Regulation of Immunopathology in *Mycobacterium tuberculosis* infection

by

Hillarie Plessner Windish

Bachelor of Arts, Arcadia University, 2003

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

2008

UNIVERSITY OF PITTSBURGH

School of Medicine

This dissertation was presented

by

Hillarie Plessner Windish

It was defended on

May 8, 2008

and approved by

Russell D. Salter, Ph.D., Department of Immunology

Penelope A. Morel, M.D., Department of Immunology

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

Ted M. Ross, Ph.D., Department of Medicine, Division of Infectious Diseases

Thesis Director: JoAnne L. Flynn, Ph.D., Department of Molecular Genetics and Biochemistry

Regulation of Immunopathology in *Mycobacterium tuberculosis* infection

Hillarie Plessner Windish, PhD

University of Pittsburgh, 2008

ABSTRACT

Approximately one third of the world's population is infected with *Mycobacterium tuberculosis,* which was responsible for about 1.6 million deaths in 2005. In spite of continuing advances in understanding host response to this infection, generation and maintenance of the host immune response remains unclear. In this thesis, we investigate molecules involved in the generation and maintenance of the host response, specifically the granuloma, to *M. tuberculosis*. We investigated the role of TNF antagonists in reactivation of tuberculosis, and showed that while anti-TNF antibody is superior to TNFR2-Fc fusion molecule in penetrating the granuloma, any blockade of TNF compromises control of acute tuberculosis. We hypothesized that TNF is required for priming T cell responses and that TNF-inducible chemokine receptors function redundantly, allowing one chemokine to compensate in the absence of another. Here, we show that TNF is not required to prime the adaptive immune response, and that TNF-inducible chemokines CXCR3 and CCR5 are simultaneously expendable, refuting the compensation hypothesis in these two chemokines. Reports have implicated unexplored inflammatory molecules in host response to *M. tuberculosis* infection. We hypothesized that the small chemotactic molecule LTB_4 and its receptor BLT1 increase pathology during *M. tuberculosis* infection. We also hypothesized that osteopontin is required for mediating an effective immune

response to tuberculosis by mediating Th1 priming and lymphocyte migration. We show here that neither BLT1 nor osteopontin play a significant role in the inflammatory response to *M. tuberculosis.* Finally, we investigated the role of ICAM-1 in priming effector and regulatory T cells in response to tuberculosis. We report that ICAM-1 is dispensable for priming and migration of effector T cells, but that ICAM-1 is required for production of inducible Foxp3+ T regulatory cells via TGFβ1 stimulation. We hypothesize that the reduction in T regulatory cells exacerbates the immune response, allowing greater inflammation in the lungs, potentially causing overwhelming inflammation. This body of work contributes to the understanding of the host response to tuberculosis by investigating activity of cytokines, chemotactic molecules and adhesion molecules in balancing the host response to *M. tuberculosis* infection.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

During the time I have spent in the Flynn laboratory, I have benefited tremendously from the training and mentorship I have received from JoAnne. I am grateful for her patience, guidance and understanding. I am also grateful to all of the past and present members of the Flynn laboratory, whose camaraderie, training and advice are indispensible.

Thank you to the members of my thesis committee: Russ Salter, Penny Morel, Karen Norris and Ted Ross, provided me with technical and scientific guidance. I am also fortunate to have had the help of all of the supportive staff in the molecular genetics and biochemistry and immunology departments and in the Interdisciplinary program.

I am especially fortunate to have a strong network of supportive family and friends. Thank you to my husband Craig, who has been my best friend since I started graduate school. Thank you to my parents, step-parents and brothers, who have always been encouraging. I am grateful for all of my friends in Pittsburgh and back home who have always been available to me when I needed them, and for my senseis who have provided training and a great outlet for frustration.

1.0 INTRODUCTION

1.1 EPIDEMIOLOGY AND THE COURSE OF INFECTION WITH MYCOBACTERIUM TUBERCULOSIS

1.1.1 History and infection with *Mycobacterium tuberculosis*

One third of the world population is infected with the acid fast bacillus *Mycobacterium tuberculosis*. The World Health Organization (WHO) estimates that in the year 2005, 8.8 million people worldwide were newly infected, and 1.6 million people died from tuberculosis [1]. Transmission of *M. tuberculosis* occurs when an actively infected person expels aerosol droplets containing mycobacteria by coughing, talking or sneezing; it has been estimated that a single cough can aerosolize as many as 600,000 bacteria-containing droplets [2]. Of those newly infected with *M. tuberculosis*, about 10% develop active disease within the first two years, while 90% of infections enter a state of latency in which the individual is neither infectious or symptomatic [3]. Over the lifetime of a latently infected individual there is a 10% risk of reactivation tuberculosis in those who are immunocompetent, but this risk rises to 7-10% per year in those immunocompromised with HIV infection [4, 5], underlining the necessity of a functional adaptive immune system in protection from this pathogen.

Mycobacterium tuberculosis has evolved with humans over thousands of years, modifying its genome to become a specialized human pathogen. Prior to genetic analysis, it had been suggested that mycobacteria were first introduced into the human population as a result of close association with *Mycobacterium bovis*-infected farm animals and by consuming infected animal byproducts in the first human settlements about 8,000 years ago [6]. However, genetic analyses using restriction fragment length polymorphism comparing several pathogenic mycobacteria (*M. tuberculosis, M. africanum, M. bovis*, and *M. microti*) showed that *M. tuberculosis* is most closely related to *M. africanum* (a proposed progenitor to modern mycobacteria) and may have begun to evolve into a specialized human pathogen more than 15,000 years ago [2, 7]. The discovery of mycobacteria most closely related to *M. tuberculosis* in the bone of a 17,000+ year old long-horned bison fossil [8] supports the hypothesis that *M. tuberculosis* began to diverge from other mycobacteria more than 15,000 years ago, and DNA analysis of bone lesions found in ancient Egyptian mummies dating back more than 4,000 years [9-11] confirms that *M. tuberculosis* has been present within the human population for thousands of years. In more recent human history, tuberculosis was the cause of death of as much as a quarter of the population in the 17^{th} and 18^{th} centuries [2].

Infection with *M. tuberculosis* can be detected by the tuberculin skin test [12] in which a mixture of mycobacterial proteins is injected into the top layer of the skin [12]. In individuals who have been exposed to *M. tuberculosis*, or who have received the BCG vaccine a delayed type hypersensitivity reaction to the purified proteins can be detected 48 to 72 hours after the injection [12]. In an immunocompetent person who is not at particular risk of exposure, a hard swelling (induration) at the site of injection with a diameter of at least 15 millimeters indicates a positive skin test [12, 13]. The swelling at the site of injection is due to the specific recruitment

of adaptive immune system cells, and indicates that the immune system has been previously exposed to mycobacterial antigens. In the case of an immunocompromised patient, a swelling 5 millimeters in diameter is sufficient to diagnose a positive skin test, and in healthcare professionals or others at higher risk of exposure, 10 millimeters is sufficient [12, 13].

Individuals with a positive skin test, but lacking clinical symptoms of tuberculosis are assumed to be latently infected with *M. tuberculosis* [14, 15]. One of the most common symptoms of active tuberculosis is a cough, which helps the mycobacteria to infect new hosts as the infection progresses. In patients with active disease, *M. tuberculosis* bacilli can sometimes be visualized by acid fast bacillus staining in the sputum or mycobacteria can be cultured from the sputum in the laboratory [15]. Latent tuberculosis lesions can sometimes be visualized on chest X-rays, but these lesions appear different from those seen in X-rays of individuals with active tuberculosis and can usually be differentiated by a radiologist. In the United States, whether patients are latently or actively infected with *M. tuberculosis*, they are candidates for antibiotic treatment [15], and active tuberculosis is treated using directly observed therapy in countries worldwide [1].

1.1.2 Treatment of tuberculosis

Until the development of modern antibiotics, the most common treatment of tuberculosis was admission to a specialized sanatorium [2, 16]. Streptomycin was the first drug to be used to treat tuberculosis in 1946, followed by isoniazid in 1954 and rifampin in 1970 [2, 16]. Unfortunately, in the decades following the 1970s, development of new anti-tuberculosis therapeutics was neglected. The current short course of anti-tuberculosis therapy requires six months of

administration of four of the first line antibiotics: rifampin, isoniazid, pyrazinamide and ethambutol [16, 17].

The lengthy antibiotic treatment schedule against *M. tuberculosis* infection has contributed to poor patient compliance, and poor compliance is thought to be the factor most responsible for development of antibiotic resistance [16, 17]. It is estimated that at the onset of antibiotic treatment in the 1950s, only 1-2% of tuberculosis patients in New York City were infected with *M. tuberculosis* resistant to the antibiotics, but by 1991, 23% of tuberculosis cases in New York City were resistant to at least one first line drug [17]. Multidrug resistant strains (MDR-TB) are defined by the WHO as having minimally acquired resistance to isoniazid and rifampin [1], two first line antibiotics. To combat continuing development of antibiotic resistance, WHO implemented directly observed therapy (DOTS), in which patients receive treatments in the presence of healthcare workers, better ensuring compliance. According to WHO, in the year 2005, 187 countries encompassing 89% of the world population had applied the DOTS program, likely contributing to the modest decline reported by WHO in new tuberculosis diagnoses and deaths worldwide [1]. Despite the success of the implemented WHO programs, in 2006, there were 8.8 million new cases of tuberculosis [1], showing the battle against tuberculosis is far from over.

MDR-TB [1] requires a second line of drugs that have a lower cure rate, which results in higher morbidity than in drug susceptible tuberculosis [17]. In some cases, strains of *M. tuberculosis* are resistant to so many antibiotics that they are termed XDR-TB, extensively drug resistant tuberculosis [1]. XDR-TB received media attention in 2007, when a patient thought to have XDR-TB took two international airplane flights: from Atlanta, Georgia, to Paris, France, then from Prague, Czech Republic to Montreal, Canada, exposing passengers on those flights to

his dangerous strain of *M. tuberculosis* [18]. Whether those exposed were infected with XDR-TB has not been reported, but in a previous incident, a patient with MDR-TB flying from Honolulu, Hawaii to Chicago, Illinois infected 150 airplane passengers [19]. This underlines the potentially serious consequences of exposing the public to a single person with active tuberculosis. Exposure to MDR *M. tuberculosis* strains is still more dangerous than exposure to drug susceptible tuberculosis, because second line drugs are associated with a far lower cure rate and higher mortality [17]. Upon repeated failure of antibiotic treatments, continued positive sputum cultures and chronic disease, a final therapy could be surgical resection of infected lung, in which 80% of patients experienced no recurrence of tuberculosis up to 36 months post-surgery [17]. The WHO is advocating continued development of new antibiotics, and further research into MDR-TB treatment [1].

1.1.3 Tuberculosis vaccination strategies

Robert Koch, known as the father of tuberculosis, was the first to identify the organism that causes tuberculosis. He was also the first to isolate mycobacterial proteins ("tuberculin"), and reportedly attempted to use tuberculin to vaccinate against infection with *M. tuberculosis* [20]. Unlike vaccination against such viruses as smallpox, his vaccination against *M. tuberculosis* proved unsuccessful at preventing infection [20]. In a phenomenon first reported by Koch, it has been confirmed that when an immune response to mycobacterial antigens has already been established, pathology may be exacerbated in a phenomenon known as the "Koch-reaction," [21]. The current vaccine, Bacille Calmette-Guerin (BCG), was introduced in 1921 and is a live attenuated strain of *M. bovis* [22]. Although BGC does not prevent *M. tuberculosis* infection, it does reduce the incidence of disseminated disease [22]. However, BCG vaccination in absence

of boosting does not result in persistent PPD reactions [20, 23], suggesting that lasting adaptive immunity is suboptimal.

Several new candidate vaccines have entered into clinical trials, but none have yet been shown to be efficacious in humans, and have only moderate effectiveness in some animal models. There are two current approaches to the development of a new vaccine against *M. tuberculosis:* to modify or boost the existing BCG vaccine, or to deliver *M. tuberculosis* antigens using a vector and an adjuvant [24]. MVA85A is a modified vaccinia virus engineered to express the mycobacterial protein antigen 85A [25]. When administered with BCG MVA85A is designed to boost induction of T cells against this antigen with the aim of conveying added protection to the host [25, 26]. A fusion protein of mycobacterial proteins ESAT6 and antigen 85b was highly successful in animal models such as non-human primates [27], mice [28] and guinea pigs [29], showing the promising results of initiating the T lymphocyte response directly during vaccination. Data are yet unavailable regarding the efficacy of these new vaccines, but all have been developed with the intention of generating a stronger adaptive immune response to mycobacterial antigens than achieved by the current BCG vaccine. As T lymphocytes are required for the control of *M. tuberculosis* infection (described in section 1.3.4 and 1.3.5), the outcome of these clinical trials will provide an interesting insight into the control of *M. tuberculosis* infection, and potentially provide more efficacious vaccine protection against tuberculosis.

1.2 THE INTERACTION OF THE HOST AND MYCOBACTERIUM TUBERCULOSIS

Infection with *M. tuberculosis* is established in the lungs after inhalation of the bacteria. Mycobacteria are thought to be phagocytosed by resident alveolar or lung macrophages by a variety of receptors (reviewed in [30, 31]). Immature dendritic cells also phagocytose mycobacteria, but unlike infected macrophages, maturing dendritic cells lose their phagocytic potential [32]. *M. tuberculosis* infected macrophages and dendritic cells produce inflammatory cytokines and chemokines [32, 33], resulting in recruitment and migration of additional leukocytes to the site of infection. Infected dendritic cells migrate to the lymph nodes [34, 35], where they present mycobacterial antigens to and prime naïve T lymphocytes. In mice, the adaptive response to *M. tuberculosis* can be visualized by three weeks post-infection [34]. Primarily macrophages and lymphocytes aggregate to form the granuloma [36-38], but other cells of the immune system such as neutrophils [36, 38] may also be present. It has been suggested that during latent tuberculosis, *M. tuberculosis* previously replicating in macrophages, may cease to replicate within the granuloma, entering a truly dormant state seen in viral latency [39] or, mycobacterial replication may continue at a low level or sporadically [40]. In either case a low level of bacteria persists within the latently infected person [41].

The granuloma provides a central focus for cell to cell interactions, such as CD4 and CD8 T cell-mediated induction of apoptosis of infected macrophages, or activation of macrophages through IFN-γ and TNF cytokine signaling [42]. Cytotoxic T cells can also kill infected macrophages by releasing lytic granules containing perforin and granzymes, or directly kill mycobacteria within the macrophage through granulysin release [43]. In addition to providing a

locus for cell to cell interactions, the granuloma is also thought to provide a barrier to bacterial dissemination [44].

Phagocytosis of bacteria by macrophages and dendritic cells occurs through a variety of receptors including complement receptors 1 and 3, Toll-like, scavenger and mannose receptors (reviewed in [31]. New phagosomes mature over time, upregulating molecules that allow fusion of lysosomes to phagosomes and acidification of the vacuole that kills susceptible bacteria [45]. Many successful pathogens have evasion mechanisms of either escaping the phagosome, or preventing phagolysosome fusion [45]. Among them, *M. tuberculosis* is able to persist within the macrophage [46], because it is able to prevent acidification that causes phagolysosome fusion (reviewed in [47]).

It has been shown that *M. tuberculosis* produces proteins that can prevent phagosome-lysosome fusion. The *M. tuberculosis* protein lipoarabinomannam (ManLAM) blocks phagosome maturation by inhibiting calcium influx within the macrophage [48] required for cellular signaling. A less glycosylated version of the LAM protein backbone, phosphatidylinositol mannoside (PIM) prevents the acidification of phagosome [49]. These two *M. tuberculosis* proteins, ManLAM and PIM work at two different points of the endosome pathway to prevent phagosome-lysosome fusion and acidification of the intracellular *M. tuberculosis* compartment. It has also been shown that *M. tuberculosis* sulfated trehalose glycolipids can directly prevent phagosome-lysosome fusion [50, 51].

Not only can *M. tuberculosis* synthesize its own proteins to bypass phagosome-lysosme fusion, but this pathogen can also take advantage of macrophage machinery. The Rab family of small GTPase proteins modulate intracellular transport within mammalian cells (reviewed in [52, 53]). An early endosome marker Rab5 and a late endosome marker, Rab7 are part of a larger

family of Rab endosome trafficking molecules. The simultaneous exchange of Rab5 for Rab7 is required for the maturation of a phagosome to allow docking of the lysosome [54]. In phagosomes containing mycobacteria, Rab5 remains on the phagosome surface, and Rab7 is absent [55]. This suggests that *M. tuberculosis* in some way modifies the expression of these molecules on the phagosome surface. It was recently shown that *M. tuberculosis* recruits another member of the Rab protein family, Rab22a to the phagosome surface, and that it is Rab22a that prevents Rab7 from being recruited to the phagosome surface [56]. It is important to note that while *M. tuberculosis* possesses many mechanisms for evasion of the innate immune response to infection, many mycobacteria can be killed in the context of the granuloma, and while some bacteria do persist, bacterial burden remains quite low during latent infection [41].

In response to phagocytosis of *M. tuberculosis,* macrophages can produce antibacterial reactive oxygen and reactive nitrogen species [57] such as hydrogen peroxide and nitric oxide. At low concentrations, nitric oxide inhibits mycobacterial replication and at high concentrations can be mycobacteriocidal [58]. Nitric oxide is required for survival of *M. tuberculosis* infection in mice [59, 60]. Due to its ability to produce peroxidase, *M. tuberculosis* is not as susceptible to reactive oxygen species [61], although exposure to millimolar concentrations of hydrogen peroxide has been shown to kill mycobacteria *in vitro* [62]. Macrophage phagocytosis of *M. tuberculosis* can occur through complement receptors 1 and 3 [63] allowing bacillus entry without activating the macrophage. This allows evasion of the oxidative burst that produces reactive oxygen and nitrogen intermediates [64]. However, *M. tuberculosis* can also be bound by other receptors such as toll-like receptors that do activate macrophage responses [31, 65], showing that *M. tuberculosis* entry into macrophages is not necessarily undetected.

1.3 LEUKOCYTES AND THEIR ROLE IN THE IMMUNE RESPONSE TO MYCOBACERIUM TUBERCULOSIS INFECTION

1.3.1 Macrophages

Lung resident, alveolar macrophages are theoretically the first cells to encounter *M. tuberculosis* upon inhalation of infectious droplets. Phagocytes within lung resident and monocyte-derived macrophages become the primary home of *M. tuberculosis* within the host, and eventually infected macrophages are surrounded by T lymphocytes and other leukocytes to form the granuloma [37]. Phagocytosis and macrophage defense mechanisms against *M. tuberculosis* are described in section 1.2.

Depending upon the route of entry, macrophages may be activated upon uptake of the mycobacteria. Activation of macrophages also occurs through signaling by the pro-inflammatory cytokines TNF and IFN-γ [33, 66, 67]. IFN-γ is necessary for macrophage control over bacterial burden [66, 67]. TNF and IFN-γ can synergistically act on a macrophage to result in optimal activation of macrophages to trigger the oxidative burst that produces reactive oxygen and nitrogen intermediates [66]. TNF can also induce macrophage expression of chemoattractant cytokines (chemokines) such as CCL5, CXCL9 and CXCL10, which recruit circulating macrophages, dendritic cells and effector T lymphocytes [33].

Macrophages are one of few cells types known as antigen presenting cells (APC), which present antigens in the context of MHC class II in addition to class I [68]. While macrophages are capable of presenting antigen they express lower levels of costimulatory molecules than specialized dendritic cells [32], making them unlikely to be a significant source of T lymphocyte priming. During *M. tuberculosis* infection, macrophages were far less efficient than dendritic

cells at stimulating T lymphocyte proliferation or IFN-γ production [32]. This may be due to factors induced by *M. tuberculosis* infection, which reduces the ability of macrophages to efficiently present antigens through mechanisms that are not yet understood [69]. So, while macrophages are strong producers of chemokines, and provide a location for *M. tuberculosis* to survive, they are not the best antigen presenting cells.

1.3.2 Dendritic cells

Dendritic cells are the professional antigen presenting cells of the immune system, capable of expressing high levels of costimulatory molecules for T lymphocyte priming [35]. Dendritic cells can be infected with *M. tuberculosis,* and increase costimulatory molecules when infected [70], suggesting that they are capable of priming an adaptive immune response. Upon uptake of a foreign antigen, the dendritic cell modifies its chemokine receptor surface expression, upregulating CXCR4 and CCR7 to initiate migration into the T cell regions of secondary lymphoid organs [71]. Within the secondary lymphoid organs, dendritic cells present antigen in the context of MHC class I for CD8+ T cells, MHC class II for CD4+ T cells and on non-classical MHC molecules that are recognized by non-classical T cell receptors [72]. This cellular communication primes the T cell response [34] that is required for control of *M. tuberculosis* infection. Depletion of dendritic cells in mice during *M. tuberculosis* infection, delays priming of CD4+ T cells resulting in an elevated bacterial burden during acute infection [73]. However, dendritic cells do not appear to be required to stimulate a memory response [73].

Like macrophages, dendritic cells are able to support *M. tuberculosis* growth [70]. During *M. tuberculosis* infection, dendritic cells activated with IFN-γ and LPS, or another second signal such as TNF, produce reactive nitrogen intermediates and express NOS2 in similar amounts as

activated macrophages [32], suggesting that dendritic cells can control bacterial burden. However, while activated macrophages actually decreased the number of live *M. tuberculosis* within the cell, dendritic cells were capable only of maintaining the same number of live bacilli [32]. This suggests that while macrophages are capable of mycobacteriocidal activities, dendritic cells can only perform mycobacteriostatic function.

As dendritic cells migrate into the lymph nodes upon the onset of maturation, infected dendritic cells are a primary mechanism for dissemination of live *M. tuberculosis* [34, 74], although extrapulmonary tuberculosis is also caused by hematogenous spread [75, 76]. BCG mycobacteria labeled with green florescent protein and delivered to the lungs by an intranasal route are phagocytosed by both macrophages and dendritic cells, but only fluorescent dendritic cells were present in the lymph nodes of recipient mice [74]. So, while dendritic cells are the primary antigen presenting cells that prime the T lymphocyte response, they also act as an agent of *M. tuberculosis* dissemination, due to an impaired ability to kill this intracellular bacterium.

1.3.3 Neutrophils

Polymorphonuclear neutrophils are phagocytic leukocytes capable of killing intracellular bacteria, and generating inflammatory cytokines and chemokines, as well as damaging surrounding tissue. Few neutrophils are present in the lungs in a non-inflammatory situation, but these are the first leukocytes recruited to the site of lung infection (reviewed in [77]). So, although alveolar macrophages are thought to be the first cells to encounter and be infected by invading *M. tuberculosis* bacilli, neutrophils could also be among the first cells to respond by migrating to the site of infection.

Once at the site of infection, neutrophils phagocytose bacteria and produce antimicrobial factors such as toxic chloramines, defensins, lysozymes and other proteases to kill internalized bacteria. Neutrophils can produce pro-inflammatory cytokines such as TNF, IL-1β and IL-12 [78] and chemokines like CXCL8 (IL-8) [79] and CXCL9 [80]. Neutrophil-secreted cytokines can activate macrophages, and chemokines recruit additional cells to the site of infection, perpetuating the response [79-81].

Neutrophils can be detected in the lungs of mice and guinea pigs during *M. tuberculosis* [80, 81] and can also be found in granulomas in humans and in the non-human primate model of *M. tuberculosis* (Lin, Klein and Flynn unpublished and [36, 82]. Although granuloma formation is delayed in mice depleted of neutrophils prior to *M. tuberculosis* infection, bacterial burden is unaffected [80, 83], and neutrophil-depleted mice form normal granulomas by 120 days post-infection [80]. This transient inhibition of granuloma formation may be due to reduction in CXCR3 chemokines, particularly CXCL9, which is secreted by neutrophils early in infection [80]. This supports a non-phagocytic role in the early response to *M. tuberculosis.* BCG infected macrophages are assisted in killing intracellular bacilli by the presence of neutrophils in a contact independent manner [79]. This is likely to be due to neutrophil cytokine production [79], which further supports a non-killing role for neutrophils during the immune response to *M. tuberculosis*.

1.3.4 CD4+ T Lymphocytes

CD4+ T cells are members of the adaptive immune system, which must be activated within secondary lymphoid organs by dendritic cells to play a role in immune responses [72]. T cell receptors (TCR) on an individual CD4+ T lymphocyte recognizes one specific antigen, and the

CD4 molecule must interact with another portion of the MHC class II molecule in order to successfully activate a naïve cell [72]. CD4+ T cells are essential during the immune response to *M. tuberculosis*, as seen in mice deficient in CD4 or MHC class II molecules, in adoptive transfer models of *M. tuberculosis* and in the CD4 T cell depletion effected by antibodies in animal models and by HIV infection in humans [84-89].

During priming of T cells in *M. tuberculosis* infection, dendritic cells produce IL-12, resulting in a TH1 response characteristic of intracellular pathogens [32, 70, 90]. IFN-γ, a cytokine required to control *M. tuberculosis* infection, is produced by CD4+ T cells in response to *M. tuberculosis* infection [84, 91]. In the absence of CD4+ T cells, IFN-γ production is delayed [84], but the delay is transient as IFN-γ production recovers after 3 weeks of infection [84]. Similarly, depletion of CD4+ T cells during chronic murine infection had no apparent effect on IFN-γ levels in the lungs of infected mice. These studies suggested that CD4+ T cells have important functions in addition to production of IFN-γ in controlling *M. tuberculosis* infection [84]. NOS2 expression, and therefore nitric oxide mediated control over *M. tuberculosis,* was delayed along with the delay in IFN-γ, and this could be responsible for increasing, perhaps overwhelming, bacterial burden [84] from which the mice could not recover.

Another possible role for CD4+ T cells is help in priming CD8+ T cell responses. CD8+ T cells are responsible for the recovery of IFN-γ in the absence of CD4+ T cells [84], suggesting that CD8+ T cells are primed to some degree. However CD8+ T cell cytotoxicity requires CD4+ T cell help during priming [92], as does the development of effective CD8+ T cell memory [93, 94]. This suggests that in the absence of CD4+ T cells, the CD8+ adaptive response may be insufficient to control *M. tuberculosis* infection. Although much is known about the function of

CD4+ T cells during *M. tuberculosis* infection, further studies are required to understand how CD4+ T cell truly contribute to control of infection.

1.3.5 T regulatory cells

T regulatory cells have received much recent attention in the literature. Identified most reliably by their expression of Foxp3, but originally identified by high expression of CD25, CD4+ T regulatory cells play key a role in suppressing autoimmunity [95]. During chronic infectious disease caused by such pathogens as *Leishmania major* and *Helicobacter pylori*, compelling data suggest that T regulatory cell function actually inhibits pathogen clearance and dampens the immune response [96, 97].

Natural CD4+ CD25+ Foxp3+ T regulatory cells are produced in the thymus. It has been suggested that T regulatory cells play a strong role in control over autoimmunity due to thymic expression of self-antigens [98], which may explain why a very strong T cell receptor signaling response could turn a developing T cell down the path to become a T regulatory cell [99]. Naïve CD4+ T cells in the periphery can also become Foxp3+ through T cell receptor stimulation in conjunction with activated TGFβ1 cytokine *in vitro* and *in vivo* [100-104]. Inducible T regulatory cells have suppressive function in response to specific non-self antigens [100, 101, 104]. Since natural and TGFβ1-induced T regulatory cells have the same phenotype *in vivo* during an inflammatory response (CD4+ CD25+ Foxp3+), it is difficult to discriminate between the roles of these two phenotypes.

Several studies have investigated the role of T regulatory cells during *M. tuberculosis* infection. Depletion of CD25+ CD4+ T cells *ex vivo* improved human and murine CD25- T lymphocyte production of IFN-γ, supporting a suppressive role for the CD25+ CD4+ T cells in

tuberculosis [105-107]. While CD25 depletion in *M. tuberculosis* infected mice was unable to decrease bacterial burden *in vivo*, higher IFN-γ expression was detected [107]. When Foxp3 expressing cells were specifically depleted, or when RAG-/- mice were reconstituted with lymphocytes, but not T regulatory cells, the bacterial burden was significantly reduced compared to mice with functional T regulatory cells [108, 109]. According to available data, T regulatory cells are likely to dampen the immune response to *M. tuberculosis* infection, preventing bacterial clearance. Elimination of *M. tuberculosis* specific T regulatory cells could potentially allow clearance of the pathogen. However, there may be a role for T regulatory cells in controlling pathology related to chronic infection with *M. tuberculosis*, and loss of T regulatory cells may result in exacerbation of pathology and disease in humans.

1.3.6 CD8+ T Lymphocytes

Like CD4+ T cells, CD8+ T cells are naïve, and travel through secondary lymphoid organs until their TCR recognizes a specific antigen presented by MHC Class I molecules [72]. CD8+ T lymphocytes are not only capable of producing cytokines such during *M. tuberculosis* infection [110], but can also act as cytotoxic T lymphocytes (CTLs) that use a variety of mechanisms to kill *M. tuberculosis* or cells infected with mycobacteria [111]. The combined results of a series of gene knock out experiments in mice support the idea that CD8+ T lymphocytes may be necessary for optimal control of *M. tuberculosis* infection (reviewed in [112]).

CD8+ T lymphocytes are likely to play several roles in control over *M. tuberculosis* infection in the lungs. These cells are capable of producing TNF and IFN-γ to activate infected macrophages [110]. In addition to cytokine production, *M. tuberculosis* responsive CD8+ T cells can induce apoptosis of infected macrophages by releasing cytotoxic molecules [111], or using

the Fas/FasL pathway [113]. It has been suggested that these mechanisms could cause death of the intracellular pathogens along with the host cell [40] and help to control bacterial burden [114].

1.4 CYTOKINES, CHEMOKINES AND CHEMOTAXIS IN THE IMMUNE RESPONSE TO MYCOBACTERIUM TUBERCULOSIS

Upon infection with *M. tuberculosis,* macrophages and dendritic cells begin to produce cytokines such as TNF [33] and IL-12 [115, 116], which help to shape the immune response. IL-12, made by macrophage or dendritic cells during T cell priming, helps to drive a strong Th1 CD4+ T cell response [116], which results in IFN-γ production in the granuloma. IFN-γ and TNF synergize to activate macrophages [66], and optimal macrophage activation probably helps to control *M. tuberculosis* infection by killing the intracellular pathogen with greater efficiency. TNF expression is also partially responsible for induction of macrophage chemokine production [33] that helps leukocytes migrate to the focus of infection. It has been suggested that chemokine production may be required for formation of a granuloma able to contain *M. tuberculosis* and allow appropriate cellular communication.

1.4.1 Cytokines

1.4.1.1 Tumor Necrosis Factor

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine produced by macrophages, dendritic cells, activated T cells and natural killer cells (reviewed by [39]). This cytokine can be

expressed on the cell surface, or may be cleaved by TNF-alpha converting enzyme (TACE) to a soluble form. Both the transmembrane and soluble cytokine are capable of inducing responses through two TNF receptors: TNFR1 (p55) or TNFR2 (p75) [117]. Among these responses are lymphocyte activation, production of cytokines and chemokines, angiogenesis, and upregulation of cell adhesion molecule expression [118]. TNFR1 deficient mice, TNF deficient mice, and mice treated with anti-TNF antibody are unable to form granulomas and do not survive *M. tuberculosis* infection [33, 119-123], suggesting that TNF expression is required for granuloma formation and that granuloma formation is required for protection from infection. On the other hand it has also been suggested that systemic TNF may contribute to their wasting [124].

The function of TNF signaling most important in dictating granuloma formation and control of infection remains unclear. During *M. tuberculosis* infection *in vitro* and *in vitro,* TNF induces the expression of chemotactic cytokines (chemokines) such as CXCL9, CXCL10, CCL3 and CCL5 [33] that may play a role in recruitment of leukocytes that form the granuloma. TNF can also synergize with interferon-γ to optimally activate macrophages, which could be another important function for TNF [66]. Other roles for TNF, such as help in priming the adaptive immune response or the induction of adhesion molecule expression during *M. tuberculosis* infection, have not yet been established.

The effects of TNF are protective during infections, but can become harmful by contributing to chronic inflammatory diseases such as rheumatoid arthritis. Since its introduction in 1998, Infliximab (Remicade), a TNF neutralizing antibody, has been used successfully to treat auto-inflammatory diseases such as Crohn's disease [125, 126]. By 2002, over 300 cases of *M. tuberculosis* reactivation had been linked to treatment with Infliximab [127]. Of the 70 original cases of Infliximab-related tuberculosis reactivation, close to a quarter of patients developed

disseminated disease and the majority had extrapulmonary disease, supporting the hypothesis that TNF is required for localizing and controlling *M. tuberculosis* infection [128]. Immunocompetent, *M. tuberculosis*-infected people not treated with Infliximab on average develop extrapulmonary disease 15% of the time, and dissemination occurs in only 1% [127]. Another anti-TNF treatment, Etanercept (Enbrel) is not an antibody, but a fusion protein of the human hinge and IgG1 Fc and two TNFRp75 components [129]. In 2004, the reactivation risk of tuberculosis was found to be statistically lower in patients taking Etanercept than in those taking Infliximab [130]. As both of these molecules bind to and neutralize TNF [131], the reason for this difference in tuberculosis reactivation remains unclear.

1.4.1.2 Interferon-γ

Interferon-γ (IFN-γ) is an inflammatory cytokine primarily produced by effector CD4+ and CD8+ T lymphocytes during *M. tuberculosis* infection [110], although it is also expressed by NK cells [132]. In IFN-γ deficient mice, *M. tuberculosis* bacterial burden is 10-100 fold higher than control mice, and mice succumb to infection within about three weeks [133]. Unlike mice deprived of TNF signaling, however, IFN-γ deficient mice do form granulomas [133, 134], so IFN-γ is not required for leukocyte aggregation at the focus of infection. IFN-γ synergizes with TNF to optimally activate macrophages [66], and can trigger macrophage synthesis of nitric oxide intermediates [66, 67], which is one of the key roles for this cytokine in the protective response to *M. tuberculosis*.

1.4.1.3 Interleukin-12

Interleukin-12 (IL-12) is a proinflammatory cytokine produced by macrophages and dendritic cells. This molecule is made up of two subunits, IL-12p35 and IL-12p40 to make a functional IL-

12p70 cytokine (reviewed in [116]). Signaling by IL-12 induces CD4+ T cells to follow the Th1 pathway preferable for the response to intracellular pathogens, which can be characterized by production of IFN-γ. IL-12 is a multifunctional cytokine that may also play a role in inducing T cell, NK and NKT cell proliferation and increase cytotoxic potential [116].

Mice lacking IL-12p40 have a significant deficit of IFN-γ expression, likely accounting for increased viable *M. tuberculosis* bacilli, and higher morbidity [135]. It should be noted, that not only is IL-12p40 a subunit of IL-12, but it can also pair with a p19 subunit to make another proinflammatory, cytokine IL-23 [116]. IL-12p40 deficient mice are more susceptible to *M. tuberculosis* infection than IL-12p35 deficient mice [136], because deficiency in IL-12 can be compensated for by IL-23 expression [137].

BALB/c mice are more susceptible to *M. tuberculosis* infection; particularly intravenous infection. When susceptible BALB/c mice received exogenous IL-12, their survival of intravenous *M. tuberculosis* infection improved [138], while exogenous IFN-γ was unable to rescue BALB/c mice [138]. This suggested that IL-12 plays a role in control over *M. tuberculosis* infection in addition to driving the production of IFN-γ. Administration of low dose IL-12 to an *M. tuberculosis* resistant mouse, C57Bl/6, surprisingly reduced the overall inflammation seen in the lungs by reducing cellularity and delaying priming of an adaptive immune response [139]. IL-12 administration in C57Bl/6 mice did not impair control of bacterial burden or survival [139]. This suggests that IL-12 can not only play a role in driving the Th1 immune response, but may have an immunoregulatory affect under certain circumstances.

1.4.1.4 Osteopontin

Osteopontin is an adhesive component of the extracellular matrix, and also a pro-inflammatory cytokine encoded by early T cell activation gene 1 (eta1) [140-144]. T lymphocytes, NK cells

and macrophages can express varying levels of osteopontin [140], and its functions range from stimulating adhesion to CD44 and α_4 integrin adhesion molecules, to induction of leukocyte chemotaxis, costimulation of T cells during priming and enhancing the production of IL-12 by macrophages [140, 143].

Osteopontin plays a role in shaping the Th1 immune response to infections such as *Listeria monocytogenes,* where this cytokine was required for optimal production of IL-12 and IFN-γ [145]. On the other hand, osteopontin was not required to protect mice from infections with either *Borrelia burgdorferi* [146, 147] or influenza virus (strain PR8) [148]. As it can be detected in high amounts by humans either administered the BCG vaccine or patients with active *M. tuberculosis* infection, and greater illness increases systemic osteopontin, this cytokine could be used as an inflammatory marker of tuberculosis [140-142]. Not only has osteopontin been detected systemically, but many cells within granulomas from patients with tuberculosis express it, and stain positively by immunohistochemistry [149]. This suggests that osteopontin may play a role in attracting leukocytes to the site of infection, shaping the immune response within the granuloma, or even in mediating cellular adherence to form and maintain the granuloma. Osteopontin deficient mice infected intraperitoneally with BCG had more and larger granulomas than controls, and there seemed to be more bacteria present overall within the liver [150]. This supports a role for osteopontin in controlling the immune response to mycobacterial infection, possibly by controlling bacterial burden.

1.4.1.5 Transforming Growth Factor-beta

Transforming Growth Factor-beta (TGFβ) is an anti-inflammatory cytokine that can mediate inflammatory responses in CD4+ T cells and macrophages [151-153]. TFGβ can be detected in the human tuberculosis granuloma [154], and it has been shown that ManLAM, a component of

the *M. tuberculosis* cell wall can directly stimulate TGFβ expression [155]. Blockade of TGFβ can decrease *M. tuberculosis* bacterial burden in human tissue culture [151] and increases T cell and macrophage activities, suggesting that its activities inhibit control of bacterial replication or survival. Suppression of T lymphocytes in response to TGFβ might be due, at least in part, to its role in inducing Foxp3 expression in post-thalamic naïve CD4+ T lymphocytes [100-102, 106, 156, 157], making inducible regulatory T cells. T regulatory cells are capable of directly suppressing proliferation and IFN-γ production of effector T cells primed in the context of an infection [96, 105, 158]. Several studies have shown that T regulatory cells reduce the host response to *M. tuberculosis* [106-109], as discussed in section 1.3.5.

1.4.2 Chemokines

Chemokines are small cytokines that drive leukocyte chemotaxis through G-protein coupled receptor signaling (reviewed in [44]). Signaling by homeostatic chemokines occurs in the absence of infection, such as those that drive migration of naïve T cells into secondary lymphoid organs. The surface expression of chemokine receptors is modified in an inflammatory situation [159]. Migration of leukocytes in response to chemokines is dependent on the expression of specific chemokine receptors on the cell surface, which can be modified upon activation of a leukocyte [71]. For example, upon phagocytosis of an invading pathogen, dendritic cells will downregulate CCR5 and CCR1 and upregulate lymphoid homing receptors CXCR4 and CCR7, which allow the cell to migrate to the lymphoid organs. Similarly, upon activation of a T cell, CCR1, CCR2, CCR3 and CXCR3 are upregulated, while naïve T cell receptors CXCR4 and CCR7 are downregulated, which allows effector T cells to migrate to the site of infection [71].

Inflammatory chemokine production in macrophages infected with *M. tuberculosis* partially depends on the presence of TNF, in the absence of which granulomas do not form. This suggests that chemokines could play a role in granuloma formation [33]. Neutralization of TNF decreases macrophage production of CCR5 and CXCR3 ligands in response to *M. tuberculosis* infection [33]. Mice deficient in individual receptors for TNF-inducible chemokines do not develop the same pathology observed in TNF deficient mice [33, 80, 160-162]. For example, in absence of CCR2, macrophages migrate to *M. tuberculosis* infected mouse lungs more slowly than in control lungs [162]. While granuloma formation in CCR2 deficient mice lags behind wild type mice, by 33 days post-infection, normal granulomas are observed. Unlike TNFRp55 deficient mice, CCR2 deficient mice survive a low dose *M. tuberculosis* infection [162], but are highly susceptible to a high dose infection [162, 163].

