 T cell migration during *M.tb* infection, the levels and kinetics of CCL4 (Figure 4.10A) and CCL3 (Figure 4.10B) were determined during acute and chronic infection. To control for background levels detected in non-infected mice, both chemokines were expressed as pg/lung above controls. The results showed that CCL4 protein levels in the lungs peaked earlier (day 21) and achieved higher levels in CCL5 KO mice than in WT controls during *M.tb* infection. Therefore, rapid and abundant CCL4 was produced when CCL5 was absent. This showed that upregulation of CCL4 partially compensated for the lack of CCL5, and may have been responsible for the numerous T cells and antigen specific lung cells at day 21 and 35 of *M.tb* infection in CCL5 KO mice (Figure 4.7).

In contrast to CCL4, the lung levels of CCL3 were not increased in CCL5 KO mice and no significant differences were detected in the first three weeks (Figure 4.10B). By day 35 and continuing until day 100 of *M.tb* infection, lung CCL3 increased in WT mice, but remained low and stable in CCL5 KO lungs. These results indicate that CCL3 production did not compensate for the lack of CCL5 during early *M.tb* infection as did CCL4. Furthermore, the low CCL3 during chronic *M.tb* infection in CCL5 KO mice may have been a consequence of reduced lung CD11c⁺ cell influx (Figure 4.7), as
Macrophages are a major cell source of CCL3 (660). The absence of CCL5, therefore, may have secondary consequences on cellular recruitment during chronic \textit{M.\text{tb}} infection. To determine whether the lack of CCL5 affected TB disease outcome, survival was monitored in a small group of CCL5 and WT mice for 300 days. No significant differences in survival were detected (data not shown), suggesting that the lack of CCL5 or possible secondary immunological consequences do not alter TB disease outcome.

These data illustrate that \textit{M.\text{tb}} protective T cell migration to lung granulomas follow temporal patterns that may not be fully compensated by functional ligand-receptor redundancies. For example, in our experiments with CCL5 KO mice, CCL4 production was up-regulated but not fast enough to compensate for the lack of CCL5 as evident by low cells numbers at day 14 of \textit{M.\text{tb}} infection and delayed granuloma formation. In contrast, CCL3 may be dispensable during early \textit{M.\text{tb}} infection but important for T cell migration and granuloma maintenance during chronic stable \textit{M.\text{tb}} infection as the levels increased over time in WT mice. Finally, our results suggested that CCR5-CCL5 interactions mediate early lung T cell migration between two and three weeks, followed by CCR5-CCL4 interactions between three and five weeks, and finally CCR5-CCL3 interactions after five weeks of \textit{M.\text{tb}} infection.
Figure 4.10. CCL4 and CCL3 levels in M. tb infected lungs. CCL5 KO mice and C57BL/6 WT controls were aerogenically infected with 50-100 CFU of M. tb Erdman, euthanized at the indicated time points, and CCL4 (A) and CCL3 (B) protein measured by ELISA in homogenized lung tissue. Data are the mean ± SEM of 2 independent experiments each with 4-5 mice per group per time point, except for day 100 for which one experiment has been performed. Data were analyzed pairwise by Student’s t-tests (***p<0.01) at each time point and by one-way ANOVA by group over time (++p<0.01, +++p<0.001). No differences were noted between non-infected CCL5 KO and WT mice (data not shown).

CCL5 is not required for T cell priming and proliferation during M. tb infection.

Our experiments using CCL5 KO mice provided valuable information regarding the kinetics and contribution of CCL5, CCL4, and CCL3 and their receptor interactions in M. tb infected lungs. It was possible however, that lung responses in CCL5 KO mice may have been diminished as a consequence of altered T cell priming. Although CCL5 is not known to be essential for T cell homeostatic trafficking, T cell priming could have been delayed in CCL5 KO mice by unknown or indirect mechanisms. To address this possibility, the lung-draining mediastinal lymph nodes were evaluated in CCL5 KO and WT mice. T cell migration, proliferation and the priming of antigen specific responses
were not impaired by the absence of CCL5. To estimate the antigenic load arriving in the mediastinal lymph nodes, *M.tb* CFU were calculated from homogenized lymph nodes (Figure 4.11A). No significant differences were detected between CCL5 KO mice and WT controls indicating that *M.tb* infected lung dendritic cells efficiently migrated to the lymph nodes.

