Effector and Regulatory CD4 T Cells During *Mycobacterium tuberculosis* Infection

by

Angela Marie Green

Susquehanna University, Bachelors of Science 1994

Submitted to the Graduate Faculty of

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

School of Medicine

This dissertation was presented

by

Angela Marie Green

It was defended on

October 7, 2010

and approved by

Robert L. Hendricks, Ph.D., Department of Opthomology

Gerard J. Nau, M.D. Ph.D., Department of Microbiology and Molecular Genetics

Karen A. Norris, Ph.D., Department of Immunology

Anuradha Ray, Ph.D., Department of Immunology

Dissertation Advisor: JoAnne L. Flynn, Ph.D. Department of Microbiology and Molecular Genetics

Effector and Regulatory CD4 T cells During *Mycobacterium tuberculosis* Infection

Angela Marie Green PhD

University of Pittsburgh, 2010

ABSTRACT

Mycobacterium tuberculosis continues to be a leading cause of death by an infectious agent world wide with approximately two million deaths attributable to it annually. Once infected, the host mounts a robust Th1 type immune response that contains, but does not eliminate the bacteria. A small percentage (5-10%) fail to contain the infection, develop active disease, and are contagious. Most remain asymptomatic and are considered latently infected. BCG, while the gold standard for vaccination, ultimately appears to limit disease, but not prevent infection. In addition, chemotherapeutic treatment is long, requires multiple agents, and compliance is difficult to maintain. With one third the world's population infected, inadequate vaccine efficacy, and difficult treatment regimen, it is imperative that a better understanding of which factors are responsible for containment of the infection be achieved. CD4 T cells are an essential component of the immune response that controls *M. tuberculosis*. CD4 T cells are able to both promote inflammation and dampen its effect. As a pro-inflammatory Th1 cell, these cells secrete pro-inflammatory cytokines, participate in macrophage and dendritic cell activation and help prime CD8 T cells. As regulatory T cells, CD4 T cells prevent autoimmunity and may act to protect surrounding tissue from immunopathologic damage. One main function of pro-inflammatory CD4 T cells is IFNγ production, however other cells can and do produce IFNγ. In addition, the role of regulatory T cells during infection and how they relate to disease progression has yet to be elucidated. The work presented in this dissertation provides a new model for addressing the role of IFNγ from sources other than CD4 T cells, and addresses what

pro-inflammatory functions they may have in addition to IFNγ production. In addition, these studies address regulatory T cells and the anti-inflammatory effects of long term IL-12 treatment as well as Treg's relationship to disease outcome in non-human primates.

TABLE OF CONTENTS

PREFACE ..... **XVI**

1.0 INTRODUCTION **1**

1.1 PREVALENCE **2**

1.2 INFECTION OUTCOME **4**

1.3 TREATMENT **5**

1.3.1 Sanitariums and Surgeries **5**

1.3.2 Chemotherapeutics **6**

1.3.3 Direct observational therapy **7**

1.4 ANIMAL MODELS OF TB **8**

1.4.1 Non-human primates **8**

1.4.2 Mice **9**

1.4.3 Rabbits (*Mycobacterium bovis*) **10**

1.4.4 Guinea pigs **10**

1.4.5 Zebra fish (*Mycobacterium marinum*) **10**

1.5 IMMUNE RESPONE TO *M. TUBERCULOSIS* INFECTION **11**

1.5.1 Infection and innate immune response **11**

1.5.2 Bridging the gap innate to adaptive immunity **13**

1.5.3 Adaptive immune response **15**

1.5.3.1 CD4 T cells 16

1.5.3.2 CD8 T cells 23

1.5.3.3 Other cells involved in the immune response 23

1.5.4 Granuloma 25

2.0 STATEMENT OF THE PROBLEM 27

3.0 IFNγ FROM CD4 T CELLS IS REQUIRED FOR BACTERIAL CONTROL AND HOST SURVIVAL DURING ACUTE *M. TUBERCULOSIS* INFECTION 29

3.1 INTRODUCTION 29

3.2 MATERIALS AND METHODS 31

3.2.1 Animals 31

3.2.2 Probiotic pre-treatment of mice 31

3.2.3 Isolation of donor cells 31

3.2.4 Adoptive transfer to recipient mice 32

3.2.5 Aerosol infection of the mice 32

3.2.6 Harvest of organs for analysis 33

3.2.7 Flow cytometry 33

3.2.8 RNA Isolation 34

3.2.9 Real time quantitative PCR 35

3.2.10 Macrophage killing assay 35

3.3 RESULTS 36

3.3.1 Building the adoptive transfer model 36

3.3.2 IFNγ from CD8 T cells is not sufficient to control *M. tuberculosis* infection in the absence of IFNγ from CD4 T cells 40

3.3.2.1 IFNγ from CD4 T cells is required for host survival..... 40

3.3.2.2 In the absence of CD4 T cells that produce IFNγ, bacterial growth is unchecked 41

3.3.2.3 CD4 T cells are required for an effective immune response..... 41

3.3.2.4 In the absence of CD4 T cells, CD8 T cells are activated, but fewer are present in the lungs. 42

3.3.2.5 Adoptively transferred CD4 T cells produce pro-inflammatory cytokines 43

3.3.2.6 The quality of the CD8 T cell function is altered in the absence of IFNγ from CD4 T cells 44

3.3.2.7 IL-2 from CD4 T cells may account for the poor immune response in animals lacking CD4 T cells. 45

3.3.3 IFNγ from CD4 T cells is necessary for antigen specific responses and directly affects the quality of the CD8 T cell response 46

3.3.3.1 IFNγ from CD4 T cells is required for control of bacterial burden 47

3.3.3.2 Without CD4 T cells, fewer cells are found at the site of infection 47

3.3.3.3 Antigen specific CD4 and CD8 T cells can be detected in the lungs of experimental animals. 47

3.3.3.4 Cytokine production by antigen specific CD4 T cells..... 50

3.3.3.5 Antigen specific CD8 T cell functions 51

3.3.3.6 The effects of whole spleen equivalents on host survival 53

3.3.4 T cell activation of macrophages to kill intracellular bacilli in the presence or absence of IFNγ 55

3.4 DISCUSSION..... 57

4.0 DELETION OF CD4+CD25+ REGULATORY T CELLS PRIOR TO *M. TUBERCULOSIS* INFECTION DOES NOT CHANGE INFECTION OUTCOME..... 60

4.1 INTRODUCTION..... 60

4.2 MATERIALS AND METHODS..... 62

4.2.1 Animals 62

4.2.2 Probiotic Pre-treatment of RAG1-/- mice..... 62

4.2.3 Reconstitution of RAG 1-/- mice in the presence and absence of CD4+CD25+ cells 63

4.2.4 Reconstitution of RAG1-/- mice with only CD4+ T cells +/- CD4+CD25+ population 63

4.2.5 Antibody mediated depletion of CD25+ cells from C57BL/6 mice prior to infection..... 64

4.2.6 Aerosol infection..... 64

4.2.7 Harvest of organs for analysis..... 65

4.2.8 Flow cytometric analysis of cellular infiltrate in lungs..... 65

4.3 THE PRESENCE OR ABSENCE OF CD25+ CELLS DOES NOT CHANGE OUTCOME OF INFECTION WHEN LYMPHOPENIC MICE ARE RECONSTITUTED WITH NAÏVE SPLENOCYTES..... 66

4.3.1 Bacterial burden is not significantly different between animals that received splenocytes with Tregs and those that did not receive Tregs..... 66

4.3.1.1 The absence of Tregs does not affect survival 67

4.3.1.2 Cellular infiltrate 69

4.4 RECONSTITUTION WITH ONLY CD4 T CELLS +/- CD25 CELLS 71

4.5 DEPLETION OF CD25+ T CELLS DURING ACUTE *M. TUBERCULOSIS* INFECTION 75

4.5.1 Experimental set up 75

4.5.2 Depletion of CD25+ cells does not affect bacterial burden over time 75

4.5.3 Depletion of CD25+ cells does not change the number of cells in each organ 76

4.5.4 Lack of CD25+ cells did not change activation state or IFNγ production in either CD4 or CD8 T cells 78

4.6 DISCUSSION 80

5.0 LONG TERM IL-12 TREATMENT DURING EARLY *M.TUBERCULOSIS* RESULTS IN AN INCREASED FREQUENCY OF REGULATORY T CELLS AT SITES OF INFECTION 82

5.1 INTRODUCTION 82

5.2 MATERIALS AND METHODS 83

5.2.1 Animals 83

5.2.2 IL-12 treatment 84

5.2.3 Anti-TGFβ treatment 84

5.2.4 Aerosol infection 84

5.2.5 Organ harvest and cellular isolation 85

5.2.6 Bacterial burden 85

5.2.7 Flow Cytometry 85

5.2.8 Statistics 86

5.3 RESULTS 86

5.3.1 Long term IL-12 treatment during early *M. tuberculosis* infection results in a greater frequency of CD4+Foxp3+ T regulatory cells in wild type mice 86

5.3.1.1 Cellular infiltrate at the site of infection is decreased in IL-12 treated animals when compared to those that received PBS 87

5.3.1.2 IL-12 treatment does not change bacterial burden 87

5.3.1.3 IL-12 treatment results in fewer CD4 T cells producing IL-2 88

5.3.1.4 IL-12 and Tregs 90

5.3.1.5 TGFβ and IL-12 treatment 92

5.3.2 IL-12 immune suppression is independent of IL-10 93

5.3.2.1 IL-12 treatment of IL-10-/- mice did not affect bacterial burden .. 93

5.3.2.2 IL-12 treatment decreases cellular infiltrate 93

5.3.2.3 Tregs and TGFβ+ CD4 T cells in IL-10-/- mice 95

5.3.3 Neutralization of TGFβ does not change IL-12 immune suppression 96

5.4 DISCUSSION 97

6.0 REGULATORY T CELLS IN A CYNOMOLGUS MACAQUE MODEL OF *M. TUBERCULOSIS* INFECTION 100

6.1 INTRODUCTION 100

6.2 MATERIALS AND METHODS 102

6.2.1 Experimental animals. 102

6.2.2 Infection of NHP 102

6.2.3 PBMC isolation 103

6.2.4 BAL Cells 103

6.2.5 Necropsy of animals 103

6.2.6 Isolation of cells from necropsy tissue 103

6.2.7 CD25 depletion from PBMCs 104

6.2.8 Lymphocyte proliferation assay 104

6.2.9 Flow cytometry 104

6.2.10 Immunofluorescence tissue staining 105

6.2.11 ELISPOT for IFNγ 105

6.2.12 Statistics 106

6.3 RESULTS 107

6.3.1 Infection outcome is independent of age, gender and weight 107

6.3.2 CD4+ Foxp3+ cells are found within involved and not uninvolved lung and draining lymph node tissue 108

6.3.3 CD4+Foxp3+ T cells are found within the lymphocyte cuff of the granuloma 110

6.3.4 Tregs migrate from peripheral blood to airways during early *M. tuberculosis* infection 111

6.3.5 Pre-infection levels of Tregs in PBMC correlate with clinical outcome . 112

6.3.6 Foxp3+ cells affect proliferative responses in peripheral blood 114

6.4 DISCUSSION 116

7.0 SUMMARY OF THE DISSERTATION 120

BIBLIOGRAPHY 127

LIST OF TABLES

Table 1. Summary of vital statistics and immunologic assays of all non-human primates used in this study. 107

LIST OF FIGURES

Figure 1 Design of adoptive transfer models and time line for set up. 39

Figure 2 Murine survival after adoptive transfer and subsequent *M. tuberculosis* infection 40

Figure 3 Bacterial burden and cellular infiltrate in the lungs of experimental animals over time 42

Figure 4. The frequency and activation status of T cells within the lung 43

Figure 5. Cytokine production by T cells 45

Figure 6. IL-2 expression in the lungs of reconstituted mice 46

Figure 7. Bacterial burden, total cell numbers and T cells in an adoptive transfer model using whole spleen equivalents 49

Figure 8. Antigen specific cytokine production by CD4 T cells 51

Figure 9. Antigen specific CD8 T cell function 53

Figure 10. Survival of mice reconstituted +/- CD4 T cells and splenic equivalents 54

Figure 11. Macrophage killing assay 57

Figure 12. Adoptive transfer, bacterial burden and survival 69

Figure 13. T cell frequency and activation in the lungs of mice reconstituted with naïve splenocytes 70

Figure 14. Monocytes found within the lung of reconstituted *M. tuberculosis* infected mice 71

Figure 15. Adoptive transfer of CD4 T cells +/- CD25+ cells 74

Figure 16. Bacterial burden and cellular infiltrate in lungs of RAG1-/- mice reconstituted with CD4 T cells +/- Tregs 74

Figure 17. Depletion of CD25+ cells in C57BL/6 mice 76

Figure 18. CFU and total cell number per organ 77

Figure 19 T cell activation in the lungs and draining lymph node 79

Figure 20. IFNγ production by CD4 and CD8 T cells in lung and lymph node 79

Figure 21. Cellular infiltrate and bacterial burden in IL-12 and PBS treated wild type mice 88

Figure 22. IL-2 producing CD4 T cells in the lungs and lymph nodes 89

Figure 23. Regulatory T cells and IL-12 treatment 92

Figure 24. CD4+TGFβ+ cells and IL-12 treatment 93

Figure 25. CFU and total cell numbers in IL-12 treated IL-10-/- mice 94

Figure 26. Frequency of Tregs and CD4+TGFβ+ cells in IL-12 treated IL-10-/- mice 96

Figure 27. Regulatory T cells are enriched in involved lung tissue and lymph node 109

Figure 28. Cellular surface markers on Tregs 110

Figure 29. Regulatory T cells in granulomas 111

Figure 30. Tregs in PBMC and airways during *M. tuberculosis* infection 113

Figure 31. Tregs inhibit PBMC proliferation 116

PREFACE

Thank you to my mentor Joanne Flynn, without whom this work would not have been possible. Her direct, straightforward and passionate approach to life and science is awe-inspiring. She is truly training independent scientist! Thank you to the current and former members of the Flynn lab. You challenge me every day to be a better scientist.

I am very grateful for the time and commitment of my committee, Robert Hendricks, Jerry Nau, Karen Norris and Anuradha Ray. Their input and suggestions have proved invaluable, especially when the data weren't as exciting as I had hoped.

No one embarks on this journey and finishes without support. I'd like to thank my family and friends for their continued love and support. In particular, my MoM and Dad, Kathy and Don Green, thanks for all the Sunday dinners and fun times. My brothers Jason and Ben, my sister-in-law Mary, and nephew Brad, thank you for putting up with the mad scientist. I'd like to thank "The Tribe" you know who you are I'm looking forward to more outings this fall. Thank you to Kris and Gibby and Patricia for all the times I was climbing the walls, you listened and knew when it was time to make me go skiing and running.

1.0 INTRODUCTION

Mycobacterium tuberculosis (*M. tuberculosis*), the acid-fast bacillus responsible for human tuberculosis (TB) is an exquisite pathogen that has been evolving with the human race for millennia. Called "phthisis" by the ancient Greeks, the long history of tuberculosis and man has worked its way into art and literature from the art of the Egyptians to such well known authors as Emily Bronte and Jane Austen [1]. At one point it was even considered desirable and fashionable to look as if one had "consumption." Numerous sufferers of TB were used as muses for artists' renderings of ancient scenes such as Botticelli's "The Birth of Venus" [1]. *M. tuberculosis* DNA has been isolated from mummies found with bone disease, dating back 9000 years [2]. In addition to its long history with man, TB was also found on almost every continent. *M. tuberculosis*-mediated bone disease, such as Potts disease, has been found in mummies from Israel (9000 y/o) [2], Egypt (5000 y/o), and Peru (1000 y/o) [3] and descriptions of TB have been found in texts from India and China dating back as long as 3300 and 2300 years ago respectively (reviewed in [4]). With such an extensive distribution among so many different cultures for thousands of years, it is not hard to understand why one third of the world is estimated to harbor this infection today [5]. As *M. tuberculosis* continued to persist throughout human evolution, in western cultures (Western Europe and North America), TB reached it zenith during the 18th and first half of the 19th centuries. Its primary victims were young adults. Their untimely death is perhaps what fueled the romanticized ideal of the wan, pale victim of TB. The "White plague"

began to wane in the middle of the 19th century. The reduction in the number of people with TB at that time has been attributed to natural selection of a more resistant population as well as better sanitation and nutrition [6]. However, a definitive answer has never been determined.

Until 1882, the cause of TB was largely unknown, on one hand thought to be inheritable since multiple members seemed to be succumb within a family unit, on the other hand hypothesized to be caused by an infectious agent [4]. Robert Heinrich Koch settled the debate when he presented his "Die Aetiologie der Tuberculose" at the Berlin Physiological Society. He had identified the tubercle bacillus, shown that it could and did cause disease and was able to isolate it again from the infected tissues. Thus, not only did he discover the cause of tuberculosis, he also presented the criteria still used today to determine a disease is caused by an infection, Koch's postulates [4].

In the early 20th century, Clemens Freiherr von Pirquet documented reactions to subcutaneously administered *M. tuberculosis*-derived proteins in asymptomatic people, thus coining the term latent tuberculosis [7]. Charles Mantoux's use of a cannulated needle to provide the tuberculin derivative, in conjunction with the development of purified protein derivative (PPD) by Florence Seibert, was the advent of what is our modern day TB skin test [4]. The development of this test allowed for more wide spread surveillance of *M. tuberculosis* prevalence among varied populations.

1.1 PREVALENCE

The decline in TB related deaths that started in the mid 19th century continued, particularly in Western Europe and North America, until the advent of the HIV/AIDS epidemic in the 1980's

[8]. With the HIV epidemic, global rates of TB rose for the first time in decades. The WHO estimates are 9 million new cases of TB in 2007, with 15% due to coinfection with HIV [9]. Globally, the WHO estimates that one third of the world's population (~2 billion people) are currently infected with *M. tuberculosis.* Of those individuals infected with the bacilli, 1.7 million died from the disease in the 2007 [5]. It is the developing world that bears the brunt of the burden of TB. Thirteen of the 15 countries with the greatest incidence of TB infection are in Africa and a direct correlation with HIV co-infection has been shown [10]. HIV infection increases the risk of developing active disease from 10% per life time to 10% per year [5]. While approximately 15% of those who get active disease are HIV+, those with co-infection account for almost 23% of the mortality rate attributed to TB [10].

In addition to the complication of HIV/TB co-infection, the incidence of multi-drug resistant (MDR)-TB and extensively drug resistant (XDR)-TB has continued to increase, particularly in Asia and the former Soviet Union. Multi-drug resistance is defined as resistance to the two front-line drugs isoniazid (INH) and rifampin (RIF) and extensively drug resistance defined as *M. tuberculosis* that is resistant to INH, RIF, the fluoroquinolones and one of the three second-line injectable drugs [11]. Many cases of XDR-TB are essentially untreatable. In the US and Western Europe, MDR-TB accounts for fewer than 3% of cases, whereas in Africa rates as high as 14% of cases are MDR [11, 12]. In Asia, specifically certain parts of China, MDR-TB accounts for up to 7.3% of the total TB cases. In the former Soviet Union, the incidence of MDR-TB ranges from 6.8% in Georgia to as high as 22% in Azerbaijan. Even more alarmingly, in the former Soviet Union, the incidence of XDR-TB among the MDR-TB accounts for from 4% in Armenia to almost 24% in Estonia [9]. WHO estimates that 40,000 new cases of XDR-TB emerge each year. MDR-TB and XDR-TB arise when a course of chemotherapy is not

completed and are thought to be indicators of TB control failures at the national level. Lack of infrastructure to maintain the supply of chemotherapeutic agents, patient non-compliance with such a long course of treatment and loss of adequate public health services have been shown to be the root cause of the rise in global MDR and XDR-TB [8, 12-14].

1.2 INFECTION OUTCOME

Exposure to *M. tuberculosis* does not guarantee infection, since only 20-30% of those exposed will develop a positive tuberculin skin test (TST). Infection is defined as a positive result for either a TST or IFNγ release assay (IGRA) [15]. TST-negative exposed persons must somehow prevent establishment of infection (and the resulting T cell response that is detected by TST). A few of the individuals that become infected (5-10%), will develop signs and symptoms of active disease within the first two years post infection (primary TB) and are contagious. Active disease is classified as a positive TST or IGRA and clinical signs of disease (positive sputum culture, coughing, weight loss, or evidence of disease by chest x-ray). 90-95% of *M. tuberculosis* infected individuals mount an effective, non-sterilizing immune response that walls off the bacilli within discrete structures called granulomas. When infection is controlled, with no clinical manifestation of disease, it is considered a latent infection. A majority will control the infection for life, and carry a 10% per year risk of developing active disease later in life (reactivation TB). Immune compromise by infection (HIV), immunomodulatory therapies (e.g. TNF-neutralizing antibodies as a treatment for inflammatory diseases) and advanced age increases the risk for reactivation [5].

In the simplest interpretation, *M. tuberculosis* infection outcome appears to result in either an active or latent infection as first proposed by von Pirquot when he described latent infection [7]. When the various manifestations of disease are evaluated however, a picture emerges that more closely resembles those proposed in a review by Barry *et al*. and Lin *et al.* [16, 17]. It seems that active disease and latent infection are not two distinct outcomes, but are a spectrum of outcomes ranging from clearance or dormant infection to the development of fulminant disease.

1.3 TREATMENT

1.3.1 Sanitariums and Surgeries

Prior to the development of the first chemotherapies to treat tuberculosis, patients with TB were quarantined in sanitariums. The prescription was for rest, plentiful rich food, sunshine and isolation from the stresses of everyday life. The first of these sanitariums in the United States was set up by Edward Livingston Trudeau at Saranac Lake, New York [18].

The quest for a mechanical solution was also sought and it was hypothesized that if rest for the body was beneficial perhaps so would be rest for the diseased lungs. Patients were subjected to pulmonary collapse therapy in which sandbags were used to splint one side of the chest [4]. Others attempted surgical intervention included therapeutic pneumothorax by artificially introducing air into the pleural space to collapse the lung so it could “rest”. This procedure may have been successful if only because it resulted in cavity closure and conversion to sputum negative (i.e. no bacilli were seen in sputum samples) [19]. In yet another surgical

variation, thoracoplasty (removal of part of the rib cage) was also tried. This was at the time considered safer since it did not require entering the pleural cavity. Again the success of this procedure may be attributable to cavity closure and not a decreased bacterial load [20]. Still today, in areas where MDR-TB is endemic, lung resection of cavitary lesions or destroyed tissue, in combination with chemotherapy is the treatment of choice [21].

1.3.2 Chemotherapeutics

In the 1940s the first chemotherapeutic compounds, streptomycin and para-amino salicylic acid (PAS), were discovered. Streptomycin was reported by Schatz *et al* in 1944 as having antibiotic activity against bacteria [22] and it was the first drug to be used to successfully treat TB. It was quickly apparent that *M. tuberculosis* could become resistant, but this could be overcome by using it in combination with PAS [23]. Streptomycin inhibits protein synthesis where as PAS most likely interferes with cell wall synthesis. Today, both streptomycin and PAS are considered second line drugs for treatment of TB [14].

While the discovery of streptomycin and PAS revolutionized the treatment of TB, both were only bacteriostatic. It was not the discovery of isoniazid (INH) in 1952 that the first bactericidal compound was introduced. An added benefit of INH was that it is administered orally [24]. In combination with Streptomycin and PAS, INH became part of the standard drug regimen [25]. INH remains a front line drug and in the developed world is given prophylacticly to patients with latent infections [14].

Pyrazinamide was also introduced in 1952. It is active against non-replicating bacteria, but is unable to provide a sterilizing course of treatment by itself. The mechanism of action

against the bacillus is not well defined, but the drug is thought to disrupt the membrane transport of Pyrazinoic acid [25].

In the 1960s further strides were made with the introduction of Ethambutol. Ethambutol interferes with construction of the arabinogalactan layer of the cell wall, but the exact mechanism has yet to be defined. It replaced PAS in the standard drug therapy combination, reducing toxicity and decreasing the length of treatment from 24 to 18 months [18]

In 1966, rifampicin (RIF) was shown to be effective against *M. tuberculosis*. Today it is a cornerstone of the modern first line drug cocktail and inhibits transcription [26]. Addition of RIF decreased treatment from 18 months to 9 months. Treatment length was further reduced to 6 months by the addition of PZA to the cocktail of INH and RIF [18].

Currently, the cornerstone of MDR-TB treatment is the fluoroquinolones. A class of broad-spectrum antibiotics, these compounds have good oral bioavailability and are bactericidal against *M. tuberculosis*. In addition, these compounds have not been shown to have cross resistance with the anti-mycobacterial drugs and newer classes of these compounds are being investigated as a replacement in first line anti-TB drug combination therapy [27].

1.3.3 Direct observational therapy

The advent of chemotherapeutic interventions revolutionized the treatment of TB. However, the large number of patients, the length of treatment that required hospitalization, and lack of enough hospital beds in some endemic areas presented a need for outpatient care. In randomized trials, those treated at home had a higher rate of non-compliance with treatment. Therefore directly observed therapy (DOT) was implemented. Those being treated for TB were directly observed taking their medicine by trained personnel. This is a WHO program, where

commitment from the government of each nation is required and criteria for drug delivery must be met. DOT therapy became direct observational therapy short course (DOTs) when the treatment cocktails regimens reduced time of treatment [28].

1.4 ANIMAL MODELS OF TB

Humans are genetically heterogeneous and this diversity results in a spectrum of disease from asymptomatic latent infection, subclinical infection, active pulmonary disease to extrapulmonary TB (reviewed in [17]). The study of TB in humans is limited by how the disease can be visualized (X-ray, MRI, CT-PET scans) and the types of samples that are safely and easily obtained such as peripheral blood and bronchial alveolar lavage (BAL). In addition, the duration of infection, size of inoculum, strain with which they were infected and the immune and co-infection status of the host at the time of *M. tuberculosis* infection are not known, all factors that can influence disease outcome. Fortunately multiple animal models are available for studying the immune response to *M. tuberculosis*.

1.4.1 Non-human primates

Non-human primate is the only animal model of *M. tuberculosis* infection that can recapitulate the spectrum of latent and active disease seen in human [29]. The desired disease outcome can be manipulated by the size of the inoculum at the time of infection (i.e. the higher the dose, the more likely the NHP will develop active disease) [30, 31]. In this model, *M. tuberculosis* is introduced to the lung by bronchoscope, but aerosol delivery is also used by some groups [32].

NHP infected with *M. tuberculosis* also develop the entire range of diverse granulomas seen in humans [30]. Sufficient reagents are available for research analysis of immunologic factors and many human reagents cross react. This model has two main drawbacks: the animals are as genetically diverse as the human population, there is both inter- and intra- monkey variability is not uncommon. In addition, this model is expensive to maintain.

1.4.2 Mice

The murine model of *M. tuberculosis* is the most widely used model for studying TB. The availability of inbred, knockout, knock-in and transgenic animals in conjunction with a large array of reagents make this an ideal system to manipulate. This system has proven useful for identification of immune factors that are essential for control of *M. tuberculosis*. All mice are susceptible to *M. tuberculosis* meaning they can be infected and develop disease that eventually proceeds to death. Some strains can control infection for longer periods of time and are considered more resistant (e.g.C57BL/6 mice control infection for longer than DBA mice on a C3H background [33].) Similar to humans, immune competent mouse strains are able to control the infection without signs of disease for up to a year. However, mice carry a high bacterial burden in the lungs (~$1x10^6$ CFU/lung) and have a chronic progressive infection and no latent form. Murine granulomas differ from those found in humans and the NHP model. In addition granulomas in this model do not result in caseous necrosis or cavitary lesions. Murine granulomas are loose aggregates of cells. Multiple routes of infection have been used, but the most physiologically relevant is by aerosolization of virulent *M. tuberculosis* directly into the airways [34].

1.4.3 Rabbits (*Mycobacterium bovis*)

Rabbits are naturally resistant to *M. tuberculosis* and while rabbits develop granulomas that can become cavitary, the lesions eventually heal. However, they are very susceptible to *Mycobacterium bovis* (*M. bovis*). Rabbits infected with *M. bovis* develop granulomas that resemble human granulomas in structure and environment and are the only model besides NHP to develop cavitary lesions [35]. Fewer reagents are available for studies in rabbits than are available in the mouse model. In addition, the rabbits require more space and are more costly to maintain than mice [36].

1.4.4 Guinea pigs

Guinea pigs are highly susceptible to *M. tuberculosis* and were used to identify *M. tuberculosis* as the etiologic agent of TB [27]. In addition, it was the model used to show the antibacterial properties of INH and ethambutol [27]. Granulomas formed can be highly organized and caseous necrosis is often seen [37]. A majority of animals used are out-bred; inbred strains are limited and few immunologic reagents are available for research in this model [34].

1.4.5 Zebra fish (*Mycobacterium marinum*)

Zebra fish are naturally susceptible to *Mycobacterium marinum* (*M. marinum*) a close relative of *M. tuberculosis*. Upon infection with *M. marinum*, zebra fish mount an adaptive immune response and develop caseating granulomas [38]. However, the greatest contributions of the model have been the ability to visualize interactions between the mycobacteria and innate

immune cells in the transparent juvenile zebra fish. This model has been used to show that *M. marinum* is capable of super-infection [39] and infected macrophages migrate toward each other and can swap intracellular bacteria [40, 41]. As this model has developed, inbred and knockout strains have been established, and forward genetics can be used to identify host factors important in control of infection. This model is relatively inexpensive to maintain and the generation time for new mutants is very short.

1.5 IMMUNE RESPONE TO *M. TUBERCULOSIS* INFECTION

1.5.1 Infection and innate immune response

When a person with active tuberculosis coughs, *M. tuberculosis* bacilli are released in aerosolized droplets. The aerosolized droplets are inhaled and the bacilli enter the airways. Inside the airways, innate immune cells such as macrophages (Mϕ) and dendritic cells (DC) phagocytose *M. tuberculosis* and begin a complex of series of host-pathogen interactions.

Phagocytosis of *M. tuberculosis* is mediated by multiple receptors such as complement receptor type 1 and 3, [42, 43], the mannose receptor [44, 45], and by directly interacting with surfactant protein-A and binding its receptors [46, 47]. The reasons why *M. tuberculosis* has developed multiple mechanisms of entry into host cells are not well understood. It may be that by using different receptors, entry into specific cells may benefit the bacilli by initiating unique cytokine secretion patterns or facilitating spread of the bacteria via migration of macrophages or dendritic cells [48].

Once inside the phagosome, *M. tuberculosis* has evolved several mechanisms to circumvent the host immune response and make the intracellular environment more hospitable. *M. tuberculosis* can escape the phagosome and this requires the expression of the ESX bacterial secretion system [49]; however, the concept that M. tuberculosis can escape to the cytosol is controversial at this time, and the physiological relevance of the in vitro data on this issue is not known. Multiple components of the cell wall, such as trehalose 6,6'-dimycolate (TDM, also known as cord factor) and lipoarabinomannan, induce specific cytokines, inhibit polymorphonuclear leukocyte migration, scavenge oxygen free radicals, inhibit protein kinase C and inhibit transcriptional activation of IFNγ inducible genes [50-52].

Fusion of the phagosome to lysosome is required for bactericidal activity in Mϕs. *M. tuberculosis* cell wall contains anionic trehalose glycolipids that can prevent phago-lysosome fusion (reviewed in [53]). *M. tuberculosis* can alter the acidity of phagosomes and lysosomes. *M. tuberculosis* can produce excess ammonia, which acts as an alkalizing agent, thus reducing the ability of lysosomal enzymes to properly function [54, 55]. In addition, the bacilli have been reported to inhibit proton ATPase-dependent acidification of the phagosome and phago-lysosome [56]. Another strategy of the bacillus is to block phagosome maturation. *M. tuberculosis* promotes retention of Rab5A on the surface of phagosomes but has impaired recruitment of Rab5 effectors EEA1 and hVPS34 preventing phagosome maturation [57-59]. In addition, phagosomes containing the bacilli recruit and retain tryptophan-aspartate containing coat protein (TACO), and this inhibits trafficking to the lysosome [60]. Reactive oxygen species and reactive nitrogen intermediates are required for killing intracellular bacteria within Mϕs. *M. tuberculosis* is able to neutralize oxidative radicals by producing superoxide dismutase in response to the sensor oxyR [61, 62]. Finally, once activated, Mϕs initiates the inflammasome,

resulting in IL-1β processing and phagosome maturation. *M. tuberculosis* directly interferes with IL-1β processing by secreting the zinc metalloprotease (ZmpA) [63, 64]. Together these mechanisms of immune modulation allow the bacteria to grow for an extended period of time within phagocytes.

In the mouse model of *M. tuberculosis* infection, the bacilli expand exponentially in the lungs, reaching a level of ~$5x10^6$ CFU/lung, until initiation of the adaptive immune response at approximately 21 days post infection (p.i.) [65]. *M. tuberculosis* infection is eventually recognized. Recognition of the infection is mediated by toll-like receptors (TLRs) and it has been shown that the TLR adaptor molecule, myeloid differentiation factor 88 (MyD88) is required for host survival [66]. In particular the heterodimer of TLR2 with TLR1 or TLR6 as well as TLR9 and TLR4 have been implicated [67-71]. Interestingly, TLR2 has been shown to directly interact with ESAT6 [72]. Recognition of bacterial products by TLRs results in the initiation of the immune response and an increased production of reactive oxygen species, reactive nitrogen intermediates, anti-microbial peptides and cytokine production.[73-75]. However, innate immunity is not sufficient to control infection without the initiation and priming of an adaptive response.

1.5.2 Bridging the gap innate to adaptive immunity

Progression from an innate immune response to initiation of the adaptive response begins at the site of infection where infected Mϕs and DCs produce multiple cytokines and chemokines [69, 76]. The character and quality of the adaptive immune response is influenced by the cytokines present during priming. Pro-inflammatory cytokines IL-1β (processed during initiation of the inflammasome), IL-12, TNF and IFNγ promote a T- helper 1 phenotype that is optimal for

infection control in both mice and humans [77-81]. During early infection, an influx of IL-17 producing cells (γδ T cells and Th17 cells) has been reported [82, 83]. They are induced by IL-23 and thought to play a role in leukocyte recruitment to the infection site, but are not required for host survival [82, 83]. Additional cytokines such as IL-4, IL-13 and IL-10 skew the immune response toward a Th2 phenotype. Th2 type responses are associated with less than optimal control of *M. tuberculosis* infection [84-86], although most persons (and mice) with TB disease have strong Th1 responses so this is not strictly a Th1/Th2 disease. In addition, it has been hypothesized that the immune response to mycobacterial infection in children is skewed toward a Th2 type response and this may be the reason for an increased risk of developing disseminated disease [87], however, this is controversial.

Multiple factors influence the type of immune response to *M. tuberculosis*. One determinant of infection control is the immune status of the host, for instance the immune response may differ between an immune competent adult, a very young child and an individual co-infected with HIV [87-89]. Additionally, bacterial components also influence the type of immune response elicited with some that promote pro-inflammatory cytokine production, and several that promote anti-inflammatory cytokine production [69, 90]. The combination of these factors and their influence on the balance between pro-inflammatory and anti-inflammatory may mean the difference between active disease and latent infection in humans.

Resident alveolar Mϕs are presumably one of the first cell types to be infected with *M. tuberculosis* and in response produce CCL2, CCL3 and CCL5, where as monocyte derived Mϕs (those thought to have migrated from the periphery) produce CCL2, CCL3, CCL4 And CCL5 [91]. Mϕs are thought to remain at the site of infection, however studies of *M. marinum* challenge this strict view. Infected Mϕs are able to migrate toward other infected Mϕs and are

able to exchange intracellular bacteria and may contribute to dissemination of the bacilli [39, 41]. One can hypothesize that chemokines may contribute to infected Mϕ migration that may be beneficial for the establishment of a persistent infection.

While Mϕs remain at the site of infection and possibly contribute to bacterial dissemination, DCs may also contribute to dissemination but also bridge the gap between innate and adaptive immunity. Once infected, DCs up regulate the expression of CXCR4 and CCR7 which are chemokine receptors necessary for migration to draining lymph nodes [92]. Recently an alternate splice variant of IL-12 receptor β1 has been shown to enhance *M. tuberculosis* infected DC migration from the lung to the lymph nodes [93]. As DCs migrate and mature the ability to phagocytose is down regulated, expression of costimulatory molecules (i.e. MHCII, CD80, CD86) increased, and cytokines that aid in T cell priming produced [94].

1.5.3 Adaptive immune response

When compared to the immune response to other infections, the initial response to *M. tuberculosis* is delayed [95, 96]. Despite this delay, exponential bacterial growth (at least in the murine model) is halted when primed T cells migrate back to the site of infection indicating the adaptive immune response acts to control and contain the bacterium [97].

Control of *M. tuberculosis* infection is dependent upon initiation of the adaptive immune response in both mice and humans [98-100]. DC migration from the site of infection to the draining lymph nodes both contributes to bacterial dissemination and initiates the priming of the adaptive response [101]. Delaying the DC migration (by DC depletion) exacerbates disease outcome and decreases control of the infection by CD4 T cells [102].

1.5.3.1 CD4 T cells

CD4 T cells are required for successful containment of *M. tuberculosis* infection. Mice lacking CD4 T cells (CD4-/- or MHCII-/- mice) carry higher bacterial burdens and die significantly sooner than wild-type mice [103]. Chronically infected mice lose control of mycobacterial infection and succumb when CD4 T cells are depleted [104]. Humans and non-human primates also require CD4 T cells for control of infection. Co-infection with HIV (or SIV in the case of NHP) increases the risk of active TB and is associated with decreasing CD4 T cell numbers [105, 106]. When CD4 T cells are depleted in non-human primates during the initial stage of infection, macaques present with more disease by 8 weeks post-infection (p.i.). Depletion of CD4 T cells during latent infection in macaques causes reactivation of the infection in 50% of the animals (Lin, Flynn, unpublished). Taken together, these studies underscore the importance of CD4 T cells in the immune response to *M. tuberculosis* infection.