CCR5 is upregulated on T lymphocytes upon activation, and CCR5 ligands CCL3, CCL4 and CCL5 are secreted by lung macrophages during *M. tuberculosis* infection [164, 165]. These chemokines attract macrophages and activated T lymphocytes expressing CCR5 on their surface [44]. Mice deficient in CCR5 controlled *M. tuberculosis* infection in the lungs, liver and spleen, but had a higher bacterial burden in the lymph nodes [164]. Granulomas formed normally in the lungs, but there was higher T cell infiltration in the lungs of CCR5 deficient mice than control mice by nine weeks post-infection, accompanied by an increase in pro-inflammatory cytokines [164]. The authors concluded that the higher cellular infiltrate was due to greater dendritic cell migration into the lymph nodes, causing the higher lymphocytic bacterial infection, and increased T cell priming [164]. This hypothesis makes sense, because dendritic cells ordinarily downregulate CCR5 upon antigen uptake, which helps them migrate to the lymph nodes [71]. Therefore, in the absence of CCR5, more dendritic cells could migrate to the lymph node.

CXCR3 is upregulated on T lymphocytes upon activation, and CXCR3 ligands CXCL9 and CXCL10 are produced upon macrophage infection with *M. tuberculosis* [33]. Mice treated with anti-CXCL9 antibody reportedly control bacterial burden and survive *M. tuberculosis* infection like control mice, but with an observable reduction in granuloma number and size early in infection [80]. Data from our laboratory using anti-CXCL9 and anti-CXCL10 double antibody neutralization did not result in any discernible differences from wild type mice (Algood and Flynn, unpublished). Surprisingly, *M. tuberculosis* infection of susceptible BALB/c mice deficient in CXCR3 maintained a lower lung and spleen bacterial burden than control BALB/c mice [161]. C57Bl/6 M. tuberculosis resistant mice deficient in CXCR3, also had a lower bacterial burden in their spleens, but not lungs. Although granulomas formed normally in CXCR3 deficient BALB/c mice, higher CD4+ T cell infiltrate was present during an established M. tuberculosis infection than in control mice [161]. This increase in CD4+ T cells found in the lungs of BALB/c mice may help to control the bacterial burden in the lungs.

Interestingly, although CCR5 and CXCR3 chemokine signaling is believed to be important for migration of activated T lymphocytes into the sites of inflammation, neither of these chemokine receptors were required for accumulation of T lymphocytes, in fact in each case, higher T cell infiltrate was detected. One possible explanation for these findings is that a redundant chemokine receptor can act in the place of one that is absent [166]. In other words, CXCR3 might compensate for the absence of CCR5, but if both of these chemokines receptors were absent, T lymphocytes may be unable to traffic to the site of infection. A transcriptome comparison showed a five-fold increase in CCL5 expression in CXCR3 deficient mice over wild type mice, with a smaller increase in CCR5 [80], suggesting that in the absence of CXCR3 signaling, CCR5 signaling increases to provide adequate leukocyte recruitment to control

infection. Whether mice deficient in both CXCR3 and CCR5 would still be able to control infection remains untested.

1.4.3 Other chemotactic molecules

In addition to chemokines, other chemotactic molecules may be involved in recruiting leukocytes to the site of *M. tuberculosis* infection. Arachidonic acid metabolism catalyzed by 5-lipoxygenase (5-LO) results in the production of lipoxin A_4 (LXA_4) and leukotriene B_4 (LTB_4) chemotactic molecules (arachidonic acid metabolism reviewed by [167]). Deficiency in the 5-LO enzyme improves survival of acute murine infection with *M. tuberculosis* [168], due to decreased inflammation and decreased tissue necrosis. This suggests that at least one chemoattractant molecule produced by 5-LO assists in the inflammatory response, or prevents regulation of the inflammatory response. Both LXA_4 and LTB_4 are produced during *M. tuberculosis* infection, but only LXA_4 continues to be expressed throughout chronic infection [168]. LXA_4 mediates inflammation and can downregulate IL-12 production in dendritic cells [169]. Downregulation of IL-12 may be responsible for a decrease in observed inflammation. Administration of an analog of LXA_4 to 5-LO deficient mice resulted in elimination of the protection seen in the absence of 5-LO [168]. Leukotriene B_4 (LTB_4) recruits leukocytes to an inflammatory site [168]. The specific role of LTB_4 in the immune response to *M. tuberculosis* was not further investigated, so it is unknown whether it is the effect of LXA_4 alone that exacerbates inflammation during *M. tuberculosis,* or whether this can be affected by either LXA_4 or LTB_4.

LTB_4 can signal through one of two G-protein-coupled, membrane-bound receptors. BLT1 can be found mainly on the surface of circulating leukocytes [170, 171], while BLT2 is ubiquitously expressed on cells within organs [172, 173]. LTB_4 is mainly produced by mast cells

and neutrophils [174-176], and can induce firm arrest of cells during migration and increase neutrophil survival [177]. The expression of BLT1 on the surface of circulating leukocytes, and the ability of LTB_4 to signal firm arrest suggests that this molecule might play a role in migration of leukocytes into enflamed tissues. An increase in survival of neutrophils could lead to overabundant cellular infiltrate, due to a decrease in apoptosis. Therefore, it is possible that LTB_4 could exacerbate the early immune response to *M. tuberculosis*, and that its elimination in the 5-LO deficient mouse contributed to improved host response.

1.5 ADHESION MOLECULES IN THE IMMUNE RESPONSE TO MYCOBACTERIUM TUBERCULOSIS

1.5.1 The role of adhesion molecules in the immune system

Adhesion molecules associate with one another to facilitate leukocyte migration into infected tissues and secondary organs of the immune system [72]. Members of the selectin family of adhesion molecules mediate initial interactions of leukocytes with endothelium to allow leukocyte rolling despite rapid flow in blood vessels. Selectin mediated rolling makes firm adhesion by activated integrins and cell adhesion molecules possible [178]. LFA-1 integrin interaction with the cell adhesion molecule ICAM-1 mediates cellular migration into tissues, known as extravasation [72]. Absence of either L-selectin or ICAM-1 on leukocytes leads to impaired delayed-type hypersensitivity reactions [179]. Decreased leukocytes can be detected in the lungs when mice are treated with blocking monoclonal anti-ICAM-1 antibody, YN1, after antigen inhalation [180], demonstrating that ICAM-1 is at least partially required for leukocyte

migration into the lungs. Elimination of L-selectin and ICAM-1 expression together severely reduces leukocyte rolling and adhesion in response to TNF stimulation and significantly delays the rejection of allogeneic skin grafts [179]. The work of these two adhesion molecules can therefore be influenced by the presence of TNF and might mediate entry into *M. tuberculosis* infected lungs.

1.5.2 Adhesion molecules in tuberculosis

The role of adhesion molecules in control of *M. tuberculosis* infection, or their potential role in granuloma formation, has not been fully defined. L-selectin ligation is not required to control *M. tuberculosis* infection ([181] and Windish and Flynn unpublished). Two published studies addressed the role of CD44 in the protective immune response against *M. tuberculosis*. A high dose of *M. tuberculosis* infection, $1x10^5$ colony forming units, led to less organized cellular infiltrate, less definitive granuloma formation, an impairment of cellular infiltration into the lungs, and a lack of control over bacterial burden in CD44 deficient mice [182]. On the other hand, a low dose of aerosol *M. tuberculosis*, 100 colony forming units, resulted in no difference in lung pathology, cellular infiltrate or bacterial burden in CD44 deficient mice [183]. These data imply that the dependence of specific adhesion molecules could be affected by the dose of infection.

The interaction of immunoglobulin-like adhesion molecules such as VCAM-1 and ICAM-1 with their integrin ligands, LFA-1 and VLA-4 respectively, mediate firm adhesion to endothelium, allowing cellular migration into *M. tuberculosis* infected lungs [72]. VCAM-1 is upregulated on enflamed endothelium during *M. tuberculosis* infection [184] and when

monoclonal antibodies neutralized lymphocyte VLA-4, lung cellular infiltrate was less organized 14 and 28 days after low dose aerosol infection than in untreated mouse lungs [184].

ICAM-1 can be expressed either on inflamed endothelium, or on migrating leukocytes [72]. This adhesion molecule could therefore be involved in either extravasation into enflamed tissue, or the aggregation of two leukocytes at the focus of infection. Mice deficient in ICAM-1 reportedly survived a mean of 136 days after aerosol *M. tuberculosis* infection, while wild type mice survived longer than 270 days [185]. Lung pathology in these mice was observed only on day 126 post-infection when half of the mice were moribund. At the time of morbidity, immune cell infiltrate was disorganized, with more neutrophils and fewer lymphocytes present at the site of infection [185]. Furthermore, ICAM-1 deficient mice had a significantly higher lung bacterial burden than control mice with more dissemination of mycobacteria to the spleens and livers [185]. Although the study showed ICAM-1 is required for control of *M. tuberculosis* infection, the question remains whether their interactions are required for extravasation or for leukocyte to leukocyte associations. Further, although mice did succumb to infection, they persisted around 4 months post-infection [185], indicating that some degree of control over infection does occur in these mice, but the source of this control has not been addressed. Conflicting data were published showing that although granulomas are not formed in the absence of ICAM-1 expression, mice survive similarly to controls [186]. Overall, studies in immunoglobulin-like adhesion molecule deficiency during *M. tuberculosis* highlight a potential role for cell adhesion molecules in control of infection, the details of which are yet to be determined.

1.6 MODELING MYCOBACTERIUM TUBERCULOSIS INFECTION

1.6.1 Mouse

Multiple strains of inbred mice are available, and different strains have varying abilities to control mycobacterial infection [187-189]. Characterization of susceptible and resistant strains was primarily in response to intravenous infection. Resistant mice maintain a lower level of splenic bacterial burden [187, 188], and cell numbers [189], compared to susceptible mice [187]. C57BL/6 mice are relatively resistant to *M. tuberculosis* delivered via aerosol or intravenously [188] [190].

In response to aerosol infection, bacteria enter the lungs of mice, and replicate within the macrophages[188]. After 2-4 weeks of infection, bacterial replication is controlled by the immune system [191]. Following stabilization of infection (~4 weeks), bacterial burden in the lungs is maintained as a chronic infection at 10^5-10^6 colony forming units/lung [191]. Numbers of CD4+ and CD8+ T lymphocytes present in the lung after about 4 weeks also are maintained at relatively steady numbers throughout infection, and these numbers are maintained by a consistent turn over, in which the number of cells undergoing apoptosis is equivalent to the number of cells either proliferating or migrating into the lung [191].

Granulomas are formed in the mouse lung during mycobacterial infection [192], but murine granulomas differ in organization and cellular recruitment from those seen in human tuberculosis. The murine granuloma consists of clusters of T cells and macrophages, with a low level of organization [190]. On the other hand, the primate granuloma appears more organized,

and has a higher prevalence of caseous necrosis [82]. Further, the human granuloma has several defined morphological structures that are not seen in the murine granulomas [82].

Although the mouse model has the advantages of availability of immunologic reagents for studies, inbred and genetically engineered strains, and tractability, *M. tuberculosis* infection in mice does not quite parallel human tuberculosis [3]. This is perhaps not surprising as the mouse is not the natural host of *M. tuberculosis* [193]. The mouse model does not model latency in human infection, in which there is a very low bacterial burden present in 90% of the infected population, instead maintaining a relatively high bacterial burden chronically [3]. Although chronic infection with *M. tuberculosis* is not a perfect model of human tuberculosis, it has been predictive of human tuberculosis, and has been useful in determining the role of host and mycobacterial factors during infection [40].

1.6.2 Non-human primates

Unlike mice, *M. tuberculosis* infection in macaques mimics the pathology observed in humans [82]. Macaques inoculated by bronchoscope with a low dose of infection will develop a positive tuberculin skin test within about 2 months of infection [36], which confirms that an adaptive immune response to *M. tuberculosis* has occurred. Macaques can develop latent or active infections, characterized based on the presence or absence of clinical symptoms of tuberculosis [36]. Actively infected macaques will also have radiologic signs of tuberculosis, and may begin to lose weight. On the other hand, latent monkeys will have no signs of tuberculosis. In a cohort of cynomolgus macaques, ~50% will develop latent infection, and this latency appears to truly mimic human latency [82]. Granulomas found in the lungs of monkeys with latent or active

tuberculosis also appear similar to human granulomas, and different types of granulomas have been observed in macaques, as has been seen in humans [36].

Non-human primates are expensive to obtain and maintain, and they are not inbred, which leads to a high degree of variability in results, contributing to the lack of use of this model until recently [36]. However, due to the study of HIV in macaques, many immunologic reagents have become available for studying the host response to infections, making it possible to develop a non-human primate model of tuberculosis [3].

1.6.3 Guinea pigs

The guinea pig is extremely susceptible to *M. tuberculosis* [29], making it useful for the study of vaccines or efficacy of drugs [29]. Guinea pig granulomas share some similarities with certain human granulomas, but clearly lack the same ability to control bacterial replication [194]. The guinea pig infection has been broken into three stages: the acute, sub-acute and chronic stages. During the acute phase, bacteria replicate rapidly, until granulomas are formed. Following granuloma formation, the sub-acute phase marks a period in which *M. tuberculosis* disseminates to extrapulmonary locations, but replication is controlled in the lungs [194]. Guinea pig infection differs from human cases of tuberculosis, in which only about 18% of patients with tuberculosis develop extrapulmonary disease [195], despite similar morphology [196, 197]. Finally, in the chronic phase of guinea pig tuberculosis, the lung is re-infected by extrapulmonary bacteria, and guinea pigs succumb to high bacterial burden [194]. Aside from the differences between guinea pig and human tuberculosis, few immunologic reagents are available for the guinea pig, making it a difficult model in which to study immune responses, although more reagents are now becoming available [194].

1.6.4 Rabbits

Rabbits are resistant to *M. tuberculosis* infection [198], making this model useful for study of susceptibility to various strains of *M. tuberculosis*. In spite of being resistant to *M. tuberculosis* infection [198] with possible clearance [199], rabbits succumb rapidly to high dose *M. bovis* infection [200]. [198-201]. While high doses of *M. tuberculosis* are required in order to see a tuberculous lesion [199], *M. bovis* granulomas appear similar to human *M. tuberculosis* granulomas [199]. It has been suggested that *M. tuberculosis* infection in rabbits may be similar to a latent infection in humans, while *M. bovis* infection is more similar to an active infection [82]. Few immunologic reagents are available for studying host-pathogen interaction in rabbits and rabbits are more expensive to maintain in a BSL3 facility, making this a difficult model in which to study tuberculosis [202].

1.6.5 Computational biology

On the whole, biological scientists find it difficult to accept that mathematics could be useful for asking biological questions [203]. However, it has been pointed out that many commonly relied upon systems are already predicted by mathematical modeling, such as weather and economic trends [203]. Mathematical models can, in fact, be generated by computer programmers by compiling existing wet bench data from the literature, biological collaborators and statistical uncertainty analyses, converted into mathematical equations, and entered into a computer [203]. This *"in silico"* model can then be used to make predictions about a system, for example a host-pathogen interaction, that could not be tested in animal models, or to make predictions that can later be tested in an animal model [82].

Several *in silico* models of human infection with *M. tuberculosis* have been developed by the Kirschner group in collaboration with the Flynn laboratory [203]. These models have been validated by comparing results with animal data collected by the Flynn laboratory. For example, when granuloma formation is examined in the mathematical model, two different granuloma types are predicted: either a small, solid granuloma, or a larger, necrotic granuloma may result. Multiple structures have been observed in the non-human primate, confirming that the computer model agrees with an existing animal model of tuberculosis [203].

Not only have the computer simulations been capable of replicating animal model data, but new predictions have been made that can now be tested *in vivo*. One such prediction is that the anti-inflammatory cytokine IL-10 is required for control over *M. tuberculosis* infection in humans, and that reactivation of tuberculosis would occur in its absence [204]. This result is surprising, because mice deficient in IL-10 have no observable differences when compared with control mice [204]. As the mouse model of tuberculosis differs from human tuberculosis in ways described in section 1.5.1, this unanticipated prediction can now be investigated in the non-human primate model of tuberculosis [82], where the immune response more closely resembles that of humans.

While *in silico* models are useful for making unintuitive predictions, it will be important for individuals working with computer generated models to work with wet bench laboratories. These models are dependent on the data that are already in the literature, and on validation by animal models. On the other hand, *in silico* models have a great advantage of compiling vast amounts of data collected in various in vivo and in vitro systems, as well as in humans, perhaps providing a broader picture of the problem.

2.0 STATEMENT OF THE PROBLEM

The goal of this study was to investigate the immunopathology of *Mycobacterium tuberculosis* infection. The granuloma prevents bacterial dissemination and allows control of bacterial burden without completely eliminating the pathogen. The cellular interactions that yield a functional granuloma are unclear, but elucidation of these interactions might provide clues about why some infected individuals develop active disease, while the majority maintains latency.

Previous work in our laboratory and others has shown that TNF is required for the formation and maintenance of the granuloma, and that in the absence of an organized granuloma mice succumb quickly to *M. tuberculosis* infection. Therefore, identification of the critical role(s) of TNF in granuloma formation would identify cellular interactions required for controlling tuberculosis. Neutralization of TNF is an effective treatment for such chronic inflammatory diseases as rheumatoid arthritis and Crohn's disease. Reports in the literature suggest that anti-TNF antibody treatment (infliximab) increases the risk of reactivating tuberculosis. On the other hand, the risk of tuberculosis during treatment with a TNF-receptor-antibody fusion protein (etanercept) might be lower than during antibody treatment. It is currently unknown whether patients might truly be at less risk of tuberculosis when TNF is neutralized by the differing reagents, nor the mechanism behind a difference in TNF reagent effects.

The granuloma provides a local environment in which leukocytes can interact with one another to control *M. tuberculosis* infection. In order for successful granulomas to be formed, T lymphocytes must be primed and migrate to the site of infection, where primed T lymphocytes, infected macrophages and other cells interact to form the granuloma. The molecules required for sufficient T cell priming and the attraction of primed T lymphocytes to the lungs of *M. tuberculosis* infected mice have not been fully defined. The potential roles of the pro-inflammatory cytokines TNF and osteopontin in the priming of functional T cells has not yet been explored. Although the expression of CXCR3 and CCR5 chemokine receptor ligands has been detected in the lungs of *M. tuberculosis* infected mice, deficiency one of these receptors does prevent lymphocyte migration. The possibility exists that one of these molecules could compensate for the absence of the other, but this has not yet been explored. Migration of leukocytes to the site of infection may be facilitated by other chemoattractants, such as the small chemoattractant molecule, LTB_4, a molecule whose role in tuberculosis is currently undefined.

Migration is facilitated by the interaction of leukocyte adhesion molecules with those on the surface of endothelium. It is therefore possible that adhesion molecules such as ICAM-1 may be required for the successful migration of leukocytes into *M. tuberculosis* infected lungs. It is further possible that once cells have located the site of infection, in order to form a functional granuloma, adhesion molecules must interact with one another. Some important adhesion molecules may even require TNF expression for their expression, which could explain why TNF is required for granuloma integrity. The role of adhesion molecules in the host response to *M. tuberculosis* infection, particularly the role of ICAM-1, has not been fully defined.

In this study, we dissected aspects of the immune system that may be required for granuloma formation. We compared the ability of anti-TNF reagents in the reactivation of

tuberculosis. We tested the requirement of the pro-inflammatory cytokines TNF and osteopontin in the priming of an effective immune response to *M. tuberculosis,* and compared the expression of several adhesion molecules in the presence and absence of TNF expression. We examined the roles of CXCR3 and CCR5 ligands, as well as LTB_4 in chemoattraction during infection. Finally, we defined the role of the adhesion molecule ICAM-1 in the migration of leukocytes into the site of infection. We further investigated the role of the adhesion molecule ICAM-1 in the formation of granulomas, and in control of tuberculosis.

3.0 CHAPTER 1 TNF NEUTRALIZATION BY ANTI-TNF ANTIBODY, BUT NOT TNF RECEPTOR FUSION MOLECULE EXACERBATES CHRONIC MURINE TUBERCULOSIS

The work presented in this chapter was published (1) or referenced (2) in the following articles:

1. **Plessner, H.**, Lin, PL., Kohno, T., Louie, J., Kirschner, D., Chan, J., Flynn, JL. Neutralization of TNF by antibody but not TNF receptor fusion molecule exacerbates chronic murine tuberculosis. *Journal of Infectious Disease*. 2007 Jun 1; 195(11):1643-50.

2. Lin, PL., **Plessner, H.,** Flynn, JL. Tumor Necrosis Factor and tuberculosis. *Journal of investigative dermatology symposium proceedings.* 2007 May; 12(1):22-5.

3.1 INTRODUCTION

TNF inhibition during chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease using anti-TNF antibodies, such as adalimumab and infliximab, or the soluble TNF receptor fusion molecule etanercept has reduced inflammation and improved quality of life [205]. However, TNF inhibition compromises the immune system, increasing the risk of infections in the respiratory tract, skin, bones and joints [206]. By 2002, over 300 cases of *M. tuberculosis* disease had been linked to infliximab treatment [207]. In 2004, voluntary data collected worldwide showed the risk of reactivation tuberculosis was statistically lower in

patients receiving etanercept (28 of 100,000 patients) compared to infliximab (54 of 100,000 patients) [130]. While not yet elucidated, it has been proposed that the mechanisms of action between these anti-TNF molecules differ.

Infliximab and etanercept associate with the trimeric TNF molecule by different mechanisms. This may account for their discordant effects reported in patients infected with *M. tuberculosis*. Each infliximab antibody has two TNF binding sites that bind two individual molecules of TNF, and more than one antibody can bind to each TNF trimer. This may result in a complex of infliximab and TNF, neutralizing all available molecules. On the other hand, a single molecule of etanercept can bind to only one molecule of TNF, enclosing the cytokine between the two p75-TNF receptors bound to an IgG1-Fc. This interaction utilizes two of the three available active sites of the trimeric TNF molecule, leaving an active site with potential to interact with host TNF receptors. *In vitro* competition experiments have shown that more than 50% of TNF bound to etanercept dissociated within 10 minutes, while TNF bound to infliximab could not be competed away for at least 90 minutes [131]. During treatment of chronic inflammatory disease, a difference in TNF neutralization may still allow a therapeutic threshold to be met, making both treatments effective. Less aggressive blockage of TNF, on the other hand, may decrease the relative risk of reactivating tuberculosis.

To model and compare the effects of anti-TNF antibody and soluble TNFR2-Fc in the primary and chronic phases of *M. tuberculosis* infection, we used the murine system and reagents modeled after infliximab and etanercept human TNF neutralizing agents. Our results demonstrate that during primary infection, both drugs exacerbated tuberculosis with similar kinetics. However, when treatment was initiated during an established infection, only those mice treated with anti-TNF antibody developed overwhelming disease. In contrast, the majority of chronically

infected mice treated with murine TNFR2-Fc (mTNFR2-Fc) survived with no evidence of exacerbated disease. We explored the effects of these test molecules during each stage of infection to identify mechanisms by which they differ. These results have important public health implications for the use of TNF neutralizing drugs in global regions where tuberculosis is endemic, as rates of tuberculosis reactivation and primary disease may increase substantially upon TNF neutralization without appropriate education, testing, and therapies.

3.2 MATERIALS AND METHODS

3.2.1 Animals

C57BL/6 female and C3 deficient mice (Jackson Laboratory, Bar Harbor, ME) were housed in a BioSafety Level 3 specific pathogen-free facility and monitored for murine pathogens. All animal protocols used were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.2 Mycobacteria and murine infection

Low dose aerosol infection of mice (20-100 colony forming units) of *M. tuberculosis* strain Erdman (Trudeau Institute, Saranac Lake, NY) has been previously described [110]. Briefly, mice were loaded into an InTox aerosol unit (Albuquerque, NM), with ~4.4 million colony forming units in 4mL in the nebulizer. Mice were allowed to breathe aerosolized bacteria for 20 minutes, then allowed to breathe aerosolized sterile phosphate buffered saline for 5 minutes prior to removal from the apparatus. Bacterial burden was determined as previously described [208]. Briefly, one day post-infection, the whole lung of representative mice from each round of infection were homogenized in phosphate buffered saline with 0.5% Tween80. Whole lung homogenates were plated on 7H10 agar plates and incubated at 37ºC for three weeks in order to count the total colony forming units received by mice in each round of infection. During organ harvests, aliquots of organ homogenates were diluted in phosphate buffered saline and 0.5%

Tween80 and allowed to incubate at 37ºC for three weeks on 7H10 plates. The total number of colony forming units per lung was calculated by multiplying the dilution factor to the total counted units on 7H10 plates and the original volume of the homogenate.

3.2.3 Chemicals and reagents

All chemicals were purchased through Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Middlebrook 7H10 agar and 7H9 liquid media for growth of *M. tuberculosis* were purchased from Difco (Detroit, MI). Flow cytometry antibodies were purchased from BD Pharmingen, except anti-C3 and its isotype control (Cedarlane Laboratories, Burlinton, NC). MP6-XT22 (rat IgG1) [208] was purified from cell culture of a hybridoma obtained from DNAX by the National Cell Culture Center (Minneapolis, MN). TNFR2-Fc [209, 210] and control IgG1 antibody were a kind gift from Amgen (Thousand Oaks, CA). Murine anti-TNF molecules or IgG1 were diluted in phosphate buffered saline and injected intraperitoneally into mice one day prior to acute infection with *M. tuberculosis* or 4 months following *M. tuberculosis* infection for chronic studies, and biweekly for the duration of the study.

3.2.4 WEHI assay for TNF bioactivity

The bioactivity of TNF was measured by sensitive WEHI 164 subclone 13 (American Type Culture Collection) as previously described [211]. Briefly, WEHI 164 subclone 13 cells were seeded on a 96 well, flat bottom plate overnight 1 day prior to the assay. Test media or sera were diluted in cell culture media and added to the WEHI cells to incubate at 37ºC for 24 hours. Bioluminescent substrate was added to the wells. Breakdown of luminescent material indicated

higher WEHI cell survival, and less killing due to TNF interaction. To detect systemic TNF neutralization, blood was collected by retro-orbital bleed using non-heparinized capillary tubes (Drummond Scientific, Broomall, PA) and centrifuged at 13xg in a serum separator tube (Becton Dickinson, Franklin Lakes, NJ). Serum was diluted in WEHI assay media (1:6) with a final concentration of 1000pg/mL TNF and incubated overnight with WEHI cells.

3.2.5 Flow cytometry

Lung infiltration was examined at predetermined time points as previously described [33, 162]. Briefly, ~10^6 cells were obtained from organ homogenates or *in vitro* culture. Cells were washed in FACS Buffer (phosphate buffered saline with 0.5% BSA, 0.5% sodium acetate) and resuspended in staining buffer (FACS buffer with 20% mouse serum). Cells were stained with ant-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD3 (clone 17A2), anti-CD11c (clone HL-3), anti-CD11b (clone M1/70), anti-GR1 (clone RB6-8C5), and anti-Annexin V (BD Pharmingen clone not provided). Diluted antibodies were added to cells and incubated at room temperature for 15 minutes. Cells were washed twice in FACS buffer after staining and resuspended in 4% paraformaldehyde.

3.2.6 Histopathology

Samples of lung were fixed in 10% normal buffered formalin and embedded in paraffin. 5-6μm sections were H&E stained. Slides were examined in a blinded fashion. TUNEL staining was performed using the Apoptag kit (Chemicon, Temecula, CA) per manufacturer's protocol. To track labeled molecules, lung blood and airways were cleared by perfusion and bronchoalveolar

lavage. Lungs were fixed in 2% paraformaldehyde and infused with 30% sucrose. Sections were stained 30 minutes with rhodamine phalloidin (Invitrogen, Carlsbad, CA) and Draq5 (Biostatus, Leicestershire, UK). Images were captured using Olympus Flowview 500 software (Olympus Inc., Mellville, NY).

3.2.7 Preparation of fluorescein (SFX)-labeled proteins

A 10-fold molar excess of 5(6)-SFX (6 Fluorescein-5-(and -6)-carboximido hexanoic acid, succinimidyl ester in DMSO was added to mTNFR2-Fc [210], MP6-XT22 or a control murine antibody (IgG1) and incubated for one hour with a final protein concentration of 3 mg/ml. Unincorporated dye was removed by size exclusion column chromatography, NAP 5 (GE Healthcare) and eluted with saline. Protein concentrations and degrees of labeling were calculated using absorbance at 280 nm and 495 nm, respectively.

3.2.8 Statistical analysis

Bacterial burden and cell infiltration were analyzed by a one way ANOVA, while the log rank test was used to compare survival. A value of $p<0.05$ was considered significant. Experiments were repeated to demonstrate reproducibility.

3.3 RESULTS

3.3.1 Systemic TNF neutralization is similar with anti-TNF antibody and mTNFR2-Fc

MP6-XT22, a rat IgG1 monoclonal antibody, neutralizes murine TNF and is the standard anti-TNF antibody used in murine studies [208, 212]. The human reagent infliximab is a chimeric monoclonal antibody (mouse-human IgG1) that is also extremely effective at neutralizing TNF [131]. We used MP6-XT22 as a model of infliximab. Murine TNFR2-Fc was modeled directly on human etanercept. Etanercept is a human soluble p75TNF receptor-human IgG1, while murine etanercept is a murine soluble p75-TNF receptor murine IgG1 [210].

To compare TNF neutralization by MP6-XT22 and mTNFR2-Fc, increasing doses of each molecule were added to WEHI clone 13 cells (a TNF bioassay) [213] in the presence of 1000pg/mL recombinant murine TNF. TNF bioactivity was neutralized equivalently *in vitro* by both mTNFR2-Fc and MP6-XT22 (Figure 1A). TNF was not reliably detectable in the serum of infected mice (data not shown), so to test the potential for systemic TNF neutralization, mice chronically infected with *M. tuberculosis* were administered one intraperitoneal injection of 0.1 mg MP6-XT22, mTNFR2-Fc or IgG1. Serum was collected four hours post-injection and every 24 hours for three days. Added TNF (1000 pg/ml) was completely neutralized by serum (diluted 1:6) from infected mice treated with either MP6-XT22 or mTNFR2-Fc, but not mice treated with IgG1 (Figure 1B).

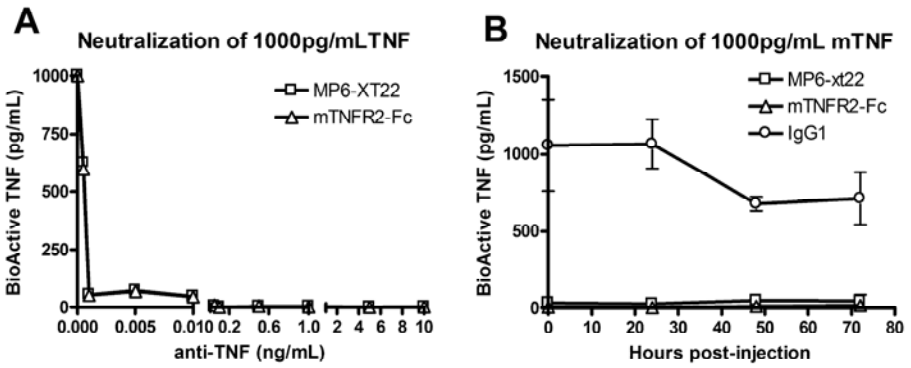

Figure 1 Efficient neutralization of tumor necrosis factor by both MP6-XT22 and mTNFR2-Fc.

WEHI bioassay was used to assess the bioactivity of TNF in the presence of MP6-XT22 or mTNFR2-Fc.

(A) Increasing MP6-XT22 or mTNFR2-Fc were added to WEHI clone 13 cultures with 1000pg/mL TNF

(B) Mice were injected at time 0 with 0.1mg MP6-XT22, mTNFR2-Fc or IgG1. Serum was collected and incubated with WEHI clone 13 and 1000pg TNF/mL.

3.3.2 Both anti-TNF antibody and TNFR2-Fc exacerbate acute infection with *M. tuberculosis*

To test whether anti-TNF antibody and soluble receptor fusion molecule treatment differentially affect control of the primary phase of *M. tuberculosis* infection, MP6-XT22 (0.5 mg), mTNFR2-Fc (0.2 mg) or IgG1 (0.5 mg) was administered intraperitoneally 1 day prior to infection, and continued twice weekly for the duration of the study. Both reagents exacerbated disease, and mice became moribund with a mean survival time of 22 $\pm$ 1 days post-infection (Figure 2A) Control mice did not succumb to infection, surviving significantly longer than both anti-TNF groups ($p<0.0001$). Even at a reduced dose (0.1 mg twice weekly), MP6-XT22 and mTNFR2-Fc treated mice succumbed to infection with mean survival times of 19 $\pm$ 5 days and 22 $\pm$ 0 days post-infection, respectively (Figure 2A).

Although slightly higher in mice treated with anti-TNF reagents, bacterial burden among all groups of mice did not differ significantly up to ~3 weeks post-infection when anti-TNF treated mice succumbed to infection (Figure 2B) as reported in previous studies [33, 120, 128]. By four weeks, granulomas were present in control mice (Figure 3A). In contrast, mice treated with either MP6-XT22 or mTNFR2-Fc had disorganized cellular infiltration and increased inflammation in the lungs (Figure 3A). The degree of lung inflammation among anti-TNF treated mice likely resulted in their poor survival. These data are consistent with previous findings that TNF is required for granuloma formation and survival [33, 120].

To examine whether low dose anti-TNF treatment during acute *M. tuberculosis* infection affected survival, mice were treated with either drug at 0.01mg twice weekly, and these mice survived > 6 months post-infection. Serum from these mice neutralized TNF in the WEHI assay, with results similar to Figure 1B (data not shown), indicating that systemic levels of reagent were sufficient for TNF neutralization.

3.3.3 Treatment with anti-TNF antibody, but not TNFR2-Fc, exacerbated chronic tuberculosis

We hypothesized that both MP6-XT22 and mTNFR2-Fc would impair control over established infection. Mice were infected with *M. tuberculosis*; after four months, during the chronic stage of infection, intraperitoneal injections of MP6-XT22 (0.5mg), mTNFR2-Fc (0.2 and 0.5 mg), IgG1 (0.5mg) or PBS were administered twice weekly. MP6-XT22 treated mice succumbed to infection with a mean survival time of 33 $\pm$ 6 days post-treatment (Figure 2C). Mice given mTNFR2-Fc or control injections survived significantly longer than MP6-XT22 treated mice ($p<0.0001$).

Three weeks of MP6-XT22 treatment in chronically infected mice resulted in a significantly higher bacterial burden than mTNFR2-Fc treated or control mice ($p<0.05$), as previously published [33, 128] (Figure 2D). Consistent with previous findings [119], lung sections revealed loss of granuloma organization after four weeks of MP6-XT22 treatment, whereas controls and mTNFR2-Fc treated mice maintained granuloma structure (Figure 3B) beyond 4 months of mTNFR2-Fc treatment (Figure 3C).

To investigate the difference between MP6-XT22 and mTNFR2-Fc treatment during chronic *M. tuberculosis* infection, leukocytes isolated from the lungs of treated mice at serial time points were analyzed by flow cytometry. No differences in the percentage of CD4 and CD8 T lymphocytes, macrophages or neutrophils were observed.

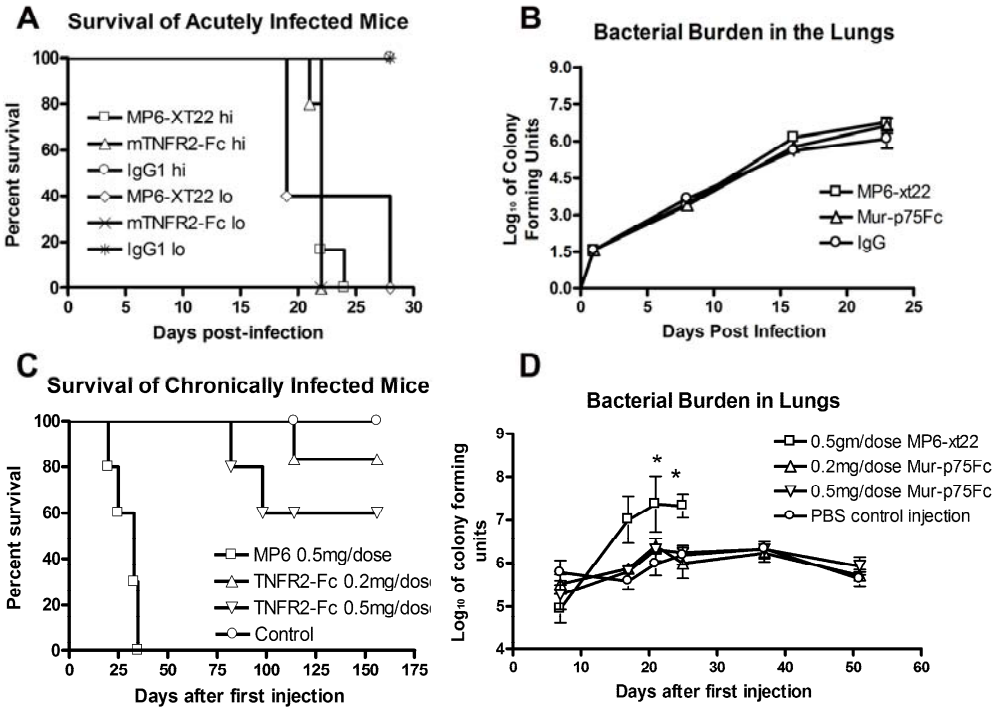

Figure 2 Survival and bacterial burden in mice treated with anti-TNF.

To determine whether MP6-XT22 or mTNFR2-Fc treatment inhibit control of *Mycobacterium tuberculosis* infection, mice were acutely (A, B) or chronically (C, D) infected with *M. tuberculosis* and treated with 0.5mg of MP6-XT22, 0.2 or 0.5mg of murine TNFR2-Fc, or control injections twice weekly. The ability of treated mice to survive infection

(A,C) and control bacterial burden (B, D) was assessed. (A) To determine whether a lower dose would also prevent control over *M. tuberculosis,* mice were acutely infected and treated with a low dose (0.1mg/dose). *p<0.05

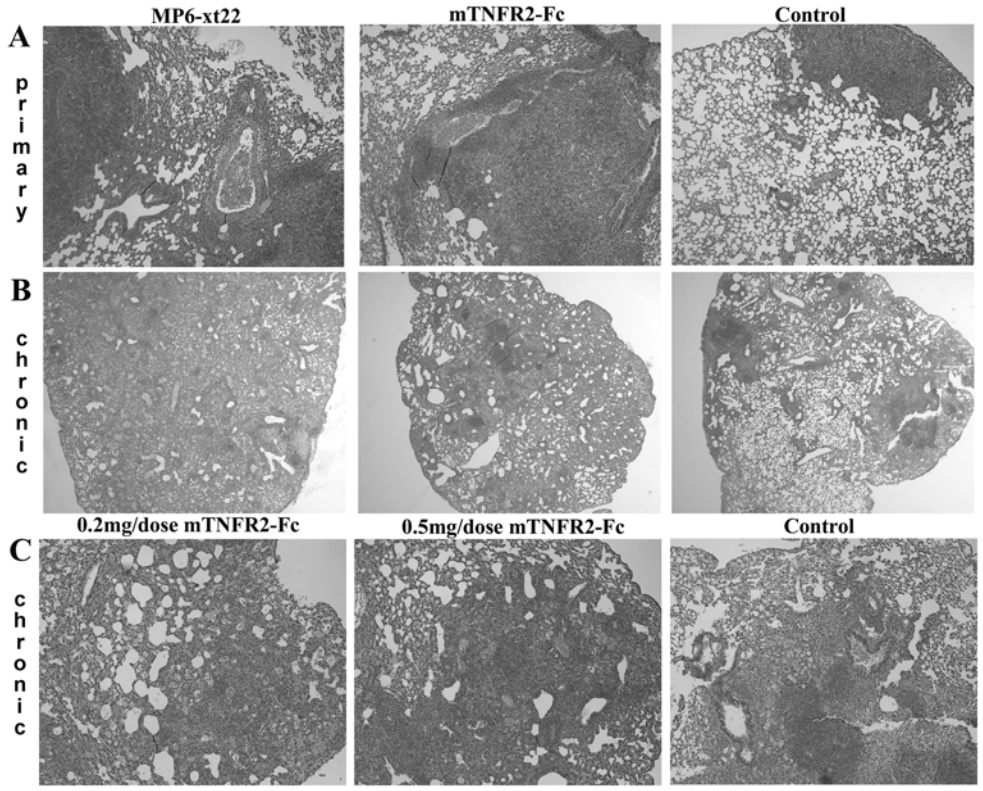

Figure 3 Granuloma formation and maintenance during anti-TNF treatment.