Lymph node CD11c<sup>+</sup> dendritic cells were also quantified (Figure 4.11B). In CCL5 KO and WT mice, dendritic cell numbers increased over time, and inconsistent differences were detected at day 21 and day 35 of *M.tb* infection. It was possible that the small differences resulted in functional disparity, despite the lack of statistical significance. To estimate the capacity of dendritic cells to present antigen, MHCII expression was determined at day 14 of *M.tb* infection. CD11c<sup>+</sup> dendritic cells expressed similar levels of surface MHCII<sub>lo</sub> (Figure 4.11C) or MHCII<sub>hi</sub> (Figure 4.11D), suggesting that mature (MHCII<sub>hi</sub>) dendritic cells from CCL5 KO mice were fully capable of activating and priming naïve T cells for *M.tb* antigen specific responses. These MHCII expression experiments, however, were from a single experiment and should be repeated.
Figure 4.11. \(M.\text{tb}\) growth and dendritic cells in mouse mediastinal lymph nodes. CCL5 KO and C57BL/6 WT mice were infected with 50-100 CFU of \(M.\text{tb}\) Erdman by aerosol, euthanized at the indicated time points and \(M.\text{tb}\) burden determined in the lung-draining mediastinal lymph nodes (A). In separate experiments, mediastinal lymph nodes were harvested, fixed and cells labeled with anti-CD11c (B) and anti-MHCII (C, D) for flow cytometry. Data are the mean ± SEM of 2 independent experiments (A, B) or 1 experiment (C, D) each with 4-5 mice per group per time point, analyzed pairwise by Student’s \(t\)-tests (*\(p\)<0.05) at each time point. MHCII\(^{\text{hi}}\) MFI was calculated by subtracting the MFI of the isotype control. This calculation was not necessary for MHCII\(^{\text{lo}}\) CD11c\(^{+}\) cells because the MFI of this bright population did not overlap with the MFI of the isotype control.

We next determined whether the absence of CCL5 affected T cell populations within the mediastinal lymph nodes. Because the absence of CCL5 decreased lung T cell recognition.
numbers at day 14 (Figures 4.7 and 4.9), we hypothesized that CCL5 KO mice would have equal or more mediastinal lymph node T cells than WT. As expected, the total numbers of cells (Figure 4.12A), CD4 T cells (Figure 4.12B) and CD8 T cells (Figure 4.12C) were increased in CCL5 KO mice lymph nodes as compared to WT. These differences were lost at day 21 and day 35 of *M.tb* infection, corresponding to T cell recruitment to the lungs (Figure 4.7) and granuloma formation (Table 4.2) in CCL5 KO mice. These results indicated that CCL5 was not required for T cell accumulation in mediastinal lymph nodes. Furthermore, CCL5 KO mice had significantly more BrdU\(^+\) CD4 T cells (Figure 4.12D) and BrdU\(^+\) CD8 T cells (Figure 4.12E), suggesting that many cells had recently encountered their antigen and had undergone clonal expansion. Therefore, *M.tb* infected CCL5 KO mice were not deficient in T cell trafficking, priming and proliferation. We expect that future analyses of lymph node cell cultures will confirm intact T cell priming cytokine responses in CCL5 KO mice.

Based on our *in vivo* results, we favor the argument that CCL5 deficiency resulted in lung cell migration defects which inhibited *M.tb* granuloma formation. We cannot conclusively rule out, however, that CCL5 was required for T cell egress from lymph node sinuses or T cell extravasation across lung vascular endothelium. However, the migratory functions of CCL5 do not overlap with molecular requirements for T cell egress (661). Therefore, a significant role for CCL5 in mediating these alternate scenarios is unlikely. When all of our results are considered in context, they support a lung migration defect in CCL5 KO mice.
**Figure 4.12. Cells in *M.*tb infected mediastinal lymph nodes.** CCL5 KO mice and C57BL/6 WT controls were aerogenically infected with *M.*tb Erdman and euthanized at the indicated time points. Lung-draining mediastinal lymph nodes were harvested and cells fixed and labeled with anti-CD3, anti-CD4, and anti-CD8. Total cells (A), absolute numbers of CD4 T cells (B) and absolute numbers of CD8 T cells (C) were determined. In separate experiments, mice were injected with BrdU 24 hours prior to euthanasia, and mediastinal lymph node cells fixed and labeled with anti-CD3, anti-CD4, anti-CD8, permeabilized and labeled with anti-BrdU. The numbers of BrdU+ CD4 (D) and CD8 (E) T cells were calculated. Data shown are the combined average ± SEM of two experiments with 4-5 mice per group analyzed by Student’s t-tests, *p<0.05.

**Discussion**

Chemotactic cytokines (chemokines) promote concentration dependent leukocyte migration (650) mediated by surface receptors and calcium dependent signaling which results in cytoskeletal re-arrangements (487-490). Chemokines can be broadly classified in two distinct categories: homeostatic chemokines and inflammatory chemokines.
Inflammatory chemokines are produced following infection, immune mediated diseases, and tissue damage (662).

The chemokine CCL5 has been detected during M.tb infection, but its role in T cell recruitment to M.tb lung granulomas has not been fully established. CCL5 has been detected in TB patients in vivo and induced T cell migration in vitro (487, 488, 491, 499, 637, 638, 646). Furthermore, in TB patients, high levels of CCL5 were associated with numerous CD4 T cells (499, 643, 663), showing a proportional relationship between T cell recruitment and CCL5 abundance as expected since chemokines function in concentration gradients. Finally, identical Ccl5 gene polymorphisms have been identified in populations of human patients with active TB disease (664, 665). All together, these results suggest that CCL5 recruits protective CD4 T cells to M.tb granulomas in vivo, while reduced or dysfunctional CCL5 is associated with M.tb susceptibility.

