DISSERTATION

CELLULAR MIGRATORY ASPECTS IN THE LUNGS OF NAÏVE AND BCG IMMUNIZED MICE INFECTED WITH *Mycobacterium tuberculosis*

Submitted by

Andre Kipnis

Department of Microbiology, Immunology, and Pathology

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ANDRE KIPNIS ENTITLED CELLULAR MIGRATORY ASPECTS IN THE LUNGS OF NAÏVE AND BCG IMMUNIZED MICE INFECTED WITH *Mycobacterium tuberculosis* BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Dr. Randall J. Basaraba

Dr. James C. DeMartini

Dr. Michael M. Fox

Dr. Joanne Turner

Adviser, Dr. Ian M. Orme

Department Head, Dr. Jeffrey Wilusz

ABSTRACT OF DISSERTATION

CELLULAR MIGRATORY ASPECTS IN THE LUNGS OF NAÏVE AND BCG IMMUNIZED MICE INFECTED WITH *Mycobacterium tuberculosis*

Granuloma is the hallmark lesion formed in pulmonary tuberculosis and it is important for both the protective immunological response as well as the development of chronic disease. In this study, I evaluated different aspects of the immune response thought to be important in the trafficking of leukocytes during *Mycobacterium tuberculosis* infection in mice.

In order to understand the role of some chemokines and adhesion molecules in pulmonary tuberculosis in mice, I describe in Chapter 2 the role of Chemokine Ligand 2 (CCL2, Monocyte Chemoattractant Protein-1) and in Chapter 3 the role of the adhesion molecule CD44. CCL2 is thought to be primarily responsible for recruiting monocytes, dendritic, NK and activated T cells, all of which have critical roles in the effective control of tuberculosis infection in mice. I show here that in mice in which the CCL2 gene was disrupted, animals exposed to low dose aerosol infection with *Mycobacterium tuberculosis* had fewer macrophages entering the lungs but only exhibited a minor and transient increase in bacterial load in the lungs, and were still able to establish a state of chronic disease. Such animals showed similar numbers of activated T cells as determined by their expression of the $CD44^{hi}CD62L^{lo}$ phenotype, but a transient reduction in cells secreting IFN-γ indicated that the primary deficiency in mice unable to produce CCL2 is a transient failure to focus antigen-specific T lymphocytes into the infected lung, whereas other elements of the acquired host response are compensated by other ligands interacting with chemokine receptor CCR2.

Upregulation of expression of the cell surface marker CD44 is a major characteristic of T lymphocytes responding in the lungs of mice infected with *Mycobacterium tuberculosis*.These lymphocytes express an activated/memory phenotype as seen by their high expression of the CD44 molecule and low expression of CD62L and CD45RB cell surface molecules. Based on increasing evidence that the CD44 molecule participates in several aspects of the inflammatory response, I evaluated its role in the response to infection with *M. tuberculosis* using gene-disrupted mice. I show that CD44 expression is not necessary for the proper trafficking of protective cells to the lungs of mice infected with *M. tuberculosis*, or the direct expression of protective immunity leading to control and containment of the bacterial load in this organ. However, although there were no differences in the bacterial load or migration of activated T lymphocytes to the inflamed lung, the absence of the CD44 molecule resulted in a substantially increased accumulation of neutrophils in the lung. These data indicate that loss of CD44 expression does not alter expression of TH1 immunity to tuberculosis in the lungs, but has major effects on the overall cellular composition of the immunopathological response.

Finally, in Chapter four I approached cellular trafficking into the lungs when a memory adaptive response is generated by vaccinating mice with *M. bovis* BCG. I investigated the cellular response in the lungs to an aerosol infection with *Mycobacterium tuberculosis*, comparing BCG immunized and non-immunized control mice. Following exposure to pathogenic bacteria, immunized mice showed a significant increase in T cell influx in the lungs as early as 10 days post challenge, while non-vaccinated mice showed a significant T cell influx only at 20 days post challenge. As a consequence of this early cellular recruitment to the lungs, immunized mice could control the bacterial load in the lungs at ten times lower levels than control mice. Further investigation of vaccinated mice prior to *M. tuberculosis* challenge provided evidence that T lymphocytes with an effector/activated phenotype are present in the lungs of those mice at higher numbers as compared to control mice. There was also a significantly higher level of T cells that responded to stimulatory antibodies by producing IFN-γ. The presence of elevated

numbers of T lymphocytes in the lungs of immunized mice allowed the host to respond sooner to an aerosol infection and maintain the pathologic response to infection in a reduced state as compared to control mice. Analyses of mRNA levels for IFN-γ in the lungs of BCG immunized mice showed that they are as low as mRNA levels from naïve mice. Taken together, the data suggest that BCG immunized mice have increased number of T cells capable of producing IFN-γ but that are not doing so. Thus the T cells present in the lungs of immunized mice fit the category of memory effector phenotype and are important for the early control of the pulmonary tuberculosis infection.

Andre Kipnis
Department of Microbiology,
Immunology, and Pathology
Colorado State University
Fort Collins, CO 80523
Summer 2004

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CHAPTER 1

Literature Review

1.1 A brief history of tuberculosis

Tuberculosis is a disease as old as mankind and its history is closely related with the history of our civilization. One of the oldest historical evidences of tuberculosis in humans is dated back to 5000 BC where acid- and alcohol-fast bacilli could be detected from human skeletons in Heidelberg, Germany. Similar proof has been obtained from Egyptian mummies from around 3500 BC. The world wide distribution of tuberculosis in those early ages is also evident from sources such as Chinese writings of 2700 BC describing lung fever and cough associated with generalized wasting[1].

Although as early as 350 BC Aristotle recognized the contagious nature of the disease, it still remained a mysterious disease for hundreds of years, with many believing that tuberculosis was hereditary, and one of the curious treatments during the middle ages was the "Touch of the King" such that in one single ceremony Philip of Valois (1328-1350) touched 1500 patients. The controversy of the etiological origin was elucidated in 1865, when Jean-Antoine Villemin proved that tuberculosis could be transmitted from man to rabbit by inoculation of tuberculous material. Finally on March 24, 1882 Dr. Robert Koch presented his findings at the Berlin Physiological Society meeting, at which he affirmed to the scientific community that *Mycobacterium tuberculosis* was the causal agent of tuberculosis. Amongst many important discoveries Dr. Koch made in the bacteriology field, many were particularly important for the study of tuberculosis. In this

regard he devised a method to stain mycobacteria using alkaline methylene blue dye solution and counterstaining with vesuvin. Koch realized the peculiar staining properties of the tubercle bacillus indicated something unusual about the cellular properties of the organism and, more specifically, related to its wall properties. He knew that in order to prove that tuberculosis was caused by the tubercle bacillus, the bacilli must be isolated from the body and cultured *in vitro*. For this purpose he developed a method to obtain pure culture of *M. tuberculosis* that used coagulated blood serum in tubes. Using this procedure Koch removed tuberculous tissues from experimental animals and inoculated serum slants. After weeks of incubation, pure cultures could be seen on the slants. In order to prove that those cultures caused the disease, Koch inoculated guinea pigs and they became progressively ill and died after four to six weeks with all the pathological characteristics of tuberculosis infection. Koch thus managed to perform all experiments necessary to fulfill his own postulates [2].

Trudeau demonstrated scientifically during the late 1800s the significance of food diet in the outcome of the disease. In his experiments he inoculated rabbits with equivalent doses of tubercle bacilli and while one group was set with plentiful food and water the other was confined in sunless cages with poor diet. The group with no restriction, when sacrificed later, showed features of healing from their inoculation, but those confined with poor diet all died of tuberculosis [1].

According to the World Health Organization it is estimated that one third of the entire world population is infected with tuberculosis. Although tuberculosis has a high infectivity, only about 10 percent of infected individuals will develop the disease

sometime during his or her entire life. Another impressive number is that every year 8.4 million new cases of tuberculosis are expected and 2 million people are expected to die of tuberculosis worldwide [3, 4], making this disease the main cause of death by an infectious agent. Tuberculosis incidence worldwide had a significant decline after the discovery and implementation of the antibiotic drugs in the late 1940s through the 1980s when acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) started to increase globally. The HIV infection increases the risk of a person developing disease during their lifetime from one in six people infected with tuberculosis to at least one in three who are also infected with HIV can develop active tuberculosis [1]. Increasing the number of infectious rates consequently increases the rate of transmission to others.

1.2 *Mycobacterium tuberculosis* etiology and physiology

Etymologically, "mycobacterium" is derived from the Greek for fungus (myces) and small rod (bacterion). The fungus component of the name derives from the tendency of these microorganisms to spread diffusely over the surface of liquid medium in a mold-like growth pattern, and because mycobacteria resist staining with conventional bacterial stains. This resistance to standard dyes and the avidity with which the mycobacteria retain certain dyes, once impregnated, are both due to the high lipid content of their cell walls. Mycolic acids are found almost exclusively among the mycobacteria and, as fatty acids, are believed to be essential to, but not the sole source of, the unique staining properties of the mycobacteria.

M. tuberculosis and other mycobacteria are biologically part of the gram-positive group, but have some distinctive features that include capsular properties that interfere with the Gram stain. Although all gram-positive bacteria have peptidoglycan associated with proteins and polysaccharides, mycobacteria have predominantly lipids associated with this polymer. The peptidoglycan layer is responsible for the shape-forming properties of the wall. Attached to this molecule by phosphodiester bond is a branched-chain polysaccharide, the arabinogalactan, whose distal ends are esterified with high-molecular-weight fatty acids (mycolic acids). Mycolic acids in turn are associated by their hydrocarbon chains with other lipids such as cord factor (dimycolyltrehalose), the sulfolipids, phthiocerol dimycocerosate (DIM/PDIM), the phosphatidylinositol mannosides (PIMs), and others. The envelope of *M. tuberculosis* consists of two distinct parts: the plasma membrane and the wall. Although *M. tuberculosis* has a normal bilayer of plasma membrane, they also have some distinctive components, such as lipopolysaccharides lipoarabinomannan (LAM), lipomannan, and the phosphatidylinositol mannosides [5]. The mycobacteria cell wall determines the size and shape of the bacterial cell, and besides these basic functions it is responsible for controlling the access to the plasma membrane of molecules in the environment of the cell as well as interfering with the fate of the host immune response as will be discussed later.

LAM consist of an a(1-6)-linked D-mannose backbone to which are attached short side chains of a(1-2)-linked D-Mannose residues and a(1-5)-linked D-arabinofuranosyl residues [6, 7]. The mannose core is linked to a phosphatidylinositol (PI) moiety which contains palmitic and tuberculoestearic acids attached to its reducing end. The acylated PI

is responsible for anchoring LAM to the plasma membrane. The non-reducing terminus of LAM can be linear or branched, and in *M. tuberculosis* Erdman strain it was shown that all termini are capped with mannose residues, which are called ManLAM [8].

Capsular proteins of *M. tuberculosis* may be of important interest, since they can come into contact with the host immune receptors and mediate a wide range of responses. They are of complex nature and it is difficult to differentiate true capsular proteins from secreted proteins caught in the process of transport to the extracellular environment. Approximately 20 proteins have been identified as being true capsular proteins based on gene fusion expression of those proteins and assessment of their localization, such that proteins as β-lactamase, MPT53, CFP29, ESAT-6, 16 kDa alpha-crystallin HSP, and superoxide dismutase among others are considered to reside in the capsular space [9].

In the same manner, proteins that are secreted by the *M. tuberculosis* comprise an interesting pool of potential antigens to induce an immune response by the host. In this regard hundreds of proteins have been found in the supernatant of bacterial cultures (culture filtrate proteins - CFP). Of these, several have been characterized [10], including the antigen 85 protein complex and the HSP60 secreted proteins, both of which have subsequently been used in different strategies of immunizations against tuberculosis [11, 12].

1.3 *M. tuberculosis* phylogeny and adaptation to the human host

The pathogen *M. tuberculosis* is estimated to be 15,000 years old. It used to be thought that the human tubercle bacillus was derived from the zoonotic pathogen *M.*

bovis, the idea being that the tubercle bacillus may have been introduced to humans when cattle were first domesticated. During all those subsequent years the bacteria underwent subtle mutations that would be responsible for its current distinct reservoirs, transmission patterns, and pathogenicity. Recent studies have now proved that this is not the case and that *M. tuberculosis* and *M. bovis* have a common ancestor strain. It is also believed that this ancestor strain was a human pathogen because 6 of the closest strains to the ancestor are human pathogens (*M. canettii*, *M. tuberculosis* strains and *M. africanum*). In this recent evolution theory, *M. bovis* species is the result of loss of segments of genomic DNA referred to as regions of difference (RD). RD 7 through 10 are missing in all *M. bovis* strains, RD1 is deleted from all *M. bovis* BCG strains [13] and depending on the *M. bovis* BCG strain, RD2, 4, 12, 13, and 14 may also be missing [14].

No matter what the origin of *M. tuberculosis* is, the adaptation achieved over thousands of years can be seen as the reason why today tuberculosis is among us as one of the leading causes of death by an infectious agent. Some of the adaptive features are: Five to ten percent of infected people develop disease, which can occur over years and decades, ensuring that diverse populations, staggered in time, are exposed and newly infected. The bacillus has adapted to reside in the cytoplasmic compartments of host cells, ensuring longer persistence within the host, emerging when the host immune system is compromised. During the active phase of the disease the bacteria can promote liquefaction and cavitation of pulmonary lesions, resulting in aerosol formation when the individual coughs or sneezes, allowing transmission and propagation of the infection to new human hosts.

Mycobacteria growth *in vivo* and *in vitro* is oxygen dependent but when grown in liquid media, bacilli may sink to the bottom of the liquid and remain viable but not replicating. Wayne suggested that such microbes may assume an analogous state in chronic lesions within human body, and he demonstrated that such bacilli resume replication soon after reexposure to oxygen [15].

1.4 Physical mechanisms of lung defense

The main function of the lung is to promote gas exchange from the interior of the body with the exterior. In order to perform such a function it has developed an enormous surface area where external air comes in close contact with the circulatory system. The counter benefit is that because large volumes of air are inhaled and exhaled constantly many other small but potentially harmful particulate materials may be deposited on the alveoli surface, which may result in damage to the host. The physical mechanisms by which particles may be cleared from the lung can be divided in three types: mucociliary clearance, cough and alveolar clearance.

Mucociliary clearance is achieved by the constant beat from the cilia and the mucus secreted by the epithelial cells that line the surface of the respiratory tract from the nose to the terminal bronchi. The beating of the cilia results in mucus and any trapped particle within, being transported from terminal bronchi toward the body's exterior.

Cough is induced by mechanical, chemical, thermal, and inflammatory stimuli of cough receptors within the respiratory tract. It starts with a rapid inspiration followed by glottal closure for a short period, a rise in pleural pressure and then the explosive event of

coughing. Coughing causes the expiratory flow to increase up to 5 times. The high linear expiratory airflow interacts with secretions to create two-phase air-liquid flow in which energy is transferred from the air to the liquid, shearing and moving liquid and finally leading to expectoration of sputum.

Lastly, small particles that escape the mucociliary clearance may be deposited in the alveolar region of the lungs. The alveolar clearance is accomplished by either a nonabsorptive mechanism, where particles are transported along the airway from the alveolar region to the ciliated region and then removed by mucociliary clearance, or by absorptive mechanisms. Of the absorptive mechanisms there may be a direct penetration into epithelial cells, with cell death and mucociliary clearance or penetration into interstitial space and either deposition or clearance by lymph or vascular drainage. A second absorptive mechanism is phagocytosis and destruction prior to transport to lymphatics. The optimum particle size for phagocytosis appears to be about 2 to 3 nm. Small particles, because of their larger total surface area, may exert a greater effect on the macrophage than large particles. Macrophages in the alveolar space move from the alveolar wall to the ciliated region because of differences in surface tension between these two regions. Additionally alveolar liquid, cells and uningested particles present in the interstitial space may be transported to the lymphatic system that is drained toward the hilar lymph nodes [16].