Immunpathology in mice treated with anti-TNF reagents was followed by staining of lung sections with hemotoxylin and eosin. Representative sections (magnification 20x) are shown.

(A) 4 weeks post-infection

(B) 4 weeks of injections in chronically infected mice

(C) 4 months of injections in chronically infected mice

3.3.4 Anti-TNF antibody increases complement protein C3 on T cells in the lungs

It has been hypothesized that if trans-membranous TNF is bound by anti-TNF antibody, the Fc portion could cause complement deposition (C3b), resulting in death of TNF-expressing cells [214, 215]. Although TNF could not be detected on the surface of cells (data not shown), flow

cytometry revealed increased C3 deposition on CD4+ T cells in lungs of mice treated with MP6-XT22 compared to mTNFR2-Fc treated or control mice during acute infection (Figure 4B). There were significantly fewer CD4+ T cells, but not macrophages in the lungs of MP6-XT22 treated mice compared to control or mTNFR2-Fc treated mice during primary infection, but not chronic infection (Figure 4A and data not shown). We did not observe a similar loss of CD4 T cells in the C3 deficient mice after MP6-XT22 treatment (Figure 4C), supporting that C3b deposition contributes to loss of CD4+ T cells. However, the C3 deficient mice were equally susceptible to MP6-XT22-induced exacerbation of tuberculosis.

Conflicting reports in the literature suggest that anti-TNF antibody may affect apoptosis of T cells and macrophages [214-217]. In both acutely and chronically infected mice, Annexin V staining by flow cytometry (Figure 4D) and TUNEL staining of lung sections (Figure 4E) revealed no obvious differences in apoptotic cells between groups.

3.3.5 More MP6-XT22 is present in established granulomas compared to TNFR2-Fc

Although the potential for systemic neutralization of TNF was similar with both MP6-XT22 and mTNFR2-Fc, drug penetration and local neutralization within the lung tissue may differ. Fluorescently labeled mTNFR2-Fc or MP6-XT22 was injected into chronically infected mice. Labeling of these molecules did not impair the TNF neutralization ability *in vitro* (data not shown). After 2 days, both molecules were detectable by confocal microscopy (representative pictures shown in Figure 5A). Occasional aggregations of fluorescently labeled MP6-XT22 were observed in sections from lung lobes of all mice from this group. In contrast, few sections of lung had visible mTNFR2-Fc-SFX or IgG1-SFX (Figure 5A and data not shown). To quantitatively compare levels of each molecule in lung, random sections of lobes were assessed,

and the ratio of labeled molecule to the number of nuclei in each section was calculated using Metamorph software (Figure 5B). Significantly more fluorescently labeled anti-TNF antibody was detected in the lungs of mice than fluorescently labeled mTNFR2-Fc or IgG1. We conclude from these findings that there was reduced penetration or retention of mTNFR2-Fc compared to MP6-XT22 into the granulomas during established infection. This may lead to less efficient neutralization of TNF within the granuloma, and improved outcomes among the chronically infected mice given mTNFR2-Fc compared to MP6-XT22.

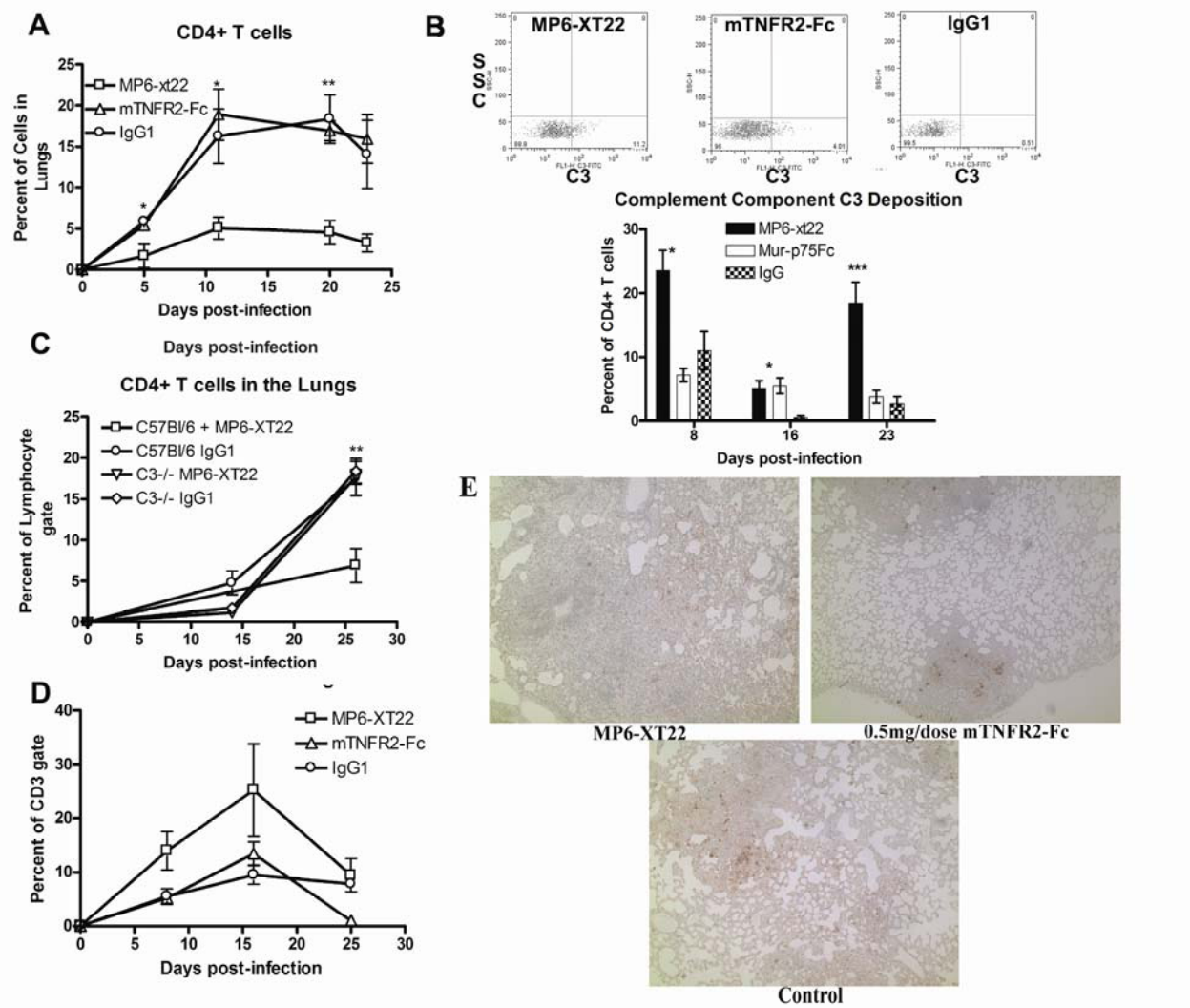

Figure 4 Complement C3 deposition and apoptosis of CD4+ T cells

(A) Percentage of CD4+ T cells in lung infiltrate collected by flow cytometry) of mice treated with MP6-XT22 of mTNFR2-Fc or infected acutely with *M. tuberculosis.* *p<0.05; **p<0.001

(B) Complement C3 staining collected by flow cytometry and gated on CD4+ lymphocytes in MP6-XT22, mTNFR2-Fc and control treated mice. p<0.05; **p<0.001

(B) CD4+ T cells in C3-/- or control mice treated with MP6-XT22 or IgG1 assessed by flow cytometry.

(C) Flow Cytometry analysis of Annexin V staining in lungs of mice injected with MP6-XT22, mTNFR2-Fc or control.

(D) TUNEL staining of lung sections of mice chronically infected with *M. tuberculosis*. (20x magnification)

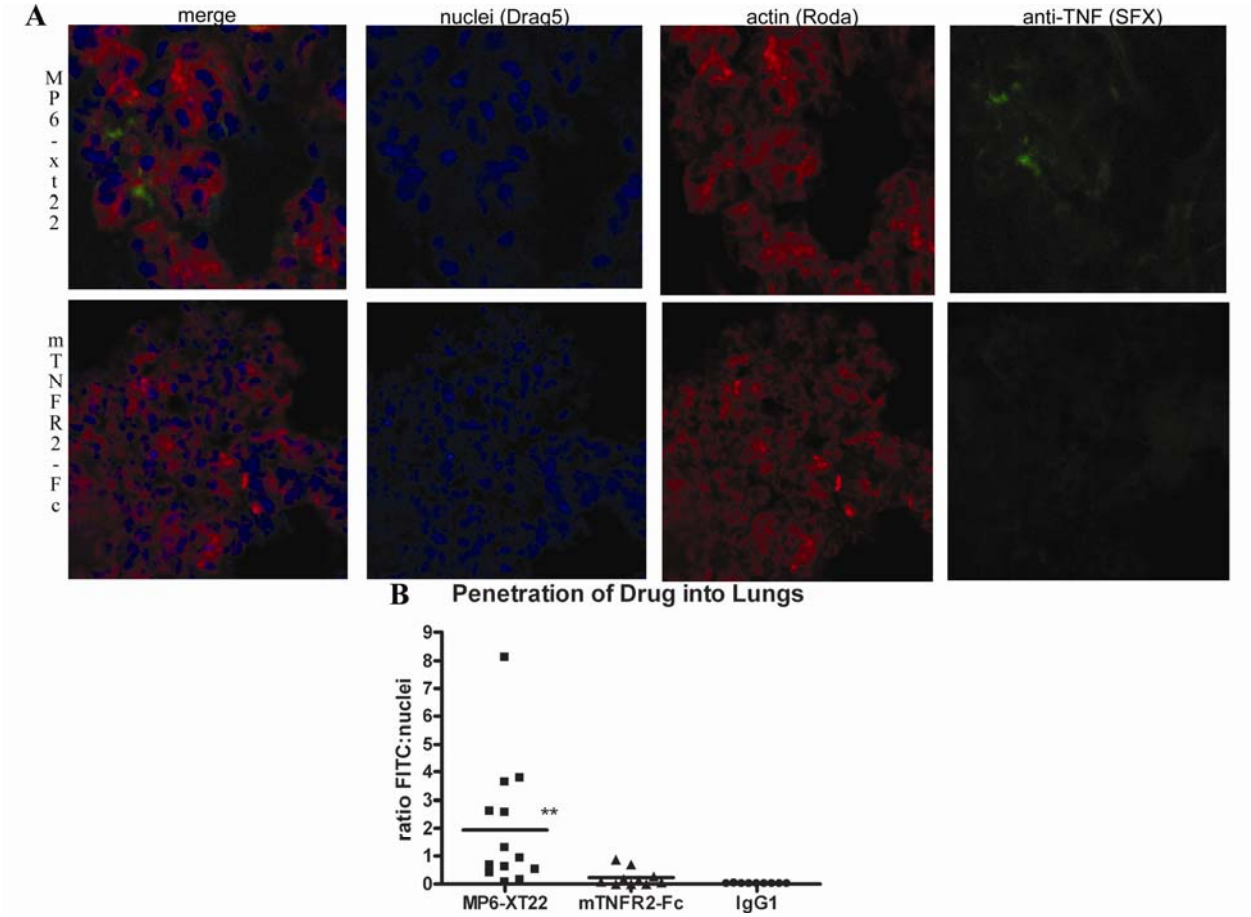

Figure 5 Penetration of treatment into the lungs with established *M. tuberculosis* infection.

To determine whether MP6-XT22, mTNFR2-Fc or IgG differentially penetrate the lungs, these reagents were labeled fluorescently (with SFX), injected into mice chronically infected with *M. tuberculosis* and tracked to the lungs 3 days post-injection.

(A) Representative lung sections (magnification 20x) from mice receiving SFX-labeled drug and staining for actin (red) and nucleus (blue).

(B) Random sections of lung from mice injected with anti-TNF or IgG1-SFX analyzed for the presence of labeled drug. Data are reported as the SFX:nuclei ratio. **p<0.001

3.4 DISCUSSION

We compared the ability of soluble TNFR2-Fc fusion protein and anti-TNF antibody treatment in C57BL/6 mice during *M. tuberculosis* infection. Murine TNF neutralizing agents were used as surrogates for the human reagents: MP6-XT22 is a rat IgG1 monoclonal antibody against murine TNF that efficiently neutralizes TNF *in vitro* and *in vivo* [208]. The human anti-TNF antibodies are human-mouse chimeric monoclonal IgG1 (infliximab) or humanized monoclonal IgG1 (adalimumab). Murine TNFR2-Fc was modeled after and is functionally similar to human etanercept [209, 210]. Despite both compounds providing systemic neutralization of circulating TNF, disease outcomes were dependent on the stage of infection and the therapeutic agent. During primary infection, treatment with either reagent was equally detrimental, resulting in disorganized granuloma formation and poor survival. This suggests that during establishment of tuberculosis, neutralization of circulating TNF prevents control of infection.

Disease was exacerbated in chronically infected mice treated with anti-TNF antibody, but not with mTNFR2-Fc. Despite equivalent systemic neutralization of circulating TNF, significantly more MP6-XT22 was detected in granulomas compared to mTNFR2-Fc. The disparity in detection of these two reagents may be due to less penetration by mTNFR2-Fc into the granuloma, and/or could be due to the formation of immune complexes of TNF and anti-TNF antibody. Maintenance of granuloma structure and increased survival in mTNFR2-Fc treated mice may be due to incomplete neutralization of TNF within the granuloma. Systemic expression of TNF appears not to be required to control chronic *M. tuberculosis* infection, highlighting the

localized nature of tuberculosis and critical function of granulomas once *M. tuberculosis* infection has been established.

These findings in the mouse model are consistent with the human literature in which higher rates of tuberculosis are associated with patients receiving anti-TNF antibody (infliximab) compared to TNFR2-Fc (etanercept) [130, 195, 207]. These data have important public health implications for the use of these drugs in regions with high endemic tuberculosis rates. Based on our data, we hypothesize that an increased rate of reactivation tuberculosis could occur among patients treated with TNF neutralizing antibody rather than soluble TNF receptor. In addition, therapies with either TNF antibody or TNF receptor may pose an increased risk for dissemination and perhaps for developing primary tuberculosis upon exposure to persons with active disease.

The pharmocodynamic differences between these two drugs likely play an important role in the overall activity against systemic versus localized diseases. Although the increased dissociation of TNF from TNFR2-Fc compared to antibody [131] may not be relevant in the periphery where the drug is in higher concentration relative to TNF, TNF dissociation may be a very important factor in the microenvironment of the granuloma. If TNF dissociates from the TNFR2-Fc in the granuloma, it may rapidly be bound again by the drug. However, TNF is just as likely to be bound to the large numbers of TNF receptors present on the leukocytes in close proximity in the granuloma. This is consistent with the fact that infliximab and etanercept are both effective in the treatment of rheumatoid arthritis but only infliximab is effective in ameliorating the symptoms of Crohn's disease [218]. Perhaps penetration of the two drugs into the inflammatory foci (similar to granulomas) of the intestines, as seen many cases of Crohn's

disease, resembles that seen in our murine studies, where the antibody penetrates tissue better than the TNF receptor fusion protein.

There are other differences between these two anti-TNF agents that might contribute to reactivation of tuberculosis. Membrane-bound TNF is reported to be bound more tightly by anti-TNF antibody compared to mTNFR2-Fc [131]. Mice that only express membrane-bound TNF were similar to wild type mice in control of acute infection, but had exacerbated disease during the chronic phase [219]. We were unable to detect membrane-bound TNF on cells from the lungs of infected mice (data not shown), suggesting that membrane-bound TNF is transient and quickly cleaved by TNF-α converting enzyme (TACE). We also did not detect antibody binding to cells from lungs of mice treated with SFX-labeled TNF inhibiting drugs by flow cytometry (data not shown). Although it is possible that differential binding of TNF by anti-TNF antibody compared to mTNFR2-Fc accounts for the different outcomes in chronic infection, we did not observe increased cell death, which would be expected for reverse signaling [220] or antibody binding to cells. While others have demonstrated that TNF itself can induce apoptosis of *M. tuberculosis*-infected macrophages [221], and neutralization of TNF may result in increased survival of infected macrophages in the granuloma, differences in apoptosis were not observed in acutely or chronically infected mice with any treatment. Immune complex formation with anti-TNF antibody treatment (which would not occur with TNFR2-Fc) may lead to increased inflammation, or even increased IL-10 production if these immune complexes interact with Fc-receptors on alternatively activated macrophages [222]. This is an area of further investigation, as increased IL-10 production in mice exacerbates chronic tuberculosis [204].

TNF is a major contributor to many facets of the immune response, including macrophage activation, granuloma formation and maintenance, regulation of cytokines and

chemokines [3, 39, 223]. It is likely that the neutralization of TNF affects several pathways important in the control of tuberculosis. The data presented here suggest that TNF inhibiting molecules may have different effects during acute compared to chronic or perhaps even latent tuberculosis, and that incomplete neutralization of TNF allows the host to maintain control of the infection. These agents provide models for understanding the mechanisms by which TNF is important in the control of tuberculosis.

4.0 CHAPTER 2 TNF EXPRESSION IS NOT REQUIRED DURING PRIMING OF THE IMMUNE RESPONSE TO MYCOBACTERIUM TUBERCULOSIS

4.1 INTRODUCTION

TNF is required to control acute infection with *Mycobacterium tuberculosis*, but the mechanism remains unclear. Granuloma formation during *M. tuberculosis* infection requires expression of TNF, as shown by disorganized lung infiltration and impaired granuloma formation in TNF or TNFR1-deficient mice or those treated with anti-TNF antibody [33, 119-121, 219].

In response to *M. tuberculosis* infection, TNF expression can induce chemokine production in macrophages [33] and dendritic cells (Algood, Windish and Flynn, unpublished), and endothelial cell expression of cell adhesion molecules (CAMs) in other systems [179, 224-226]. Whether TNF stimulation in the presence of *M. tuberculosis* can similarly upregulate expression of adhesion molecules on leukocytes has not yet been shown. In the absence of LFA-1 [227] or VCAM-1 [184] expression, granulomas are formed less efficiently; suggesting adhesion molecules may play a role in the formation of the granuloma. We hypothesize that TNF induction of adhesion molecules is required for formation of the granuloma by mediating leukocyte migration, priming of the immune response to *M. tuberculosis,* or by facilitating adhesion within the granuloma to keep leukocytes in tight contact, and that in the absence of

TNF expression, decreased expression of adhesion molecules impairs granuloma formation or function and inhibits survival of *M. tuberculosis* infection.

Since TNF activates myeloid leukocytes, the ability to present antigen and costimulatory factors to prime the adaptive immune response could be initiated or enhanced by TNF. Since functional CD4+ T lymphocytes are required to control acute *M. tuberculosis* infection [84], a role for TNF in priming the adaptive immune response might explain the failure of granuloma formation and survival in the absence of this proinflammatory cytokine. It has been suggested that TNF expression may be required during T cell priming when limited antigen is available, or when antigens are low in immunogenicity [228]. TNF is not required for priming an adaptive immune response to lymphocyte choriomeningitis virus infection, where antigen is relatively high and the infection is immunogenic. On the other hand, TNF must be present to prime tumor antigen specific responses, where the antigens are not highly immunogenic [228]. No studies to date have addressed whether TNF is required for priming the T cell response to *M. tuberculosis.* Increasing bacterial burden in dendritic cells reduces their capacity to present antigen [34], probably due interference of the innate response by *M. tuberculosis*. As in presentation of tumor, the less than optimal antigen presentation by dendritic cells may require TNF stimulation to increase the ability of these cells to prime *M. tuberculosis* specific T cell responses. Alternatively, TNF can directly signal through T lymphocyte TNF receptor to increase T cell proliferation during priming [229-231]. If TNF is required for proliferation of T cells, this could contribute to an insufficient response that prevents granuloma formation and results in exacerbated tuberculosis.

We used the murine model of tuberculosis to address whether TNF is required to induce adhesion molecule expression or to prime the adaptive immune response. We followed specific T

cell responses to *M. tuberculosis* antigens in TNF deficient mice, control mice treated with IgG1 antibody, or wild type mice injected with anti-TNF antibody (MP6-XT22) during the initial phase of infection with *M. tuberculosis.* We followed the expression of adhesion molecules during the first four weeks of *M. tuberculosis* infection in the presence or absence of TNF expression and were unable to detect any differences in the expression of adhesion molecules. Migration of dendritic cells into the lymph nodes in the absence of TNF expression was similar in the presence or absence of TNF expression. As has been previously reported [33, 119], bacterial burden in the lungs and lymph nodes were similar in all groups over the first 2-3 weeks of infection, providing similar levels of antigen for presentation. Within the lymph nodes, T cell specific responses were detectable by 15 days post-infection, and were similar in all groups. We conclude from these studies that TNF is not required for modification of adhesion molecule expression, the migration of *M. tuberculosis* infected dendritic cells in to the lymph nodes, or for priming of the adaptive immune response.

4.2 MATERIALS AND METHODS

4.2.1 Animals

C57BL/6 female and TNF deficient mice (Jackson Laboratory, Bar Harbor, ME) were maintained in a BioSafety Level 3 specific pathogen-free facility and monitored for murine pathogens. All animal protocols used were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

4.2.2 Mycobacteria and murine infection

Low dose aerosol infection of mice (20-100 colony forming units) of *M. tuberculosis* strain Erdman (Trudeau Institute, Saranac Lake, NY) has been previously described [110]. Bacterial burden was determined as previously described [208]. For a description of these protocols, refer to section 3.2.2 of Chapter 3.

4.2.3 Chemicals and reagents

All chemicals were purchased through Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Middlebrook 7H10 agar and 7H9 liquid media for growth of *M. tuberculosis* were purchased from Difco (Detroit, MI). Flow cytometry antibodies were purchased from BD Pharmingen, except anti-C3 and its isotype control (Cedarlane Laboratories, Burlinton, NC). MP6-XT22 (rat IgG1) [208, 212] was purified from cell culture of a hybridoma obtained from DNAX by the

National Cell Culture Center (Minneapolis, MN). IgG1 was diluted in phosphate buffered saline and antibodies were injected intraperitoneally into mice one day prior to acute infection with *M. tuberculosis* and twice weekly for the duration of the study.

4.2.4 Flow cytometry

Lung infiltration was examined at predetermined time points as previously described [33, 162]. For a description of this protocol, refer to section 3.2.5 of Chapter 3. Cells were stained with anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD3 (clone 17A2), anti-CD11c (clone HL-3), anti-CD11b (clone M1/70), anti-GR1 (clone RB6-8C5), anti-CD62L (clone RB40.34), anti-ICAM-1 (clone 1A29), anti-CD11a (clone 2D7) and anti-VCAM-1 (clone MVCAM.A).

4.2.5 IFN-γ Detection by ELISpot

Expression of IFN-γ was determined by the ELISpot assay performed as previously described [90, 232]. Briefly, filtered plates were coated overnight at 4ºC with anti-murine IFN-γ antibody (BD Pharmingen). 80,000 lung cells or 160,000 lymph node cells were taken from organ homogenates and incubated with media only, 10μg/mL ConA, uninfected bone marrow derived dendritic cells or *M. tuberculosis* infected bone marrow derived dendritic cells infected for 48 hours in filtered plates. After 48 hours of incubation, cells were flicked out and the plates were washed repeatedly with phosphate buffered saline and 0.01% Tween20. Secondary anti-IFN-γ antibody (BD Pharmingen) was incubated in the plates for 2 hours at 37ºC and plates were washed repeatedly. ABC reagent from Vector Laboratories (Burlingame, CA) was incubated on plates for 1 hour at room temperature and plates were washed repeatedly. Plates were developed

for 7 minutes with BD Pharmingen AEC reagent and spots were counted on an ELISpot reader. ESAT6 peptide for measuring CD4 specific responses was obtained from Genscript (Piscataway, NJ), 5μg/mL ESAT6 peptide was incubated in the ELISpot wells with uninfected dendritic cells at a 1:1 ratio with lung or lymph node cells. GAP peptide, MT133 was obtained from Genescript Corporation (Piscataway, NJ). 4 μg/mL GAP peptide was incubated with uninfected bone marrow derived dendritic cells at a 1:1 ratio with lung or lymph node cells. Peptides were incubated with lung or lymph node cells for 48 hours. The assay was performed as previously described [90, 232].

4.2.6 Production of bone marrow derived antigen presenting cells

Production of bone marrow derived dendritic cells used as antigen presenting cells in the ELISpot assay was done as previously described [90, 232]. Briefly, bone marrow was obtained from wild type C57Bl/6 mice and incubated in Dulbeccos Modified Eagle's Medium based dendritic cell media including fetal bovine serum, sodium pyruvate, L-glutamine, non-essential amino acids with IL-4 and GMCSF cytokines.

4.2.7 Statistical analysis

Bacterial burden and cell infiltration were analyzed by a one way ANOVA, while the log rank test was used to compare survival. A value of $p<0.05$ was considered significant. Experiments were repeated to demonstrate reproducibility.

4.3 RESULTS

4.3.1 TNF does not modify the expression of certain adhesion molecules during *Mycobacterium tuberculosis* infection

C57Bl/6 mice were injected with 0.2mg MP6-XT22 or 0.2mg IgG1 control antibody one day prior to *M. tuberculosis* infection. On Day 0, TNF deficient mice, MP6-XT22 and IgG1 treated mice were aerosol infected with a low dose of *M. tuberculosis* (~50 colony forming units/lung). As it had been previously published that they were required for control over *M. tuberculosis* infection, we followed the lung expression of the following adhesion molecules: VCAM-1 [184], LFA-1 (CD11a) [227] and ICAM-1 [185]. As L-selectin is required for leukocyte rolling and migration into the lungs [179], we also followed expression of L-selectin during acute *M. tuberculosis* infection. In naïve and *M. tuberculosis* infected mice, LFA-1 could be detected on >99% of lymphocytes (data not shown). Although ICAM-1 expression increased throughout infection in lymphocytes (Figure 6A) and macrophages (Figure 6B), there were no differences in expression of ICAM-1 in the presence or absence of TNF. Expression of L-selectin decreased during *M. tuberculosis* infection on the lymphocytes (Figure 6A), and increased on macrophages (Figure 6B), but again, there were no differences in expression of L-selectin in the presence of absence of TNF. Due to low recovery of lymphocytes early in *M. tuberculosis* infection, VCAM-1 expression could only be examined on Day 21 of *M. tuberculosis* infection, and there was no difference in the expression of this adhesion molecule in the presence or absence of TNF expression on lymphocytes (Figure 6A) or macrophages (Figure 6B). We conclude that TNF

expression is not required for the expression of ICAM-1, LFA-1, L-selectin or VCAM-1 during *M. tuberculosis* infection.

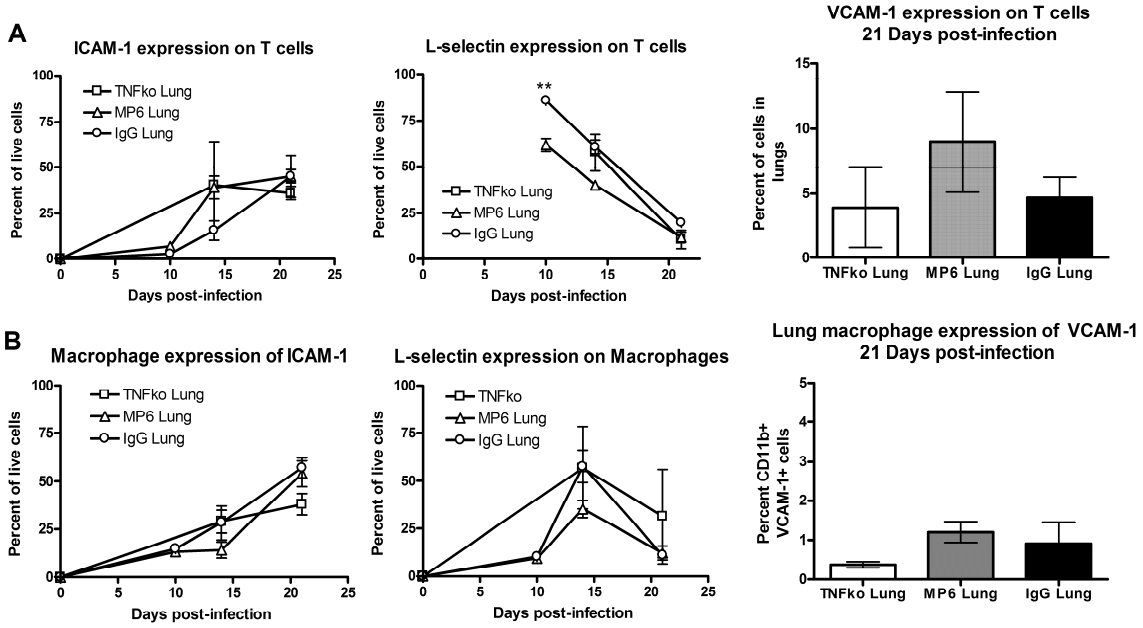

Figure 6 TNF is not required to induce adhesion molecule expression

Flow cytometry detection of adhesion molecules on the cell surface during acute *M. tuberculosis* infection in TNFko, MP6-XT22 treated or control treated mice.

(A) Expression of ICAM-1 (left), L-selectin (middle) and VCAM-1 (right) in on lymphocytes (CD3+ cells) in TNFko (□), MP6-XT22 treated (Δ) and IgG treated (○) mouse lungs.

(B) Expression of ICAM-1 (left), L-selectin (middle) and VCAM-1 (right) on macrophages (CD11b+ cells) in TNFko (□), MP6-XT22 treated (Δ) and IgG treated (○) mouse lungs.

4.3.2 TNF expression is not required for dissemination of bacteria to the lymph nodes

As previously observed [33, 119], bacterial burden in the lungs of TNF deficient, anti-TNF antibody treated or control mice remained similar throughout the first three weeks of infection (Figure 7A). This was not surprising, as it has been previously published that although mice

lacking TNF signaling succumb to infection within the first four weeks of infection, bacterial burden does not reach a lethal level in these mice, suggesting that mice succumb to overwhelming pathology [33, 119]. Indeed, during each repetition of in this study, all anti-TNF treated and TNF deficient mice succumbed to infection with similar kinetics, between 25 and 31 days post-infection (data not shown).

M. tuberculosis infected dendritic cells traffic to the lymph nodes with the highest frequency during the first three weeks of infection [34]. TNF may play a role in modifying trafficking by modifying chemokine receptors on dendritic cells that allow the migration to the lymph nodes. As dendritic cells are most likely to be the cells that allow carry *M. tuberculosis* to the lymph nodes [34], bacterial burden in the lymph nodes was followed over time. *M. tuberculosis* infection could not be detected in the lymph nodes until 13 days post-infection, at which time similar bacterial burden was observed in all groups (Figure 7B). Dendritic cell migration into the lymph nodes was also followed over time. Two subsets of dendritic cells were followed: those that express CD11b (myeloid derived) or do not express CD11b (lymphoid derived). There were no significant differences in migration of dendritic cells from either subset in the presence or absence of TNF (Figure 7C, 7D). These data suggest that TNF is not required for migration if *M. tuberculosis* infected dendritic cells into the lung draining lymph nodes.

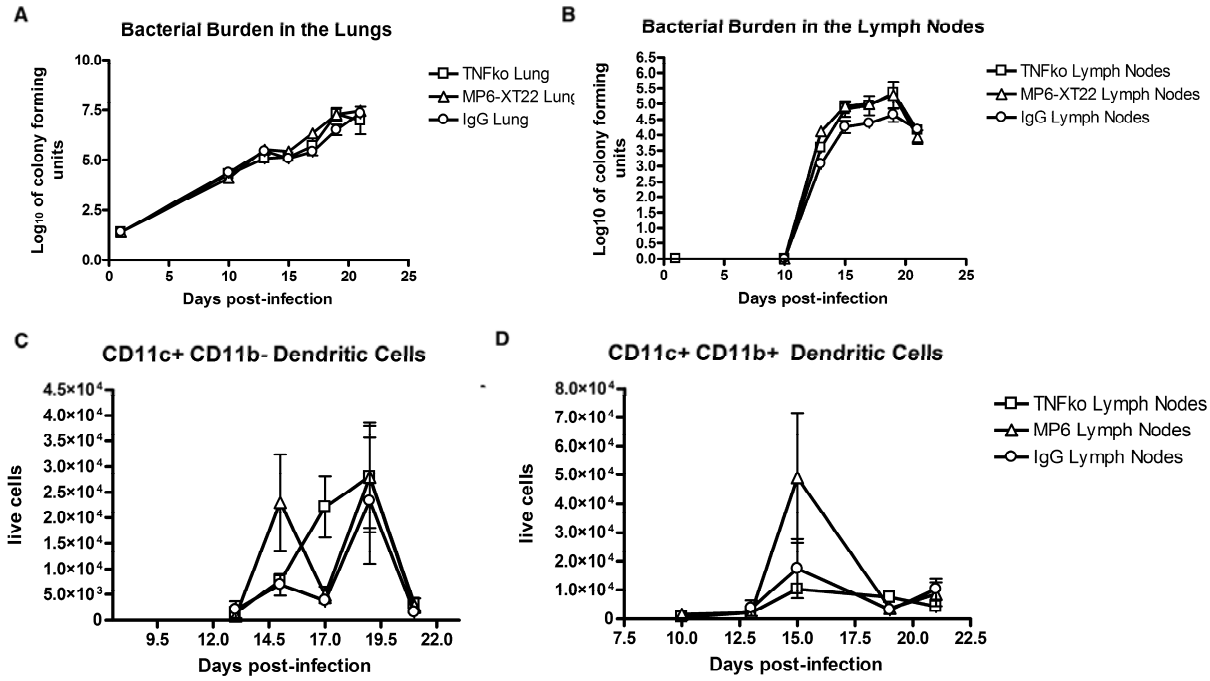

Figure 7: TNF is not required for migration of infected *M. tuberculosis* dendritic cells to the lymph nodes

(A, B) The bacterial burden (counted colony forming units) in the lungs and lymph nodes in *M. tuberculosis* infected TNFko (□), MP6-XT22 treated (Δ) or IgG1 treated (○) mice.

(C, D) The migration of dendritic cells into the lymph nodes was determined by counting the total number of live cells present in the lymph nodes, and multiplying by the fraction that were CD11c+ and either CD11b+ or CD11b- (by flow cytometry) in TNFko (□), MP6-XT22 treated (Δ) or IgG1 treated (○) mice.

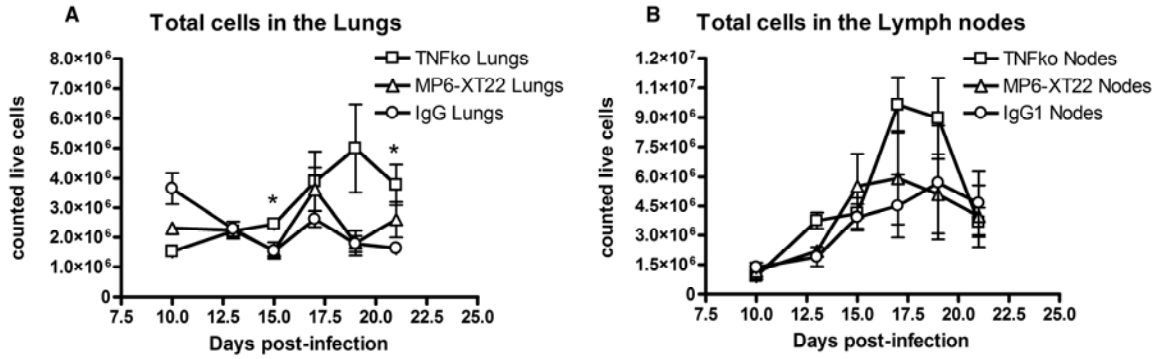

Figure 8 Equivalent leukocytes in the lungs and lymph nodes in the presence or absence of TNF

The total number of leukocytes in the lungs and lymph nodes were counted by trypan blue exclusion under a microscope in TNFko (□), MP6-XT22 treated (Δ) or IgG1 treated (○) mice.

.

4.3.3 TNF is not required leukocyte localization or dendritic cell maturation

The number of live cells in the lungs or lymph nodes between groups were counted and compared over time. Although TNF deficient mice appeared to have higher total cells at days 15 and 21 post-infection, the overall number of cells in the lungs of *M. tuberculosis* infected mice was relatively similar between control mice and anti-TNF treated mice (Figure 8A). Previous studies have confirmed that the administered dose of anti-TNF antibody successfully neutralizes TNF during acute *M. tuberculosis* infection (Figure 1A, 1B), so it is unlikely that this difference between anti-TNF treated and TNF deficient mice is due to low level TNF expression. The observed difference in lung infiltrating cells between TNF deficient and antibody treated mice might be an artifact caused by structural differences in the absence of TNF during development. In the lymph nodes, the total number of cells increased similarly in all groups of mice, regardless of the presence of TNF (Figure 8B), which suggests that TNF is not required for either the migration or production of leukocytes in the lungs or lymph nodes.

Although the total number of cells present in the lymph nodes was similar, TNF might be required for priming *M. tuberculosis* specific responses. The expression of surface molecules CD80 and CD86 indicates maturity of dendritic cells [32], and act as costimulatory molecules during T cell priming [233, 234].. In the presence or absence of TNF expression, CD80 and CD86 levels were are similar on dendritic cells from all groups (Figure 9A). This suggests that TNF is not required for the maturation of the dendritic cell that allows T cell priming.

Furthermore, CD4+ and CD8+ T cell compartments represented equivalent percentages of live cells found in the lymph nodes (Figure 9B, 9C), suggesting that TNF is not required for the expansion of T lymphocytes during the priming of the adaptive immune response against *M. tuberculosis* antigens.

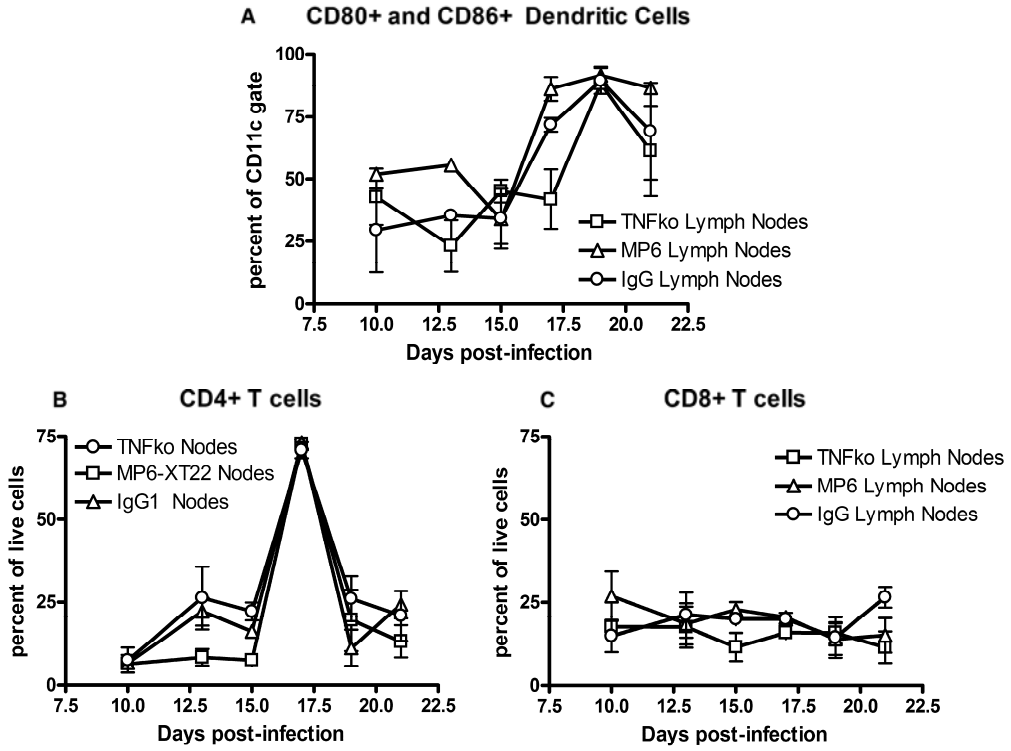

Figure 9 Dendritic cell maturation and T cell expansion do not require TNF expression

(A) The percent of dendritic cells expressing costimulatory molecules CD80 and CD86 was determined by flow cytometry staining in TNFko (□), MP6-XT22 treated (Δ) or IgG1 treated (○) mice.