Prior to our work in this Chapter, a small number of studies in mice hinted at a role for CCL5 mediated T cell recruitment to M.tb granulomas. High levels of CCL5 had been associated with mycobacterial resistance, including to M.tb (637, 639, 666). In contrast, low CCL5 gene expression was reported in M.tb susceptible DBA/2 mice (519). Furthermore, Chensue et. al., 1999 (639) blocked CCL5 in vivo and proved that functional CCL5 was necessary for cellular recruitment to M. bovis antigen coated beads. These results provided a strong foundation for determining the role of CCL5 during M.tb infection, and supported our hypothesis that CCL5 promoted T cell migration in the lungs.
We focused on CCR5 because it binds CCL5 with high affinity (658) and is expressed on effector T cells and Th1 cells (662, 667). CCR5 promotes chemotaxis towards two additional ligands which are in the same beta-chemokine family as CCL5: CCL3 and CCL4 (487, 488, 491, 493, 668-671). Furthermore, there is evidence in humans TB patients that CCR5 drives T cell migration. For example, CCR5⁺ T cell lines migrated towards M.tb antigen stimulated macrophages (672) and activated CCR5⁺ T cells have been detected at sites of infection/antigenic stimulation in human TB patients (525, 642). All of these studies suggest that CCR5 directs lymphocytes to pulmonary M.tb granulomas in vivo, a concept that has been tested in CCR5 KO mice. Granuloma size was comparable between wild-type and CCR5 KO mice at six weeks of infection (637), but unexpected granuloma structure and composition differences emerged after nine weeks of infection (666). Instead of cell-poor infiltrates (as would be expected in the absence of CCR5), the lungs of CCR5 KO mice contained increased numbers of macrophages, T cells, and neutrophils (666). Furthermore, photomicrographs (666) showed altered granuloma organization with large perivascular and peribronchiolar distribution of lymphocytes in the absence of CCR5. One explanation for these observations was that T cells entered the lung tissue, but could not migrate effectively towards CCR5 ligands to form effective compact granulomas.

Similar to previous chapters, we used the well established differences in C57BL/6 and CBA/J mouse strains to identify specific immunologic factors associated with increased M.tb susceptibility. Because we observed differences in the numbers and function of protective M.tb protective lung cells (Chapter 2) and granuloma structure, we focused on mechanisms that control lung cell migration to granulomas. Overall the data
established three important results. First, using CBA/J mice, we established that early and persistently low CCL5 was associated with increased M.tb susceptibility. These results provided an essential foundation for justifying future experiments that focused on early time points. Second, using CCL5 KO mice we confirmed that CCL5 primarily contributed to early protective immunity by rapidly localizing antigen specific IFN-γ producing cells to the lungs which helped control M.tb growth. Third, we reconstructed both the T cell chemokine receptor usage (CCR5) and CCL5-related ligand (CCL4) that likely contributed to early M.tb granuloma formation in vivo. This allowed us to develop an updated M.tb granuloma model which accounts for temporal regulation of beta chemokines. This latter point is novel because we are suggesting that the extensive functional redundancy in chemokines and chemokine receptor interactions in vitro may not be important in vivo. Therefore, despite functional overlap in vitro, the temporal expression of chemokines may direct sequential waves of migrating immune cells to M.tb granulomas in vivo in a manner that is minimally impacted by redundancy.

In Chapter 2, we showed that M.tb susceptible CBA/J mice localized fewer CD4 T cells and significantly fewer antigen specific IFN-γ producing CD4 T cells to the lungs throughout acute and chronic infection than C57BL/6 mice. In this chapter, we have expanded these findings to include lymphocyte-poor granulomas during early infection which corresponded to poor CD4 and CD8 T cell lung recruitment by day 14, the earliest manifestation of protective acquired immunity detected in our model. Furthermore, we showed that CBA/J mice did not have defects in recruiting CD11c+ lung cells (macrophages) during the first five weeks of M.tb infection. We therefore hypothesized that CBA/J T cells had defective migration abilities, which resulted in low numbers of T
cells and lymphocyte poor granulomas. We determined that low Ccl5 gene expression and low CCL5 protein production from lung macrophages was associated with increased M. tb susceptibility in CBA/J mice. Low Ccl5 mRNA has also been reported in DBA/2 mice, another M. tb susceptible mouse strain (519). Together these results indicate that low CCL5 is a common correlate of M. tb susceptibility in mice, which may also be true in humans (664, 665).

In our CBA/J mouse model, we ruled out CXCL10 and CCL12 as potential correlates of M. tb susceptibility as the levels were equivalent in M. tb susceptible CBA/J mice and M. tb resistant C57BL/6 mice. Although TNF is a potent inducer of chemokines during in response to mycobacteria (166, 168), the marginally decreased TNF in CBA/J mice, therefore, was not responsible for a general reduction in lung chemokines in this mouse strain. The slight differences in TNF may have diminished mycobacteriostatic or – cidal effects in CBA/J macrophages but these possibilities have not been addressed.