CD4 T cells develop in the thymus as either natural regulatory T cells (Tregs) [107] or naïve CD4 T cells (reviewed in [108]). Naïve CD4 T cells can be primed to become one of three pro-inflammatory T-helper phenotypes: Th1, Th2 and Th17. Each of these lineages is defined by a signature cytokine profile and requires a different series of cytokines present to activate lineage specific transcription factors (reviewed in [108]).

Th1 CD4 T cells

Th1 CD4 T cells are primed to produce IFNγ, TNF, IL-2 and GMCSF [109]. Expression of these cytokines depends on the expression of IL-12 by DCs that act on the CD4 T cell to upregulate the transcription factor T-bet and signal through Stat-4 [110]. T-bet and Stat-4 synergize to induce IFNγ, and upregulate expression of IL-12rβ2 and IL-18 receptor [111]. Thus enhancing the T cells ability to respond to pro-Th1 polarizing cytokines. Interestingly, T-bet

knockout animals are more susceptible to *M. tuberculosis* infection than wild-type mice. This vulnerability correlated to an increased production of the Th2 associated cytokine IL-10 and decreased IFNγ production, underscoring the importance of the Th1 response to this infection [112]. In addition to promoting Th1 differentiation, T-bet blocks expression of Gata-3 (the transcription factor associated with Th2 differentiation) [113].

During the course of infection, naïve CD4 T cells encounter *M. tuberculosis* infected IL-12 secreting DCs in the lymph node and recognize antigen in the context of MHC II. Under these conditions, CD4 T cells are primed as Th1 cells and are a primary source of IFNγ during the acute phase of the infection with peak production around 3-4 weeks p.i. in the murine model [114]. IFNγ is required for control of *M. tuberculosis* infection. Mice lacking IFNγ are the most vulnerable strain, dying significantly earlier p.i. than any other immune compromised strain [77]. In addition, humans with loss of function genetic mutations in IFNγ signaling are more susceptible to mycobacterial infections (reviewed in [115]). IFNγ activates Mϕs to kill intracellular pathogens and increases the expression of MHC and co-stimulatory molecules on DCs [116]. IFNγ enhances pro-inflammatory cytokine production in T cells and directly affects CD8 T cell expansion and differentiation into memory cells [117]. Mice lacking CD4 T cells exhibit a delay in IFNγ production, but eventually reach levels equivalent to wild type, however this is insufficient to rescue the host [103]. When CD4 T cells were depleted from chronically infected mice, no change in IFNγ levels in the lungs were observed [104]. These data indicate that during the course of the immune response to *M. tuberculosis*, cells other than CD4 T cells can and do produce IFNγ. This leads to the hypothesis that CD4 T cells have functions in addition to IFNγ production (addressed in Chapter 3).

CD4 T cells influence the immune response to *M. tuberculosis* by the production of cytokines. In addition to IFNγ, Th1 CD4 T cells can produce TNF, IL-2, and GMCSF [118] (reviewed in [108]). Production of IFNγ and TNF by CD4 T cells activates Mϕs to induce bactericidal activity against intracellular pathogens [119] by inducing NOS2 to produce reactive nitrogen intermediates [120] and induction of the small GTPase LRG-47 [121]. Whereas IL-2 directly impacts T cell expansion and survival [122], GMCSF has been shown to modify DC function improving IL-12 production and increasing expression of co-stimulatory molecules on the surface [123].

CD4 T cells influence the quality of the CD8 T cell response. CD4 T cells interact with DCs to facilitate better antigen presentation and provide cytokines required for growth, survival and enhanced function. In the absence of CD4 T cells, CD8 T cells can be primed, but are less cytotoxic [124]. Some data suggest this is due to the production of IL-2 by CD4 T cells [125]. While CD4 T cells capable of secreting several cytokines simultaneously have been shown to be beneficial in other models of infection [126, 127], the correlation of protection in *M. tuberculosis* has yet to be established [128-130].

Th2 CD4 T cells

Th2 responses are essential for an effective immune response against extra cellular pathogens [131] and are also mediators of asthma (reviewed in [132]). IL-4, IL-5 and IL-13 are the signature cytokines for Th2 CD4 T cells. However it has been shown that Th2 CD4 T cells can also make TNF and IL-2, but in smaller quantities when compared to Th1 cells [133]. The transcription factor Gata-3 is the master regulator for Th2 differentiation [134] and promotes the priming of a Th2 response in the presence of both IL-2 and IL-4 [135]. Gata-3 promotes IL-4

production, instructing Th2 commitment, selectively promoting growth of Th2 cells while simultaneously suppressing Th1 differentiation [136].

Th2 responses are not effective in response to *M. tuberculosis*. Mϕs alternatively activated with IL-4 fail to kill intracellular bacteria [85]. Studies in the murine model of *M. tuberculosis* show that when mice are put under stress, they lose control of bacterial growth. The increased bacterial growth is associated with a shift from Th1 type responses to Th2 type cytokine production [137]. In addition, humans with active disease have an increased production of IL-4 by CD4 T cells and CD8 T cells [138, 139] and increased Th2 type cytokine production was associated with the presence of cavitary lesions [138].

Bacillus Calmette-Guerin (BCG) is an attenuated *Mycobacterium bovis* that has been used as a vaccine since 1921 with variable efficacy (0-80% protection depending upon the study) [140]. In areas endemic for helminth (worm) infection, the efficacy of BCG is greatly reduced [141]. This reduction is associated with a shift toward Th2 phenotype that is induced in people colonized with helminthes [142]. Subsequent studies in mice colonized with *Schistosoma mansoni* prior to BCG vaccination had reduced benefit from vaccination when challenged with virulent *M. tuberculosis* [143]. These data indicate that Th2 type responses cannot control *M. tuberculosis* infection and may abrogate an effective immune response against *M. tuberculosis*.

Th17 CD4 T cells and other IL-17 producing cells

IL-17 is a pro-inflammatory cytokine that recruits cells into parenchymal tissue, and is induced by IL-23 (reviewed in [144]). The majority of IL-17 is produced by γδ T cells during the initial phases of *M. tuberculosis* infection [83]. However a small percentage of Th17 CD4+ T cells are primed to secrete this cytokine and thought to contribute to the recruitment of cells into the lung parenchyma [82, 145]. Interestingly, a decreased frequency of Th17 cells is

reported in patients with active TB, which was attributed to down regulation of the IL-6 receptor (the cytokine that in conjunction with IL-23 is needed to prime Th17 cells) [146]. However, it should be noted that IL-17 is not required for control of *M. tuberculosis* infection [82], at least in the murine model.

CD4 Regulatory T cells

CD4 T cells can also function as immune suppressive regulatory T cells (Tregs) and were first identified as potent inhibitors of autoimmunity [147]. Several different types of Tregs have been described within the literature. Natural Tregs (nTregs) are thymically selected, express the IL-2 receptor α chain (CD25) and the transcription factor Foxp3 [107, 148, 149]. Inducible Tregs (iTregs) have a phenotype similar to nTregs, but are induced from the naïve CD4+CD25- T cell population in the presence of T cell receptor (TCR) signal, IL-2 and TGFβ1 [150-153]. In mice, iTregs have immunosuppressive activity, but in humans iTregs have not been demonstrated to have this function even when tested *in vitro* [154, 155]. Finally, two other types of Tregs are described in the literature as Tr1 and Th3 cells. Both are defined by the cytokine produced with Tr1 secreting IL-10 and Th3 cells producing TGFβ1 [156, 157]. While each of the Treg sub groups described here are referred to in the literature, their functions are not mutually exclusive since nTregs and iTregs have been reported to secrete IL-10 or TGFβ1 to modulate the immune response.

Both nTregs and iTregs require the transcription factor Foxp3 for suppressive activity [148, 158]. Scurfin-/- mice and humans with IPEX (immunodeficiency, polyendocrinopathy and enteropathy, X-linked syndrome) lack Foxp3, have no Tregs and present with multiple types of autoimmune responses [159, 160]. Furthermore, transduction of naïve CD4 T cells with a constitutively active Foxp3 induces a Treg phenotype capable of immunosuppression and similar

to nTregs [148]. TGFβ is important for maintenance of nTregs [161]. TCR signaling in the presence of TGFβ activates NFAT and Smad-3 which work in conjunction with Foxp3 to impart the immunosuppressive phenotype [162]. Regardless of origin, Tregs down-regulate the immune response by cell to cell contact and secretion of anti-inflammatory cytokines such as IL-10 and TGFβ (reviewed in [163]).

Tregs have been shown to play a role in the establishment of persistent infections [164-166]. Whether the presence of Tregs is beneficial or detrimental to the infection outcome remains controversial, and may be dependent on the pathogen and the stage of infection. Tregs have been shown to facilitate immune evasion by *Plasmodium spp*. [166-168] and suppress HCV specific CD8 T cells [165, 169]. In contrast, in an experimental model of *Leishmania major* infection, Tregs facilitated pathogen persistence, however this was of benefit to the host because the presence of antigen was required for sustained immune protection [164]. The role of Tregs in *M. tuberculosis* infection is just as complex. Multiple studies report Tregs are present in the lungs and lymph nodes of *M. tuberculosis* infected mice [170, 171]. Antigen specific Tregs expand first in the draining lymph node and then migrate to and accumulate at the site of infection [172]. These Tregs expand in response to the pro-inflammatory environment of the mycobacterial immune response [173]. However, whether Tregs are beneficial or detrimental to the host during *M. tuberculosis* infection has yet to be determined.

To determine what role Tregs may contribute to the immune response to *M. tuberculosis,* initial studies in mice used antibody depletion of CD25+ T cells prior to *M. tuberculosis* infection. Depletion of the nTreg population prior to infection had no effect on bacterial burden and disease course in the murine model, however it did result in increased IFNγ production [174, 175]. This approach was limited because CD25 is expressed on T cells as they become

activated, thus cells could be depleted only prior to infection and not continuously. To overcome this issue, a bone marrow chimera model was used. Mice received a mixture of bone marrow from scurfin-/- mice (Thy1.2+) and Thy1.1 wild type mice. Thus, the bone marrow reconstitution provided a Treg population that was Thy1.1. Tregs were depleted from the mice at different times post infection. In this model depletion of Tregs resulted in a decreased but not eliminated bacterial burden. Thus Tregs were found to limit antimycobacterial responses in the lungs [171]. Interestingly when lymphopenic mice were reconstituted with CD4 T cells only with and without CD4+CD25+ Treg population, mice lacking the initial Treg population had significantly less bacterial burden in the lungs or no culturable bacteria in the lung [176]. The reasons for this bimodal distribution remain unknown. Finally, to determine whether Tregs were antigen specific and from which population (nTreg CD4+CD25+ or naïve CD4+CD25-) CD4 T cells transgenic for *M. tuberculosis* antigen 85A cells were used. When adoptively transferred into Thy1.1 wild type mice, Tregs expanded first in the draining lymph node and migrated to the site of infection (i.e. the lung). In addition these cells were antigen specific and expanded from the nTreg population [172]. Tregs may help *M. tuberculosis* establish a persistent infection or they may be acting to protect healthy tissue from immunopathologic damage. (The role of Tregs in the murine model of *M. tuberculosis* infection is addressed more fully in chapters 4 and 5).

Finally, Tregs may influence the development of active or latent infection in humans. Evaluation of persons with active disease showed an association between increased frequencies of Tregs in the peripheral blood when compared to healthy uninfected or latently infected controls [177-180]. Less antigen specific IFNγ was produced by those with active disease, but was reversed when CD25+ Cells were depleted from the peripheral blood [177-181]. However, whether Tregs predispose an individual toward active disease or whether Tregs increase in

response to the inflammation associated with active disease is not understood (addressed in chapter 6).

1.5.3.2 CD8 T cells

CD8 T cells are primed in the lymph node, accumulate at the site of infection and appear to have a similar delay in priming in *M. tuberculosis* infection, as reported for CD4 T cells [96, 182]. Functionally, CD8 T cells are primed to be either cytotoxic or secrete cytokines [183], with IFNγ secreting CD8 T cells reportedly involved in control of bacilli during chronic infection [184]. CD8+ cytotoxic cells express perforin and have been shown to lyse infected macrophages [185]. While CD4 T cells are essential for an effective immune response to *M. tuberculosis*, the role of CD8 T cells has been harder to elucidate at least in the murine model of infection. However, the importance of CD8 T cells in TB was demonstrated in the NHP model, where antibody depletion of CD8 T cells results in loss of control of the mycobacterial infection (Lin, Flynn unpublished data). CD8 depletion also impaired control of vaccine-induced immunity in a macaque model [186]. One hypothesis is that CD8 T cells eliminate cells that no longer respond to cytokines [187]. However, CD8 T cells can also produce cytokines such as IFNγ and TNF and may contribute to Mϕ activation [188].

1.5.3.3 Other cells involved in the immune response

In addition to T cells, other cell types are found within the granuloma such as B cells, γδ T cells, Natural killer (NK) cells, and NK T cells. However the roles of these cells have not been as well defined as T cells.

B cells

In addition to T cells, DCs and Mϕs, B cells are present within the granuloma, however their function are not as well understood as those of T cells in particular. Studies in B cell deficient mice indicate that B cells may be acting as antigen presenting cells, and responsible for the generation of germinal centers within the infected lung [189-191]. In addition, several studies have suggested that B cells help to modulate the inflammation found at the infection site [191, 192].

In mice lacking B cells (μMT), infection with low dose aerosol *M. tuberculosis* resulted in increased inflammation and recruitment of neutrophils. This was associated with an increased production of IL-10. Furthermore, when infected with a higher dose, mice lacking B cells had an increased bacterial burden and susceptibility when compared to wild-type counterparts. The increased susceptibility of B cell deficient mice infected with higher inoculum could be rescued by adoptive transfer of B cells prior to infection [191]. Thus B cells may contribute to the control of *M. tuberculosis* by modulating cytokine production at the site of infection.

Traditionally, immune responses have been separated into humoral (antibody mediate) and cellular mediated immunity. However, antibodies are able to modulate cellular mediated immunity through Fcγ receptors (FcγR). FcγRs activate or inhibit the immune response depending on the threshold of activating or suppressing signals [193]. Activating FcγRs share a γ chain that signals through an intracellular ITAM whereas the inhibiting receptor contains a cytoplasmic ITIM [193]. The balance between the two signals determines the amount of cellular activation [193]. When mice lacking the inhibitory receptor FCγRIIB were infected with *M. tuberculosis*, decreased bacterial burden and less immunopathology were observed at the site of infection. Conversely, mice lacking the common γ chain (Fcγchain-/-), the loss of activating

signals resulted in an increased IL-10 production, increased bacterial burden, dissemination and susceptibility [194]. Taken together these studies indicate that B cells may play a role in modulating the immune response to *M. tuberculosis* by influencing both the cytokine environment and cellular activation state.

1.5.4 Granuloma

Initiation of the adaptive immune response results in CD4 T cells, CD8 T cells and B cells migrating from the draining lymph node to the site of infection and surrounding infected Mϕs and DCs. Cell migration is mediated by various chemokines produced by infected cells and surrounding tissue [195, 196]. This discrete structure is called a granuloma. It effectively walls off the bacteria that cannot be eliminated even in the presence of a robust Th1 response, thus benefiting the host [197]. The granuloma may also benefit the bacillus by providing a protected place for the mycobacteria to reside, in a non-replicative or occasionally replicating state for decades [198, 199]. Interestingly, bacterial components facilitate granuloma formation by interacting with Mϕs, DCs and lung epithelium to produce inflammatory chemokines [94, 200].

Granulomas in humans, NHPs, guinea pigs and rabbits are highly organized. Infected Mϕs and DCs are surrounded by uninfected monocytes encased in a lymphocytic cuff. In contrast, in mice the granulomatous structure appears as loosely organized aggregates of cells [37]. The phenotype of granulomas in humans and NHPs can range from a calcified (healing) granuloma to a necrotic cavitary lesion that can erode into the airways and cause spread of the bacilli [29].

The granuloma serves as a barrier and is a dynamic interaction between the host immune response and the tubercule bacilli. Fluctuation in factors that influence the immune response can

lead to loss of control of the bacilli and development of exacerbated disease. Chemokines are required for granuloma formation and are induced by mycobacterial infection and by TNF. Depletion of TNF in mice, humans and NHP can result in loss of control of bacterial growth, and in primates, development of active disease [196, 201, 202]. Similarly, loss of T cells because of infection (HIV, SIV), immunosuppressive treatment [203] or due to antibody depletion can result in worsening infection [104, 106].

2.0 STATEMENT OF THE PROBLEM

Mycobacterium tuberculosis (*M. tuberculosis*) continues to be a threat to global public health because of a large potential reservoir of infection, lack of an effective vaccine to prevent infection or disease, and a long chemotherapeutic regime that is difficult to maintain. Following infection, the host mounts a vigorous Th1 type immune response that contains, but does not eliminate the bacilli. The hallmark of *M. tuberculosis* infection is the granuloma, a collection of immune cells that serves the host by walling off the bacteria, but also provides an environment in which the bacilli can persist insulated from the host. Immunologic studies of *M. tuberculosis* infection have shown that an adaptive immune response is required for bacterial containment, of which CD4 T cells are an essential component. In addition CD4 T cells can function to both promote and dampen inflammation. The goal of this study was to examine the pro-inflammatory and regulatory role of CD4 T cells during the course of *Mycobacterium tuberculosis* infection.

The murine model of *M. tuberculosis*, through the use of knockout and transgenic animals as well as antibody depletions, has been invaluable for discovering which components of the immune response are required for bacterial containment. However the usefulness of this approach is limited to understanding the loss of function or gain of an immune component at a systemic level. Thus one is not able to discern where and at what time these factors are critical. With the work presented in this dissertation, we developed a new model of adoptive transfer to address whether IFNγ from sources other than CD4 T cells was sufficient to contain bacilli. In

addition, we provide new insight into the role of regulatory T cells and the anti-inflammatory effects of long term IL-12 treatment in murine models.

In humans, unlike mice, *M. tuberculosis* infection results in the development of either active disease or latent infection and the factors that contribute to such disparate disease outcomes are not well understood. Most likely in humans, infection outcome is a spectrum between those with full-blown primary TB and those classified as having latent infection. Studies in humans suggested an association between an increased frequency of regulatory T cells (Tregs) and development of active disease. Here I explored that association in a non-human primate model. This is the only current animal model that mimics human active and latent *M. tuberculosis* infection. We examine the association between Treg frequency prior to and during the first 6 months of infection in the blood and airways of *M. tuberculosis* infected cynomolgus macaques and infection outcome. These studies address an important question in human TB: is control of initial infection dependent simply on a robust TH1 or inflammatory response, or is a more balanced immune response necessary for initial, and perhaps long term, containment of infection?

3.0 IFNγ FROM CD4 T CELLS IS REQUIRED FOR BACTERIAL CONTROL AND HOST SURVIVAL DURING ACUTE *M. TUBERCULOSIS* INFECTION

3.1 INTRODUCTION

The pro-inflammatory cytokine interferon γ (IFNγ) promotes the development of a T-helper (Th) 1 T cell response [204]. In addition, it synergizes with TNFα to activate macrophages (Mϕ), promoting the induction of nitric oxide synthase (NOS2). NOS2 produces nitric oxide that forms reactive nitrogen intermediates and in conjunction with reactive oxygen species can kill intracellular pathogens [205]. Humans with loss-of-function genetic mutations in either IFNγ or its receptor are very susceptible to mycobacterial infections [115]. Mice lacking IFNγ or NOS2 are two of the most vulnerable strains, failing to control *M. tuberculosis* and succumbing within weeks of challenge [77, 206]. Thus, mice require NOS2 and IFNγ and humans require IFNγ for containment of *M. tuberculosis* infection and host survival.

CD4 T cells are a primary source of IFNγ during the adaptive immune response to *M. tuberculosis* infection and are required for host survival during both the acute and chronic stages of infection [103, 104]. CD4-/- and MHCII-/- mice are unable to control bacterial growth and succumb to infection significantly sooner than wild-type counterparts [103], yet these mice survive at least twice as long as those lacking IFNγ or NOS2 [74, 77, 103, 206]. Lack of CD4 T cells during initial infection results in delayed IFNγ and NOS2 production, but eventually levels

comparable to wild type are reached, however this cannot rescue the mice from the infection [103]. When CD4 T cells are depleted during chronic infection, exponential bacterial growth and eventual host death occurs, despite maintenance of pre-depletion levels of IFNγ and NOS2 [104]. As Th1 cells, CD4 T cells also produce IL-2 and TNF, interact with dendritic cells to help with T cell priming and provide T cell help to B cells [108]. CD8 T cells as well as other cells can and do produce IFNγ during *M. tuberculosis* infection. Taken together, these data suggest that CD4 T cells may have roles in addition to IFNγ production. These data lead to the hypotheses that IFNγ from sources other than CD4 T cells is sufficient for bacterial containment and CD4 T cells have functions in addition to IFNγ production. To test the hypotheses, an adoptive transfer model was designed to permit manipulation of the CD4 T cell population. The adoptive transfer model was designed in two ways. The first was a stream-lined model that specifically assessed the ability of CD8 T cells as a source of IFNγ and second, a more comprehensive selection of the immune system components for reconstitution to assess the role of CD4 T cells in control of infection.

The data from this study indicate that CD4 T cells are necessary as a source of IFN-γ, as well as influencing the function of CD8 T cells in the immune response against *M. tuberculosis*. The adoptive transfer systems developed provide an opportunity to manipulate the immune response to identify specific factors important in control of M. tuberculosis infection.

3.2 MATERIALS AND METHODS

3.2.1 Animals

8-12 week old RAG1-/- (B6.129S7-Rag1^{tm1Mom}/J), Thy1.1 (B6.PL-Thy1^{a}/CyJ), IFNγ -/- (GKO) (B6.129S7-Ifng^{tm1Mom}/J), IFNγreceptor-/- (B6.129S7-Ifngr^{tm1agt}/J) and wild-type (WT) C57BL/6 mice were obtained from Jackson laboratories (Bar Harbor, ME) or maintained in an in-house breeding facility. Mice were maintained in a biosafety level 3 facility in micro-isolator cages, free fed a diet of mouse chow and autoclaved water. All animals were maintained as per the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.2 Probiotic pre-treatment of mice

To prevent the development of reconstitution induced inflammatory bowel disease [207, 208], RAG 1-/- mice were prophylactically treated with probiotics. Prior to adoptive transfer, mice received one scoop of Bene-Bac Powder (Pet Ag Inc. Hampshire, IL) (25 million Colony Forming Units (CFU) per gram, *Lactobacillus fermentum, Enterococcus faecium, Lactobacillus plantarum, Lactobacillus acidophilus*) in 350 ml of fresh, autoclaved water every day for 14 days. Water bottles were changed daily.

3.2.3 Isolation of donor cells

Thy1.1 mice were pretreated with 1mg/ mouse anti-CD4 antibody (clone GK1.5, NCCC, NIH Bethesda, MD) 7 days prior to splenocyte harvest. GKO and WT mice did not receive

pretreatment with antibody. Naïve donor mice were anaesthetized by isoflurane and euthanized by cervical dislocation. Spleens were isolated under sterile conditions. A single cell suspension was obtained by crushing each spleen individually through a 40μm cell strainer in Dulbecco's phosphate buffered saline (Sigma-Aldrich, St. Louis, MO) with the back of a sterile 5ml syringe. Erythrocytes were lysed with red blood cell lysis buffer (90ml 0.16M NH_4, 10ml 0.17M Tris pH 7.65) for two minutes at room temperature. Following erythrocyte lysis and wash, remaining splenocytes from like animals were combined. Individual cell populations were isolated via Miltenyi MACS bead separation (Miltenyi Biotech, Auburn, CA) as per manufacture's instructions. For the first adoptive transfer model, CD8+ and B220+ cells were positively selected from Thy1.1 mice depleted of CD4+ cells. In addition, CD4+ cells were positively selected from either WT or GKO mice. For the second adoptive transfer model, naïve Thy1.1 mice were depleted of CD4 T cells by antibody neutralization and then positive selection of CD4 T cells by magnetic bead separation as per manufacturer's protocol prior to adoptive transfer.

3.2.4 Adoptive transfer to recipient mice

Recipient RAG1-/- mice received purified splenocytes via a tail vein injection in 300μl of PBS per mouse.

3.2.5 Aerosol infection of the mice

M. tuberculosis was passed through mice, grown in culture once and frozen in 7H9 complete media 10% glycerol. Immediately prior to infection, an aliquot was thawed and diluted in PBS 0.05% Tween 80. Bacteria were disaggregated by cup horn sonication. Mice were aerosol

infected with low dose (<50 CFU, Erdman strain) using a nose-only exposure aerosolizer (InTox Products, Albuquerque, NM). Mice were exposed to aerosolized *M. tuberculosis* for 20 minutes then room air for 5 minutes. One day following infection, lungs of one mouse per round were crushed in 5 ml PBS-0.5% Tween-80 and plated neat on 7H10 or 7H11 plates (Difco Laboratories, Detroit, MI) to determine inoculum.

3.2.6 Harvest of organs for analysis

At serial time points following infection, mice were anesthetized with isoflurane and euthanized by cervical dislocation. Under sterile conditions, organs were crushed in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) through a 40μm cell strainer with the plunger from a sterile 5ml syringe. An aliquot of cellular homogenate was plated on 7H10 agar plates for CFU determination. Cells were then pelleted and washed. Erythrocytes were lysed as described previously and cells were resuspended for enumeration and assay set up.

3.2.7 Flow cytometry

For intracellular cytokine staining, cells were stimulated with media only or with anti-CD3 (10μg/ml, clone 145-2C11, BD Pharmingen) and anti-CD28 (10μg/ml, clone, BD Pharmingen) and monensin (3μM, for 5 hours at 37°C 5% CO_2) (Sigma-Aldrich). For antigen specific responses, cells were incubate with 1μl of either $ESAT6_{1\text{-}20aa}$ MHC Class II multimer or GAP MHC Class I tetramer ($Mtb32_{aa309\text{-}318}$ (GAPINSATAM) NIAID tetramer facility, Bethesda, MD) per 100μl at 37°C 5%CO_2 for 60 minutes in media then washed. Once stained with tetramers, cells were stimulated with either $ESAT6_{1\text{-}20aa}$ peptide or GAP peptide in the presence of

monensin for 5 hours. During staining with GAP tetramer, anti-CD107 (1D4B, BD Pharmingen) antibody was present during the stimulation period to assess degranulation. Following stimulation, cells were surfaced stained for CD3, CD4 (clone L3T4, BD Pharmingen), and CD8 (clone 53-6.7, BD Pharmingen) at room temperature for 15 minutes in PBS 0.5% BSA 20% Mouse serum, washed and then fixed with 2% paraformaldehyde (Sigma-Aldrich) for one hour. Following fixation, cells were permeablized then washed with PBS 0.5% BSA 0.2% Saponin (Sigma-Aldrich). Cells were stained for intracellular cytokines IFNγ (clone XMG-6.1, BD Pharmingen) and TNF (clone MP6-XTT22, BD Pharmingen) by incubating antibodies in PBS 0.5% BSA 0.2% Saponin and 20% mouse serum for 15 minutes at room temperature. Cells were washed and resuspended in 1% BSA. Data were collected on a FACS Aria and analyzed using FloJo software 8.6.3 (Treestar).

3.2.8 RNA Isolation

At the time of harvest, a lobe of lung was isolated under sterile conditions and immediately flash frozen in liquid nitrogen in an RNAse free, sterile cryovial, and stored at -80°C until RNA harvest. For RNA isolation, frozen tissue was homogenized in TRIzol reagent protocol with modifications. Tissue was homogenized using a dounce homogenizer in 3ml of TRIzol and extracted with chloroform twice. RNA was precipitated with isopropanol and washed with 70% Ethanol. RNA was further purified on an RNAeasy Mini Kit column (Qiagen).

3.2.9 Real time quantitative PCR

As reported previously, the RNA was reversed transcribed by Superscript II enzyme as per manufacturer's instructions (Invitrogen, Carlsbad, CA)[201]. The relative gene expression method was used to report the data. Data were normalized to hypoxanthine phosphoribosyltransferase with uninfected lung as a calibrator. Primer probe sets were tested for efficiency with efficiency >97% for all sets. Triplicates of each sample were run along with a no RT control on an ABI PRISM Sequence Detector 7700. Data are reported as relative gene expression to uninfected samples. To calculate the relative gene expression ($2^{(-\Delta\Delta \text{ cycle threshold (Ct)})}$), ΔCt=Ct (Gene of interest)-Ct(normalizer) and the $\Delta\Delta$Ct= ΔCt (sample)- ΔCt (calibrator).

3.2.10 Macrophage killing assay

Bone marrow derived macrophages were obtained by culturing bone marrow from the long bones of mice with 25% L929-cell supernatant containing media for 5 days in 10cm culture dishes ($2.5x10^6$ cells/ plate in 10 ml media). Cells by this time had formed a monolayer of macrophages. Macrophages were removed by incubation with PBS on ice for 20 minutes followed by vigorous pipetting to dislodge any remaining adherent cells. Once removed, the cells were pelleted, resuspended, counted and diluted to a concentration of $2.5x10^7$ cells/ml with macrophage infection media (DMEM, 1% FBS, 1% sodium pyruvate, 1% L-glutamine, 1% non-essential amino acids). Macrophages were plated at 100μl/well in a 96 well plate and allowed to adhere for 45 minutes. Subsequently, macrophages were infected with *M. tuberculosis* at an MOI≤1 for 4 hours at 37°C 5% CO_2. After the macrophage infection, supernatant from three wells per macrophage type was saved and the macrophages in all wells washed. Three wells per

each macrophage type were lysed with 1% Saponin and the saved supernatant and macrophage lysis were diluted and plated on 7H10 plates to determine the “input” bacterial numbers.

T cells from the lungs of wild type mice infected with *M. tuberculosis* for 4 weeks Infected mice were anesthetized with isoflurane and euthanized by cervical dislocation. Lungs were retrieved under sterile conditions and crushed through a 40μm cell strainer with the back of a sterile 5 ml syringe. Once a single cell suspension was obtained and erythrocytes lysed, CD4 and CD8 T cells were purified by magnetic bead separation (Miltenyi) as per manufacturer’s instructions. T cells were incubated in wells with infected macrophages at a 1:1 ratio of T cell to macrophage (n=3 wells per condition) Media without T cells in wells with infected macrophages was used as a control for growth of *M. tuberculosis*. As a positive control, wells were treated with IFNγ (250U/ml, Invitrogen) and LPS (3μg/ml, Sigma-Aldrich). Cells were incubated for 72 hours at 37°C 5% CO_2. Following incubation, supernatant was removed and saved and cells were lysed with 1% Saponin. Supernatants and cell lysates were serially diluted and plated on 7H10 agar plates for determination of bacterial burden per well.

3.3 RESULTS

3.3.1 Building the adoptive transfer model

Knockout and transgenic animals as well as antibody depletion have been powerful tools for determining which factors are necessary for control of *M. tuberculosis.* However, these techniques are limited by studying the global effect of the factors, and it can be difficult to determine which specific function of a cell type, for example, is causing the *in vivo* phenotype in

a murine model. To determine whether IFNγ from sources other than CD4 T cells were sufficient to contain bacterial growth, a new model system was needed. An adoptive transfer model was developed so that the presence or absence of CD4 T cells from IFN-g-/- or wild type (WT) mice could be compared for ability to control M. tuberculosis infection. Three experimental groups were needed: 1) a control group lacking CD4 T cells (CTLAT), 2) a group in which CD4 T cells were from IFNγ-/- mice (γAT) and 3) a group in which CD4 T cells were from WT mice (WTAT). In addition, the remainder of the immune system components had to be capable of producing IFNγ.

Several initial attempts to adoptively transfer CD4 T cells into CD4 deficient mice were not successful. Adoptively transferred cells were often hard to find and did not remain within the host (data not shown). In addition, CD4-/- mice have been reported to have double negative, MHCII restricted T cells that are able to respond in certain infection models [209]. Thus, RAG 1 deficient mice were chosen as recipients. RAG 1-/- mice lack B cells and T cells, but approach wild-type levels of innate immune responders, yet do not produce excessive amount of IFNγ, as has been reported in other immune compromised strains [210].

The development of this model was approached in two distinct ways. The first was a streamlined model in which CD8 T cells and B cells from Thy 1.1 congenic mice were adoptively transferred with or without CD4 T cells from WT or IFNγ-/- mice (both of which had the Thy1.2 marker) at a ratio of 4:1 (CD8 T cells and B cells to CD4 T cells) (Figure 1A). The second approach was to create a more natural context for immune response with the hope of extending the length of time infection could be sustained. For this, we reconstituted RAG1-/- mice with and without CD4 T cells from WT or IFNγ mice in conjunction with whole spleen

equivalents. The whole spleen equivalents were derived from CD4-depleted naïve Thy1.1 mice and adoptively transferred at a ration of 20:1 (CD4- splenocytes to CD4 T cells). (Figure 1B).

In addition to varying the type and number of cells used to reconstitute the recipients, we also experimented with the length of time between reconstitution and *M. tuberculosis* infection. Initially, animals were reconstituted one day prior to infection. However, the responses to infection, primarily cytokine production, were extremely vigorous. A majority of cells examined, when stimulated with anti-CD3 and anti-CD28, produced large amounts of IL-2, TNF and where applicable IFNγ. We hypothesized that one possible cause of the exacerbated cytokine production was the homeostatic proliferation that occurs when RAG 1-/- mice are reconstituted with lymphocytes [211]. By extending the time between reconstitution and infection from one day to 18 days, the model better approximated the conditions found in wild type mice.

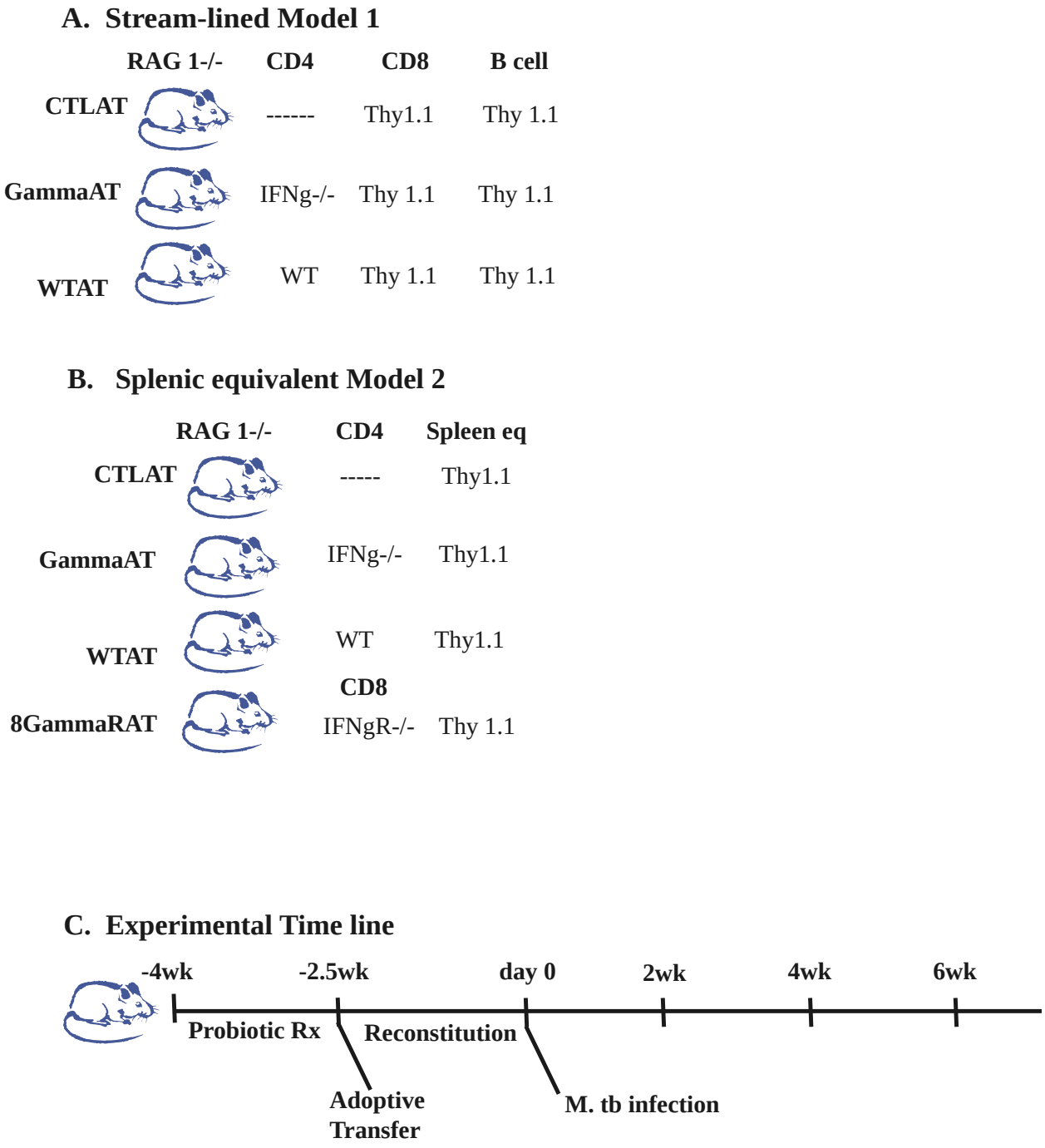


Figure 1 Design of adoptive transfer models and time line for set up.

Two models of adoptive transfer are described here. A) the streamlined model in which only B cells; CD4 and CD8 T cells were transferred into RAG1-/- mice. B) reconstitution with CD4 depleted splenic equivalents. C) the time line of experimental setup.