(B,C) The percentage of the total lymph node populated by CD4+ or CD8+ T lymphocytes was determined by flow cytometry in TNFko (□), MP6-XT22 treated (Δ) or IgG1 treated (○) mice.

4.3.4 TNF expression is not required for *Mycobacterium tuberculosis* specific T cell priming

To determine whether TNF is required to prime T cell responses to *M. tuberculosis*, lymph node leukocytes were re-stimulated with *M. tuberculosis* infected bone marrow derived dendritic cells in an IFN-γ ELISpot assay. Similar IFN-γ responses were observed in response to *M. tuberculosis* infected dendritic cells in T lymphocytes from TNF deficient mice, mice treated with anti-TNF antibody and IgG1 treated control lymph nodes (Figure 10A). CD4+ and CD8+ T lymphocyte responses were further investigated by comparing IFN-γ responses to ESAT6 CD4 antigen or GAP CD8 antigen presented by uninfected bone marrow derived dendritic cells. CD4 and CD8 peptide stimulation elicited similar IFN-γ secretion whether the T cells came from mice expressing or not expressing TNF (Figure 10B, 10C).

TNF can induce chemokine production by macrophages that can induce migration of primed T cells to the lungs of *M. tuberculosis* infected mice [235]. To determine whether *M. tuberculosis* specific T lymphocytes were able to function in *M. tuberculosis* infected lungs in the absence of TNF, IFN-γ ELISpots were used to detect specific IFN-γ responses. *M. tuberculosis* specific responses to infected dendritic cells (Figure 11A), ESAT6 (Figure 11B), and GAP (Figure 11C) peptides were all similar in T cells from TNF deficient, anti-TNF antibody treated or IgG1 treated control mice. This confirms that TNF is not required for the migration of *M. tuberculosis* specific T lymphocytes into the lungs of infected mice.

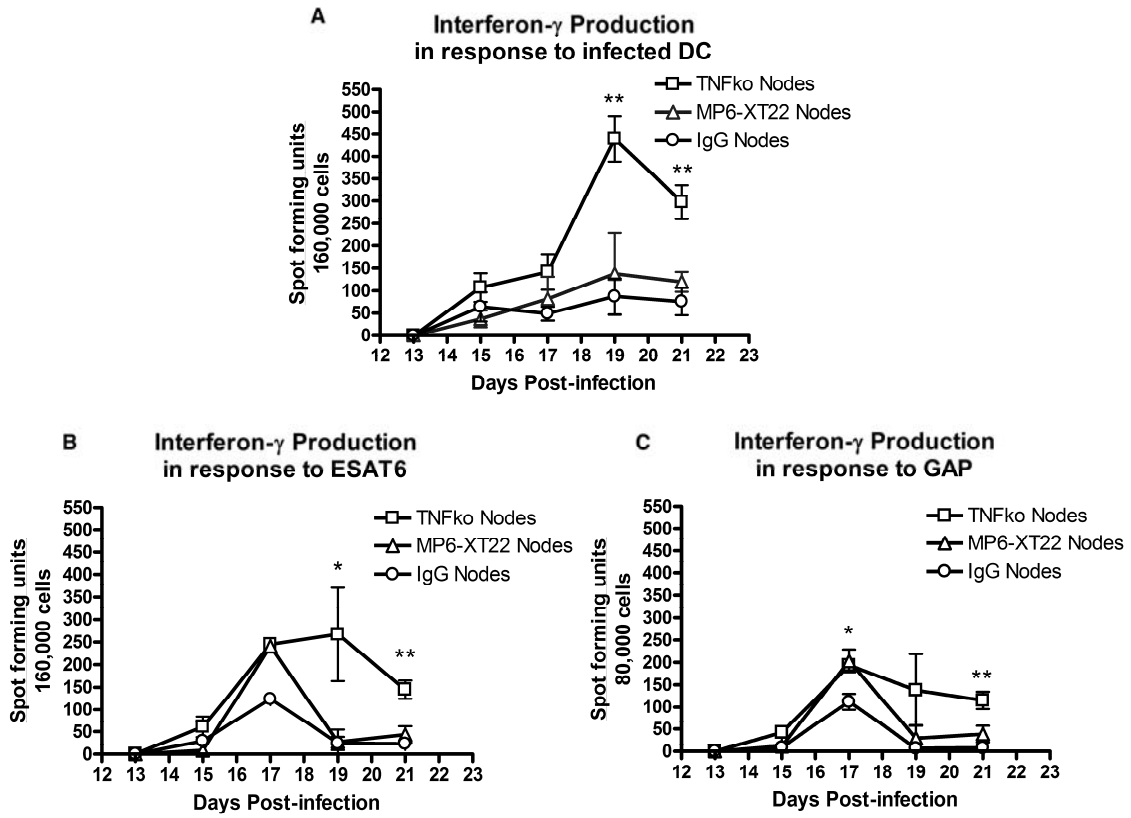

Figure 10 *Mycobacterium tuberculosis* specific T lymphocyte priming does not require TNF expression

Total cellular aliquots from lymph nodes of TNFko (□), MP6-XT22 (Δ) and IgG1 (○) treated mice infected with *M., tuberculosis* were taken at specific time points for an IFN-γ ELISpot, and incubated with stimulators indicated below.

(A) Bone marrow derived dendritic cells were infected with *M. tuberculosis*, washed with media and counted prior to incubation at a 1:1 ratio with lymph node cells.

(B) Uninfected bone marrow derived dendritic cells were incubated at a 1:1 ratio with lymph node T cells and 5μg/mL ESAT6 peptide.

(C) Uninfected bone marrow derived dendritic cells were incubated at a 1:1 ratio with lymph node T cells and 4μg/mL GAP peptide.

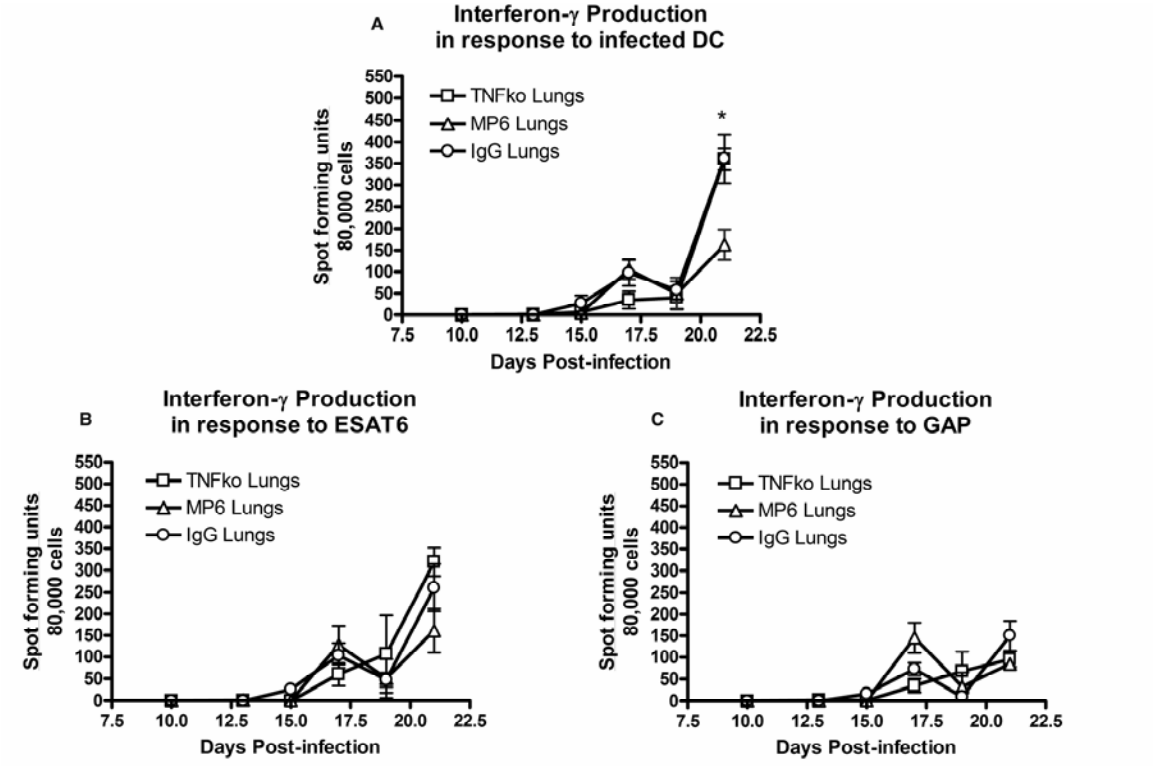

Figure 11 *Mycobacterium tuberculosis* specific T lymphocytes do not require TNF expression to migrate to and function within the lungs

Total cellular aliquots from lungs of TNFko (□), MP6-XT22 (Δ) and IgG1 (○) treated mice infected with *M., tuberculosis* were taken at specific time points for an IFN-γ ELISpot, and incubated with stimulators indicated below.

(A) Bone marrow derived dendritic cells were infected with *M. tuberculosis*, washed with media and counted prior to incubation at a 1:1 ratio with lung cells.

(B) Uninfected bone marrow derived dendritic cells were incubated at a 1:1 ratio with lung cells and 5μg/mL ESAT6 peptide.

(C) Uninfected bone marrow derived dendritic cells were incubated at a 1:1 ratio with lung cells and 4μg/mL GAP peptide.

4.4 DISCUSSION

TNF is required for control of infection with *Mycobacterium tuberculosis* [33, 119-121, 123, 219], and data in the literature suggest this cytokine is also required to control human tuberculosis. Although it is apparent that TNF is required for granuloma formation and maintenance [119, 235], the specific functions of TNF are responsible are unclear. We have investigated the role of TNF in adhesion molecule expression and in priming the adaptive immune response to *M. tuberculosis.* We hypothesized that since adhesion molecules mediate cell migration, and certain adhesion molecules are required for optimal granuloma formation [182, 184, 227], insufficient expression of adhesion molecules would be detrimental to granuloma formation. Tumor antigens are low in antigenicity, and TNF is required to prime specific responses [228], while viral antigens are highly immunogenic and TNF is not required [228]. Presentation of the *M. tuberculosis* antigens by dendritic cells is below their potential ability to present antigens [34], probably due to *M. tuberculosis* interference of the innate immune response. TNF may be required to help prime the immune response to *M. tuberculosis*, as is the case in tumor responses.

TNF signaling increases expression of cell adhesion molecules on endothelial cells [179, 224-226]. Expression of VCAM-1, CD44 and LFA-1 is required for optimal granuloma formation, and survival of infection with *M. tuberculosis* [182, 184, 227]. TNF induces expression of chemokines during *M. tuberculosis* infection [33], and we hypothesized that adhesion molecule expression might also be TNF-inducible. Although expression of LFA-1

(CD11a) was detectable on all lymphocytes in the presence or absence of TNF (data not shown), in these studies we were unable to determine whether LFA-1 was in an active conformation. TNF could be required to activate LFA-1, and further investigation will be required to examine this possibility. Expression of ICAM-1, L-selectin and VCAM-1 were unchanged by the presence or absence of TNF early in *M. tuberculosis* infection. Although TNF induces their expression in other systems and expression of adhesion molecules increased during *M. tuberculosis* infection, other factors induced this increase. We were unable to confirm the hypothesis that TNF-inducible adhesion molecules are required for granuloma formation during *M. tuberculosis* infection, but we examined only a subset of adhesion molecules in this study. Microarray or RNase protection assays would allow a more complete analysis of adhesion molecules at the transcription level, and may highlight other adhesion molecules that are TNF-inducible in the presence of *M. tuberculosis* infection.

The data presented here show that TNF is not required for the migration of dendritic cells into the lymph nodes, or the maturation that allows them to prime naïve T lymphocytes (Figure 7C, 7D, 9A). Bacterial burden in the lymph nodes is equivalent in the presence and absence of TNF, indicating that similar amount of antigen is available for priming the adaptive immune response. IFN-γ production in response to re-stimulation with dendritic cells infected with *M. tuberculosis,* ESAT6 and GAP antigens are similar in the lymph nodes of TNFko, MP6-XT22 treated and IgG1 treated mice (Figure 10), showing that TNF is not required for the induction of functional *M. tuberculosis* specific T cells. In the lungs, a sufficient T lymphocyte response to *M. tuberculosis* antigens is comparable in the presence or absence of TNF expression, showing that T lymphocytes are functional in the absence of TNF. All of the data presented here suggest that

TNF does not play an indispensable role in priming of specific T lymphocyte responses to *M. tuberculosis.*

TNF antagonist treatment in humans with chronic inflammatory disease reportedly reactivates tuberculosis [130, 207]. We have shown that during acute *M. tuberculosis* TNF antagonism either by anti-TNF fusion molecule or by antibody neutralization prevents control of initial infection in mice (Chapter 1) and [236], and mathematical models predict that this would be true in humans [237]. That TNF is not required for priming of the specific immune response to *M. tuberculosis* suggests that T cell responses would be sufficient during TNF antagonism, and that TNF must play a different role in protection from *M. tuberculosis* infection. Although T lymphocytes are primed appropriately in the lymph nodes in the absence of TNF, they are unable to aggregate with other leukocytes that allows formation of granulomas in the lungs during *M. tuberculosis* infection [33, 119]. Granuloma formation is also not dependent on individual TNF inducible chemokines such as ligands of CCR2 [162], CCR5 [164] or CXCR3 [80, 161], although the absence of one chemokine receptor might be compensated for by another chemokine receptor in these models. Granuloma formation is abnormal in the absence of expression of the adhesion molecules LFA-1 and VLA-4, suggesting that adhesion molecules could play a role in granuloma formation. TNF expression can induce expression of adhesion molecules [238], suggesting that TNF induction of adhesion molecules might be required for granuloma formation.

5.0 CHAPTER 3 CXCR3 IS NOT RESPONSIBLE FOR THE OVERABUNDANCE OF T LYMPHOCYTES FOUND IN CCR5 DEFICIENT MICE

5.1 INTRODUCTION

When infected with *Mycobacterium tuberculosis,* macrophages upregulate leukocyte attracting chemokines such as the ligands that bind to chemokine receptors CCR2, CCR5 and CXCR3. Production of these chemokines partially depends on TNF, as neutralization of TNF results in decreased, but not absent, chemokine production [33]. Granuloma formation is required for control over *M. tuberculosis* infection, and in the absence of TNF granuloma formation is impaired, resulting in rapid morbidity [33, 119-121, 123, 219, 239]. It has been hypothesized that TNF-inducible chemokine production guides granuloma formation, but elimination of no individual chemokine receptor has been linked to the impaired granuloma formation and pathology observed in the absence of TNF. In CCR2 deficient mice, granuloma formation lags behind wild type, but by 33 days post-infection, normal granulomas are observed. Unlike mice lacking TNF signaling, CCR2 deficient mice survive low dose *M. tuberculosis* infection [162]. So, while CCR2 signaling chemokines appear to play a role in early granuloma formation, which may be related to the demonstrated impaired migration of macrophages to the lungs in these mice [163], other mechanisms must be able to compensate in the absence of CCR2 signaling.

The chemokine receptor CCR5 is upregulated on macrophages and T lymphocytes upon activation [240], and CCR5 ligands CCL3, CCL4 and CCL5 are secreted by macrophages during *M. tuberculosis* infection [33]. CCR5 chemokines attract leukocytes to regions of inflammation [241], such as the focus of *M. tuberculosis* infection. While mice deficient in CCR5 controlled *M. tuberculosis* infection in the lung, liver and spleen, they harbored higher bacterial burden in the lymph nodes [164]. In absence of CCR5 expression, granulomas formed normally, but there was higher T cell infiltration in the lungs of CCR5 deficient mice compared to control mice by nine weeks post-infection, accompanied by an increase in pro-inflammatory cytokines [164]. Bacterial numbers were increased in the lymph nodes of CCR5-/- mice compared to wild type mice. The authors concluded that the higher cellular infiltrate was probably due to greater dendritic cell migration into the lymph nodes, bringing more bacteria to the lymph nodes and causing more cells to be primed [164]. This hypothesis makes sense, because dendritic cells ordinarily downregulate CCR5 upon antigen uptake [71].

CXCR3 is induced on T lymphocytes upon activation, and CXCR3 ligands CXCL9 and CXCL10 are made upon macrophage infection with *M. tuberculosis* [33]. Mice treated with anti-CXCL9 antibody control bacterial burden and survive *M. tuberculosis* infection similarly to control mice, but with a reduction in granuloma number and size early in infection [80]. In contrast, data from our laboratory using anti-CXCL9 and anti-CXCL10 double neutralization did not result in any discernible differences from wild type mice [33]. Surprisingly, *M. tuberculosis* infection in susceptible CXCR3-/- BALB/c mice maintained a lower bacterial burden than control BALB/c mice in the lungs and spleen [161]. CXCR3-/- C57Bl/6 mice also had a lower splenic, but not lung bacterial burden than control mice [161]. Although granulomas formed normally in CXCR3-/- BALB/c mice, higher CD4+ T cell infiltrate was detected during an

established *M. tuberculosis* infection than in control mice [161]. This increase in the CD4+ T cells in the lungs of BALB/c mice may have helped to control the bacterial burden in the lungs.

Although CCR5 and CXCR3 chemokine signaling is thought to play a role in migration of activated T lymphocytes into sites of inflammation, neither of these chemokines receptors were required individually for accumulation of T lymphocytes in the lungs. In contrast, in each case, higher T cell infiltrate was detected in the absence of the chemokine receptor. One possible explanation for these findings is a redundancy in chemokine receptor usage in the setting of M. tuberculosis infection. This means that CXCR3 might compensate for the absence of CCR5. If both of these chemokines receptors were absent, T lymphocytes may be unable to traffic to the site of infection. A transcriptome comparison showed a five-fold increase in CCL5 expression in CXCR3 deficient mice along with increased CCR5 expression [80], suggesting that in the absence of CXCR3 signaling, CCR5 signaling increases, possibly providing adequate leukocyte recruitment to control infection.

To address this question, we infected C57Bl/6 CXCR3xCCR5 doubly deficient mice with a low dose *M. tuberculosis* infection. We found that infiltration of the lungs was significantly greater in CXCR3xCCR5-/- mice than in WT mice, as had previously been reported in BALB/c CXCR3-/- [161] and CCR5-/- [164] mice. Within the lymph nodes, higher bacterial burden, accompanied by higher IFN-γ production in CXCR3xCCR5-/- mice, which also mimicked results reported in CCR5-/- mice [164]. In low dose *M. tuberculosis* infection, granulomas began to form before 5 weeks post-infection in both knock out and control mice, which indicated that the absence of both CXCR3 and CCR5 chemokine receptors did not interfere with granuloma formation. Overall, the data collected in CXCR3xCCR5-/- mice was very similar to findings

reported in CCR5 deficiency alone, and we concluded that CXCR3 does not compensate for the absence of CCR5 to control low dose *M. tuberculosis* infection.

5.2 MATERIALS AND METHODS

5.2.1 Animals

C57BL/6 female and CCR5xCXCR3-/- mice (a kind gift from Dr. John Chan) were maintained in a BioSafety Level 3 specific pathogen-free facility and monitored for murine pathogens. All animal protocols used were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

5.2.2 Mycobacteria and murine infection

Low dose aerosol infection of mice (20-100 colony forming units) of *M. tuberculosis* strain Erdman (Trudeau Institute, Saranac Lake, NY) has been previously described [110]. Bacterial burden was determined as previously described [208]. For a description of these protocols, refer to section 3.2.2 of Chapter 3.

5.2.3 Chemicals and reagents

All chemicals were purchased through Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Middlebrook 7H10 agar and 7H9 liquid media for growth of *M. tuberculosis* were purchased from Difco (Detroit, MI). Flow cytometry antibodies were purchased from BD Pharmingen, except anti-C3 and its isotype control (Cedarlane Laboratories, Burlinton, NC).

5.2.4 Flow cytometry

Lung infiltration was examined at predetermined time points as previously described [33, 162]. For a description of this protocol, refer to section 3.2.5 in Chapter 3. Cells were stained with ant-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD3 (clone 17A2), anti-CD11c (clone HL-3), anti-CD11b (clone M1/70) and anti-GR1 (clone RB6-8C5).

5.2.5 IFN-γ Detection by ELISpot

Expression of IFN-γ was determined by the ELISpot assay performed as previously described [191, 232]. For a description of the protocol, see section 4.2.5 of Chapter 4.

5.2.6 Production of bone marrow derived antigen presenting cells

Production of bone marrow derived dendritic cells used as antigen presenting cells in the ELISpot assay was done as previously described [191, 232]. For a description of the protocol, refer to section 4.2.6 in Chapter 4.

5.2.7 Histopathology

Samples of lung were fixed in 10% normal buffered formalin and embedded in paraffin. 5-6μm sections were H&E stained. Slides were examined in a blinded fashion. TUNEL staining was performed using the Apoptag kit (Chemicon, Temecula, CA) per manufacturer's protocol. To track labeled molecules, lung blood and airways were cleared by perfusion and bronchoalveolar

lavage. Lungs were fixed in 2% paraformaldehyde and infused with 30% sucrose. Sections were stained 30 minutes with rhodamine phalloidin (Invitrogen, Carlsbad, CA) and Draq5 (Biostatus, Leicestershire, UK). Images were captured using Olympus Flowview 500 software (Olympus Inc., Mellville, NY).

5.3 RESULTS

5.3.1 Lymph node bacterial burden in CXCR3xCCR5 doubly deficient mice is higher than in wild type mice

Mice treated with anti-TNF antibody express significantly less CCR5 and CXCR3 ligands than control mice [33]. Whether loss of signaling through these individual molecules is responsible for impaired granulomas in anti-TNF treated mice has been previously addressed. CCR5-/- mice and CXCR3-/- mice (on a C57BL/6 background) were able to form granulomas, and survive low dose *M. tuberculosis* infection [161, 164], in contrast to anti-TNF treated mice that succumb to acute infection within a month [33]. In fact, CXCR3-/- mice on the BALB/c background actually had improved control of infection. It has been hypothesized that CXCR3 signaling could compensate for deficient CCR5 signaling, or vice versa [71]. If these chemokine receptors are truly redundant, mice deficient in both CXCR3 and CCR5 might be impaired in granuloma formation, and could succumb to *M. tuberculosis* infection more rapidly than control mice. To determine whether CXCR3 and CCR5 double deficiency compromises granuloma formation and survival of *M. tuberculosis* infection, C57Bl/6 CXCR3xCCR5-/- and wild type (WT) control mice were infected with low dose *M. tuberculosis* and bacterial burden was followed over time. Like CCR5-/- mice[164], bacterial burden in the lungs (Figure 12A) and spleens (Figure 12B) of CXCR3xCCR5-/- mice was similar to that found in WT mice. However, in the mediastinal lymph node there was a significant increase in bacterial burden by 2 months post-infection (Figure 12C). As was the case in singly deficient mice [33, 161], there was no difference in the

ability of CXCR3xCCR5 doubly deficient mice and control mice to survive low dose *M. tuberculosis* infection (data not shown).

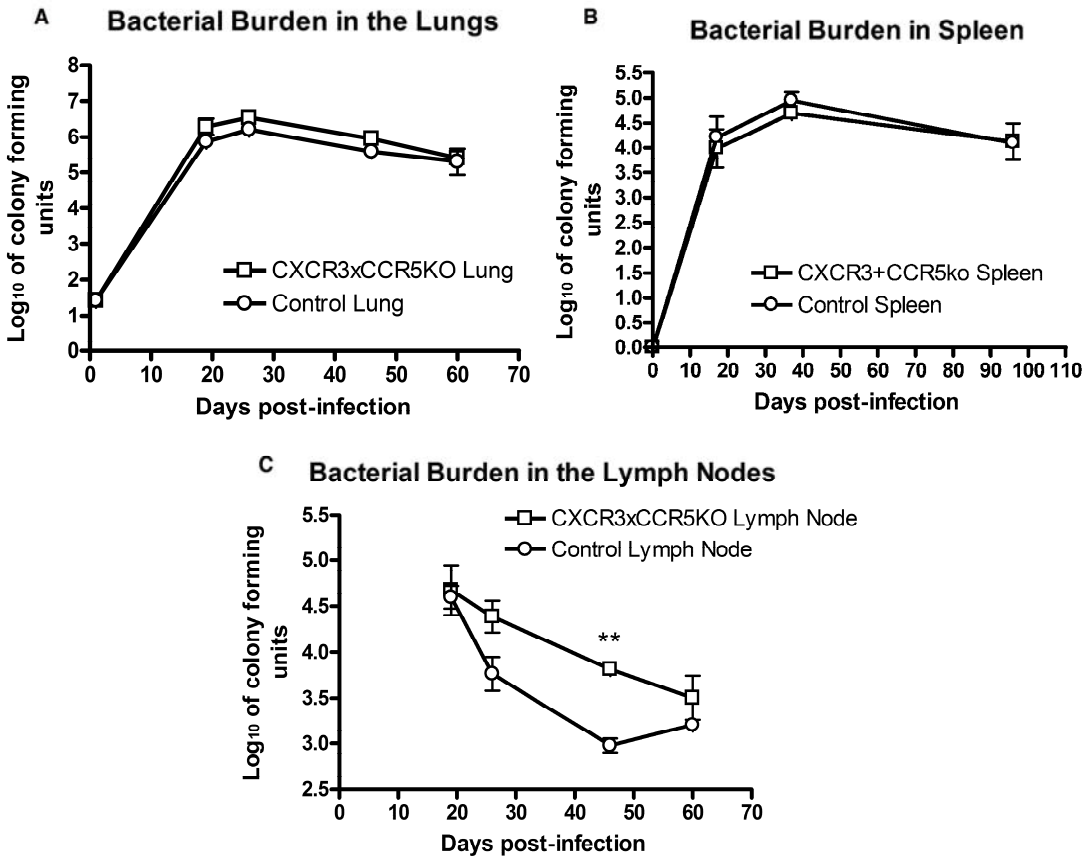

Figure 12 Increased bacterial burden in the lymph nodes of CXCR3xCCR5-/- mice

CXCR3xCCR5-/- (□) and WT (○) mice were infected with a low dose of *M. tuberculosis.* Bacterial burden was determined by7H10 plating lung (A), spleen (B) or lymph node (C) homogenates at specific time points of infection.

5.3.2 Mice doubly deficient in CXCR3 and CCR5 formed granulomas normally

Granulomas formed normally in the absence of CXCR3 [161] or CCR5 [164], and with similar kinetics to wild type mice. However, in CXCR3xCCR5-/- and WT mice, granulomas had formed

by 5 weeks post-infection (Figure 13A). By 7 weeks post-infection in the absence of both CXCR3 and CCR5, greater lymphocyte infiltration could be observed by histology compared to controls (Figure 13B), which is similar to observations using CCR5-/- mice [164].

5.3.3 Higher lymphocytic infiltration of the lungs of CXCR3xCCR5 deficient mice could be due to greater lymph node priming

The make up of the increased leukocyte infiltration in the lungs of CXCR3xCCR5-/- mice was further investigated by flow cytometry. The number of CD4+ and CD8+ T lymphocytes present in the lungs of CXCR3xCCR5-/- mouse lungs was significantly higher than those present in wild type lungs (Figure 14A, 14C). There was no reproducible difference in the number of dendritic cells or macrophages either in the lungs or lymph nodes of CXCR3xCCR5-/- and WT mice (data not shown) infected with low dose *M. tuberculosis*. No significant difference in the number of lymphocytes was detected in the lymph nodes of CXCR3xCCR5-/- and WT mice (Figure 14B, 13D). In CCR5-/- mice compared to WT, higher bacterial burden in the lymph nodes was accompanied by higher IFN-γ production in the lymph nodes, suggesting that T cell priming was greater in absence of CCR5. A similar increase in IFN-γ production was detected in the lymph nodes of CXCR3xCCR5-/- mice (Figure 14F), while there was not a significant difference in IFN-γ in the lungs (Figure 14E).

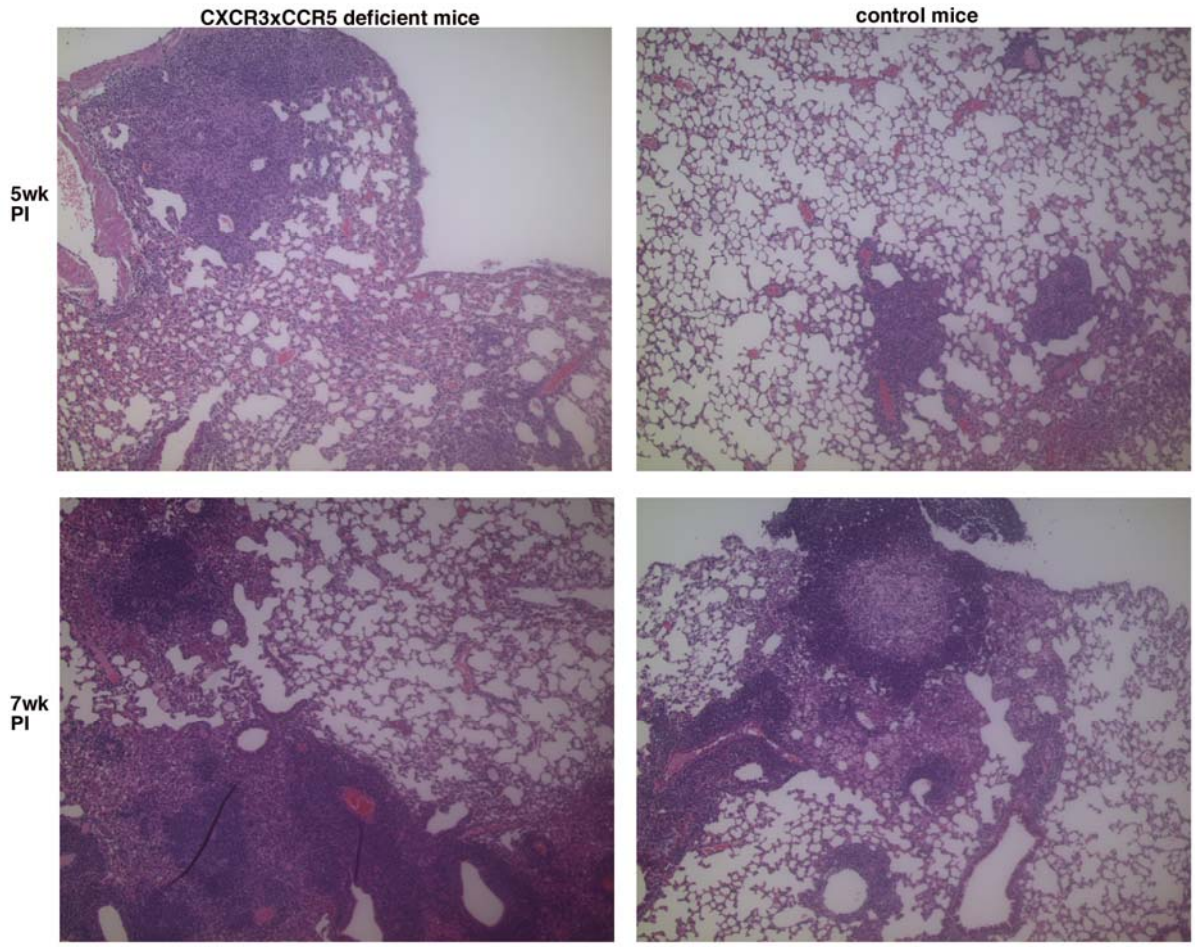

Figure 13 Intact granuloma formation in the absence of CXCR3 and CCR5 expression

To observe granuloma formation, paraformaldehyde-fixed lung sections were stained with hemotoxylin and eosin. Representative sections (20x magnification) are shown at 5 weeks (top) and 7 weeks (bottom) post-infection in CXCR3xCCR5-/- (left) and WT mice (right).

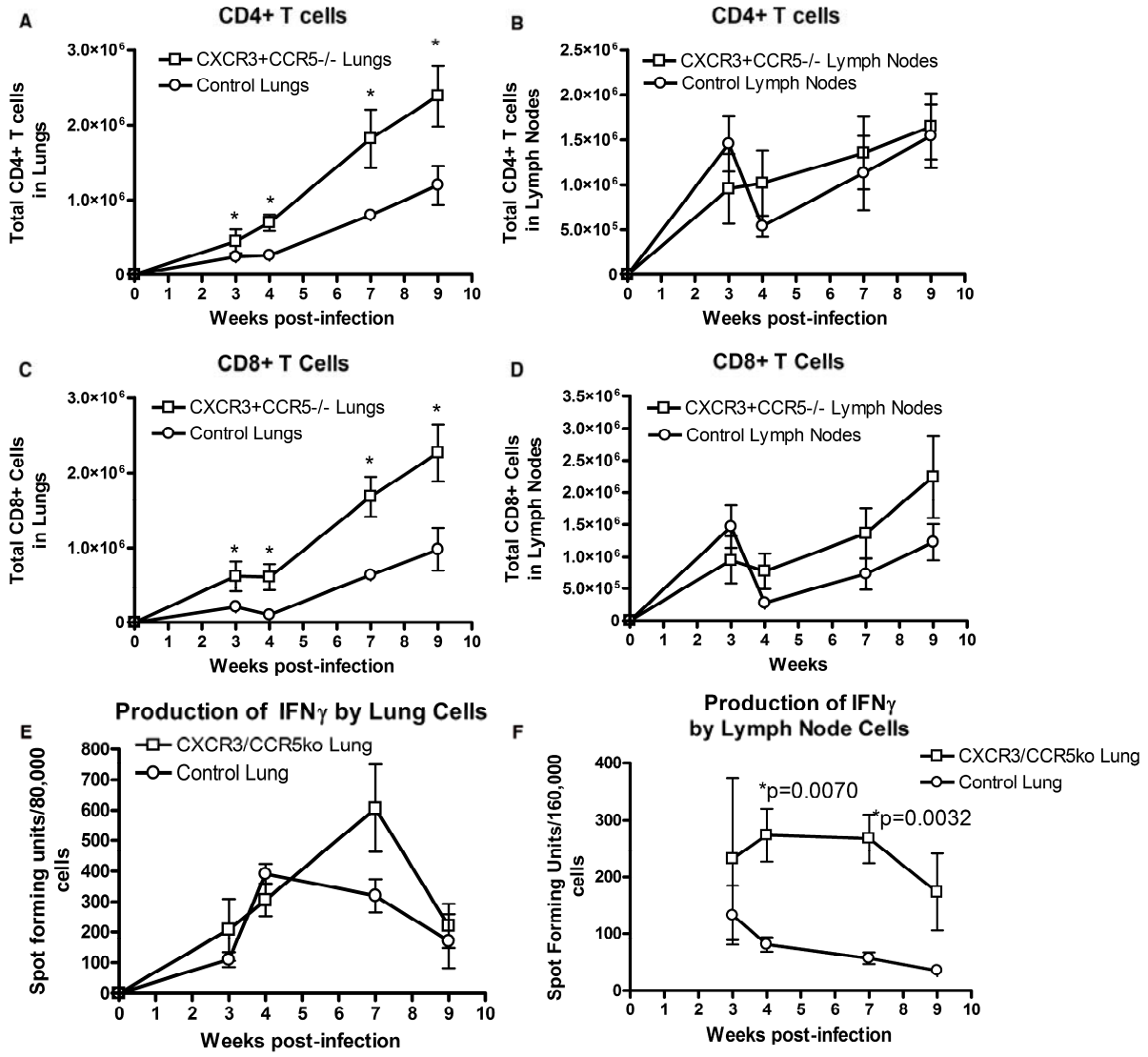

Figure 14 Higher T lymphocyte infiltration in the lung in the absence of CXCR5 and CCR5

(A-D) Cells in the organs were counted by trypan blue exclusion. The number CD4+ and CD8+ T lymphocytes in CXCR3xCCR5-/- (□) and WT (○) organs was determined by multiplying total lung or lymph node cells with the percentage of live cells (by flow cytometry) that were CD4 (A, B) or CD8 (C, D) positive.

(E-F) IFN-γ ELISpot formation by CXCR3xCCR5-/- (□) and WT (○) lung or lymph node T cells form *M. tuberculosis* infected mice in response to stimulation with *M. tuberculosis* infected bone marrow derived dendritic cells.

5.4 DISCUSSION

The expression of TNF by *Mycobacterium tuberculosis* infection increases the production of leukocyte attracting chemokines like CCL3, CCL4, CCL5, CXCL9 and CXCL10 [33]. The production of these chemokines could recruit macrophages and lymphocytes to the site of *M. tuberculosis* infection, to contribute to forming the granuloma. The granuloma is absent in mice deficient in TNF or treated with anti-TNF antibody [33, 119, 123, 195], and is thought to be required for survival of *M. tuberculosis*. It seems reasonable that chemokine signaling through receptors such as CCR5 and CXCR3 recruits leukocytes to stimulate formation of the granuloma. However, although anti-TNF treated mice express less TNF-inducible chemokines [33] than controls, no studies to date have shown that the expression of an individual chemokine receptor is required for granuloma formation or to control low dose *M. tuberculosis* infection [80, 161, 162, 164]. It is possible that one chemokine receptor might compensate for the absence of another during inflammatory situations [71].

In this study, we have investigated possible redundancy of CXCR3 and CCR5 recruitment of dendritic cells involved in priming lymphocytes in the lymph nodes, and recruitment of primed lymphocytes into the lungs of *M. tuberculosis* infected mice. In spite of deficiency in both CXCR3 and CCR5, granuloma formation remained intact and survival of low dose *M. tuberculosis* infection was unaffected. As was observed during CCR5 deficiency (C57Bl/6 *M. tuberculosis* resistant background) [164] and in CXCR3 deficiency on a *M. tuberculosis* susceptible BALB/c background [161], CXCR3xCCR5 double deficiency (on a

C57Bl/6 background) resulted in higher lung lymphocytic infiltration than in control mice (Figure 14A, 14C). As was previously hypothesized [164], the increase of lymphocytes in the lungs during CCR5 deficiency could be due to the loss of CCR5 on the surface of dendritic cells, increasing migration into the lymph nodes. Although no difference in CXCR3xCCR5-/- and WT dendritic cells in the lymph nodes could be detected (data not shown), higher *M. tuberculosis* bacterial burden was present in CXCR3xCCR5-/- mice. Higher bacterial burden could result in increased T lymphocyte priming. Alternatively, the absence of CXCR3 with CCR5 expression may have caused less recruitment of dendritic cells to the lymph nodes, and the increase in bacterial burden could be incidental. In either case, higher IFN-γ responses in the lymph nodes of CCR5-/- [164] and CXCR3xCCR5-/- (Figure 14E) mice over WT were both observed in the presence of higher lymph node bacterial burden, and could be due to the presence of more antigen available for presentation. Higher priming responses in the lymph nodes could correlate to the higher infiltration of lungs in CCR5-/- [164] and CXCR3xCCR5-/- mice over WT controls.

The response of CXCR3xCCR5-/- mice to infection with low dose *M. tuberculosis* was very similar to the responses of CCR5-/- mice [164]. Higher lung infiltration by lymphocytes, higher bacterial burden and IFN-γ production in the lymph nodes, and sufficient granuloma formation were not different between these two groups of chemokine receptor deficient mice. The differences between C57Bl/6 CXCR3-/- and WT mice were more limited, resulting only in a modest increase in bacterial burden in the spleen [161], while the differences in BALB/c CXCR3-/- mice were more significant. Taken together, data from CXCR3-/- mice and CXCR3xCCR5-/- suggest that CXCR3 ligation plays a minimal or insignificant role in protection from *M. tuberculosis* infection, and no role in granuloma formation. On the other

hand, while CCR5 is not required for granuloma formation [164], this chemokine receptor seems to prevent the migration of a fraction of the dendritic cells from the lung to the lymph nodes. Chemokine ligation in this case may function in the host response to *M. tuberculosis* by modulating the amount of antigen available for presentation, and therefore the robustness of the immune response. This modulation may serve to keep inflammation at a manageable level during the chronic phase of *M. tuberculosis* infection.