Additional experiments showed fewer CCR5+ T cells in CBA/J lungs than C57BL/6, suggesting that CCR5 mediated T cell recruitment to the lungs. As expected this likely resulted in directly proportional relationship between CCL5 abundance and CCR5 usage, i.e. both were abundant in C57BL/6 lungs and low in CBA/J lungs. In our experiments in CBA/J mice the additional CCR5 ligands, CCL3 and CCL4 were not investigated. Therefore, whether M. tb susceptible CBA/J mice have a single deficiency in CCL5 production as we are suggesting, or whether there is a more global deficiency in production of the beta chemokines remains unanswered but would be important to address in future studies.
The underlying mechanism of reduced CCL5 in *M.tb* infected CBA/J mice was not fully addressed in this Chapter. We did show that CBA/J mice had low lung *Ccl5* mRNA indicating transcriptional regulation was reduced in comparison to C57BL/6 mice. Complex control of *Ccl5* gene expression has been shown to differ in multiple cell types involving JNK MAPK pathways in human NK cells (673), the transcription factor AP-1 in epithelial and endothelial cell lines (674) and the transcription factors IRF-8, IRF-1, and PU.1 in macrophages (675). Since we identified low CCL5 from CBA/J lung macrophages, future experiments could focus on IRF-8, IRF-1, and PU.1.

Identifying molecular mechanisms underlying *Ccl5* gene expression differences are valid pursuits that may lead to a better understanding of immune responses associated with *M.tb* susceptibility. We believe, however, that the most significant application of low CCL5 as a correlate of *M.tb* susceptibility is the potential for delivering exogenous CCL5 as an immunological therapy in CBA/J (or DBA/2) mice that may alter protective immunity, granuloma formation or disease outcome. Although the complete lack of CCL5 did not accelerate TB disease progression in C57BL/6 mice (data not shown), this finding does not exclude the possibility that CCL5 supplementation may be beneficial. We envision CCL5 could be administered as an intrapulmonary instillation or into the systemic circulation. Systemic delivery of chemokines may be counterintuitive as it would be undesirable to produce a general inflammatory response focused in or around blood vessels. It has been established, however, that activated endothelial cells under conditions of inflammation bind chemokines, including CCL5 (676) sites other than those responsible for leukocyte migration (667). This suggests that activated endothelial cells
within *M. tb* granulomas could accumulate exogenous CCL5 and be positioned to promote beneficial extravasation and migration of effector T cells directly at the sites of disease.

To further define the role of CCL5 during *M. tb* infection and granuloma formation we moved away from *M. tb* susceptible CBA/J mice and used CCL5 KO mice. We showed that CCL5 rapidly recruited antigen specific IFN-γ cells, CD4 and CD8 T cells and promoted early granulomas which controlled *M. tb* growth. We also identified that CCR5-CCL5 interactions mediated most T cell migration, while few cells used the CCR1. In WT mice, the CCL5-related ligand CCL4 peaked at five weeks, while CCL3 increased significantly during chronic *M. tb* infection. In CCL5 KO mice, we showed that CCL4 partially compensated for the absence of CCL5 by an earlier peak in production (three weeks *versus* five weeks), which corresponded to rising numbers of lung T cells and more IFN-γ producing cells in CCL5 KO mice. In contrast, CCL3 production did not compensate for the lack of CCL5.

Overall, these results indicate that the temporal expression of chemokine and chemokine receptor may be more important in *M. tb* granuloma formation and/or maintenance. Numerous chemokines and chemokine receptors have been implicated models of *M. tb* granuloma formation, including CCR2, CCL2, CXCR3, and CXCL10. Few studies, however, have been able to define clear roles for single chemokines or chemokine receptors during *M. tb* infection. Exceptions include, the requirement of CCR2 for resistance to *M. tb* in mice (677, 678), while its ligand, CCL2 was not essential (679). In these publications using KO mice, the results were often complex and/or contradictory to the initial hypotheses. For example, when CCR5 was absent, instead of reduced lymphocytes as expected, the lung cellularity was markedly increased (666).
Additionally, although CXCR3 is highly expressed by effector T cells, its absence increased neutrophils in the lungs of \textit{M.\textit{tb}} infected mice (680). Overall these examples highlight the complexity and redundancy in chemokine and chemokine receptor interactions complicate the understanding of their individual functions \textit{in vivo} particularly with regard to granuloma formation.

By systematically evaluating related chemokines and their shared receptors during \textit{M.\textit{tb}} infection, we believe that the chemokine network inducing T cell migration to granulomas \textit{in vivo} may be simpler than previously assumed due to temporal regulation. Our results indicated that the contribution of the chemokines CCL5, CCL4, and CCL3 to T cell recruitment and \textit{M.\textit{tb}} granuloma formation change over time and that CCR5 usage dominates over CCR1. Therefore the extensive functional redundancies documented \textit{in vitro} using migration assays may not accurately reflect \textit{in vivo} expression and inflammatory chemokine usage during \textit{M.\textit{tb}} infection. If similar temporal patterns of chemokine expression could be identified in \textit{M.\textit{tb}} infected people or in TB patients, chemokines could be used to devise disease stage specific immunotherapies that enhanced granuloma formation or function \textit{in vivo}. 