3.3.2 IFNγ from CD8 T cells is not sufficient to control *M. tuberculosis* infection in the absence of IFNγ from CD4 T cells

3.3.2.1 IFNγ from CD4 T cells is required for host survival

To determine whether IFNγ from non-CD4 T cell sources was sufficient for host survival, the first adoptive transfer model shown in Figure 1A was used. Five groups of mice were infected with an n=5 mice per group: wild type (WT), RAG1-/- that did not receive lymphocytes, CTLAT (received only CD8 T cells and B cells), γAT (received CD4 T cells from IFNγ-/- mice) and WTAT (received CD4 T cells from WT mice). Following infection, mice lacking IFNγ from CD4 T cells failed to survive longer than non-reconstituted RAG 1-/- mice, while 80% of mice reconstituted with CD4 T cells from wild type mice survived until the end of the experiment (Figure 2). This indicated that IFNγ from CD4 T cells is essential for host survival.

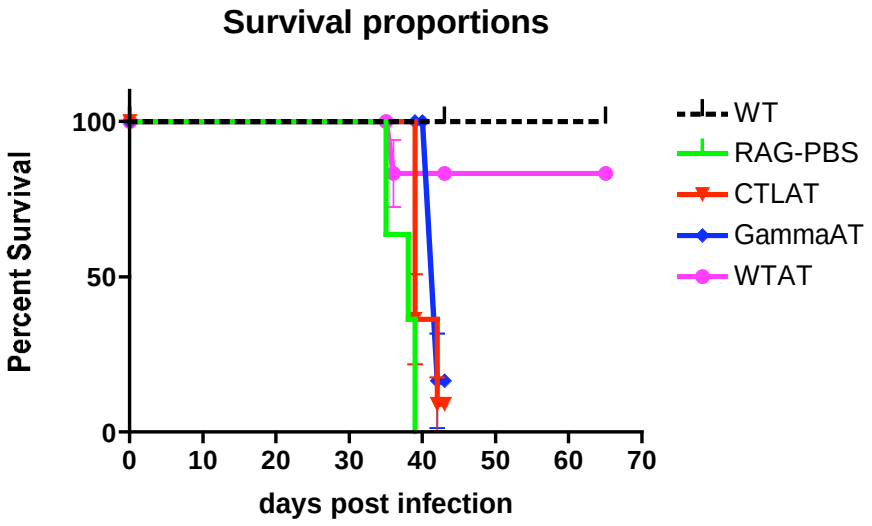


Figure 2 Murine survival after adoptive transfer and subsequent *M. tuberculosis* infection

RAG1-/- mice were adoptively transferred with CD8 T cells and B cells from naïve Thy 1.1 mice in the presence and absence (CTLAT) of CD4 T cells from either IFNγ -/- (γAT) or wild type (WTAT) mice. As controls, a group of RAG1-/- mice were sham reconstituted with PBS. (n=5 per group).

3.3.2.2 In the absence of CD4 T cells that produce IFNγ, bacterial growth is unchecked

Given that lack of IFNγ from CD4 T cells was necessary for survival of the host, it was not surprising to find that the bacterial burden in lungs was significantly higher in animals that had not received CD4 T cells or had received IFNγ-/- CD4 T cells by 4 weeks post infection (Figure 3A).

3.3.2.3 CD4 T cells are required for an effective immune response

In general, as antigenic burden increases one expects a proportional increase in the number of cells at the site of infection [97]. However, in the absence of CD4 T cells, almost no cells were recovered in the lungs of CTLAT animals until 6 weeks post infection. In contrast, animals that received CD4 T cells, even in the absence of CD4-derived IFNγ, had an increase in the number of cells in the lung as the bacterial burden increased over time, indicating that CD4 T cells are necessary for either induction or maintenance of the immune response and this is independent of IFNγ production (Figure 3B)

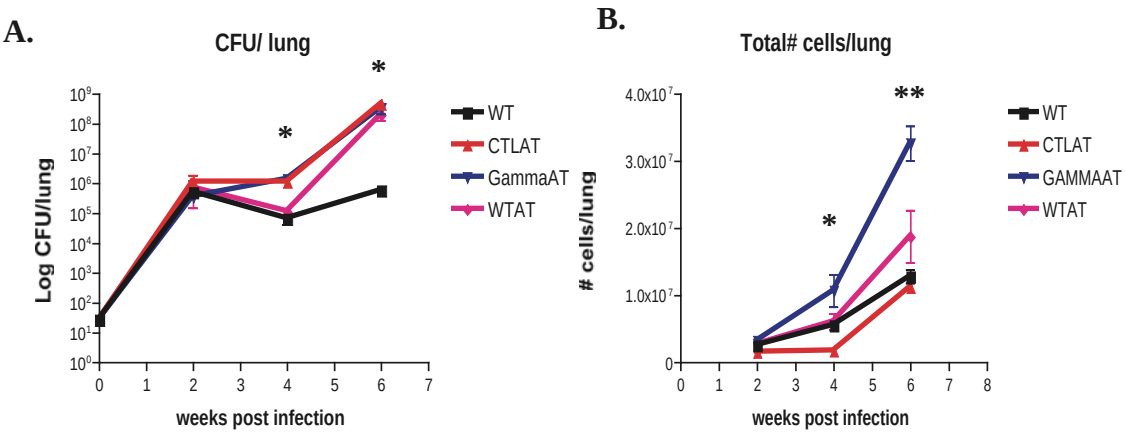


Figure 3 Bacterial burden and cellular infiltrate in the lungs of experimental animals over time

Lungs of experimental animals were harvested at serial time points post infection and homogenized. An aliquot of organ homogenate was serially diluted and plated on 7H10 agar plates for enumeration. Following crushing, erythrocytes were lysed and the remaining cells counted by trypan blue exclusion. Statistical significance was determined by ANOVA analysis and a $p<0.05$ considered statistically significant. At 4 weeks post infection in A, $p<0.05$ for γAT and CTLAT when compared to WTAT and wild type and $p<0.05$ for γAT CTLAT and WTAT when compared to WT at 6 weeks post infection. In B $p<0.05$ for γAT when compared to CTLAT at 4 weeks and compared to all other groups at week 6 p.i.

3.3.2.4 In the absence of CD4 T cells, CD8 T cells are activated, but fewer are present in the lungs.

Analysis of lymphocytes recovered from the lung showed that, as expected, CTLAT mice lacked CD4 T cells, while γAT and WTAT mice had comparable frequencies of CD4 T cells (Figure 4A). When CD4 T cells were not present, the frequency of CD8 T cells found within the lung was significantly lower compared to experimental groups that had received CD4 T cells (CTLAT vs. WTAT or γAT, Figure 4B). However, it is interesting to note that while the CD8 T cell frequency was lower, the activation profile of the cells present was comparable in all groups (Figure 4D). Taken together, these data suggest that CD4 T cells, independent of IFNγ production, provide help for CD8 T cell expansion or survival during *M. tuberculosis* infection. In addition, the presence of CD4 T cells does not seem to affect whether the CD8 T cells are activated or not.

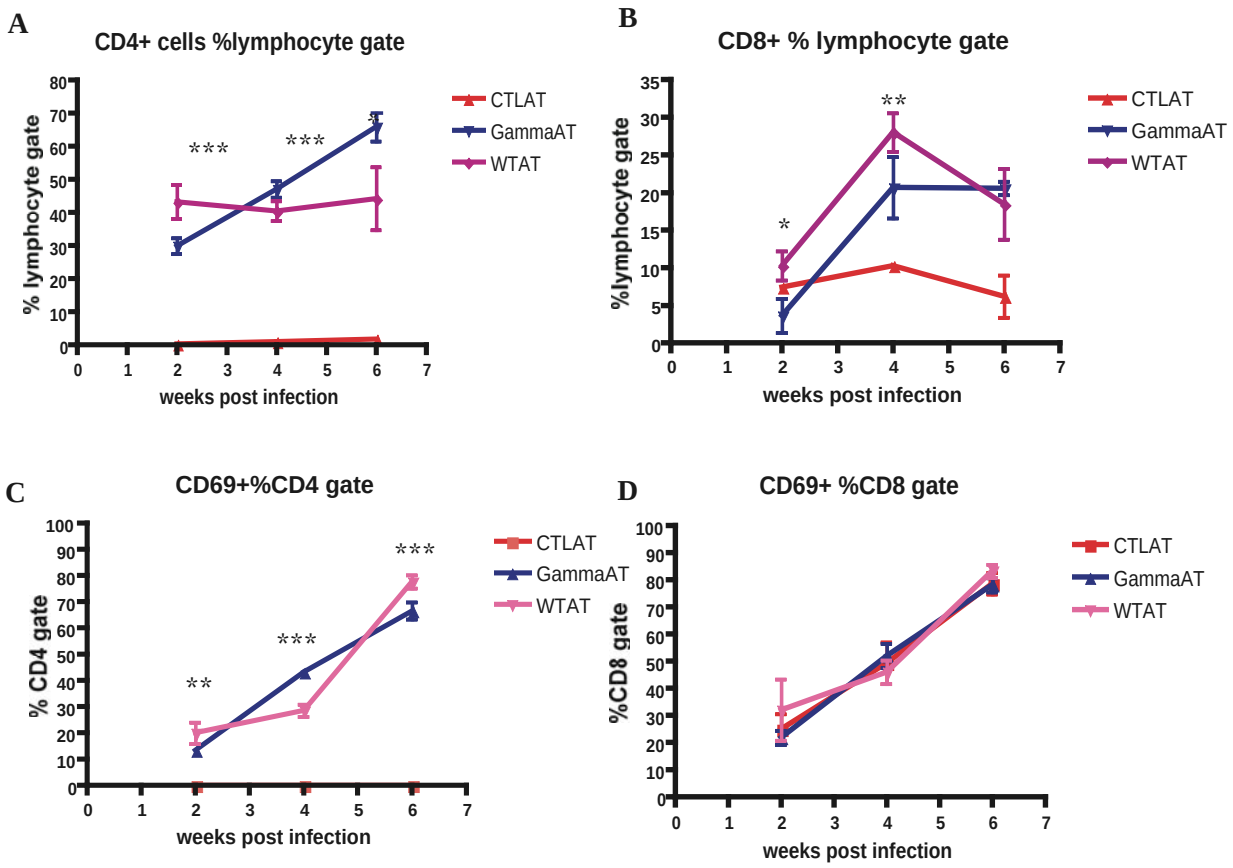


Figure 4. The frequency and activation status of T cells within the lung

To determine the frequency of each cell type present in the lung, cells were surface stained with CD3, CD4, CD8 and CD69 as previously described. The data are reported as a frequency of CD4 T cells (A) and CD8 T cells (B) within the lymphocyte gate. The activation state of the CD4 (C) and CD8 (D) T cell populations are reported as the percent CD69+ within each population. Overall statistical significance was determined by ANOVA, while significance between pairs was determined by bonferroni and a $p<0.05$ was considered statistically significant. In A, $p<0.05$ for CTLAT when compared to both γAT and WTAT at 2 and 4 weeks p.i. where as in C the $p<0.05$ for CTLAT compared to the other experimental groups for all three time points. Finally, $P<0.05$ between WTAT and CTLAT at 2 weeks and between CTLAT and the other two groups at week 4 in B.

3.3.2.5 Adoptively transferred CD4 T cells produce pro-inflammatory cytokines

In wild type mice, as Th1 cells, CD4 T cells produce pro-inflammatory cytokines during the course of *M. tuberculosis* infection. To determine whether adoptively transferred CD4 T cells

were capable of producing pro-inflammatory cytokines, cells isolated from lungs of infected animals were stimulated and then stained for intracellular cytokines. As expected CD4 T cells from WTAT animals produced IFNγ while those from γAT did not (Figure 5A) while CD4 T cells from both groups produced TNF (Figure 5B).

3.3.2.6 The quality of the CD8 T cell function is altered in the absence of IFNγ from CD4 T cells

Studies of *M. tuberculosis* infection in CD4-/- and MHCII-/- mice have shown that a CD8 T cells can be a significant source of IFNγ [103, 124]. In the adoptive transfer model, we sought to determine the quality of the CD8 T cell response generated. Animals lacking CD4 T cells had a reduced frequency of CD8 T cells that produced either IFNγ (Figure 5C) or TNF (Figure 5D). Interestingly, when CD4 T cells could not make IFNγ, the frequency of CD8 T cells capable of producing cytokine was reduced by approximately 20% at the peak of the infection (4 wks). These data indicate that CD4 T cells are required for a quality CD8 T cell response and that IFNγ from CD4 T cells may act directly on CD8 T cells to boost the frequency of cells capable of producing cytokine.

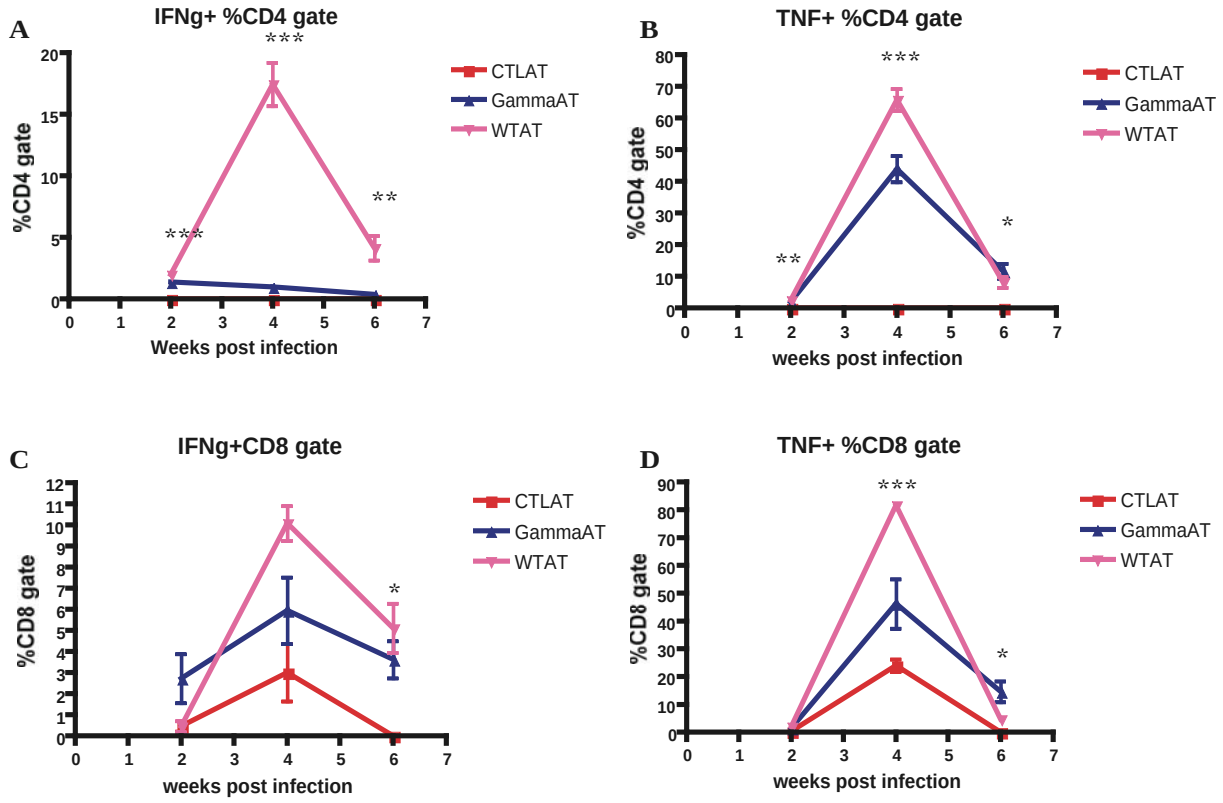


Figure 5. Cytokine production by T cells

Cells isolated from the lungs of experimental animals and stimulated with anti-CD3 and anti-CD28 in the presence of monensin for 5 hours at 37°C 5% CO_2. Following stimulation, the cells were surface stained for CD3, CD4, CD8 and intracellularly stained for IFNγ and TNF. The frequency of CD4+IFNγ+ (A), CD4+TNF+ (B), CD8+IFNγ+(C) and CD8+TNF+(D) cells are reported as a percent of their T cell population. Statistical significance was determined by ANOVA and a $p<0.05$ was considered statistically significant. Significance between pairs was determined by a bonferroni posttest when ANOVA was statistically significant. In all the above figures, $p<0.05$ was between WTAT and CTLAT values at all time points.

3.3.2.7 IL-2 from CD4 T cells may account for the poor immune response in animals lacking CD4 T cells.

As helper cells, CD4 T cells produce IL-2 during the course of an immune response. However CD8 T cells are able to make IL-2 as well. To address whether the poor immune response seen

in mice lacking CD4 T cells may be due to a lack of IL-2, we performed quantitative REAL-Time PCR on RNA from the lungs of adoptively transferred and infected mice. Mice lacking CD4 T cells had no IL-2 gene expression (Figure 6) indicating that lack of IL-2 may be responsible for the poor immune response.

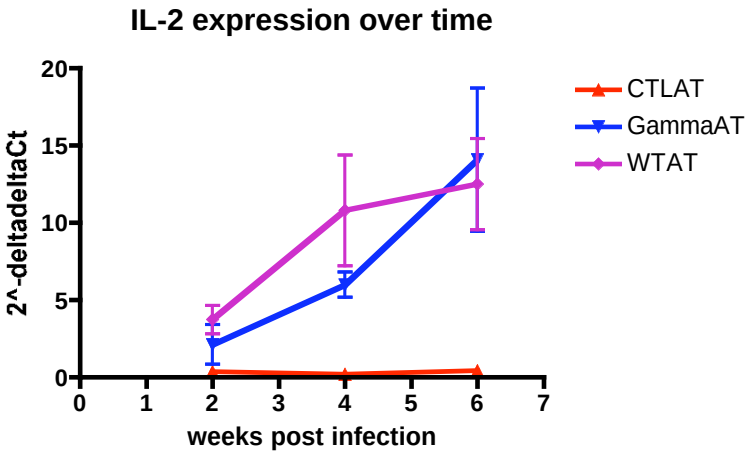


Figure 6. IL-2 expression in the lungs of reconstituted mice

At the time of necropsy, a lobe of lung was taken and flash frozen in liquid nitrogen for RNA isolation and analysis of IL-2 expression over time.

3.3.3 IFNγ from CD4 T cells is necessary for antigen specific responses and directly affects the quality of the CD8 T cell response

The "stream-lined" adoptive transfer model evaluated whether IFNγ from CD8 T cells was sufficient in the absence of IFNγ from CD4 T cells. In this model, CD4 T cells were a necessary source of IFNγ to control M. tuberculosis infection. However, T cells are not the sole immune cells capable of producing IFNγ, therefore the model was further developed to approximate a more wild type immune system. Using half splenic equivalents in which naïve splenocytes were depleted of CD4 T cells and adoptively transferred into RAG1-/- mice with and without isolated

naïve CD4 T cells from IFNγ-/- or WT mice. An additional experimental group was added to evaluate the direct effects of IFNγ signaling CD8 T cells. This group of RAG1-/- mice (8γrAT) was reconstituted with CD8-depleted splenic equivalents and CD8 T cells from IFNγ receptor -/- mice were added back (Figure 1B). With the use of tetramers, we were able to determine whether the deficits in the response on a global T cell scale were also happening at an antigen specific level.

3.3.3.1 IFNγ from CD4 T cells is required for control of bacterial burden

Even when other components of the immune system capable of producing IFNγ are present, experimental animals lacking IFNγ from CD4 T cells fail to contain bacterial growth (Figure 7A). The CTLAT group, which lack CD4 T cells completely, had the highest bacterial burden, however the increased bacterial burden differs only slightly from the groups that have CD4 T cells. This is different than the bacterial burden of mice adoptively transferred without splenic equivalents (i.e. the first adoptive transfer model described).

3.3.3.2 Without CD4 T cells, fewer cells are found at the site of infection

Similar to previous results, the group lacking CD4 T cells had 4 fold fewer cells found in the lungs during the first weeks post infection (Figure 7 B), indicating that CD4 T cells are needed to initiate or sustain an adaptive immune response to *M. tuberculosis*.

3.3.3.3 Antigen specific CD4 and CD8 T cells can be detected in the lungs of experimental animals.

Over the course of infection, CD4 T cells continue to expand within experimental groups (Figure. 7C) whereas CD8 T cells peak in the lungs at week 2.5 post infection in all experimental

groups (Figure. 7E). Using tetramers to track antigen specific cells, CD4+ ESAT-6+ (Figure 7D) and CD8+GAP+ (Figure 7E) T cells were found within the lungs of infected animals.

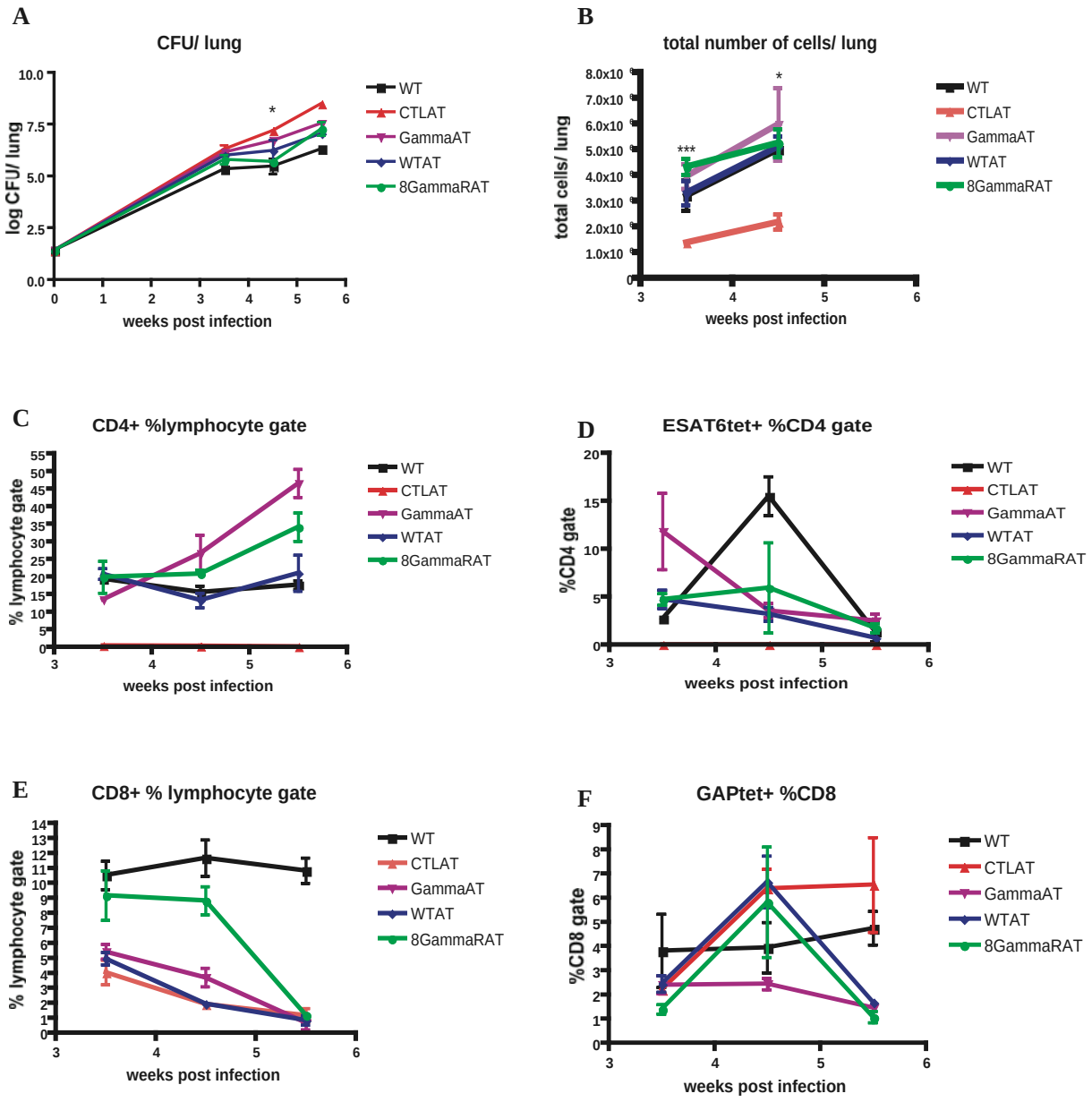


Figure 7. Bacterial burden, total cell numbers and T cells in an adoptive transfer model using whole spleen equivalents

Using a more physiologic mix of cells, RAG1-/- mice received CD4 depleted naïve Thy1.1 half spleen equivalents in the presence of absence of CD4 T cells from IFNγ-/- or WT mice (n=4 mice per group per time point). One additional group received CD8 depleted half spleen equivalent and CD8 T cells from IFNγ receptor -/- mice. Bacterial load was determined by plating serial dilution of organ homogenates on 7H10 agar plates (A). The total

number of cells per lung (B) was determined by trypan blue exclusion. The frequency of CD4 T cells within the lymphocyte gate (C), CD4+ESAT6tet+ cells within the CD4 T cell gate (D), of CD8 T cells within the lymphocyte gate (E) and the frequency of CD8+GAPtet+ cells within the CD8 gate (F) were determined by flow cytometry.

3.3.3.4 Cytokine production by antigen specific CD4 T cells

During the course of infection we sought to determine whether CD4 T cells produced cytokine in response to antigen-specific stimulation. CD4 T cells from the lungs were stained with ESAT-6 tetramer and stimulated with $ESAT6_{1\text{-}20aa}$ peptide in the presence of monensin. Interestingly, in groups that received CD4 T cells capable of producing IFNγ, a delay was detected until approximately 5.5 weeks post infection within the tetramer positive population (Figure 8A). In contrast, both TNF (Figure 8B) and IL-2 (Figure 8C) were produced at even the earliest time point. These data indicate that at least within this population, CD4 T cells are functional and producing cytokine.

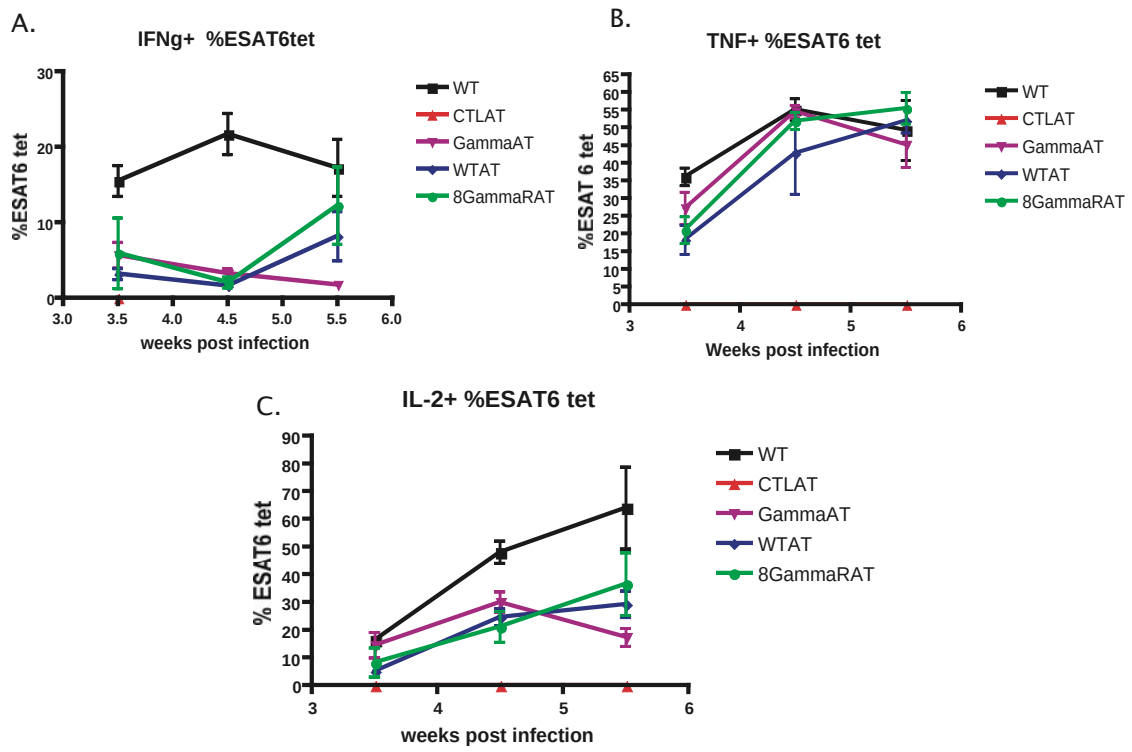


Figure 8. Antigen specific cytokine production by CD4 T cells

Lung cells were stained with ESAT6 tetramer and subsequently stimulated with $ESAT6_{1\text{-}20aa}$ peptide in the presence of monensin. Following stimulation, cells were stained for surface markers and intracellular cytokines.

3.3.3.5 Antigen specific CD8 T cell functions

In the previously described experiment using only CD4 T cells, CD8 T cells and B cells, we described attenuated CD8 T cell response in the absence of CD4 T cells and a slightly truncated production of cytokines in groups lacking IFNγ from CD4 T cells. In these experiments, we sought to determine whether lack of IFNγ directly affects CD8 T cells. To do this an additional experimental group was added, where Thy 1.1 splenocytes depleted of CD8 T cells and CD8 T cells from IFNγ receptor deficient mice were used to reconstitute the animals. First, we wanted to determine whether IFNγ directly affected cytotoxic CD8 T cells by using CD107 staining as a measure of exocytosis/ degranulation. To follow *M. tuberculosis* specific

responses, we followed the CD8 T cell responses using tetramer techonology. GAPtet is a fluoroflor linked MHC I molecule that holds the peptide GAPINSATAM. Interestingly it appeared that neither lack of CD4 T cells nor the inability to respond to IFNγ played a role in the development of the ability of the CD8+GAPtet+ cells to degranulate (Figure 9A). This is in contrast to previous work in the CD4-/- mouse that showed lack of CD4 T cells resulted in a decreased cytotoxic precursor frequency [124]. This may be explained by the differences in cytotoxic T cell detection, i.e. limited dilution assay versus flow cytometry. Lack of CD4 T cells or IFNγ signal did not significantly affect IL-2 production within this cell population (Figure 9B). However, at 4.5 weeks post infection, lack of IFNγ signal for CD8 T cells resulted in slightly fewer cells producing TNF (Figure 9C) and significantly fewer CD8 T cells producing IFNγ (Figure 9D). The reduction seen at this time point is similar to the reduction reported at the peak of infection in our other model of adoptive transfer. Taken together these data suggest that at the peak of the inflammatory response (~4-5 weeks p.i.) IFNγ from CD4 T cells directly enhances the CD8 T cells production of both TNF and IFNγ.

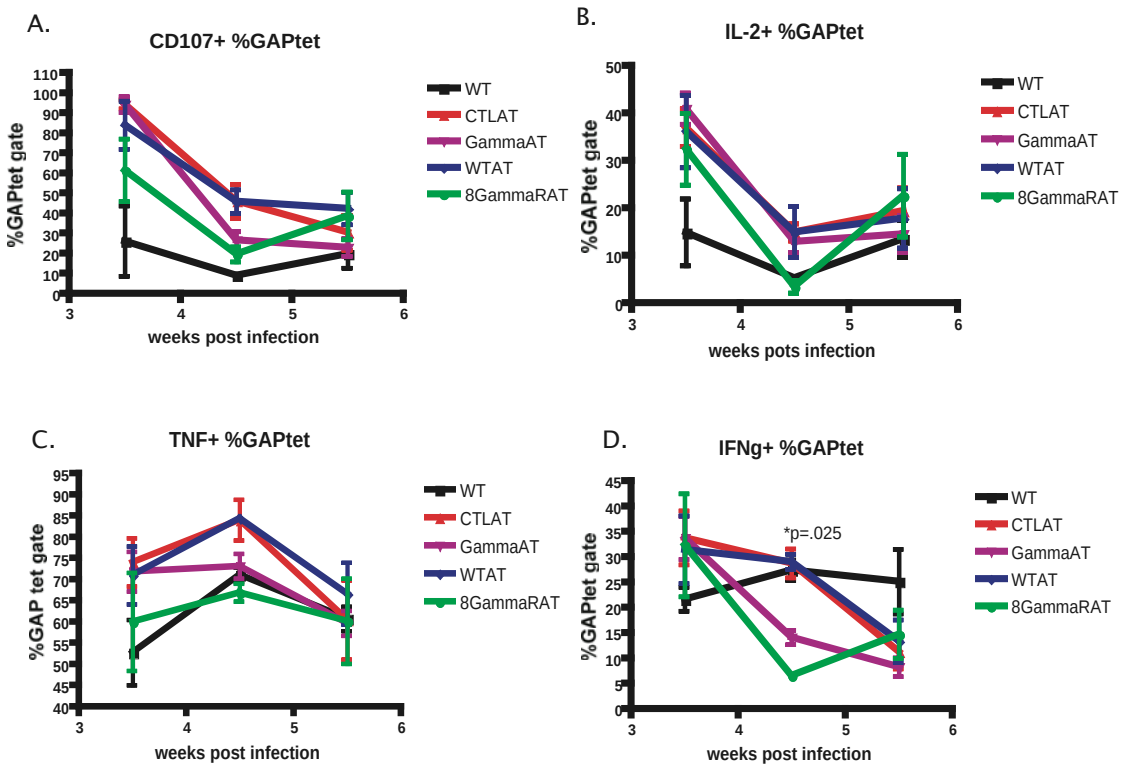


Figure 9. Antigen specific CD8 T cell function

Cells from the lungs of *M. tuberculosis* infected animals were stained with GAP tetramer and stimulated with GAP peptide in the presence of monensin and CD107. Following stimulation cells were stained for surface markers and intracellular cytokines. Cells are gated on live cell gate, lymphocyte gate, CD8 and finally GAPtet. Data was analyzed by flow cytometry and reported as a frequency of the CD8+GAPtet+ population. Statistical significance was determined by analysis of variance and a $p<0.05$ considered statistically significant.

3.3.3.6 The effects of whole spleen equivalents on host survival

In experiments using our first model of adoptive transfer, mice lacking CD4 T cell and mice that had received IFNγ-/- CD4 T cells failed to control bacterial burden and succumbed to infection as quickly as RAG1-/- mice that had not been reconstituted. In addition, the mice in both groups had exponentially higher bacterial burdens by 4 weeks post infection. Interestingly, when bacterial burden was assessed using the second model of adoptive transfer using splenic

equivalents to reconstitute, mice that received IFNγ-/- CD4 T cells had bacterial burdens in the lungs that were similar to those mice that had received wild type CD4 T cells. In a subsequent experiment, we sought to determine whether the presence of other cell types in these adoptive transfer mice were able to increase survival in mice with or without CD4 T cells. Interestingly, the group lacking CD4 T cells succumbed to infection just as quickly as previously reported. However, mice with IFNγ-/- CD4 T cells survived twice as long (Figure 10). In addition, at the time of death, mice lacking CD4 T cells carried a 1000-fold higher bacterial burden (average log CFU 9.7±0.1 SEM) than did those with CD4 T cells (day 78p.i. γAT log CFU 6.5±0.01 SEM, WTAT log CFU 6.0±0.18 SEM). These data indicate that CD4 T cells in the presence of whole spleen equivalents were able to extend the life of *M.tuberculosis* infected mice, suggesting that CD4 T cells have integral functions in addition to IFNγ production during the early stages of infection.

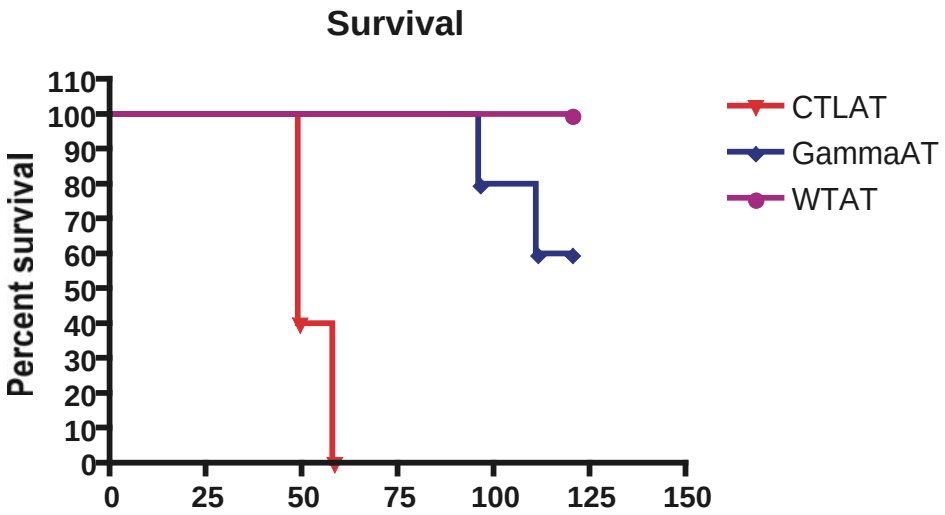


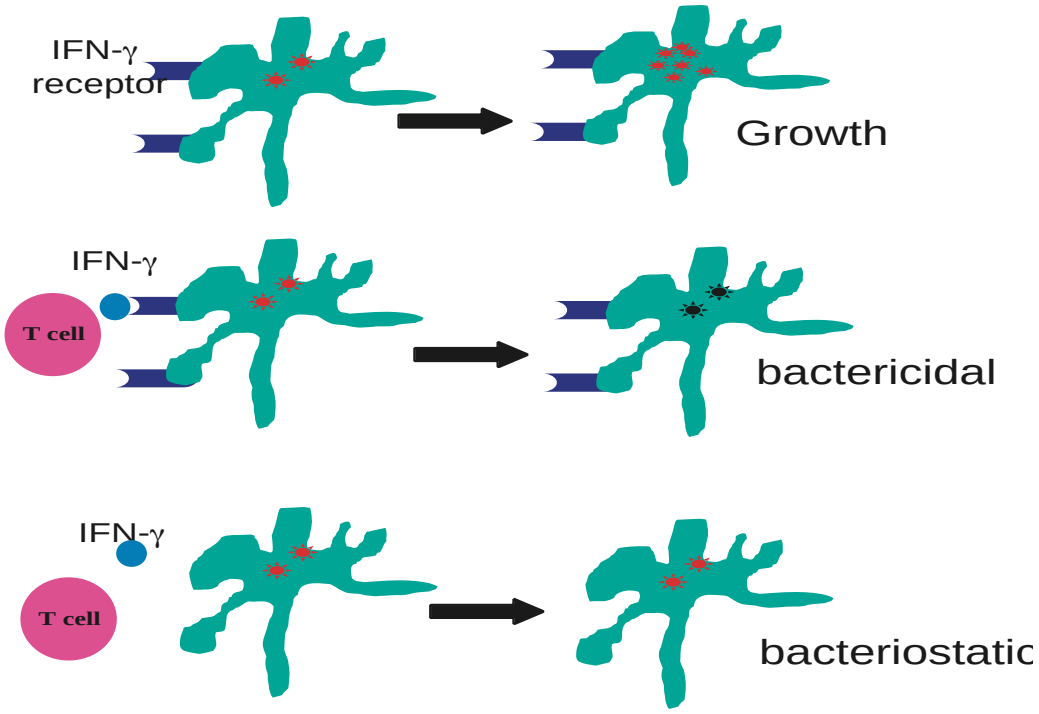
Figure 10. Survival of mice reconstituted +/- CD4 T cells and splenic equivalents

RAG1-/- mice were reconstituted as previously described (adoptive transfer model 2) and infected with low dose *M. tuberculosis* to assess survival. (n=5 mice per group).