1.5 Innate defense of the lung against *M. tuberculosis*

Transmission of tuberculosis starts by the elimination of infectious particles, in the majority of cases by the sputum of individuals with active pulmonary tuberculosis

infections, although rare cases of elimination of infectious material from cuetaneous lesions have been reported [17]. Infection occurs after initial inhalation of bacteria contained within droplet particles of appropriate size (usually 0.5–3 μm range) reach the alveolar spaces and ultimately are engulfed by alveolar macrophages and possibly dendritic cells. Bacteria may promote their adhesion to and uptake by macrophages through different receptors.

Although the mucus layer acts as a barrier against pathogens, bacteria can adhere to and subsequently multiply within it. Bacteria growth within the mucus will induce a range of tissue responses as well as promote further mucus secretion that will add to the airway surface liquid. The accumulation of liquid may promote greater clearance from the airway by cough but also may result in blockage of the smaller airways and a further chance to allow bacterial multiplication. Infection may then become established due to a malfunction of a defense mechanism, which may be inherited (for example cystic fibrosis), acquired (for example viral infection), or because of the virulence of the microorganism [18].

The first immunological line of defense against microbial invasion in the lung is provided by innate mechanisms, which are non-antigen specific, do not require a prolonged period for induction, and begin functioning within minutes of infection. If these innate immune defenses are not sufficient to eliminate the pathogen an antigen specific immune response will take place. Alveolar resident macrophages are probably the primary cell type involved in the initial uptake of *M. tuberculosis*. Alveolar macrophages avidly phagocytose inert particles, but ingest viable bacteria considerably

less efficiently. Endocytosis of *M. tuberculosis* is greately enhanced when receptors on the phagocytic cell bind to either opsonized or nonopsonized *M. tuberculosis [19].*

Collectins represent a related group of proteins that includes surfactant proteins, mannose-binding lectins (MBL), complement-reactive proteins, and C1q among others with the characteristic of forming high order oligomers. Besides being able to activate complement, some collectins can bind to receptors on the surface of phagocytes and consequently mediate pathogen uptake. Surfactants are a multicomplex of proteins and lipids that are secreted by type II pneumocytes and serve to reduce the surface tension of the alveoli, allowing expansion of the lung during inspiration. There are four types of surfactants proteins (SP): SP-A, SP-B, SP-C, and SP-D. The hydrophobic surfactants SP-B and SP-C do not seem to play a predominant role in innate immune responses. In contrast, SP-A and SP-D, which are hydrophilic C-type lectin proteins, have a carbohydrate-recognition domain allowing them to participate in innate immune responses. Surfactant protein A facilitates the uptake of *M. tuberculosis* through binding to either the macrophages [20], type II pneumocytes [21], or neutrophils [19]. In contrast, surfactant protein D has been found to block the uptake of pathogenic strains of *M. tuberculosis* in macrophages [22].

Opsonization of pathogens as a consequence of complement activation is an important first line of defense against many extracellular microbes. C3b components are deposited on the surface of the microorganism, which together with C5 initiates the lytic pathway. Phagocytic leukocytes bind to C3b via the C3b receptor enhancing phagocytosis by polymorphonuclear neutrophils (PMN). As a consequence of

complement activation, C3a and C5a promote the release of histamine from mast cells and basophils, contraction of smooth muscle, and increased capillary permeability. C5a has chemotactic properties for phagocytic leukocytes. *M. tuberculosis* can induce complement activation by the alternative complement pathway by binding to MBL [23, 24].

The ability of macrophages to interact with microbes in an opsonin-independent manner is mediated by surface receptors capable of binding specific ligands including toxins, polysaccharides, lipopolysaccharides, complement proteins and immunoglobulins. The ability to recognize surface lectins on microbes is crucial to innate immune defenses. Nonopsonized *M. tuberculosis* can bind directly to complement receptor 3 (CR3) [25] and complement receptor 4 (CR4) [26]. In experiments in which CR3 was blocked, the phagocytosis of *M. tuberculosis* was reduced by approximately 70 percent [27, 28]. The best known mechanism for non-opsonin mediated phagocytosis of *M. tuberculosis* is the macrophage manonose receptor, which recognizes terminal mannose residues on bacteria [28, 29]. The type A scavenger receptor can also play a role in the internalization of *M. tuberculosis* as it was shown that blocking both complement and manose receptors did not abrogate binding of *M. tuberculosis* to macrophages, but the residual binding capacity was completely abrogated when an antibody to type A scavenger receptor was included [30].

The fact that *M. tuberculosis* preferentially enters macrophages suggests that *M. tuberculosis* has found the intracellular environment of macrophages advantageous. One of the possible advantages is that macrophages provide mechanism for dissemination of

M. tuberculosis by assisting transport of bacteria across pulmonary epithelial barriers. Different routes of entry of *M. tuberculosis* may result in differences of signal transduction, immune activation, and intracellular survival of *M. tuberculosis*. For example Fcγ-receptor mediated phagocytosis is directly linked to an inflammatory response, while binding to CR is not. Survival of *M. tuberculosis* after binding to CR1 is better than that after binding to CR3 or CR4 [31]. Also phagocytosis of SP-A opsonized mycobacteria by alveolar macrophages suppresses reactive nitrogen intermediates [32], one of the mechanism by which phagocytosed mycobacteria are thought to be killed.

Recent findings have shown that virulent mycobacteria lyse lung epithelial cells by the concerted action of the secreted proteins culture filtrate protein 10 (cfp10) and 6-kDa early secretory antigenic target (ESAT-6), which are secreted with the aid of Rv3871, Rv3876 and Rv3877 all of which are located within the RD1 region of virulent mycobacteria [33]. Deletion of any of these genes from a virulent strain, as is the case of BCG strains, results in attenuation as well as diminished cell lysis and interstitial infiltration. These observations can explain how mycobacteira can actively enter the lung interstitium and gain the blood circulation to disseminate to other tissues.

1.6 The innate inflammatory response to *M. tuberculosis*

During the interaction between microbe and host cell there are several molecules involved in the process of recognizing microbial molecules that will not only alert the host about a potential infection but will result in promoting an immune response. Toll-like receptors (TLRs) are phylogenetically conserved mediators that can bind to

microbial compounds that are not normally found in the host, referred to as pathogen-associated molecular patterns (PAMP). Mammalian TLRs derive their name from the *Drosophila* Toll protein, which was shown to have a critical function against fungal infection in this species [34]. TLRs are type I membrane proteins containing an extra cellular domain with leucine-rich repeats and a cytoplasmic portion with homology to the IL-1 receptor (IL-1R) family. The cytoplasmic domain of TLRs interacts with myeloid differentiation factor 88 (MyD88), IL-1R-associated protein kinase (IRAK) and TNFR-activated factor 6 [35], which lead to the activation of transcription factors like NFkB, to signal the production of cytokines and other proteins that mediate the link between innate and adaptive immune responses (Reviewed in [36]). To date there are 10 known TLRs that recognize distinct PAMP, so that TLR1 together with TLR2 recognizes bacterial lipoproteins, TLR2 recognizes lipoteichoic acid and together with TLR6 recognizes peptidoglycan from Gram-positive bacteria, and zymosan from yeast. TLR3 recognizes viral double stranded RNA making it important for viral infection recognition. TLR4 recognizes bacterial LPS. TLR5 recognizes flagellin, and TLR9 recognizes cytosine-phosphodiester-guanine (CpG) DNA. TLRs 7 and 8 have recently been shown to recognize single stranded bacterial and/or viral RNA [37, 38].

TLRs have an important role in the control of tuberculosis infection. MyD88 deficient mice were shown to be highly susceptible to different pathogens, including *Toxoplasma gondii[39]*, *Listeria monocytogenes[40]*, and *Staphylococcus aureus[41]*. Within the mycobacteria group of pathogens, mice deficient for the MyD88 adaptor molecule have increased susceptibility to *M. avium* infection, succumb to infection after about 9-14 weeks post infection, and have deficient IFN-γ responses compared to wild

type mice [42]. Similarly MyD88-deficient mice on a C57BL/6 strain background succumbed to infection at about 50 days after a low dose aerosol infection. They had increased bacterial numbers in the lung and liver compared to wild type mice, as well as low levels of expression of the important protective proinflammatory cytokines IL-12, IFN-γ, and TNF-α [43]. Those findings were in part corroborated by another study where MyD88-deficient mice, when infected with a clinical isolate of *M. tuberculosis*, had higher bacterial load in the lung and spleen following aerosol infection but they did not succumb to infection [44]. The discrepancy between these two experiments may be due to the difference in the strain of *M. tuberculosis* used. More specifically, TLR2, TLR4, and TLR9 are the main receptors believed to have an important role in the cellular responses to *M. tuberculosis*. TLR2, but not TLR4, was shown to be necessary for signaling in response to mycobacterial LAM [45, 46] and the 19-kDa *M. tuberculosis* lipoprotein [47]. Recognition of mycobacterial LAM involved the cell surface receptor CD14 which can recognize LAM and other glycolipids [48]. It has been shown that LAM binds to CD14 on the cell surface, but intracellular signaling is only accomplished by the combined action of the adapter molecule CD14 and the signal transducing molecule TLR2 [49]. A role for TLR4 in macrophage response to the *M. tuberculosis* bacilli has been shown [45], but the actual role of this receptor in providing an important role in protection has yet to be elucidated. In this regard C3H/HeJ mice, which are devoid of TLR4 expression, where shown to be more susceptible to *M. tuberculosis* aerosol infection than the TLR4 sufficient C3H/HeN mice[50]. The previous study was put in question regarding the role of TLR4 when it was shown that the C3H mouse may be more susceptible to *M. tuberculosis* infection due to genetic heterogeneity, rather than the

defect in TLR deficiency. *In vivo* the relative contribution of CD14, TLR2 and TLR4 in the immune response to *M. tuberculosis* infection seems to be redundant. Mice defective for each one of these receptors were as resistant as its congenic controls to a low dose aerosol infection. The important role attributed to TLR2 was only apparent when high levels of infecting dose was used [51].

Signaling through TLRs may induce the activation of diverse cytokine repertoires depending on the nature of the TLR ligand that will ultimately favor the control or the persistence of tuberculosis infection. In this manner the binding of the the 19-kDa lipoprotein to TLR-2 induced the production of IL-12 by monocytes [47] and also elicited the production of high levels of IL-12 but little IL-10 by monocyte-derived dendritic cells [52]. In contrast to the 19-kDa lipoprotein signaling through TLR2, LAM was shown to inhibit LPS-induced IL-12 production by human DCs [53]. There is evidence that signaling through TLR4 and TLR2 induces the production of TNF-α, while signaling through TLR4 but not TLR2 induces NO production by murine macrophages [54].

1.7 Effector mechanisms of mycobacteria killing and mycobatcerial evasion mechanisms.

Once phagocytosed by mononuclear phagocytes, a series of events are initiated that will ultimately potentially lead to the killing of the bacteria. In the same manner pathogens have also evolved, along many years of coexistence, different strategies to avoid being killed by its host. Immediately following phagocytosis the phagosome becomes alkaline for a short time so that defensins and other basic proteins can act, and later the phagosome becomes acidic as a result of acquisition of vacuolar adenosine

triphosphate-dependent proton pumps [55]. The phagolysosome formed as the result of fusion between phagosome and lysosome is a late event in which lysosomal enzymes will degrade mainly already-killed bacteria. Lysosomal acidic hydrolases function optimally at acidic pH, a condition that has to be maintained within the phagolysosome in order to have efficient bactericidal activity. Mycobacteria have overcome this deadly mechanism of defense by developing several strategies to avoid being killed at this early event of infection. There is evidence that mycobacterial sulfatides [56], and other components have the ability to inhibit phagolysososmal fusion. A more recent discovery of fusion inhibition mechanism accounts for the retaining of the TACO (for tryptophane aspatate-containing coat protein) protein within phagolysosomes [57]. TACO is present in the phagosomal membrane for a transient period after phagocytosis of dead BCG, while it remains for longer periods in live BCG infected phagosomes. Retention of TACO by mycobacteria prevents lysosomal delivery and consequently phagolysosomal fusion. The inhibiton promoted by TACO is independent from the phagocytic machinery since transfection of TACO in nonphagocytic cells also inhibits mycobacterial delivery to late lysosomes. Prevention of acidification of mycobacterial phagosomes is thought to be another mechanism by which the bacteria can avoid being killed by the host environment. This mechanism has been shown to occur in phagosomes containing both *M. tuberculosis* and *M. avium* [55, 58].

Killing of bacteria is accomplished by highly reactive toxic molecules, particularly reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs), reviewed in [59]. The importance of these toxic intermediates in the control of tuberculosis infection has been demonstrated in different models. Using cell lines

deficient for ROI production it was speculated that the antibacterial effect of macrophages infected with *M. tuberculosis* was RNI but not ROI dependent [60-62]. ROI production is activated by a membrane bound NADPH oxidase, which is activated by IFN-γ and by IgG/FcR binding. The O_2^- and OH^- radicals are powerful oxidants with high bactericidal activity, causing damage to DNA, membrane lipids and proteins. Another approach involved the use of $p47^{phox}$ gene knock out mice which are unable to produce superoxide. Aerosol infection with *M. tuberculosis* showed that these mice suffer a transient increase in susceptibility during the initial phase of infection when the acquired immune response is not yet in full force [63].

Nitric oxide (NO) is derived from the terminal guanidino-nitrogen atom of L-arginine, a reaction catalyzed by iNOS and is regulated by the synergistic action of IFN-γ and an agonist of the nuclear factor κB (NFκB) pathway [64]. RNIs exert their action by directly inactivating iron-sulfur-containing enzymes, by S-nitrosilating proteins, by damaging DNA, or by synergizing with ROIs. The importance of RNI in infection have been demonstrated by using either iNOS inhibitors or iNOS knock out mice. The use of aminoguanidine or NG-nonomethyl-L-arginine as inhibitors of iNOS have rendered mice highly susceptible to *M. tuberculosis* infections as assessed by mortality, bacterial burden and pathology of infected organs [65] . Inhibition of iNOS by aminoguanidine in a latent infection mouse model [66] resulted in reactivation of the disease demonstrating its importance not only in the initial stages, but in the control throughout the entire course of infection. In *M. tuberculosis* infection of iNOS gene knockout mice the increase in susceptibility seen is not as drastic as seen in IFN-γ knockout, suggesting that although

NO is a required mechanism for the control of tuberculosis, there are other not yet known IFN-γ dependent mechanisms by which macrophages may compensate for the absence of the NO in the control of infection [67]. Overall bacteria are more resistant to ROI than RNI, which may be in part due to their production of superoxide dismutase [68] and catalase [69] in addition to some components of their cell wall such as LAM, which can scavenge the oxygen radicals [70].

Bacteria can avoid being killed by the action of ROIs and RNIs. Binding to CR1/CR3 does not induce the respiratory burst and ROI production [30]. Some bacterial phenolic glycolipidic compounds can scavenge ROIs [71]. Interfering with proteinase C activity can block the respiratory burst and such a mechanism also affects iNOS activity. Many intracellular bacteria including *M. tuberculosis* produce superoxide dismutase and catalase that can neutralize the effect of those portent oxide radicals.

Intracellular bacteria require iron, and production of ROIs and RNIs in the phagolysosome also depends on iron. Thus competition for the intracellular iron pool between the intracellular pathogen and the host cell may influence the outcome of their relationship. IFN-γ activated macrophages downregulate transferrin receptor expression and intracellular ferritin [72], resulting in reduced available iron within the phagosome. This is another mechanism by which macrophages can become more competent in killing intracellular bacteria, including *M. tuberculosis*.

Additionally mycobacteria can inhibit MHC class-II antigen presentation. This has been shown to be achieved by the mycobacterial 19-kDa lipoprotein [73]. The same

lipoprotein that induces an inflammatory response when signaling through TLR2 can result in inhibition of antigen presentation if the stimulation occurs for a prolonged period of time as would be the case of chronic infection.

1.8 Cell migration into the inflamed lung

Alveolar macrophages have an important role in the recruitment of additional phagocytic cells and effector molecules to the site of inflammation by releasing cytokines and chemokines. Alveolar macrophages once stimulated produce numerous proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 upon recognition of microbial constituents. Those pro-inflammatory cytokines will promote the increase in vascular diameter of the local blood vessels leading to an increase in blood flow and permeability, and also increase the expression of adhesion molecules on the surface of the endothelium. The overall result of these initial inflammatory events is an increase in the local accumulation of immunoglobulins, complement factors and other blood proteins as well as an increase in the rate of migration of leukocytes across blood vessel walls into the inflamed tissue.