CHAPTER 5: SUMMARY AND SIGNIFICANCE OF RESULTS

In Chapter 1, we introduced the TB disease states that occur in adults: progressive primary TB, latent TB, and reactivation TB; and the immune mediators that are protective against \textit{M.tbc}. The most important immune mediators for resistance to \textit{M.tbc} are cytokines that activate macrophages, for example, TNF; molecules that promote acquired immunity and Th1 antigen specific responses, for example, IL-12, MHCII, MHCI, TCR, CD4, CD8, IFN-\(\gamma\), IFNGR; and anti-mycobacterial molecules, for example, ROI and RNI. The necessity for these immune mediators is known from \textit{M.tbc} infection of KO mice, transfer of purified immune cell populations, and \textit{in vivo} depletion of immune cells. With few exceptions (230, 681) nearly all of these results were obtained using C57BL/6 mice, a mouse strain that is relatively resistant to \textit{M.tbc} infection and survives into old age before succumbing to TB disease.

C57BL/6 mice, and KO mice on that genetic background, provide critical information regarding the requirements for resistance to \textit{M.tbc}. These mediators of resistance, however, may not be sufficient to prevent TB disease. The facts, that 1) a large proportion of \textit{M.tbc} infected humans do not develop active TB and 2) the majority of active TB patients do not have known immunodeficiencies, indicate that additional factors contribute to TB disease progression. In Chapters 2, 3, and 4, we identified and manipulated immune mediators that could contribute to \textit{M.tbc} susceptibility. This is an
important concept because accurate prediction of disease outcomes would allow earlier intervention in patients likely to transmit *M.tbc* organisms.

It is unlikely that C57BL/6 mice can reflect the breadth of immune responses in the genetically diverse human population. To model variation in the natural susceptibility of humans to *M.tbc*, we generated hypotheses and identified *M.tbc* susceptibility correlates using multiple inbred mouse strains. By taking advantage of differences in *M.tbc* infection and TB disease progression in C57BL/6, CBAJ, DBA/2, and C3H/H3J inbred strains, the results obtained may better reflect the genetic diversity and immune responses of the human population. The majority of the studies focused on two mouse strains, *M.tbc* susceptible CBA/J mice, and relatively resistant C57BL/6 mice. In Chapter 2, however, the significance of these findings was expanded to additional *M.tbc* susceptible mouse strains. We recognize that even these expanded results may not capture all of the potentially relevant immunologic responses in humans that develop active TB disease. Nevertheless, the concept of using of *M.tbc* susceptible CBA/J mice, or other susceptible mouse strains, to identify and/or manipulate immune mediators provides a valid tool to understand *M.tbc* infection and TB disease progression.

It has been shown in mice and in humans that IFN-γ and the IFNGR are both crucial for *M.tbc* resistance and when either are absent, mycobacterial diseases progress rapidly (138, 139, 211). In most active pulmonary TB cases, which are the source of infectious organisms for naïve individuals, however, such severe immunodeficiencies are not evident (2, 4). In fact, in cases of reactivation TB, protective immunity is not absent and *M.tbc* organisms are successfully controlled for years to decades. We hypothesize, as do others, that reactivation TB may result from subtle deficiencies in resistance that may
wane over time, from the production of immunosuppressive factors, or both. Our results suggest that both mechanisms may play a role in \textit{M.tb} susceptibility and TB disease progression in CBA/J mice and, furthermore, that reactivation TB may be prevented.

In Chapter 2, the magnitude of antigen specific IFN-\gamma production and the number of IFN-\gamma responding cells were significantly reduced from lungs and blood of \textit{M.tb} susceptible CBA/J mice as compared to more resistant C57BL/6 mice. These results clearly showed that quantitative differences in this protective cytokine were detected as early as three weeks post \textit{M.tb} infection, maintained at low levels throughout chronic infection, and present in asymptomatic mice. Furthermore, we showed that the blood responses were similar to the lung responses, which has particular relevance to man, where access to lung cells is limited and requires invasive procedures. Persistently low amounts of antigen specific IFN-\gamma or a reduced frequency of IFN-\gamma producing blood cells, therefore, might also predict TB disease progression in humans. In some CBA/J mice it was apparent that very low levels of IFN-\gamma secreted in cell culture supernatants overlapped with the responses from non-infected individuals. Therefore, under some test conditions, diagnosis of \textit{M.tb} infection or attempts to estimate risk of TB disease progression could be inaccurate for the human population most likely to develop reactivation TB. Fortunately, the use of a more sensitive test to detect the numbers of antigen specific IFN-\gamma responding cells could provide a noninvasive, simple and accurate solution.

In CBA/J mice, the exact immunologic mechanisms underlying diminished antigen specific IFN-\gamma remain unknown. We showed that T cells from \textit{M.tb} infected CBA/J mice were capable of maximal IFN-\gamma secretion when stimulated by cross-linking
the TCR with anti-CD3 and anti-CD28, but fewer of these cells were generated in response to *M.tb* infection as compared to C57BL/6 mice. When stimulated with *M.tb* antigens (*M.tb* Ag85, *M.tb* ESAT-6, and *M.tb* CFP) both the frequency of responding cells and the amount of IFN-γ per cell were reduced in CBA/J mice as compared to C57BL/6 mice. Finally, we showed that CBA/J mice generated protective immunity to single protein (*M.tb* Ag85 and *M.tb* ESAT-6) vaccines. These data indicate that CBA/J lung T cells function well with artificial TCR stimulation in the absence of antigen presenting cells. This suggests a deficiency in CBA/J antigen presenting cells at the level of dendritic cell/T cell priming or at the level of infected macrophages interacting with T cells.