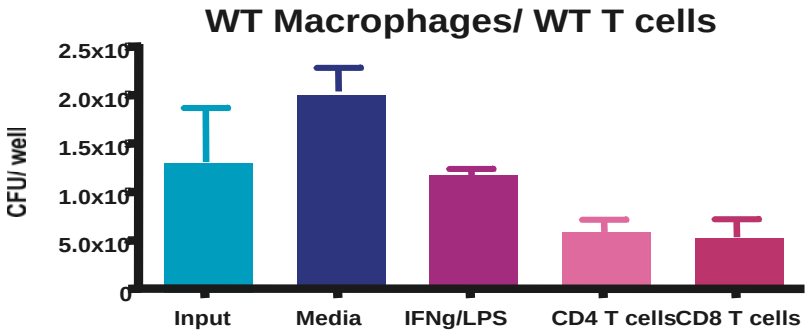
3.3.4 T cell activation of macrophages to kill intracellular bacilli in the presence or absence of IFNγ

T cells that produce IFNγ are capable of interacting with macrophages, via cytokine production (i.e. IFNγ) and cell-to-cell contact. IFNγ can synergize with TNF to activate the macrophage and mobilize mechanisms of intracellular killing. We sought to determine whether T cell-macrophage interactions were sufficient in the absence of IFNγ signal to activate the macrophage to kill intracellular *M. tuberculosis*. We hypothesized that one of three possible outcomes were possible in the absence of IFNγ signal between macrophage and T cell (Figure 11A): 1) Cell-to-cell contact would be sufficient to activate bactericidal activity, 2) in the absence of IFNγ signal, bacterial growth would be limited (bacteriostatic), or 3) T cells would fail to slow intracellular bacterial growth. To test our hypothesis, we utilized the macrophage killing assay. Bone marrow derived macrophages from either wild type or IFNγ receptor-/- mice were infected with *M. tuberculosis* and incubated with media only, LPS/IFNγ, CD4 T cells or CD8 T cells isolated from the lungs of *M. tuberculosis* infected wild type mice for 72 hours. Following incubation, the bacterial burden per well was calculated and compared to the initial input. Wild type T cells (either CD4 or CD8 T cells) were able to activate macrophage killing of intracellular bacteria when macrophages were from WT mice (Figure 11B). Interestingly in the absence of IFNγ signal, T cells were able to limit bacterial growth, but not induce bactericidal macrophage activity (Figure 11C).

A.


IFN-γ
receptor
Growth
IFN-γ
T cell
bactericidal
IFN-γ
T cell
bacteriostatic

B.


WT Macrophages/ WT T cells
CFU/ well
2.5x10
2.0x10
1.5x10
1.0x10
5.0x10
0
Input
Media
IFNg/LPS
CD4 T cells
CD8 T cells

C.

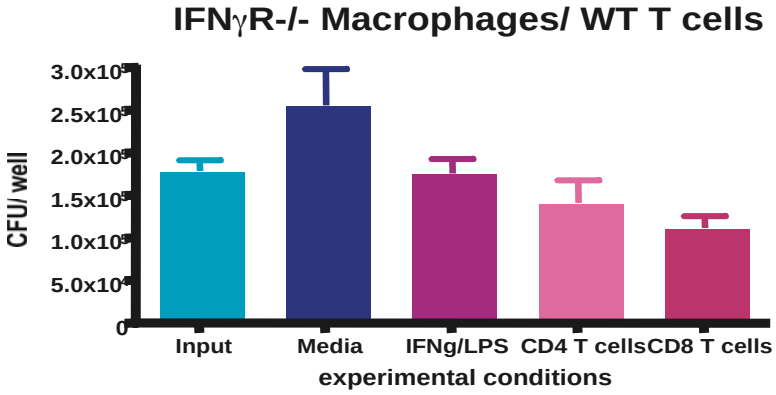

IFNγR-/- Macrophages/ WT T cells
CFU/ well
3.0x10
2.5x10
2.0x10
1.5x10
1.0x10
5.0x10
0
Input
Media
IFNg/LPS
CD4 T cells
CD8 T cells
experimental conditions

Figure 11. Macrophage killing assay

Bone marrow derived macrophages were infected with *M. tuberculosis*, incubated with T cells isolated from the lungs of wild type *M. tuberculosis* infected mice and evaluated for the ability to kill intracellular bacilli after 72 hours of incubation.

3.4 DISCUSSION

IFNγ and NOS2 are required for control of mycobacterial infections [77, 115, 206]. CD4 T cells are a primary source of IFNγ during the initial phases of *M. tuberculosis* infection. Literature reports of studies in knockout mice and antibody depletions have shown that CD4 T cells are required for an effective response against *M. tuberculosis* [103, 104]. In addition, they provided valuable insight as to the functions CD4 T cells may have in addition to IFNγ production. However, the traditional approaches of knockout and transgenic mice, as well as antibody mediated depletion, only provide information about the global effects of loss or gain of the CD4 T cell subset and fail to evaluate the source of these factors. With data presented in this chapter, we sought to investigate the role of IFNγ from CD4 T cells during *M. tuberculosis* infection. To do that, I developed two models of adoptive transfer. First, we used a more stream-lined model that allowed us to specifically evaluate whether CD8 T cells were a sufficient source of IFNγ when CD4 T cells were present, but unable to make IFNγ. The second model utilized a plethora of splenocyte cell types and permitted us to evaluate if in a more physiologic environment whether the lack of IFNγ from CD4 T cells could be overcome and host survival and bacterial burden contained.

In both experimental adoptive transfer models, the data indicate that CD4 T cells are essential for the control of *M. tuberculosis* infection and host survival since both groups lacking CD4 T cells failed to live longer than RAG1-/- mice that had not received CD4 T cells. These data agree with published literature from both knockout and antibody depletion studies. In addition, mice reconstituted without CD4 T cells had a truncated or abortive immune response in which higher bacterial burden failed to elicit an expected dramatic increase in cellular infiltrate. This may be because CD4 T cells are the initial and primary source of IL-2 as was demonstrated by the lack of IL-2 measured by real-time quantitative PCR. An additional explanation, untested in these experiments, is that in the absence of CD4 T help, dendritic cells do not receive the proper signals to activate the available T cells.

These experiments showed that CD8 T cells in the presence of only B cells and CD4 T cells are not a sufficient source of IFNγ for bacterial control or host survival. Interestingly, when an entire splenic complement of cells was provided in the presence of CD4 T cells that didn't produce IFNγ, animals were able to better able to control bacterial and survive twice as long as animals that did not have CD4 T cells. These data indicate that cell types other than CD4 T cells are capable of producing enough IFNγ to initially stunt growth of the bacteria, but long term, IFNγ from CD4 T cells is necessary survival.

We showed that independent of IFNγ production, CD4 T cells are required for an appropriate activation and cellular infiltrate into the site of infection. However, IFNγ is required for maximal quality CD8 T cell cytokine production, but not necessarily for a cytotoxic T cell response. These data differ from studies completed in CD4 deficient mice that reported lack of CD4 T cells resulted in a defect in cytotoxic T cell precursors [124]. The disparity in these

results may be due to the methods of measuring the frequency of cytotoxic T cells, here by flow cytometry and in literature by limiting dilution assay.

Finally, we evaluated the ability of CD4 T cells and CD8 T cells from the lungs of infected mice to activate macrophages and kill intracellular pathogens in the presence and absence of an IFNγ signal. Here the data indicate that IFNγ signal is required for bactericidal activity in macrophages. This was expected as the literature reports that NOS2 and LRG47 (both induced by IFNγ) are required for mycobactericidal macrophage activity. Surprisingly, T cells were able to halt growth of intracellular bacilli, but not kill the bacteria, which leads naturally to questions of mechanism that are a focus of subsequent future studies.

With the development of this adoptive transfer model, we've provided a basis upon which we can begin to ask questions about the specific origin and function of cytokines from particular sources. It provides a powerful tool to move beyond global systemic neutralization or loss of function and begin to discover when and from where the factors required for an effective immune response against *M. tuberculosis* originate.

4.0 DELETION OF CD4+CD25+ REGULATORY T CELLS PRIOR TO *M. TUBERCULOSIS* INFECTION DOES NOT CHANGE INFECTION OUTCOME

4.1 INTRODUCTION

When controlled, *M. tuberculosis* infection results in discrete areas of organized immune cells called granulomas that contain the bacteria, yet do not affect surrounding areas of healthy tissue. In order to contain the bacilli a robust Th1 response is required. To maintain the integrity of the healthy tissue, presumably the Th1 response must be limited to the areas of infection-involved tissue, and that likely requires factors that balance the inflammation caused by Th1 responses. The factors that contribute to the regulation of the immune response are largely unknown. One hypothesis is that CD4+ T regulatory (Treg) cells limit immunopathologic tissue damage, but may also function to help *M. tuberculosis* establish a persistent infection.

In addition to pro-inflammatory actions, CD4 T cells can also be immunoregulatory. CD4+CD25+ T regulatory cells were first defined by Sakaguchi *et al* and shown to be necessary to prevent autoimmune diseases [147]. Tregs dampen the immune response by cell to cell contact, secretion of anti-inflammatory cytokines such as IL-10 and TGFβ and can limits IL-2 availability [212]. In addition to the expression of CD25, Tregs have also been reported to express CTLA-4 (CD152), glucocorticoid-induced TNFR related protein, and CD39 [163]. However, all of these surface markers are also associated with activated pro-inflammatory CD4

T cells and are expressed during T cell activation. To date the most definitive marker of Tregs is the transcription factor Foxp3 [148]. Tregs are reported to play a role in the establishment of persistent viral [165], parasitic [164, 168] and bacterial infections [213, 214]. However the role they play in the immune response to *M. tuberculosis* remains unclear.

Studies in humans show that immediately following *M. tuberculosis* infection, the frequency of Tregs in peripheral blood decreases and they are hypothesized to migrate to areas of infection and limit tissue damage [215]. As infection progresses, a higher frequency of Tregs in peripheral blood is associated with individuals who have active disease when compared to either healthy uninfected or latently infected controls [177-179, 216, 217]. In both humans and mice, Tregs have been found at the site of *M. tuberculosis* infection [170, 218]. In addition, Tregs expand in the draining lymph nodes, and when depleted in a bone marrow chimera model have been associated with modest decreases in bacterial burdens in the lungs [171]. In yet another study, bacterial burden was reduced below the limit of detection or significantly decreased when lymphopenic animals were reconstituted with naïve non-Treg CD4 T cells when compared to animals that had received both Tregs and non-Tregs [176]. We sought to determine whether Tregs present during early *M. tuberculosis* infection function to limit the immune response to the bacilli and subsequently contribute to persistence of the infection.

Foxp3 has been shown to be the definitive marker for Tregs. It is an intracellular transcription factor, and therefore cannot be used to deplete the Treg populations within a mouse. Thus CD25 was the marker I chose to target Tregs for depletion in naïve mice. Three distinct approaches to eliminate the CD4+CD25+ Tregs from mice prior to infection were used. First, an adoptive transfer method in which RAG1-/- mice were reconstituted with whole splenocyte populations with or without CD25+ cells. In a subsequent experiment RAG1-/- mice received

CD4+ T cells only, with or without the CD4+CD25+ population. Finally, CD25+ cells were depleted with anti-CD25 antibody prior to infection in wild type mice. Regardless of the experimental design, no significant reduction in the bacterial burden was found indicating that at least in the murine model of *M. tuberculosis* infection, global elimination of CD25+ Tregs prior to infection does not alter the immune response sufficiently to prevent *M. tuberculosis* from becoming a persistent chronic infection in mice.

4.2 MATERIALS AND METHODS

4.2.1 Animals

As previously described in chapter 3, RAG1-/- and C57BL/6 mice were obtained from Jackson laboratories or maintained in an in-house breeding facility. Animals were free fed a diet of mouse chow and autoclaved water and maintained as per the University of Pittsburgh Institutional Animal Care and Use Committee.

4.2.2 Probiotic Pre-treatment of RAG1-/- mice

To prevent the development of reconstitution induced inflammatory bowel disease[207, 208], RAG 1-/- mice were prophylactically treated for 14 days with probiotics. Prior to adoptive transfer, mice received one scoop of Bene-Bac Powder(Pet Ag Inc. Hampshire, IL) (25 million Colony Forming Units (CFU) per gram, *Lactobacillus fermentum, Enterococcus faecium,*

Lactobacillus plantarum, Lactobacillus acidophilus) in 350ml of fresh, autoclaved water everyday. Water bottles were changed daily.

4.2.3 Reconstitution of RAG 1-/- mice in the presence and absence of CD4+CD25+ cells

Splenocytes were isolated from naïve C57BL/6 mice under sterile conditions. A single cell suspension was achieved by crushing the spleens through a 40μm cell strainer with the back of a sterile 5ml syringe. Erythrocytes were lysed by RBC lysis buffer (previously described in chapter 3) and cells washed and pelleted. Following enumeration by trypan blue exclusion, the splenocytes were split into two portions. CD4+CD25+ cells were depleted by magnetic bead separation (Miltenyi) from half of the splenocytes as per manufacturer's directions. CD25 depletion was confirmed by flow cytometry. Once depleted, cells were washed once in PBS and resuspended in PBS at $4x10^6$ cells/ ml. RAG1-/- mice received $1x10^6$ cells/200 μl PBS by i.v. tail vein injection. One group received splenocytes without CD4+CD25+ cells and one group received undepleted splenocytes the day before *M. tuberculosis* infection.

4.2.4 Reconstitution of RAG1-/- mice with only CD4+ T cells +/- CD4+CD25+ population

C57BL/6 mice were treated with either anti-CD25 antibody (0.5mg/mouse, clone PC61, NCCC, NIAID, Bethesda, MD) or an equivalent volume of PBS (300μl/ mouse) by intra-peritoneal (i.p.) injection 3 days prior to CD4 T cell isolation. Splenocytes from each treatment group (anti-CD25 or PBS) were harvested as previously described. Following enumeration, CD4+ cells were positively selected by magnetic bead separation (Miltenyi) as per manufacture's instructions. Cells were washed once in PBS and resuspended in PBS at $5x10^6$ cells /ml. One

day prior to infection, RAG 1-/-mice were reconstituted with CD4+ only or CD4+and CD4+CD25+ cells at a concentration of $1x10^6$ cells/ mouse in 200μl PBS by i.v. tail vein injection.

4.2.5 Antibody mediated depletion of CD25+ cells from C57BL/6 mice prior to infection

Three days prior to *M. tuberculosis* infection, C57BL/6 mice were treated with either 0.5mg/mouse anti-CD25 antibody (Clone PC61, NCCC, NIAID Bethesda MD) or PBS as injection control via interperitoneal injection. CD25+ cell depletion was confirmed by flow cytometry.

4.2.6 Aerosol infection

M. tuberculosis was passed through mice, grown in liquid culture once and frozen in 7H9 complete media 10% glycerol. Immediately prior to infection, an aliquot was thawed and diluted in PBS 0.05% Tween 80. Bacteria were disaggregated by cup horn sonnication. Mice were aerosol infected with low dose (<50 CFU, Erdmann strain) using a nose only exposure aerosolizer (InTox Products, Albuquerque, NM). Mice were exposed to aerosolized for 20 minutes then room air for 5 minutes. One day following infections, lungs of one mouse per round were crushed in 5 ml PBS-0.5% Tween-80, plated neat on 7H10 or 7H11 plates (Difco Laboratories, Detroit, MI) to determine the inoculum dose.

4.2.7 Harvest of organs for analysis

At serial time points following infection, mice (n=4 mice per group per time point) were anesthetized with isoflurane and euthanized by cervical dislocation. Organs were isolated under sterile conditions. Organs were crushed in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) through a 40μm cell strainer with the plunger from a sterile 5ml syringe. An aliquot of cellular homogenate was used for CFU determination. Cells were then pelleted and washed. Erythrocytes were lysed as described previously in chapter 3 and cells were resuspended, counted by trypan blue exclusion and used for assay set up.

4.2.8 Flow cytometric analysis of cellular infiltrate in lungs

Cells were surfaced stained for CD3 (Clone 145-2C11, BD Pharmingen), CD4 (Clone RM4-5, BD Pharmingen), CD8 (Clone 53-6.7, BD Pharmingen) and CD69 (Clone H1.2-F3, BD Pharmingen) at room temperature for 15 minutes in PBS 0.5% BSA 20% mouse serum, washed and then fixed with 2% Paraformaldehyde (Sigma-Aldrich). In the event that cells were stained for intracellular cytokine analysis, cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of monensin as previously described in chapter 3. Following stimulation cells were stained for surface markers and fixed in 2% PFA for 20 minutes. To stain intracellular IFNγ (Clone XMG1.2, BD Pharmingen), cells were permeabilized in FACS buffer with 0.2% saponin (Sigma-Aldrich) and stained in the same buffer for 15 minutes at room temperature. Cells were washed in the permeabilization buffer and fixed in 2%PFA. The Data were collected on a FACS aria (BD bioscience) and analyzed using FloJo software 8.6.3 (Treestar).

4.3 THE PRESENCE OR ABSENCE OF CD25+ CELLS DOES NOT CHANGE OUTCOME OF INFECTION WHEN LYMPHOPENIC MICE ARE RECONSTITUTED WITH NAÏVE SPLENOCYTES

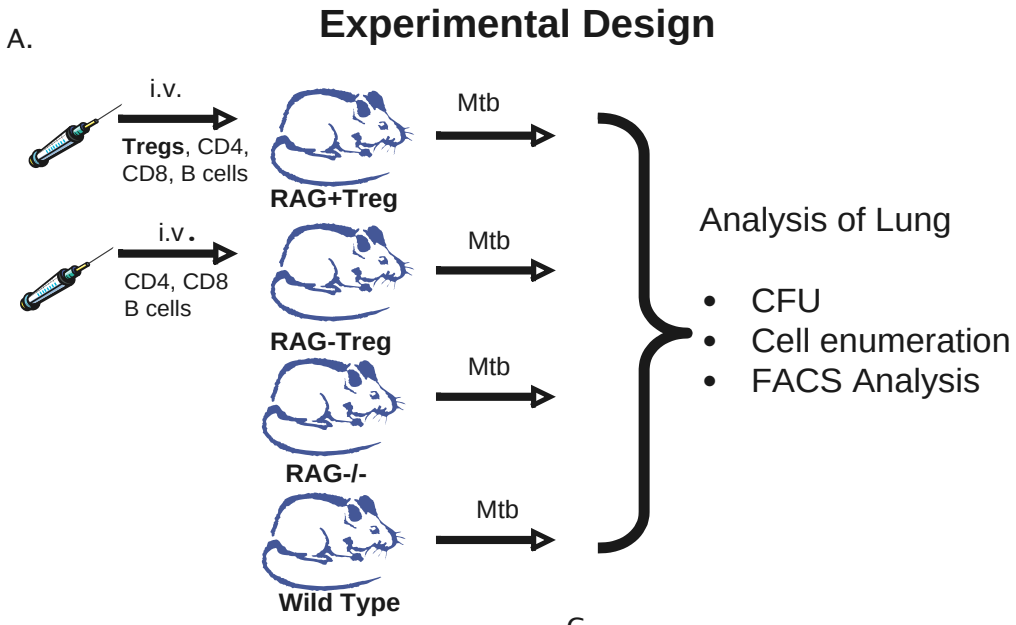
4.3.1 Bacterial burden is not significantly different between animals that received splenocytes with Tregs and those that did not receive Tregs

We hypothesized that Tregs may be acting to limit the immune response to *M. tuberculosis* infection and thus preventing a sterilizing response. First to determine whether the elimination of Tregs prior to infection would result in decreased bacterial burden, RAG1-/- mice were reconstituted with naïve splenocytes with CD25+ cells (RAG+Treg) or splenocytes depleted of CD25+ cells (RAG-Treg) and compared to wild type (C57BL/6) and non-reconstituted RAG 1-/- mice. Mice were aerosol infected with low dose (<50 CFU) *M. tuberculosis* one day after adoptive transfer (Figure 12A). Bacterial burden was assessed by serial dilution of organ homogenate and plated on 7H10 agar plates. A slightly though not significantly, higher bacterial burden was found in the animals that received Tregs during reconstitution compared to those that did not. However, this slight disparity is resolved by nine weeks (Figure 12B). Analysis of an independent repeat of these test groups shows that at 6 months post infection (Figure 12C), both experimental adoptive transfer groups have a similar bacterial numbers per lung and both are higher than WT controls. These data indicate that when Tregs are depleted prior to adoptive transfer, a transient, but not significant difference is found within the first month of infection. As the infection progresses the difference is quickly resolved and not maintained during chronic infection.

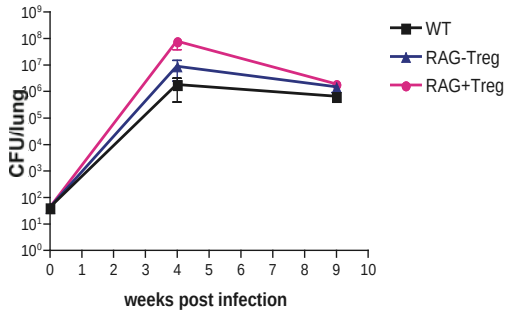
4.3.1.1 The absence of Tregs does not affect survival

We hypothesized that the lack of Tregs in the lungs may result in an aberrant immune response that would damage the surrounding lung and thus result in premature death in mice lacking Tregs. This was not the case as both groups survived significantly longer than RAG1-/- mice and as long as wild type (6 months post infection) (Figure 12D), indicating that in this model the presence of Tregs at the beginning of infection did not affect survival.

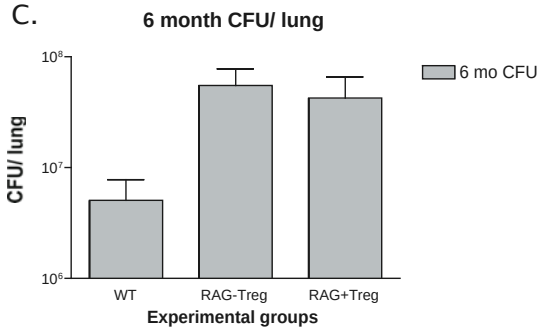
A.


Experimental Design
i.v.
Tregs, CD4, CD8, B cells
RAG+Treg
Mtb
i.v.
CD4, CD8 B cells
RAG-Treg
Mtb
RAG-/-
Mtb
Wild Type
Mtb
Analysis of Lung
CFU
Cell enumeration
FACS Analysis

B.


CFU/lung
weeks post infection
WT
RAG-Treg
RAG+Treg

C.


6 month CFU/ lung
CFU/ lung
WT
RAG-Treg
RAG+Treg
Experimental groups
6 mo CFU

D.

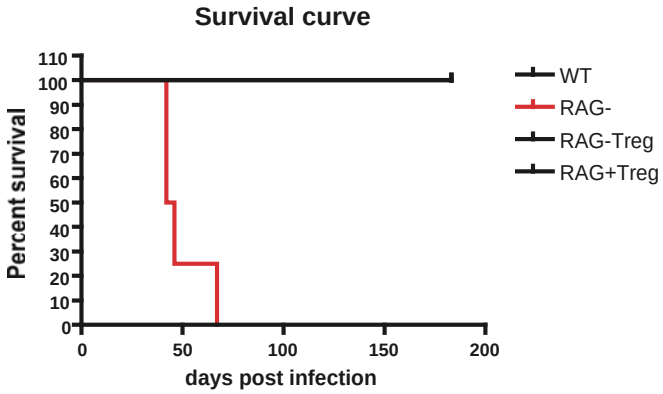

Survival curve
Percent survival
days post infection
WT
RAG-
RAG-Treg
RAG+Treg

Figure 12. Adoptive transfer, bacterial burden and survival

RAG1-/- mice were reconstituted with naïve splenocytes in the presence or absence of CD4+CD25+ T cells and compared to non-reconstituted RAG-/- and wild type mice (A). Bacterial burden was determined by serial dilutions of lung homogenate plated on 7H10 plates and is shown for early infection (B) as well as at 6months post infection (C). Murine survival (D) was assessed for 6 months post infection.

4.3.1.2 Cellular infiltrate

Analysis of the lung cellular infiltrate revealed that both CD4 (Figure 13A) and CD8 T cells (Figure 13B) are found in similar numbers in the reconstituted experimental groups. The T cells exhibited an identical activation profile when both groups were compared (Figures 13C and 13D). Furthermore, macrophages (Figure 14A), dendritic cells (Figure 14B) and neutrophils (Figure 14C) were also found in equal numbers. Taken together these data suggest that depletion of CD25 prior to infection did not change the type or quantity of cells that infiltrated the lungs during *M. tuberculosis* infection.

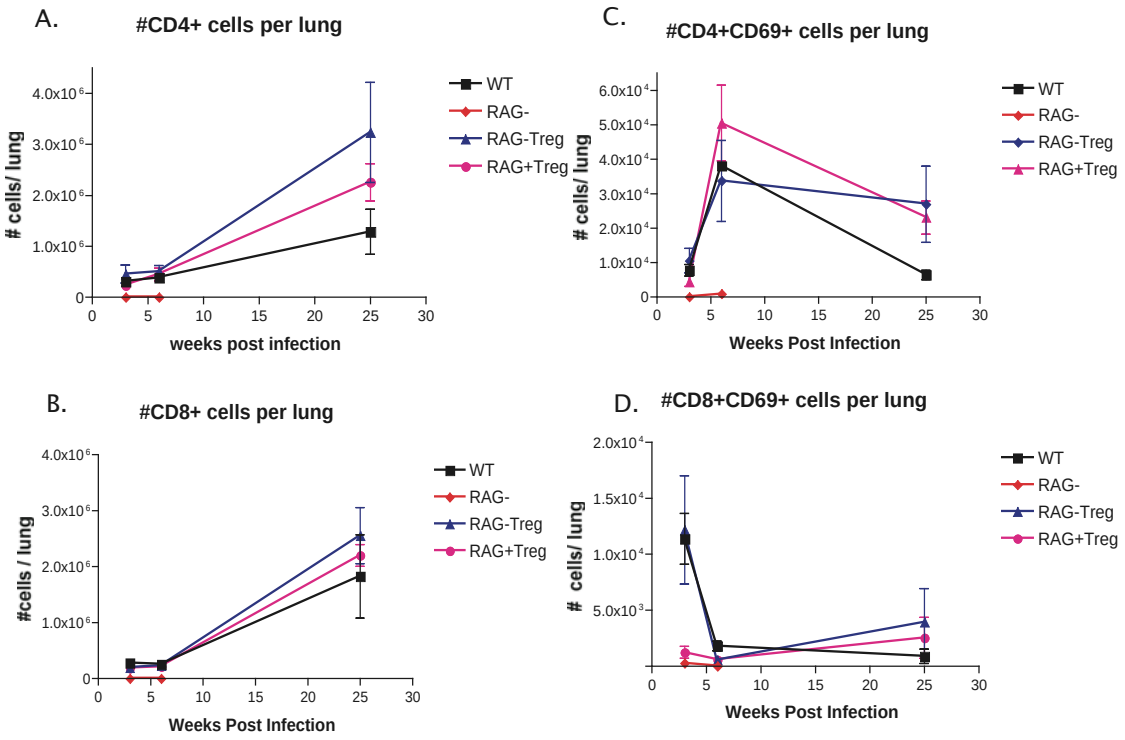


Figure 13. T cell frequency and activation in the lungs of mice reconstituted with naïve splenocytes

T cell frequency and activation were determined by flow cytometry. CD4 T cells (A), CD8 T cells (B), CD4+CD69+ T cells (C) and CD8+CD69+ T cells (D) are reported as the number of cells per lung (n=4 mice per group).

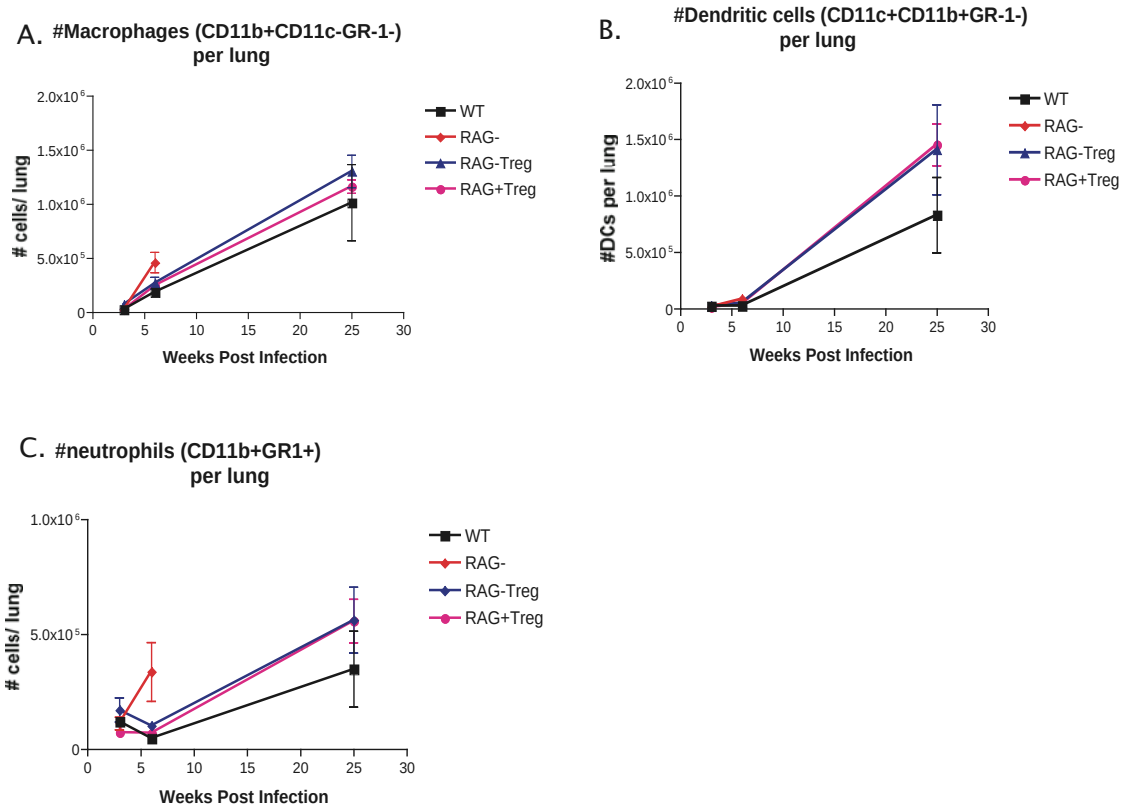


Figure 14. Monocytes found within the lung of reconstituted *M. tuberculosis* infected mice

The frequency of macrophages (A), dendritic cells (B) and neutrophils (C) was determined by flow cytometry and reported as the total number of cells per lung (n=4 mice per group).

4.4 RECONSTITUTION WITH ONLY CD4 T CELLS +/- CD25 CELLS

CD4 Tregs are not the only mechanism by which the immune response is controlled and we hypothesized that other immune control mechanisms may be present in complete splenocytes that were able to control the inflammation. To further dissect the regulatory role of these cells, we modified the adoptive transfer model and only transferred CD4+ T cells with or without the CD4+CD25+ population into RAG1-/- mice (Figure 15). Again we analyzed the bacterial burden (Figure 16A), cellular infiltrate (Figure 16B and 16C) and activation state of the cells

(Figure 16D) and found no significant difference in any parameter analyzed. These data indicate that the CD4+CD25+ population is not necessary during the initial stages of infection even when no other mechanisms for regulation are present.

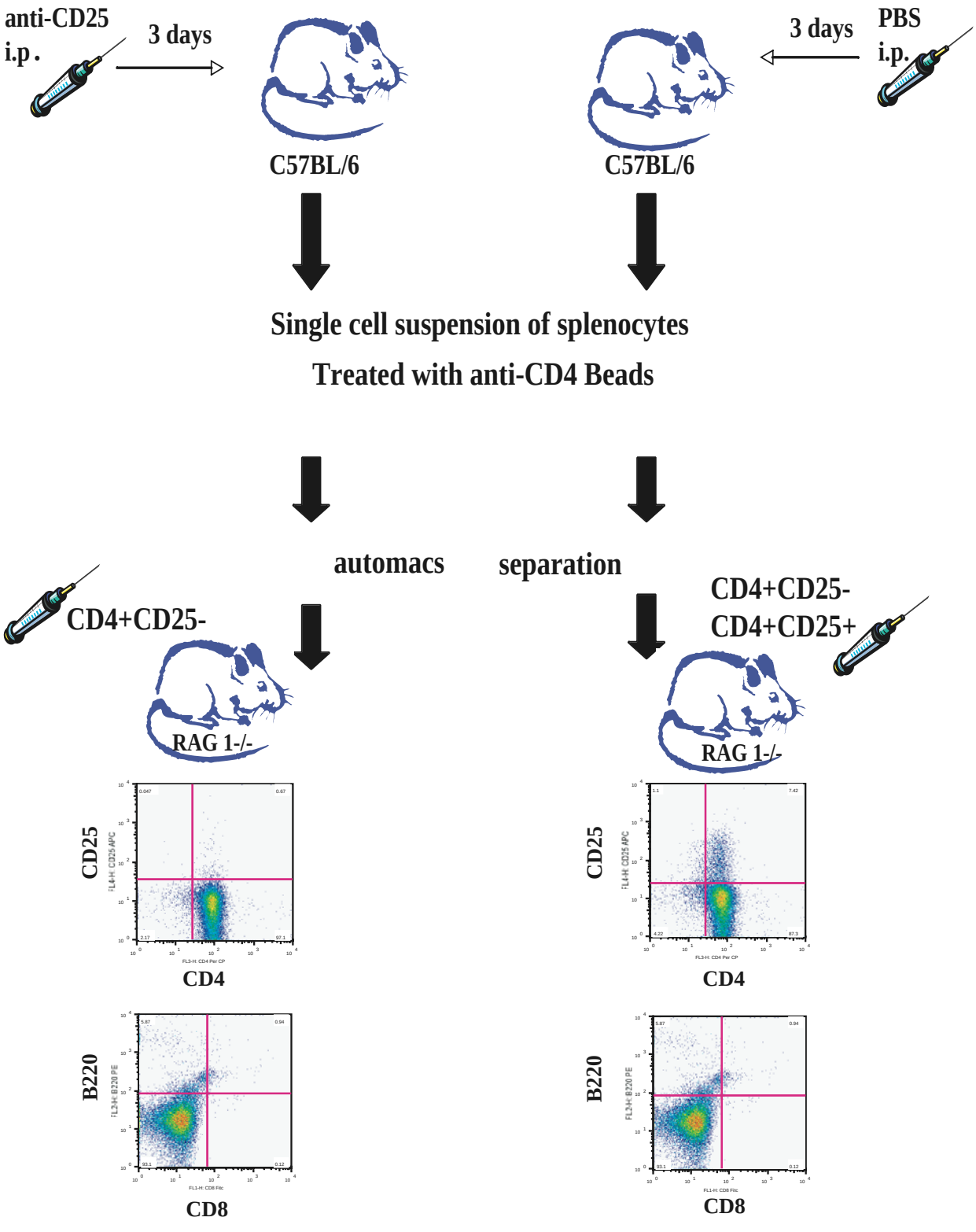

anti-CD25
i.p .
3 days
C57BL/6
3 days
PBS
i.p.
C57BL/6
Single cell suspension of splenocytes
Treated with anti-CD4 Beads
automacs
separation
CD4+CD25-
RAG 1-/-
CD4+CD25-
CD4+CD25+
RAG 1-/-
CD25
CD4
B220
CD8
CD25
CD4
B220
CD8

Figure 15. Adoptive transfer of CD4 T cells +/- CD25+ cells

C57BL/6 mice were treated with either anti-CD25 antibody or PBS i.p. for 3 days prior to isolation of CD4 T cells. CD4 T cells were isolated from both groups by anti-CD4 magnetic bead separation. RAG1-/- mice were reconstituted with either CD4CC4 T cells +/- the CD25+ population. (n=4 mice per group per time point). Representative flow cytometry plots are shown to depict the purity of the isolation.