Leukocyte migration into the lung involves several steps. There is an initial weak binding of cells to the vascular endothelium through interactions between carbohydrate ligands on leukocytes and selectins on the endothelium. TNF-α induces the expression of P- and E-selectins on endothelial cells within a few hours that are able to recognize the blood group antigen sialyl Lewis X moiety on the surface of leukocytes. Although this initial adhesion provides a transient contact between leukocyte and endothelial cells, it is

enough to slow the rolling leukocyte allowing it to sample the local environment. The second step in leukocyte migration is dependent on the interaction between intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and the leukocyte function antigen-1 (LFA-1) on the surface of leukocytes. TNF-α is involved in the induction of ICAM-1 on endothelial cells. Tight binding between LFA-1 and ICAM-1 arrest the rolling leukocyte and allows it to attach firmly to the endothelium. The next step involves the transmigration of leukocytes through the venule wall and their subsequent chemotaxis towards inflamed tissue. Several molecules have been shown to be important chemotaxins in the lung during an inflammatory response to *Staphylococcus aureus*, *Streptococcus pneumoniae*, *H. influenza* and *P. aeruginosa* such as the complement component C5 and its fragments. Alveolar macrophages generate products of arachidonic acid such as leukotrienes that induce increases in vascular permeability, leukocyte migration, and aggregation. Chemokines synthesized by a wide variety of immune and non-immune parenchymal cells in the lung also recruit leukocytes. Mononuclear phagocytes, endothelial cells and epithelial cells have the ability to synthesize chemokines in response to TNF-α and IL-1 and to microbial products such as LPS. Chemokines bind to proteoglycan molecules both in the extracellular matrix and on parenchymal cells, so they are displayed on a solid substrate along which leukocytes migrate [18].

1.9 Granuloma formation in tuberculosis

The hallmark of tuberculosis is the development of granulomas, a focal aggregate of cells that form around sites of mycobacteria infection. Infection with *M. tuberculosis* is accomplished by inhalation of bacteria and their deposition in the lung alveolar space. In humans primary pulmonary tuberculosis granuloma begin to develop with the influx of macrophages at the site of infection, which under the influence of T lymphocytes, undergo differentiation into epithelioid-like histiocytes or fuse to become Langhans' giant cells. The histiocytes aggregate in small clusters, which eventually undergo necrosis. The initial parenchymal focus of tuberculosis is known as the Ghon focus, which together with the hilar lymphadenopathy comprise the primary complex. As the infection progresses, the individual granulomas enlarge and coalesce resulting in a focus of necrotic lung surrounded by granulomatous tissue. The granuloma may develop to a larger and macroscopically visible entity or it may undergo healing by deposition of fibrous tissue. The healing process of granuloma is often accompanied by calcification of the necrotic tissue, which becomes evident in radiographic x-rays. Infected individuals that recover from the disease do not become sterile in terms of the presence of viable bacteria in their organism, but remain with viable bacteria in a latent or dormant phase. Post primary tuberculosis may result from reinfection or probably more often from reactivation of primary infection. Such reactivation is frequently associated with immunosupression, malnutrition, and/or debilitation. Disease reactivation is characterized by necrotizing granulomatous formation, with coalescence and enlargement of multiple inflammatory foci, leading to destruction of the lung parenchyma, and consequent fibrosis. The degenerative process results in the formation of cavities that allow the

necrotic material to reach the airway and circulatory systems with consequent spread to other areas of the lung as well as to dissemination throughout the body respectively. [74].

In the mouse model of tuberculosis infection, the disease progression in the infected lungs does not have the formation of the classical granuloma as defined in guinea pig and humans. Nevertheless it has provided a great amount of information about granuloma formation and characteristics. After a low dose bacterial infection of the resistant C57BL/6 strain of mice, the bacterial load in the lungs increases exponentially in the first three weeks when it stabilizes, coinciding with the influx of protective T cells and the onset of the acquired immune response. Although the bacterial load in the lungs of mice remains constant or only slowly rises throughout the chronic phase of infection, there is a progressive modulation of the pathological findings. The first signs of pathological changes in the lungs are seen about a week following a low dose aerosol infection, when discrete interstitial pneumonia develops resulting in the accumulation of a few mononuclear phagocytes and alveolar macrophages. During the second and third week post infection, lymphocytes appear organized in perivascular and peribronchiolar cuffs. About a month after infection there is an accumulation of epithelioid macrophages that, together with lymphocytes, start to fill the lumens of alveoli. About two months later sheets of epithelioid and foamy macrophages are formed and the perivascular lymphocytes are replaced by large wedges of lymphocytes in the epithelioid cell granulomas. In some cases the macrophage rich granuloma is surrounded by an incomplete rim of lymphocytes resembling a rudimentary tubercle. Due to a central rather than a peripheral fibrosis and lack of central necrosis at this stage, these granulomas do not fit the true definition of a tubercle. The granulomatous foci continue to spread and

eventually begin to coalesce with other nearby granuloma leading to consolidation with large proportions of the lungs. During the chronic stages of infections small necrotic foci often associated with clefts of accumulated cholesterol are visible. The later stages of infection have extensive areas of the lung compromised by granulomatous inflammation where the original individual lesions cannot be distinguished, consisting mainly of epithelioid and foamy macrophages with fewer lymphocytes either individually spread or in tight aggregations resembling follicles[75-77].

The spatial arrangement of the major subpopulations of lymphocytes within the mouse granuloma have been studied by immunohistochemistry by Gonzalez-Juarrero *et al.* [78]. Following aerosol infection there was a gradual increase of CD3 T cells, which consisted of proportional increases of CD4 and CD8 T cells, CD4 T cells infiltrate were always present in 1.5 times higher numbers than CD8 T cells. A smaller percentage of B cells were present during infection, but their relative percentage remained constant. In the chronic stages of infection (later than 100 days post infection) CD4 T cells co-localized with the lymphocytic aggregates within the granulomas, while CD8 T cells seemed to be more closely associated with the periphery of the granuloma. The authors suggested that this arrangement of the CD8 T cells supports the idea that CD8 T cells play a role in immunosurveillance, and that their strategic location would allow them to detect any possible dissemination of bacteria through infected macrophages [78].

What is the function of the granuloma? Although there is not a direct answer to this question, three different roles can be attributed to the granuloma formation in tuberculosis. First it provides a framework where effector cells such as macrophages

come in close proximity to T lymphocytes that will activate the former by the release of cytokines. A second role is to limit the inflammatory response and consequently protect the lung tissue. A third function of the granuloma is to prevent dissemination of the infection to other sites of the lung as well to other tissues [79]. The protective role for granuloma formation during infection has been questioned by experiments using mice deficient for the expression of ICAM-1 which had reduced monocyte influx into tissues. ICAM-1 deficient mice were equally resistant to infection as were wild type mice, and had normal cell mediated response, but did not form granuloma in the lungs nor could they mount a delayed type hypersensitivity (DTH) response when challenged with culture filtrate protein (CFP) antigens [80]. These mice died about 140 days after infection due to an extensive cellular infiltration and widespread tissue necrosis [81]. These data support the idea that the protective response as mediated by production of important cytokines such as IL-12 and IFN-γ to activate effector cells such as macrophages to kill bacteria does not necessarily require the formation of granuloma or the development of DTH.

1.10 Chemokines and their participation in granuloma formation:

Leukocyte migration into tissues can be mediated in a constitutive fashion or induced by inflammation. Constitutive recruitment occurs in secondary lymphoid tissues such as lymph nodes and the spleen and is responsible for the recruitment of naïve and central memory T cells and B cells. In a similar fashion tissue resident macrophages and dendritic cells migrate in a constitutive manner. Inflammatory recruitment accounts for the migration of phagocytes, lymphocytes and effector cells at sites of local infection or inflammation. Constitutive recruitment of naïve T cell from the high endothelial venules

(HEVs) into secondary lymphoid lymphoid organs is carried out through CCL21 (6Ckine/SLC) expression on the HEV and CCR7 on the T cells [82]. In contrast many chemokines can recruit effector T cells into inflammatory tissues. For example Th1 cells express CCR1, CCR2, CCR5 and CXCR3 among other chemokine receptors, which allow them to respond to CCL2 (MCP-1), CCL7 (MCP-3), CCL8 (MCP-2), CCL5 (RANTES) and CXCL10 (IP-10) (reviewed in [83].

Today there are approximately 40 known chemokines among which there is a great deal of redundancy. Chemokines can be grouped on the basis of the arrangement of their amino terminal cysteine residues. Accordingly, CXC (also known as alpha chemokines) and CC chemokines (also known as gamma chemokines) have the first two cysteine residues separated by a single amino acid or adjacent one to another respectively. Additionally there are two other classes of chemokines that do not fall into the previous description, which are lymphotactin (C chemokines) that lack the first cysteine residue of the typical chemokine structure, and fractalkine (CX3C chemokines) that have 3 amino acids between the first two cysteines (reviewed in [84]).

Infection with *M. tuberculosis in vitro* and *in vivo* induces the production of a variety of chemokines, including CCL5 (RANTES), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL2 (MCP-1), CCL7 (MCP-3), CCL12 (MCP-5), and CXCL10 (IP10) in the murine model [79, 85, 86]. In humans the evidence of the role of chemokines are indirectly inferred by observations that patients with active disease have increased levels of certain chemokines as compared to healthy controls [87, 88]. Despite the relatively few models of mice available to study the role of chemokines, some studies using chemokine-

deficient or chemokine receptor-deficient mice have been done and, probably due to the redundancy of their function, their effects during infection are not clear. One important observation was obtained by the use of mice deficient for the chemokine receptor 2 (CCR2). CCR2 is expressed on monocytes, macrophages, dendritic cells as well as in activated T cells and it binds to CCL2, CCL7 and CCL12 endowing this receptor a major role in the recruitment of monocyte/macrophage recruitment in response to intracellular pathogens. When infected with *M. tuberculosis* CCR2 deficient mice had an impaired migration of monocytes/macrophages to the lungs, developing atypical granulomas [89, 90]. In this model it was shown that the outcome of the disease was dependent on the dose and route of infection. While mice infected with a high i.v. dose ($5x10^5$ CFU/mouse) succumbed to infection due to an immediate defect in cellular recruitment [89], using a smaller dose either i.v (10^4 CFU/mouse) or via aerosol (50 CFU/mouse) the CCR2 KO mice controlled infection similar to wild type mice [90]. Infecting mice with a lower dose resulted in similar defects as seen with the high dose such as decreased monocyte/macrophage influx to the lungs, with abnormal granuloma formation, and delayed but not deficient production of the protective cytokine IFN-γ. Despite the defects in the inflammatory response seen in the CCR2 KO mice, the response was still protective if the initial bacterial load was low.

In contrast, mice with disruption of the *CCL2* gene, which codes for CCL2 protein one of the known ligands for CCR2, were shown to have abnormalities in monocyte trafficking, altered cytokine secretion by splenocytes sensitized to Schistosome egg antigen, but normal DTH responses [91] demonstrating that although redundancy among chemokines may occur, the location and temporal production of particular chemokines

may play subtle and yet unknown roles in the immune response. Lu *et al.* reported that CCL2 KO mice infected i.v. with *M. tuberculosis* had no difference in susceptibility when compared to wild type mice, suggesting that despite deficiencies in monocyte migration, the absence of CCL2 did not compromise the protective response against *M. tuberculosis* during a high load i.v. infection.

1.11 Antigen presentation

The expression of antibacterial activities by mononuclear phagocytes depends on appropriate stimulation by cytokines, with IFN-γ produced mainly by T lymphocytes being of major importance. Mycobacterial protein antigens are mainly presented by major histocompatibility complex (MHC) class II molecules that are recognized by CD4 αβ T cells. In the same manner some mycobacterial antigens may be presented in a MHC class I context as a result of cytoplasmic "escape" from the phagosome; in this case CD8 αβ T cells may participate in the recognition of infected cells. Other molecules may be involved in mycobacterial antigen presentation, like MHC class Ib and CD1. MHC class Ib is specialized for presentation of bacterial N-formyl-methionine-containing peptides to CD8 T cells [92]. CD1 molecules are capable of presenting mycobacterial lipids and glycolipids to NK T cells [93, 94]. The presentation process by CD1 molecules starts at the late endosomal compartment [95].

1.12 Cell subpopulations and tuberculosis

1.12.1 CD4 T cells

CD4 T cells play an important role in the control of intracellular bacterial infections. Immunological surveillance of intracellular pathogens is accomplished by the detection of antigens presented on MHC molecules. Antigens that are processed in the phagolysosomal compartments are loaded on class II MHC molecules and presented to CD4 T cells.

One of the first pieces of evidence on the importance of T cells in the control of *M. tuberculosis* infection was made by North [96] who showed that thymectomized and gamma-irradiated mice were more susceptible to IV infection with H37Rv strain of *M. tuberculosis*. The requirement of T cells to contain the tuberculosis infection was further demonstrated by Orme and Collins [97] by recovering T cell enriched splenocytes from *M. tuberculosis* infected mice and adoptively transferring protection to subsequent mycobacterial challenge in thymectomized, gamma-irradiated T cell-deficient mice.

Mice depleted of CD4 prior to infection with *M. bovis* BCG are unable to control mycobacterial growth [98] and Orme [99] demonstrated that CD4 T cells, and to a lesser degree CD8 T cells, derived from *M. tuberculosis* infected mice could transfer resistance to subsequent challenge of recipient mice. The important role of CD4 T cells in the control of *M. tuberculosis* was further evidenced with the tragedy of human immunodeficiency virus infection, where infected individuals with diminished levels of

CD4 T cells become significantly more susceptible to primary tuberculosis infection as well as reactivation [100].

The role of CD4 T cells in reactivating tuberculosis infection in mice was shown by Scanga *et al.* where CD4 T cells were depleted by antibody treatment in mice chronically infected with *M. tuberculosis.* In such mice the infection reactivated resulting in increased bacterial burden and transformation of necrotic regions that were not seen in the control group [101].

CD4 T cells proliferate and produce cytokines in response to *M. tuberculosis* infection of which IFN-γ has been shown to be essential to control *M. tuberculosis* infection [102, 103]. The evident association that the protective role of CD4 T cells was due to its production of IFN-γ during the course of infection was made. To further elucidate the T cell subpopulation contributions, experiments were done using different gene knock out mice models.

Intravenous infection of MHC class II or CD4 deficient mice demonstrated increased susceptibility to *M. tuberculosis* and while MHC class II deficient mice were more susceptible than CD4 deficient mice as measured by survival, both mutants had only transient diminished levels of IFN-γ compared to their wild type counterparts [104]. CD4 deficient mice infected with *M. tuberculosis* by the aerosol route were also more susceptible than wild type mice although to a lesser degree when compared to the i.v. infection. In the aerosol infected mice infection was controlled for the first 50 days at similar levels, but after this time CD4 KO mice had higher bacterial numbers in the lung

and spleen as well as began to die. The major defect in CD4 deficient mice was a defect in attracting cells into the lung and generating the typical mononuclear granulomatous lesions [105]. Therefore although CD4 T cells were the major T cell subpopulation responsible for control of infection by producing IFN-γ, in their absence other cell types could produce IFN-γ and provide a partial compensation for their loss.

1.12.2 CD8 T cells

CD8 lymphocytes recognize antigens through the interaction of their TCRs and specific antigens presented in the context of MHC class I molecules. Upon TCR ligation and the proper costimulatory signals such as interaction between CD28 on lymphocytes and B7 on APCs, and IL-2 interaction with IL-2 receptor, they become activated, rendering both effector and memory CD8 T cells. CD8 effector T cells can produce cytokines such as IFN-γ [106] as well as have cytotoxic activity, both of which are important functions in the control of *M. tuberculosis* infection. The cytolytic activity of CD8 T cells involves the release of perforin and granzymes once engaged to target cells that will eventually lead to apoptosis of the target cell [107]. Apoptosis can also be induced by CD8 T cells through the interaction of the Fas ligand (CD95L) present on CD8 T cells and Fas (CD95) bearing target cells.