When CBA/J mice were vaccinated with immunodominant antigens (Ag85 and ESAT-6), however, they generated specific protective immunity; showing that processing, presentation, and the generation of antigen specific memory responses were intact and functional. Overall, these data show that many antigen presenting cell functions may be intact in CBA/J mice, yet, during primary *M.tb* infection, these mice remain more susceptible than C57BL/6 mice. Additional unidentified *M.tb* infection-specific conditions *in vivo* may affect the function of antigen presenting cells in CBA/J mice that, in turn, could lead to less efficient T cell priming during primary *M.tb* infection and diminished amounts of protective cytokines. Future experiments will address some of these questions, starting with the availability of Ag85 *in vivo* and identification of antigen specific cells that do not secrete IFN-γ.

Two issues that we did not directly address in Chapter 2 include whether IFN-γ is truly a correlate of protection or whether increasing the amounts of IFN-γ in CBA/J mice
could control *M.tb* better or lengthen survival. Based on improvement following exogenous IFN-γ to TB patients (213, 214), it is reasonable to assume that similar results would occur in CBA/J mice. Further experiments, however, need to be performed to formally test this hypothesis. The results of such experiments, either confirming or refuting the protective role of boosting IFN-γ in this mouse strain, however, would not detract from the overall significance of our findings in Chapter 2. Low antigen specific IFN-γ from the blood would remain an important practical application of this work, predicting eventual TB disease, even if low IFN-γ were not a single direct cause.

To explore causal immunologic mechanisms of TB disease progression, in Chapter 3 we used CBA/J mice to model reactivation TB in humans, as characterized by long periods of symptom-free controlled *M.tb* infection, followed by bacterial growth and TB disease. CBA/J mice recapitulated clinical and microscopic features of human reactivation TB that may be under-appreciated in other murine models. We were able to show that morbid CBA/J mice had extensive lung inflammatory infiltrates with large regions of caseous necrosis, cavitation, fibrosis and airways filled with cellular debris and *M.tb* organisms, which are all characteristics of active human TB (511).

Although mice with TB disease were used to justify the CBA/J strain as a model of reactivation TB, to identify potential causes of disease progression, we focused on the immune responses that occurred prior to disease onset. To confirm integrity of the CBA/J reactivation TB model, we repeated previous work at another BSL-3 facility. This work associated increasing IL-10 with TB disease progression in CBA/J mice and in mice that over-expressed IL-10 (326). By re-analyzing our data from CBA/J mice that were not ill, we identified 10-fold *M.tb* re-growth in the lungs between 90 and 120 days of infection.
and a narrow time frame in which IL-10 could be targeted *in vivo* for blockade. IL-10 function *in vivo* was inhibited by weekly administration of anti-IL10R1 or control antibodies (both whole rat IgG and rat IgG1) starting at day 90, prior to *M.tb* re-growth. When IL-10 was blocked, *M.tb* re-growth was prevented and the lung bacterial burden stabilized at a level that was tolerated by the CBA/J mice. Furthermore, survival was improved. These results showed that IL-10 contributed to reactivation TB and also suggested that IL-10 may be a viable target for immunotherapy in humans. Additional studies need to be conducted to optimize anti-IL10R1 as a therapy that address dosage and timing regimes, minimize the formation of neutralizing antibodies, and select appropriate patient populations for therapy. Patient selection would be critical for successful use of IL-10 blockade as an immunotherapy, limited to *M.tb* infected individuals who would likely develop reactivation TB. Anti-IL10R1 therapy would have to be initiated prior to *M.tb* re-growth. We would suggest that patients at risk for reactivation TB be first identified through the use of blood IGRAs as described in Chapter 2. Patients could be sequentially monitored to confirm low antigen specific IFN-γ secretion or a low frequency of responding cells in the blood. Since low IFN-γ was consistently low in mouse populations at risk for TB disease, IGRA results could be used to estimate a general risk of reactivation TB in humans, but would not be able to precisely determine the timing of disease onset. As seen in our mouse model, however, increasing levels of IL-10 were detected in the lungs of CBA/J mice 30 to 60 days prior to *M.tb* re-growth. Therefore, in patients initially identified by low IGRAs, IL-10 could be monitored as a second biomarker. As demonstrated in the CBA/J model of reactivation TB, rising IL-10 levels predict disease progression within a much shorter time frame than
IGRAs. Furthermore, increasing IL-10 could also determine the optimal timing of IL-10 blockade in at-risk humans. Finally, these studies would best translate to man if rising IL-10 levels could be detected from blood assays or serum of *M. tb* infected individuals, creating a prognostic blood test that could simultaneously be used to calculate the long- and short-term risks of reactivation TB.