TNF is a pro-inflammatory cytokine capable of carrying out various functions. Mice deficient in TNF do not form functional granulomas thought to be required for control and survival of *M. tuberculosis* infection [33, 119]. How TNF expression controls granuloma formation has not yet been determined. It seems likely that since TNF expression can upregulate expression of chemokines such as those that signal through CXCR3 and CCR5, these chemokines may participate in formation of the granuloma. However, evidence available in the literature in mice deficient in a single molecule [80, 161, 162, 164] and the data presented here in double deficiency of CXCR3 and CCR5 suggest that these two receptors do not play an essential role in granuloma formation. Although induction of chemokines during *M. tuberculosis* infection is a function of TNF expression [235], this does not seem to be a necessary role for TNF during *M. tuberculosis* infection. Although TNF synergizes with IFN-γ for optimal activation of the macrophage [66, 67], could be required to control bacterial burden, it seems unlikely that this induces granuloma formation. We have also confirmed that TNF is not required for the priming of a *Mycobacterium tuberculosis* specific immune response (Chapter 2). Another possible mechanism by which TNF might guide formation of the granuloma is by induction of adhesion molecule expression or activation of adhesion molecules like integrins to allow leukocytes to adhere to and communicate with one another in the context of the granuloma. With the

availability of antibodies to stain for, or block adhesion of specific adhesion molecules currently available, the role of various adhesion molecules can be investigated, and these studies have begun in our and other laboratories. The most likely possibility is that several functions are carried out by TNF, and that no individual function is required, but that an combination of several functions of TNF leads to the successful formation of the granuloma and containment of infection. Currently, the mechanism by which TNF controls to granuloma formation remains unclear.

6.0 CHAPTER 4 LTB_4 SIGNALING IS NOT REQUIRED FOR MICE TO CONTROL MYCOBACTERIUM TUBERCULOSIS INFECTION

6.1 INTRODCUTION

Arachidonic acid metabolism catalyzed by 5-lipoxygenase (5-LO) results in the production of lipotoxin A_4 (LXA_4) and leukotriene B_4 (LTB_4) chemotactic molecules (arachidonic acid metabolism reviewed by [167]). Deficiency in the 5-LO enzyme improves survival of acute murine infection with *M. tuberculosis* [168], due to decreased inflammation and decreased tissue necrosis. This suggests that at least one arachidonic acid derived chemoattractant molecule prevents regulation of the inflammatory response. Both LXA_4 and LTB_4 are produced during *M. tuberculosis* infection, but only LXA_4 continues to be expressed throughout chronic infection [168]. LXA_4 mediates inflammation and can downregulate IL-12 production in dendritic cells [169]. Downregulation of IL-12 may be responsible for a decrease in observed inflammation. Administration of an analog to LXA_4 to 5-LO deficient mice resulted in elimination of the protection seen in the absence of 5-LO [168]. The specific role of LTB_4 in the immune response to *M. tuberculosis* has not been further investigated, so it is unknown whether it is the effect of LXA_4 alone that exacerbates inflammation during *M. tuberculosis,* or whether this can be affected by either LXA_4 or LTB_4.

LTB_4 can signal through one of two G-protein-coupled, membrane-bound receptors. BLT1 can be found mainly on the surface of circulating leukocytes [170, 171], while BLT2 is expressed within organs [172, 173]. LTB_4 is mainly produced by mast cells and neutrophils [174-176], and can both induce firm arrest of cells during migration and increase neutrophil survival [177]. The expression of BLT1 on the surface of circulating leukocytes, and the ability of LTB_4 to signal firm arrest suggests that this molecule might play a role in migration of leukocytes into *M. tuberculosis* infected lungs. Increased neutrophil survival could lead to overabundant lung infiltration by neutrophils. Thus, it is possible that LTB_4 could exacerbate the early immune response to *M. tuberculosis*, and that its elimination in the 5-LO deficient mouse contributed to improved host response.

Recent murine studies have outlined the role of BLT1 and LTB_4 interaction in joint inflammation modeling rheumatoid arthritis [242], and in murine models of asthma [243]. LTB_4 is overproduced in the lungs of cystic fibrosis patients, which may cause neutrophils to become less responsive, possibly contributing to difficulty in fighting bacterial colonization [244]. LTB_4 expression also contributes to pathology in chronic obstructive pulmonary disease [245].

BILL 284 is a human biologic agent that inhibits LTB_4 signaling and neutrophil recruitment to areas of inflammation [246]. Symptoms were not reduced during a two week period of observation of rheumatoid arthritis patients receiving BILL 284, but Mac-1 (CD11b/CD18) adhesion molecule expression decreased [247]. Additional clinical trials using BILL 284 have confirmed that no improvement of rheumatoid arthritis symptoms resulted when treatment was extended over a longer period of time [248]. LTB019 is another LTB_4 blocking agent that similarly failed to improve the outcome of chronic obstructive pulmonary disease, but interestingly also resulted in decreased Mac-1 expression [249]. However, their efficacies in

other diseases that are exacerbated by LTB_4 have not yet been investigated, and warrant careful investigation. Perhaps during infectious disease, LTB_4 antagonism could improve lung pathology during tuberculosis, to give a better prognosis during active or chronic disease.

In this study, we investigated the role of BLT1 in leukocyte recruitment into the lungs during low dose *M. tuberculosis* infection, and the role of BLT1-mediated chemotaxis. Throughout a three month course of observation, no difference in lung or lymph node bacterial burden between BLT1-/- and wild type (WT) control mice was observed. Analysis of leukocyte infiltration provides evidence that BLT1 does not play a significant role in leukocyte recruitment into *M. tuberculosis* infected lung, at least during a low dose infection. Histological examination of mice showed that granuloma formation had begun by 21 days post-infection in the presence or absence of BLT1 signaling, and LTB_4-BLT1 mediated chemotaxis was not required for survival of low dose *M. tuberculosis* infection. Overall, these data suggest that LTB_4 or BLT1 antagonism would be unlikely to improve control of *M. tuberculosis* infection.

6.2 MATERIALS AND METHODS

6.2.1 Animals

C57Bl/6 female mice (Jackson Laboratory, Bar Harbor, ME) and BLT1-/- (a kind gift from Andrew Tager, MD and Andrew Luster MD/PhD) were transferred from Harvard Medical School to a specific pathogen-free facility at the University of Pittsburgh. Mice infected with *M. tuberculosis* were housed in a BioSafety Level 3 laboratory for the duration of experiments and monitored for murine pathogens by serological and histological methods. The University of Pittsburgh Institutional Animal Care and Use Committee approved all procedures used in this study.

6.2.2 Mycobacteria and murine infection

Low dose aerosol infection of mice (20-100 colony forming units) of *M. tuberculosis* strain Erdman (Trudeau Institute, Saranac Lake, NY) has been previously described [250]. Bacterial burden was determined as previously described [208]. For a description of these protocols, refer to section 3.2.2 of Chapter 3.

6.2.3 Chemicals and reagents

All chemicals were purchased through Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Middlebrook 7H10 agar and 7H9 liquid media for growth of *M. tuberculosis* were purchased

from Difco (Detroit, MI). Flow cytometry antibodies were purchased from BD Pharmingen (Cedarlane Laboratories, Burlinton, NC).

6.2.4 Flow cytometry

Lung infiltration was examined at predetermined time points as previously described [33, 162]. For a description of this protocol, refer to section 3.2.5 of Chapter 3. Cells were stained with ant-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD3 (clone 17A2), anti-CD11c (clone HL-3), anti-CD11b (clone M1/70) and anti-GR1 (clone RB6-8C5).

6.2.5 IFN-γ Detection by ELISpot

Expression of IFN-γ was determined by the ELISpot assay performed as previously described [191, 232]. For a description of the protocol, see section 4.2.5 of Chapter4.

6.2.6 Production of bone marrow derived antigen presenting cells

Production of bone marrow derived dendritic cells used as antigen presenting cells in the ELISpot assay was done as previously described [191, 232]. For a description of the protocol, refer to section 4.2.6 of Chapter4.

6.2.7 Histopathology

Samples of lung were fixed in 10% normal buffered formalin and embedded in paraffin. 5-6μm sections were H&E stained. Slides were examined in a blinded fashion.

6.2.8 Statistical Analysis

Two groups were compared by a student's T test, while three or more groups were analyzed by an ANOVA statistical analysis. A value of $p<0.05$ was considered significant. Experiments were repeated to demonstrate reproducibility.

6.3 RESULTS

6.3.1 BLT1 and LTB_4 interaction does not control of *M. tuberculosis* bacterial burden

In the absence of 5-LO expression, bacterial burden declined, and this decline was abrogated in during LXA_4 antagonism [168]. To determine whether LTB_4 signaling through BLT1 similarly affects *Mycobacterium tuberculosis* bacterial burden, BLT1-/- and wild type (WT) control mice were infected with low dose *M. tuberculosis.* BLT1-/- mice regulated bacterial burden in the lungs similarly to WT mice for up to 12 weeks (Figure 15A). Dissemination of mycobacteria was assessed by observing the bacterial burden in the lymph nodes of *M. tuberculosis* infected mice. Throughout the observation period, bacterial burden was equivalently controlled in lymph nodes by BLT1-/- and control mice (Figure 15B). Infection was allowed to persist for 9 months following infection, with no difference in the ability of BLT1-/- mice or WT mice to survive.

6.3.2 Infiltration of the *M. tuberculosis* infected lung does not require ligation of BLT1

As BLT1 is expressed on circulating leukocytes [170, 173], and LTB_4 signaling leads to the arrest that allows extravasation [177], their interaction may be required for entry of leukocytes into the lungs during *M. tuberculosis* infection. Total lung leukocyte infiltration was similar between BLT1-/- and WT mice through 12 weeks of low dose *M. tuberculosis* infection (Figure 16A). The percentage of macrophages (CD11b+, CD11c-, and GR1-) and neutrophils (CD11b+,

CD11c- and GR1+) were also comparable between BLT1-/- and control mice (Figure 16A). The percent of CD4+ and CD8+ lymphocytic lung infiltrate was also similar between BLT1-/- and WT control mice (Figure 16B). Although LTB_4 can play a role in migration, it was not required for entry of leukocytes into the lungs during *M. tuberculosis* infection.

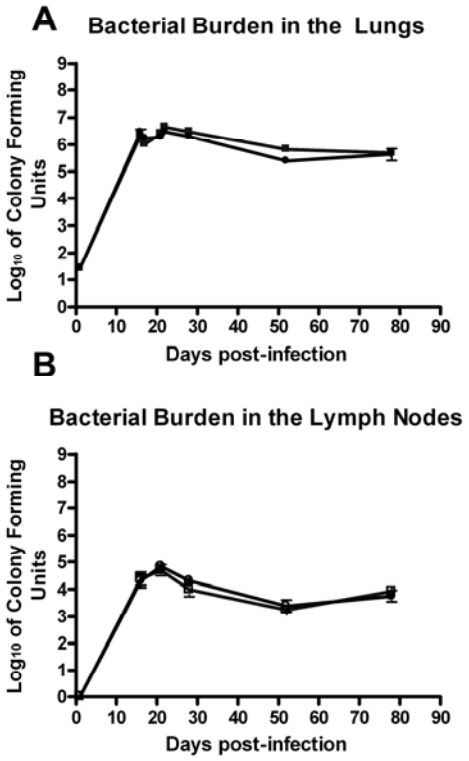

Figure 15 BLT1 signaling is not required to control low dose *M. tuberculosis* bacterial burden

(A, B) Bacterial burden (counted colony forming units) was followed over time in BLT1-/- (■) and WT (●) lungs (A) and lymph nodes (B) in mice infected with *M. tuberculosis.*

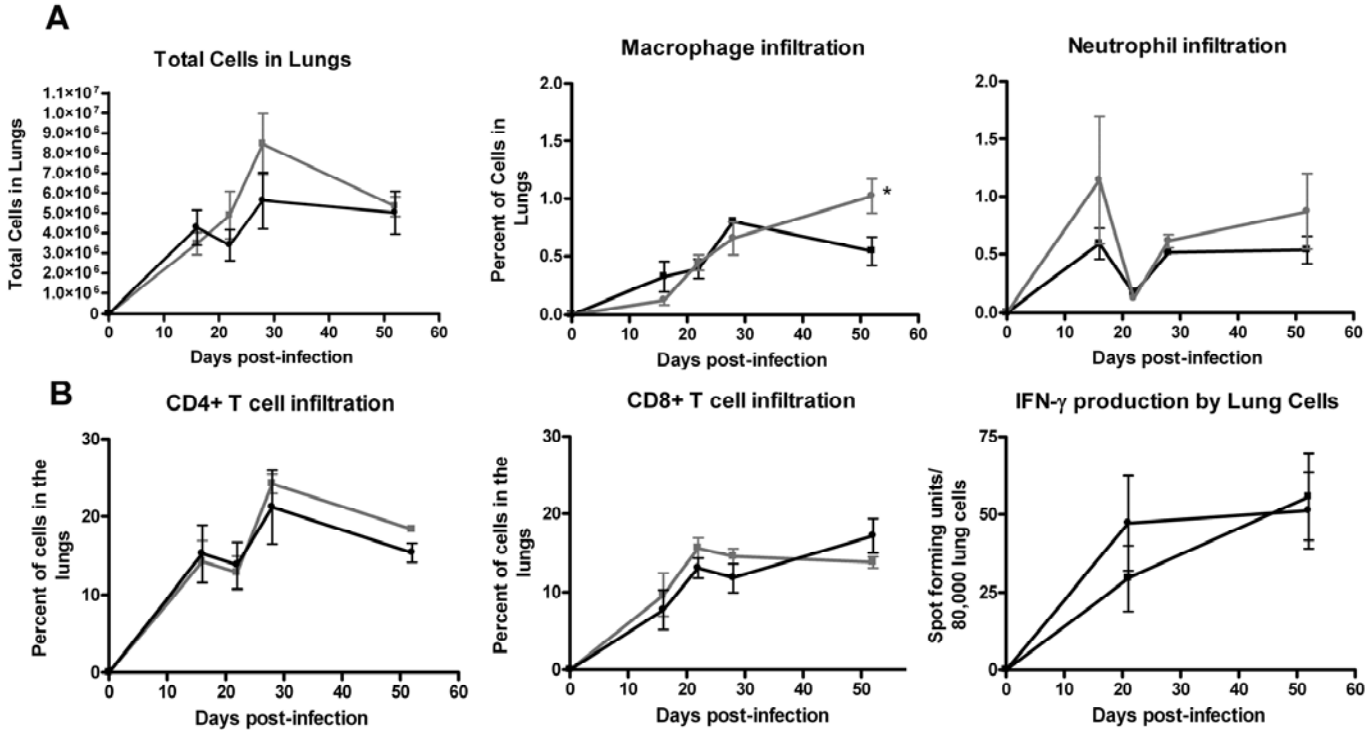

Figure 16 BLT1 and LTB4 interaction are not required for lymphocyte migration or function

(A) Cells from lung homogenates from BLT1 (grey, ■) or control mice (black, ●) were counted by trypan blue exclusion. Flow cytometry was used to determine what percentage of live cells were macrophages (CD11b+CD11c-GR1-) or neutrophils (CD11b+CD11c-GR1+).

(B) Cells from lung homogenates collected by flow cytometry to determine how many CD4+ or CD8+ T lymphocytes were present in the lungs of BLT1-/- (grey, ■) or WT (black, ●) *M. tuberculosis* infected mice. Aliquots from the same lung homogenates were assessed for IFN-γ production in response to *M. tuberculosis* infected dendritic cells in the ELISpot assay.

6.3.3 Signaling by LTB_4 is not required for the adaptive immune response to *M. tuberculosis* infection

Production of interferon gamma (IFN-γ) by T lymphocytes is required to control *M. tuberculosis* infection [133]. The ability of lung infiltrating leukocytes to produce IFN-γ in response to re-

stimulation with *M. tuberculosis* infected dendritic cells was assessed by ELISpot. No difference in the ability to respond to *M. tuberculosis* infected dendritic cells was found between lymphocytes from the lungs of BLT1 deficient and control animals (Figure 16B).

6.3.4 Granuloma formation is not dependant on LTB_4 inducible chemotaxis

The granuloma provides a physical barrier against dissemination of mycobacteria as well as a local environment for cells of the immune system to interact [44]. Granuloma formation and maintenance are deficient in the absence of TNF signaling, despite similar infiltration of leukocytes into the lungs of anti-TNF treated or TNFR1 deficient mice and control mice [44]. Therefore, although leukocyte migration is sufficient in BLT1-/- mice, changes in granuloma formation are possible. The role of BLT1 in forming the granuloma was assessed by H&E staining of fixed lung sections from BLT1-/- and control mice. Granuloma formation appeared similar after 21 days of *M. tuberculosis* infection and was maintained through 3 months following *M. tuberculosis* infection (Figure 17).

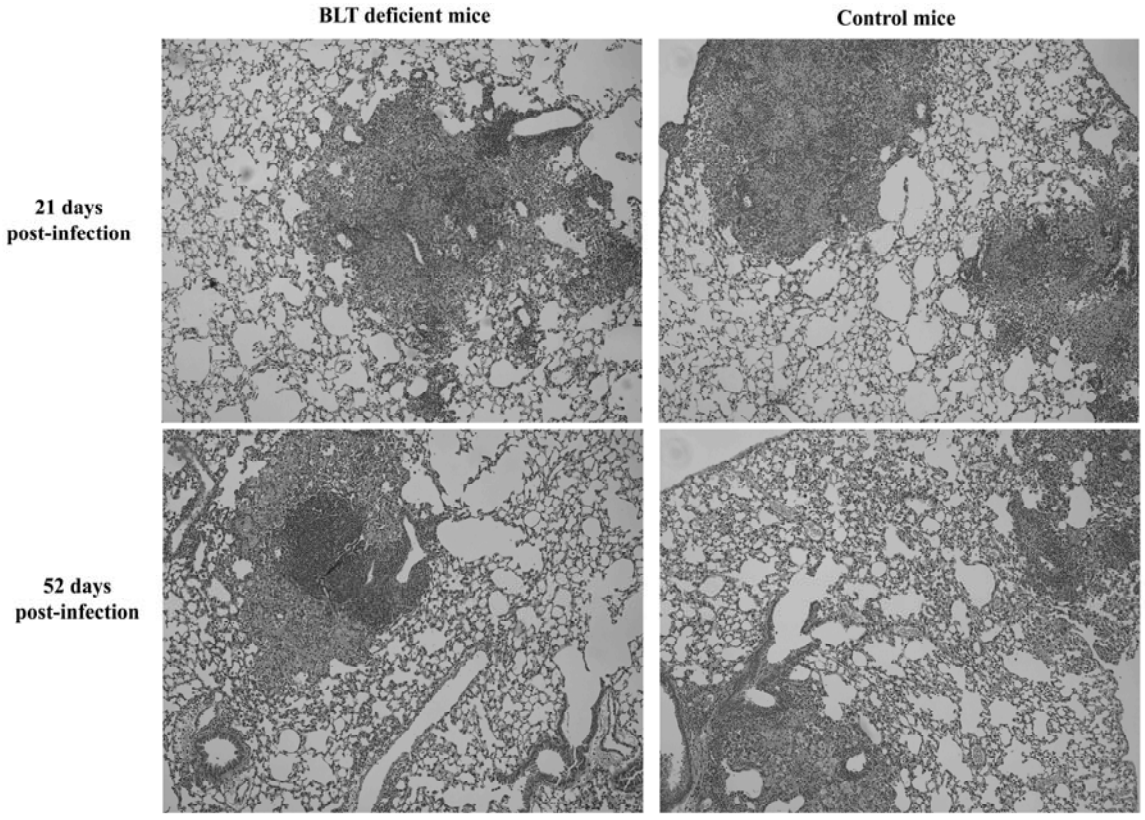

Figure 17 Granuloma formation does not require signaling through BLT1

To observe granuloma formation and lung pathology, lung sections were stained with hemotoxylin and eosin 3 weeks (top) and 7 weeks (bottom) post-infection. Representative sections are shown (20x magnification).

6.4 DISCUSSION

In this study, we have assessed the role of BLT1 and LTB_4 in the mouse model of *M. tuberculosis* infection. The data presented here suggest that although LTB_4 is present during early acute *M. tuberculosis* low dose infection in mice [168], signaling of this lipid chemoattractant through its leukocyte receptor is not required for entry into the lungs or for migration for granuloma formation. Bacterial burden remained similar through chronic infection in BLT1-/- and WT control mice, suggesting that LTB_4 expression does not contribute to pathology in the same manner as LXA_4 [168].

Anti-TNF treatment has greatly reduced the inflammation of synovial joints in many patients with auto-inflammatory disease [205]. Patients latently infected with *M. tuberculosis* risk reactivation of tuberculosis if treated with anti-TNF drugs [251]. Patients that are not candidates for anti-TNF biologic therapy could benefit from treatment by inhibiting other components of the immune system involved in autoimmune inflammation. In mice, the absence of LTB_4-BLT1 interaction prevented acute and improved chronic rheumatoid arthritis in the K/BxN serum transfer mouse model [242, 252]. Blocking LTB_4 signaling in an asthma model also decreased inflammation [243]. These data suggest that treatment of inflammation with an inhibitor of LTB_4 could decrease inflammatory symptoms.

Clinical trials in rheumatoid arthritis [248] and chronic obstructive pulmonary disease [249] were unsuccessful in treating these diseases using two different LTB_4 antagonists. The failure of LTB_4 antagonism to decrease the immune response during rheumatoid arthritis and chronic obstructive pulmonary disease is disappointing when compared to the success in mouse

models of these diseases. Despite clear similarities between human rheumatoid arthritis and the K/BXN murine model of arthritis, the mouse develops arthritis in response to a single antigen [253]. So, the difference in response to antagonism of LTB_4 may be due to the difference in complexity between the human disease and the simpler mouse model. On the other hand, the data presented here suggest that absence of LTB_4 signaling has no measurable effect on the immune response to low dose *M. tuberculosis,* in spite of improved immune responses in the absence of 5-LO.. This suggests that LTB_4 is not the most potent regulator of the immune response, at least in the case of low dose *M. tuberculosis* infection, although higher doses of *M. tuberculosis* might be more affected by the absence of LTB_4. In contrast, antagonism of TNF as a treatment for chronic inflammatory diseases has been shown to have a negative affect on control of low dose *M. tuberculosis* infection in humans and mice [33, 119-122, 195, 254-256]. In the event that antagonism of LTB_4 is shown to be efficacious in treating any chronic inflammatory diseases, our data do not predict exacerbation or reactivation of tuberculosis.

7.0 CHAPTER 5 OSTEOPONTIN IS NOT REQUIRED TO CONTROL MYCOBACTERIUM TUBERCULOSIS INFECTION

7.1 INTRODUCTION

More recently characterized as a pro-inflammatory cytokine, but known an adhesive component of the extracellular matrix, osteopontin mediates cellular mechanisms such as chemotaxis and production of the cytokine IL-12 [140-143]. T lymphocytes, NK cells and macrophages can express this cytokine [140], and its functions can vary from adhering to CD44 and α_4 integrin adhesion molecules, induction of leukocyte chemotaxis, costimulation of T cells during priming and enhancing the production of IL-12 by macrophages [140, 143].

Osteopontin plays a role in shaping the Th1 immune response to infections such as to *Listeria monocytogenes,* where osteopontin was required for optimal production of IL-12 and IFN-γ [257], but is not required to protect mice from infections with either *Borrelia burgdorferi* [146, 147] or influenza virus (strain PR8) [148]. Expression of osteopontin can be found in granulomas produced in response to sarcoidosis, a T cell mediated granulomatous disease for which the cause is unclear [143], and the production of granulomas in response to *Schistosoma mansoni* is delayed in the absence of this cytokine [258].

Osteopontin is made in large amounts by humans given the BCG vaccine and by patients with active *M. tuberculosis* infection. Since severity of disease correlates with increased systemic

levels of osteopontin, it has been suggested that this cytokine could be used as a biomarker marker for tuberculosis [140-142]. Not only has it been found in the circulatory system of those with tuberculosis, but many cells within granuloma are producing osteopontin as determined by immunohistochemistry [149]. It has been reported that expression of CD44 adhesion molecule, which plays a role in granuloma formation and is required to control high dose *M. tuberculosis* [182], is increased by osteopontin expression [259]. Osteopontin may play a role in attracting leukocytes to the site of infection, in shaping the immune response within the granuloma, or by enabling cells to adhere to one another to form and maintain the granuloma.

Osteopontin deficient mice infected intraperitoneally with BCG formed more and larger granulomas, and there seemed to be more bacteria present overall within the liver [150], although actual bacterial numbers were not determined. This supports a role for osteopontin in controlling the immune response to mycobacterial infection, regulating bacterial burden. The immune response to intraperitoneal infection with BCG could differ from the immune response to an aerosol infection with *M. tuberculosis*, due to the route of infection and differences in virulence, so the ability of the immune system of osteopontin deficient mice to respond to *M. tuberculosis* warrants further investigation.

To further investigate the role of osteopontin in the shaping of the immune response to *M. tuberculosis* infection, we infected osteopontin deficient (OPN-/-) and wild type (WT) control mice with low dose infection *M. tuberculosis*. We were surprised to find similar bacterial burden in both groups, with no impairment in survival of the mice. T lymphocyte induction, migration into the lungs and function within the lungs were similar in both groups. Although osteopontin can stimulate macrophage migration [143], similar numbers of macrophages and neutrophils were detected between OPN-/- and WT mice throughout infection. We conclude from these

studies that while osteopontin may be elevated in patients with tuberculosis and although there may be a deficiency in controlling high dose BCG in OPN-/- mice, there is no requirement for this cytokine in developing an effective immune response to a low dose aerosol infection with *M. tuberculosis*.

7.2 MATERIALS AND METHODS

7.2.1 Animals

C57BL/6 female and osteopontin deficient mice Jackson Laboratory (Bar Harbor, ME) were maintained in a BioSafety Level 3 specific pathogen-free facility and monitored for murine pathogens. All animal protocols used were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

7.2.2 Mycobacteria and murine infection

Low dose aerosol infection of mice (20-100 colony forming units) of *M. tuberculosis* strain Erdman (Trudeau Institute, Saranac Lake, NY) has been previously described [250]. Bacterial burden was determined as previously described [208]. For a description of these protocols, refer to section 3.2.2 of Chapter 3.

7.2.3 Chemicals and reagents

All chemicals were purchased through Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Middlebrook 7H10 agar and 7H9 liquid media for growth of *M. tuberculosis* were purchased from Difco (Detroit, MI). Flow cytometry antibodies were purchased from BD Pharmingen, except anti-C3 and its isotype control (Cedarlane Laboratories, Burlinton, NC).

7.2.4 Flow cytometry

Lung infiltration was examined at predetermined time points as previously described [33, 162]. For a description of this protocol, refer to section 3.2.5 of Chapter 3. Cells were stained with ant-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD3 (clone 17A2), anti-CD11c (clone HL-3), anti-CD11b (clone M1/70) and anti-GR1 (clone RB6-8C5).

7.2.5 IFN-γ Detection by ELISpot

Expression of IFN-γ was determined by the ELISpot assay performed as previously described [191, 232]. For a description of the protocol, see section 4.2.5 of Chapter 4.

7.2.6 Production of bone marrow derived antigen presenting cells

Production of bone marrow derived dendritic cells used as antigen presenting cells in the ELISpot assay was done as previously described [191, 232]. For a description of the protocol, refer to section 4.2.6 of Chapter 4.

7.2.7 Statistical Analysis

Two groups were compared by a student's T test, while three or more groups were analyzed by an ANOVA statistical analysis. A value of $p<0.05$ was considered significant. Experiments were repeated to demonstrate reproducibility.

7.3 RESULTS

7.3.1 Osteopontin expression is not required for control over bacillary burden in mice infected with *M. tuberculosis*

In osteopontin deficient (OPN-/-) mice intravenously infected with BCG, bacterial burden increased, and survival was diminished [150]. To determine whether the control over bacterial burden to aerosolized *Mycobacterium tuberculosis* was similarly inhibited in the absence of osteopontin, OPN-/- mice and control mice were infected with a low dose aerosol infection. Bacterial burden was monitored for up to 7 months post-infection, and was not significantly different between OPN-/- and WT control mice in the lungs, lymph nodes, spleen or liver (Figure 18). Survival of mice infected with *M. tuberculosis* was allowed to persist for up to a year to determine whether osteopontin was required for long term survival of chronic infection. There was no difference in survival of OPN-/- or WT mice (data not shown), showing that osteopontin expression is not required for control over bacterial burden or ultimately for survival of a low dose infection with *M. tuberculosis*.

7.3.2 Leukocyte migration does not require osteopontin expression

Osteopontin can induce chemotaxis of leukocytes, and can stimulate leukocyte adhesion [140-143], so this cytokine could play a role in migration of primed T cells or of myeloid cells into *M.*

tuberculosis infected tissues. However, no reproducible differences were observed CD4+ or CD8+ lymphocytes, macrophages or neutrophils infiltrating the lung (Figure 18). After about 2 months of infection, there was a transient increase in neutrophils and macrophages within the lungs of OPN-/- mice over WT mice, but this resolved at the next time point, and was not reproducible. This suggests that osteopontin is not required for migration of T lymphocytes into lungs during low dose *M. tuberculosis* infection. Granuloma formation in the lungs, liver and spleen was similar at all time points in OPN-/- and WT mice, suggesting that osteopontin is not required for granuloma formation (data not shown).

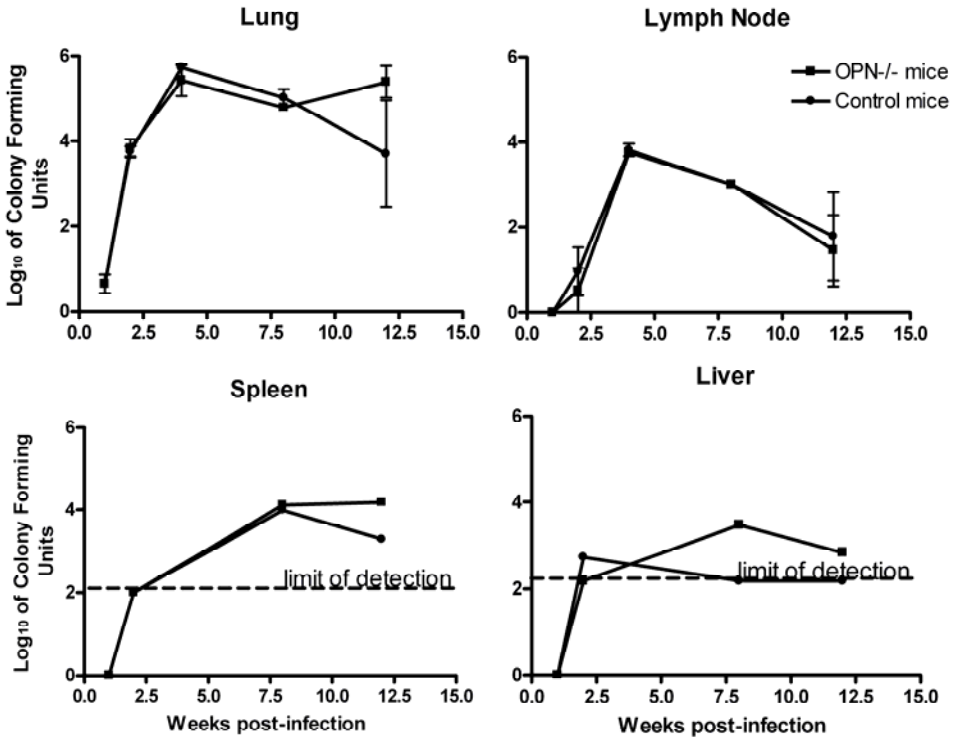

Figure 18 Bacterial burden is sufficiently controlled in absence of osteopontin

Bacterial burden (colony forming units) in the organs of OPN-/- (■) and WT (●) mice was similar in the lungs (top left), lymph nodes, (top right), spleen (bottom left) or liver (bottom right).

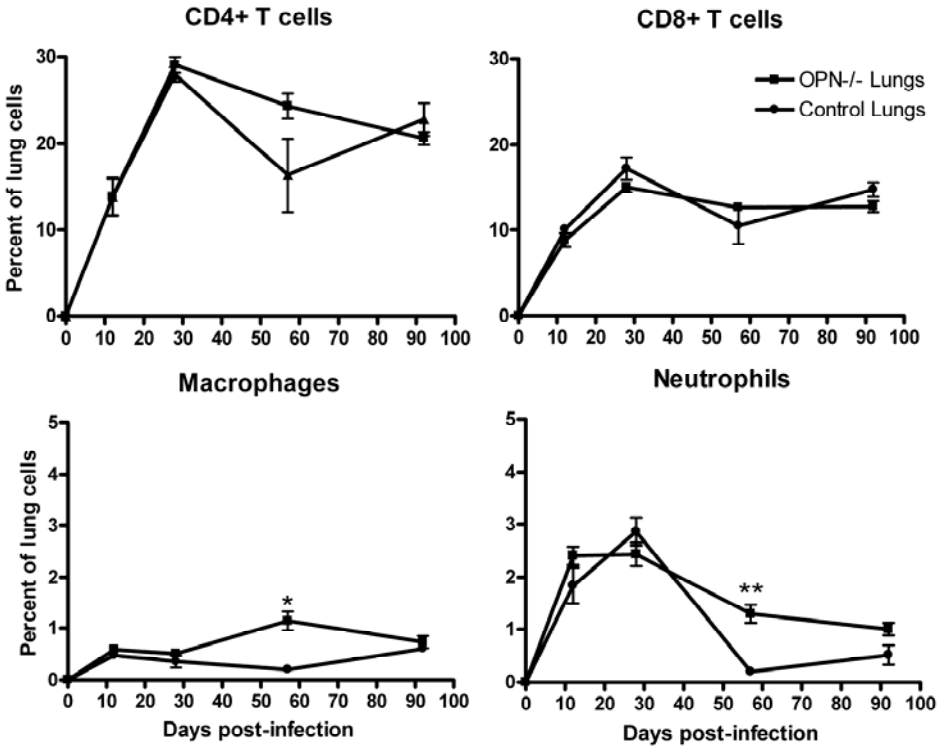

Figure 19 Leukocyte migration is independent of osteopontin expression

The percent of lung infiltrate that were CD4+ T cells, CD8+ T cells, macrophages (CD11b+CD11c-GR1-) and neutrophils (CD11b+CD11c-GR1+) OPN-/- (■) and WT (●) was detected by flow cytometry.

7.3.3 Osteopontin cytokine activity is not required for priming of the Th1 T lymphocyte response

Osteopontin was required to induce IL-12 expression during *Listeria monocytogenes* infection, which is necessary for priming a Th1 CD4+ T lymphocyte response characterized by IFN-γ expression [257]. IFN-γ expression is essential for survival of infection with *M. tuberculosis* [133], so if osteopontin could be required for the priming of the Th1 response to *M. tuberculosis* infection by inducing IL-12 expression. To determine whether the Th1 immune response to *M.*

tuberculosis infection requires osteopontin we performed IFN-γ ELISpot assays in which T cells from infected lungs or lymph nodes respond by producing IFN-γ if they recognize antigens presented by *M. tuberculosis* infected dendritic cells. There was no significant difference in the production of IFN-γ in cells from the lungs or lymph nodes of OPN-/- and WT control mice (Figure 20). Because there were no differences in IFN-γ production, the expression of IL-12 was not compared, but IL-12 expression was clearly sufficient to induce a sufficient Th1 immune response during a low dose *M. tuberculosis* infection.

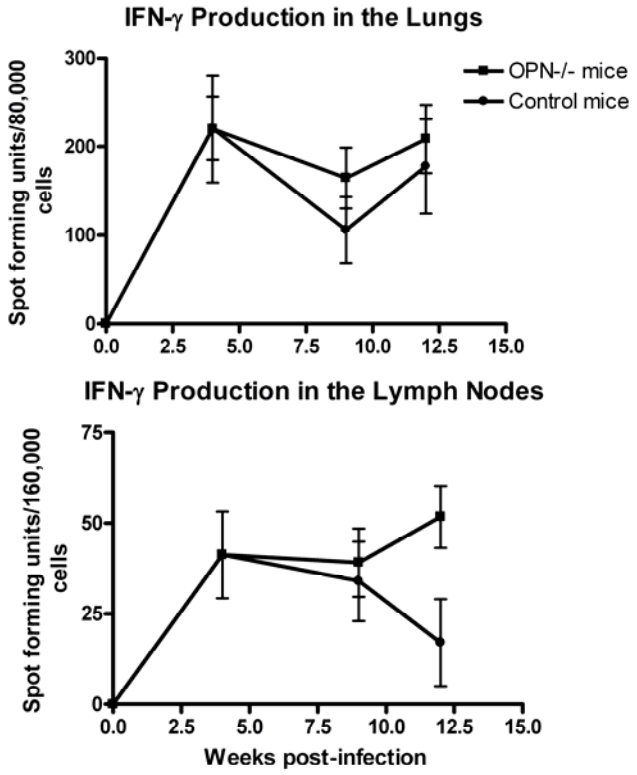

Figure 20 Priming *M. tuberculosis* specific responses does not require osteopontin expression

IFN-γ ELISpots were used to assess the ability of T lymphocytes isolated from lungs or lymph nodes of *M. tuberculosis* OPN-/- (■) or WT (●) to respond to *M. tuberculosis* antigens presented by infected dendritic cells.

7.4 DISCUSSION

Studies in humans have shown that osteopontin expression is upregulated during active tuberculosis and it has been suggested that systemic osteopontin could be a good indicator of disease [140-142]. It is tempting to assume that since osteopontin is expressed abundantly during *M. tuberculosis* infection, it modulates the immune response to infection. Indeed, osteopontin deficient mice infected with BCG had higher bacterial burdens, larger and more numerous granulomas and were more susceptible mycobacterial infection. There are several mechanisms by which this cytokine might drive the host immune response to infection. First, osteopontin can act as a mediator of migration through chemotactic signaling in the extracellular matrix. Second, it could act as an adhesion molecule, which might aid in migration, or might allow two cells to adhere to one another to prime an immune response or form a granuloma. Finally, it has been shown that osteopontin can stimulate IL-12 production required for the function of the Th1 immune response to *Listeria monocytogenes* [257] required for control over *M. tuberculosis* infection.

In this study, we have addressed the role of osteopontin in controlling aerosol infection with *M. tuberculosis.* In spite of abundant human expression of systemic osteopontin during tuberculosis [140-142], and the susceptibility of OPN-/- mice to BCG [150], this cytokine was not required for control over low dose aerosol *M. tuberculosis* infection. Bacterial burden, granuloma formation, survival of mice, recruitment of leukocytes and priming of *M. tuberculosis* specific immune response were similar between OPN-/- and WT mice.

The difference between OPN-/- mouse control over BCG and *M. tuberculosis* infections could be due to the route of administration or dose of inoculum. The immune response to intravenous and aerosol infection differ, even when the same bacterial burden initially reaches the lungs [260]. Mice are more susceptible to an aerosol infection than an intravenous infection with *M. tuberculosis* [260], which supports the administration of fewer bacteria to establish an aerosol infection. Overall, fewer bacteria were administered in the low dose aerosol infection presented here than in the BCG infection of OPN-/- mice [150], which suggests that OPN-/- mice may simply be susceptible to a higher dose of bacteria. The low dose *M. tuberculosis* infection leads to fulminant disease and decreased survival in the absence of important inflammatory mediators such as TNF [33] and nitric oxide [60], supporting the validity of the low dose model. This indicates that osteopontin is not required to control a low dose *M. tuberculosis* infection.

Another possible reason for the difference between the data presented here and the findings in OPN-/- mice infected with BCG is that BCG differs genetically from *M. tuberculosis.* The RD1 and RD3 regions of *M. bovis* and *M. tuberculosis* are completely absent from attenuated BCG mycobacteria [261]. Deletion of the RD1 locus mimics the attenuated phenotype of BCG, suggesting that the RD1 region of *M. tuberculosis* encodes genes associated with virulence [262]. Elimination of these virulence factors can also eliminate epitopes recognized by the adaptive immune system, such as ESAT6 which is recognized by CD4+ T cells [263]. Perhaps in the absence of antigens encoded by the RD1 region of *M. tuberculosis* genetic material, osteopontin expression is required to prime an effective adaptive immune response, but in the presence of RD1 encoded epitopes like ESAT6, T cell priming does not require osteopontin, although this seems unlikely.

The data presented here suggest that osteopontin expression is not required for control over bacterial burden or priming an immune response to low dose *M. tuberculosis* infection. Expression of osteopontin during *M. tuberculosis* infection may not play a physiologic role in attempting to control the infection, but might still be a good indicator or biomarker of active tuberculosis.