Although the major T cell subset involved in control of infection are the CD4 T cells, CD8 T cells were shown to have an important role in the protection against tuberculosis. In the mouse model of tuberculosis, CD8 T cells probably contribute to protective immunity, since *M. tuberculosis* infection results in a markedly increased

bacterial burden and death in mice with disrupted β–2microglobulin (β2m) genes [108]. As a component of class I MHC molecules, β2m is required for CD8 T cells, as they are not positively selected during T cell maturation in the thymus. β2m is also a component of other antigen-processing molecules, including H2-M3, Qa-1, Qa-2, and CD1d all of which, with the exception of CD1d, bind peptide antigens loaded in a transporter-associated with antigen processing (TAP)-dependent fashion. Behar *et al.* have shown that mice deficient of TAP present similar susceptibility seen in β2m deficient mice, ruling out the possible contribution of CD1d molecules to the increase in susceptibility [109]. Although the data from β2m deficient mice pointed to a possible protective role for CD8 T cells, more detailed studies that included CD8 deficient mice showed that these mice were no more susceptible to infection than the controls over the first 50 days post infection, and the increased susceptibility seen in the β2m-KO group was then attributed to other possible β2m related factors, ruling out the CD8 T cell deficiency [110].

CD8 T cells harvested from immune mice adoptively transferred to athymic mice prolonged survival in an acute aerosol infection model [111]. The role of these cells in human antimycobacterial defenses *in vivo* remains uncertain, since CD8 T cells are not selectively concentrated at the site of disease in patients with tuberculosis, and the severity of tuberculosis in HIV-infected patients is unaffected by the CD8 cell count [112]. The role of CD8 T cells in mice is not yet defined as perforin and granzyme deficient mice infected with *M. tuberculosis* aerogenically showed no difference in susceptibility to the control group, suggesting that cytokine secretion may be the major

mechanism of protective immunity expressed by CD8 T cells [113]. On the other hand perforin-deficient mice were shown to have more activated T cells than control [114] reflecting in higher production of cytokines, such as IFN-γ, by CD8 T cells in the perforin-KO mice that may compensate for the deficiency in one of the effector functions of CD8 T cells.

Mycobacteria-specific CD8 T cells were shown to be present in the lungs and lung-draining lymph nodes of *M. tuberculosis* infected mice [115]. These CD8 T cells specifically lysed *M. tuberculosis*-infected macrophages in a perforin-dependent manner (in mice infected via aerosol this effect was seen only at 6 weeks post infection compared to 2 weeks in i.v. infected mice). Recognition of mycobacterial antigen by those CTLs was β2m dependent, MHC class I dependent, and CD1 independent [115].

CD8 T cells may not be as critical as CD4 T cells in the initial stages of infection but were shown to play an important role during the chronic phase of the disease. When chronically infected mice were antibody-depleted of either CD4 or CD8 T cells, the bacterial load increased significantly only in the latter group compared to isotype antibody treated mice [116], suggesting that in the chronic stage the importance of these two cell subsets interchange, with CD8 becoming of greater importance. A supportive observation [117] was made in a study with CD8-KO mice where it was shown that in the chronic phase of infection (after 100 days) these mice became more heavily infected than control, which means that CD8 T cells are necessary to control infection in these later stages. CD8 T cells mediated protection was shown to be CD95 mediated in the chronic stages, in that CD95 deficient mice were also unable to control chronic infection [117].

These data are consistent with previous data suggesting a role for CD8 T cells to control infection during the chronic phase by monitoring possible bacterial escape from the granuloma.

1.12.3 Other cell types: CD1 restricted cells, γδ cells, and neutrophils

CD1 is a family of cell surface molecules that participate in the recognition of lipid-based antigens by T cells. CD1 molecules are structurally related to MHC molecules, but are nonpolymorphic. The human CD1 family is encoded by five different genes located on chromosome 1, of which four have been shown to be expressed as protein products, CD1A, B, C, and D. CD1 molecules can be grouped based on their homologies in group I (CD1a, CD1b and CD1c) and group II (CD1d), whereas mouse and rat express only the latter group. CD1-restricted T cells are mostly $CD4^{neg}$ and include $\alpha\beta^{+}CD8$, $\alpha\beta CD4^{-}$ $CD8^{-}$, NK T cell and $\gamma\delta^{+}$ T cells [118]. They recognize nonpeptide lipid and glycolipid antigens of *M. tuberculosis* that are thought to contribute to virulence [93, 119]. CD1 restricted T cells may contribute to the protection against intracellular pathogens by two important mechanisms. Firstly mycobacteria-specific group I CD1-restricted T cells may release high levels of IFN-γ and low levels of IL-4 [120], and secondly they show a high degree of cytolytic activity *in vitro* against antigen-pulsed $CD1^{+}$ mononuclear phagocytes and they were also shown to recognize and lyse $CD1^{+}$ targets infected with live virulent *M. tuberculosis* bacilli [121]. It is interesting to note that the mechanism of cellular lysis promoted by CD1-restricted T cells can be either by Fas-FasL interaction (in the case of $CD4^{-}/CD8^{-}$ cells) or perforin/granulysin mediated (in the case of $CD8^{+}$ cells). One important piece of evidence that CD1 plays a significant role in mycobacterial infections

came from the findings that the expression of CD1 correlated directly with effective immunity to *M. leprae* in humans [122]. The mouse model for studying the role of CD1 in *M. tuberculosis* infections is limited because this species expresses only the group II molecules and may explain why no differences were seen in mice deficient in CD1d infected with *M. tuberculosis* [109]. Nevertheless mice treated with antibody against CD1 and infected with *M. tuberculosis* were more susceptible than controls [123]. One of the possible explanations for the different results seen in these experiments may be the compensatory effect in CD1 gene-depleted mice. In another approach it was shown that α-galactosylceramide, a molecule known to be presented in the CD1d context and to be recognized by NKT cells, could induce NKT cell activation resulting in the production of substantial amounts of IFN-γ, as well as confer protection to otherwise susceptible BALB/c and C3H/HeJ mice [124]. The guinea pig model approximates more to humans in this matter, since they have the group I CD1 molecules not present in the mouse, and in the future they may give more light into the role of this pathway of antigen presentation in the response to *M. tuberculosis*.

Gamma delta (γδ) T cells are a subset of T cells bearing a T cell receptor (TCR) composed of the rearranged γ and δ chain genes instead of the more common α and β seen in the majority of T cells. The nature of the ligand that binds to the γδ TCR is not completely elucidated but some of the strong natural ligands include isopentenyl pyrophosphate (IPP), geranylpryophosphate, uridine and thymidine [125]. It is of interest to note that IPP is highly abundant in mycobacterial supernatant and represents a potential antigen that can prompt γδ T cells to respond during tuberculosis infection. Its

distinct function may lie in recognition of nonpeptide phenyl pyrophosphate derivatives that are not recognized by the most abundant αβ T cells. The percentage of γδT cells is markedly elevated in draining lymph nodes and lungs of mice after initial infection with *M. tuberculosis* [126], and γδ T cells from the cord blood of newborn babies proliferate in response to *M. tuberculosis*, indicating that these cells recognize mycobacterial antigens without prior exposure to mycobacteria [127]. They can produce Th1 cytokines and lyse *M. tuberculosis*-infected monocytes similar to CD4 T cells [128] in a mechanism that is mediated by the perforin/granzyme exocytosis pathway [129]. Studies using gene disrupted mice revealed different results in susceptibility to *M. tuberculosis* infection depending on the route and antigen dose. In a high dose i.v. infection the TCRδ$^{-/-}$ mice died earlier than TCRβ$^{-/-}$ suggesting that γδ T cells contribute to the innate protection against *M. tuberculosis* [130]. Aerosol infection of γδ TCR-KO mice showed that there was no difference between KO and wild-type controls in their ability to contain growth of bacilli in the lungs. However, the granulomatous response seen in KO mice was a more pronounced one, with a higher amount of polymorphonuclear cells not seen in controls [131]. These findings additionally attribute to γδ T cells the function of maintaining an appropriate granulomatous inflammatory response.

1.12.4 Polymorphonuclear (PMN) cells

While alveolar macrophages can clear small inocula of some microbes, the recruitment of granulocytes is required to clear most extracellular pulmonary pathogens effectively. PMN cells are professional phagocytes with the ability to mediate bacterial

killing through the oxidative burst. The importance of PMN in early bacterial clearance of extracellular pathogens has been shown in animal models where, PMN depleted animals were unable to clear inocula of *Pseudomonas aeruginosa, Klebsiella pneumoniae and Haemophilus influenzae*. PMNs also play an important role in the control of infection of certain intracellular pathogens such as infection with *Listeria monocytogenes* [132, 133]. Neutrophils have a short life span and because of the chronic nature of tuberculosis it is believed that they have no major role in protection. Conversely neutrophils have been shown to be present in the peritoneum of mice infected with either *M. avium* [134] or *M. tuberculosis* [135] intraperitoneally. Studies where neutrophils were antibody depleted were not conclusive. Neutrophil depletion in the first 5 days of *M. tuberculosis* infection rendered mice more susceptible to infection during the first 25 days of infection [135]; however, others have not seen any difference between wild type and neutropenic mice [136]. In addition, neutrophil depletion in later periods of infection with either *M. tuberculosis* or *M. bovis* when the acquired immune response had already started to take place, showed no difference when compared to antibody treated mice [135, 137]. It is possible that neutrophils do play a role for fast growing bacteria which can reach high numbers rapidly, but for slow growing bacteria like pathogenic mycobacteria the role of neutrophils is not evident.

1.13 Memory T cells

During and after an infection, the goal of the immune system is to generate a memory for that particular infection to prevent or lessen the severity of a disease upon re-encounter with that pathogen. This is accomplished by the generation of memory B cells

with the ability to promptly differentiate into plasma cells and secrete large amounts of neutralizing antibodies. These mechanisms are extremely important for extracellular pathogens but also provides an important protection against intracellular pathogens such as virus and intracellular bacteria. On the other hand, T cells evolved ways to promote protection against intracellular infections, where CD4 memory cells will produce cytokines that will aid the production of antibodies as well as the generation of CD8 T cells. CD8 memory cells will kill infected cells and/or secrete cytokines. Although microbial pathogenicity, as well the host immunological responses, can be described according to the type of microorganism involved (e.g. virus, extracellular bacteria, intracellular bacteria), the type of memory responses necessary for protection, whether it will be humoral, cellular or a combined type, varies from each individual pathogen.

Studies of the humoral response to tuberculosis infection have shown little evidence for any protective effect by antibodies [138]. On the other hand the much stronger data relating to cellular transfer of immunity against tuberculosis infection has led to the more comprehensive study of memory response emphasizing T cells. In a detailed study of the phenotypic characterization of $CD4^+$ T cells following *M. tuberculosis* infection [139], it was proposed that naïve T cells with moderate expression of the molecule CD45RB and low expression of the molecule CD44 (CD45RB moderate high/ $CD44^{lo}$) would undergo activation and up-regulate the expression of CD44, and have a larger size because they are actively undergoing proliferation, and for this same reason these cells present a transiently high CD45RB expression. These effector cells continue to differentiate and start to down-regulate expression of CD45RB. The $CD44^{hi}/CD45RB^{lo}$ cells were considered by the authors as short-lived effector cells that were able to confer a protective

response to *M. tuberculosis*. A third group of T cells presented the phenotype $CD44^{hi}/CD45RB^{neg}$ cells which were thought to be long-lived memory cells.

Although of great importance, this study was limited in the markers analyzed. Memory and active/effector cells cannot be exclusively characterized based solely on those markers. Recent studies [140] have shown that human memory T cells can be divided in two groups, effector memory T cells and central memory T cells. Effector memory T cells retain the markers of activation such as $CD45RA^{neg}$ together with markers such as $CD62L^{lo}$ and $CCR7^{neg}$ that allow them to circulate in the periphery looking for possible sites of infection. These cells, when stimulated, can produce IL-4, IFN-γ and moderate levels of IL-2 as well up-regulate the expression of CD40L. $CD8^{+}$ cells with this phenotype were shown to produce IFN-γ and perforin when properly stimulated. In contrast central memory T cells have a phenotype similar to naïve T cells, in which they express $CD45^{+}/CCR7^{-}/CD62L^{lo}$, which confer on them the ability to home into secondary lymphoid tissues. Effector memory cells were also shown to express high levels of integrins required for their mobilization into tissues like β1 and β2 integrins as well as CCR1, CCR3 and CCR5, while central memory T cells express intermediate levels of β1/β2 integrins and as well as CCR4, CCR6 and CXCR3. Central memory cells, when stimulated, produce high levels of IL-2, no detectable IL-4 or IFN-γ, but up regulate the expression of CD40L [141], in contrast to naïve cells that fail to up-regulate CD40L. Overall effector memory cells can respond promptly to invading pathogens in tissues promoting a quick response. In BCG infected mice, a higher degree of $CD44^{hi}/CD45RB^{lo}$ cells capable of IFNγ production is seen in the lungs of mice (Orme,

unpublished data), which is consistent with the description for effector memory cells. Central memory cells would be responsible to monitor possible infection by scanning secondary lymphoid tissues for the presence of antigens being presented by APCs, possibly dendritic cells, and once they encounter their specific antigen they would undergo differentiation capable of generating activated and effector memory cells. In memory T cell studies regarding tuberculosis infection and or BCG vaccination, a detailed analysis of the type of memory cells generated is required. Similarly a study is required to determine if the memory population is lost that can be correlated with the loss of protection, as was observed in vaccinated mice that were challenged 25 months later [142]. A detailed study to identify the population of memory cells that is lost could then address possible approaches to sustain their survival for prolonged periods of time.

In a recent study addressing memory T cell subsets where mice were infected i.v. with *M. tuberculosis*, treated with antibiotics and rechallenged 6 months later [143], a population matching the phenotypic description for memory effector T cells, that is $CD44^{hi}/CD45RB^{lo}/CD62L^{lo}/CD69^{-}$ was identified. This population rapidly appeared in the rechallenged mice, increased in size (blasting), expressed CD69, as well as produced IFNγ. To show which of the T cell population phenotypes were responsible for protective memory, $CD45RB^{hi}$ and $CD45RB^{lo}$ cells populations were sorted and adoptively transferred to nude mice. It was shown that cells with a high expression of CD45RB, cells with phenotype similar to naïve and central memory T cells could confer protection, while cells with low expression (memory effector T cells) conferred little or no protection at all, as was the case of protection in the lungs.

1.14 Cytokines

In order to have an efficient response against invading pathogens, the host must recognize the pathogen, leukocytes need to be recruited and an appropriate immune response must be mounted. Cytokines play an important role in this process by orchestrating the interaction among the different cell types involved in a particular immunological process. Of particular interest are the cytokines produced by T helper cells, in which it is accepted that in order to mount an efficient protection against an intracellular pathogen infection the type 1 pattern of cytokines, such as IL-2 and IFN-γ must be produced. Following is a description of the roles of the most important cytokines known to be involved in the immune response to *M. tuberculosis*.

1.14.1 IFN-γ

Gamma-interferon (IFN-γ) is the primary cytokine known to activate macrophages and render them competent with antimycobacterial properties. Following infection with *M. tuberculosis* this cytokine was shown to be produced by CD4 [144] and CD8 T cells [106, 145] as well as by NK cells [146]. The influx of CD4 T cells capable of producing IFN-γ following infection peaked at the same time as maximal protection was observed, around the third week, suggesting the CD4 T cells can confer protection via the secretion of IFN-γ. The essential role of IFN-γ in the control of infection in mice was shown by the use of IFN-γ knockout (GKO) mice. Cooper *et al.* infected GKO mice with 10^5 CFUs of *M. tuberculosis* intravenously and showed that these mice succumbed to infection very rapidly with high bacterial load in the liver, lung, and spleen [102]. The same pattern of

infectivity was also seen in a low dose aerosol infection model. While both wild type and GKO mice developed similar granulomatous response in the first two weeks post infection, there was a dramatic increase in necrotic areas within the granuloma of the GKO mice 4 weeks after infection, with increased influx of granulocytes [102]. Flynn *et al.* observed similar characteristics when they infected GKO mice intravenously with a high dose of *M. tuberculosis* those mice survived were less than 20 days, while the wild type mice survived for more than 2 months [103]. Flynn *et al.* also showed that the GKO mice failed to express nitric oxide synthase following infection, which is necessary for the production of bactericidal RNI, confirming observation of others that peritoneal macrophages from BCG-infected GKO mice required addition of exogenous IFN-γ to generate RNI *in vitro* [147].