In Chapter 3, we determined the immunological mechanisms that were altered by anti-IL10R1 treatment and improved TB disease outcome. By focusing on mediators known to be inhibited by IL-10, we showed that IL-10 blockade increased the numbers of lung T cells capable of IFN-γ production, improved *ex vivo* antigen specific IFN-γ secretion and increased IFN-γ protein levels in the lungs. The overall interpretation of these results indicates that IL-10 blockade enhanced Th1 immune responses which are crucial for *M. tb* resistance. When individually scrutinized, however, changes in IFN-γ levels varied between the two time points measured, and sometimes across treatment groups. For example, quantifying the numbers of IFN-γ⁺ T cells relied on TCR cross-linking, a method which produced maximal stimulation of each cell and eliminated the requirement for antigen presenting cells. Thus, at day 150 of *M. tb* infection, IL-10 blockade resulted in more cells that had the potential to respond to TCR mediated signals with IFN-γ production. In contrast, *ex vivo* antigen specific stimulation significantly increased IFN-γ secretion at day 115 but minimal levels were detected at day 150. These results appear to contradict the TCR cross-linking findings. It is possible, however, that T cells from anti-IL10R1 treated mice secreted more IFN-γ/cell at day 115 than control mice and more IFN-γ accumulated in supernatants while the numbers of IFN-γ⁺ cells was the same. Further experiments could be performed to address this possibility.
IL-10 blockade had many small effects on other immune molecules that may have been dependent or independent of increased IFN-γ. Our *in vivo* experiments did not allow us to distinguish between effects purely due to a lack of functional IL-10 versus those that were enhanced by more IFN-γ. The point, however, may be of academic interest only with little biological relevance *in vivo*, as release from IL-10 mediated inhibition may have the same effects as IFN-γ. For example, inhibition of IL-10 should increase MHCII and iNOS (262) and additional IFN-γ also increased MHCII and iNOS (207). In our experiments we saw a trend for increased CIITA gene expression and increased surface MHCII in anti-IL10R1 treated mice which could have been attributed to IL-10 blockade or IFN-γ or both.

Overall, IFN-γ and IL-10 appear to play major roles in balancing and counterbalancing inflammatory/immune responses. In fact, IFN-γ and IL-10 frequently act as antagonists (207, 262), so it may be difficult to cleanly separate their functions *in vivo*. Based on our experiments in the context of *M.tb* disease progression, each may contribute to dichotomous roles with abundant antigen specific IFN-γ predicting optimal resistance, while IL-10 promotes reactivation TB.

In Chapter 4, we again used CBA/J mice to identify additional correlates of *M.tb* susceptibility but focused on early immune responses. It was clear from Chapter 2 that CBA/J mice had fewer protective T cells in the lungs during early *M.tb* infection which may have allowed bacteria to achieve higher levels than the more resistant C57BL/6 mice. Furthermore, CBA/J mice had lymphocyte poor granulomas, suggesting these mice were less capable of localizing T cells to the lungs. By focusing on factors that could contribute to cellular migration and granuloma formation, we determined that the
chemokine CCL5 was significantly reduced in CBA/J mice compared to C57BL/6, while other types of chemokines and cytokines important for granuloma formation were minimally different. These experiments comparing mouse strains identified low CCL5 as a correlate of \( M.\text{tb} \) susceptibility and we focused in particular on CCL5 mediated T cell recruitment. Furthermore, increasing levels of CCL5 in \( M.\text{tb} \) susceptible populations may be able to promote \( M.\text{tb} \) resistance and future studies will address this possibility. Again, it must be emphasized that even if lack of CCL5 does not in and of itself result in TB disease progression it may still be a therapeutic target.

The final sets of experiments were conducted in C57BL/6 CCL5 KO mice, which allowed us to more cleanly dissect the role of this chemokine in T cell migration to \( M.\text{tb} \) infected lungs. As expected, the absence of CCL5 resulted in fewer antigen specific IFN-\( \gamma \) producing CD4 and CD8 T cells recruited to the lungs, delayed granuloma formation, and increased \( M.\text{tb} \) growth. CCL5 KO mice also had fewer CD11c\(^+\) cells in lungs, a feature that was not identified in CBA/J mice but had been detected in other inflammatory models (682, 683). CCL5 therefore contributes to the localization of both T cells and macrophages to the lungs during \( M.\text{tb} \) infection. We further showed that the majority of CD4 and CD8 T cells used CCR5 to enter the lungs during \( M.\text{tb} \) infection, and not CCR1, a receptor that also binds CCL5.

An important finding was that the increased \( M.\text{tb} \) burden in CCL5 KO mice detected at three and five weeks of \( M.\text{tb} \) infection was transient as levels were equivalent as WT controls by day 100. These results may have important implications for humans infected with \( M.\text{tb} \), showing that CCL5 is necessary for optimal early \( M.\text{tb} \) resistance. The transient increased \( M.\text{tb} \) susceptibility, however, indicates that the lack of this single
chemokine does not cause rapid TB disease progression in mice. Therefore the ccl5 gene polymorphisms in some TB patients (210, 664) may contribute to *M.tb* susceptibility but alone are unlikely to cause reactivation TB.