8.0 CHAPTER 6 THE ADHESION MOLECULE ICAM-1 IS NOT REQUIRED FOR CONTROL OF MYCOBACTERIUM TUBERCULOSIS INFECTION, BUT IS REQUIRED FOR THE PRODUCTION OF INDUCIBLE T REGULATORY CELLS

8.1 INTRODUCTION

CD4+ T regulatory cells are identified most reliably by their expression of Foxp3 [264-266], but were originally identified by high expression of CD25. T regulatory cells play a key role in suppressing autoimmunity [95]. During chronic infections with pathogens such as *Leishmania major* and *Helicobacter pylori*, T regulatory cell function inhibits pathogen clearance and dampens the immune response [96, 97, 267], thus these cells are responsible for persistence of certain pathogens.

Natural CD4+CD25+Foxp3+ T regulatory cells are produced in the thymus. A very strong T cell receptor signaling response could turn a developing T cell down the path to become a T regulatory cell [99], and it has been suggested that these cells play a strong role in control over autoimmunity [98]. Naïve CD4+ T cells in the periphery can also become Foxp3+ through T cell receptor stimulation in conjunction with activated TGFβ1 cytokine *in vitro* and *in vivo* [100-104]. Inducible T regulatory cells have suppressive function in response to specific non-self antigens [100, 101, 104]. Since natural and TGFβ1-induced T regulatory cells have the same

phenotype *in vivo* during an inflammatory response (CD4+CD25+Foxp3+), it is difficult to discriminate between the roles of these two phenotypes.

Several studies have investigated the role of T regulatory cells during *M. tuberculosis* infection. Depletion of CD4+CD25+ T cells *ex vivo* increased human and murine effector T cell production of IFN-γ, a proinflammatory cytokine required for control over *M. tuberculosis* infection [105-107], supporting a suppressive role for CD25+CD4+ T cells in tuberculosis. While *in vivo* CD25 depletion in *M. tuberculosis* infected mice did not to decrease bacterial burden [107], specific depletion of Foxp3+ cells or reconstitution of RAG-/- mice with leukocytes lacking CD4+CD25+ cells did reduce bacterial burden [108, 109]. Thus, the available data support that, as in other infectious diseases, T regulatory cells dampen the immune response to *M. tuberculosis* infection and help to prevent clearance of the infection.

Adhesion molecules associate with one another to facilitate leukocyte migration into infected tissues and secondary organs of the immune system (reviewed in [268]). Members of the selectin family of adhesion molecules mediate interactions of leukocytes with endothelium to allow leukocyte rolling in blood vessels. When leukocytes are rolling along the endothelial surface, activated integrins such as LFA-1 (CD11a/CD18) can bind to cellular adhesion molecules such as ICAM-1 (CD54) mediating the firm adhesion that allows extravasation into inflamed tissues. ICAM-1 antibody neutralization results in fewer lymphocytes entering the lungs [180]. ICAM-1 can be expressed not only on endothelium, but also on leukocytes, and its absence impairs delayed-type hypersensitivity reactions [179]. Adhesion molecules are also involved in cell to cell communication. ICAM-1 interaction with LFA-1 allows T cells to adhere to thymic stroma during development [269], and assists in thymic positive selection of CD4+ or CD8+ T lymphocytes [269, 270]. ICAM-1 can be found in association with LFA-1 at the

immunological synapse [271-273], where their interaction likely keeps the antigen presenting cell in close association with the lymphocyte to allow priming. Not only do they mediate adhesion, but ICAM-1 and LFA-1 can also transmit co-stimulatory signals independent of CD28 that facilitate priming of the T lymphocyte [274-278]. It has been reported in a review that unpublished data suggest that ICAM-1 deficient mice develop fewer T regulatory cells in the periphery than controls [279], but the cause and effect of the deficiency have not yet been explored. Thus, ICAM-1 has the potential to play a role in migration of leukocytes into inflamed tissues, adhesion of lymphocytes to antigen presenting cells, in providing a costimulatory signal during T cell priming, and possibly in the generation of T regulatory cells.

Two studies addressing the role of ICAM-1 in *M. tuberculosis* infection had conflicting results. One study reported that mice required ICAM-1 to form granulomas, with higher bacterial burdens and lower survival in ICAM1-/- mice [185]. However another study suggested that ICAM-1 is not required for survival of mycobacterial infection [186]. Lack of expression of the ICAM-1 ligand LFA-1 results in decreased T cell priming, and aberrant granuloma formation [227], while another ligand for ICAM-1, Mac-1 (CD11b/CD18) is not required to control *M. tuberculosis* [227]. The immunologic function of ICAM-1 during *M. tuberculosis* infection thus remains unclear.

In this study, we investigated the role of ICAM-1 in the production of T regulatory cells. In spite of similar Foxp3 expression in the thymus, ICAM-1-/- mice had significantly fewer T regulatory cells in peripheral organs such as the lung and spleen. Further investigation showed that ICAM-1 is required for the induction of CD4+CD25+Foxp3+ T regulatory cells in response to active TGFβ1 and T cell receptor stimulation, and that this was due to a functional defect in the TGFβ1 signaling pathway. In the presence of an inflammatory response to *Mycobacterium*

tuberculosis, the deficit in expression of Foxp3+ T regulatory cells was exacerbated, leading to a significantly more robust effector T lymphocyte response. Despite the increased response to *M. tuberculosis* when T regulatory cells were significantly reduced, there was no difference in the bacterial burden of mice infected with *M. tuberculosis,* so unlike in the case of complete elimination of T regulatory cells, T regulatory cell reduction was not sufficient to enhance clearance of the pathogen.

8.2 MATERIALS AND METHODS

8.2.1 Animals

Breeding pairs of B6.129S4-*ICAM-1*^{tm1jcgr}/J (ICAM-1 deficient) mice were obtained from Jackson Laboratory (Bar Harbor, ME), were housed and bred in a specific pathogen free facility and monitored for murine pathogens. Prior to and throughout *M. tuberculosis* infection, ICAM-1 deficient mice, the recommended control mice (C57BL/6J) and B6.PL-*Thy1*^{a}/CyJ (Thy1.1) mice (Jackson Laboratory, Bar Harbor, ME) were housed in a BioSafety Level 3 specific pathogen-free facility and monitored for murine pathogens. All animal protocols used were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

8.2.2 Mycobacteria and infection of mice

Low dose aerosol infection of mice (50-100 colony forming units) of *M. tuberculosis* strain Erdman (Trudeau Institute, Saranac Lake, NY) has been previously described [250]. Bacterial burden was also determined as previously described [208].

8.2.3 Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Middlebrook 7H10 agar and 7H9 liquid media for growth of *M. tuberculosis* were purchased from Difco (Detroit, MI). Flow cytometry antibodies were purchased from BD Pharmingen,

except anti-active caspase 3 (Cell Technology, Inc. Mountain View, CA), anti-KI-67 (PD Pharmingen, San Diego, CA) and anti-Foxp3 antibodies, fix/perm buffer and permeabilization buffer (EBioscience Inc. San Diego, CA).

8.2.4 IFN-γ Detection by ELISpot

Expression of IFN-γ was determined by the ELISpot assay performed as previously described [191, 232].

8.2.5 Production of bone marrow derived antigen presenting cells

Production of bone marrow derived dendritic cells used as antigen presenting cells in the ELISpot assay was done as previously described [191, 232].

8.2.6 Flow cytometry

Expression of T lymphocytes, macrophages, neutrophils and dendritic cells was examined as previously described [33, 162]. Cells were stained with ant-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD3 (clone 17A2), anti-CD11c (clone HL-3), anti-CD11b (clone M1/70), anti-GR1 (clone RB6-8C5), anti-CD25 (clone PC61), anti-KI-67 (clone B56) and anti-Annexin V (clone information not provided).

8.2.7 Histopathology

Samples of lung were fixed in 10% normal buffered formalin and embedded in paraffin. 5-6μm sections were H&E stained. Slides were examined in a blinded fashion.

8.2.8 In vitro survival assays

Leukocytes were collected from the spleens of *M. tuberculosis* infected ICAM-1 deficient or WT in the same manner as for ELISpot and flow cytometry assays. The manufacturer's protocol was followed to enrich CD4+ and CD8+ T lymphocytes using combined CD4 and CD8 MACS beads (Miltenyi Biotec, Auburn, CA). Enriched T lymphocytes and non-T lymphocyte flow through were counted. WT or ICAM-1 deficient T lymphocytes were combined in a 1:1 ratio with WT or ICAM-1 deficient non-T lymphocytes to determine whether ICAM-1 on either the T lymphocyte or antigen presenting cell was required for activation induced cell death. Exogenous 30units/mL IL-2 (Peprotec, Rocky Hill, NJ) was provided to ensure that T lymphocyte death was an active process. At predetermined time points, cells were collected, counted per milliliter, and flow cytometry was performed to determine the percent of T lymphocytes surviving under each condition.

8.2.9 In vivo migration assays

Leukocytes were collected from the spleens of ICAM-1 deficient or wild type (WT) *M. tuberculosis* infected mice. CD4+ CD25+ T lymphocytes were separated using the MACS CD4+ CD25+ Regulatory T cell Isolation kit for mouse (Miltenyi Biotec, Auburn, CA) according to the

manufacturer's protocol. After isolation, T regulatory cells were counted and fluorescently labeled using PKH26 (red, ICAM-1 deficient) or PKH67 (green, WT) according to the manufacturer's instructions. Labeled T regulatory cells were counted and combined in a 1:1 ratio. *M. tuberculosis* infected recipient ICAM-1 deficient or WT mice were intravenously (IV) injected with labeled T regulatory cells and rested overnight. 18 hours following injection, lung perfusion and bronchioalveolar lavage were performed on each mouse to ensure that cells detected in the lungs were located in the tissue, and not in the blood or alveolar spaces. Tissue from the lung, liver, lymph nodes and spleen was used for immunofluorescent detection of migrating cells and flow cytometry.

8.2.10 In vivo survival assay

Naïve Thy1.2+ ICAM-1 deficient and WT Thy1.2+ T regulatory cells were collected as described for the *in vivo* migration assay. Enriched ICAM-1 deficient or WT T regulatory cells were counted and IV injected unlabeled into naïve WT Thy1.1 recipient mice. After 1-2 weeks of resting, recipient mice were sacrificed, and tissue from the lungs and spleens were used for flow cytometry and immunofluorescent detection of surviving T regulatory cells.

8.2.11 Cell lysates for immunoblots

Expression of TGFβR1, phosphorylated Smad3 and β-actin loading control were detected by immunoblotting. Whole lung homogenates of spleen, lung, thymus or liver were made in sterile phosphate buffered saline (Sigma-Aldrich, St. Louis, MO), red blood cells were lysed using a hypotonic solution of ammonium chloride and Tris, and the remaining homogenate was washed

thoroughly. Cells recovered from the organs were counted using trypan blue exclusion, and lysed in cell lysis buffer (20mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40) for 10 minutes on ice in the presence of phosphatase and protease inhibitors: leupeptin, aprotinin, pepstatin A, AEBSF, beta-GLP and orthvan (kind gifts from the laboratory of Dr. Larry Kane, PhD). Cell lysates were centrifuged at 13,000 rpm for 10 minutes, and the supernatant was removed. Sample running buffer (Tris-Cl, SDS, Glycerol, DTT, Bromophenol blue) was added to each sample, and the mixture was boiled for 5 minutes prior to loading on the gel.

8.2.12 Immunoblotting

Cell lysates were loaded on a 10% acrylamide mini-gel, using a Bio-Rad Precision Protein Dual Color Standard (Bio-Rad, Hercules, CA) as a protein standard. After running, proteins were wet transferred onto a 0.45μm PVDF membrane (Pall Life Sciences, East Hills, NY) in the presence a Tris-Glycine-Methanol buffer. Following transfer, membranes were blocked using a 4% bovine serum albumin solution. Primary antibody against phosphorylated Smad3 or TGFβR1 protein (Cell Signaling Technology, Boston, MA) was diluted in 0.5% bovine serum albumin in TBS/Tween20 and incubated at 4ºC overnight. After washing the immunoblot, secondary antibody conjugated to HRP was diluted and incubated at room temperature for at least 1 hour. Bands were visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL) according to the manufacturer instructions and images were captured using a Kodak Image station. β-actin (antibody a kind gift from Dr. Larry Kane, PhD) was detected by incubating immunoblots with primary anti- β-actin at room temperature for one hour, followed by washing and a secondary for one hour, and detection with the SuperSignal West

Pico Chemiluminescent Substrate. Quantification of detected proteins was completed on the Kodak Image station.

8.2.13 Statistical Analysis

Two groups were compared by a student's T test, while three or more groups were analyzed by an ANOVA statistical analysis. A value of $p<0.05$ was considered significant. Experiments were repeated to demonstrate reproducibility.

8.3 RESULTS

8.3.1 Fewer Foxp3+ T regulatory cells are in the periphery of ICAM-1-/- mice

A review suggested that fewer T regulatory cells could be found in the absence of ICAM-1 expression [279], but the supporting data were not published. If T regulatory cells are less abundant in the absence of ICAM-1, this molecule must play a role in their production or maintenance. To explore whether ICAM-1 is required for production of T regulatory cells, the presence of Foxp3+CD4+ T regulatory cells was assessed by flow cytometry in the periphery of C57BL/6 ICAM-1-/- and wild type (WT) control mice (Figure 21A). Significantly fewer Foxp3+ T regulatory cells were detected in the lungs and spleens of ICAM-1-/- mice compared to WT controls. On the other hand, in the thymi of ICAM-1-/- and WT mice, Foxp3 expression was similar, suggesting that ICAM-1 is not required for thymic production of T regulatory cells.

ICAM-1 might play several roles in localizing T regulatory cells in peripheral organs. First, since both ICAM-1 and its lymphocyte ligand, LFA-1, can produce internal signals that cause proliferation, apoptosis or costimulation [274-278], ICAM-1 ligation may be a required survival signal in the T regulatory cell. Next, ICAM-1 might be required for firm adhesion that allows migration of T regulatory cells into the peripheral organs. Finally, although natural T regulatory cells are produced equivalently in the thymi of ICAM-1-/- and control mice, ICAM-1 might be required to produce inducible T regulatory cells in the periphery.

8.3.2 ICAM-1 is not required for the survival of T regulatory cells

To test the hypothesis that ICAM-1 is required to send survival signals to regulatory T cells, flow cytometry staining of activated caspase 3 was used as a marker for apoptosis. Apoptosis of Foxp3+ T regulatory cells in the thymi of ICAM-1-/- and WT mice was similar (Figure 21B), suggesting that ICAM-1 is not required for natural T regulatory cells to survive within the thymus. Foxp3+ T regulatory cells in the lungs and in the spleens of ICAM-1-/- and WT mice showed similar percentages of activated caspase 3+ cells within the Foxp3 population (Figure 21B), suggesting that Foxp3+ T regulatory cells do not require ICAM-1 ligation to survive.

To confirm that ICAM-1 is not required for survival of T regulatory cells in the periphery, Thy1.2+CD4+CD25+ T regulatory cells were enriched from ICAM-1-/- and WT controls. $5x10^6$ T regulatory cells were adoptively transferred into WT Thy1.1+ recipients. Two weeks following transfer, lungs and spleens of Thy1.1 recipients were harvested, and the survival of injected ICAM-1-/- or WT T regulatory cells was assessed. Similar levels of Thy1.2+ donor T regulatory cells were detected in the lungs and spleens of WT recipients (Figure 21C), confirming that ICAM-1 is not required on T regulatory cells for their survival in the periphery.

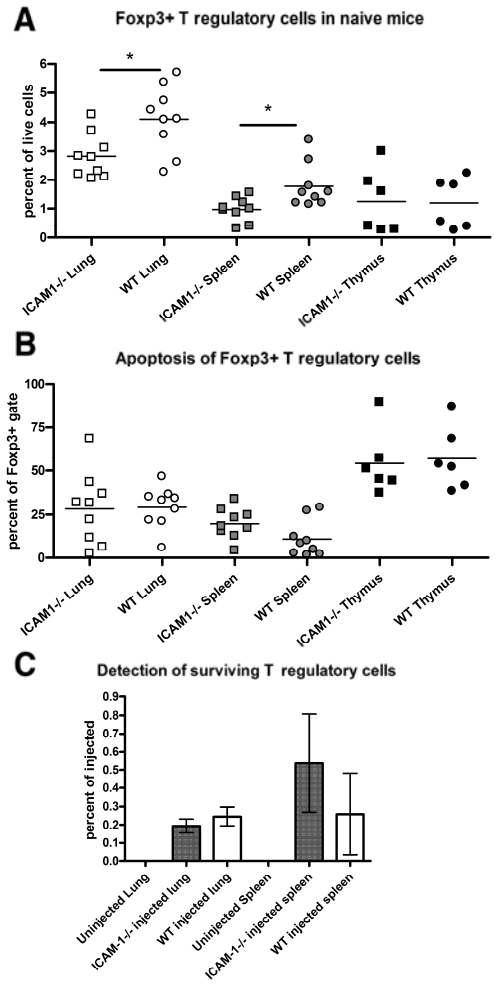

Figure 21 Foxp3 expression is significantly reduced in ICAM-1-/- mice

(A) Expression of Foxp3 was determined in naïve mice reported as a percentage of CD4+ T cells. *$p<0.05$

(B) The percentage of T regulatory cell (Foxp3 gate) undergoing apoptosis (active caspase 3).

(C) The percentage of injected T regulatory cells (Thy1.2+) in the recipient (Thy1.1+) organs.

8.3.3 ICAM-1 is not required for the migration of T regulatory cells in the periphery

T regulatory cells could be dependent on ICAM-1 for migration in the periphery. To test this hypothesis, we used an antigen specific system to follow the migration of T regulatory cells into

the tissues during an inflammatory response. ICAM-1-/- or WT mice were infected with *Mycobacterium tuberculosis* for at least 4 weeks. CD4+CD25+ T regulatory cells from infected ICAM-1-/- or WT spleens were enriched using MACS columns. In ICAM-1-/- mice and WT mice, ~1.8% and ~99.9% of CD25+ splenic T cells were Foxp3+, respectively. As depicted in Figure 22, ICAM-1-/- CD4+CD25+ T cells were labeled red (PKH26), and WT CD4+CD25+ T cells were labeled green (PKH67). Equal numbers of ICAM-1-/- and WT T regulatory cells were combined and injected into *M. tuberculosis*-infected ICAM-1-/- or WT recipient mice. Donor CD4+CD25+ T cells were allowed to migrate for 18 hours, at which point recipient organs were harvested to assess the migration of ICAM-1-/- and WT T regulatory cells. Figure 22A shows representative dot plots of flow cytometry detection of labeled T regulatory cells in recipient lungs. WT T regulatory cells were equally capable of migrating into the lungs (Figure 22B) and spleens (data not shown) of ICAM-1-/- and WT recipient mice, suggesting that ICAM-1 expression is not required on the endothelium in order for T regulatory cells to migrate into tissues. Similar percentages of ICAM-1-/- and WT T regulatory cells were detected in the lungs (Figure 22C) and spleens (data not shown) of WT mice, suggesting that ICAM-1 is not required on the T regulatory cell for the migration to tissues from the periphery. Similar experiments performed with total CD4 T cell populations demonstrated that ICAM-1 expression on T cells or endothelium was not required for normal migration of cells into the lungs of infected mice, nor was there an increase in migration into ICAM1-/- lungs of total CD4 T cells (data not shown).

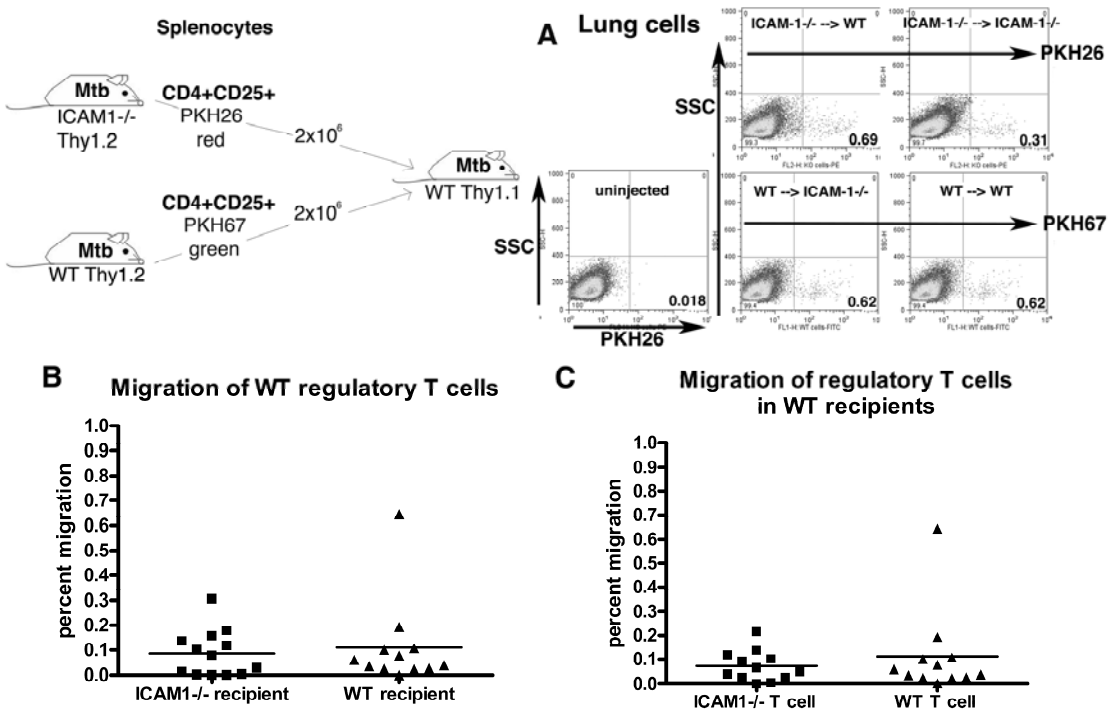

Figure 22 ICAM-1 expression is not required for migration of T regulatory cells in the periphery

The *in vivo* migration of T regulatory cells from *M. tuberculosis* infected mice stained red (ICAM-1-/-) or green (WT) in *M. tuberculosis* infected recipients.

(A) Representative dot plots: detection of ICAM1-/-1 (PKH26, red) or WT (PKH67, green) transferred cells in the lungs of recipient mice by flow cytometry.

(B) To determine whether migration requires ICAM-1 on the endothelium, the percentages of injected WT T regulatory cells detected in lungs of ICAM-1-/- (squares) or WT (triangles) recipients (cells detected in organs/injected cells*100) were compared. No significant differences were observed.

(C) To determine whether migration requires ICAM-1 on the T regulatory cells, the percentages of transferred WT (triangles) or ICAM-1-/- (squares) T regulatory cells detected in the lungs of WT mice were compared (cells detected in organs/injected cells*100). No significant differences were found.

8.3.4 Induction of regulatory T cells by TGFβ1 partially requires ICAM-1 expression

Foxp3 expression that results in functional T regulatory cells can be induced in peripheral CD4+ T cells by TGFβ1 stimulation *in vitro* [102, 104, 252] and *in vivo* [101, 103]. To determine whether ICAM-1 is required to induce Foxp3 expression, CD4+CD25- (non-T regulatory) cells were enriched from naïve ICAM-1-/- or WT spleens using MACS columns. CD4+CD25- T cells were incubated for up to 72 hours with recombinant active TGFβ1, IL-2, anti-CD3 and anti-CD28 antibodies as previously described by others as a method of inducing T regulatory cells [102, 104, 105, 157]. CD25 expression was upregulated on 100% of ICAM-1-/- and WT T cells by 48 hours of incubation and maintained through 72 hours (data not shown). After 72 hours, Foxp3 expression was detected in significantly fewer ICAM-1-/- CD4+ T cells compared to WT (Figure 23A), suggesting a functional difference between ICAM-1-/- and WT CD4+ T cells in their ability to respond to TGFβ1. Addition of soluble ICAM-1-Fc recovered Foxp3 induction, supporting a role for ICAM-1 in Foxp3 induction (Figure 23A).

Active TGFβ1 interacts with TGFβRII, which phosphorylates TGFβRI. In turn, TGFβR1 recruits and phosphorylates Smads [280, 281] that translocate to the nucleus to act as transcription factors [282]. To assess whether TGFβ1 signaling was equivalent in ICAM-1-/- and WT CD4+ T cells, phosphorylation and translocation of Smad2 and Smad3 was compared by immunoblotting. Protein was collected from the cytoplasmic and nuclear fractions of T cells in the *in vitro* culture system described above, with representative blots shown in Figure 23B. Early expression of phosphorylated Smad2 during TGFβ1 stimulation was similar in ICAM-1-/- and WT T cells (data not shown). ICAM-1-/- and WT T cells also had similar levels of total Smad3 protein prior to treatment with TGFβ1 (data not shown), and similar levels of phosphorylated Smad3 were detected in the cytoplasm of ICAM-1-/- and WT CD4+ T cells during in vitro

culture (Figure 23C). However, significantly more phosphorylated Smad3 was detected in the nucleus of WT CD4+ T cells compared to ICAM-1-/- CD4+ T cells (Figure 23D). Greater overall phosphorylated Smad3 (determined by adding relative nuclear and cytoplasmic expression in immunoblots, and by flow cytometry) was present in WT compared to ICAM-1-/- CD4+ T cells (Figure 23E). Addition of soluble ICAM-1-Fc to ICAM-1-/- CD4+ T cells resulted in a significant increase in phosphorylated Smad3 (Figure 23E), which correlated with recovery of WT levels of Foxp3 expression. In WT CD4+ T cells, the relative ratio of phosphorylated Smad3 in the nucleus compared to the cytoplasm was higher than in ICAM-1-/- CD4+ T cells (Figure 23F), supporting that there is a defect in translocation of phosphorylated Smad3 into the nucleus of ICAM-1-/- T cells.

8.3.5 ICAM-1-/- T regulatory cell deficit results in more severe inflammation

We hypothesized that during an inflammatory response there would be a more pronounced difference due to the ICAM-1-/- defect in induction of Foxp3. To test this, ICAM-1-/- and WT mice were infected with of *M. tuberculosis* (either ~25 or ~100 colony forming units). After seven weeks of infection, the total number of Foxp3+ T regulatory cells increased in both WT and ICAM-1-/- mice, but there were significantly fewer T regulatory cells in the lungs of ICAM-1-/- mice than in WT lungs (Figure 24A). Foxp3+ T regulatory cells in the lungs of WT mice represented a significantly greater percentage of total CD4+ T cells than in ICAM-1-/- mice (data not shown).

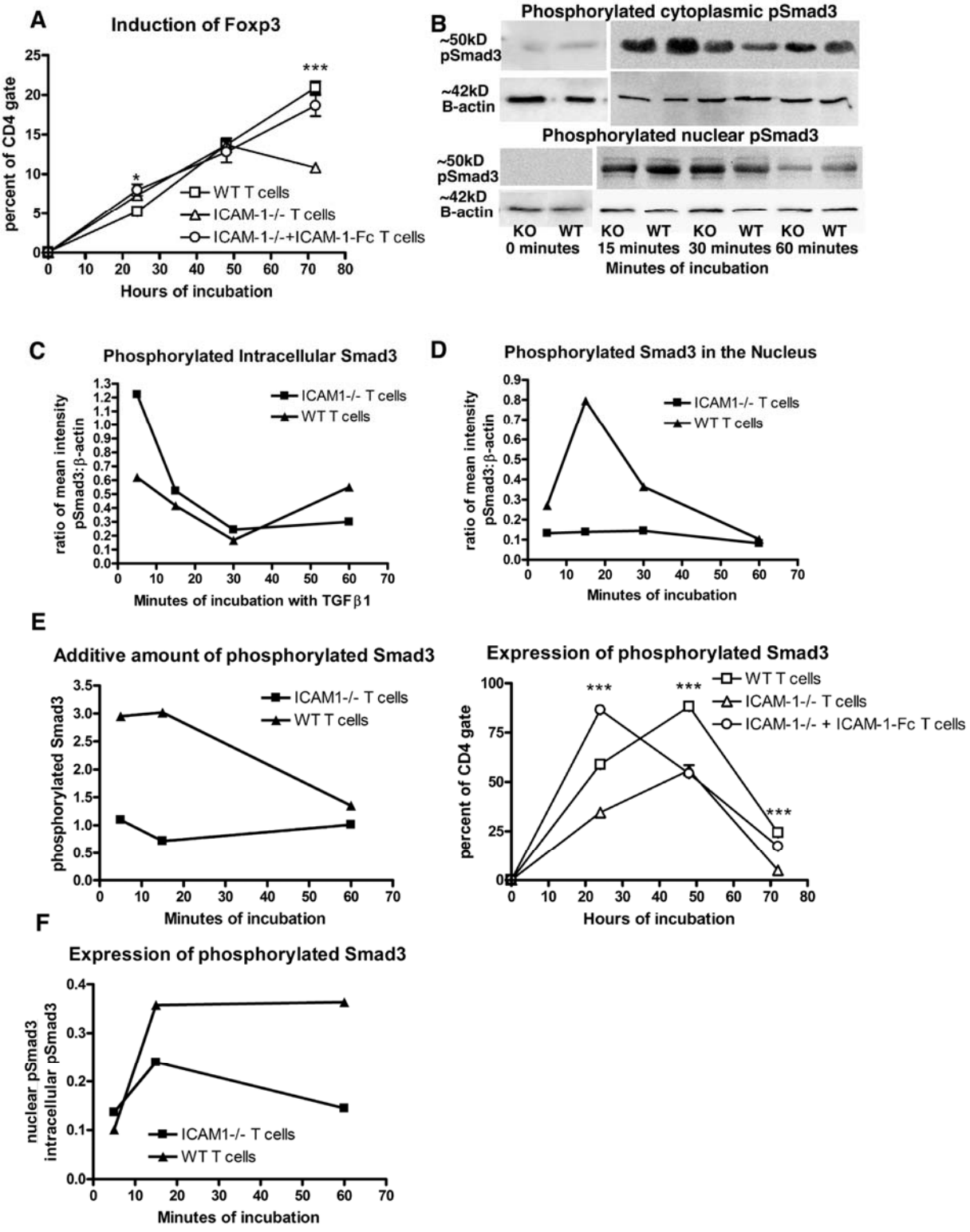

Figure 23 Aberrant TGFβ1 signaling is responsible for reduced Foxp3 induction in ICAM-1-/- T cells

Naïve CD4+CD25- spleen cells from WT or ICAM1-/- mice were incubated in vitro with anti-CD3, IL-2 and TGF-β1 to generate inducible T regulatory cells, identified as Foxp3+.

(A) Flow cytometry was used to detect expression of Foxp3 in naïve CD4+CD25- WT (□), ICAM-1-/- (Δ) or ICAM-1-/- + soluble ICAM-1-Fc (○) T cells up to 72 hours of culture. ***p<0.0001

(B) Representative immunoblot detection of phosphorylated Smad3 in cytoplasmic and nuclear T cell compartments.

(C, D) Quantified immunoblot detection of phosphorylated Smad3 relative to β-actin in proteins isolated from the cytoplasm (C) or nucleus (D). ICAM-1-/- (■), WT (▲).

(E) Additive expression of phosphorylated Smad3 from immunoblots (left) and total CD4+ T cells expressing phosphorylated Smad3 detected by flow cytometry. WT (■; ○), ICAM-1-/- (▲, Δ), ICAM-1-/- +ICAM-1-Fc (○)

(F) Relative ratio of phosphorylated Smad3 detected in the nucleus:cytoplasm. ICAM-1-/- (■), WT (▲).

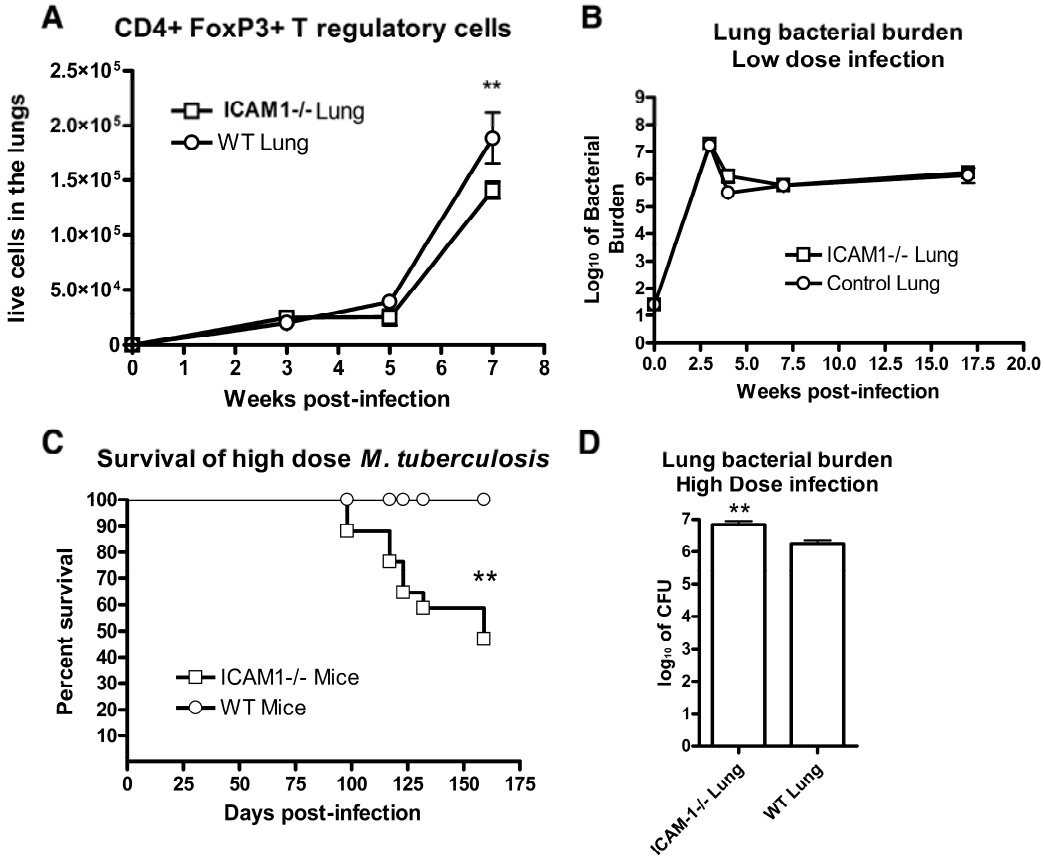

Figure 24 Reduced levels of T regulatory cells are found in the lungs in M. tuberculosis-infected ICAM1-/- mice, and at higher inoculum, survival of the ICAM-1-/- mice is impaired

(A) Total Foxp3+ T regulatory cells in ICAM-1-/- (□) or WT (▲) lungs determined by multiplying the total number of cells in the lungs by the fraction of live cells that were CD4+ and Foxp3+ by flow cytometry **p<0.001 (representative of 8 separate experiments)

(B) Bacterial numbers in the lungs of ICAM1-/- (square) and WT (triangle) mice infected with a low dose of M. tuberculosis (~25 CFU) are not substantially different over the course of infection. Experiment was repeated 8 times.

(C) Survival of ICAM-1-/- (squares) and WT (circles) infected with higher dose (~100 CFU/mouse). p<0.001. Experiment was repeated twice.

(D). Bacterial numbers were slightly higher in ICAM1-/- lungs compared to WT lungs at 18 weeks post infection when mice were infected with ~100 CFU/lungs (p<.001).

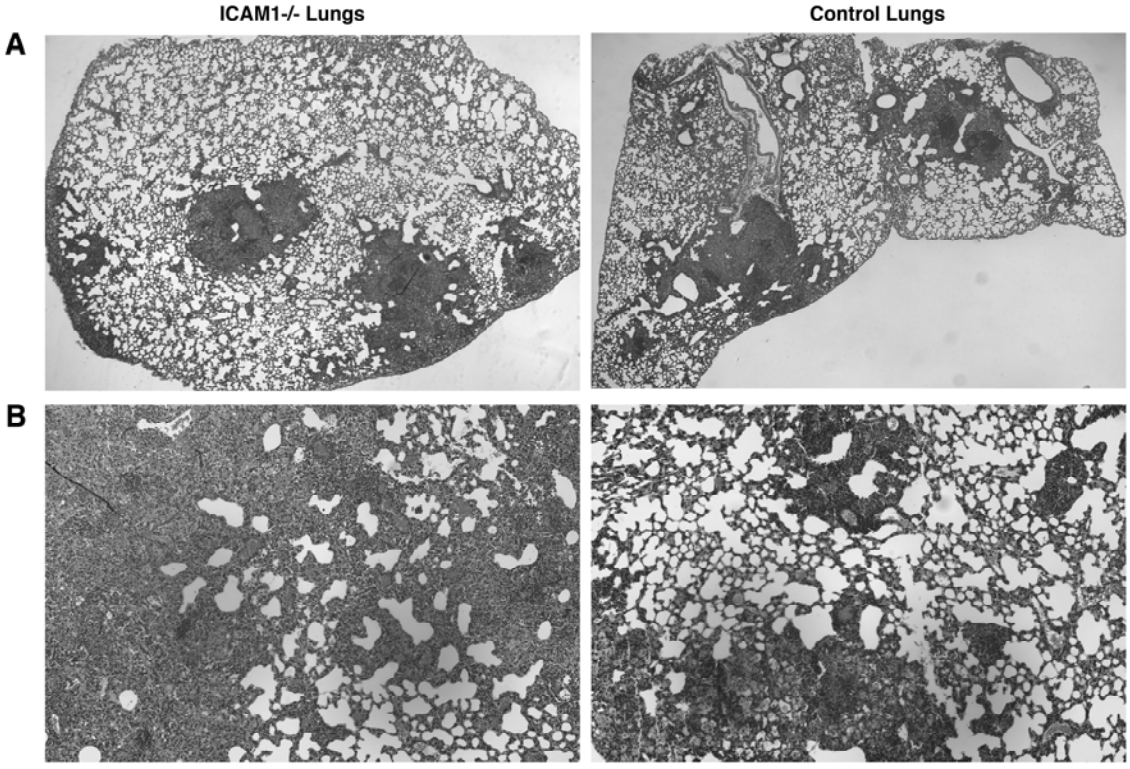

Figure 25 ICAM-1 is not required for granuloma formation, but in absence of ICAM-1, inflammation is increased in M. tuberculosis-infected lungs

Representative sections of H&E stained ICAM-1-/- (left) or WT (right) lungs.

(A) 4x magnification of lung sections from mice infected 4 weeks with *M. tuberculosis*

(B) 10x magnification of lung sections from mice infected 16 weeks with *M. tuberculosis*

Recent studies suggest that in the absence of T regulatory cells, the cytokine response to *M. tuberculosis* is enhanced [105, 107-109, 156] and bacterial control is improved [108, 109]. There was no difference in survival of ICAM-1-/- and WT mice infected with ~25 CFU of *M. tuberculosis* (both strains survived more than a year post-infection), and bacterial numbers in the lungs were similar over the course of infection (Figure 24B). Histological examination of the lungs of ICAM-1-/- and WT mice infected with *M. tuberculosis* revealed that organized granulomas were detectable in the lungs by 4 weeks post-infection (Figure 25A). However, after 16 weeks of infection, lung infiltration appeared much higher by histological observation in ICAM-1-/- animals than controls (Figure 25B).

In light of previous publications [185, 186] in which ICAM-1-/- mice were more susceptible to *M. tuberculosis* infection, we infected ICAM-1-/- and WT mice with a higher dose of infection (~100 CFU), and followed survival. ICAM-1-/- mice began to succumb to infection by 14 weeks post-infection (Figure 24C). Further investigation revealed that while bacterial burden was slightly higher in ICAM-1-/- mice than WT mice (~5 fold in the lungs) (Figure 24D), it was not at a level that is usually fatal. Histological examination revealed higher inflammation and pathology in the lungs of ICAM-1-/- mice, but that granulomas were still intact (Figure 26). The lack of effector T cell regulation in lungs of ICAM-1-/- mice likely contributed to overwhelming inflammation and increased morbidity in the absence of ICAM-1.