Other defects are also present in the GKO mice during infection, shown early in infection when bacterial loads between gene disrupted and wild type mice were similar, but GKO mice presented an increased proportion of activated lymphocytes with a concomitant increase in the recruitment of granulocytes to the granuloma formation [148]. The increase of activated lymphocytes in the GKO mice suggests a possible role for IFN-γ in inhibiting lymphocyte activation or expansion, which the authors suggest to be a consequence of the absence of nitric oxide.

Human genetic defects either in the IFN-γ or IFN-γ receptor (IFN-γR) genes cause severe susceptibility to BCG infection as well as recurrent nontuberculous mycobacteria infection in children [149]. The IFN-γR is composed of two chains, IFN-γR1 that binds to the cytokine, and IFN-γR2 required for signal transduction. Mutations in the IFN-γR1

gene resulting in the expression of an altered form that does not bind to IFN-γ [150], as well as mutations where this chain is not expressed on the surface of the cell, have been reported [151]. In the same manner, mutations in the IFN-γR2 gene are associated with different degrees of susceptibility to BCG and *M. avium* infections [152-154].

1.14.2 IL-12, IL-23, and IL-18

Interleukin 12 (IL-12) is a heterodimeric cytokine composed of two different peptides, p35 and p40, linked by a disulfide bond forming the biologically active p70 IL-12 protein. IL-12 acts through a high affinity receptor complex made of IL-12Rβ1 and β2 [155]. The major producers of IL-12 are dendritic cells and macrophages in response to bacterial cell wall components such as carbohydrates, lipids and glycolipids. LPS from gram-negative bacteria, lipoteichoic acids of gram-positive bacteria as well as LAM from mycobacteria have been shown to induce IL-12 secretion [156, 157]. IL-12 provides the link between innate and adaptive immune responses by inducing $CD4^+$ T cells to differentiate into TH1/IFN-γ-producing phenotype [158]. The p40 subunit can also form a homodimer with affinity to the IL-12 receptor complex but is incapable of intracellular signaling induction, and consequently is considered an antagonist of the bioactive IL-12 p70 [159]. IL-12 acts not only on Th1 cells but also on NK cells and γδ T cells to produce IFN-γ [160], which are important cells as an early source of Th1 inducing cytokine in response to pathogens before activated T helper cells can reach the site of infection. The association of IL-12 and protective response to intracellular pathogens have been described for several bacteria species [161-163].

The role of IL-12 in the control of infection against *M. tuberculosis* was first shown by treating the highly susceptible BALB/c strain with exogenous IL-12, which improved their survival rates, resulting in lower bacterial loads in tissues, and less severe pathological development. The protective effect of exogenous IL-12 was abrogated when the same experiment was performed in a IFN-γ gene-disrupted mouse model, suggesting that the effect is IFN-γ dependent[164]. Further proof of the important role of IL-12 during infection with *M. tuberculosis* was demonstrated by Cooper *et al.* who treated C57BL/6 mice with neutralizing antibodies against IL-12 resulting in a reduction in granuloma integrity as well as reduction in the ability for the mice to control bacterial growth [165]. Finally, the use of mice genetically deficient for IL-12 cytokine production due to the disruption of the IL-12 p40 gene gave unconditional evidence of the role of this cytokine in the protection against tuberculosis. IL-12 $p40^{-/-}$ mice were extremely susceptible to *M. tuberculosis* I.V infection [166]. The main defect in the IL-12 p40 deficient-mice was a pronounced decrease in IFN-γ and TNF-α production following infection, resulting in the absence of macrophage activation, delayed T cell activation and IL-12Rβ2 expression. It was also demonstrated that the IL-12Rβ2 expression by T cells following infection was IL-12 p40 dependent.

Although the bioactive IL-12 p70 is the most important cytokine to induce T cell to produce IFN-γ, studies using mice deficient for either the p40 or p35 subunits proved to result in different levels of susceptibilities to aerosol infection with *M. tuberculosis* [167]. Mice devoid of p40 subunits are highly susceptible to infection, with decreased production of IFN-γ and increased mortality. On the other hand, $p35^{-/-}$ mice were able to

control infection at an intermediate level, when compared to WT and $p40^{-/-}$ mice, generate an antigen-specific IFN-γ response, as well as having longer survival. The observed difference between the two models suggested that in the absence of the p35 molecule, other factors could be mediating an IL-12 like response, and the recent discovery of the IL-23 cytokine [168] prompted the authors to analyze the expression of this cytokine. Mice deficient for p35 retained high levels of mRNA expression for the p19 subunit, which when complexed with p40 forms IL-23, after infection with *M. tuberculosis* as compared to wild type mice. These findings suggested that in the absence of the p35 subunit, IL23 may be available to compensate for the absence of the bioactive IL-12 p70 [167].

IL-18, also know as interferon-gamma inducing factor, due to having similar properties as IL-12 in inducing IFN-γ production, has been implicated in the development of protective immunity to infections where Th1 cytokines play an important role, such as in tuberculosis. In humans, IL-18 was found to be expressed in higher levels in the sera of individuals with pulmonary tuberculosis compared to healthy individuals [169]. It was also shown that peripheral blood mononuclear cells (PBMC) as well as alveolar macrophages produce IL-18 in response to *M. tuberculosis* infection [170]. IL-18 knock out mice infected with *M. tuberculosis* had increased bacterial loads in the lungs with concomitant reduced splenic levels of IFN-γ compared to wild type [171]. IL-18 and IL-12 seem to act through similar and diverse mechanisms since double knock out mice are much more susceptible to *M. tuberculosis* infection than either IL-12 or IL-18 knock out mice alone [172].

1.14.3 Tumor Necrosis Factor (TNF)

Tumor necrosis factor -α was discovered in 1975 by Carswell *et al.*, who demonstrated that a protein released into the circulation following stimulation with LPS could cause rapid necrotic regression of certain forms of tumors [173]. Many other biological functions have since been attributed to TNF-α, and these include but are not limited to immunostimulation, resistance to infection agents, resistance to tumors, sleep regulation, and embryonic development. On the other hand increased TNF-α expression has also been associated with a deleterious effect in parasitic, bacterial, and viral infections [174]. Functional TNF-α exists as a trimer, and it exerts its action through one of the two receptors, p55 (TNFR-I) or p75 (TNFR-II), although most of TNF-α biological effects are mediated by the TNFR-I. Binding of TNF-α to its receptor activates a series of secondary signaling molecules that includes G proteins, transcription factors such as NF-κB and AP-1 and protein kinases that ultimately will lead to the activation of transcription of genes such as those coding for MHC and reactive oxygen intermediates or nitrogen radicals. TNF receptors have cytoplasmic motifs known as death domains that interact with adaptor proteins that will activate serine and cysteine caspases, inducing cell apoptosis.

TNF-α is produced mainly by macrophages in response to bacterial LPS, mycobacterial LAM, virus infections, protozoa and other microorganisms. TNF production can also be upregulated by several cytokines including TNF itself, IL-1, IFN-γ, granulocyte stimulationg factor , IL-2 and TGF-β [175].

In human tuberculosis, inhibition of *M. tuberculosis* replication in human alveolar macrophages was associated with increased production of TNF-α, and neutralization of TNF-α enhanced mycobacterial growth [176]. *In vitro* assays showed that apoptosis of human mononuclear phagocytes inhibited intracellular growth of *M. bovis* [177].

The important role of TNF-α in animals was first shown by Kindler *et al.* by treating mice with neutralizing anti-TNF antibody following an i.v. BCG infection and showing that the treatment resulted in a dramatic change in the granuloma structure formation as well in the capacity of the treated group to control infection [178]. Using antibody treatment as well as mice that did not have the functional TNFR-I gene, Flynn *et al.* showed that survival of *M. tuberculosis* infected mice was markedly diminished, when compared to control mice [179]. In mice without bioactive TNF-α due to gene disruption or antibody treatment, granulomas showed extensive necrosis and large amounts of mycobacteria, indicating that TNF-α played a central role in containing mycobacterial infection and preventing widespread tissue damage. In the absence of TNF-α, the increased susceptibility correlated with a delay in iNOS expression in the lungs, indicating that in order to have an efficient production of reactive nitrogen, it is necessary to have intact TNF-α stimulation. TNF gene disrupted mice infected with a low dose via the aerogenic route resulted in an acute widespread dissemination of *M. tuberculosis*, associated with extensive areas of necrosis in the lungs [180]. Although the absence of TNF-α did not affect the numbers of CD4+ and CD8+ T cells infiltrating in the infected lung, it was evident that these cells were restricted to the perivascular and peribronchial areas instead of being present within the macrophage rich areas of the lung granulomas as

seen in the wild type mice. In contrast to the findings of Flynn *et al.*, macrophages within the lungs of $TNF^{-/-}$ mice showed similar levels of iNOS expression when analyzed by immunohistochemistry. It seems very clear that TNFα plays an important role in the resistance to mycobacterial infection and is probably achieved by the formation of a functional granuloma that restrains the infection. In the absence of a characteristic granuloma the bacteria multiply unrestricted and tissue necrosis develops. The possible role of iNOS induction by TNF-α mentioned earlier has been questioned in other models and remains to be further analyzed.

TNF-α was also shown to be required for the control of chronic infection in mice. Mice treated with pentoxifylline, an inhibitor of TNF-α, at chronic stages of the infection resulted in no increase in the bacterial load in the lungs, but presented increased lung damage [181]. Similar findings were seen in antibody neutralized infected mice [182] providing further evidence for the role of TNF-α granuloma maintenance during infection with *M. tuberculosis*.

1.14.4 Lymphotoxin

Lymphotoxin α (LTα) is a member of the TNF superfamily, and its active trimeric form LTα3 is produced by T lymphocytes after antigenic or mitogenic stimulation, and binds to both TNF receptors. *In vitro* studies showed that LTα has the same activities as TNF, and for this reason was considered as functionally redundant. *In vivo* studies have shown that LTα plays an important role in the formation of secondary lymphoid organs during development [183]. To overcome the lack of peripheral lymph nodes in the

$LT\alpha^{-/-}$, chimeras were constructed by transferring bone marrow cells from LTα deficient mice onto irradiated mice [184]. The absence of LTα did not cause any deficiency in the T cell recruitment to the *M. tuberculosis* infected lung, but those cells were restricted to the perivascular and peribronchiolar areas similarly to that seen in $TNF^{-/-}$ infected mice. The $LT\alpha^{-/-}$ chimeras were highly susceptible to aerosol infection with *M. tuberculosis*, and did not form the granuloma as seen in the wild type chimeras. A large influx of neutrophils associated with necrotic areas in the lung was also seen. Since the TNF-α levels of expression seemed to be normal in the infected LTα chimeras it was proposed that LTα may signal through a yet undefined receptor [184] leading to the theory that both TNF-α and LTα contribute to granuloma formation by independent mechanisms.

1.14.5 TNF-α and chemokines

The important role of TNF-α in the formation and maintenance of granulomas as discussed earlier is likely related to its role in regulating the expression of chemokine and chemokine receptors. TNF-α can induce the expression of CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL2 (MIP-2), CCL2 (MCP-1), and CCL5 (RANTES) [185, 186]. The chemokine induction by TNF-α and its importance in the clearance of pathogens have been demonstrated by infecting TNF-α knock out mice with *M. smegmatis*. In the absence of TNF-α the avirulent bacteria is cleared at lower rates coinciding with a delay in the expression of CCL11 (eotaxin), CCL5 (RANTES), CCL4 (MIP-1β), CCL3 (MIP-1α), CXCL2 (MIP-2), and CCL2 (MCP-1). The delay in chemokine expression was reflected

in the formation of diffuse granulomas in the liver of TNF-α KO mice as compared to a tight aggregation seen in wild type mice [187].

1.14.6 IL-10

IL-10 is an anti-inflammatory cytokine produced mainly by macrophages and T cells that inhibits cytokine synthesis in monocytes and suppresses antigen-specific T-cell proliferation by down-regulation of macrophage class II MHC expression. Through its anti-inflammatory actions, IL-10 may prevent excessive local inflammation and tissue damage from an uncontrolled inflammatory response. At the systemic level, IL-10 may contribute to anergy in patients with tuberculosis by suppression of antigen-specific T-cell proliferation [188]. Another suppressive effect induced by IL-10 is by limiting apoptosis mediated by TNF-α by inducing the expression of soluble TNFR-II [189] that lacks a death domain like TNFR-I and competes for available TNF-α. Virulent strains of *M. tuberculosis* induce the expression of IL-10 *in vitro* and *in vivo* [190, 191]. Transgenic mice constitutively producing IL-10 were shown to be more susceptible to BCG i.v. infection in the chronic phase despite the fact that T cell proliferation and IFN-γ production were normal, suggesting that increased susceptibility may be due to suppression of macrophage function [192]. In another experiment using transgenic mice expressing IL-10, *M. tuberculosis* infected mice showed increase in susceptibility only in the chronic phase of infection with decreased TNF-α and IL-12p40 mRNA expression as well as decreased antigen-specific IFN-γ secretion [193]. Mice with disrupted IL-10 gene had no difference in susceptibility to BCG [194] or *M. tuberculosis* infection [195] despite the demonstration that in IL-10 KO mice infected with BCG had higher levels of

IFN-γ. The lack of additional protection that was expected for IL-10 KO mice may be due to the genetic background of the mouse strains used. In these experiments the gene-disrupted mice had as genetic background the naturally resistant C57BL/6 strain, which are able to generate a strong immune response that had little if any improvement with the absence of IL-10 expression. These studies suggest that IL-10 exerts an anti-inflammatory function by a mechanism that is not necessarily by IFN-γ inhibition.

1.14.7 Other cytokines

IL-1 is an important mediator of inflammatory response that causes fever, hypotension, and weight loss. It is produced by macrophages [190] including alveolar macrophages. It is produced upon stimulation with *M. tuberculosis* and specific mycobacterial components, including lipoarabinomannan and mycobacterial proteins with molecular weights of 20 and 46 kDa. Others have shown it to contribute to immunosuppression in human tuberculosis, where it is hypothesized that excessive IL-1 production induces IL-2 receptor expression by monocytes, which consume IL-2 and therefore inhibit T-cell proliferation/activation [196].

IL-6 can cause fever and hypotension. IL-1 and TNF-α stimulate mononuclear phagocytes to produce IL-6, which is a late component of the proinflammatory cytokine cascade. Human monocytes exposed to *M. tuberculosis* or lipoarabinomannan produce IL-6 through transcriptional activation of the IL-6 gene, mediated by nuclear factors NF-IL6 and NF-kB [190, 197]. Mice with disrupted IL-6 gene are highly susceptible to disease from *Listeria monocytogenes* and *Candida albicans*, suggesting that IL-6

contributes to immunity against intracellular pathogens [198, 199]. These knockout mice showed an early increase in bacterial load with a concurrent delay in the induction of IFN-γ when infected with *M. tuberculosis,* but were able to contain and control bacterial growth and develop a protective memory response to secondary infection [200]. High IL-6 concentrations enhance intracellular growth of *M. avium* in human monocytes by antagonizing the antimycobacterial activity of TNF-α in murine macrophages infected with *M. avium*. These data suggests that low levels enhance antimycobacterial immune defenses, whereas high IL-6 levels inhibit protective immunity [201].

TGF-β is an anti-inflammatory cytokine that downregulates monokyne synthesis, monocyte class II expression, and T-cell proliferation. PPD induces TGF-β production by monocytes *in vitro* [202]. TGF-β enhances intracellular growth of *M. tuberculosis* in human monocytes and abrogates the mycobacteriostatic effects of TNF-α and interferon-γ [203]. Its production is associated with depressed antigen-induced lymphocyte proliferation and interferon-γ production [204].

IL-8 is a chemoattractant for neutrophils and activated T-cells, which like IL-6 is part of the secondary cascade of cytokines whose production is induced by TNF-α and IL-1. IL-8 is produced by monocytes and alveolar macrophages when exposed to *M. tuberculosis* or to lipoarabinomannan [197]. Treatment of *M. tuberculosis* i.v. infected mice with monoclonal antibody to deplete neutrophils resulted in increased bacterial loads in the lungs, spleen and liver as well as lower levels of mRNAs for IFN-γ and iNOS in the liver compared to the nondepleted.