In Chapter 4, we quantified the lung tissue levels of chemokines CCL4 and CCL3 that share receptors with CCL5. We hypothesized that CCL4 or CCL3 could compensate for the lack of CCL5, and thus explain the transient loss of *M.tb* resistance and explain the increased numbers of antigen specific IFN-γ producing cells. In the lungs, CCL4 peaked earlier when CCL5 was absent, resulting in partial but not complete compensation. No differences were detected in early CCL3 lung levels. During chronic *M.tb* infection, however, CCL3 levels continued to increase in WT mice but were stable in CCL5 KO mice, suggesting that CCL3 does not compensate for the lack of CCL5. Integration of these results allowed us to propose an updated model of chemokine mediated granuloma formation whereby waves of CCR5⁺ T cells enter the lungs in response to sequential expression of CCL5, CCL4, and CCL3. More specifically, these data suggest that CCL5 is important for T cell migration between weeks two and three of *M.tb* infection, followed by CCL4 between weeks four and six, while CCL3 may be important during the chronic phases of *M.tb* infection. Finally, it was apparent that the complete lack of one chemokine (CCL5) had secondary effects on the long term production of other chemokines (CCL3), but that there was no impact on *M.tb* burden.

Overall, this body of work has attempted to model the complexity of the immune response to *M.tb* infection by exploiting differences in inbred mouse strains. This approach has allowed us to identify three immune correlates of *M.tb* susceptibility, low antigen specific IFN-γ, high levels of IL-10, and low CCL5. Based on human IGRA
literature reviews and responses to IFN-γ supplementation (213, 214) low antigen specific IFN-γ likely causes *M. tb* susceptibility and indicates poor acquired immunity. Although causality was not addressed in our experiments, it would be possible to deliver exogenous IFN-γ to CBA/J mice to formally prove this hypothesis. Instead we chose to broaden the implications of our findings to enhance the usefulness of current human diagnostic tests (IGRAs). Across genetically diverse populations, we showed that blood IGRAs truly reflected the lung responses. Furthermore, low antigen specific IFN-γ was evident soon after *M. tb* infection, predicting a poor outcome months prior to reactivation TB. Thus in mice, we defined a simple blood test that may translate to *M. tb* infected humans.

The second immune correlate of *M. tb* susceptibility, IL-10 was shown *in vivo* to cause reactivation TB in our CBA/J mouse model. By blocking IL-10, *M. tb* re-growth was prevented and survival was prolonged. This was a highly significant finding as it is rare that loss of one immune mediator improved TB disease progression. To our knowledge, only the absence of IL-4 or IL-13 improved *M. tb* bacterial loads (230) but survival of these mice was not reported. Our inability to define a single enhanced immune function likely reflected the pleotropic effects of IL-10 *in vivo*. Thus IL-10 blockade would also result in multiple complex immune alterations that may have been biologically beneficial, but not statistically significant. With anti-IL10R1 treatment, the most profound changes occurred in cell populations and responses of acquired immunity especially with recruitment of Th1 cells to the lungs. Like Chapter 2, these results may also translate to human TB patients in particular that IL-10 could be used as a biomarker predicting a short term risk of TB disease progression (within two months). Additionally,
immunotherapy targeting IL-10 may prevent reactivation TB, which could improve outcomes for individual patients and decrease the chances of \( M.\text{tb} \) transmission.

The third immune correlate of \( M.\text{tb} \) susceptibility, CCL5, may also be used to predict TB disease outcome, as low levels were detected throughout \( M.\text{tb} \) infection in susceptible CBA/J mice. Similar to low IFN-\( \gamma \) results in Chapters 2, this suggests that CCL5 may be boosted to improve \( M.\text{tb} \) resistance. By studying CCL5 KO mice, it was clear that CCL5 deficiency alone did not cause \( M.\text{tb} \) susceptibility, and therefore is unlikely to be a single cause of active TB in humans despite the presence of functional gene polymorphisms (664, 665). We showed, however, that CCL5 was indeed important for control of \( M.\text{tb} \) growth with early localization of antigen specific cells to the lungs. We further expanded the relevance of these findings by developing a new granuloma theory to account for the kinetics of CCL5, CCL4 and CCL3 expression in \( M.\text{tb} \) infected lungs. Finally, the results of our data may be summarized as outlined below in Table 5.1.

<table>
<thead>
<tr>
<th>Predictor of ( M.\text{tb} ) susceptibility</th>
<th>Cause of Reactivation TB</th>
<th>Approximate time prior to disease onset</th>
<th>Organ/Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-( \gamma )</td>
<td>Yes</td>
<td>Probably</td>
<td>4 months</td>
</tr>
<tr>
<td>IL-10</td>
<td>Yes</td>
<td>Yes</td>
<td>1 month</td>
</tr>
<tr>
<td>CCL5</td>
<td>Yes</td>
<td>No</td>
<td>4 months</td>
</tr>
</tbody>
</table>

Table 5.1. Immunologic mechanisms and predictors of susceptibility to \( M.\text{tb} \) in mice.
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