Flow cytometry analysis confirmed that significantly higher CD4+ and CD8+ T lymphocytes were present in the lungs of ICAM-1-/- mice infected with both low dose or high dose beginning ~7 weeks post-infection (Figure 27A, B). Interestingly, levels of CD4+Foxp3- T cells tended to be higher in naïve ICAM-1-/- lungs and spleen compared to WT (data not shown). Overall, in the ICAM-1-/- mice in organs where fewer T regulatory cells were present,

more non-regulatory CD4+ T cells could be detected, both in naïve mice, and during *M. tuberculosis* infection, suggesting that Foxp3+ T regulatory cells are required to maintain T cell homeostasis.

The increased numbers of T cells in the lungs of ICAM-1-/- mice accordingly led to higher IFN-γ production 7 weeks post-infection in an ELISpot assay (data not shown). Flow cytometry confirmed that the percentage of CD4+ and CD8+ T cells producing IFN-γ were similar, but the numbers of these cells in ICAM-1-/- lungs were increased (data not shown). At all time points, lung infiltrating macrophages, neutrophils and dendritic cells were equivalent between ICAM-1-/- and control mice (data not shown). No differences in any cell populations were detected in the lung draining lymph nodes (data not shown), suggesting that similar numbers of T lymphocytes were produced in the secondary lymphoid organs.

8.3.6 ICAM-1 is required for T lymphocyte apoptosis in the lungs of M. tuberculosis infected mice

As similar T cell populations were detected in the lymph nodes of ICAM-1-/- and control mice (data not shown), differences in priming the total CD4+ and CD8+ lymphocyte population is unlikely. Increased effector T lymphocytes detected in ICAM-1-/- lungs could be due to greater proliferation or decreased apoptosis. Expression of the nuclear antigen Ki-67 was used to compare proliferation of T lymphocytes in the lungs of *M. tuberculosis* infected mice by flow cytometry; there was no difference in proliferation of ICAM-1-/- and WT lung T cells (data not shown). A significant decrease in ICAM-1-/- T lymphocyte apoptosis was consistently detected 7 weeks post-infection ICAM-1-/- lungs (Figure 27D), detected by Annexin V staining and

confirmed by activated caspase 3 staining (data not shown). These data suggest that optimal contraction of the effector T lymphocyte population in the lungs during *M. tuberculosis* infection requires ICAM-1 expression.

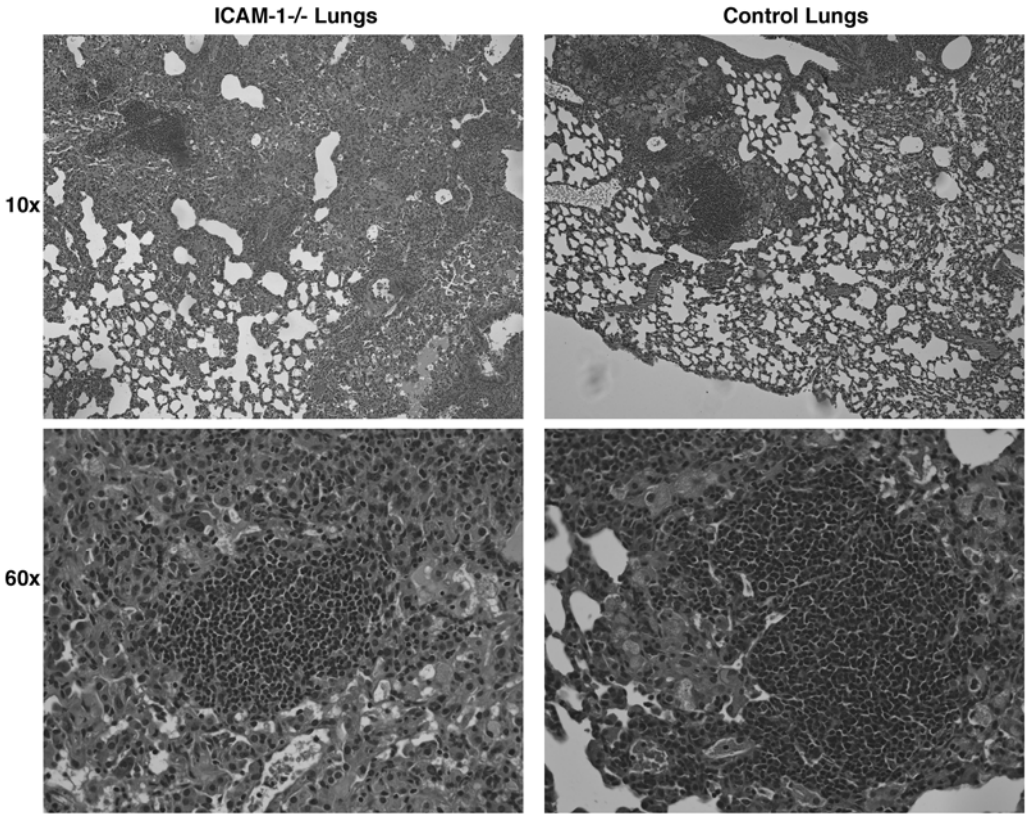

Figure 26 Increased pathology, but present granulomas, in ICAM-1-/- mice that succumb to high dose *M. tuberculosis* infection

Representative lung tissue sections from ICAM-1-/- mice and WT mice infected with high dose (~100 CFU) *M. tuberculosis* was stored when ICAM-1-/- mice were succumbing to infection. Sections were stained with hemotoxylin and eosin. 10x and 60x magnification shows that granuloma structure was similar in ICAM-1-/- and WT mice.

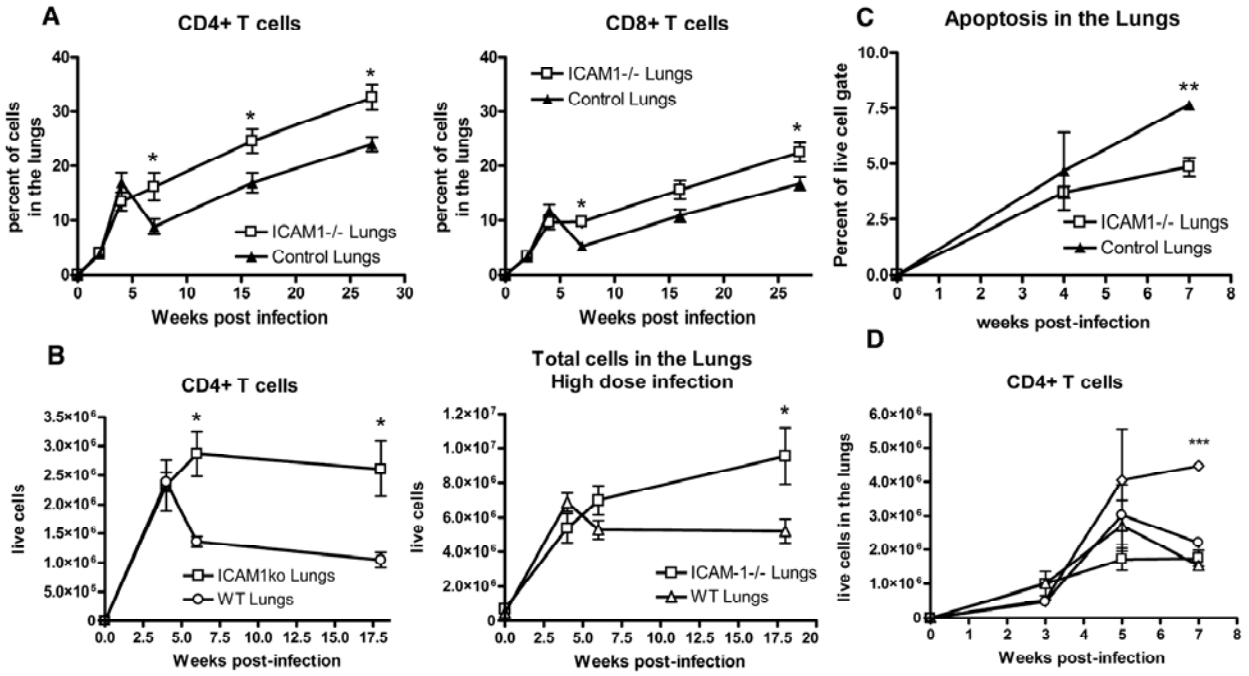

Figure 27 ICAM-1 deficient mice infected with M tuberculosis have increased functional effector T cells in the lungs despite a similar bacterial burden to WT

(A) ICAM-1-/- (□) or WT (▲) CD4+ and CD8+ T lymphocytes in the lungs by flow cytometry during low dose *M. tuberculosis* infection. *p<0.05. Representative of 8 experiments.

(B) Total cells and CD4+ T cells in the lungs increase in response to high dose M. tuberculosis infection *p<0.05.

(C) Apoptosis of ICAM-1-/- (□) or WT (▲) T lymphocytes by Annexin V staining and detection by flow cytometry. **p<0.001

(D) Mice received $8x10^6$ adoptively transferred naïve splenic WT CD4+CD25+ cells. CD4+ T lymphocytes in the lungs of ICAM-1-/- (◊), WT (○), adoptive transfer (AT) recipient ICAM-1-/- (□) and recipient WT (○) mice following *M. tuberculosis* infection. Transfer of T regulatory cells corrected the increased inflammation in ICAM1-/- lungs.

8.3.7 Adoptive transfer of naïve CD4+ CD25+ T regulatory cells prior to *M. tuberculosis* infection decreases total effector T cells in the lungs over time

In naïve and *M. tuberculosis*-infected ICAM-1-/- mice, fewer T regulatory cells are present than in WT mice (Figure 21). The total number of T lymphocytes in naïve spleen and *M. tuberculosis* infected lung was also higher in ICAM-1-/- mice compared to WT mice (data not shown). To determine whether these phenotypes are related, naïve CD4+CD25+ T cells were enriched from WT spleens (99% were also Foxp3+) and adoptively transferred into ICAM-1-/- or WT mice one day prior to *M. tuberculosis* infection. We hypothesized supplemental WT CD4+CD25+ T cells would decrease the ICAM-1-/- adaptive response to *M. tuberculosis* infection. At 7 weeks post-infection, the total number of CD4+ (Figure 27D) and CD8+ (data not shown) T cells in lungs of ICAM1-/- mice that received WT CD4+CD25+ cells was reduced significantly compared to non-recipient ICAM-1-/- mice. These data support that WT regulatory T cells complement the defect in ICAM-1-/- lungs, and restore normal T cell and inflammatory responses to *M. tuberculosis*.

8.4 DISCUSSION

The data presented here demonstrate that ICAM-1 is required for the induction of Foxp3+ T regulatory cells in the periphery, which impacts T cell homeostasis. The reduced responsiveness to TGFβ1 signaling in ICAM-1 deficient CD4+ T cells resulted in impaired Foxp3 expression, and a subsequent reduction in T regulatory cells in the tissues of ICAM-1-/- mice. Reduction of inducible T regulatory cells was more pronounced during an inflammatory response to *Mycobacterium tuberculosis* infection, and was the cause of increased effector T lymphocytes in infected lungs, as addition of regulatory T cells to the ICAM1-/- mice restored normal numbers of T cells in the lungs. Rather than controlling proliferation, T regulatory cells may play a role in contraction of T lymphocytes, as apoptosis was reduced during the contraction phase of the immune response. Contrary to previous reports [185, 186], in the absence of ICAM-1, granulomas formed in response to low dose *M. tuberculosis* infection, allowing mice to control bacterial burden and survive equivalently to WT mice. However in mice infected with higher doses of *M. tuberculosis*, lung inflammation and pathology increased substantially late in infection, contributing to the decreased survival of ICAM1-/- mice. The data presented here support that the reduction in regulatory T cells in ICAM1-/- mice contributed to increased inflammation and pathology, and a worsened outcome in M. tuberculosis infected mice. This suggests a previously unappreciated role for ICAM-1 in regulation of immune responses.

Although there was a significant reduction in T regulatory cells in peripheral organs in naïve ICAM-1-/- mice, similar expression of Foxp3 in the thymus of ICAM-1-/- and WT mice was observed, suggesting that thymic production of natural T regulatory cells does not require

ICAM-1. Fewer Foxp3+ T regulatory cells in the tissues of naïve ICAM-1-/- mice and in lungs of ICAM-1-/- *M. tuberculosis* infected mice support a requirement for ICAM-1 for peripheral T regulatory cell induction. Survival or migration of T regulatory cells, or migration of all T cells, were not dependent on ICAM-1.. ICAM-1-/- CD4+CD25- splenocytes cultured *in vitro* had significantly reduced induction of Foxp3 in response to TGFβ1 compared to WT cells, suggesting that post-thymic induction of T regulatory cells is impaired in the absence of ICAM-1.

During *Mycobacterium tuberculosis* infection in WT mice, Foxp3 expression increased up to 7 weeks post-infection (Figure 24A). Foxp3 expression in ICAM-1-/- CD4+ T cells remained much lower *in vitro* and *in vivo*, which suggests that the same mechanism could be responsible for this deviation from WT responses. The expression of activated TGFβ1 was very similar in the lungs and lymph nodes of ICAM-1-/- and WT mice throughout infection (data not shown), suggesting that it is the induction of Foxp3 that is defective *in vivo*, and not availability of active TGFβ1. While the phenotype of natural and inducible T regulatory cells *in vivo* is indistinguishable (CD4+CD25+Foxp3+), it seems more likely that inducible T regulatory cells are produced during the inflammatory response, rather than increased production of natural T regulatory cells. T regulatory cells may also be proliferating *in vivo* during *M. tuberculosis* infection, but Ki-67 staining of CD3+ T from lungs of ICAM-1-/- and WT mice was similar, supporting that *de novo* expression of Foxp3 in peripheral CD4+ T cells is responsible for increased T regulatory cell expression. Transferring additional T regulatory cells to ICAM1-/- mice reduced the inflammation in the lungs during *M. tuberculosis* infection to wild type levels, strongly implicating the reduction in T regulatory cells in the ICAM1-/- mice as the responsible factor for increased numbers of lymphocytes in the lungs of these mice.

We have observed that during low dose *M. tuberculosis* infection in ICAM-1-/- mice, granuloma formation occurs with similar kinetics seen in WT mice. Bacterial burden was maintained at a similar level to WT, and ICAM-1-/- mice are able to survive low dose *M. tuberculosis* infection equivalently. However, ICAM1-/- mice succumb to higher dose infection at ~14 weeks, most likely due to increased pathology in the lungs. Although LFA-1 deficiency resulted in insufficient granuloma formation due to decreased T lymphocyte priming [227], ICAM-1-/- antigen presenting cells clearly primed effector cells efficiently, and mounted a sufficient immune response. The findings reported here show that ICAM-1 expression is not required to control *M. tuberculosis* infection, although it is involved in control of inflammation.

TGFβ1 signaling requires transport of phosphorylated Smads into the nucleus, where transcription responses rely on prolonged TGFβ-receptor signaling [283, 284]. TGFβ1 induces Foxp3 expression requisite for CD4+CD25+ T regulatory cells [104]. In the absence of ICAM-1 expression, induction of Foxp3 through TGFβ receptor signaling was reduced. A defect in the TGFβ receptor signaling machinery caused reduced translocation of phosphorylated Smad3, but not phosphorylated Smad2 from the cytoplasm into the nucleus. These results complement findings in Cbl-b deficient mice, in which Cbl-b was required for phosphorylation of Smad2, but not phosphorylated Smad3 [285]. Together the findings reported here and previously suggest that regulation of TGFβ1 signaling is mediated on several levels by molecules as diverse as ubiquitin ligases and adhesion molecules.

The relationship between ICAM-1 expression and TGFβ receptor signaling is not intuitive. The observed differences between ICAM-1-/- and WT CD4+ T cell induction of Foxp3 could be due to a requirement for signaling between ICAM-1 and LFA-1 during TGFβ1 stimulation. We were unable to detect ICAM-1 expression on the vast majority of WT T

regulatory cells (data not shown), but ICAM-1 shedding and/or secretion has been well documented [286-289]. Addition of soluble ICAM-1 to the *in vitro* cultures restored the ability of ICAM-1-/- cells to express Foxp3 (Figure 23A). Soluble ICAM-1 does not lose its ability to bind LFA-1 [286], and, if present, could transmit a signal through LFA-1 on CD4+ T cells that assists in TGFβ1 signaling. LFA-1 ligation activates actin polymerization [290], myosin motor activity [291] and rearrangement of the microtubule network [292, 293]. The microtubule network is responsible for controlling Smad localization in the cytoplasm, such that destabilization of microtubles allows the release of Smads into the nucleus [294]. The role of LFA-1 in rearranging the microtubule network might therefore allow phosphorylated Smads to be released from the cytoplasm into the nucleus. Loss of ICAM-1/LFA-1 interaction could inhibit cytoskeletal rearrangements required for translocation of nucleus-bound transcription factors such as phosphorylated Smad3 from the cytoplasm. Alternatively, ICAM-1 may play a role in translocation of phosphorylated Smad3 through influencing its association with the common mediator Smad, Smad4, or by inhibiting Smad3 interactions with the inhibitory Smads that work on several levels to prevent signaling (reviewed in [295].

Further studies are required to investigate the role of ICAM-1/LFA-1 signaling in microtubule reorganization that may allow translocation of transcription factors into the nucleus. It is of interest to determine whether it is only TGFβ receptor signaling that is affected by the absence of ICAM-1, or whether there are additional signaling pathways that are negatively affected by ICAM-1 deficiency. Finally, TGFβ1 signaling may be impaired in somatic cells other than T lymphocytes, and this has not yet been investigated.

9.0 SUMMARY

Mycobacterium tuberculosis bacilli are inhaled into the lung, where they are thought to be engulfed by resident macrophages [30, 31]. Infected macrophages produce proinflammatory cytokines and chemokines that recruit additional leukocytes to the site of infection [33]. Dendritic cells infected with *M. tuberculosis* migrate to the lung draining lymph nodes to prime *M. tuberculosis* specific T lymphocytes which migrate to the site of infection [32, 35, 296]. T cells, macrophages and other leukocytes aggregate within the lungs to form the hallmark of *M. tuberculosis,* the granuloma. Priming of a CD4+ T lymphocyte response is required for control of *M. tuberculosis* infection as has been shown in mice lacking CD4+ T cells [84]. Formation of a functional granuloma is also required for survival, as has been shown in mice deficient in TNF or treated with anti-TNF antibody [33, 119-121, 123], in the absence of which granulomas are not formed, and mice succumb to infection. The overall goal of this thesis research was to identify factors important in recruitment of cells to the lungs and formation of a granuloma in response to *M. tuberculosis* infection.

TNF neutralization has been effective in treating chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease in humans, but as in mice, anti-TNF treatment in humans increases the risk of tuberculosis [122, 130, 297]. Reports in the literature suggested that treatment with two different TNF-neutralization reagents, anti-TNF antibody (infliximab) and soluble TNF receptor fusion molecule (etanercept), may differentially increase

the risk of tuberculosis [122, 130, 297], but this is difficult to study in humans. In Chapter 1, we used murine models of infliximab [anti-TNF antibody (MP6-XT22)] and etanercept [soluble receptor fusion molecule (mTNFR2-Fc)] to directly compare their ability to neutralize systemic TNF, prevent granuloma formation during acute *M. tuberculosis* infection and disturb previously formed granulomas when administered during chronic infection. We report that anti-TNF antibody and soluble receptor fusion molecule equivalently neutralize TNF in the periphery, and that mice acutely infected with *M. tuberculosis* failed to form granulomas when treated with either molecule, which resulted in mortality with either treatment. However, mice treated with soluble receptor fusion molecule maintained previously formed *M. tuberculosis* granulomas during chronic infection, while anti-TNF antibody disrupted chronic granulomas, resulting in rapid mortality. Further investigation suggested that significantly less soluble receptor fusion molecule than anti-TNF antibody could be detected within established granulomas, suggesting that less granuloma TNF could be neutralized by TNF receptor fusion molecule than anti-TNF antibody during chronic infection. Overall, the work presented in Chapter 1 suggests that TNF neutralization using etanercept during established tuberculosis may not cause reactivation as rapidly as neutralization by infliximab, but that during primary tuberculosis, control of infection will be impaired using either treatment. In locations where tuberculosis prevalence is high, TNF neutralization as a treatment for chronic inflammatory disease should be considered to be a major risk factor for development of tuberculosis.

As TNF is required for granuloma formation, determining the critical role of TNF in granuloma formation may provide clues about how the granuloma is formed. In Chapter 2, we test the hypotheses that TNF is required for optimal priming of the T cell response or for induction of adhesion molecules that could play a role in T cell priming, migration of leukocytes,

or in cellular adhesion that allows formation of the granuloma. We compared priming of T lymphocytes and the upregulation of adhesion molecules on all leukocytes in the lungs and lymph node during low dose acute *M. tuberculosis* infection in TNFko, anti-TNF antibody treated and control mice. We report that whether T cells were primed in the presence or absence of TNF, IFN-γ production in response to *M. tuberculosis* antigens was equivalent. The expression of VCAM-1, LFA-1, L-selectin and ICAM-1 adhesion molecules during the initial infection was similar on all leukocytes studied in the presence or absence of TNF activity. We conclude from this work that TNF is not required for T lymphocyte priming or to modify expression of VCAM-1, LFA-1, L-selectin or ICAM-1 during low dose acute infection with *M. tuberculosis*. The role of TNF in granuloma formation and control over acute infection remains unidentified.

To further investigate the formation of the tuberculosis granuloma, and the role of TNF in this process, we addressed the role of chemokines in recruitment of T lymphocytes to the lungs and focus of infection. Induction of macrophage chemokines that signal through such receptors as CCR2, CCR5 and CXCR3 is partially dependant on TNF expression [33], but the absence of these chemokines individually does not replicate the overwhelming pathology and poor survival seen in the absence of TNF during low dose aerosol *M. tuberculosis* infection [161, 162, 164]. Chemokine receptors may compensate for the absence of one another, so that leukocytes are recruited similarly in the presence or absence of a single chemokine receptor, but if multiple receptors were absent simultaneously, leukocyte recruitment might be impaired. It has been shown that during *M. tuberculosis* infection in the absence of either CXCR3 or CCR5 alone, bacterial burden in the lung is maintained and mice survive similarly to controls [161, 162]. In Chapter 3, we investigated whether elimination of CXCR3 and CCR5 chemokine receptors

simultaneously would impede migration of T lymphocytes and survival of acute *M. tuberculosis* infection. We found that CXCR3xCCR5 doubly deficient mice maintained lung bacterial burden similarly to control mice and survived infection. Like CCR5 deficient mice [162], CXCR3xCCR5 doubly deficient mice had higher numbers of recruited lymphocytes, higher lymph node bacterial burden, and an increase in IFN-γ production in the lymph nodes, suggesting that higher bacterial burden leads to greater priming of T lymphocytes in the lymph nodes. The results reported in Chapter 3 confirm those reported in CCR5 deficient mice [162], but did not reveal compensatory mechanisms that require expression of either CCR5 or CXCR3.

In the first three chapters of this work, we report that while TNF expression is required for control of acute *M. tuberculosis* infection, TNF is not required for priming of T lymphocytes or expression of adhesion molecules on leukocytes. We further showed that detection neither CXCR3 nor CCR5 TNF-inducible chemokines is required for formation of the granuloma, even when both receptors are absent. In addition to TNF and TNF-inducible chemokines, other cytokines and chemokines may play a role in T cell priming and recruitment of leukocytes that allows successful granuloma formation. We have investigated the role of LTB4-BLT1 interaction in recruitment of leukocytes to the site of infection (Chapter 4) and osteopontin (Chapter 5) in T lymphocyte priming and cellular recruitment during *M. tuberculosis*. The work presented in Chapters 4 and 5 shows that neither BLT1 nor osteopontin expression were required in mice to control low dose *M. tuberculosis* infection. In this body of work, we have therefore eliminated these molecules from the vast number of those that might be required for tuberculosis granuloma formation.

To continue investigating how a granuloma is formed, we reasoned that although TNF is not required for their expression, adhesion molecules may play a role in the priming of *M.*

tuberculosis specific T lymphocytes, recruitment of leukocytes to the site of infection, or in forming a functional granuloma that prevents bacterial growth and dissemination. In support of this idea, recent work has shown that adhesion molecules such as VCAM-1 and VLA-4 are required for the recruitment of T cells to *M. tuberculosis* infected lungs [184], and LFA-1 is required for priming of *M. tuberculosis* specific T lymphocytes [227]. The immunologic role of ICAM-1, which binds to LFA-1, is less clearly defined in the literature, as the two published studies [185, 186] disagree about whether ICAM-1 is required for survival of infection, and did not investigate the cellular immunology. We hypothesized that ICAM-1 is required for priming *M. tuberculosis* specific T lymphocytes, migration of leukocytes to the site of infection, for adhesion/communication within the granuloma or a combination of these potential roles. In Chapter 6, we report the surprising finding that T lymphocytes in ICAM-1 deficient mice are not only functionally primed, but that they are found in greater abundance in the lungs of *M. tuberculosis* infected mice. Further investigation revealed that ICAM-1 is partially required for the production of inducible T regulatory cells, and that the reduction in T regulatory cells likely resulted in the significantly greater detection of effector T cells in the lungs of ICAM-1 deficient mice. Western blotting and flow cytometry data suggest that the transcription factor phosphorylated Smad3 that is activated by the TGFβ1 signaling pathway requires ICAM-1 for translocation into the nucleus, which results in reduced transcription of Foxp3. The data presented in Chapter 6 suggest novel role for ICAM-1 in TGFβ signaling that allows post-thymic T regulatory cell induction, and suggests many new lines of ICAM-1 research. It is also significant that when T regulatory cells are reduced during higher dose *M. tuberculosis* infection, mice succumb to overwhelming inflammation that seems to result from reduced regulation of effector T cell numbers. This is of particular interest, because recent reports have suggested that

elimination of T regulatory cells may allow a greater immune response to *M. tuberculosis,* and reduction of bacterial burden [108, 109]. Although we did not find a role for ICAM-1 in the formation of the tuberculosis granuloma, the findings in ICAM-1 mice are still significant, as T regulatory cells are apparently necessary during *M. tuberculosis* infection to reduce excessive inflammation, and in their absence, mice may succumb not to bacterial burden, but to an overabundant immune response.

The data presented in this body of work expand the previous understanding of the immunopathology of *M. tuberculosis* infection. We have shown that TNF neutralization by either infliximab or etanercept is detrimental to control of primary *M. tuberculosis* infection and granuloma formation. To determine how the essential tuberculosis granuloma is formed, we exploited the requirement of TNF. We conclude that TNF is not required for priming of a functional T lymphocyte response to *M. tuberculosis,* nor is TNF required for the expression of adhesion molecules such as LFA-1, VCAM-1 and VLA-4 that have been shown to be required to control tuberculosis. We have shown that while ICAM-1, which binds the required LFA-1 adhesion molecule, is not required for granuloma formation or T lymphocyte priming, ICAM-1 is partially required for expression of regulatory T cells that keep inflammation at a manageable level during the immune response. Considered together, the work presented here expands our understanding of how the infection with *M. tuberculosis* is controlled by defining several molecules such as BLT-1 (LTB4 receptor), osteopontin and ICAM-1, and several roles for TNF such as adhesion molecule expression and T cell priming that are not required for formation of the granuloma. We have also defined a novel role for ICAM-1 in induction of the regulatory T cell. The idea that T regulatory cells are required to modulate the immune response to *M.*

tuberculosis is not trivial, as it has been suggested by others that elimination of T regulatory cells might lead to better control of bacterial burden through a more robust immune response.

BIBLIOGRAPHY

1. Organization, W.H., *Global tuberculosis control: surveillance, planning, financing.* WHO report 2007, 2007.
2. Griffith, D.E. and C.M. Kerr, *Tuberculosis: disease of the past, disease of the present.* J Perianesth Nurs, 1996. **11**(4): p. 240-5.
3. Tufariello, J.M., J. Chan, and J.L. Flynn, *Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection.* Lancet Infect Dis, 2003. **3**(9): p. 578-90.
4. Selwyn, P.A., et al., *Clinical manifestations and predictors of disease progression in drug users with human immunodeficiency virus infection.* N Engl J Med, 1992. **327**(24): p. 1697-703.
5. Selwyn, P.A., et al., *A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection.* N Engl J Med, 1989. **320**(9): p. 545-50.
6. Prat, J.G.i. and S.M. Mendonca de Souza, *Prehistoric Tuberculosis in America: Adding Comments to a LIterature Review.* Mem Inst Oswaldo Cruz, rio de Janeiro, 2003. **98**(Suppl 1): p. 151-159.
7. Sreevatsan, S., et al., *Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9869-74.
8. Rothschild, B.M., et al., *Mycobacterium tuberculosis complex DNA from an extinct bison dated 17,000 years before the present.* Clin Infect Dis, 2001. **33**(3): p. 305-11.
9. Konomi, N., et al., *Detection of mycobacterial DNA in Andean mummies.* J Clin Microbiol, 2002. **40**(12): p. 4738-40.
10. Zink, A., et al., *Molecular analysis of skeletal tuberculosis in an ancient Egyptian population.* J Med Microbiol, 2001. **50**(4): p. 355-66.
11. Zink, A.R., et al., *Characterization of Mycobacterium tuberculosis complex DNAs from Egyptian mummies by spoligotyping.* J Clin Microbiol, 2003. **41**(1): p. 359-67.
12. Prevention, C.f.D.C.a., *Tuberculin Skin Testing.* http://www.cdc.gov/TB/pubs/tbfactsheets/skintesting.pdf, 2007.
13. Khan, E.A. and J.R. Starke, *Diagnosis of tuberculosis in children: increased need for better methods.* Emerg Infect Dis, 1995. **1**(4): p. 115-23.
14. Ayub, A., et al., *Testing for Latent Tuberculosis.* CLINICAL MEDICINE & RESEARCH, 2004. **2**(3): p. 191-194.
15. *Targeted Tuberculin Testing and Treatment of Latent Tuberculosis Infection . This Official Statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. This is a Joint Statement of the American Thoracic Society (ATS) and the Centers for Disease Control and Prevention (CDC). This Statement was*

endorsed by the Council of the Infectious Diseases Society of America (IDSA), September 1999, and the sections of this Statement as it relates to infants and children were endorsed by the American Academy of Pediatrics (AAP), August 1999. Am. J. Respir. Crit. Care Med., 2000. **161**(4): p. 221S-247.

16. Iseman, M.D., *Tuberculosis therapy: past, present and future.* Eur Respir J, 2002. **20**(36_suppl): p. 87S-94.
17. Lalloo, U.G., R. Naidoo, and A. Ambaram, *Recent advances in the medical and surgical treatment of multi-drug resistant tuberculosis.* Curr Opin Pulm Med, 2006. **12**(3): p. 179-85.
18. Altman, L.K., *TB Patient is isolated after taking two flights.* New York Times, 2007. **May 30, 2007**.
19. Kenyon, T.A., et al., *Transmission of multidrug-resistant Mycobacterium tuberculosis during a long airplane flight.* N Engl J Med, 1996. **334**(15): p. 933-8.
20. Waddington, K., *To stamp out "so terrible a malady": bovine tuberculosis and tuberculin testing in Britain, 1890-1939.* Med Hist, 2004. **48**(1): p. 29-48.
21. Moreira, A.L., et al., *Mycobacterial Antigens Exacerbate Disease Manifestations in Mycobacterium tuberculosis-Infected Mice.* Infect. Immun., 2002. **70**(4): p. 2100-2107.
22. Bannon, M.J., *BCG and tuberculosis.* Arch Dis Child, 1999. **80**(1): p. 80-3.
23. Roth, V.R., et al., *A multicenter evaluation of tuberculin skin test positivity and conversion among health care workers in Brazilian hospitals.* Int J Tuberc Lung Dis, 2005. **9**(12): p. 1335-42.
24. Kaufmann, S.H., S. Baumann, and A. Nasser Eddine, *Exploiting immunology and molecular genetics for rational vaccine design against tuberculosis.* Int J Tuberc Lung Dis, 2006. **10**(10): p. 1068-79.
25. McShane, H., et al., *Enhanced immunogenicity of CD4(+) t-cell responses and protective efficacy of a DNA-modified vaccinia virus Ankara prime-boost vaccination regimen for murine tuberculosis.* Infect Immun, 2001. **69**(2): p. 681-6.
26. McShane, H., et al., *Protective immunity against Mycobacterium tuberculosis induced by dendritic cells pulsed with both CD8(+)- and CD4(+)-T-cell epitopes from antigen 85A.* Infect Immun, 2002. **70**(3): p. 1623-6.
27. Langermans, J.A., et al., *Protection of macaques against Mycobacterium tuberculosis infection by a subunit vaccine based on a fusion protein of antigen 85B and ESAT-6.* Vaccine, 2005. **23**(21): p. 2740-50.
28. Derrick, S.C., A.L. Yang, and S.L. Morris, *A polyvalent DNA vaccine expressing an ESAT6-Ag85B fusion protein protects mice against a primary infection with Mycobacterium tuberculosis and boosts BCG-induced protective immunity.* Vaccine, 2004. **23**(6): p. 780-8.
29. Olsen, A.W., et al., *Protective effect of a tuberculosis subunit vaccine based on a fusion of antigen 85B and ESAT-6 in the aerosol guinea pig model.* Infect Immun, 2004. **72**(10): p. 6148-50.
30. Ernst, J.D., *Macrophage receptors for Mycobacterium tuberculosis.* Infect Immun, 1998. **66**(4): p. 1277-81.
31. Underhill, D.M. and A. Ozinsky, *Phagocytosis of microbes: complexity in action.* Annu Rev Immunol, 2002. **20**: p. 825-52.
32. Bodnar, K.A., N.V. Serbina, and J.L. Flynn, *Fate of Mycobacterium tuberculosis within murine dendritic cells.* Infect Immun, 2001. **69**(2): p. 800-9.

33. Algood, H.M., et al., *TNF influences chemokine expression of macrophages in vitro and that of CD11b+ cells in vivo during Mycobacterium tuberculosis infection.* J Immunol, 2004. **172**(11): p. 6846-57.
34. Wolf, A.J., et al., *Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo.* J Immunol, 2007. **179**(4): p. 2509-19.
35. Hickman, S.P., J. Chan, and P. Salgame, *Mycobacterium tuberculosis induces differential cytokine production from dendritic cells and macrophages with divergent effects on naive T cell polarization.* J Immunol, 2002. **168**(9): p. 4636-42.
36. Capuano, S.V., 3rd, et al., *Experimental Mycobacterium tuberculosis infection of cynomolgus macaques closely resembles the various manifestations of human M. tuberculosis infection.* Infect Immun, 2003. **71**(10): p. 5831-44.
37. Gonzalez-Juarrero, M., et al., *Disruption of granulocyte macrophage-colony stimulating factor production in the lungs severely affects the ability of mice to control Mycobacterium tuberculosis infection.* J Leukoc Biol, 2005. **77**(6): p. 914-22.
38. Tsai, M.C., et al., *Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension.* Cell Microbiol, 2006. **8**(2): p. 218-32.
39. Co, D.O., et al., *Mycobacterial granulomas: keys to a long-lasting host-pathogen relationship.* Clin Immunol, 2004. **113**(2): p. 130-6.
40. Flynn, J.L. and J. Chan, *Immunology of tuberculosis.* Annu Rev Immunol, 2001. **19**: p. 93-129.
41. Fenhalls, G., et al., *Distribution of IFN-gamma, IL-4 and TNF-alpha protein and CD8 T cells producing IL-12p40 mRNA in human lung tuberculous granulomas.* Immunology, 2002. **105**(3): p. 325-35.
42. Flad, H.D., et al., *The role of cytokines in monocyte apoptosis.* Pathobiology, 1999. **67**(5-6): p. 291-3.
43. Stenger, S., et al., *An antimicrobial activity of cytolytic T cells mediated by granulysin.* Science, 1998. **282**(5386): p. 121-5.
44. Algood, H.M., J. Chan, and J.L. Flynn, *Chemokines and tuberculosis.* Cytokine Growth Factor Rev, 2003. **14**(6): p. 467-77.
45. Meresse, S., et al., *Controlling the maturation of pathogen-containing vacuoles: a matter of life and death.* Nat Cell Biol, 1999. **1**(7): p. E183-8.
46. Armstrong, J.A. and P.D. Hart, *Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes.* J Exp Med, 1971. **134**(3 Pt 1): p. 713-40.
47. Rohde, K., et al., *Mycobacterium tuberculosis and the environment within the phagosome.* Immunol Rev, 2007. **219**: p. 37-54.
48. Vergne, I., J. Chua, and V. Deretic, *Mycobacterium tuberculosis phagosome maturation arrest: selective targeting of PI3P-dependent membrane trafficking.* Traffic, 2003. **4**(9): p. 600-6.
49. Vergne, I., J. Chua, and V. Deretic, *Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade.* J Exp Med, 2003. **198**(4): p. 653-9.
50. Goren, M.B., O. Brokl, and B.C. Das, *Sulfatides of Mycobacterium tuberculosis: the structure of the principal sulfatide (SL-I).* Biochemistry, 1976. **15**(13): p. 2728-35.

51. Goren, M.B., et al., *Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of Mycobacterium tuberculosis.* Proc Natl Acad Sci U S A, 1976. **73**(7): p. 2510-4.
52. Novick, P. and M. Zerial, *The diversity of Rab proteins in vesicle transport.* Curr Opin Cell Biol, 1997. **9**(4): p. 496-504.
53. Zerial, M. and H. McBride, *Rab proteins as membrane organizers.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 107-17.
54. Rink, J., et al., *Rab conversion as a mechanism of progression from early to late endosomes.* Cell, 2005. **122**(5): p. 735-49.
55. Via, L.E., et al., *Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7.* J Biol Chem, 1997. **272**(20): p. 13326-31.
56. Roberts, E.A., et al., *Higher order Rab programming in phagolysosome biogenesis.* J Cell Biol, 2006. **174**(7): p. 923-9.
57. May, M.E. and P.J. Spagnuolo, *Evidence for activation of a respiratory burst in the interaction of human neutrophils with Mycobacterium tuberculosis.* Infect Immun, 1987. **55**(9): p. 2304-7.
58. Firmani, M.A. and L.W. Riley, *Reactive nitrogen intermediates have a bacteriostatic effect on Mycobacterium tuberculosis in vitro.* J Clin Microbiol, 2002. **40**(9): p. 3162-6.
59. Chan, J., et al., *Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis.* Infect Immun, 1995. **63**(2): p. 736-40.
60. Scanga, C.A., et al., *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**(12): p. 7711-7.
61. Manca, C., et al., *Mycobacterium tuberculosis catalase and peroxidase activities and resistance to oxidative killing in human monocytes in vitro.* Infect Immun, 1999. **67**(1): p. 74-9.
62. Laochumroonvorapong, P., et al., *Mycobacterial growth and sensitivity to H2O2 killing in human monocytes in vitro.* Infect Immun, 1997. **65**(11): p. 4850-7.
63. Schorey, J.S., M.C. Carroll, and E.J. Brown, *A macrophage invasion mechanism of pathogenic mycobacteria.* Science, 1997. **277**(5329): p. 1091-3.
64. Velasco-Velazquez, M.A., et al., *Macrophage--Mycobacterium tuberculosis interactions: role of complement receptor 3.* Microb Pathog, 2003. **35**(3): p. 125-31.
65. Faure, E., et al., *Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells.* J Biol Chem, 2000. **275**(15): p. 11058-63.
66. Flesch, I.E., et al., *Growth inhibition of Mycobacterium bovis by IFN-gamma stimulated macrophages: regulation by endogenous tumor necrosis factor-alpha and by IL-10.* Int Immunol, 1994. **6**(5): p. 693-700.
67. Rook, G.A., et al., *Activation of macrophages to inhibit proliferation of Mycobacterium tuberculosis: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages.* Immunology, 1986. **59**(3): p. 333-8.
68. Hilburger, C.L., et al., *Regulation of metallothionein mRNA in human hepatoma (HEP3B) cells.* Int J Radiat Oncol Biol Phys, 1994. **29**(2): p. 397-402.
69. Gercken, J., et al., *Defective antigen presentation by Mycobacterium tuberculosis-infected monocytes.* Infect Immun, 1994. **62**(8): p. 3472-8.