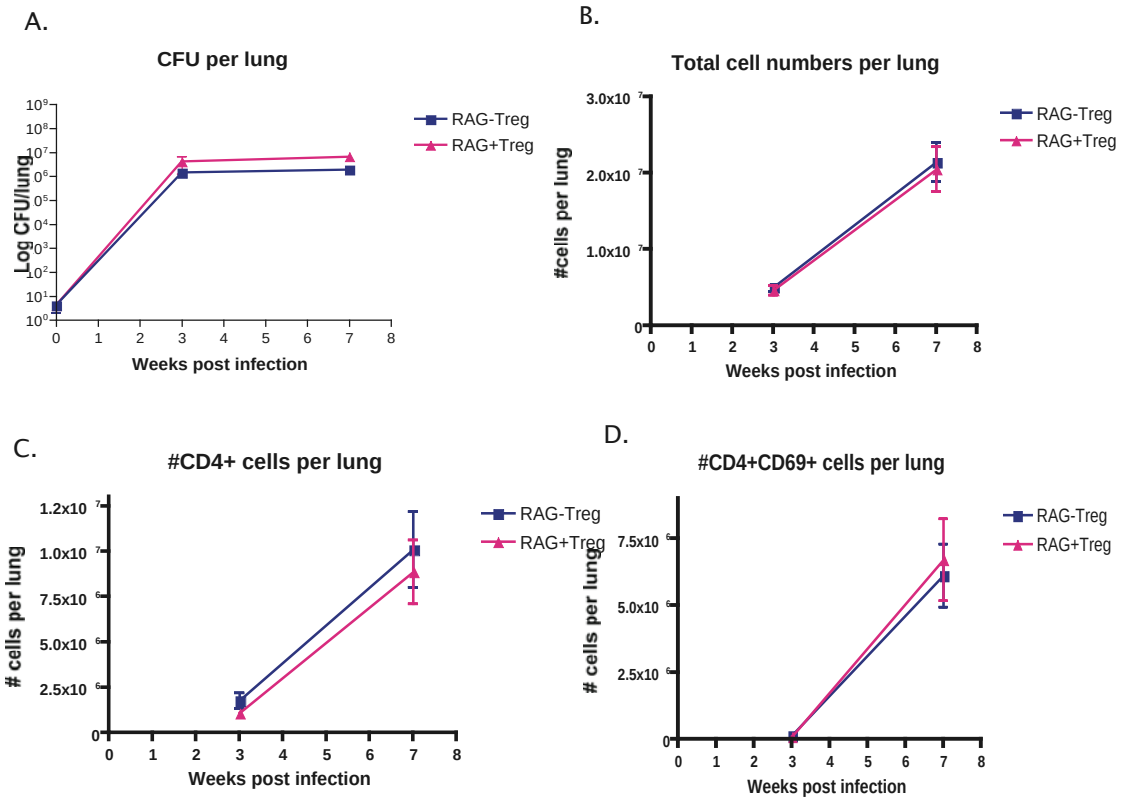


Figure 16. Bacterial burden and cellular infiltrate in lungs of RAG1-/- mice reconstituted with CD4 T cells +/- Tregs

The total bacterial burden per lung was determined by plating serial dilutions of lung homogenate on 7H10 agar (A). The total number of cells per lung (B) was determined by trypan blue exclusion. The total number of CD4+ (C) and CD4+CD69+ (D) cells per lung was determined by flow cytometry.

4.5 DEPLETION OF CD25+ T CELLS DURING ACUTE *M. TUBERCULOSIS* INFECTION

To determine whether the presence of Tregs prevented clearance of *M. tuberculosis* infection, we depleted CD25+ T cells from mice prior to infection. CD25 is the α- subunit of the IL-2 recepter and is constitutively expressed on the surface of natural Tregs.

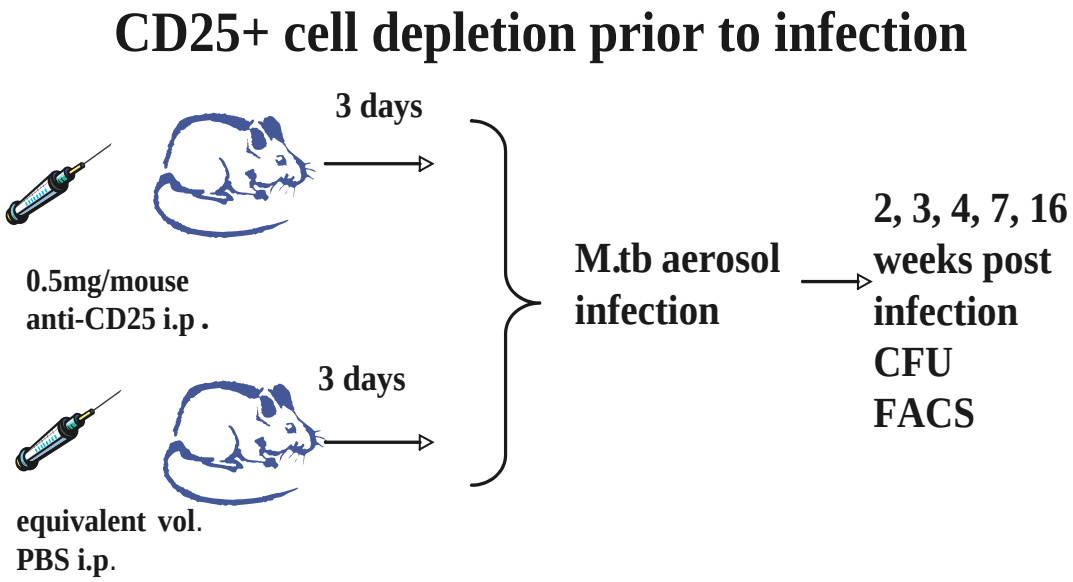
4.5.1 Experimental set up

Three days prior to *M. tuberculosis* infection, C57BL/6 mice were treated with either anti-CD25 antibody (0.5mg/mouse) or an equivalent volume of PBS via intraperitoneal injection (Figure 17A). On day 3 a mouse from each group was sacrificed and examined for the presence of CD25+ cells. As is shown in Figure 1B, CD25+ cells was not seen in animals treated with the anti-CD25 antibody.

4.5.2 Depletion of CD25+ cells does not affect bacterial burden over time

Originally we hypothesized that deletion of the CD25+ cells (Tegs) from mice prior to infection would result in a more robust anti-mycobacterial response and thus in a decreased bacterial burden in the lungs. Depletion of Tregs prior to infection did not change the bacterial burden found in the lungs (Figure 18A)

A.



B.

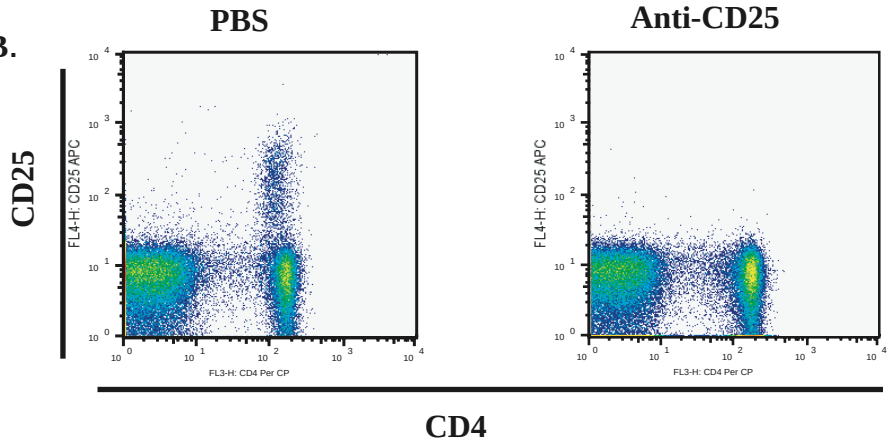


Figure 17. Depletion of CD25+ cells in C57BL/6 mice

Mice were treated with either 0.5mg/mouse of anti-CD25 antibody or an equivalent volume of PBS (A) three days prior to *M. tuberculosis* infection. (B) CD25+ cells were not visible by flow cytometry on the day of infection.

4.5.3 Depletion of CD25+ cells does not change the number of cells in each organ

Administration of anti-CD25 did not change the total number of cells found with the lungs (Figure 18B), spleen (Figure 18C) or draining lymph nodes (Figure 18D) when compared to those that had received the mock injections of PBS.

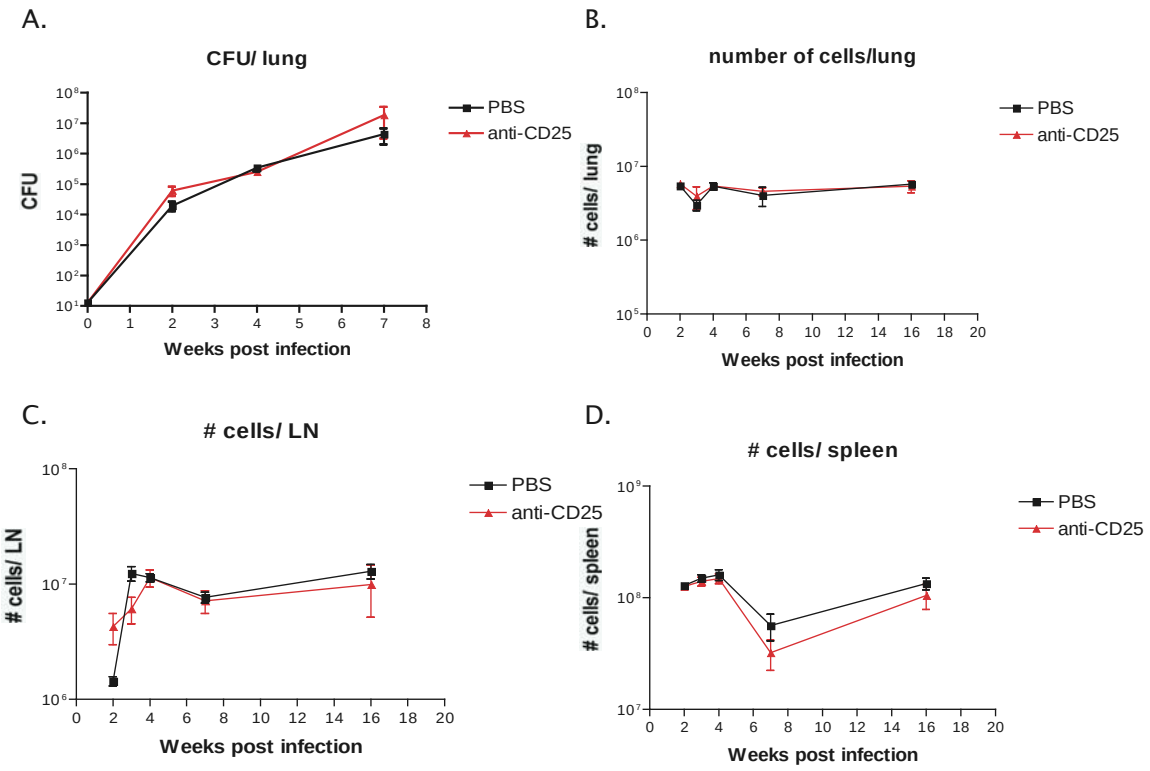


Figure 18. CFU and total cell number per organ

The bacterial burden per lung was determined by plating serial dilutions of organ homogenate on 7H10 plates (A). the total number of cells per organ was determined by trypan blue exclusion for the lung (B), draining lymph nodes (C) and spleen (D).

4.5.4 Lack of CD25+ cells did not change activation state or IFNγ production in either CD4 or CD8 T cells

We sought to determine whether depletion of Tregs would change the activation profile of T cells during the course of the immune response. The early activation marker CD69 was used as an indicator of activation on both CD4 T cells and CD8 T cells. Again no differences between groups were observed in either the lungs or lymph nodes (Figure 19A-D).

Finally we evaluated the frequency of IFNγ producing T cells within the CD4 and CD8 T cell compartments. The overall frequency of IFNγ+ CD4 or CD8 T cells remained exactly the same between the groups in both organs (Figure 20A-D). These data indicate that depletion of CD25+ cells prior to infection did not change the kinetics of the immune response or the ability of these cells to produce the pro inflammatory cytokine IFNγ.

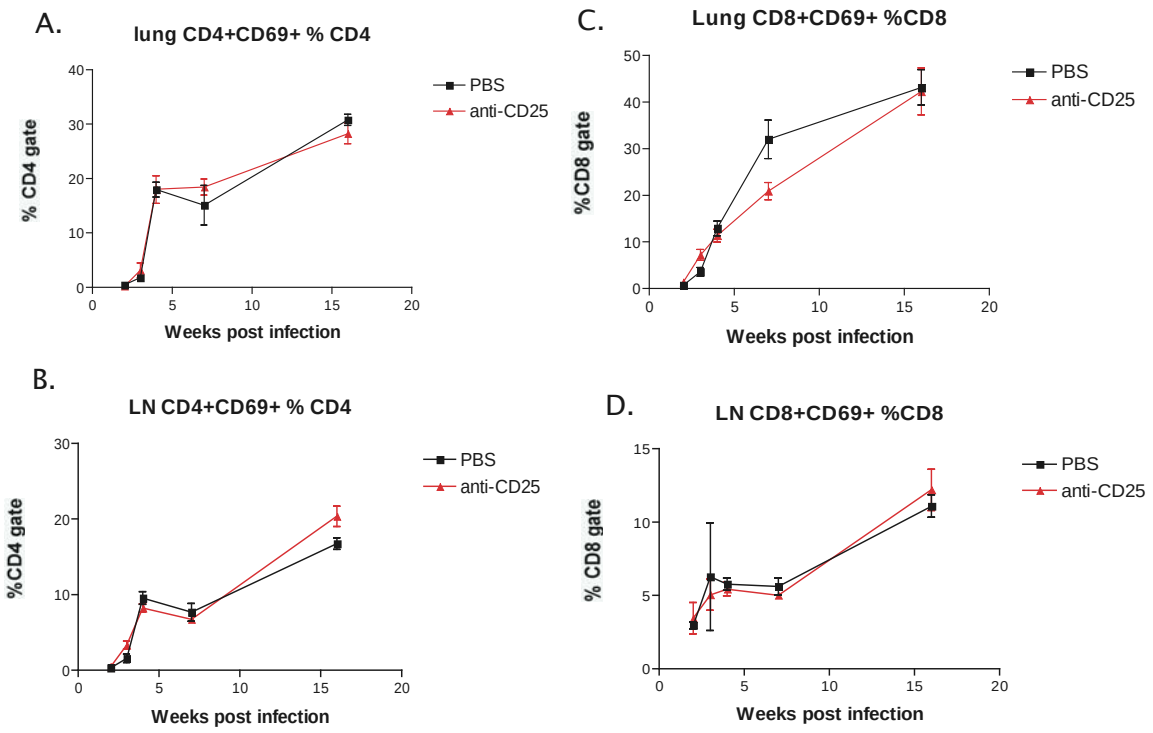


Figure 19 T cell activation in the lungs and draining lymph node

The early activation marker CD69 was used as an indicator of recent T cell activation. CD4+CD69+ cells in the lungs (A) and lymph node (B) as well as CD8+CD69+ cells in the lungs (C) and lymph nodes (D) were measured by flow cytometry and reported as a frequency within the respective T cell compartments.

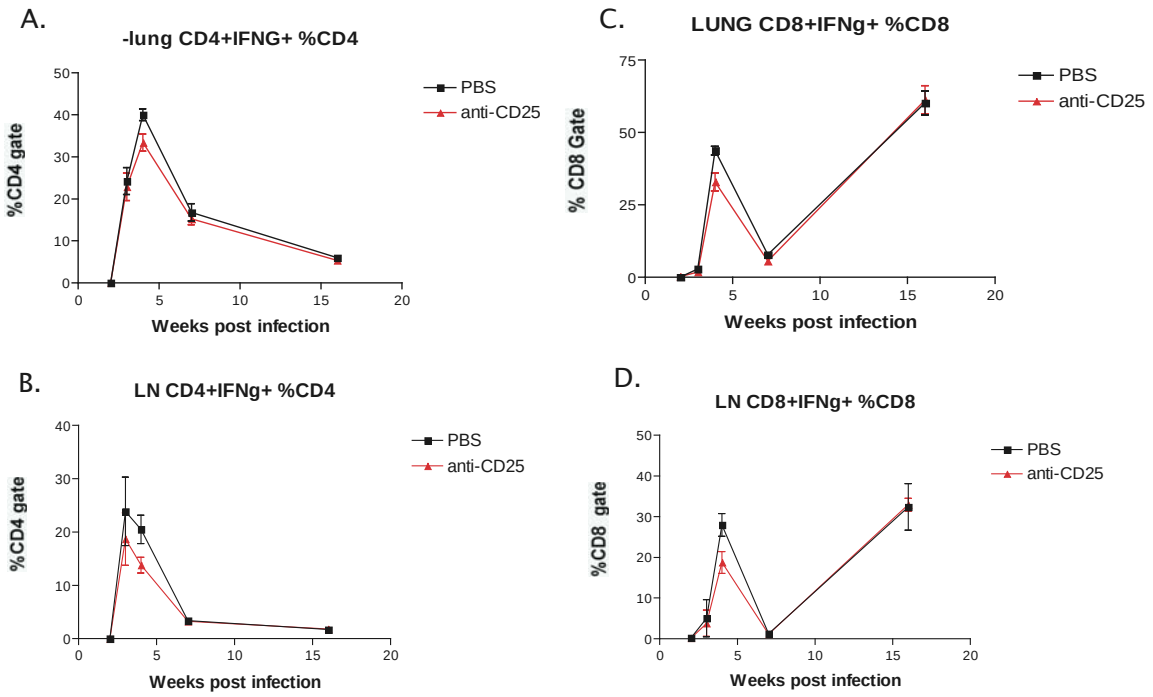


Figure 20. IFNγ production by CD4 and CD8 T cells in lung and lymph node

Cells from lungs and draining lymph nodes were stimulated with anti-CD3 and anti-CD28 antibodies for 6 hours in the presence of monensin. IFNγ production was measured by intracellular cytokine staining and flow cytometry. The frequency of CD4+IFNγ+ in the lungs (A) and lymph node (B) and the CD8+IFNγ+ cells in the lungs (C) and lymph node (D) are reported as a frequency of either the CD4 or CD8 T cell compartments respectively.

4.6 DISCUSSION

Regulatory T cells are essential for maintaining a balanced immune response and prevention of autoimmunity. Multiple studies report the role Tregs play in the establishment of persistent chronic infections from multiple pathogens [164-166, 168, 169, 213, 219]. In addition, Tregs have been found at the site of infection in both humans and mice [170, 218] and are reported to limit *M. tuberculosis* specific IFNγ production by effector cells within the blood of patients with active disease. What role Tregs have and whether they function to the benefit or detriment of the host has yet to be defined for Tregs in tuberculosis.

One hypothesis is that Tregs are detrimental to the host by limiting the pro-inflammatory Th1 response and preventing clearance of the bacilli by a sterilizing immune response. However, Tregs may benefit the host by limiting the immunopathologic damage to surrounding tissue. To address what role Tregs may play in *M. tuberculosis* infection, three distinct experimental approaches were used. My data demonstrate that global systemic elimination of CD4+CD25+ T regulatory cells prior to infection had little to no effect on the immune response or infection outcome. In one experiment, lack of Tregs resulted in a mildly transient, but not significant reduction in bacterial burden during early infection that resolved by the chronic phase of infection.

Interestingly our data are supported by data presented by Quinn *et al* [175], found that depletion of CD25+ cells prior to infection resulted in an increased IFNγ response but did not result in a change in bacterial burden or result in clearance of the bacilli. These data are in contrast to experiment in a bone marrow chimera model and used a Thy1.1/1.2 disparity to deplete Tregs. Most likely the difference in outcomes can be explained by the timing and method of Treg depletion. Since CD25 is the α subunit to the IL-2 receptor and is upregulated

on newly activated effector T cells, we were limited by when and how often we could use an anti-CD25 antibody to deplete the actual Treg population without affecting expanding effector T cells. In addition, the use of a Thy1.1/1.2 disparity to eliminate the Treg population could be used multiple time and thus keep the Treg population depleted for longer periods of time over the course of infection. Finally, the data from experiments in which only CD4 T cells were adoptively transferred showed a modest half log decrease in bacterial burden by 7 weeks p.i. in mice that did not receive Tregs. These data are in contrast to data reported by Kursar *et al* [176] in which mice that did not receive Tregs had either a 1000-fold decrease in bacterial numbers or cleared the pathogen. On possible explanation for why our data were unable to duplicate the biphasic response to infection outcome may be because we utilized a more physiologic concentration of Tregs compared to the 10X greater frequency of Tregs used by Kursar *et al*.

5.0 LONG TERM IL-12 TREATMENT DURING EARLY *M.TUBERCULOSIS* RESULTS IN AN INCREASED FREQUENCY OF REGULATORY T CELLS AT SITES OF INFECTION

5.1 INTRODUCTION

Interleukin-12 (IL-12) is an essential component of an effective immune response against *M. tuberculosis* infection. This heterodimeric cytokine (p70) is made up of the subunits p40 and p35 which signals through the IL-12 receptor (IL-12Rβ1/IL-12Rβ2). IL-12 promotes a T helper 1 phenotype immune response and is produced by dendritic cells and phagocytes following pathogen infection or exposure to inflammatory cytokines [220]. When T cells, NK cells and NKT cells become activated the IL-12Rβ2 subunit is up regulated and IL-12 promotes IFNγ production by these cells. In conjunction with other pro-inflammatory cytokines such as IL-2, IL-18 and IL-27, IL-12 increases IFNγ production by T cells [115, 220].

Humans with genetic mutations in IL-12 signaling (most commonly through loss of function mutations in the receptor complex) have an increased susceptibility to mycobacterium infections, even *Mycobacterial spp.* considered less pathogenic such as *M. avium* [115]. In addition, studies of mice lacking IL-12 (p40-/- or p35-/- or p40/p35-/-) succumb to infection [79, 221]. Neutralization of IL-12 by antibody has also been shown to correlate with a decrease in

IFNγ production and increased mortality in the host [80, 222]. In mice lacking IL-12, the susceptibility to *M. tuberculosis* can be reversed by exogenous delivery of IL-12 [80, 222].

IL-12 has been studied as an immunotherapeutic for cancer [223] and some viral infections such as HIV [224]. In addition, IL-12 administration to BALB/c mice [73] and CD4-/- infected with *M. tuberculosis* improved survival and decreased bacterial burden [225]. While IL-12 therapy had promising results during intra-tumor injections in mice and humans, long-term treatment had the potential to result in toxicity and death [223]. The reason for this moribund outcome could be linked to excessive IFNγ production [223]. However, counter-intuitively, multiple reports indicate that IL-12 may be immune suppressive [224, 226-229]. Previously, our lab reported that long term IL-12 administration during *M. tuberculosis* infection of wild type mice failed to change bacterial load in the lungs, but resulted in a decreased cellular infiltrate and less area of the lung involved in inflammation. In this chapter we attempt to discern the mechanism by which IL-12 decreases the immune infiltrate into *M. tuberculosis* infected lungs without affecting bacterial burden.

5.2 MATERIALS AND METHODS

5.2.1 Animals

8-week-old C57BL/6 wild type mice and IL-10-/- (B6.Cg-IL10^{tm1cgn}) were obtained from Jackson laboratories (Bar Harbor, ME) and housed under bio safety level 3 conditions in micro-isolator cages. Mice were free fed a diet of mouse chow and autoclaved water. All animal care

was in compliance with the University of Pittsburgh's institutional animal care and use committee.

5.2.2 IL-12 treatment

One day prior to *M. tuberculosis* infection and then three times a week (Monday, Wednesday and Friday) for up to 8 weeks p.i., mice were treated with either IL-12 (200ng/mouse, Genentech/Wyeth/Pfizer pharmaceuticals, New York, NY) or an equivalent volume of phosphate buffered saline via intra-peritoneal (i.p.) injection.

5.2.3 Anti-TGFβ treatment

In one series of experiments mice were treated, 3 times a week with the pan anti-TGFβ antibody 0.15 μg/mouse/ treatment (Clone 1D11, NCCC, Bethesda, MD) i.p. These treatments were in conjunction with IL-12 or PBS mock treatments.

5.2.4 Aerosol infection

M. tuberculosis was passed through mice, grown in culture once and frozen in 7H9 complete media 10% glycerol. Immediately prior to infection, an aliquot was thawed and diluted in PBS 0.05% Tween 80. Bacteria were disaggregated by cup horn sonnication. Mice were aerosol infected with low dose (<50 CFU, Erdman strain) using a nose only exposure aerosolizer (InTox Products, Albuquerque, NM). Mice were exposed to aerosolized *M. tuberculosis* for 20 minutes then room air for 5 minutes. One day following infections, lungs of one mouse per round were

crushed in 5 ml PBS-0.5% Tween-80, plated neat on 7H10 plates (Difco Laboratories, Detroit, MI) to determine inoculum.

5.2.5 Organ harvest and cellular isolation

At serial time points, mice were anaesthetized with isoflurane and euthanized by cervical dislocation. Organs were recovered under sterile conditions. To obtain single cells suspensions, organs were crushed in Dulbecco's modified eagle's medium, through a 40μm cell filter with the back of a sterile 5ml syringe. Erythrocytes were eliminated from the cell pellet by incubating in RBC lysis buffer (described in chapter 3) for 2 minutes at room temperature. Followed by washing and trypan blue enumeration as previously described in chapter 3.

5.2.6 Bacterial burden

Bacterial burden was determined by plating serial dilutions of organ homogenate on 7H10 agar plates (Difco). Plates were read after 21 day incubation at 37°C 5% CO_2. Bacterial burden was calculated and reported as CFU/ organ. For statistical analysis, CFU were log transformed to normalize the data.

5.2.7 Flow Cytometry

Cells were stained as previously described for both surface and intracellular cytokine staining in chapters 3 and 4. Foxp3 staining was carried out as per eBioscience Foxp3 staining kit

instructions. Data was collected on a FACS aria (BD bioscience) and analyzed using FlowJo (8.6.3, Treestar).

5.2.8 Statistics

To determine significance between two groups a student-t test was used. When more than two groups were compared the significance was determined by Analysis of variance. If ANOVA was determined to be significant, Bonferroni post-hoc analysis was used to determine pair significance. Significance was assigned when $p<0.05$.

5.3 RESULTS

5.3.1 Long term IL-12 treatment during early *M. tuberculosis* infection results in a greater frequency of CD4+Foxp3+ T regulatory cells in wild type mice

Previously data published by our laboratory reported that IL-12 treatment during the first two months of *M. tuberculosis* infection resulted in a decreased cellular response at the site of infection without a decreased bacterial burden. We hypothesized the decreased cellular response may be due to an increased presence of Tregs.

5.3.1.1 Cellular infiltrate at the site of infection is decreased in IL-12 treated animals when compared to those that received PBS

To test our hypothesis, one day prior to *M. tuberculosis* infection, C57BL/6 mice were treated with either IL-12 (200ng/mouse) or an equivalent volume of PBS via i.p. injection. At serial time points following infection, lungs and draining lymph nodes were harvested and the total number of cells per organ determined (n=4 mice per group). As previously reported, mice treated with IL-12 had significantly fewer cells in both the lungs (Figure 21A) and lymph nodes (Figure 21B) [225].

5.3.1.2 IL-12 treatment does not change bacterial burden

At the time of harvest an aliquot of organ homogenate was serially diluted and plated on 7H10 agar plates to determine the bacterial burden in the lungs and lymph nodes of infected animals. With most infections, the presence of more antigen often results in increased inflammation with an enhanced immune response and has been shown in *M. tuberculosis* infection [97]. Thus one would expect a decreased bacterial burden in mice treated with IL-12, since fewer cells were recovered from the infected tissues. However this was not the case. Bacterial burdens were the same during the first weeks post infection in the lungs (Figure 21C). During later time points, PBS recipient mice exhibit a statistically significant lower bacterial burden. However, it was only a decrease of 5-fold, which is relatively modest in the murine tuberculosis model. Bacterial burden was comparable in the draining lymph nodes (Figure 21D). These data indicate that IL-12 treatment decreases inflammation without affecting the amount of antigen (bacteria) present.

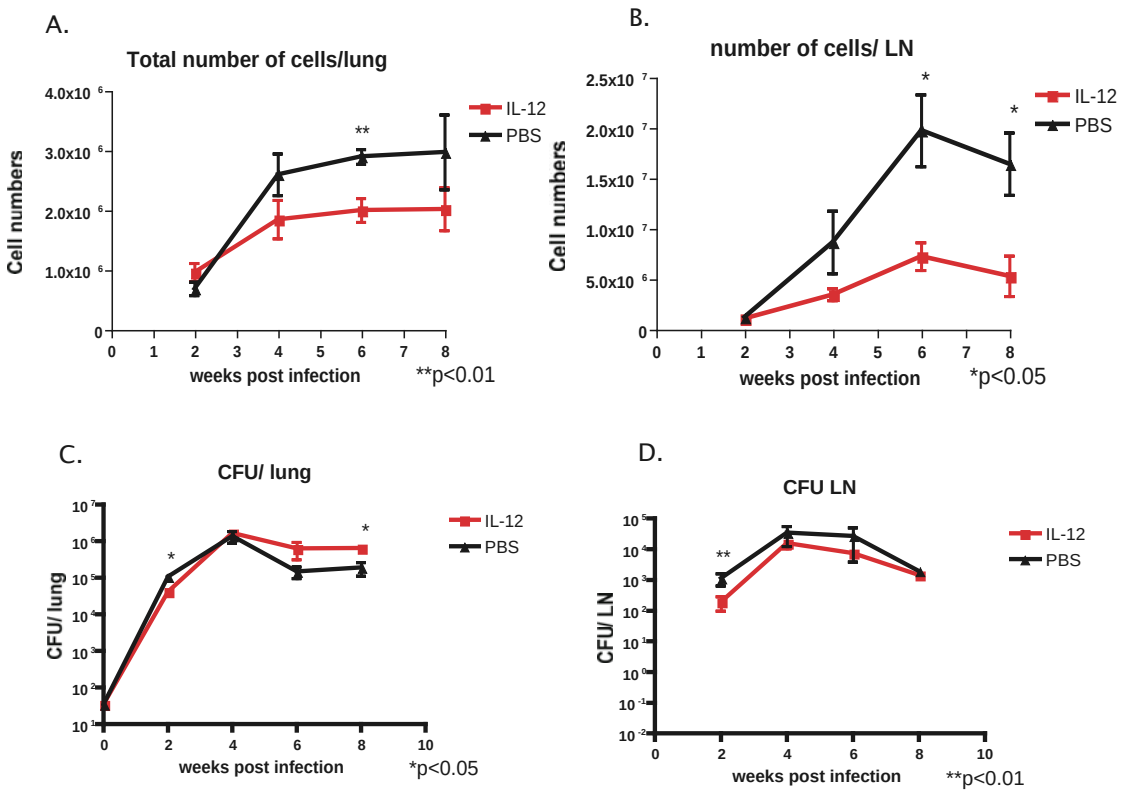


Figure 21. Cellular infiltrate and bacterial burden in IL-12 and PBS treated wild type mice

Wild type C57BL/6 mice were treated one day prior to *M. tuberculosis* infection and then three times per week p.i. with 200ng/mouse of IL-12 or and equivalent volume of PBS. To determine the bacterial burden and total number of cells per organ, lungs and lymph nodes were harvested at serial time points. The total number of cells per lung (A) and lymph node (B) were determined by trypan blue exclusion. Bacterial burden was determined by plating serial dilutions of organ homogenate on 7H10 agar plates and are reported as colony forming units per lung (C) or lymph node (D). Statistical significance was determined by student-t test and a $p<0.05$ was considered significant.

5.3.1.3 IL-12 treatment results in fewer CD4 T cells producing IL-2

IL-2 is a critical factor needed to initiate and sustain an adaptive immune response. Thus to determine whether the decreased cellular response may be due to fewer cells producing IL-2, the frequency of CD4+IL-2+ T cells were analyzed by flow cytometry. Mice that received IL-12 had fewer CD4 T cells that produced IL-2 first in the lymph nodes (Figure 22A) and at later

time points in the lungs (Figure 22B), suggesting that IL-2 production may be limited with IL-12 treatment.

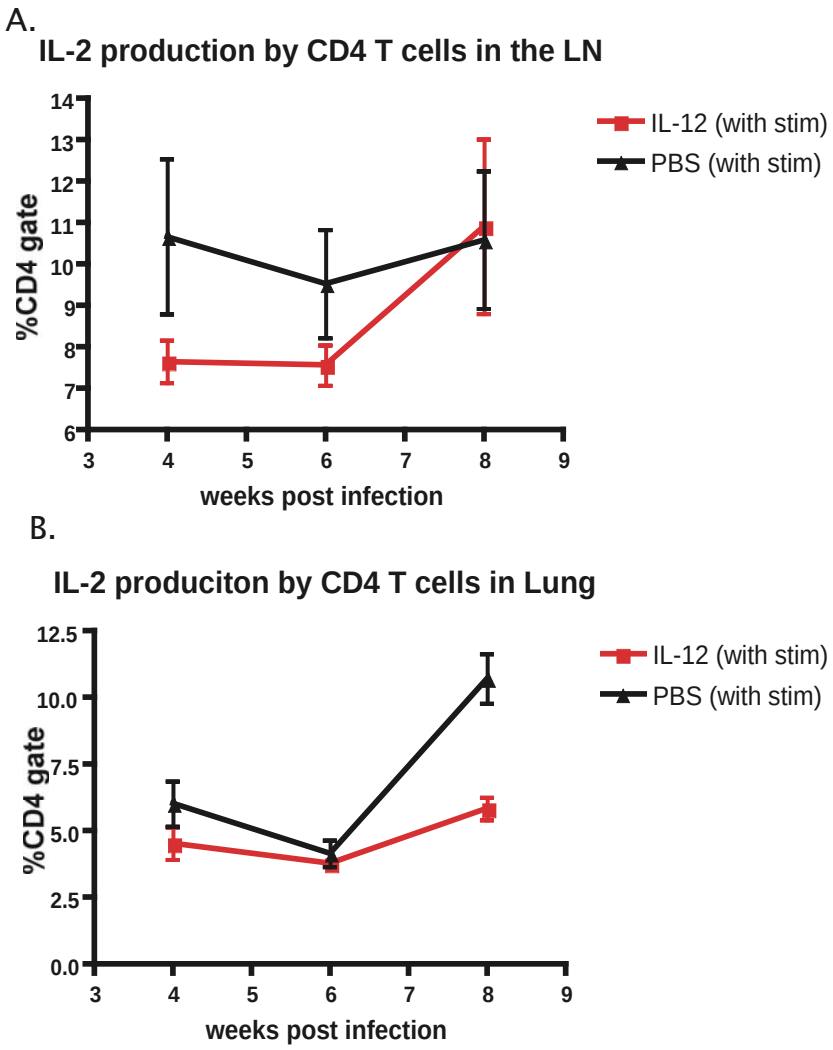


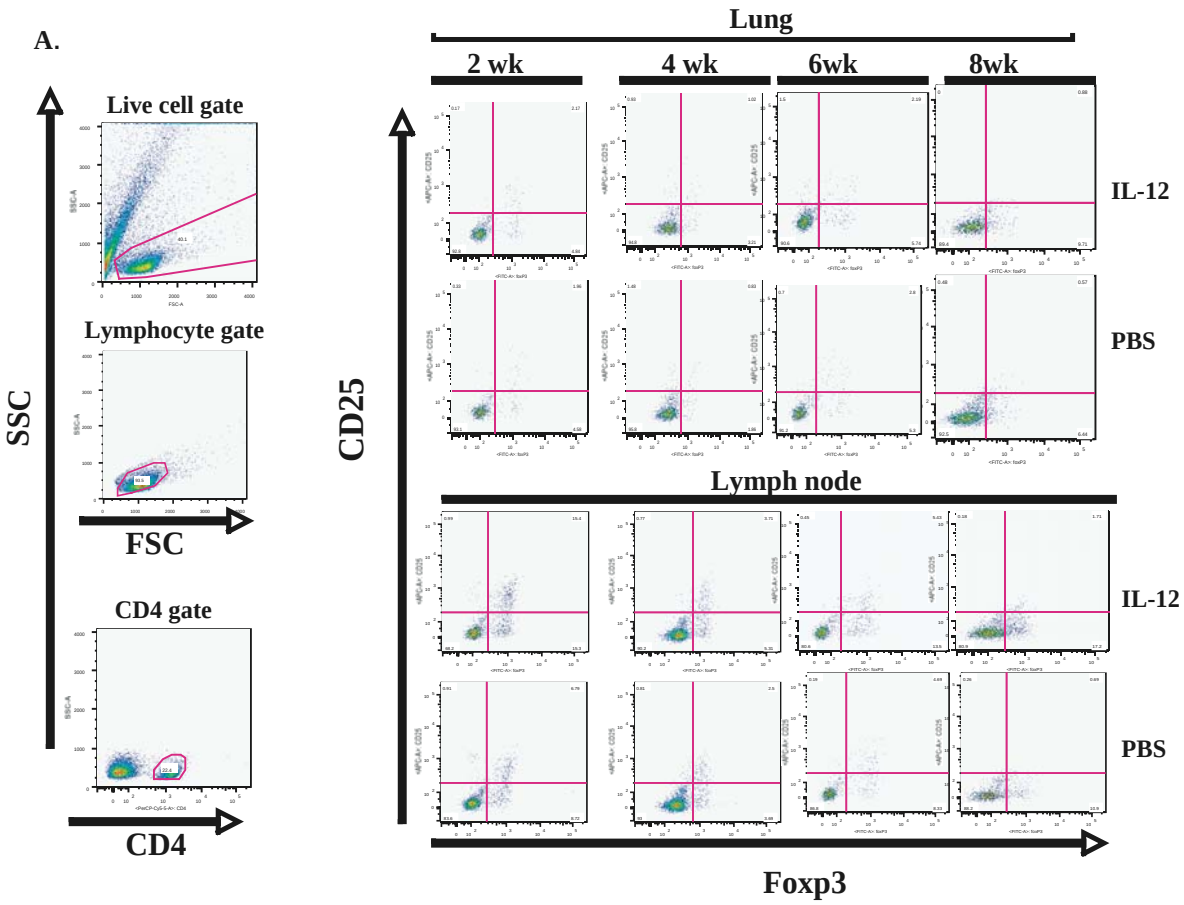
Figure 22. IL-2 producing CD4 T cells in the lungs and lymph nodes

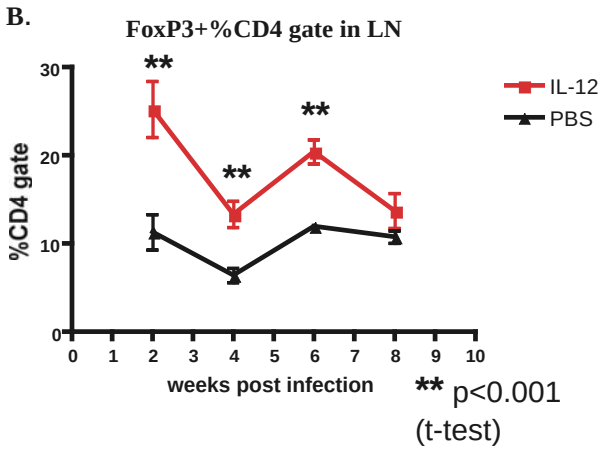
Cells from the lymph nodes (A) and lungs (B) of infected mice were stimulated with anti-CD3 and anti-CD28 in the presence of monensin for 5 hours. Following stimulation cells were stained for surface markers and intracellular cytokines. The frequency of IL-2+ CD4 T cells was obtained by gating cells on live cell gate, lymphocyte gate, and then CD4+ gate. Data are reported as a frequency of CD4 T cell gate.