CHAPTER 2

Role of Chemokine CCL2 in the Protective response to Early Murine Pulmonary Tuberculosis

Introduction

Leukocyte trafficking in the host tissues is governed mainly by the action of chemokines and adhesion molecules. Chemokines are small proteins of 70 to 130 amino acids (8-14 kDa) that are secreted, with the exception of two chemokines, CXCL16 and CX3CL1, which are membrane bound. Chemokines are an extensive family of homologous related proteins grouped in four subfamilies based on the positioning of conserved cysteine residues. While most of the chemokines fall within two of the four subfamilies designated as CXC or alpha and CC or beta chemokines, there are also two other minor groups of chemokines consisting of C or gamma, and CX3C or delta chemokines. Since the discovery of IL-8 or human monocyte-derived neutrophil chemotatic factor as the first chemokines in the late 1980's [205], more than 40 different molecules have been characterized. A new nomenclature has been proposed to circumvent the confusion of the different names given to the same chemokine in the past, and this nomenclature is based on the two cysteine amino acids arrangements. To date more than 50 chemokines have been described and a comprehensive list is seen on Table 2.1.

Table 2.1: Classification of chemokines according to their role in the host and the receptors to which they bind.

	Homeostatic		Dual-function	
	Ligand	Receptor	Ligand	Receptor
	CXCL12 (SDF-1)	CXCR4	CXCL9 (MIG)	CXCR3
	CXCL13 (BCA-1)	CXCR5	CXCL10 (IP-10)	CXCR3
	CXCL14 (BRAK)	Unknown	CXCL11 (I-TAC)	CXCR3
	CCL18 (DC-CK1)	Unknown	CXCL16	CXCR6
	CCL19 (ELC, exodus-3)	CCR7	CCL1 (I-309)	CCR8
	CCL21 (SLC)	CCR7	CCL17 (TARC)	CCR4
			CCL20 (exodus-1)	CCR6
			CCL22 (MDC)	CCR4
			CCL25 (TEK)	CCR9

Adapted from Moser *et al.* [84], Zlotnik and Yoshie [206], and Rossi and Zlotnik [207].

Chemokines bind to extracellular matrices by binding to glycosaminoglycans [208] and by doing so they can generate a gradient of increasing concentration toward the site where they are being produced. The biologic effects of chemokines are mediated by seven-transmembrane-domain receptors coupled to G proteins. The primary role of the chemokine receptor is to attract leukocytes [206]. One of the immediate actions of chemokines on leukocytes is the formation and retraction of lamellipodia as result of polymerization and breakdown of actin filaments respectively [209]. Other important responses by leukocytes are the upregulation and activation of integrins which enable leukocytes to adhere to endothelial cells before migrating into the tissues. Chemokine diverse functions include hemopoietic cell migration, lymphoid cell development and trafficking, wound healing, angiogenesis/angiostasis, metastasis, and inflammation among others [207]. Accordingly chemokines can be grouped into three functional subfamilies known as inflammatory, homeostatic, and those with dual function (Table 2.1) [84].

The process of cellular extravasation occurs in a stepwise fashion. First leukocytes weakly interact with the cells of the endothelium through the action of adhesion molecules such as selectins and integrins, in a process known as rolling. If the circumscribing tissue is intact the leukocytes will remain in the blood circulation but in the case of a pathological condition the blood vessels will become dilated and consequently the flow is proportionally slowed down. The presence of chemokines released from the inflammatory process will promote the activation of leukocytes which is characterized by changes in the integrins to become with higher affinity. This results in

a step of the process known as arrest or adhesion where leukocytes remain attached to the endothelial cells for prolonged periods and eventually will lead to leukocyte extravasation to the tissues [210], and migration toward the site of inflammation following an increasing gradient of chemokines.

The nature of the host immune response to *M. tuberculosis* in the lungs is still poorly understood. Although the central T cell populations have been identified [211-213] and the key role of the cytokine interferon gamma (IFN-γ) established [102, 103], the formation of the granulomatous response in the lungs and its control by host molecules remains minimally analyzed. For instance, although the pulmonary tuberculosis granuloma in the mouse has been described in detail [77], it was surprising to observe that blocking the influx of macrophages from the bloodstream into the lungs did not affect the capacity of the animal to express protective immunity [80]. As a result of this, it has been proposed that this mechanism was cytokine driven, whereas the slower elements of the granulomatous response was predominantly controlled by chemokines [214].

A chemokine believed to be central to macrophage influx is CCL2, which binds to the CCR2 on macrophages, T cells, and natural killer cells [215-219]. Expression of this chemokine is markedly increased, both in the mouse model [85, 89] and in patients infected with tuberculosis [87]. However, it is unclear how important this chemokine is to the outcome of the acquired response, since mice lacking CCL2 were not found to be more susceptible to an intravenous infection with *M. tuberculosis* [91].

To address the issue of whether this also applied to direct infection of the lungs, I exposed CCL2 gene knockout mice to an aerosol infection with *M. tuberculosis*. As shown below, while a considerable reduction in inflammation and macrophage influx into the lungs was observed, only a transient increase in the bacterial load was seen, which was concomitant with a reduction in antigen-specific IFN-γ secreting cells entering this organ. These data suggest that recruitment of activated T cells into the lungs, rather than macrophage recruitment, is the key role of CCL2, at least in terms of the efficient expression of protective immunity.

Material and methods

Mice

CCL2 gene disrupted mice were generated by the disruption of the gene SCYA2 gene as described[91] and kindly provided by Dr. Barrett Rollins from the Dana Farber Cancer Institute. CCL2-KO mice were backcrossed to C57BL/6 mice for at least 6 generations. All mice were maintained in specific pathogen free conditions and were used between 8 and 10 weeks of age. C57BL/6 mice of matching age and sex purchased from The Jackson Laboratory (Bar Harbor, ME) were used as the wild type controls.

Bacterial Strains

M. tuberculosis strain Erdman, originally obtained from Trudeau Institute (Saranac Lake, NY), was grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and then frozen in aliquots at -70^0C until needed.

Aerosol Infection

Mice were infected using a Glas-Col aerosol generator (Glas-Col, Terre Haute, IN) such that 100 bacteria were deposited in the lungs of each animal. The number of viable bacteria in target organs was determined at various time points by plating serial dilutions of partial organ homogenates on nutrient Middlebrook 7H11 agar and counting colonies after 21 days of incubation at 37°C.

Media and reagents

Lung and spleen cell suspensions were cultured in Dulbecco's minimal essential medium (DMEM Cellgro, Herndon, VA) containing 10 mM *N*-2-hydroxy-ethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES, Sigma-Aldrich, St Louis, MO), 2mM L-glutamine (Sigma-Aldrich), and MEM nonessential amino acids (Sigma-Aldrich) supplemented with 10% heat-inactivated, endotoxin-low fetal calf serum (GIBCO BRL; Invitrogen, Carlsbad, CA).

Cell culture

Infected animals were killed by CO_2 asphyxiation and lung, spleen and mediastinal lymph nodes were harvested from infected animals at indicated times after infection. Lungs were perfused through the heart with cold saline containing 30U/mL of heparin. After removal, the lungs were sectioned in ice-cold media using sterile razor blades. Dissected lung tissue was then incubated in DMEM containing collagenase IX (0.7 mg/ml; Sigma-Aldrich) and deoxyribonuclease (30 μg/ml; Sigma-Aldrich) at 37°C for 30

min. Single-cell suspension from digested lung, spleen or lymph nodes was obtained by passing the organs gently through a 70μm nylon cell strainer. Red blood cells were lysed using Gey's solution (0.15M NH_4CL, 10mM $KHCO_3$). Cells were counted and were either cultured with antigen (at $5x10^6$ cells/ml) or analyzed by flow cytometry.

ELISA for IFN-γ

T cells were cultured with purified culture filtrate proteins of *M.tuberculosis* (10μg/ml; provided by NIH Contract AI-75320) for five days and then assayed for the presence of IFN-γ by ELISA using capture antibodies (BD Pharmingen, San Diego, CA). Cells were also cultured in the presence of 5μg/mL of concanavalin A (Sigma-Aldrich) as positive control. Briefly, the primary antibody (clone R4-6A2) was incubated overnight in 96-well flat bottom plates (Immulon, Thermo Labsystems, Chantilly, VA) in carbonated coating buffer. Excess antibody was washed away using PBS-Tween 20 (PBS-T) and samples dispensed in triplicate into wells. The presence of cytokine was detected by the addition of a secondary biotinylated antibody (clone XMG1.2), followed by avidin peroxidase and substrate (2,2'-azinobis[3-ethylbenz-thiazline-6-sulfonic acid]). The amount of cytokine was determined by plotting the OD readings on a standard curve made with serial dilutions of recombinant IFN-γ.

Flow cytometry

A single cell suspension of either spleen or lung was prepared as described above and resuspended in deficient RPMI (Irvine Scientific, Santa Ana, CA) containing 0.1%

azide. Some cells were stimulated with anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51) and monensin for four hours prior to staining. Cells were incubated for at least 1 hour with dRPMI containing 0.1% azide and stained with specific antibody for 30 min at 4°C and in the dark. Cells were stained with antibodies recognizing CD4 (Allophyocyanin (APC) or Phycoerythrin (PE) labeled clone (GK1.5), CD8 (APC labeled clone 53-6.7), CD44 (FITC labeled clone IM7), and CD62L (PE labeled clone MEL-14) (all from BD Pharmingen, San Diego, CA). Control cells labeled with isotype antibodies were also prepared. After being stained on their surface, stimulated cells were permeabilized with Perm Fix/Perm Wash (BD Pharmingen) and the presence of intracellular IFN-γ was probed with labeled antibody (FITC-labelled clone XMG1.2). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA). All antibodies were purchased from BD Pharmingen, including appropriate isotype control antibodies that were included throughout the analysis. Cells were collected in a FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA), dual laser, flow cytometer with excitation at 488nm and 633nm, and analyzed using CellQuest software (BD Biosciences). Lymphocytes were gated based on their forward and side scatter characteristics. Ten thousand CD4 or CD8 positive lymphocytes were then analyzed for their expression of the activation markers CD44 and CD62L.

Histology

The posterior lobe of the right lung was perfused with 10% paraformaldehyde in PBS and used for histological analysis. Hematoxylin and eosin staining was performed on sectioned tissues. Slides were coded, evaluated by a pathologist, and graded according to degree of lesion present.

RT-PCR analysis of gene expression in the lungs

The anterior lobe of the right lung was removed and placed into test tubes with 1 ml of Ultraspec (Biotecx, Friendswood, TX). Samples were homogenized and immediately frozen at -70°C until extraction. Extraction of total RNA was performed by adding 200 μl of chloroform to each sample for organic extraction. The aqueous phase was precipitated first with isopropanol and second by using the combination of 70% ethanol and 0.3M acetic acid. Total RNA was quantified by OD 260nm spectrophotometric readings and 1 μg of total RNA was used for the reverse transcriptase reaction using murine Maloney-leukemia virus reverse transcriptase (Invitrogen Life Technologies, Grand Island, NY) and random primers (Roche Applied Science, Indianapolis, IN). Following the reverse transcription, 175 μl of ultra pure water was added to each sample to yield a final volume of 200 μl. Five microliters of each cDNA sample were used for amplification by PCR with program of 23 cycles for HPRT and 30 cycles for iNOS each consisting of denaturation at 95°C for 1 min., annealing at 54°C for 1 min and extension at 72°C for 1 min. PCR products were subject fo a final extension period of 7 minutes at 72°C and stored at -20°C. All primers were used at a final concentration of 0.2 mM in a

50 ml-volume PCR mixture containing 0.2 mM DNTP mix, 1X PCR buffer containing MgCl2 for a final concentration of 1.5 mM, and 1 U of Taq DNA polymerase. PCR products were subject to eletrophoresis in 1% agarose, Southern blotting, and hybridization with fluorescein-conjugated specific probes which were visualized using a commercial chemiluminescent detection kit (ECL detection system, Amersham Life Sciences, Arlington Heights, IL) as described elsewhere[220]. The sequences of the primers and probes used are listed in table 2.2. The densities of the chemiluminescent signals on the detection film were measured in pixels using a flatbed scanner (Epson Expression Scanner 800) and quantified using Image Software (shareware; National Institutes of Health, Bethesda, MD). The housekeeping HPRT pixel values were used to normalize any discrepancies in the amount of starting cDNA.

Table 2.2: Sequence information for the primers and probes used.

Gene product	Accession number	Sense (S) and antisense (AS) primers	Probe sequence
HPRT	K01507	(S) GTT GGA TAC AGG CCA GAC TTT GTT G (AS) GAT TCA ACT TGC GCT CAT CTT ACG C	GTT GTT GGA TAT GCC CTT GAC
iNOS	M84373	(S) CTG GAG GAG CTC CTG CCT CAT G (AS) GCA GCA TCC CCT CTG ATG GTG	CTG GAT GAG CTC ATC TTT GCC

Statistical analysis

Four mice per group of three different experiments were used for all studies. Student's t test was used for comparisons between wild type and knock out groups.

Results:

Mice lacking CCL2 are more susceptible to pulmonary tuberculosis during the early stage of infection.

To assess possible differences in susceptibility, control and CCL2-KO mice were exposed to approximately 100 bacteria by aerosol (Fig.2.1). The uptake and initial bacterial load were similar but by day 20 the CCL2-KO mice showed a 10 fold higher bacterial load compared to control animals. Despite this, the infection was then controlled, and at later time points did not differ significantly between the two groups. Mice lacking CCL2 showed similar numbers of bacteria escaping to the spleen and liver, indicating that this event was not affected by the absence of this molecule.

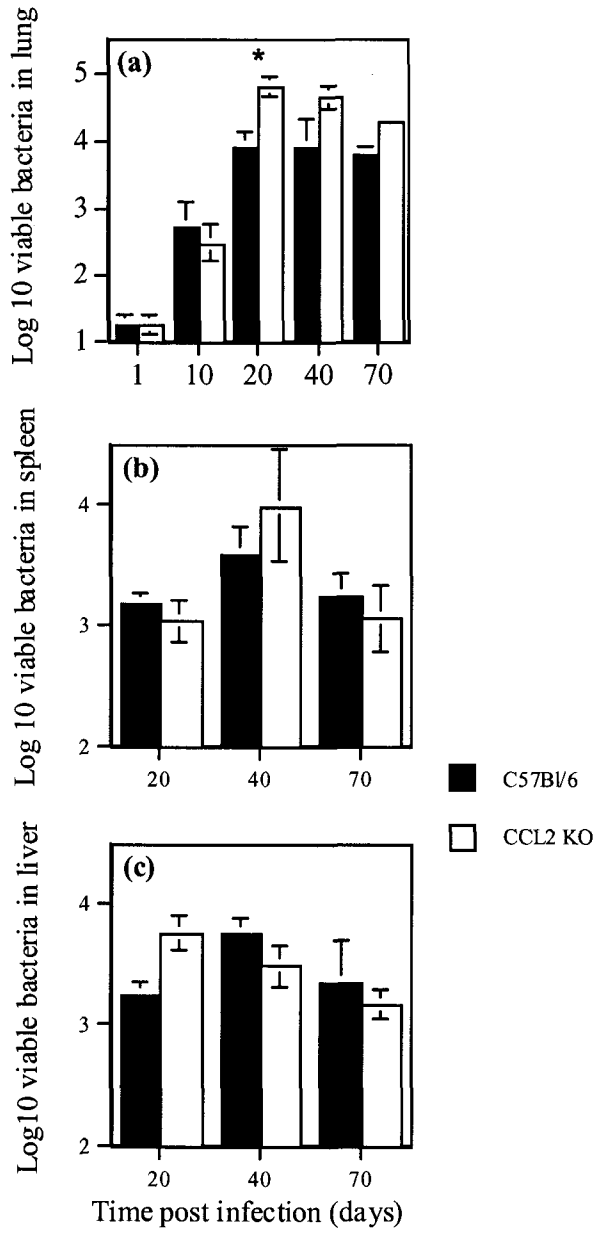

Figure 2.1 Mice lacking the gene for chemokine ligand 2 (CCL2) differ in their susceptibility to low dose aerosol infection with *M. tuberculosis*. Control (closed circles) and CCL2 gene disrupted (open circles) mice were infected by the aerogenic route. The lungs (a), spleen (b) and liver (c) were evaluated for bacterial growth at different time points after infection. Data points represent the mean (± standard error of the mean) values from four mice. The value marked with an asterisk is significantly different from the control mice (*p=0.019, Students's *t* test). Data are representative of three separate experiments.