70. Henderson, R.A., S.C. Watkins, and J.L. Flynn, *Activation of human dendritic cells following infection with Mycobacterium tuberculosis.* J Immunol, 1997. **159**(2): p. 635-43.
71. Allavena, P., et al., *The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues.* Immunol Rev, 2000. **177**: p. 141-9.
72. Janeway, C.A., et al., *Immunobiology*. 5 ed. Immunobiology, ed. P. Austin and E. Lawrence. 2001, New York, NY: Garland Publishing.
73. Tian, J., et al., *Mycobacterium tuberculosis appears to lack alpha-ketoglutarate dehydrogenase and encodes pyruvate dehydrogenase in widely separated genes.* Mol Microbiol, 2005. **57**(3): p. 859-68.
74. Humphreys, I.R., et al., *A role for dendritic cells in the dissemination of mycobacterial infection.* Microbes Infect, 2006. **8**(5): p. 1339-46.
75. Harbinson, J.A., *Multiple Foci of Tuberculosis Including Subcutaneous Abscesses from Hematogenous Dissemination.* Chest, 1959. **35**(5): p. 576-583.
76. Li, Y., et al., *Distribution and characteristics of hematogenous disseminated tuberculosis within the abdomen on contrast-enhanced CT.* Abdom Imaging, 2007. **32**(4): p. 484-8.
77. Zhang, P., et al., *Innate immunity and pulmonary host defense.* Immunol Rev, 2000. **173**: p. 39-51.
78. Petrofsky, M. and L.E. Bermudez, *Neutrophils from Mycobacterium avium-infected mice produce TNF-alpha, IL-12, and IL-1 beta and have a putative role in early host response.* Clin Immunol, 1999. **91**(3): p. 354-8.
79. Martineau, A.R., et al., *Neutrophil-mediated innate immune resistance to mycobacteria.* J Clin Invest, 2007. **117**(7): p. 1988-94.
80. Seiler, P., et al., *Early granuloma formation after aerosol Mycobacterium tuberculosis infection is regulated by neutrophils via CXCR3-signaling chemokines.* Eur J Immunol, 2003. **33**(10): p. 2676-86.
81. Sawant, K.V. and D.N. McMurray, *Guinea pig neutrophils infected with Mycobacterium tuberculosis produce cytokines which activate alveolar macrophages in noncontact cultures.* Infect Immun, 2007. **75**(4): p. 1870-7.
82. Lin, P.L., et al., *Early events in Mycobacterium tuberculosis infection in cynomolgus macaques.* Infect Immun, 2006. **74**(7): p. 3790-803.
83. Seiler, P., et al., *Rapid Neutrophil Response Controls Fastâ€ Replicating Intracellular Bacteria but Not Slowâ€ Replicating Mycobacterium tuberculosis.* The Journal of Infectious Diseases, 2000. **181**(2): p. 671-680.
84. Caruso, A.M., et al., *Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis.* J Immunol, 1999. **162**(9): p. 5407-16.
85. Feng, C.G. and W.J. Britton, *CD4+ and CD8+ T cells mediate adoptive immunity to aerosol infection of Mycobacterium bovis bacillus Calmette-Guerin.* J Infect Dis, 2000. **181**(5): p. 1846-9.
86. Kipnis, A., et al., *Memory T lymphocytes generated by Mycobacterium bovis BCG vaccination reside within a CD4 CD44lo CD62 Ligandhi population.* Infect Immun, 2005. **73**(11): p. 7759-64.
87. Saunders, B.M., et al., *Characterization of immune responses during infection with Mycobacterium avium strains 100, 101 and the recently sequenced 104.* Immunol Cell Biol, 2002. **80**(6): p. 544-9.

88. Scanga, C.A., 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**(3): p. 347-58.
89. Serbina, N.V., V. Lazarevic, and J.L. Flynn, *CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during Mycobacterium tuberculosis infection.* J Immunol, 2001. **167**(12): p. 6991-7000.
90. Lazarevic, V., et al., *Induction of Mycobacterium tuberculosis-specific primary and secondary T-cell responses in interleukin-15-deficient mice.* Infect Immun, 2005. **73**(5): p. 2910-22.
91. Feng, C.G., et al., *Increase in gamma interferon-secreting CD8(+), as well as CD4(+), T cells in lungs following aerosol infection with Mycobacterium tuberculosis.* Infect Immun, 1999. **67**(7): p. 3242-7.
92. Serbina, N.V. and J.L. Flynn, *CD8(+) T cells participate in the memory immune response to Mycobacterium tuberculosis.* Infect Immun, 2001. **69**(7): p. 4320-8.
93. Shedlock, D.J. and H. Shen, *Requirement for CD4 T cell help in generating functional CD8 T cell memory.* Science, 2003. **300**(5617): p. 337-9.
94. Shedlock, D.J., et al., *Role of CD4 T cell help and costimulation in CD8 T cell responses during Listeria monocytogenes infection.* J Immunol, 2003. **170**(4): p. 2053-63.
95. Sakaguchi, S., et al., *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**(3): p. 1151-64.
96. Belkaid, Y., et al., *CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity.* Nature, 2002. **420**(6915): p. 502-7.
97. Lundgren, A., et al., *Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients.* Infect Immun, 2005. **73**(1): p. 523-31.
98. Sakaguchi, S., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self.* Nat Immunol, 2005. **6**(4): p. 345-52.
99. Itoh, M., et al., *Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance.* J Immunol, 1999. **162**(9): p. 5317-26.
100. Chen, W., 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**(12): p. 1875-86.
101. Cobbold, S.P., et al., *Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants.* J Immunol, 2004. **172**(10): p. 6003-10.
102. Davidson, T.S., et al., *Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells.* J Immunol, 2007. **178**(7): p. 4022-6.
103. Knoechel, B., et al., *Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen.* J Exp Med, 2005. **202**(10): p. 1375-86.
104. Kretschmer, K., et al., *Inducing and expanding regulatory T cell populations by foreign antigen.* Nat Immunol, 2005. **6**(12): p. 1219-27.
105. Chen, X., et al., *CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease.* Clin Immunol, 2007. **123**(1): p. 50-9.

106. Hougardy, J.M., et al., *Regulatory T cells depress immune responses to protective antigens in active tuberculosis.* Am J Respir Crit Care Med, 2007. **176**(4): p. 409-16.
107. Quinn, K.M., 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**(5): p. 467-74.
108. Kursar, M., et al., *Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis.* J Immunol, 2007. **178**(5): p. 2661-5.
109. Scott-Browne, J.P., et al., *Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis.* J Exp Med, 2007. **204**(9): p. 2159-69.
110. Serbina, N. and E.G. Pamer, *Quantitative studies of CD8+ T-cell responses during microbial infection.* Curr Opin Immunol, 2003. **15**(4): p. 436-42.
111. Turner, J. and H.M. Dockrell, *Stimulation of human peripheral blood mononuclear cells with live Mycobacterium bovis BCG activates cytolytic CD8+ T cells in vitro.* Immunology, 1996. **87**(3): p. 339-42.
112. Lazarevic, V. and J. Flynn, *CD8+ T cells in tuberculosis.* Am J Respir Crit Care Med, 2002. **166**(8): p. 1116-21.
113. Oddo, M., et al., *Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis.* J Immunol, 1998. **160**(11): p. 5448-54.
114. Kaufmann, S.H., *Protection against tuberculosis: cytokines, T cells, and macrophages.* Ann Rheum Dis, 2002. **61 Suppl 2**: p. ii54-8.
115. Pompei, L., 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**(8): p. 5192-9.
116. Trinchieri, G., *Interleukin-12 and the regulation of innate resistance and adaptive immunity.* Nat Rev Immunol, 2003. **3**(2): p. 133-46.
117. Palladino, M.A., et al., *Anti-TNF-alpha therapies: the next generation.* Nat Rev Drug Discov, 2003. **2**(9): p. 736-46.
118. Keane, J., *TNF-blocking agents and tuberculosis: new drugs illuminate an old topic.* Rheumatology (Oxford), 2005. **44**(6): p. 714-20.
119. Flynn, J.L., et al., *Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice.* Immunity, 1995. **2**(6): p. 561-72.
120. Bean, A.G., et al., *Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin.* J Immunol, 1999. **162**(6): p. 3504-11.
121. Botha, T. and B. Ryffel, *Reactivation of latent tuberculosis infection in TNF-deficient mice.* J Immunol, 2003. **171**(6): p. 3110-8.
122. Ehlers, S., *Tumor necrosis factor and its blockade in granulomatous infections: differential modes of action of infliximab and etanercept?* Clin Infect Dis, 2005. **41 Suppl 3**: p. S199-203.
123. Saunders, B.M., et al., *Transmembrane TNF is sufficient to initiate cell migration and granuloma formation and provide acute, but not long-term, control of Mycobacterium tuberculosis infection.* J Immunol, 2005. **174**(8): p. 4852-9.
124. *Tuberculosis* Second Edition ed. Tuberculosis, ed. W. Rom, N. and S.M. Garay. 2004.

125. Hanauer, S.B., *Efficacy and safety of tumor necrosis factor antagonists in Crohn's disease: overview of randomized clinical studies.* Rev Gastroenterol Disord, 2004. **4 Suppl 3**: p. S18-24.
126. Sands, B.E., et al., *Infliximab maintenance therapy for fistulizing Crohn's disease.* N Engl J Med, 2004. **350**(9): p. 876-85.
127. Keane, J., *Tumor necrosis factor blockers and reactivation of latent tuberculosis.* Clin Infect Dis, 2004. **39**(3): p. 300-2.
128. Mohan, V.P., et al., *Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology.* Infect Immun, 2001. **69**(3): p. 1847-55.
129. van der Poll, T., et al., *Effect of a recombinant dimeric tumor necrosis factor receptor on inflammatory responses to intravenous endotoxin in normal humans.* Blood, 1997. **89**(10): p. 3727-34.
130. Wallis, R.S., et al., *Granulomatous infectious diseases associated with tumor necrosis factor antagonists.* Clin Infect Dis, 2004. **38**(9): p. 1261-5.
131. Scallon, B., et al., *Binding and functional comparisons of two types of tumor necrosis factor antagonists.* J Pharmacol Exp Ther, 2002. **301**(2): p. 418-26.
132. Feng, C.G., et al., *NK cell-derived IFN-gamma differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with Mycobacterium tuberculosis.* J Immunol, 2006. **177**(10): p. 7086-93.
133. Flynn, J.L., et al., *An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection.* J Exp Med, 1993. **178**(6): p. 2249-54.
134. Cooper, A.M., et al., *Disseminated tuberculosis in interferon gamma gene-disrupted mice.* J Exp Med, 1993. **178**(6): p. 2243-7.
135. Cooper, A.M., et al., *Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis.* J Exp Med, 1997. **186**(1): p. 39-45.
136. Cooper, A.M., et al., *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**(3): p. 1322-7.
137. Khader, S.A., 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**(2): p. 788-95.
138. Flynn, J.L., et al., *IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection.* J Immunol, 1995. **155**(5): p. 2515-24.
139. Nolt, D. and J.L. Flynn, *Interleukin-12 therapy reduces the number of immune cells and pathology in lungs of mice infected with Mycobacterium tuberculosis.* Infect Immun, 2004. **72**(5): p. 2976-88.
140. Inomata, S., et al., *Circulating interleukin-18 and osteopontin are useful to evaluate disease activity in patients with tuberculosis.* Cytokine, 2005. **30**(4): p. 203-11.
141. Koguchi, Y., et al., *High plasma osteopontin level and its relationship with interleukin-12-mediated type 1 T helper cell response in tuberculosis.* Am J Respir Crit Care Med, 2003. **167**(10): p. 1355-9.
142. Nau, G.J., et al., *Osteopontin expression correlates with clinical outcome in patients with mycobacterial infection.* Am J Pathol, 2000. **157**(1): p. 37-42.

143. O'Regan, A.W., et al., *Osteopontin is associated with T cells in sarcoid granulomas and has T cell adhesive and cytokine-like properties in vitro.* J Immunol, 1999. **162**(2): p. 1024-31.
144. Stier, S., et al., *Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size.* J Exp Med, 2005. **201**(11): p. 1781-91.
145. Song, C.H., et al., *Interleukin-8 is differentially expressed by human-derived monocytic cell line U937 infected with Mycobacterium tuberculosis H37Rv and Mycobacterium marinum.* Infect Immun, 2003. **71**(10): p. 5480-7.
146. Craig-Mylius, K., et al., *Borrelia burgdorferi, an extracellular pathogen, circumvents osteopontin in inducing an inflammatory cytokine response.* J Leukoc Biol, 2005. **77**(5): p. 710-8.
147. Potter, M.R., et al., *Role of osteopontin in murine Lyme arthritis and host defense against Borrelia burgdorferi.* Infect Immun, 2002. **70**(3): p. 1372-81.
148. Abel, B., et al., *Osteopontin is not required for the development of Th1 responses and viral immunity.* J Immunol, 2005. **175**(9): p. 6006-13.
149. Nau, G.J., et al., *A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis.* Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6414-9.
150. Nau, G.J., et al., *Attenuated host resistance against Mycobacterium bovis BCG infection in mice lacking osteopontin.* Infect Immun, 1999. **67**(8): p. 4223-30.
151. Hirsch, C.S., et al., *In vitro restoration of T cell responses in tuberculosis and augmentation of monocyte effector function against Mycobacterium tuberculosis by natural inhibitors of transforming growth factor beta.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3926-31.
152. Rojas, R.E., et al., *Regulation of human CD4(+) alphabeta T-cell-receptor-positive (TCR(+)) and gammadelta TCR(+) T-cell responses to Mycobacterium tuberculosis by interleukin-10 and transforming growth factor beta.* Infect Immun, 1999. **67**(12): p. 6461-72.
153. Tomioka, H., et al., *The role of tumor necrosis factor, interferon-gamma, transforming growth factor-beta, and nitric oxide in the expression of immunosuppressive functions of splenic macrophages induced by Mycobacterium avium complex infection.* J Leukoc Biol, 1995. **58**(6): p. 704-12.
154. Maeda, J., et al., *Local production and localization of transforming growth factor-beta in tuberculous pleurisy.* Clin Exp Immunol, 1993. **92**(1): p. 32-8.
155. Dahl, K.E., et al., *Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of Mycobacterium tuberculosis.* Infect Immun, 1996. **64**(2): p. 399-405.
156. Hougardy, J.M., et al., *In vitro expansion of CD4+CD25highFOXP3+CD127low/- regulatory T cells from peripheral blood lymphocytes of healthy Mycobacterium tuberculosis-infected humans.* Microbes Infect, 2007. **9**(11): p. 1325-32.
157. Zheng, S.G., et al., *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**(9): p. 5213-21.
158. Mason, C.M., et al., *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**(3): p. 537-45.

159. Cyster, J.G., *Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs.* J Exp Med, 1999. **189**(3): p. 447-50.
160. Badewa, A.P., et al., *Chemokine receptor 5 and its ligands in the immune response to murine tuberculosis.* Tuberculosis (Edinb), 2005. **85**(3): p. 185-95.
161. Chakravarty, S.D., et al., *The chemokine receptor CXCR3 attenuates the control of chronic Mycobacterium tuberculosis infection in BALB/c mice.* J Immunol, 2007. **178**(3): p. 1723-35.
162. Scott, H.M. and J.L. Flynn, *Mycobacterium tuberculosis in chemokine receptor 2-deficient mice: influence of dose on disease progression.* Infect Immun, 2002. **70**(11): p. 5946-54.
163. Peters, W., et al., *Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis.* Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7958-63.
164. Algood, H.M. and J.L. Flynn, *CCR5-deficient mice control Mycobacterium tuberculosis infection despite increased pulmonary lymphocytic infiltration.* J Immunol, 2004. **173**(5): p. 3287-96.
165. Fuller, C.L., J.L. Flynn, and T.A. Reinhart, *In situ study of abundant expression of proinflammatory chemokines and cytokines in pulmonary granulomas that develop in cynomolgus macaques experimentally infected with Mycobacterium tuberculosis.* Infect Immun, 2003. **71**(12): p. 7023-34.
166. Sallusto, F., A. Lanzavecchia, and C.R. Mackay, *Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses.* Immunol Today, 1998. **19**(12): p. 568-74.
167. Werz, O. and D. Steinhilber, *Therapeutic options for 5-lipoxygenase inhibitors.* Pharmacol Ther, 2006. **112**(3): p. 701-18.
168. Bafica, A., et al., *Host control of Mycobacterium tuberculosis is regulated by 5-lipoxygenase-dependent lipoxin production.* J Clin Invest, 2005. **115**(6): p. 1601-6.
169. Pascual, D.W., et al., *Impaired mucosal immunity in L-selectin-deficient mice orally immunized with a Salmonella vaccine vector.* J Immunol, 2001. **167**(1): p. 407-15.
170. Huang, W.W., et al., *Molecular and biological characterization of the murine leukotriene B4 receptor expressed on eosinophils.* J Exp Med, 1998. **188**(6): p. 1063-74.
171. Yokomizo, T., et al., *cDNA cloning and mutagenesis study of leukotriene B4 12-hydroxydehydrogenase.* Adv Exp Med Biol, 1997. **407**: p. 151-6.
172. Tryselius, Y., et al., *Cloning and characterization of cDNA encoding a novel human leukotriene B(4) receptor.* Biochem Biophys Res Commun, 2000. **274**(2): p. 377-82.
173. Yokomizo, T., et al., *A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis.* Nature, 1997. **387**(6633): p. 620-4.
174. Jakobsson, P.J., et al., *Human B lymphocytes possess 5-lipoxygenase activity and convert arachidonic acid to leukotriene B4.* Biochem Biophys Res Commun, 1991. **178**(1): p. 302-8.
175. Jakobsson, P.J., et al., *Biosynthesis and biological effects of leukotriene B4 in B lymphocytes.* Adv Prostaglandin Thromboxane Leukot Res, 1991. **21B**: p. 1005-12.
176. Musch, M.W. and M.I. Siegel, *Antigenic stimulated release of arachidonic acid, lipoxygenase activity and histamine release in a cloned murine mast cell MC9.* Biochem Biophys Res Commun, 1985. **126**(1): p. 517-25.
177. Tager, A.M., et al., *Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment.* Nat Immunol, 2003. **4**(10): p. 982-90.

178. Ley, K., *Molecular mechanisms of leucocyte rolling and adhesion to microvascular endothelium.* Eur Heart J, 1993. **14 Suppl I**: p. 68-73.
179. Steeber, D.A., et al., *Leukocyte entry into sites of inflammation requires overlapping interactions between the L-selectin and ICAM-1 pathways.* J Immunol, 1999. **163**(4): p. 2176-86.
180. Chin, J.E., et al., *Involvement of intercellular adhesion molecule-1 in the antigen-induced infiltration of eosinophils and lymphocytes into the airways in a murine model of pulmonary inflammation.* Am J Respir Cell Mol Biol, 1998. **18**(2): p. 158-67.
181. Schreiber, T., et al., *Selectin ligand-independent priming and maintenance of T cell immunity during airborne tuberculosis.* J Immunol, 2006. **176**(2): p. 1131-40.
182. Leemans, J.C., et al., *CD44 is a macrophage binding site for Mycobacterium tuberculosis that mediates macrophage recruitment and protective immunity against tuberculosis.* J Clin Invest, 2003. **111**(5): p. 681-9.
183. Kipnis, A., et al., *Increased neutrophil influx but no impairment of protective immunity to tuberculosis in mice lacking the CD44 molecule.* J Leukoc Biol, 2003. **74**(6): p. 992-7.
184. Feng, C.G., et al., *Up-regulation of VCAM-1 and differential expansion of beta integrin-expressing T lymphocytes are associated with immunity to pulmonary Mycobacterium tuberculosis infection.* J Immunol, 2000. **164**(9): p. 4853-60.
185. Saunders, B.M., A.A. Frank, and I.M. Orme, *Granuloma formation is required to contain bacillus growth and delay mortality in mice chronically infected with Mycobacterium tuberculosis.* Immunology, 1999. **98**(3): p. 324-8.
186. Johnson, C.M., et al., *Adequate expression of protective immunity in the absence of granuloma formation in Mycobacterium tuberculosis-infected mice with a disruption in the intracellular adhesion molecule 1 gene.* Infect Immun, 1998. **66**(4): p. 1666-70.
187. Forget, A., et al., *Differences in response among inbred mouse strains to infection with small doses of Mycobacterium bovis BCG.* Infect Immun, 1981. **32**(1): p. 42-7.
188. Musa, S.A., et al., *Response of inbred mice to aerosol challenge with Mycobacterium tuberculosis.* Infect Immun, 1987. **55**(8): p. 1862-6.
189. Roch, F. and M.A. Bach, *Strain differences in mouse cellular responses to Mycobacterium lepraemurium and BCG subcutaneous infections. II. Production of interleukins 2, 4, and 6 and of interferon-gamma by draining lymph node cells.* Clin Immunol Immunopathol, 1991. **60**(3): p. 443-54.
190. Turner, J., et al., *The progression of chronic tuberculosis in the mouse does not require the participation of B lymphocytes or interleukin-4.* Exp Gerontol, 2001. **36**(3): p. 537-45.
191. Lazarevic, V., D. Nolt, and J.L. Flynn, *Long-term control of Mycobacterium tuberculosis infection is mediated by dynamic immune responses.* J Immunol, 2005. **175**(2): p. 1107-17.
192. Bourassa, D., et al., *Cellular immune response to Mycobacterium bovis (BCG) in genetically-susceptible and resistant congenic mouse strains.* Clin Exp Immunol, 1985. **62**(1): p. 31-8.
193. Pozos, T.C. and L. Ramakrishnan, *New models for the study of Mycobacterium-host interactions.* Curr Opin Immunol, 2004. **16**(4): p. 499-505.
194. Ordway, D., et al., *The cellular immune response to Mycobacterium tuberculosis infection in the guinea pig.* J Immunol, 2007. **179**(4): p. 2532-41.

195. Keane, J., et al., *Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent.* N Engl J Med, 2001. **345**(15): p. 1098-104.
196. Basaraba, R.J., et al., *Decreased survival of guinea pigs infected with Mycobacterium tuberculosis after multiple BCG vaccinations.* Vaccine, 2006. **24**(3): p. 280-6.
197. Basaraba, R.J., et al., *Pulmonary lymphatics are primary sites of Mycobacterium tuberculosis infection in guinea pigs infected by aerosol.* Infect Immun, 2006. **74**(9): p. 5397-401.
198. Dorman, S.E., et al., *Susceptibility to tuberculosis: clues from studies with inbred and outbred New Zealand White rabbits.* Infect Immun, 2004. **72**(3): p. 1700-5.
199. Manabe, Y.C., et al., *Different strains of Mycobacterium tuberculosis cause various spectrums of disease in the rabbit model of tuberculosis.* Infect Immun, 2003. **71**(10): p. 6004-11.
200. Converse, P.J., et al., *Cavitary tuberculosis produced in rabbits by aerosolized virulent tubercle bacilli.* Infect Immun, 1996. **64**(11): p. 4776-87.
201. Converse, P.J., et al., *Pulmonary bovine-type tuberculosis in rabbits: bacillary virulence, inhaled dose effects, tuberculin sensitivity, and Mycobacterium vaccae immunotherapy.* Clin Diagn Lab Immunol, 1998. **5**(6): p. 871-81.
202. Flynn, J.L., *Lessons from experimental Mycobacterium tuberculosis infections.* Microbes Infect, 2006. **8**(4): p. 1179-88.
203. Kirschner, D. and S. Marino, *Mycobacterium tuberculosis as viewed through a computer.* Trends Microbiol, 2005. **13**(5): p. 206-11.
204. Turner, J., et al., *In vivo IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice.* J Immunol, 2002. **169**(11): p. 6343-51.
205. Feldmann, M., *Development of anti-TNF therapy for rheumatoid arthritis.* Nat Rev Immunol, 2002. **2**(5): p. 364-71.
206. Listing, J., et al., *Infections in patients with rheumatoid arthritis treated with biologic agents.* Arthritis Rheum, 2005. **52**(11): p. 3403-12.
207. Cush, J.J., *Safety of New Biologic Therapies in Rheumatoid Arthritis.* Bulletin on the Rheumatic Diseases, 2003. **52**(8).
208. Scanga, C.A., et al., *Reactivation of latent tuberculosis: variations on the Cornell murine model.* Infect Immun, 1999. **67**(9): p. 4531-8.
209. Hu, Y.L., et al., *Pharmacodynamic effects of the murine p75-Fc fusion protein in mice.* J Investig Dermatol Symp Proc, 2007. **12**(1): p. 50-1.
210. Kim, H.Y., et al., *Construction and purification of the murine p75-murine IgG1 fusion protein.* J Investig Dermatol Symp Proc, 2007. **12**(1): p. 48-9.
211. Sud, D., et al., *Contribution of CD8+ T cells to control of Mycobacterium tuberculosis infection.* J Immunol, 2006. **176**(7): p. 4296-314.
212. Abrams, J.S., et al., *Strategies of anti-cytokine monoclonal antibody development: immunoassay of IL-10 and IL-5 in clinical samples.* Immunol Rev, 1992. **127**: p. 5-24.
213. Eskandari, M.K., et al., *WEHI 164 Subclone 13 Assay for TNF: Sensitivity, Specificity and Reliability.* Immunological Investigations, 1990. **19**(1): p. 69-79.
214. Catrina, A.I., et al., *Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages, but not lymphocytes, in rheumatoid arthritis joints: extended report.* Arthritis Rheum, 2005. **52**(1): p. 61-72.

215. Van den Brande, J.M., et al., *Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease.* Gastroenterology, 2003. **124**(7): p. 1774-85.
216. Lugering, A., et al., *Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway.* Gastroenterology, 2001. **121**(5): p. 1145-57.
217. Smeets, T.J., et al., *Tumor necrosis factor alpha blockade reduces the synovial cell infiltrate early after initiation of treatment, but apparently not by induction of apoptosis in synovial tissue.* Arthritis Rheum, 2003. **48**(8): p. 2155-62.
218. Mpofu, S., F. Fatima, and R.J. Moots, *Anti-TNF-alpha therapies: they are all the same (aren't they?).* Rheumatology (Oxford), 2005. **44**(3): p. 271-3.
219. Saunders, B.M., H. Briscoe, and W.J. Britton, *T cell-derived tumour necrosis factor is essential, but not sufficient, for protection against Mycobacterium tuberculosis infection.* Clin Exp Immunol, 2004. **137**(2): p. 279-87.
220. Eissner, G., et al., *Critical involvement of transmembrane tumor necrosis factor-alpha in endothelial programmed cell death mediated by ionizing radiation and bacterial endotoxin.* Blood, 1995. **86**(11): p. 4184-93.
221. Keane, J., et al., *Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis.* Infect Immun, 1997. **65**(1): p. 298-304.
222. Anderson, C.F., J.S. Gerber, and D.M. Mosser, *Modulating macrophage function with IgG immune complexes.* J Endotoxin Res, 2002. **8**(6): p. 477-81.
223. Co, D.O., et al., *T cell contributions to the different phases of granuloma formation.* Immunol Lett, 2004. **92**(1-2): p. 135-42.
224. Munro, J.M., J.S. Pober, and R.S. Cotran, *Tumor necrosis factor and interferon-gamma induce distinct patterns of endothelial activation and associated leukocyte accumulation in skin of Papio anubis.* Am J Pathol, 1989. **135**(1): p. 121-33.
225. Lombardi, A., et al., *A New Mechanism Involving Erk Contributes to Rosiglitazone Inhibition of Tumor Necrosis Factor-{alpha} and Interferon-{gamma} Inflammatory Effects in Human Endothelial Cells.* Arterioscler Thromb Vasc Biol, 2008: p. ATVBAHA.107.160713.
226. Xu, H., et al., *Involvement of up-regulated CXC chemokine ligand 16/scavenger receptor that binds phosphatidylserine and oxidized lipoprotein in endotoxin-induced lethal liver injury via regulation of T-cell recruitment and adhesion.* Infect Immun, 2005. **73**(7): p. 4007-16.
227. Ghosh, S., et al., *The LFA-1 adhesion molecule is required for protective immunity during pulmonary Mycobacterium tuberculosis infection.* J Immunol, 2006. **176**(8): p. 4914-22.
228. Calzascia, T., et al., *TNF-alpha is critical for antitumor but not antiviral T cell immunity in mice.* J Clin Invest, 2007. **117**(12): p. 3833-45.
229. Tartaglia, L.A., et al., *Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor.* J Immunol, 1993. **151**(9): p. 4637-41.
230. Tartaglia, L.A., D. Pennica, and D.V. Goeddel, *Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor.* J Biol Chem, 1993. **268**(25): p. 18542-8.
231. Tartaglia, L.A., et al., *The two different receptors for tumor necrosis factor mediate distinct cellular responses.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 9292-6.

232. Lazarevic, V., S. Pawar, and J. Flynn, *Measuring T-cell function in animal models of tuberculosis by ELISPOT.* Methods Mol Biol, 2005. **302**: p. 179-90.
233. Lenschow, D.J., et al., *CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes.* Immunity, 1996. **5**(3): p. 285-93.
234. Lenschow, D.J., T.L. Walunas, and J.A. Bluestone, *CD28/B7 system of T cell costimulation.* Annu Rev Immunol, 1996. **14**: p. 233-58.
235. Algood, H.M., P.L. Lin, and J.L. Flynn, *Tumor necrosis factor and chemokine interactions in the formation and maintenance of granulomas in tuberculosis.* Clin Infect Dis, 2005. **41 Suppl 3**: p. S189-93.
236. Plessner, H.L., et al., *Neutralization of tumor necrosis factor (TNF) by antibody but not TNF receptor fusion molecule exacerbates chronic murine tuberculosis.* J Infect Dis, 2007. **195**(11): p. 1643-50.
237. Marino, S., et al., *Differences in reactivation of tuberculosis induced from anti-TNF treatments are based on bioavailability in granulomatous tissue.* PLoS Comput Biol, 2007. **3**(10): p. 1909-24.
238. Neumann, B., et al., *Crucial role of 55-kilodalton TNF receptor in TNF-induced adhesion molecule expression and leukocyte organ infiltration.* J Immunol, 1996. **156**(4): p. 1587-93.
239. Roach, D.R., et al., *Tumor necrosis factor (TNF) and a TNF-mimetic peptide modulate the granulomatous response to Mycobacterium bovis BCG infection in vivo.* Infect Immun, 1999. **67**(10): p. 5473-6.
240. Ebert, L.M. and S.R. McColl, *Up-Regulation of CCR5 and CCR6 on Distinct Subpopulations of Antigen-Activated CD4+ T Lymphocytes.* J Immunol, 2002. **168**(1): p. 65-72.
241. Glass, W.G., H.F. Rosenberg, and P.M. Murphy, *Chemokine regulation of inflammation during acute viral infection.* Curr Opin Allergy Clin Immunol, 2003. **3**(6): p. 467-73.
242. Kim, N.D., et al., *A unique requirement for the leukotriene B4 receptor BLT1 for neutrophil recruitment in inflammatory arthritis.* J Exp Med, 2006. **203**(4): p. 829-35.
243. Muz, M.H., et al., *The effects of low dose leukotriene receptor antagonist therapy on airway remodeling and cysteinyl leukotriene expression in a mouse asthma model.* Exp Mol Med, 2006. **38**(2): p. 109-18.
244. Lawrence, R.H. and T.C. Sorrelli, *Decreased polymorphonuclear leucocyte chemotactic response to leukotriene B4 in cystic fibrosis.* Clin Exp Immunol, 1992. **89**(2): p. 321-4.
245. Hicks, A., et al., *Leukotriene B4 receptor antagonists as therapeutics for inflammatory disease: preclinical and clinical developments.* Expert Opin Investig Drugs, 2007. **16**(12): p. 1909-20.
246. Birke, F.W., et al., *In vitro and in vivo pharmacological characterization of BIIL 284, a novel and potent leukotriene B(4) receptor antagonist.* J Pharmacol Exp Ther, 2001. **297**(1): p. 458-66.
247. Alten, R., et al., *Inhibition of leukotriene B4-induced CD11B/CD18 (Mac-1) expression by BIIL 284, a new long acting LTB4 receptor antagonist, in patients with rheumatoid arthritis.* Ann Rheum Dis, 2004. **63**(2): p. 170-6.
248. Diaz-Gonzalez, F., et al., *Clinical trial of a leucotriene B4 receptor antagonist, BIIL 284, in patients with rheumatoid arthritis.* Ann Rheum Dis, 2007. **66**(5): p. 628-32.

249. Gronke, L., et al., *Effect of the oral leukotriene B(4) receptor antagonist LTB019 on inflammatory sputum markers in patients with chronic obstructive pulmonary disease.* Pulm Pharmacol Ther, 2007.
250. Serbina, N.V., et al., *CD8+ CTL from lungs of Mycobacterium tuberculosis-infected mice express perforin in vivo and lyse infected macrophages.* J Immunol, 2000. **165**(1): p. 353-63.
251. Moller, B. and P.M. Villiger, *Inhibition of IL-1, IL-6, and TNF-alpha in immune-mediated inflammatory diseases.* Springer Semin Immunopathol, 2006. **27**(4): p. 391-408.
252. Chen, M., et al., *Neutrophil-derived leukotriene B4 is required for inflammatory arthritis.* J Exp Med, 2006. **203**(4): p. 837-42.
253. Kouskoff, V., et al., *Organ-specific disease provoked by systemic autoimmunity.* Cell, 1996. **87**(5): p. 811-22.
254. Briscoe, H., et al., *A novel tumor necrosis factor (TNF) mimetic peptide prevents recrudescence of Mycobacterium bovis bacillus Calmette-Guerin (BCG) infection in CD4+ T cell-depleted mice.* J Leukoc Biol, 2000. **68**(4): p. 538-44.
255. Chakravarty, S.D., et al., *Tumor necrosis factor blockade in chronic murine tuberculosis enhances granulomatous inflammation and disorganizes granulomas in the lungs.* Infect Immun, 2008. **76**(3): p. 916-26.
256. Gardam, M.A., et al., *Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management.* Lancet Infect Dis, 2003. **3**(3): p. 148-55.
257. Song, F., et al., *In vitro generation of IFN-gamma-producing Listeria-specific T cells is dependent on IFN-gamma production by non-NK cells.* Cell Immunol, 1995. **160**(2): p. 211-6.
258. O'Regan, A.W., et al., *Abnormal pulmonary granuloma formation in osteopontin-deficient mice.* Am J Respir Crit Care Med, 2001. **164**(12): p. 2243-7.
259. Marroquin, C.E., et al., *Osteopontin increases CD44 expression and cell adhesion in RAW 264.7 murine leukemia cells.* Immunology Letters, 2004. **95**(1): p. 109-112.
260. North, R.J., R. LaCourse, and L. Ryan, *Vaccinated Mice Remain More Susceptible to Mycobacterium tuberculosis Infection Initiated via the Respiratory Route than via the Intravenous Route.* Infect. Immun., 1999. **67**(4): p. 2010-2012.
261. Mahairas, G.G., et al., *Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis.* J. Bacteriol., 1996. **178**(5): p. 1274-1282.
262. Lewis, K., et al., *Deletion of RD1 from Mycobacterium tuberculosis Mimics Bacille Calmetteâ€ Guérin Attenuation.* The Journal of Infectious Diseases, 2003. **187**(1): p. 117-123.
263. Ulrichs, T., et al., *Differential T cell responses to Mycobacterium tuberculosis ESAT6 in tuberculosis patients and healthy donors.* Eur J Immunol, 1998. **28**(12): p. 3949-58.
264. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
265. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
266. Yagi, H., et al., *Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells.* Int Immunol, 2004. **16**(11): p. 1643-56.
267. Stoop, J.N., et al., *Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection.* Hepatology, 2005. **41**(4): p. 771-8.

268. Vestweber, D., *Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium.* Immunol Rev, 2007. **218**: p. 178-96.
269. Revilla, C., et al., *Treatment with anti-LFA-1 alpha monoclonal antibody selectively interferes with the maturation of CD4- 8+ thymocytes.* Immunology, 1997. **90**(4): p. 550-6.
270. Paessens, L.C., et al., *Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) provide co-stimulation in positive selection along with survival of selected thymocytes.* Mol Immunol, 2008. **45**(1): p. 42-8.
271. Grakoui, A., et al., *The immunological synapse: a molecular machine controlling T cell activation.* Science, 1999. **285**(5425): p. 221-7.
272. Suzuki, J., et al., *The actin cloud induced by LFA-1-mediated outside-in signals lowers the threshold for T-cell activation.* Blood, 2007. **109**(1): p. 168-75.
273. Monks, C.R., et al., *Three-dimensional segregation of supramolecular activation clusters in T cells.* Nature, 1998. **395**(6697): p. 82-6.
274. Bachmann, M.F., et al., *Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation.* Immunity, 1997. **7**(4): p. 549-57.
275. Hubbard, A.K. and R. Rothlein, *Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades.* Free Radic Biol Med, 2000. **28**(9): p. 1379-86.
276. Lebedeva, T., M.L. Dustin, and Y. Sykulev, *ICAM-1 co-stimulates target cells to facilitate antigen presentation.* Curr Opin Immunol, 2005. **17**(3): p. 251-8.
277. Wang, Q., G.R. Pfeiffer, 2nd, and W.A. Gaarde, *Activation of SRC tyrosine kinases in response to ICAM-1 ligation in pulmonary microvascular endothelial cells.* J Biol Chem, 2003. **278**(48): p. 47731-43.
278. Van Seventer, G.A., et al., *The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells.* J Immunol, 1990. **144**(12): p. 4579-4586.
279. Sakaguchi, S., *Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.* Annu Rev Immunol, 2004. **22**: p. 531-62.
280. Yingling, J.M., et al., *Mammalian dwarfins are phosphorylated in response to transforming growth factor beta and are implicated in control of cell growth.* Proc Natl Acad Sci U S A, 1996. **93**(17): p. 8940-4.
281. Zhang, Y., et al., *Receptor-associated Mad homologues synergize as effectors of the TGF-beta response.* Nature, 1996. **383**(6596): p. 168-72.
282. Liu, F., et al., *A human Mad protein acting as a BMP-regulated transcriptional activator.* Nature, 1996. **381**(6583): p. 620-3.
283. Inman, G.J., F.J. Nicolas, and C.S. Hill, *Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity.* Mol Cell, 2002. **10**(2): p. 283-94.
284. Pierreux, C.E., F.J. Nicolas, and C.S. Hill, *Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus.* Mol Cell Biol, 2000. **20**(23): p. 9041-54.
285. Wohlfert, E.A., et al., *Cutting edge: deficiency in the E3 ubiquitin ligase Cbl-b results in a multifunctional defect in T cell TGF-beta sensitivity in vitro and in vivo.* J Immunol, 2006. **176**(3): p. 1316-20.
286. Becker, J.C., et al., *Shedding of ICAM-1 from human melanoma cell lines induced by IFN-gamma and tumor necrosis factor-alpha. Functional consequences on cell-mediated cytotoxicity.* J Immunol, 1991. **147**(12): p. 4398-401.

287. Leeuwenberg, J.F., et al., *E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro.* Immunology, 1992. **77**(4): p. 543-9.
288. Pigott, R., et al., *Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants of cytokine activated cultured endothelial cells.* Biochem Biophys Res Commun, 1992. **187**(2): p. 584-9.
289. Rothlein, R., et al., *A form of circulating ICAM-1 in human serum.* J Immunol, 1991. **147**(11): p. 3788-93.
290. Porter, J.C., et al., *Signaling through integrin LFA-1 leads to filamentous actin polymerization and remodeling, resulting in enhanced T cell adhesion.* J Immunol, 2002. **168**(12): p. 6330-5.
291. Smith, A., et al., *LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment.* J Cell Sci, 2003. **116**(Pt 15): p. 3123-33.
292. Volkov, Y., A. Long, and D. Kelleher, *Inside the crawling T cell: leukocyte function-associated antigen-1 cross-linking is associated with microtubule-directed translocation of protein kinase C isoenzymes beta(I) and delta.* J Immunol, 1998. **161**(12): p. 6487-95.
293. Volkov, Y., et al., *Crucial importance of PKC-beta(I) in LFA-1-mediated locomotion of activated T cells.* Nat Immunol, 2001. **2**(6): p. 508-14.
294. Dong, C., et al., *Microtubule binding to Smads may regulate TGF beta activity.* Mol Cell, 2000. **5**(1): p. 27-34.
295. Ross, S. and C.S. Hill, *How the Smads regulate transcription.* The International Journal of Biochemistry & Cell Biology, 2008. **40**(3): p. 383-408.
296. Wolf, A.J., 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**(1): p. 105-15.
297. Wallis, R.S. and S. Ehlers, *Tumor necrosis factor and granuloma biology: explaining the differential infection risk of etanercept and infliximab.* Semin Arthritis Rheum, 2005. **34**(5 Suppl1): p. 34-8.