5.3.1.4 IL-12 and Tregs

IL-12 treatment resulted in a decrease in the number of immune cells found at the site of infection without a decrease in antigenic burden. Tregs can inhibit IL-2 production by T cells and fewer CD4 T cells produced IL-2 in IL-12 treated animals. Taken together these data lead us to ask whether Tregs may play a role in the reduced inflammation seen in IL-12 treated mice.

Cellular infiltrates were analyzed by flow cytometry for Foxp3+CD4+ cells within the lungs and lymph nodes of infected mice over time and can be seen in representative plots (Figure 23A). Interestingly, IL-12 treated mice had a significantly higher frequency of CD4+Foxp3+ T cells first in the lymph nodes (Figure 23B) and then the lungs (Figure 23C). These data indicate that Tregs may expand in the lymph nodes and migrate to the lungs. In addition, the increased frequency of Tregs in IL-12 treated mice may contribute to the decreased inflammation in these animals.


A.
Live cell gate
Lymphocyte gate
CD4 gate
SSC
FSC
CD4
Lung
2 wk
4 wk
6wk
8wk
IL-12
PBS
CD25
Lymph node
IL-12
PBS
Foxp3


B.
FoxP3+%CD4 gate in LN
%CD4 gate
weeks post infection
IL-12
PBS
** p<0.001
(t-test)

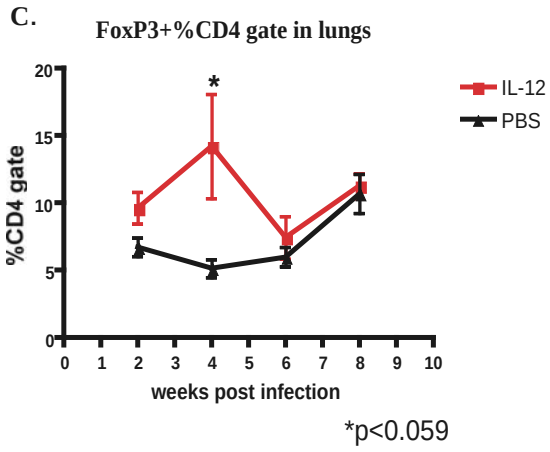

C.
FoxP3+%CD4 gate in lungs
%CD4 gate
weeks post infection
IL-12
PBS
*p<0.059

Figure 23. Regulatory T cells and IL-12 treatment

Cellular infiltrate from the lungs and lymph nodes of IL-12 and PBS treated mice were obtained at serial time points. To assess the frequency of Tregs present, cells were stained for CD3, CD4, CD25 and Foxp3. (A) Representative plots of CD25 vs. Foxp3 within the CD4 gate over time. The frequency of Tregs (CD4+Foxp3+ cells) within the CD4 gate is shown for lymph node (B) and lung (C). Statistics were determined by Student T test and a $p<0.05$ was considered statistically significant.

5.3.1.5 TGFβ and IL-12 treatment

Transforming growth factor β (TGFβ) has been shown to be important for the induction and function of Tregs [230] and TGFβ+CD4+ cells are reported to accumulate in the lungs of *M. tuberculosis* infected mice [170]. To assess whether immune regulation by IL-12 was associated an increased frequency of CD4+TGFβ+ cells, cellular infiltrate was analyzed by flow cytometry. Analysis of CD4+TGFβ+ T cells within the CD4 T cells compartment showed that at two weeks post infection, the frequency of these cells was significantly increased in the lymph nodes (Figure 24A) and lungs (Figure 24B). However this difference was transient and no difference in the expression of TGFβ was seen past two weeks (data not shown).

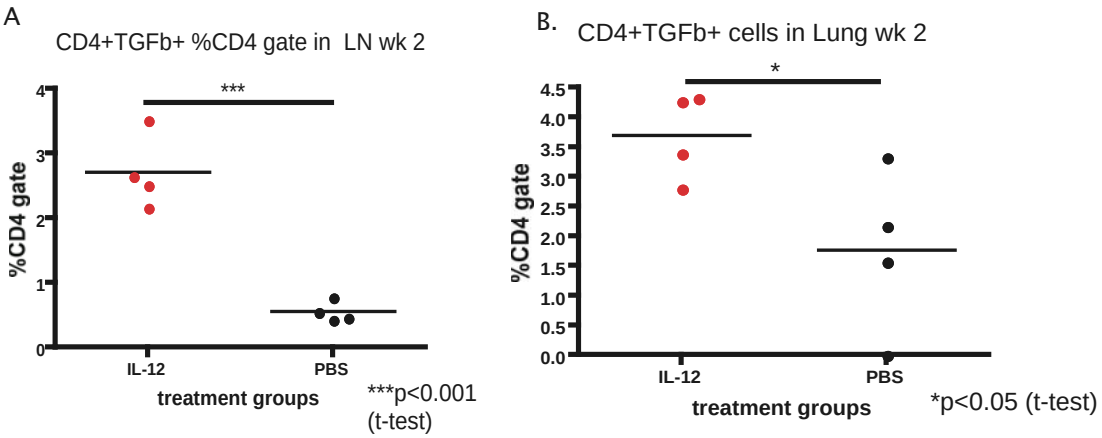


Figure 24. CD4+TGFβ+ cells and IL-12 treatment

Cellular infiltrate from the lymph nodes (A) and lungs were stained for CD3, CD4 and TGFβ expression. The frequency of CD4+TGFβ+ at two weeks post infection are reported within the CD4 T cell gate. Statistical significance was determined by student t-test and a $p<0.05$ was considered significant.

5.3.2 IL-12 immune suppression is independent of IL-10

IL-12 treated C57Bl/6 mice have fewer cells (less inflammation) at the site of infection without a decreased antigenic burden. This difference is associated with fewer CD4 T cells that produce IL-2, an increased frequency of CD4+TGFβ+ cells (at least in the beginning of the response) and an increased frequency of Tregs that start in the lymphocytes and appear to migrate to the lungs. Since IL-12 and IL-10 have been shown to each regulate the function of the other [231], we sought to determine whether IL-12 treatment associated immune regulation was dependent upon IL-10. To address this question, IL-10-/- mice were treated with IL-12 and examined for bacterial burden, total cell numbers and frequency of regulatory T cells.

5.3.2.1 IL-12 treatment of IL-10-/- mice did not affect bacterial burden

IL-12 treatment did not change the total bacterial count found in the lungs of IL-10-/- mice when compared to PBS mock treated mice (Figure 25A). In addition, a similar amount of bacteria were found within the lymph nodes of animals from both experimental groups (Figure 25B).

5.3.2.2 IL-12 treatment decreases cellular infiltrate

If IL-12 mediated immune regulation were mediated by IL-10, IL-12 treated mice would not have decreased cellular infiltrate compared to PBS treated mice. The total number of cells

per lymph node (Figure 25C) and lungs (Figure 25D) were determined by trypan blue exclusion. In both sets of organs, IL-12 treatment resulted a significant reduction in the number of cells following infection. These data indicate the IL-12 immune suppression is not mediated by IL-10.

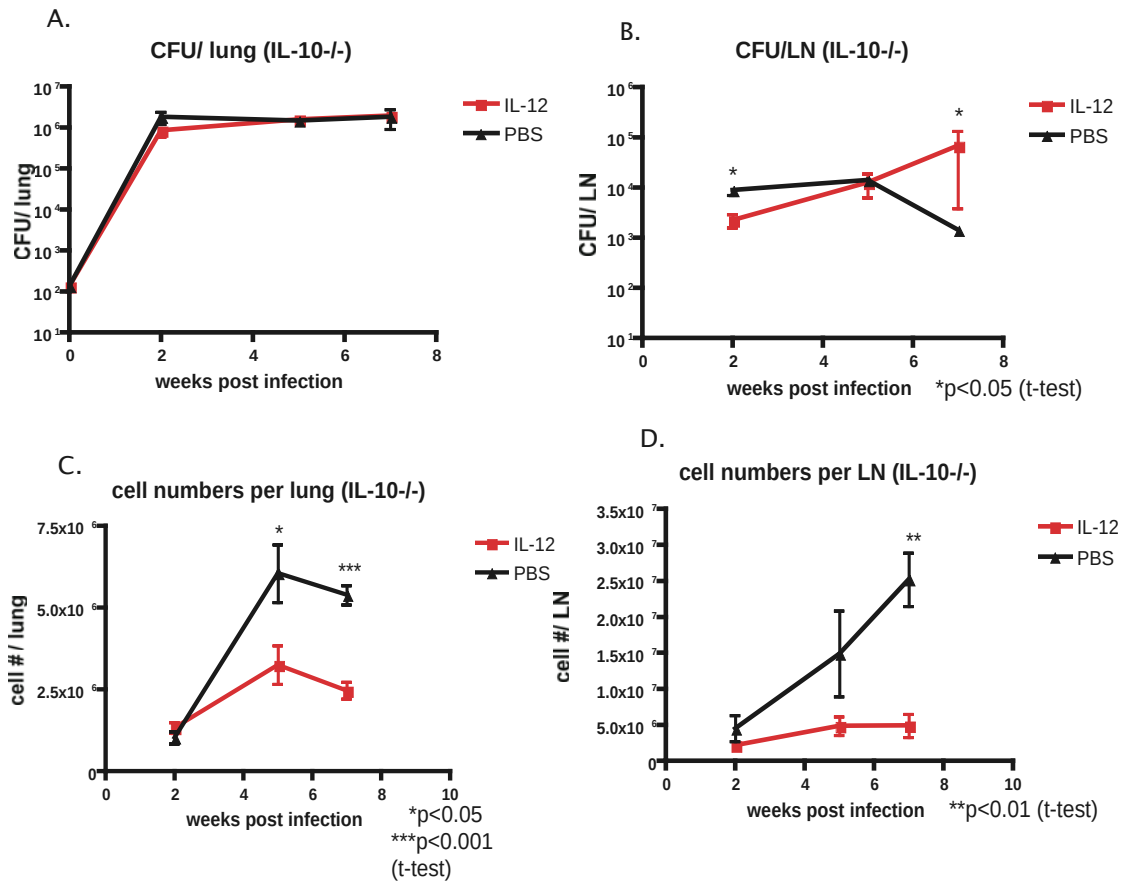


Figure 25. CFU and total cell numbers in IL-12 treated IL-10-/- mice

IL-10-/- mice were treated as described in materials and methods. CFU was determined by plating serial dilutions of organ homogenate from lung (A) and lymph node (B) on 7H10 agar plates and is reported as CFU/organ. The total number of cells recovered from each organ was determined by trypan blue exclusion and reported as total number of cells per organ. Statistical significance was determined by student-t test and $p<0.05$ was considered to be significant.

5.3.2.3 Tregs and TGFβ+ CD4 T cells in IL-10-/- mice

Finally, we sought to address whether the lack of IL-10 would affect the increased frequency of Tregs and TGFβ+ CD4 T cells found in IL-12 treated animals as compared to PBS mock treated mice. In both the lungs (Figure 26A) and lymph nodes (Figure 26B) of IL-12 treated mice, a significantly higher frequency of Treg cells were found. Interestingly, when we assessed the frequency of CD4+TGFβ+ cells in IL-10-/- mice, they were present in higher quantities than were ever found in wild type mice, regardless of IL-12 treatment (data not shown). As expected the IL-12 treated IL-10-/- mice had significantly higher frequencies of these cells than PBS treated in both the lungs (Figure 26C) and lymph nodes (Figure 26D). These data in conjunction with the results of IL-12 treatment in wild type mice suggest that IL-12 mediated immune suppression may be due to increased Tregs or possibly an increased expression of TGFβ+CD4+ T cells and is independent of IL-10.

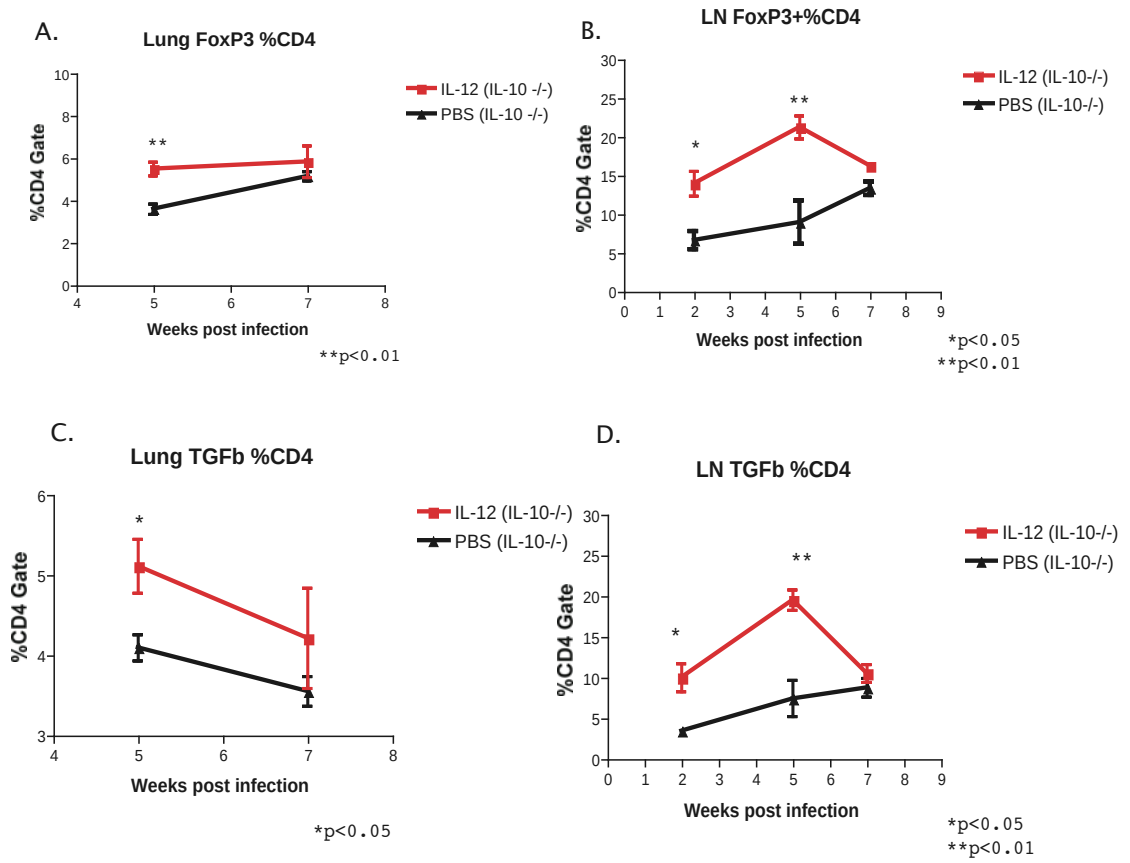


Figure 26. Frequency of Tregs and CD4+TGFβ+ cells in IL-12 treated IL-10-/- mice

The frequency of CD4+Foxp3+ cells and CD4+TGFβ+ cells were determined by flow cytometry. Statistical significance was determined by student-t test and a $p<0.05$ was considered significant. Lung cells were not available for staining at the 2-week time point for either Tregs (A) or TGFβ+CD4+cells (C).

5.3.3 Neutralization of TGFβ does not change IL-12 immune suppression

TGFβ is a pleiotropic cytokine that in very low concentrations is immune stimulatory, but in higher concentrations is immune regulatory. In addition TGFβ plays a role in the development of CD4+Foxp3+ T regulatory cells [232]. Since IL-12 treated animals presented with a higher frequency of TGFβ+CD4+cells and Tregs, we sought to determine whether TGFβ plays a role in the anti-inflammatory effects seen with IL-12 treatments during *M. tuberculosis* infection. Based

on prior experiments, the increased frequency of Tregs in IL-12 treated animals begins to peak in the lymph nodes between 2 and 4 weeks post infection. Thus we chose the time point of day 20 (week 3 p.i.) to examine the effects of TGFβ neutralization during IL-12 treatment. Four groups of wild type C57BL/6 mice were treated one day prior to *M. tuberculosis* infection and then three days a week following infection with one of the following regimen: 1) anti-TGFβ antibody (0.15μg/mouse)/IL12 (200ng/mouse), 2) IL-12 alone, 3) anti-TGFβ alone or 4) PBS. Groups were assessed for bacterial burden, total cell numbers and frequency of Tregs was assessed 20 days post infection. We hypothesized that IL-12 treatment may be suppressing the immune response through TGFβ induction of Treg cells. Neutralization of TGFβ had no effect on IL-12 treatment and results were similar to those reported in IL-12 treatment of IL-10-/- and wild type mice (Data not shown). These data indicate that IL-12 immune regulation is not mediated by TGFβ induction.

5.4 DISCUSSION

The heterodimeric cytokine IL-12 is essential for the development of a Th1 immune response and thus required for effective control of *M. tuberculosis* infection [79, 221, 222]. IL-12 is produced by antigen presenting cells upon infection with an intracellular pathogen and act on T cells to dampen Th2 cytokine production and promote the production of IFNγ [220]. In addition, IL-12 had been used as an immunotherapeutic in multiple cancer and chronic infection models [224, 233]. Conversely, IL-12 administration has also been associated with immune suppressive activity. Previous data published by our lab has indicated that IL-12 treatment during the first two months results in a decreased inflammation and cellular infiltrate in infected tissues, without

affecting the amount of antigen present. With these experiments we sought to determine the factors that contribute to IL-12 mediated immune suppression.

IL-12 administration to wild type mice resulted in a decreased frequency of IL-2 producing CD4 T cells and an increased Treg population as well as an increased CD4+TGFβ+ T cells. Based on these results we first asked whether IL-10 may be responsible for the decreased inflammation being observed in IL-12 treated mice. IL-10 is a potent cytokine capable of decreasing IL-12/Th1 mediated immune responses [231]. In addition, in multiple studies, IL-10 has been implicated in the down regulation of *M. tuberculosis* specific responses [233]. More specifically over expression of IL-10 in a murine model of *M. tuberculosis* infection resulted in premature death [86]. To address whether the immune suppressive properties of IL-12 were mediated by IL-10, IL-10 deficient mice were treated with IL-12 as previously described in wild type mice and infected with *M. tuberculosis*. Results were similar to those found in wild type mice indicating that IL-10 was not responsible for the immune suppression of IL-12 treated mice.

Finally, we chose to address the role that transforming growth factor β may be playing in the anti-inflammatory properties of IL-12. TGFβ in higher doses is anti-inflammatory and is produced by monocytes from individuals infected with *M. tuberculosis*, or upon being stimulated with purified protein derivative (PPD) [234, 235]. TGFβ has been shown to modulate the effects of IL-12 [236] and is an essential component involved in the induction and selection of Tregs *in vitro* and *in vivo* [232]. When either wild type or IL-10 deficient mice were treated with IL-12, a greater frequency of CD4+TGFβ+ cells and CD4+Foxp3+ cells were found in the lymph nodes and lungs of experimental animals. To determine whether the reduced inflammation found in IL-12 treated animals was dependent upon TGFβ, we used antibody neutralization in the

presence and absence of IL-12 treatment. Neutralization of TGFβ failed to alter the IL-12 mediated decrease in inflammation and increased Treg frequencies, indicating that TGFβ was not the mechanism of immune suppression.

Independent of both IL-10 and TGFβ, the mechanism by which IL-12 treatment results in a decreased immune response with no reduction in bacterial burden remains unknown at this point and requires further investigation. It is interesting to note that in a report by Zhao et al, mice lacking IL-12 signaling due to IL-12receptor β2 developed more severe disease and at a faster rate than control animals in a diabetes model and these outcomes were associated with less efficient and fewer regulatory T cells [237]. These data indicate that IL-12 may play a role in the development of effective immune regulation through the maintenance of Tregs. In addition, new studies of regulatory T cells have revealed these cells are not a rigidly fixed population but can and do respond to the cytokine environment around them to even express pro-inflammatory cytokines [238].

6.0 REGULATORY T CELLS IN A CYNOMOLGUS MACAQUE MODEL OF *M. TUBERCULOSIS* INFECTION

6.1 INTRODUCTION

Tuberculosis is the leading cause of death by a single infectious agent [5]. Although 5-10% of infected persons progress to primary TB, the majority control the infection, are asymptomatic and considered to be latently infected (LTBI) [29]. Factors contributing to these infection outcomes are not well understood. The extended chemotherapy, increase in drug resistant strains [239], and lack of an effective vaccine [240] make identification of factors that affect infection outcome imperative.

Once infected, the host mounts a robust Th1 response, and forms a granuloma, which can function to contain the bacilli. Presumably, the immune response must be controlled to limit damage of surrounding tissue. There is likely a fine balance in each granuloma of effector, inflammatory and regulatory mechanisms. Patients with active TB produce more anti-inflammatory cytokines such as IL-10 [231, 241] and TGFβ [241, 242], compared to LTBI, but whether aberrant immune modulation contributes to development of active disease is unknown. The CD4+ T regulatory cell (Treg) has been suggested as a possible factor in promotion of active TB.

CD4 T cells expressing CD25 are potent inhibitors of autoimmunity [147]; the transcription factor Foxp3 is a defining characteristic of these Treg cells [148]. Tregs inhibit inflammatory responses and proliferation by production of cytokines, cell-to-cell contact and inhibiting IL-2 [163]. Natural Tregs are thought to be self-reactive and prevent autoimmunity [149]. Tregs can also potentiate persistence of certain pathogens [164-166, 169, 219].

Recent data suggest a role for Tregs in *M. tuberculosis* persistence. Removal of Tregs in mice resulted in decreased bacterial burden in lungs [171, 176], indicating Tregs may down regulate *M. tuberculosis* specific immune responses. PBMCs from active TB patients had increased frequencies of Tregs and decreased IFNγ production in response to certain M. tuberculosis antigens when compared to LTBI [180, 181, 216]. *In vitro* depletion of CD25+ cells in active TB patients increased *M. tuberculosis* specific IFNγ production, suggesting Tregs are suppressing specific responses [180, 181, 216]. These studies are unable to differentiate between increased Tregs contributing to development of active TB or occurring in response to inflammation in active disease. Studies with human TB patients are complicated by difficulties in defining time of infection, extent of disease, mycobacterial strain and size of inoculum, which may contribute to the quality of immune responses and disease outcome.

To address whether an increased frequency of Tregs affects development of active disease or occurs in response to inflammation caused by active disease, we used a non-human primate (NHP) model of *M. tuberculosis* infection. This is the only established model to accurately mimic human latent infection [29]. When cynomolgus macaques are infected with a low dose via bronchoscope, ~50% of animals exhibit no signs of disease despite being tuberculin skin test positive and are considered latently infected by six months. The other 50% develop primary tuberculosis [29]. These clinical classifications were validated by pathology and bacterial

numbers at necropsy [30]. Using this model, we addressed the correlation between Tregs and outcome of infection, and the dynamics of Treg in the periphery and airways.

6.2 MATERIALS AND METHODS

6.2.1 Experimental animals.

Cynomolgus macaques (*Macaca fascicularis*) (Alpha-Genesis, Yamassee, SC; Covance, Madison, WI; Valley Biosystems, Sacramento, CA), were ≥4 years of age, 3.5-10kg, housed in a bio safety level 3 facility [243], and free of TB or other infections. The University of Pittsburgh School of Medicine Institutional Animal Care and Use Committee approved all procedures and protocols.

6.2.2 Infection of NHP

Monkeys were infected with ~25 CFU *M. tuberculosis* strain Erdman as described [30, 239] by bronchoscopic instillation. CFU were determined in the inoculum by plating on 7H10 agar (Difco Laboratories, Detroit, MI). Infection of monkeys is confirmed by tuberculin skin test, and lymphocytic proliferation and IFN-γ production in response to mycobacterial antigens. Infection outcome was independent of age, gender and weight (Table 1) [30].

6.2.3 PBMC isolation

Blood was collected by percutaneous venipuncture [243]. Peripheral blood mononuclear cells (PBMCs) were isolated by Percoll gradient (Amersham Bioscience, Piscataway, NJ).

6.2.4 BAL Cells

Cells were sampled from airways by bronchoalveolar lavage (BAL)[243].

6.2.5 Necropsy of animals

Prior to necropsy, animals were sedated, then euthanized with sodium pentobarbital (Schering-Plough Animal Health, Union, NJ), as described [243]. A veterinary pathologist conducted all necropsies; tissues and samples were obtained in a sterile fashion [30].

6.2.6 Isolation of cells from necropsy tissue

At necropsy, granulomatous and non-granulomatous lung and lymph node were excised [30]. Cell suspensions were obtained by homogenizing tissues in PBS using a MediMixer (BD Biosciences, San Jose, CA). An aliquot of each suspension was plated for enumeration of *M. tuberculosis* colonies.

6.2.7 CD25 depletion from PBMCs

PBMCs were stained with PE anti-CD25 (Clone M-A251, BD Pharmingen, San Jose, CA); CD25+ cells were isolated using anti-PE beads (Miltenyi, Auburn, CA).

6.2.8 Lymphocyte proliferation assay

PBMCs prior to, at 6 weeks p.i. and for depletion studies were suspended in AIM V media (Invitrogen, Grand Island, NY) at 200,000 cells/well in 200 μl. Cells were stimulated with phytohemagglutinin (PHA 5μg/ml), culture filtrate protein (CFP, NIH-NIAID Contract HHSN266200400091C)(10 μg/ml), or media in triplicate wells for 60 hours at 37°C, 5% CO_2; for the final 18 hours, [^{3}H]-thymidine (1μCi/well, Amersham) was added. Cells were harvested onto filters and radioactive incorporation measured. Data were reported as a stimulation index (SI): fold increase in cpm over unstimulated control.

6.2.9 Flow cytometry

PBMCs, BAL cells, and cells from tissue were surface stained for CD3 (clone SP34, BD Pharmingen), CD4 (clone SK3, BD Biosciences), CD25 (clone MA251, BD Biosciences) [243], CD39 (clone eBioA1, eBioscience), GITR (clone eBioAITR, eBioscience) and intracellular CTLA-4 (clone BNI3, BD bioscience), then for Foxp3 (150D [BioLegend, San Diego, CA], PCH101, or 236A/E7 [eBioscience]) utilizing eBioscience Foxp3 staining kit. Data were collected on a FACSAria (BD Biosciences) and analyzed using FlowJo 8.6.3 (Tree Star, Inc., Ashland, OR).

6.2.10 Immunofluorescence tissue staining

Antigen retrieval on formalin fixed, deparaffinized tissue sections was performed with High pH Antigen Retrieval Buffer (Dako, Carpinteria, CA) at 95°C, 20 min. Slides were blocked with 2% goat serum and incubated with polyclonal rabbit anti-human CD3 (Dako) and biotinylated anti-human Foxp3 (clone 236A/E7; eBioscience) antibodies. Isotypes were stained using rabbit anti-human CD3 and mouse Universal Negative Control (Dako), 4°C overnight. Sections were stained with goat anti-rabbit AlexaFluor 488 and AlexaFluor 546 streptavidin conjugates (Invitrogen), and nuclei stained with Draq5 (Biostatus Limited). Sections were imaged with a Leica TCS-SL confocal microscope (Leica Microsystems), Z projections were made with ImageJ (vs 1.4.0, http://rsb.info.nih.gov/ij/), and brightness and contrast adjusted with Adobe Photoshop 7.0 (Adobe Systems).

6.2.11 ELISPOT for IFNγ

As previously described [243], 96 well opaque filtration plates (Multiscreen$_{HTS}$, Millipore Corp., Bedford, MA) were hydrated with sterile 35% ethanol and 1X PBS and coated with anti-human/monkey IFN γ antibody (15μg/ml, Mabtech, Nacka Strand, Sweden). Plates were incubated at 4°C overnight prior to use. The following day, plates were washed with 1X PBS and blocked with RPMI and 10% human AB serum (Gemini for 1 h at 37°C 5% CO_2. Duplicate wells were used for each condition. Monoclonal mouse anti-monkey CD3 (30μg/ml, Biosource International, Camarillo, CA) was used as a positive control and media was used as a negative control. Mycobacterial culture filtrate protein (CFP) (10μg/ml), and peptide pools of M.

tuberculosis-specific proteins ESAT-6 (Rv3875) (10μg/ml) and CFP-10 (38-1/Rv3874) (2μg/ml) were used to stimulate.

PBMC ELISPOTs were performed at 6 weeks post infection and at the time of necropsy. PBMCs were added at a concentration of 2 x 10^5 cells/well in RPMI 10% human AB serum. BAL cell ELISPOTS were done monthly over the course of infection. BAL cell were added at a concentration of 1 x 10^5 cells/well with 5 x 10^3 dendritic cells/well with and without stimulators. Cells were incubated at 37°C 5% CO_2 for approximately 40 hours.

ELISPOTs were developed by serial washes with deionized water and 1X PBS 0.01% Tween-20. Plates were incubated with 100μl of biotinylated anti-human IFNγ antibody (2.5μg/ml, Mabtech, Nacka Strand, Sweden) at 37°C 5% CO_2 for 2 hours. Following the incubation, the plates were washed multiple times with 0.01% Tween-20 in 1X PBS and strepavidin-horseradish-peroxidase-Q (Mabtech) was diluted 1:100 and added to each well for 1 h at 37°C. Spots were visualized by staining with AEC peroxidase kit as per the manufacture's instructions and counted using an automatic ELISPOT reader. Data were collected by image acquisition 4.5 and analyzed by Immunospot 5.0.3 (Cellular Technology Ltd. (Shaker Heights, OH)

6.2.12 Statistics

To compare two groups when data were determined to have normal distribution, a student t-test was used. If data did not have a Gaussian distribution, Mann-Whitney was used for unpaired data and Wilcox Signed Rank for paired data. Comparison of contiguous data over time was analyzed by repeated measures ANOVA and if significant, pair-wise comparison was by Tukey-Newman tests. $p<0.05$ was considered statistically significant.

6.3 RESULTS

6.3.1 Infection outcome is independent of age, gender and weight

The NHP is the only animal model that mimics the spectrum of M. tuberculosis infection (latent and active disease) seen in humans [30]. Infection outcomes are determined by clinical criteria and severity of disease quantified at necropsy [30]. *M. tuberculosis* infection is confirmed by tuberculin skin test, antigen-specific ^{3}H-thymidine incorporation, and IFNγ production by ELISPOT. Data for the monkeys used here are summarized in Table 1. As reported in other studies, all non-human primates were positive for immunologic indicators of infection and infection outcome was independent of age, gender and weight [30].

Infection outcome	**Total #**	**#M/#F**	**Age (y/o)**	**Weight (Kg)**	**%+ TST**	**%+LPA**	**%+IFNγ**
Active	19	16/3	7.7±1.7	6.0±1.3	100	100	100
Latent	21	18/3	8.3±1.4	6.5±1.0	100	100	100

Table 1. Summary of vital statistics and immunologic assays of all non-human primates used in this study.

The table summarizes the total number of animals that developed latent infection or active disease as well as the total number of each gender. The mean age and weight and standard deviation for each group are listed. Following inoculation, non-human primates are tested for infection by skin test (TST+), lymphoproliferation (LPA) and IFNγ production by ELISPOT (IFNγ+) to *M. tuberculosis* antigen.

6.3.2 CD4+ Foxp3+ cells are found within involved and not uninvolved lung and draining lymph node tissue

Cells from involved lung and thoracic LN at necropsy were stained for CD3, CD4, CD25 and Foxp3 (Figure 27A, B). In tissues, not all Foxp3+ cells are CD25+ and not all CD25+ cells are Foxp3+. Classically, Tregs have been defined as CD4+CD25^{hi} cells. However, CD25 is also found on activated effector T cells and where immune stimulation is continuous, (e.g. chronic infection), one is unable to distinguish Tregs from effector populations with this single marker. The transcription factor Foxp3 denotes a Treg phenotype [148]. In humans, Foxp3 is transiently expressed during priming, but transfection of constitutively active Foxp3 conferred the Treg phenotype [244]. CD4+Foxp3+ cells in PBMC expressed Treg-associated cell surface markers and did not produce cytokine in response to *M. tuberculosis* antigens (Figure 28). Thus we used CD3+CD4+Foxp3+ to define the Treg population.

To address whether Tregs were preferentially localized to areas of lung containing bacilli, tissue homogenates were divided into *M. tuberculosis*+ (involved) or *M. tuberculosis*- (uninvolved), based on whether *M. tuberculosis* was cultured from the sample. Involved lung tissue (granulomas) contained a significantly higher frequency of both total CD4+ T cells (Figure 27C) and Tregs (Figure 27D) than uninvolved lung. Similar to murine studies [171], lymphocytes localized to pulmonary sites that contained *M. tuberculosis*. Interestingly, when thoracic LN from infected NHP were divided into *M. tuberculosis*+ and *M. tuberculosis*- samples, involved LN had significantly fewer CD4 T cells than uninvolved LN (Figure 27E), but there was a trend toward more Tregs within the CD4 subset (Figure 27F). It may be that fewer T cells are present in involved LN because of effacement by granulomas with caseous necrosis.

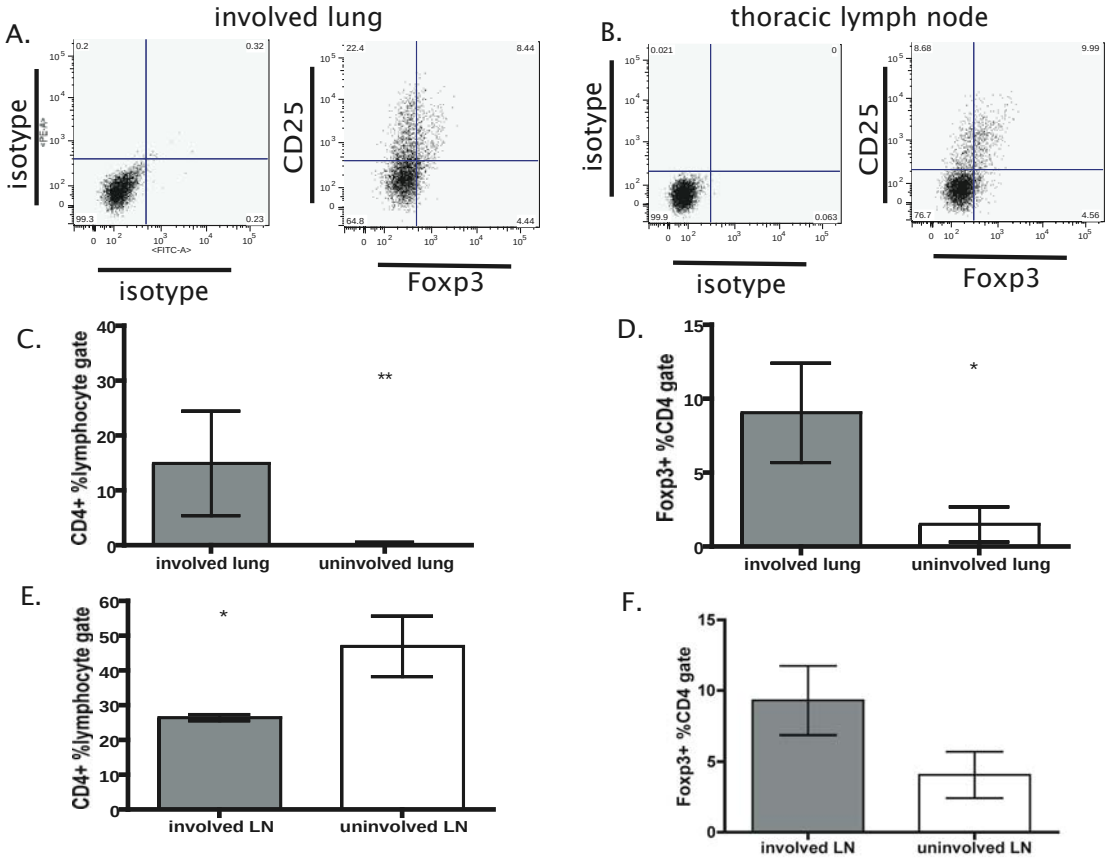


Figure 27. Regulatory T cells are enriched in involved lung tissue and lymph node

The frequency of Tregs (CD3+CD4+Foxp3+) was determined by gating on live cells, lymphocytes and CD3+CD4+ cells in involved (M. tuberculosis+) and uninvolved (M. tuberculosis-) lung and thoracic lymph node at necropsy. Representative dot plots of A) involved lung and B) lymph node from an infected monkey. The frequency of CD4+ cells (C, E) and Tregs within CD4 gate (D, F) in lung or lymph node were compared within involved and uninvolved tissue.