Mice lacking CCL2 are able to activate T cells equivalent to control mice.

To determine whether the difference in susceptibility observed was due to impairment of T cell activation, cultures of spleen and mediastinal lymph nodes cells were stimulated with mycobacterial culture filtrate proteins (CFP) for 5 days and assayed for the production of IFN-γ. As shown in Fig. 2.2 the peak of response is seen by day 20 and the response decreased at later time points in correlation with the control of bacterial growth. Uninfected mice produce minimal levels of IFN-γ [193]. In both organs analyzed the production of IFN-γ by CFP stimulated cells were similar when comparing wild type to CCL2-KO mice, indicating that both groups were equally capable of activating T cells.

Mice lacking CCL2 recruit $CD44^{hi}/CD62L^{lo}$ in comparable levels to control mice but are less able to recruit IFN-γ producing activated lymphocytes into the lungs.

The activation of T cells was analyzed by flow cytometry. As shown in Fig. 2.3, activated $CD4^+$ and $CD8^+$ T cells, defined by populations with high levels of expression of the CD44 molecule ($CD44^{hi}$) and low levels of the selectin CD62L ($CD62^{lo}$) increased as the infection progressed. This was associated with a parallel increase in the population of $CD4^+$ and $CD8^+$ that expressed IFN-γ (Figs. 2.3 c and d). CCL2 deficient mice showed a tendency to have higher numbers of activated CD4 and CD8 T cells than wild type mice, though the differences were not significant.

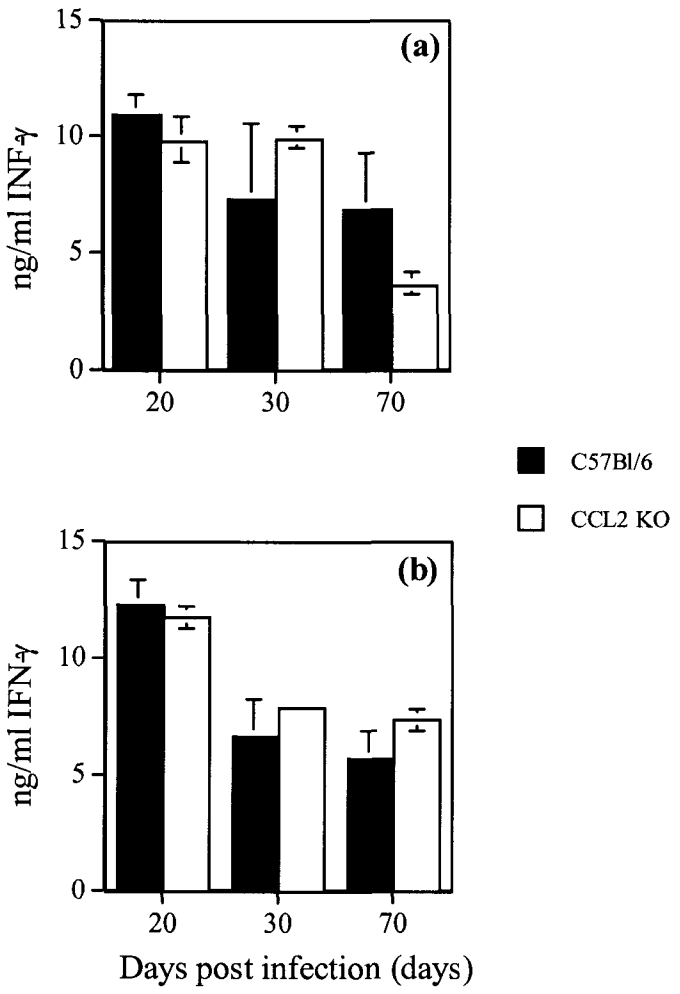

Figure 2.2 Mice lacking the gene for chemokine ligand 2 (CCL2) were capable of generating an antigen-specific IFN-γ response to mycobacterial antigen in the spleen or draining lymph node. Control (solid bars) or CCL2-KO (open bars) were infected as in Figure 1 and splenocytes (a) and mediastinal lymph node cells (b) were analyzed by ELISA for their ability to generate IFN-γ in response to mycobacterial antigen. The amount of IFN-γ produced by unstimulated cells was under the level of detection of the assay. The data shown is representative of 3 separate experiments.

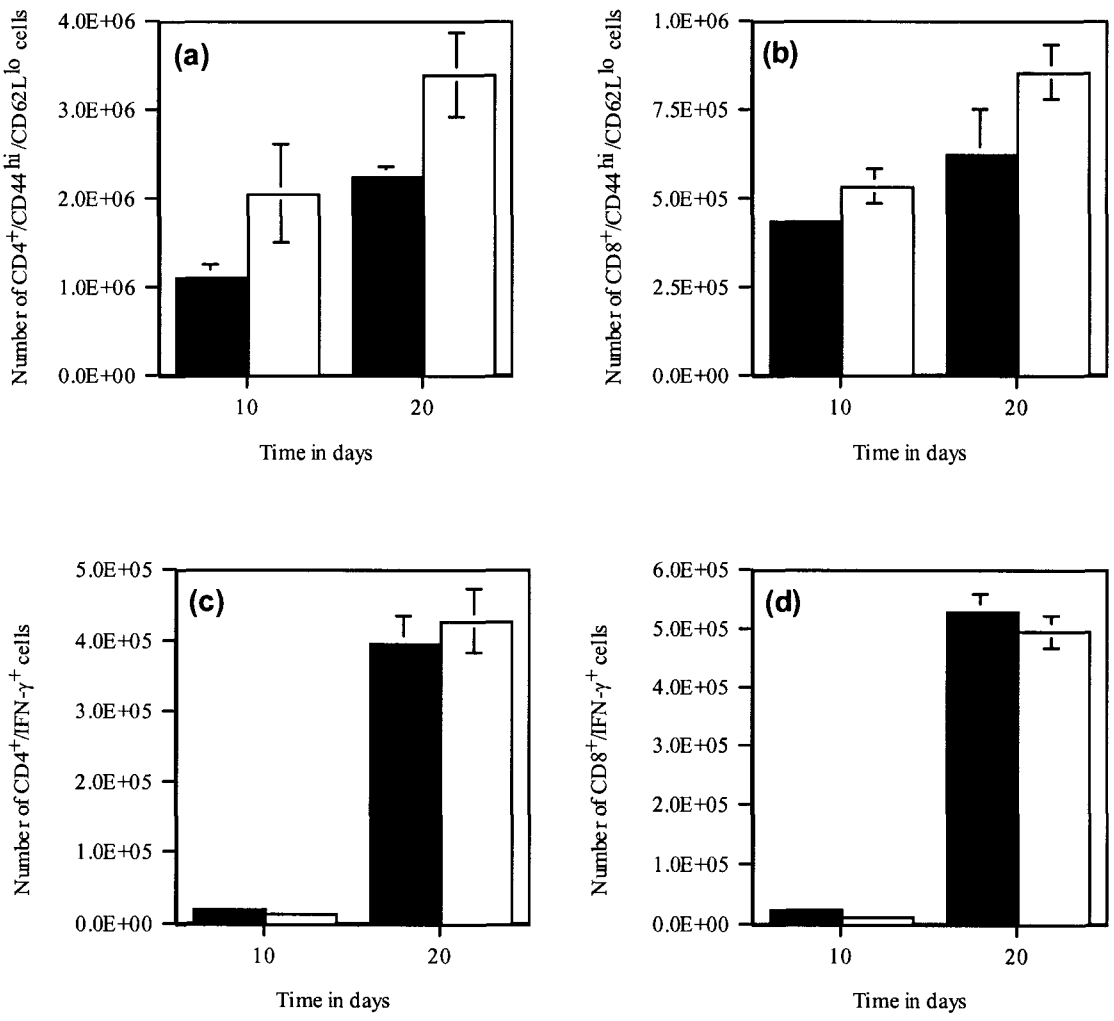

Figure 2.3. Mice lacking the gene for chemokine ligand 2 (CCL2) were able to activate T cells similar to control mice following aerogenic challenge with *M. tuberculosis*. Control (solid bars) or CCL2-KO (open bars) mice were infected as described in Fig. 1, and splenocytes were analyzed by flow cytometry for expression of $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ (a) and $CD8^+$ $CD44^{hi}$ $CD62L^{lo}$ (b). The number of cells expressing high levels of CD44 and low levels of CD62L per spleen is reported. Splenocytes were also stimulated with anti-CD3/anti-CD28 and monensin and then analyzed for intracellular IFN-γ production (c and d). The bars represent the means of four mice per group, and the graph shows one experiment representative of three.

Subsequent analysis of T cells entering the lungs revealed a similar picture in terms of the numbers of cells with an activated phenotype (Figs. 2.4 a and b). However, in contrast to the spleen cell data, significantly fewer cells present in the lungs at day 20 stained positive for IFN-γ (Figs. 2.4 c and d).

Histological analysis of CCL2KO mice.

The morphology of the infected lungs was followed over the first seventy days in order to allow the granulomatous response to fully develop. An assessment of lung lesions was done by a pathologist who evaluated the sections. Each slide was graded according to number of lesions present, lesion extension, presence of macrophages, lymphocytes and neutrophils, as well as for the development of necrosis. In Table 2.3 a compilation of such findings shows that the lesion development in the lungs of CCL2-KO mice occurs in a similar fashion as in wild type mice up to day 40 when a slight increase in macrophage numbers proportional to lymphocytes is seen in the wild type group compared to knock out. This is exacerbated by day 70 post infection and as shown in Fig.2.5, by day 70 post infection the granulomatous inflammatory response in the control mice was substantially more florid than that seen in the CCL2-KO mice. Moreover the distribution of mononuclear cells was clearly different, with much fewer macrophages seen in the CCL2-KO mice. There were no significant differences in the histopathology observed in the lungs prior to day 70.

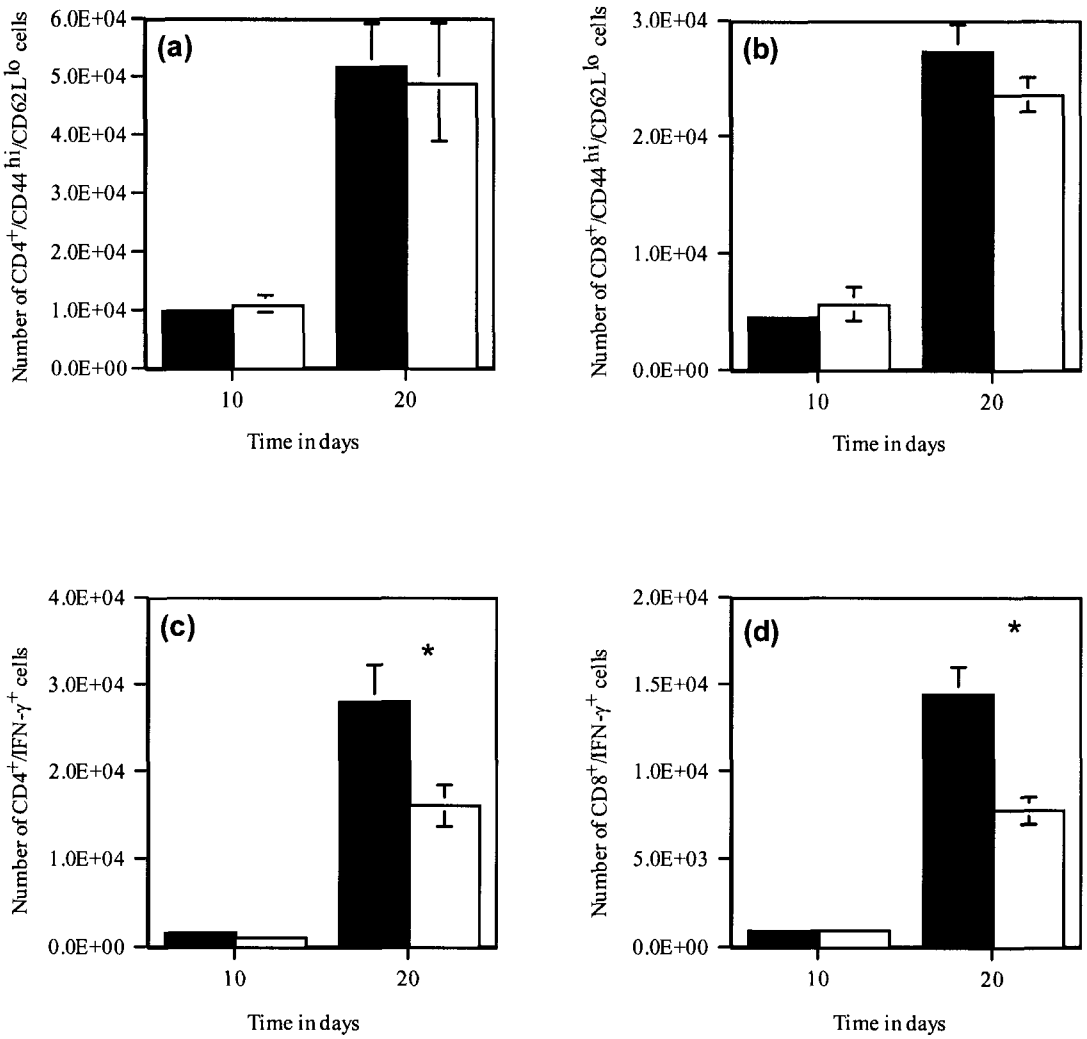

Figure 2.4 Mice lacking the gene for chemokine ligand 2 (CCL2) fail to recruit as many IFN-γ producing T lymphocytes to the lung as wild type mice. Control (solid bars) or CCL2-KO (open bars) mice were infected as described in Fig. 1.1, and lung cells were analyzed by flow cytometry for expression of $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ (a) and $CD8^+$ $CD44^{hi}$ $CD62L^{lo}$ (b). The number of cells expressing high levels of CD44 and low levels of CD62L per lung is reported. Lung cells were also stimulated with anti-CD3 and anti-CD28, and monensin and then analyzed for intracellular IFN-γ production (c and d). The bars represent the means of four mice per group, and the graph shows one experiment representative of three. Bars marked * were significantly different (* $P<0.05$, Student's *t* test).

Table 2.3 Histological evaluation of lung sections stained with hematoxylin and eosin.

	Lesion Extension[a]	Macrophage score [b]	Lymphocyte score c	PMN score [d]	Necrosis[e]	# lesions[f]
C57BL/6 day 10	nd	0	0	0	0	nd
CCL2-KO day 10	nd	0	0	0	0	nd
C57BL/6 day 20	F	0.5	0.5	0	0	1 / 4
CCL2-KO day 20	F	0.5	0.5	0	0	1 / 4
C57BL/6 day 40	MF	2	1	0.5	0	6 / 4
CCL2-KO day 40	MF-C	1.5	1	0.5	0	4 / 3
C57BL/6 day 70	MF-C	2.5	1	0.5	0.5	5 / 4
CCL2-KO day 70	MF-C	1	1.5	0.5	0.5	6 / 3

[a] Lung lesions were scored as not detected (nd), being discrete or focal (F), multiple but discrete lesions (MF), or multiple lesions that had expanded and coalesced with other nearby lesions (MF-C).

[b,c,d] Relative numbers of macrophages, lymphocytes and neutrophils respectively were determined. Scores were qualitatively designated ranging from 0 to 3, with increments of 0.5.

[e] Necrosis was absent in the first 40 days of infection and by day 40 a moderate scattered necrosis within the inflammatory lesions were seen.

[f] Number of lesions refers to number of lesions per sections examined. For example 6 / 4 represents that within 4 sections examined there were 6 lesions. If no lesion was observed it was marked as not detected (nd).