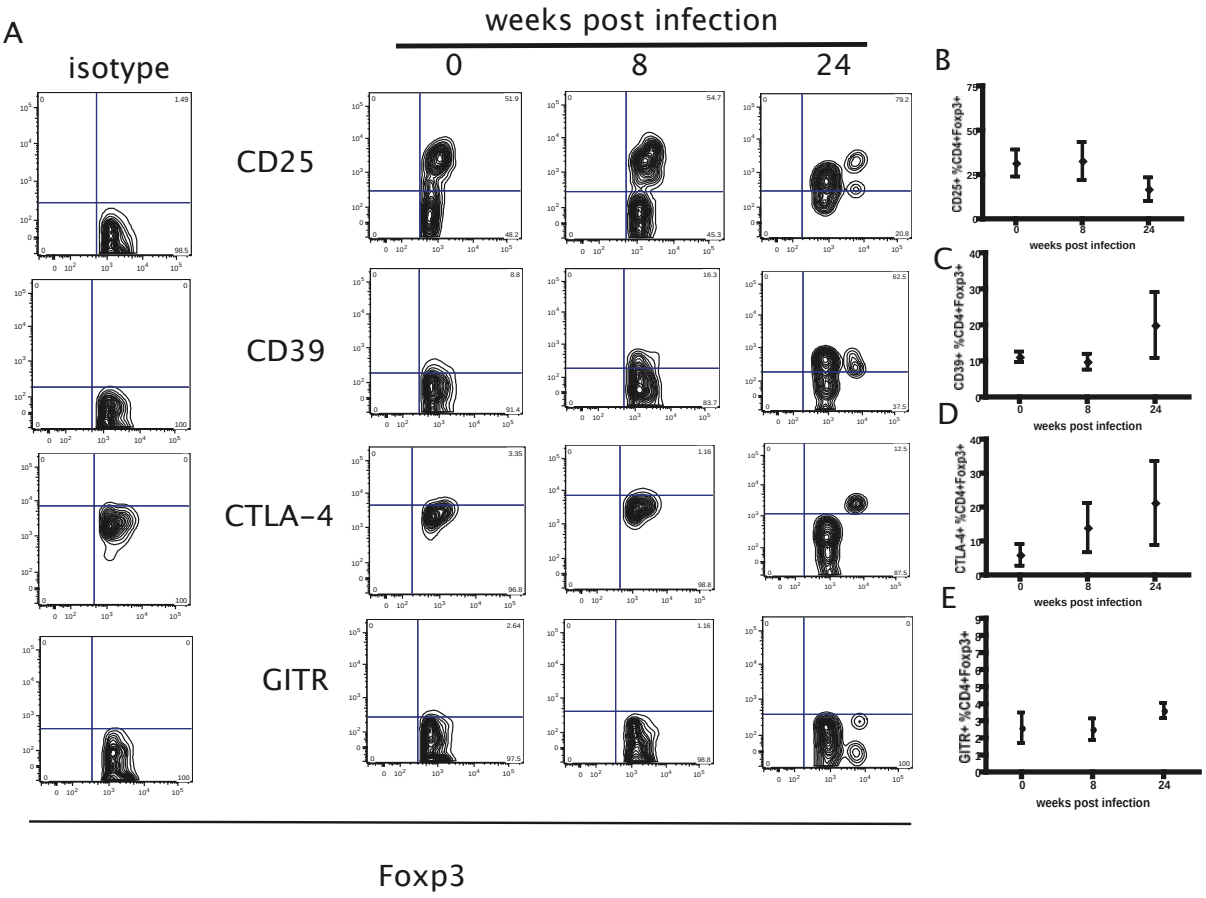


Figure 28. Cellular surface markers on Tregs

CD4+Foxp3+ Tregs were analyzed for expression of Treg associated cell surface markers at 0, 8 and 24 weeks post infection. A) Shows representative plots of CD25 (B), CD39 (C), intracellular CTLA-4 (D) and GITR (E).

6.3.3 CD4+Foxp3+ T cells are found within the lymphocyte cuff of the granuloma

Immunofluorescent staining demonstrated that CD3+Foxp3+ Tregs are abundant within the lymphocyte cuff of granulomas (Figure 29), but rare in uninvolved lung. These data support that Tregs preferentially localize to involved tissue (granulomas) and are proportionally increased in thoracic LN. Thus Tregs may control the local immune response within granulomas.

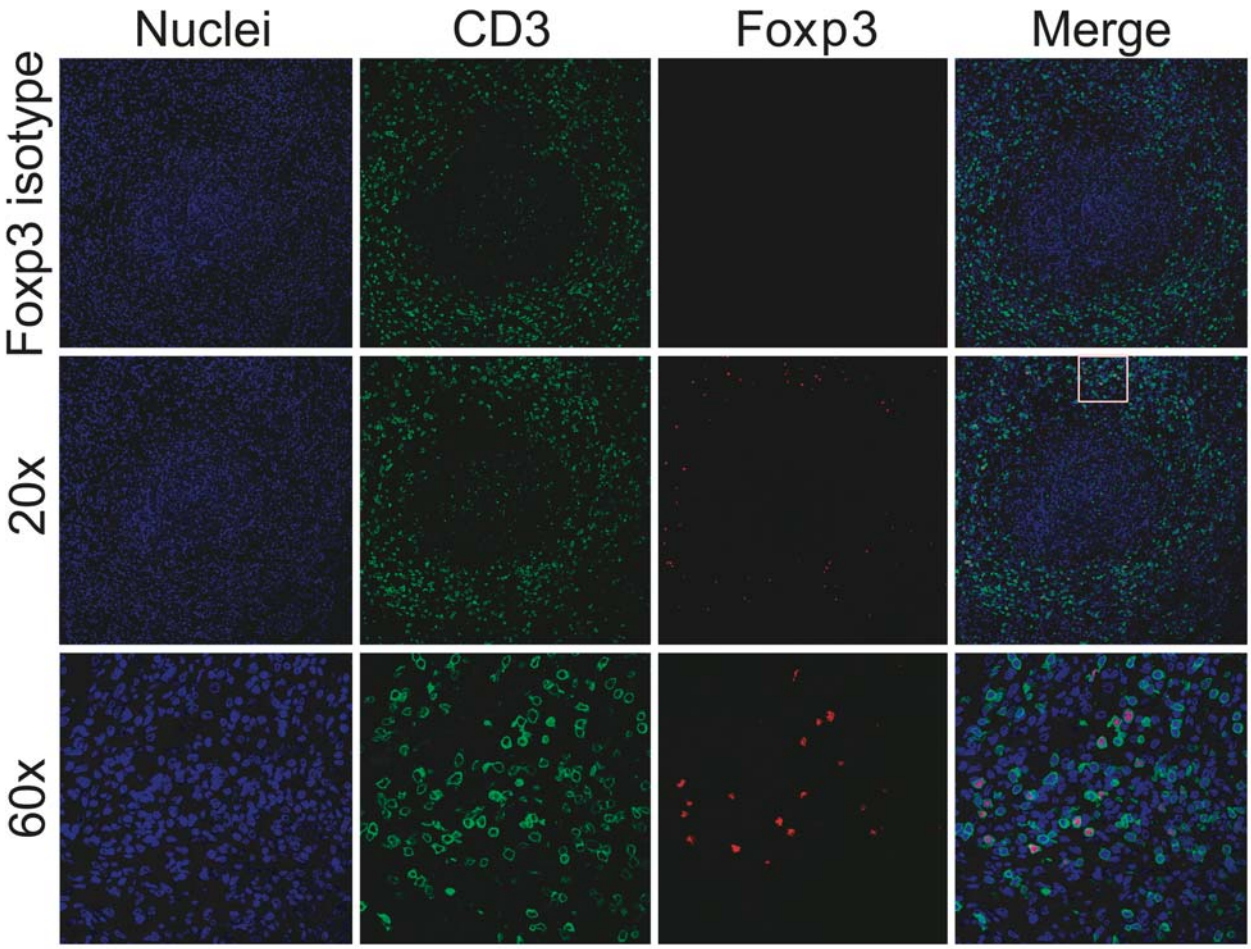


Figure 29. Regulatory T cells in granulomas

Paraffin-embedded granulomatous lung tissue was immunofluorescently stained for nuclei (blue), CD3 (green) and Foxp3 (red). The 60x section is an enlargement of box indicated on merged image from 20x.

6.3.4 Tregs migrate from peripheral blood to airways during early *M. tuberculosis* infection

We analyzed PBMCs from 41 NHPs (infected for other studies) for six months after inoculation. The frequency of Tregs decreased dramatically within the first two weeks and was significantly lower than pre-infection levels 8-12 weeks p.i. (Figure 30A), while CD4 T cell frequencies were unchanged (Figure 30C).

The reduction in Treg frequencies in the periphery during early infection suggested that Tregs were migrating to the lungs. BAL is the only relatively non-invasive way to serially sample the pulmonary environment. For 6 months, BAL cells were obtained monthly from 17 macaques and stained for Tregs (Figure 30B). By 4 weeks p.i., the frequency of Tregs increased in airways, corresponding to the reduction in the periphery. After an initial influx of Tregs into airways, the frequency of Tregs fluctuated until 16 weeks p.i., when both Tregs and CD4 T cells (Figure 30B,D) increased. These data suggest Tregs are migrating from blood to the lungs soon after *M. tuberculosis* infection.

6.3.5 Pre-infection levels of Tregs in PBMC correlate with clinical outcome

Of 41 macaques followed, 22 developed active disease and 19 had latent infections. Surprisingly, the animals that became latent had significantly higher frequencies of Tregs prior to infection in PBMCs compared to monkeys that developed active disease ($p=0.0116$)(Figure 30E). Although all NHP exhibited an initial decrease of Tregs, latently infected animals maintained higher levels of Foxp3+ cells compared to those with active disease at 8 weeks p.i. (Figure 30E). By 16 weeks, all animals returned to pre-infection levels of Tregs. Those that would develop latent infection maintained pre-infection Treg frequencies, while Foxp3+ cells continued to increase up to 28 weeks p.i. in those developing active disease. Latent NHP demonstrate a trend of more Tregs in BAL by 4 wks p.i. compared with those that would develop active disease (Figure 30F), although this was not statistically significant.

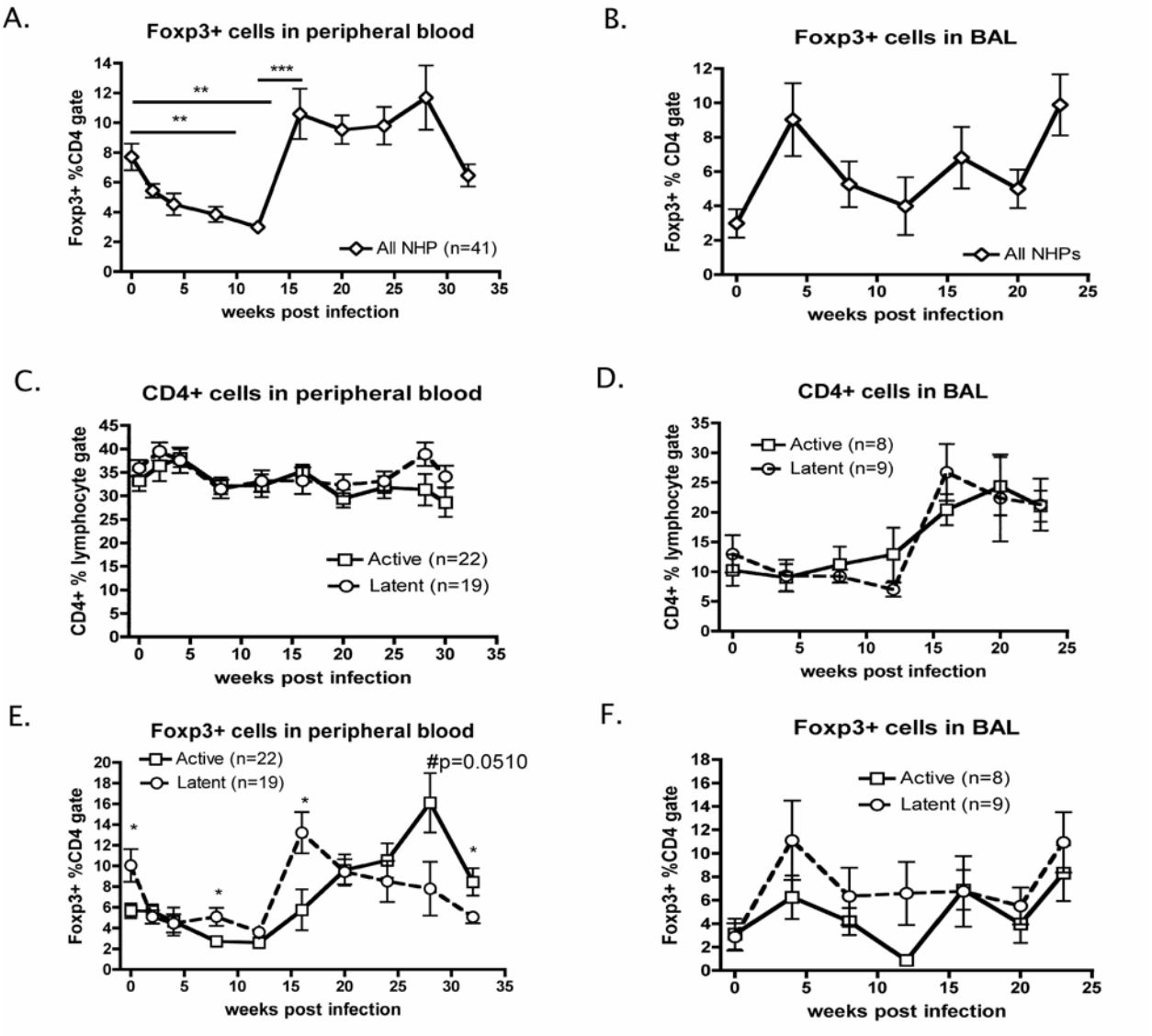


Figure 30. Tregs in PBMC and airways during *M. tuberculosis* infection

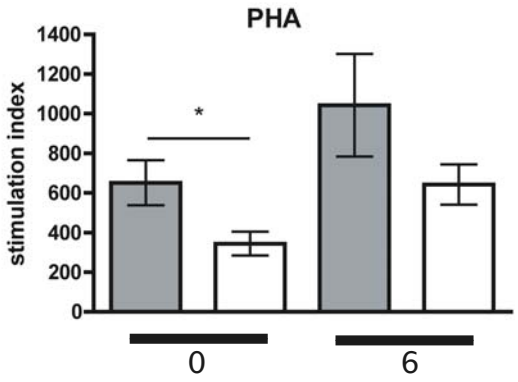
A) The frequency of Tregs (CD3+CD4+Foxp3+) in PBMC during *M. tuberculosis* infection (n=41 monkeys) was determined by gating on live cells, lymphocytes and CD3+CD4+ cells. B) BAL cells from infected NHPs (N=17) were stained and gated as above. $p < 0.05$ was considered significant. PBMCs and BAL cells were stained and compared between active disease (open squares) and latently infected (open circles) groups. The frequency of CD4 T cells within the lymphocyte gate (C, D) or Foxp3+ cells within CD4 gate (E, F) for blood (C, E) and airways (B, D) are represented. Statistical significance over time (A) was determined by repeated measure ANOVA ($p<0.0001$) with a Tukey-Newman post-test. (C-F) Mann-Whitney determined significance between each group at each time point.

6.3.6 Foxp3+ cells affect proliferative responses in peripheral blood

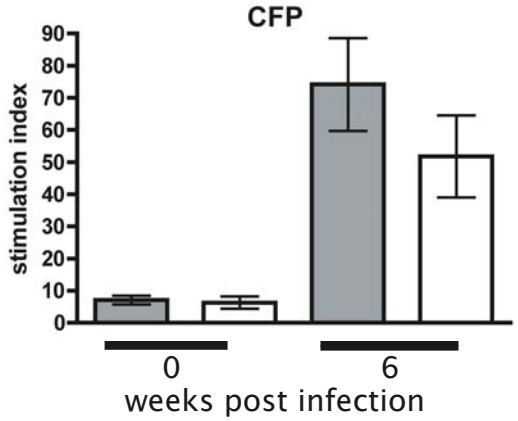
To determine whether increased Tregs correlated with a reduced proliferative response, PBMCs were stimulated with the mitogen PHA or mycobacterial CFP. Prior to infection, PBMCs from animals that would become latent demonstrated significantly less proliferation in response to PHA than PBMCs from animals destined to develop active TB (Figure 31A), correlating with more peripheral Tregs in latent monkeys (Figure 30E). By 6 weeks p.i., the latent group exhibited a trend toward decreased proliferation in response to both PHA (Figure 31A) and CFP (Figure 31B). Tregs were not measured at 6 weeks, but the frequency of Tregs is significantly increased at 8 weeks p.i. in latently infected monkeys compared to those that would develop active disease. All NHPs showed increased proliferation to both stimuli by 6 weeks p.i. and had significantly lower frequencies of Treg compared to pre-infection by 8 weeks. These data support an association between decreased proliferation and more Tregs, suggesting that Tregs may limit T cell proliferation.

We tested whether depletion of Tregs would reverse this reduced response. No convenient method for depleting Foxp3+ cells from primates exists, so CD25+ cells were depleted from PBMC (Figure 31C). Depletion of CD25+ cells resulted in increased proliferation in seven of ten animals for both stimuli (Figure 31D,E).

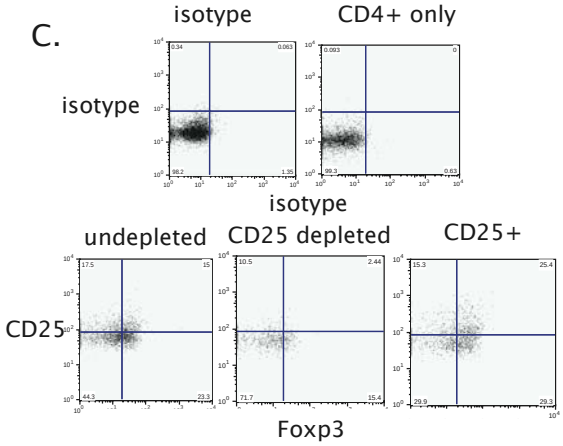
A.


PHA
stimulation index
1400
1200
1000
800
600
400
200
0
*
0
6

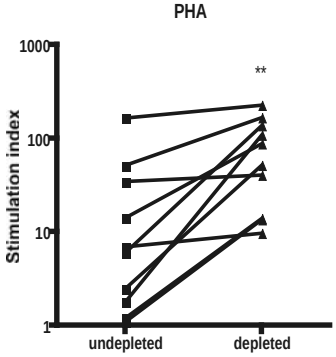
B.


CFP
stimulation index
90
80
70
60
50
40
30
20
10
0
0
6
weeks post infection

C.


isotype
CD4+ only
isotype
isotype
undepleted
CD25 depleted
CD25+
CD25
Foxp3

D.


PHA
Stimulation index
1000
100
10
1
**
undepleted
depleted

E.

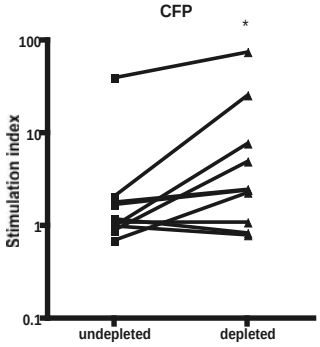

CFP
Stimulation index
100
10
1
0.1
*
undepleted
depleted

Figure 31. Tregs inhibit PBMC proliferation

PBMCs from animals that would develop active disease (shaded bars, N=22) or latent infection (open bars, N=19) at 0 and 6 weeks p.i. were stimulated with A) PHA and B) CFP, and proliferation measured. Data reported as stimulation index. Statistical significance was determined by Mann-Whitney. CD25+ cells were depleted from PBMCs (N=10) (C), and stimulated as above. Comparison of PBMC proliferative response to PHA (D) and CFP (E) before and after CD25 depletion is reported as a stimulation index statistical significance was determined by Wilcox Signed Rank.

6.4 DISCUSSION

The immune response against *M. tuberculosis* is a tightly controlled balance between sufficient inflammation to limit *M. tuberculosis* growth and regulatory factors that prevent damage of surrounding tissue. We sought to determine whether the frequency of regulatory T cells in blood and airways was associated with outcome of infection in a macaque model of TB. We hypothesized that if Tregs were involved in modulating the immune response to *M. tuberculosis* and limiting damage to tissue, Tregs would be found at the site of infection. If regulatory T cells contributed to development of active disease, we would expect the following: First, animals that develop primary TB would have a higher frequency of Tregs in blood and airways prior to or during early infection when compared to those that develop latent infection. Second, an increased frequency of Tregs would correspond with decreased proliferation when PBMCs were stimulated with antigens. Conversely, if increased Tregs were not a causative factor for active TB, but a response to inflammation, a disparity in Tregs would become apparent between the two groups when those with active disease failed to control bacterial growth.

In healthy individuals, *M. tuberculosis* infection is predominantly localized to the lung and thoracic LN. Bacilli and granulomas are most commonly found in lung parenchyma, rather than airways, making sampling involved tissues difficult. In humans, timing of infection, as well as dose and strain of inoculum are unknown, and samples prior to infection are not generally available for comparison. Since these factors can contribute to the quality of the immune response and infection outcome, we used the macaque model of TB. This model resembles human TB in disease outcome and pathology, and inoculum size, strain and timing of infection can be controlled [239]. PBMC and BAL cells can be obtained frequently, and tissue samples are available at necropsy. In these studies, not all Foxp3+ cells are CD25+ and not all CD25+ cells are Foxp3+; we defined our Treg cell population as CD3+CD4+Foxp3+. In blood we are most likely measuring Foxp3+ natural Tregs [163]; cells found within airways and tissues may be primed before migrating to the site of infection. Here we show that Tregs are present in involved lung and LN and localize to the lymphocyte cuff of granulomas, suggesting they may modulate immune responses within the granuloma.

We examined the association between Tregs in blood and airways of infected monkeys and development of active disease and found surprising results. Whereas the literature [177, 178, 180, 181, 215] supports an association between active disease and increased Tregs in blood of humans, we found that monkeys who developed latent infection had a significantly higher frequency of Tregs in PBMC prior to and during early infection than those that developed primary TB. The increased frequency of Tregs in monkeys that became latently infected correlated with decreased proliferation to mitogen prior to *M. tuberculosis* infection. When CD25+ cells were depleted from PBMCs, several NHPs exhibited a modest increase in proliferation when stimulated with CFP and PHA. Our findings do not support the hypothesis

that more Tregs and a less vigorous lymphoproliferative response during the initial phase of *M. tuberculosis* infection would predispose an individual to development of primary TB. On the contrary, our data indicate that more Tregs prior to infection correlates with a better infection outcome.

These data led us to ask whether the increased frequency of Tregs associated with active disease is a response to increased inflammation. Regardless of infection outcome the frequency of Foxp3+ cells dramatically decreased in blood within the first 8 weeks. This early reduction in Foxp3+ cells suggests that these cells are poised to sense inflammation and migrate quickly to the site of infection, and mirrors a drop reported in peripheral blood of healthy human contacts in the Gambia [215]. At the same time, the frequency of Tregs increased in airways, supporting the hypothesis that these cells migrate to the lungs. *In vitro* experiments on lymphocytes from both humans and mice indicate that natural Tregs change their chemokine receptor phenotype more quickly than naïve/resting T cells in the periphery [245]. This may be a mechanism for rapidly increasing Tregs at the infection site for expansion during the initial burst of inflammation to protect surrounding tissue from damage. While all monkeys returned to baseline levels of Tregs in PBMC by 16 weeks p.i., those with latent infection remained steady while Tregs in NHPs with active disease began to increase.

In summary, these data indicate that following infection, Tregs rapidly decrease in peripheral blood, and are maintained at low levels in the periphery for ~4 months. When bacterial growth is under control, i.e. during latent infection, peripheral levels of Tregs return to pre-infection levels. However, when bacterial growth is not contained and active disease develops, Tregs continue to increase, perhaps acting to dampen peripheral inflammation as a protective mechanism. Based on this concept, we propose a model where Tregs in peripheral

blood act as important regulators poised to protect uninvolved tissues from potentially damaging antimycobacterial immune responses. Upon infection Treg migrate to the lung and draining LN to protect "healthy" tissue and limit inflammation. Tregs are retained within granulomas and involved LN along with effector T cells. Provided the T cell response is sufficient to contain *M. tuberculosis* infection, Tregs control damage to surrounding tissue by limiting proliferation of effector T cells in granulomas. In latent infection, peripheral responses are limited due to containment of antigen within granulomas and control of bacterial growth. However if the immune response is insufficient to control bacterial replication, antigenic burden increases, resulting in increased inflammation. In this instance, Tregs in the periphery may limit the inflammation associated with active disease. The increased Treg frequency observed in blood of people with active TB is likely a response to inflammation and bacterial burden, and not a predetermining or contributing factor to active disease, at least during the initial stages of infection.

Control of *M. tuberculosis* is a dynamic balance between inflammation and immune regulation maintained by the host. Here we explored the role of Treg cells in the dynamics of this response during initial stages of infection. Further understanding of how these cells are functioning at the site of infection as well as the signals responsible for early exodus from the periphery will provide valuable information about the microenvironments needed to maintain control of *M. tuberculosis* infection.

7.0 SUMMARY OF THE DISSERTATION

Mycobacterium tuberculosis is an acid fast, obligate intracellular bacteria and the etiologic agent of TB. An exquisite pathogen that has been evolving with the human race for millennia, the effects of *M. tuberculosis*-mediated bone disease (e.g. Potts disease) have been found in the mummies of such disparate ancient cultures as Peru, Israel, Egypt and China [1-4]. Its long history with humans has enabled *M. tuberculosis* to become widely distributed throughout the global population with an estimated 1/3 of the world's population infected. While only a small percentage of those infected ever develop active disease, the potential reservoir for tuberculosis is enormous [9].

The AIDS epidemic in the 1980's brought TB back to the forefront with a vengeance [105]. The burden of this resurgence has been the developing world, a place that lacks sufficient public health infrastructure to administer DOTS therapy on a large-scale basis. Chemotherapy is long and comprised of multiple chemotherapeutic agents with a plethora of side effects. Many of those with disease fail to complete the treatment regimen and contribute to the development and transmission of the growing number of multi drug resistant and extensively drug resistant strains of *M. tuberculosis* [9, 239]. Finally lack of an effective vaccine for disease prevention and the development of relatively few new effective chemotherapeutic agents in the last 50 years make it imperative that more research is conducted to better understand what constitutes an effective immune response against this pathogen.

M. tuberculosis is spread when droplets containing the bacilli are inhaled into the airways and phagocytosed by alveolar macrophages and dendritic cells. Once inside the phagosome, their primary residence, the bacilli begin a complex series of interactions with the host. Eventually infected dendritic cells migrate from the lungs to the draining lymph nodes and prime an adaptive immune response. Once primed, antigen specific CD4 and CD8 T cells migrate back to the site of infection and activate *M. tuberculosis* infected macrophages to kill some but not all resident bacilli. In addition, T cells wall off the macrophages to form the granuloma. The granuloma, a collection of immune cells at the site of infection, protects the host by containing the bacteria, however it also provides a niche for the bacterium to persist [16, 190]. Thus the granuloma, while containing infection also becomes the site of the host: pathogen standoff.

Maintenance of this standoff is in the best interest of both the host and bacilli. If the bacillus was to kill the host, it loses the chance to spread and continue to thrive. In contrast the host must walk a fine line between sufficient inflammation to control bacterial growth and limiting damage to surrounding uninvolved healthy tissue. The work presented within this dissertation sought to better elucidate the role CD4 T cells played in the immune response to *M. tuberculosis*.

CD4 T cells are essential for an effective immune response against *M. tuberculosis*. Their importance was first shown in HIV+ individuals whose incidence of TB increases as CD4 T cell counts decrease. Similar results have also been shown in SIV infected NHPs[105, 106]. In addition, mice deficient in CD4 T cells fail to control infection and succumb much sooner than WT mice [103]. CD4 T cells are able to both promote and inhibit inflammation. Naïve CD4 T cells can be primed to be pro-inflammatory and exhibit a Th1, Th2 or Th17 phenotype [108]. During the course of *M. tuberculosis* infection, both Th1 and Th17 CD4 T cells are

primed [144]. While Th1 cells are required for control of the bacilli, the role of Th17 has yet to be completely defined. Th1 cells produce IFNγ, IL-2, TNF and GMCSF. These cells act to help prime an effective CD8 T cell response, condition DCs to be effective APCs, and produce cytokines that activate macrophages to kill intracellular bacteria. Th17 cells produce IL-17 and are thought to work to recruit lymphocytes and other cells into the parenchyma [108]. While a few CD4 T cells produce IL-17, γδ T cells are the primary source of IL-17 during the early course of *M. tuberculosis* infection [83]. Interestingly, even though both cytokines are produced, IFNγ and IL-17 are produced by separate and distinct T cell populations.

To determine how CD4 T cells contribute to the immune response *to M.* tuberculosis infection the work presented in this dissertation utilized both the murine and non-human primate models of infection. Previous studies focused on the immune response to *M. tuberculosis* have relied heavily on knockout and transgenic mouse models in conjunction with antibody depletion studies. Without question the power of these tools has been the relatively low cost and the ease with which the system can be manipulated. These studies have provided valuable insight into the identity of key players involved in infection control (e.g. CD4 T cells [103]). However powerful, these approaches are limited by the loss or gain of function on a global scale and are unable to address the timing and source of specific components of the immune system. Here we present a new adoptive transfer model that allows manipulation of individual cell populations and addresses when and from what source important mediators of the immune response are required (Chapter 3).

To determine whether IFNγ from sources other than CD4 T cells was sufficient to contain *M. tuberculosis* infection we used our new model. The data suggest that both the presence of CD4 T cells and IFNγ produced by CD4 T cells are required for host survival. In addition, the

data suggest that IFNγ from CD4 T cells directly affects the quality of the CD8 T cell response. The data indicate in the absence of IFNγ, T cells can activate macrophages to limit growth, but not kill intracellular pathogens. CD4 T cells that were unable to produce IFNγ produced IL-17 and prolonged host survival when compared to those lacking CD4 T cells. However, these animals eventually succumb, indicating that the Th17 cells are not sufficient to contain infection. In addition to IFNγ production, the presence of CD4 T cells is required to initiate or sustain an effective immune response and this is most likely due to IL-2 production. The new adoptive transfer model described in this dissertation will allow for future more detailed studies that investigate cytokine and chemokine production by specific cell types (Chapter 3).

While the adaptive immune response is required for control of bacterial growth, the host must also limit the response to involved tissue to protect surrounding uninfected lung [246]. CD4 T cells are also capable of regulating the immune response to infection as Tregs. Natural Tregs are thymically selected, expand in the draining lymph node during infection and migrate to the site of infection once primed. These cells are antigen specific and have been shown to express the Th1 specific transcription factor T-bet during priming, indicating that these cells expand in the presence of the Th1 cytokine milieu.

To determine whether Tregs were important during *M. tuberculosis* infection, we utilized antibody depletion to investigate the effects of Treg elimination prior to infection. The data presented indicate that elimination of Tregs prior to infection does not influence either survival or antigenic burden in mice (Chapter 4). Human *M.* tuberculosis infection results in either latent infection or active TB and what contributes to these disparate infection outcomes is yet unknown. *M. tuberculosis* infection in mice is a chronic progressive infection, thus what

contributes to the development of either active disease or latent infection cannot be addressed with these models.

To assess the relationship between Treg frequency and development of active disease or latent infection, we chose the non-human primate model. To date it is the only well defined animal model to recapitulate human infection outcomes of latent and active disease. Human studies have suggested that an increased frequency of Tregs contributes to the development of active disease [178-180, 247]. However, this association is limited to the comparison of individuals once infection is established, without knowing what frequency of Treg was prior to infection. Studies in humans are also limited by not knowing the size and strain of the inoculum, the length of infection and the immune status of the host, all of which can affect the immune response to *M. tuberculosis* infection. In addition, studies are limited to what types of imaging (X-ray, MRI, CT-PET scan) and samples (BAL, PBMC) can be obtained easily and safely. Thus with human studies alone, one is not able to determine whether Tregs predispose an individual to develop active disease or whether Tregs increase in frequency in response to the inflammation found in those with active disease. In NHP, regardless of infection outcome, within two weeks of infection, Tregs decrease dramatically in the peripheral blood and increase in the airways. We speculate that this early migration to infected tissues may be to limit possible damage to the lung tissue and control inflammation at the site of infection. Interestingly, a higher frequency of Tregs prior to infection correlated with the development of latent infection. However, once infection was established, NHP with active disease exhibited increasing frequency of Tregs in the peripheral blood (Chapter 6). Taken together, these data show that a higher frequency of Tregs prior to infection correlated with a better infection outcome and the increased frequency of Tregs found in those with active disease occurred once active disease was established [248].

These experiments challenge the notion that Tregs predispose the host toward development of active disease and introduce the idea that regulation of the immune system may be important in maintaining the host: bacilli standoff.

Finally, our laboratory has previously published that long term IL-12 treatment of mice during the first two months of *M. tuberculosis* infection results in decreased cellular infiltration into infected tissues without decreased bacterial burden [225]. These results suggest that IL-12, while an essential cytokine in control of this infection, may have anti-inflammatory properties in addition to pro-inflammatory ones. Here we show that IL-12 treatment results in decreased cellular infiltrate associated with an increased frequency of Tregs that is independent of bacterial burden (Chapter 5). Thus while the presence of Tregs in a chronic progressive infection may not alter host survival, they may play a role in limiting damage sustained in surrounding uninvolved tissues. These data provide new insight about what is necessary and needed for a sufficient appropriate immune response to *M. tuberculosis* infection. These results add to the growing body of literature that report that immunoregulatory responses maybe as important as the pro-inflammatory ones during infection to prevent immunopathologic damage to healthy tissues. By priming the immunoregulatory response at the same time as the pro-inflammatory immune response, the host is in effect laying the groundwork for peace while waging the war.

Based on our data and recently published literature, we propose that an effective immune response is one in which the pro-inflammatory response is balanced effectively with the immunoregulatory response, presumably resulting in latent infection. If one of the responses is out of balance, for example too much inflammation, one might expect to see damage to surrounding healthy tissue or erosion of the granuloma into an airway, thus allowing the bacilli to escape when the host coughs. In contrast if the host mounts too much of an immunoregulatory

response, one might expect that the host is unable to control bacterial growth and may develop active disease.

To date, we lack an effective vaccine for prevention of *M. tuberculosis* infection and the development of active disease. BCG, the current gold standard vaccine has been in use for 90 years and has variable efficacy. In addition, co-infections in endemic areas influence the outcome of vaccination. Thus better understanding of what constitutes an effective response against *M. tuberculosis* is imperative. Traditionally, vaccination of many diseases has resulted in sterilizing immunity; this may not be possible with vaccination against *M. tuberculosis*.

BIBLIOGRAPHY

1. Chalke HD. The Impact of Tuberculosis on History, Literature and Art. Med Hist 1962;6:301-18
2. Hershkovitz I, Donoghue HD, Minnikin DE, et al. Detection and molecular characterization of 9,000-year-old Mycobacterium tuberculosis from a Neolithic settlement in the Eastern Mediterranean. PLoS One 2008;3:e3426
3. Salo WL, Aufderheide AC, Buikstra J and Holcomb TA. Identification of Mycobacterium tuberculosis DNA in a pre-Columbian Peruvian mummy. Proc Natl Acad Sci U S A 1994;91:2091-4
4. Daniel TM. The history of tuberculosis. Respir Med 2006;100:1862-70
5. (WHO) WHO. WHO annual report on global TB control- summary, 2008:10
6. Wilson LG. The historical decline of tuberculosis in Europe and America: its causes and significance. J Hist Med Allied Sci 1990;45:366-96
7. von Piroquet CF. Frequency of tuberculosis in childhood. J Am Med Assoc 1909;52:675-678
8. Schwalbe N, Harrington P. HIV and tuberculosis in the former Soviet Union. Lancet 2002;360 Suppl:s19-20
9. WHO report 2009.
10. Glaziou P, Floyd K and Raviglione M. Global burden and epidemiology of tuberculosis. Clin Chest Med 2009;30:621-36, vii
11. Schaaf HS, Moll AP and Dheda K. Multidrug- and extensively drug-resistant tuberculosis in Africa and South America: epidemiology, diagnosis and management in adults and children. Clin Chest Med 2009;30:667-83, vii-viii
12. Migliori GB, Centis R, Lange C, Richardson MD and Sotgiu G. Emerging epidemic of drug-resistant tuberculosis in Europe, Russia, China, South America and Asia: current status and global perspectives. Curr Opin Pulm Med;16:171-9
13. Migliori GB, Richardson MD and Lange C. Of blind men and elephants: making sense of extensively drug-resistant tuberculosis. Am J Respir Crit Care Med 2008;178:1000-1
14. Migliori GB, Sotgiu G, Jaramillo E, et al. Development of a standardised multidrug-resistant/extensively drug-resistant tuberculosis assessment and monitoring tool. Int J Tuberc Lung Dis 2009;13:1305-8
15. Connell TG, Ritz N, Paxton GA, Buttery JP, Curtis N and Ranganathan SC. A three-way comparison of tuberculin skin testing, QuantiFERON-TB gold and T-SPOT.TB in children. PLoS ONE 2008;3:e2624
16. Barry CE, 3rd, Boshoff HI, Dartois V, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol 2009;7:845-55

17. Lin PL, Flynn JL. Understanding latent tuberculosis: a moving target. J Immunol;185:15-22
18. Scheindlin S. The fight against tuberculosis. Mol Interv 2006;6:124-30
19. Hjaltested O TK. Clinical aspects of Pneumothorax therapy as illustrated by the results obtained in 191 cases of completed treatment. British Journal of Tuberculosis 1939;33:4-16
20. HJ B. A review of 100 consecutive cases of other with special reference to the advanced case. British Journal of Tuberculosis and Disease of the Chest 1951;45:131-137
21. Shiraishi Y, Nakajima Y, Katsuragi N, Kurai M and Takahashi N. Resectional surgery combined with chemotherapy remains the treatment of choice for multidrug-resistant tuberculosis. J Thorac Cardiovasc Surg 2004;128:523-8
22. Schatz A, Bugie E and Waksman SA. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. 1944. Clin Orthop Relat Res 2005:3-6
23. PREVENTION of streptomycin resistance by combined chemotherapy; a Medical Research Council investigation. Br Med J 1952;1:1157-62
24. ISONIAZID in pulmonary tuberculosis. Lancet 1952;2:19-21
25. Saltini C. Chemotherapy and diagnosis of tuberculosis. Respir Med 2006;100:2085-97
26. Campbell EA, Korzheva N, Mustaev A, et al. Structural mechanism for rifampicin inhibition of bacterial rna polymerase. Cell 2001;104:901-12
27. Nuermberger EL, Spigelman MK and Yew WW. Current development and future prospects in chemotherapy of tuberculosis. Respirology
28. Bayer R, Wilkinson D. Directly observed therapy for tuberculosis: history of an idea. Lancet 1995;345:1545-8
29. Capuano SV, 3rd, Croix DA, Pawar S, et al. Experimental Mycobacterium tuberculosis infection of cynomolgus macaques closely resembles the various manifestations of human M. tuberculosis infection. Infect Immun 2003;71:5831-44
30. Lin PL, Rodgers M, Smith L, et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. Infect Immun 2009;77:4631-42
31. Walsh GP, Tan EV, dela Cruz EC, et al. The Philippine cynomolgus monkey (Macaca fasicularis) provides a new nonhuman primate model of tuberculosis that resembles human disease. Nat Med 1996;2:430-6
32. Sharpe SA, McShane H, Dennis MJ, et al. Establishment of an aerosol challenge model of tuberculosis in rhesus macaques, and an evaluation of endpoints for vaccine testing. Clin Vaccine Immunol
33. Cardona PJ, Gordillo S, Diaz J, et al. Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with Mycobacterium tuberculosis. Infect Immun 2003;71:5845-54
34. Young D. Animal models of tuberculosis. Eur J Immunol 2009;39:2011-4
35. Via LE, Lin PL, Ray SM, et al. Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. Infect Immun 2008;76:2333-40
36. Dharmadhikari AS, Nardell EA. What animal models teach humans about tuberculosis. Am J Respir Cell Mol Biol 2008;39:503-8
37. Ratcliffe HL, Palladino VS. Tuberculosis induced by droplet nuclei infection; initial homogeneous response of small mammals (rats, mice, guinea pigs, and hamsters) to human and to bovine bacilli, and the rate and pattern of tubercle development. J Exp Med 1953;97:61-8
38. Swaim LE, Connolly LE, Volkman HE, Humbert O, Born DE and Ramakrishnan L. Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. Infect Immun 2006;74:6108-17

39. Cosma CL, Humbert O and Ramakrishnan L. Superinfecting mycobacteria home to established tuberculous granulomas. Nat Immunol 2004;5:828-35
40. Davis JM, Clay H, Lewis JL, Ghori N, Herbomel P and Ramakrishnan L. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity 2002;17:693-702
41. Davis JM, Ramakrishnan L. The role of the granuloma in expansion and dissemination of early tuberculous infection. Cell 2009;136:37-49
42. Vetvicka V, Thornton BP and Ross GD. Soluble beta-glucan polysaccharide binding to the lectin site of neutrophil or natural killer cell complement receptor type 3 (CD11b/CD18) generates a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonized target cells. J Clin Invest 1996;98:50-61
43. Thornton BP, Vetvicka V, Pitman M, Goldman RC and Ross GD. Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). J Immunol 1996;156:1235-46
44. Schlesinger LS. Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. J Immunol 1993;150:2920-30
45. Taylor ME, Drickamer K. Structural requirements for high affinity binding of complex ligands by the macrophage mannose receptor. J Biol Chem 1993;268:399-404
46. Downing JF, Pasula R, Wright JR, Twigg HL, 3rd and Martin WJ, 2nd. Surfactant protein a promotes attachment of Mycobacterium tuberculosis to alveolar macrophages during infection with human immunodeficiency virus. Proc Natl Acad Sci U S A 1995;92:4848-52
47. Pasula R, Downing JF, Wright JR, Kachel DL, Davis TE, Jr. and Martin WJ, 2nd. Surfactant protein A (SP-A) mediates attachment of Mycobacterium tuberculosis to murine alveolar macrophages. Am J Respir Cell Mol Biol 1997;17:209-17
48. Ernst JD. Macrophage receptors for Mycobacterium tuberculosis. Infect Immun 1998;66:1277-81
49. van der Wel N, Hava D, Houben D, et al. M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. Cell 2007;129:1287-98
50. Indrigo J, Hunter RL, Jr. and Actor JK. Cord factor trehalose 6,6'-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. Microbiology 2003;149:2049-59
51. Devergne O, Emilie D, Peuchmaur M, Crevon MC, D'Agay MF and Galanaud P. Production of cytokines in sarcoid lymph nodes: preferential expression of interleukin-1 beta and interferon-gamma genes. Hum Pathol 1992;23:317-23
52. Chan J, Fan XD, Hunter SW, Brennan PJ and Bloom BR. Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages. Infect Immun 1991;59:1755-61
53. Goren MB. Phagocyte lysosomes: interactions with infectious agents, phagosomes, and experimental perturbations in function. Annu Rev Microbiol 1977;31:507-33
54. Gordon AH, Hart PD and Young MR. Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature 1980;286:79-80
55. Hart PD, Young MR, Jordan MM, Perkins WJ and Geisow MJ. Chemical inhibitors of phagosome-lysosome fusion in cultured macrophages also inhibit saltatory lysosomal movements. A combined microscopic and computer study. J Exp Med 1983;158:477-92

56. Crowle AJ, Dahl R, Ross E and May MH. Evidence that vesicles containing living, virulent Mycobacterium tuberculosis or Mycobacterium avium in cultured human macrophages are not acidic. Infect Immun 1991;59:1823-31
57. Clemens DL, Lee BY and Horwitz MA. Deviant expression of Rab5 on phagosomes containing the intracellular pathogens Mycobacterium tuberculosis and Legionella pneumophila is associated with altered phagosomal fate. Infect Immun 2000;68:2671-84
58. Fratti RA, Backer JM, Gruenberg J, Corvera S and Deretic V. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. J Cell Biol 2001;154:631-44
59. Fratti RA, Chua J, Vergne I and Deretic V. Mycobacterium tuberculosis glycosylated phosphatidylinositol causes phagosome maturation arrest. Proc Natl Acad Sci U S A 2003;100:5437-42
60. Ferrari G, Langen H, Naito M and Pieters J. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 1999;97:435-47
61. Sherman DR, Sabo PJ, Hickey MJ, et al. Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proc Natl Acad Sci U S A 1995;92:6625-9
62. Dussurget O, Stewart G, Neyrolles O, Pescher P, Young D and Marchal G. Role of Mycobacterium tuberculosis copper-zinc superoxide dismutase. Infect Immun 2001;69:529-33
63. Master SS, Rampini SK, Davis AS, et al. Mycobacterium tuberculosis prevents inflammasome activation. Cell Host Microbe 2008;3:224-32
64. Fremond CM, Togbe D, Doz E, et al. IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to Mycobacterium tuberculosis infection. J Immunol 2007;179:1178-89
65. Dunn PL, North RJ. Virulence ranking of some Mycobacterium tuberculosis and Mycobacterium bovis strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. Infect Immun 1995;63:3428-37
66. Feng CG, Scanga CA, Collazo-Custodio CM, et al. Mice lacking myeloid differentiation factor 88 display profound defects in host resistance and immune responses to Mycobacterium avium infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. J Immunol 2003;171:4758-64
67. Edwards AD, Manickasingham SP, Sporri R, et al. Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. J Immunol 2002;169:3652-60
68. Bafica A, Scanga CA, Feng CG, Leifer C, Cheever A and Sher A. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. J Exp Med 2005;202:1715-24
69. Pompei L, Jang S, Zamlynny B, et al. Disparity in IL-12 release in dendritic cells and macrophages in response to Mycobacterium tuberculosis is due to use of distinct TLRs. J Immunol 2007;178:5192-9
70. Reiling N, Holscher C, Fehrenbach A, et al. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. J Immunol 2002;169:3480-4
71. Jang S, Uematsu S, Akira S and Salgame P. IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on TLR2-mediated recognition. J Immunol 2004;173:3392-7

72. Pathak SK, Basu S, Basu KK, et al. Direct extracellular interaction between the early secreted antigen ESAT-6 of Mycobacterium tuberculosis and TLR2 inhibits TLR signaling in macrophages. Nat Immunol 2007;8:610-8
73. Chan J, Tanaka K, Carroll D, Flynn J and Bloom BR. Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis. Infect Immun 1995;63:736-40
74. Flynn JL, Scanga CA, Tanaka KE and Chan J. Effects of aminoguanidine on latent murine tuberculosis. J Immunol 1998;160:1796-803
75. Liu PT, Stenger S, Li H, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 2006;311:1770-3
76. Jang S, Uzelac A and Salgame P. Distinct chemokine and cytokine gene expression pattern of murine dendritic cells and macrophages in response to Mycobacterium tuberculosis infection. J Leukoc Biol 2008;84:1264-70
77. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA and Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med 1993;178:2249-54
78. Flynn JL, Goldstein MM, Chan J, et al. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity 1995;2:561-72
79. Cooper AM, Kipnis A, Turner J, Magram J, Ferrante J and Orme IM. Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present. J Immunol 2002;168:1322-7
80. Feng CG, Jankovic D, Kullberg M, et al. Maintenance of pulmonary Th1 effector function in chronic tuberculosis requires persistent IL-12 production. J Immunol 2005;174:4185-92
81. Altare F, Ensser A, Breiman A, et al. Interleukin-12 receptor beta1 deficiency in a patient with abdominal tuberculosis. J Infect Dis 2001;184:231-6
82. Khader SA, Pearl JE, Sakamoto K, et al. IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-gamma responses if IL-12p70 is available. J Immunol 2005;175:788-95
83. Lockhart E, Green AM and Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 2006;177:4662-9
84. Demissie A, Abebe M, Aseffa A, et al. Healthy individuals that control a latent infection with Mycobacterium tuberculosis express high levels of Th1 cytokines and the IL-4 antagonist IL-4delta2. J Immunol 2004;172:6938-43
85. Kahnert A, Seiler P, Stein M, et al. Alternative activation deprives macrophages of a coordinated defense program to Mycobacterium tuberculosis. Eur J Immunol 2006;36:631-47
86. Turner J, Gonzalez-Juarrero M, Ellis DL, et al. In vivo IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice. J Immunol 2002;169:6343-51
87. Lewinsohn DA, Gennaro ML, Scholvinck L and Lewinsohn DM. Tuberculosis immunology in children: diagnostic and therapeutic challenges and opportunities. Int J Tuberc Lung Dis 2004;8:658-74
88. Rook GA, Dheda K and Zumla A. Immune responses to tuberculosis in developing countries: implications for new vaccines. Nat Rev Immunol 2005;5:661-7
89. Lawn SD, Butera ST and Shinnick TM. Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to Mycobacterium tuberculosis. Microbes Infect 2002;4:635-46

90. Jang AS, Park SW, Ahn MH, et al. Impact of circulating TGF-Beta and IL-10 on T cell cytokines in patients with asthma and tuberculosis. J Korean Med Sci 2006;21:30-4
91. Sadek MI, Sada E, Toossi Z, Schwander SK and Rich EA. Chemokines induced by infection of mononuclear phagocytes with mycobacteria and present in lung alveoli during active pulmonary tuberculosis. Am J Respir Cell Mol Biol 1998;19:513-21
92. Allavena P, Sica A, Vecchi A, Locati M, Sozzani S and Mantovani A. The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. Immunol Rev 2000;177:141-9
93. Robinson RT, Khader SA, Martino CA, et al. Mycobacterium tuberculosis infection induces il12rb1 splicing to generate a novel IL-12Rbeta1 isoform that enhances DC migration. J Exp Med;207:591-605
94. Hickman SP, Chan J and Salgame P. Mycobacterium tuberculosis induces differential cytokine production from dendritic cells and macrophages with divergent effects on naive T cell polarization. J Immunol 2002;168:4636-42
95. Reiley WW, Calayag MD, Wittmer ST, et al. ESAT-6-specific CD4 T cell responses to aerosol Mycobacterium tuberculosis infection are initiated in the mediastinal lymph nodes. Proc Natl Acad Sci U S A 2008;105:10961-6
96. Wolf AJ, Desvignes L, Linas B, et al. Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. J Exp Med 2008;205:105-15
97. Lazarevic V, Nolt D and Flynn JL. Long-term control of Mycobacterium tuberculosis infection is mediated by dynamic immune responses. J Immunol 2005;175:1107-17
98. North RJ, Izzo AA. Granuloma formation in severe combined immunodeficient (SCID) mice in response to progressive BCG infection. Tendency not to form granulomas in the lung is associated with faster bacterial growth in this organ. Am J Pathol 1993;142:1959-66
99. Deerojanawong J, Chang AB, Eng PA, Robertson CF and Kemp AS. Pulmonary diseases in children with severe combined immune deficiency and DiGeorge syndrome. Pediatr Pulmonol 1997;24:324-30
100. Hugosson C, Harfi H. Disseminated BCG-osteomyelitis in congenital immunodeficiency. Pediatr Radiol 1991;21:384-5
101. Humphreys IR, Stewart GR, Turner DJ, et al. A role for dendritic cells in the dissemination of mycobacterial infection. Microbes Infect 2006;8:1339-46
102. Tian T, Woodworth J, Skold M and Behar SM. In vivo depletion of CD11c+ cells delays the CD4+ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection. J Immunol 2005;175:3268-72
103. Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR and Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. J Immunol 1999;162:5407-16
104. Scanga CA, Mohan VP, Yu K, et al. Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. J Exp Med 2000;192:347-58
105. Aaron L, Saadoun D, Calatroni I, et al. Tuberculosis in HIV-infected patients: a comprehensive review. Clin Microbiol Infect 2004;10:388-98
106. Diedrich CR, Mattila JT, Klein E, et al. Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T cell depletion and not virus load. PLoS One;5:e9611

107. Fontenot JD, Dooley JL, Farr AG and Rudensky AY. Developmental regulation of Foxp3 expression during ontogeny. J Exp Med 2005;202:901-6
108. Zhu J, Yamane H and Paul WE. Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol;28:445-89
109. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A and Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 1993;260:547-9
110. Mullen AC, High FA, Hutchins AS, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science 2001;292:1907-10
111. Thieu VT, Yu Q, Chang HC, et al. Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. Immunity 2008;29:679-90
112. Sullivan BM, Jobe O, Lazarevic V, et al. Increased susceptibility of mice lacking T-bet to infection with Mycobacterium tuberculosis correlates with increased IL-10 and decreased IFN-gamma production. J Immunol 2005;175:4593-602
113. Usui T, Preiss JC, Kanno Y, et al. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. J Exp Med 2006;203:755-66
114. Jung YJ, Ryan L, LaCourse R and North RJ. Properties and protective value of the secondary versus primary T helper type 1 response to airborne Mycobacterium tuberculosis infection in mice. J Exp Med 2005;201:1915-24
115. Casanova JL, Abel L. Genetic dissection of immunity to mycobacteria: the human model. Annu Rev Immunol 2002;20:581-620
116. Mogues T, Goodrich ME, Ryan L, LaCourse R and North RJ. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. J Exp Med 2001;193:271-80
117. Sercan O, Stoycheva D, Hammerling GJ, Arnold B and Schuler T. IFN-gamma receptor signaling regulates memory CD8+ T cell differentiation. J Immunol;184:2855-62
118. Mueller H, Detjen AK, Schuck SD, et al. Mycobacterium tuberculosis-specific CD4+, IFNgamma+, and TNFalpha+ multifunctional memory T cells coexpress GM-CSF. Cytokine 2008;43:143-8
119. Cowley SC, Elkins KL. CD4+ T cells mediate IFN-gamma-independent control of Mycobacterium tuberculosis infection both in vitro and in vivo. J Immunol 2003;171:4689-99
120. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK and Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci U S A 1997;94:5243-8
121. MacMicking JD, Taylor GA and McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science 2003;302:654-9
122. Moreira AL, Tsenova L, Aman MH, et al. Mycobacterial antigens exacerbate disease manifestations in Mycobacterium tuberculosis-infected mice. Infect Immun 2002;70:2100-7
123. Nambiar JK, Ryan AA, Kong CU, Britton WJ and Triccas JA. Modulation of pulmonary DC function by vaccine-encoded GM-CSF enhances protective immunity against Mycobacterium tuberculosis infection. Eur J Immunol;40:153-61
124. Serbina NV, Lazarevic V and Flynn JL. CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during Mycobacterium tuberculosis infection. J Immunol 2001;167:6991-7000

125. Kamimura D, Bevan MJ. Naive CD8+ T cells differentiate into protective memory-like cells after IL-2 anti IL-2 complex treatment in vivo. J Exp Med 2007;204:1803-12
126. Kannanganat S, Ibegbu C, Chennareddi L, Robinson HL and Amara RR. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. J Virol 2007;81:8468-76
127. Kannanganat S, Kapogiannis BG, Ibegbu C, et al. Human immunodeficiency virus type 1 controllers but not noncontrollers maintain CD4 T cells coexpressing three cytokines. J Virol 2007;81:12071-6
128. Lindenstrom T, Agger EM, Korsholm KS, et al. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol 2009;182:8047-55
129. Sutherland JS, Adetifa IM, Hill PC, Adegbola RA and Ota MO. Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. Eur J Immunol 2009;39:723-9
130. N. Caccamo GG, SA. Joosten, G. Gelsomino, P. Dicarlo, L. Titone, D. Galati, M. Bocchino, A, Maratrese, A. Salerno, A. Sanduzzi, WP Franken, TH Ottenhoff, F. Dieli. Multifunctional CD4+ T cells correlate with active Mycobacterium tuberculosis infection. Eur J Immunol 2010;in press
131. Miller CM, Smith NC, Ikin RJ, Boulter NR, Dalton JP and Donnelly S. Immunological interactions between 2 common pathogens, Th1-inducing protozoan Toxoplasma gondii and the Th2-inducing helminth Fasciola hepatica. PLoS One 2009;4:e5692
132. Agrawal DK, Shao Z. Pathogenesis of allergic airway inflammation. Curr Allergy Asthma Rep;10:39-48
133. Killar L, MacDonald G, West J, Woods A and Bottomly K. Cloned, Ia-restricted T cells that do not produce interleukin 4(IL 4)/B cell stimulatory factor 1(BSF-1) fail to help antigen-specific B cells. J Immunol 1987;138:1674-9
134. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997;89:587-96
135. Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD and Paul WE. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. J Exp Med 1990;172:921-9
136. Zhu J, Yamane H, Cote-Sierra J, Guo L and Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res 2006;16:3-10
137. Howard AD, Zwilling BS. Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines. Clin Exp Immunol 1999;115:428-34
138. van Crevel R, Karyadi E, Preyers F, et al. Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is related to the presence of pulmonary cavities. J Infect Dis 2000;181:1194-7
139. Baliko Z, Szereday L and Szekeres-Bartho J. Th2 biased immune response in cases with active Mycobacterium tuberculosis infection and tuberculin anergy. FEMS Immunol Med Microbiol 1998;22:199-204
140. Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. Lancet 1995;346:1339-45

141. Bentwich Z, Kalinkovich A, Weisman Z, Borkow G, Beyers N and Beyers AD. Can eradication of helminthic infections change the face of AIDS and tuberculosis? Immunol Today 1999;20:485-7
142. Resende Co T, Hirsch CS, Toossi Z, Dietze R and Ribeiro-Rodrigues R. Intestinal helminth co-infection has a negative impact on both anti-Mycobacterium tuberculosis immunity and clinical response to tuberculosis therapy. Clin Exp Immunol 2007;147:45-52
143. Elias D, Akuffo H, Pawlowski A, Haile M, Schon T and Britton S. Schistosoma mansoni infection reduces the protective efficacy of BCG vaccination against virulent Mycobacterium tuberculosis. Vaccine 2005;23:1326-34
144. Khader SA, Cooper AM. IL-23 and IL-17 in tuberculosis. Cytokine 2008;41:79-83
145. Cruz A, Khader SA, Torrado E, et al. Cutting edge: IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. J Immunol 2006;177:1416-20
146. Chen X, Zhang M, Liao M, et al. Reduced Th17 response in patients with tuberculosis correlates with IL-6R expression on CD4+ T Cells. Am J Respir Crit Care Med;181:734-42
147. Sakaguchi S, Sakaguchi N, Asano M, Itoh M and Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151-64
148. Fontenot JD, Gavin MA and Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-6
149. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG and Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 2005;22:329-41
150. Zheng SG, Wang JH, Gray JD, Soucier H and Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. J Immunol 2004;172:5213-21
151. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003;198:1875-86
152. Fu S, Zhang N, Yopp AC, et al. TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. Am J Transplant 2004;4:1614-27
153. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR and Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol 2004;172:5149-53
154. Tran DQ, Ramsey H and Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983-90
155. DiPaolo RJ, Brinster C, Davidson TS, Andersson J, Glass D and Shevach EM. Autoantigen-specific TGFbeta-induced Foxp3+ regulatory T cells prevent autoimmunity by inhibiting dendritic cells from activating autoreactive T cells. J Immunol 2007;179:4685-93
156. Faria AM, Weiner HL. Oral tolerance. Immunol Rev 2005;206:232-59
157. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K and Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006;212:28-50

158. Hori S, Nomura T and Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61
159. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 2001;27:68-73
160. Wildin RS, Ramsdell F, Peake J, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet 2001;27:18-20
161. Marie JC, Liggitt D and Rudensky AY. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity 2006;25:441-54
162. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI and Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nat Immunol 2008;9:194-202
163. Wohlfert E, Belkaid Y. Role of endogenous and induced regulatory T cells during infections. J Clin Immunol 2008;28:707-15
164. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM and Sacks DL. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature 2002;420:502-7
165. Boettler T, Spangenberg HC, Neumann-Haefelin C, et al. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. J Virol 2005;79:7860-7
166. Hisaeda H, Maekawa Y, Iwakawa D, et al. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. Nat Med 2004;10:29-30
167. Walther M, Tongren JE, Andrews L, et al. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. Immunity 2005;23:287-96
168. Couper KN, Blount DG, Wilson MS, et al. IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. PLoS Pathog 2008;4:e1000004
169. Rushbrook SM, Ward SM, Unitt E, et al. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. J Virol 2005;79:7852-9
170. Mason CM, Porretta E, Zhang P and Nelson S. CD4+ CD25+ transforming growth factor-beta-producing T cells are present in the lung in murine tuberculosis and may regulate the host inflammatory response. Clin Exp Immunol 2007;148:537-45
171. Scott-Browne JP, Shafiani S, Tucker-Heard G, et al. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. J Exp Med 2007;204:2159-69
172. Shafiani S, Tucker-Heard G, Kariyone A, Takatsu K and Urdahl KB. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. J Exp Med;207:1409-20
173. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB and Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat Immunol 2009;10:595-602
174. Quinn KM, McHugh RS, Rich FJ, et al. Inactivation of CD4+ CD25+ regulatory T cells during early mycobacterial infection increases cytokine production but does not affect pathogen load. Immunol Cell Biol 2006;84:467-74

175. Quinn KM, Rich FJ, Goldsack LM, et al. Accelerating the secondary immune response by inactivating CD4(+)CD25(+) T regulatory cells prior to BCG vaccination does not enhance protection against tuberculosis. Eur J Immunol 2008;38:695-705
176. Kursar M, Koch M, Mittrucker HW, et al. Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. J Immunol 2007;178:2661-5
177. Antas PR, Sampaio EP. Another round for the CD4+CD25+ regulatory T cells in patients with tuberculosis. Am J Respir Crit Care Med 2007;176:214-5
178. Chen X, Zhou B, Li M, et al. CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease. Clin Immunol 2007;123:50-9
179. Chiacchio T, Casetti R, Butera O, et al. Characterization of regulatory T cells identified as CD4(+)CD25(high)CD39(+) in patients with active tuberculosis. Clin Exp Immunol 2009;156:463-70
180. Hougardy JM, Place S, Hildebrand M, et al. Regulatory T cells depress immune responses to protective antigens in active tuberculosis. Am J Respir Crit Care Med 2007;176:409-16
181. Guyot-Revol V, Innes JA, Hackforth S, Hinks T and Lalvani A. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. Am J Respir Crit Care Med 2006;173:803-10
182. Chackerian AA, Alt JM, Perera TV, Dascher CC and Behar SM. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. Infect Immun 2002;70:4501-9
183. Einarsdottir T, Lockhart E and Flynn JL. Cytotoxicity and secretion of gamma interferon are carried out by distinct CD8 T cells during Mycobacterium tuberculosis infection. Infect Immun 2009;77:4621-30
184. van Pinxteren LA, Cassidy JP, Smedegaard BH, Agger EM and Andersen P. Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. Eur J Immunol 2000;30:3689-98
185. Serbina NV, Liu CC, Scanga CA and Flynn JL. CD8+ CTL from lungs of Mycobacterium tuberculosis-infected mice express perforin in vivo and lyse infected macrophages. J Immunol 2000;165:353-63
186. Chen CY, Huang D, Wang RC, et al. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. PLoS Pathog 2009;5:e1000392
187. Ting LM, Kim AC, Cattamanchi A and Ernst JD. Mycobacterium tuberculosis inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. J Immunol 1999;163:3898-906
188. Forbes EK, Sander C, Ronan EO, et al. Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. J Immunol 2008;181:4955-64
189. Tsai MC, Chakravarty S, Zhu G, et al. Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension. Cell Microbiol 2006;8:218-32
190. Ulrichs T, Kosmiadi GA, Trusov V, et al. Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung. J Pathol 2004;204:217-28

191. Maglione PJ, Xu J and Chan J. B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with Mycobacterium tuberculosis. J Immunol 2007;178:7222-34
192. Bosio CM, Gardner D and Elkins KL. Infection of B cell-deficient mice with CDC 1551, a clinical isolate of Mycobacterium tuberculosis: delay in dissemination and development of lung pathology. J Immunol 2000;164:6417-25
193. Nimmerjahn F, Ravetch JV. Fcgamma receptors: old friends and new family members. Immunity 2006;24:19-28
194. Maglione PJ, Xu J, Casadevall A and Chan J. Fc gamma receptors regulate immune activation and susceptibility during Mycobacterium tuberculosis infection. J Immunol 2008;180:3329-38
195. Algood HM, Chan J and Flynn JL. Chemokines and tuberculosis. Cytokine Growth Factor Rev 2003;14:467-77
196. Algood HM, Lin PL and Flynn JL. Tumor necrosis factor and chemokine interactions in the formation and maintenance of granulomas in tuberculosis. Clin Infect Dis 2005;41 Suppl 3:S189-93
197. Gonzalez-Juarrero M, Turner OC, Turner J, Marietta P, Brooks JV and Orme IM. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis. Infect Immun 2001;69:1722-8
198. Fenhalls G, Stevens L, Moses L, et al. In situ detection of Mycobacterium tuberculosis transcripts in human lung granulomas reveals differential gene expression in necrotic lesions. Infect Immun 2002;70:6330-8
199. Seishima M, Fujisawa T, Yamanaka S, et al. BCG granuloma appearing more than 50 years after vaccination. Arch Dermatol 2006;142:249-50
200. Volkman HE, Pozos TC, Zheng J, Davis JM, Rawls JF and Ramakrishnan L. Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. Science;327:466-9
201. Algood HM, Lin PL, Yankura D, Jones A, Chan J and Flynn JL. TNF influences chemokine expression of macrophages in vitro and that of CD11b+ cells in vivo during Mycobacterium tuberculosis infection. J Immunol 2004;172:6846-57
202. Lin PL, Plessner HL, Voitenok NN and Flynn JL. Tumor necrosis factor and tuberculosis. J Investig Dermatol Symp Proc 2007;12:22-5
203. Jick SS, Lieberman ES, Rahman MU and Choi HK. Glucocorticoid use, other associated factors, and the risk of tuberculosis. Arthritis Rheum 2006;55:19-26
204. Lighvani AA, Frucht DM, Jankovic D, et al. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. Proc Natl Acad Sci U S A 2001;98:15137-42
205. Saito S, Nakano M. Nitric oxide production by peritoneal macrophages of Mycobacterium bovis BCG-infected or non-infected mice: regulatory role of T lymphocytes and cytokines. J Leukoc Biol 1996;59:908-15
206. Scanga CA, Mohan VP, Tanaka K, Alland D, Flynn JL and Chan J. The inducible nitric oxide synthase locus confers protection against aerogenic challenge of both clinical and laboratory strains of Mycobacterium tuberculosis in mice. Infect Immun 2001;69:7711-7
207. O'Mahony L, Feeney M, O'Halloran S, et al. Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. Aliment Pharmacol Ther 2001;15:1219-25

208. Sheil B, Shanahan F and O'Mahony L. Probiotic effects on inflammatory bowel disease. J Nutr 2007;137:819S-24S
209. Pearce EL, Shedlock DJ and Shen H. Functional characterization of MHC class II-restricted CD8+CD4- and CD8-CD4- T cell responses to infection in CD4-/- mice. J Immunol 2004;173:2494-9
210. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S and Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 1992;68:869-77
211. Ge Q, Hu H, Eisen HN and Chen J. Naive to memory T-cell differentiation during homeostasis-driven proliferation. Microbes Infect 2002;4:555-8
212. Belkaid Y, Tarbell K. Regulatory T cells in the control of host-microorganism interactions (*). Annu Rev Immunol 2009;27:551-89
213. Alam MS, Kurtz CC, Rowlett RM, et al. CD73 is expressed by human regulatory T helper cells and suppresses proinflammatory cytokine production and Helicobacter felis-induced gastritis in mice. J Infect Dis 2009;199:494-504
214. Thirumalapura NR, Crossley EC, Walker DH and Ismail N. Persistent infection contributes to heterologous protective immunity against fatal ehrlichiosis. Infect Immun 2009;77:5682-9
215. Burl S, Hill PC, Jeffries DJ, et al. FOXP3 gene expression in a tuberculosis case contact study. Clin Exp Immunol 2007;149:117-22
216. Ribeiro-Rodrigues R, Resende Co T, Rojas R, et al. A role for CD4+CD25+ T cells in regulation of the immune response during human tuberculosis. Clin Exp Immunol 2006;144:25-34
217. Roberts T, Beyers N, Aguirre A and Walzl G. Immunosuppression during active tuberculosis is characterized by decreased interferon- gamma production and CD25 expression with elevated forkhead box P3, transforming growth factor- beta , and interleukin-4 mRNA levels. J Infect Dis 2007;195:870-8
218. Sharma PK, Saha PK, Singh A, Sharma SK, Ghosh B and Mitra DK. FoxP3+ regulatory T cells suppress effector T-cell function at pathologic site in miliary tuberculosis. Am J Respir Crit Care Med 2009;179:1061-70
219. Rushbrook SM, Hoare M and Alexander GJ. T-regulatory lymphocytes and chronic viral hepatitis. Expert Opin Biol Ther 2007;7:1689-703
220. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003;3:133-46
221. Cooper AM, Magram J, Ferrante J and Orme IM. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. J Exp Med 1997;186:39-45
222. Cooper AM, Roberts AD, Rhoades ER, Callahan JE, Getzy DM and Orme IM. The role of interleukin-12 in acquired immunity to Mycobacterium tuberculosis infection. Immunology 1995;84:423-32
223. Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev 2002;13:155-68
224. Gherardi MM, Ramirez JC and Esteban M. Interleukin-12 (IL-12) enhancement of the cellular immune response against human immunodeficiency virus type 1 env antigen in a DNA prime/vaccinia virus boost vaccine regimen is time and dose dependent: suppressive effects of IL-12 boost are mediated by nitric oxide. J Virol 2000;74:6278-86

225. Nolt D, Flynn JL. Interleukin-12 therapy reduces the number of immune cells and pathology in lungs of mice infected with Mycobacterium tuberculosis. Infect Immun 2004;72:2976-88
226. Asselin-Paturel C, Megherat S, Vergnon I, et al. Differential effect of high doses versus low doses of interleukin-12 on the adoptive transfer of human specific cytotoxic T lymphocyte in autologous lung tumors engrafted into severe combined immunodeficiency disease-nonobese diabetic mice: relation with interleukin-10 induction. Cancer 2001;91:113-22
227. Koblish HK, Hunter CA, Wysocka M, Trinchieri G and Lee WM. Immune suppression by recombinant interleukin (rIL)-12 involves interferon gamma induction of nitric oxide synthase 2 (iNOS) activity: inhibitors of NO generation reveal the extent of rIL-12 vaccine adjuvant effect. J Exp Med 1998;188:1603-10
228. Kurzawa H, Wysocka M, Aruga E, Chang AE, Trinchieri G and Lee WM. Recombinant interleukin 12 enhances cellular immune responses to vaccination only after a period of suppression. Cancer Res 1998;58:491-9
229. Lasarte JJ, Corrales FJ, Casares N, et al. Different doses of adenoviral vector expressing IL-12 enhance or depress the immune response to a coadministered antigen: the role of nitric oxide. J Immunol 1999;162:5270-7
230. Davidson TS, DiPaolo RJ, Andersson J and Shevach EM. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. J Immunol 2007;178:4022-6
231. Gong JH, Zhang M, Modlin RL, et al. Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression. Infect Immun 1996;64:913-8
232. Shevach EM, Tran DQ, Davidson TS and Andersson J. The critical contribution of TGF-beta to the induction of Foxp3 expression and regulatory T cell function. Eur J Immunol 2008;38:915-7
233. Boussiotis VA, Tsai EY, Yunis EJ, et al. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. J Clin Invest 2000;105:1317-25
234. Toossi Z, Gogate P, Shiratsuchi H, Young T and Ellner JJ. Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. J Immunol 1995;154:465-73
235. Toossi Z, Young TG, Averill LE, Hamilton BD, Shiratsuchi H and Ellner JJ. Induction of transforming growth factor beta 1 by purified protein derivative of Mycobacterium tuberculosis. Infect Immun 1995;63:224-8
236. Toossi Z, Mincek M, Seeholtzer E, Fulton SA, Hamilton BD and Hirsch CS. Modulation of IL-12 by transforming growth factor-beta (TGF-beta) in Mycobacterium tuberculosis-infected mononuclear phagocytes and in patients with active tuberculosis. J Clin Lab Immunol 1997;49:59-75
237. Zhao Z, Yu S, Fitzgerald DC, et al. IL-12R beta 2 promotes the development of CD4+CD25+ regulatory T cells. J Immunol 2008;181:3870-6
238. Wohlfert E, Belkaid Y. Plasticity of T reg at infected sites. Mucosal Immunol;3:213-5
239. Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs--worldwide, 2000-2004. MMWR Morb Mortal Wkly Rep 2006;55:301-5
240. Andersen P, Doherty TM. The success and failure of BCG - implications for a novel tuberculosis vaccine. Nat Rev Microbiol 2005;3:656-62
241. Othieno C, Hirsch CS, Hamilton BD, Wilkinson K, Ellner JJ and Toossi Z. Interaction of Mycobacterium tuberculosis-induced transforming growth factor beta1 and interleukin-10. Infect Immun 1999;67:5730-5

242. Hirsch CS, Hussain R, Toossi Z, Dawood G, Shahid F and Ellner JJ. Cross-modulation by transforming growth factor beta in human tuberculosis: suppression of antigen-driven blastogenesis and interferon gamma production. Proc Natl Acad Sci U S A 1996;93:3193-8
243. Lin PL, Pawar S, Myers A, et al. Early events in Mycobacterium tuberculosis infection in cynomolgus macaques. Infect Immun 2006;74:3790-803
244. Allan SE, Song-Zhao GX, Abraham T, McMurchy AN and Levings MK. Inducible reprogramming of human T cells into Treg cells by a conditionally active form of FOXP3. Eur J Immunol 2008;38:3282-9
245. Lee JH, Kang SG and Kim CH. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. J Immunol 2007;178:301-11
246. Lazar-Molnar E, Chen B, Sweeney KA, et al. Programmed death-1 (PD-1)-deficient mice are extraordinarily sensitive to tuberculosis. Proc Natl Acad Sci U S A;107:13402-7
247. Gazzola L, Tincati C, Gori A, Saresella M, Marventano I and Zanini F. FoxP3 mRNA expression in regulatory T cells from patients with tuberculosis. Am J Respir Crit Care Med 2006;174:356; author reply 357
248. Green AM, Mattila JT, Bigbee CL, Bongers KS, Lin PL and Flynn JL. CD4(+) regulatory T cells in a cynomolgus macaque model of Mycobacterium tuberculosis infection. J Infect Dis;202:533-41