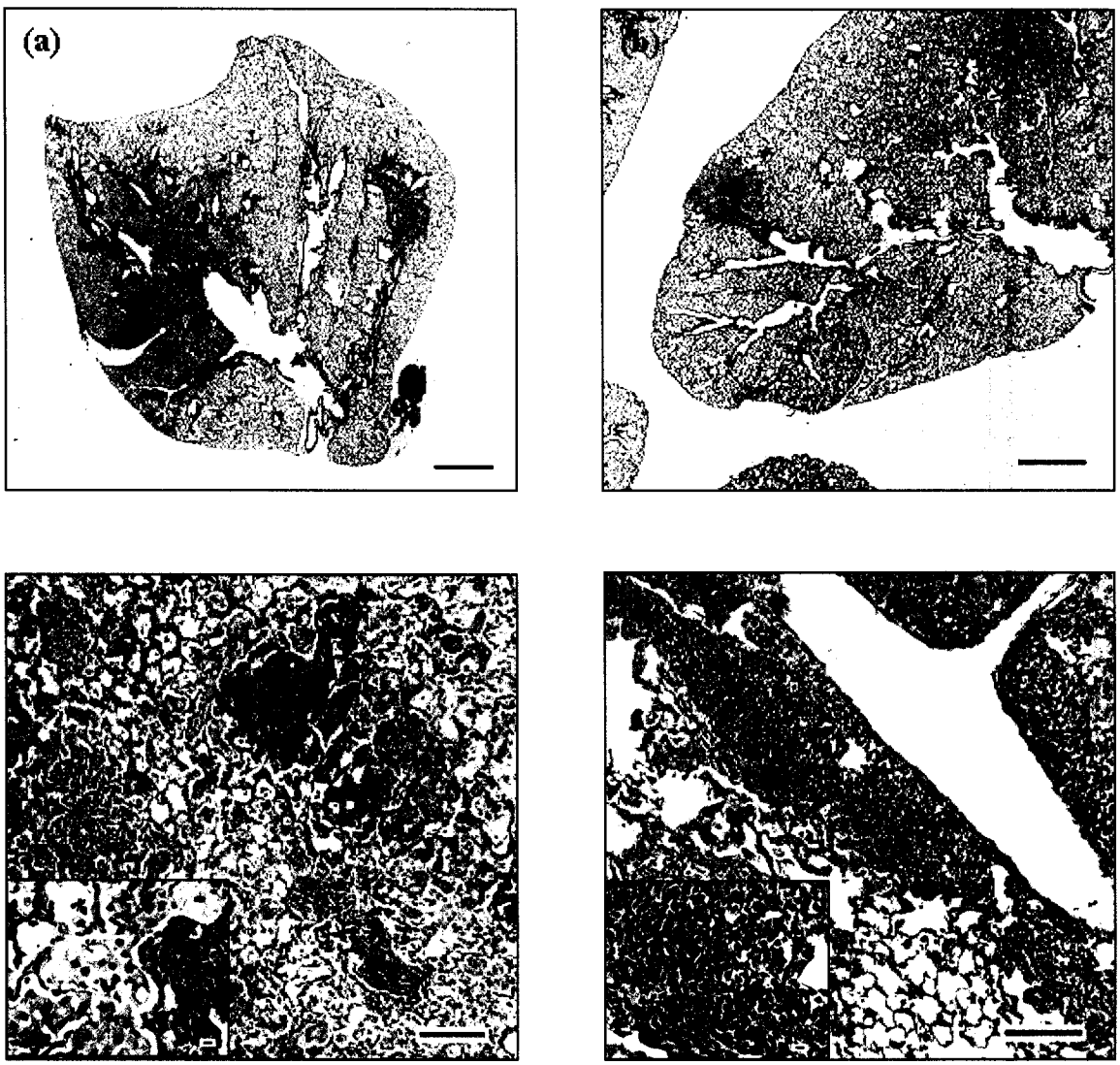

Figure 2.5. Representative micrograph showing lung lesions in C57BL/6 mice (a and c) and chemokine ligand 2 knockout CCL2-KO mice (b and d) 70 days after aerosol infection with *M. tuberculosis*. Hematoxylin and eosin staining. Size bars are 1mm a and b, 100 μm c and d, and 10 μm insets. Note macrophagic rich granulomatous response (asterisk and inset of c) in C57BL/6 mice, while in CCL2 deficient mice there is a predominant accumulation of lymphocytes (arrows and inset of c and d).

Pulmonary levels of mRNA for iNOS

In order to qualitatively determine if macrophagic function could be compromised in the CCL2-KO mice I evaluated the levels of mRNA for iNOS. As shown in Figure 2.6 expression of iNOS in the lung becomes evident after 40 days of infection, although knock out mice may have an earlier expression at day 20 (Fig. 2.6 a). After normalization for the input of cDNA using the HPRT message there were no significant differences in the expression of iNOS between the groups although there was always a trend of CCL2-KO mice having higher levels of iNOS then wild type mice. Thus macrophage function as measured by levels of iNOS mRNA was not involved in the increased bacterial load seen at 20 days post infection in CCL2-deficient mice.

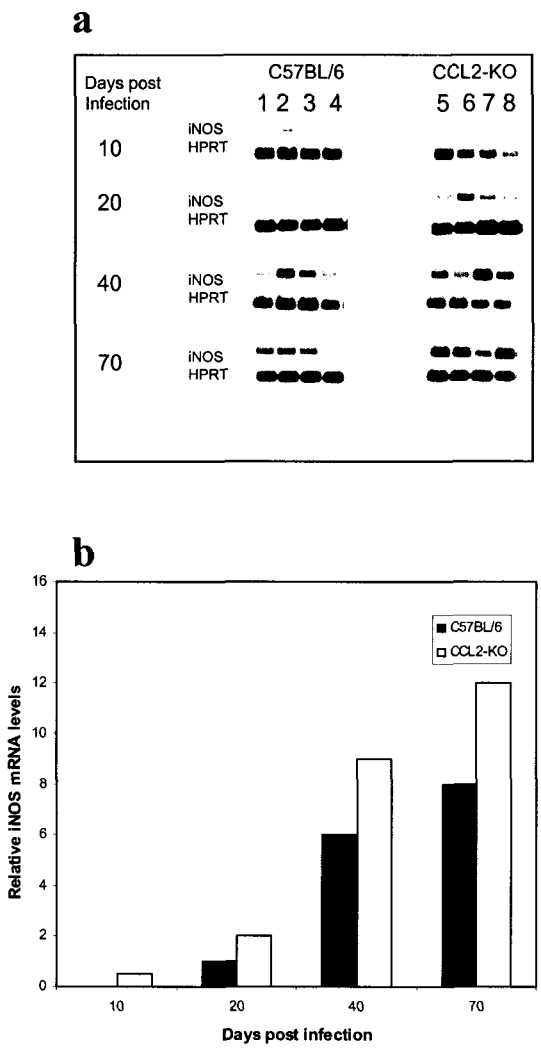

Figure 2.6 CCL2-KO mice had similar levels of iNOS mRNA expression as wild type. iNOS mRNA expression in the lungs of C57BL/6 wild type and CCL2-KO mice was detected by Southern blotting specific PCR products (a). Normalization of amount of cDNA input was done using the amplification of the housekeeping gene HPRT. Relative quantification of iNOS levels was done based on band intensities after normalization (b). The data shown in a represent samples from 4 mice in each group (lanes 1-4 are C57BL/6 and lanes 5-8 are from CCL2-KO mice). The arbitrary value of 1 was given to the group that had the least amount of message (CCL2-KO day 10) that was above threshold of detection.

Discussion

The results of this study show that mice unable to express the CCL2 chemokine exhibited a transient increase in bacterial load in the lungs over the first month of infection with *M.tuberculosis* but were still able to establish a state of chronic disease. It was notable that this event occurred despite a visually evident reduction in macrophages arriving in the lung tissues over this early period of time. Examination of the T cell response showed that the animal was fully capable of generating activated CD4 and CD8 T cells in the draining lymph nodes and spleen, but there was a transient reduction, observed on day 20, in the numbers of cells focusing in the lungs that were capable of secreting IFN-γ.

It is reasonable to hypothesize, therefore, that the transient increase in the bacterial load in the lungs was due to fewer IFN-γ+ cells accumulating at this time. The data also confirms earlier observations[80] that mice lacking the capacity to efficiently recruit monocytes from the blood into areas of infection does not in fact directly prevent the animal from expressing protective immunity. In this regard, such information has been used to propose[214] that protective immunity is a separate mechanism to the influx of monocytes and the development of the granulomatous response.

In fact, while CCL2 is regarded as a major chemokine for macrophages and monocytes, the data obtained here seem to indicate that in the context of pulmonary tuberculosis a more important role for this molecule is in the efficient recruitment of antigen-specific IFN-γ secreting T cells into the lungs. The observation that CCL2 is

involved in the attraction of T lymphocytes is not novel and has been described in other reports [216, 221].The mice used here retain fully functional CCR2 receptors, and therefore are responsive to other members of the MCP family, such as CCL7, CCL8, and CCL12[222], suggesting that CCL2 may play a selective role in recruiting T cells into the lungs. This may be organ-specific, since loss of the ability to make CCL2 has been shown[91] not to affect the course of tuberculosis in mice given a high dose intravenous infection.

In contrast to this latter study, a defect or transient delay in priming T cells to secrete IFN-γ and to focus in the lungs was observed in another high dose intravenous infection model in which mice lacking the CCR2 receptor were used[89]. Because of the high dose, these mice were rapidly killed, but defects in both macrophage influx and the capacity of T cells to secrete IFN-γ were observed. In a more realistic model using low dose aerosol infection, CCR2 deficient mice were capable of controlling the infection despite a reduced influx of macrophages, and again defects in T cell priming and influx were observed[90]. These latter observations are therefore very consistent with those of the current study.

One minor difference between CCR2-KO mice and CCL2-KO was the observation in the CCR2 deficient mice of reduced T cell priming and activation, whereas in the current study this parameter, measured in terms of the arrival of $CD44^{hi}$ $CD62^{lo}$ phenotype cells, showed no differences. A simple explanation for this is that ligands for the CCR2 other than CCL2 play a role in this event.

Other possible explanation for the transient increased susceptibility presented by CCL2-KO mice could be that the absence of CCL2 production during the first few weeks post could result in a deficiency in the expression of other chemokines and/or cytokines necessary for the control of the infection. The analysis of chemokines and cytokine expression in the lungs of infected mice was not performed in the present study but it would be interesting to know if this hypothesis may play a role during infection.

Macrophages are key elements in the control of pulmonary tuberculosis as they are the host for mycobacterial infection as well as the effector cells responsible for killing bacteria. Since the major role of CCL2 is to attract monocytes it was important for us to evaluate if the absence of this chemokine would result in decreased macrophage infiltration into the inflammation sites and consequently provide poor antimicrobial effects. For this I qualitatively analyzed histological samples from CCL2-KO and wild type mice and in the first 40 days post infection no major deficiency in macrophage influx was seen. A significant effect of CCL2 deficiency was seen on macrophage influx at day 70 post infection, where the granuloma from CCL2-KO mice contained many more lymphocytes then normally seen in wild type mice. Despite this disproportional balance of lymphocytes and macrophages, knock out mice were able to control the infection as well as wild type mice at this time point. Thus if macrophage trafficking is impaired in the CCL2-KO mice, it is in later periods of the infection when no difference in controlling infection is seen between the two groups of mice. In corroboration to this I found no significant differences in the levels of expression of mRNA for iNOS.

The controlled influx of immune cells into sites of bacterial implantation in the lungs where immunity must be expressed is clearly both a complex and sustained process, and in terms of chemoattractant ligands and their corresponding receptors there are considerable levels of redundancy that makes analysis difficult. Better understanding of such mechanisms is obviously needed however, since control of the granulomatous process by chemokines is a central event in tuberculosis, and therapeutic modulation of this process may help limit the eventual lung damage caused by these structures as well as potentially modify breakdown and the onset of reactivation disease.

Chapter 3

Mycobacterium tuberculosis infection progression in mice lacking expression of the CD44 adhesion molecule.

Introduction

Mice infected by aerosol exposure to *Mycobacterium tuberculosis* gradually develop severe granulomatous inflammation in the lungs. The granuloma formation is dependent on the secretion of cytokines such as tumor necrosis factor alpha (TNF-α) [180] and CC chemokine ligand 2 [89, 223], as well as the expression of appropriate adhesion molecules by both endothelial cells and circulating leukocytes. The role of granuloma formation encompasses both innate and acquired immunity, the latter involving the specific influx of protective T cells and macrophages, in order to prevent or limit dissemination of the infection from the lungs [79, 80]. Successful containment of the infection requires the influx of T cells and mononuclear phagocytes from the blood [224] leading to activation of the latter by interferon gamma (IFN-γ) [102, 103].

Migration of T cells into sites of inflammation where immunity must be expressed is an integral and crucial component of cell-mediated immunity. Cell extravasation and migration is accomplished in inflamed tissues as a consequence of the presence of chemokine in the tissue, as well as the expression of specific adhesion molecules and chemokines receptors on leukocytes. CD44 is an adhesion receptor involved in multiple functions including tissue remodeling and T cell extravasation [225]. Adhesion molecules are cell surface glycoproteins that have large extracellular domains, and are involved in cell-cell or cell-matrix interactions, as well as in cell motility and differentiation. CD44 is a type I transmembrane glycoprotein that is expressed by most cell types, including

leukocytes. The CD44 gene is composed of 10 standard exons encoding the standard protein isoform (CD44s), while 10 other variant exons joined in different combinations resulting in the expression of variant isoforms (CD44v). The CD44 glycoproteins have the property to bind to proteins of the extracellular matix (ECM). The major ligand of CD44 is hyaluronate (HA), but binding of CD44 to ECM proteins depends on post-translational modifications. The cytoplasmic domain of CD44 was shown to interact with ankyrin[226] and the ERM family (erzin, radixin, and moesin) of proteins[227], which in turn connect elements of the plasma membrane with the actin filament network of the cell providing the link necessary for cell movement. Several lymphocyte functions appear to be dependent upon CD44 expression. Activation of T cell induces an increase in the levels of surface expression of CD44 [228], which endow those cells with the ability to bind to vascular endothelial cell via binding of HA. The targeting of lymphocytes to inflammatory by CD44-HA interaction is enhanced by the induction of HA synthesis in vascular endothelium by proinflammatory cytokines, TNF-α, and IL-1β [229]. More recently, evidence has emerged showing an important role for CD44 in resolving inflammation in the lungs and thus preventing lung tissue damage. If CD44 is absent due to targeted gene disruption (CD44-KO mice) then injection of an inflammatory material into the lungs causes massive inflammation, which can be fatal [230]. It would be anticipated, therefore, that pulmonary infection of CD44-KO mice with *M. tuberculosis* would cause uncontrolled inflammation, as well as a reduction in the number of T cells capable of entering the lungs.

To address this, wild type and CD44-KO mice were exposed to a low dose aerosol infection with virulent *M. tuberculosis*. As the infection progressed both the alveolar

spaces and lung tissues showed abnormally high numbers of neutrophils entering these areas, but despite this no differences were seen in the bacterial load or in the T cell influx over this period of time. These data indicate that while the loss of CD44 on leukocytes results in a severe pyogenic form of granulomatous inflammation in the lungs during tuberculosis infection, its absence did not affect the generation, arrival, or expression of protective immunity. These data thus confirm the important role of CD44 in controlling and limiting lung inflammation, at least in the context of granulocyte influx, but show that CD44 is not an essential molecule needed for T cell entry into the lungs.

Materials and methods

Mice

C57BL/6 wild type and CD44 gene-disrupted mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in specific pathogen free conditions and were used between 8 and 10 weeks of age.

Bacterial strains

M. tuberculosis strain Erdman, originally obtained from Trudeau Institute (Saranac Lake, NY), was grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and then frozen in aliquots at –70°C until needed.

Bacterial infections

Mice were infected using a Glas-Col aerosol generator (Glas-Col, Terre Haute, IN) such that about 100 bacteria were deposited in the lungs of each animal. The number of viable bacteria in target organs was determined at specific time points by plating serial dilutions of partial organ homogenates on nutrient Middlebrook 7H11 agar and counting colonies after 21 days of incubation at 37°C.

Bronchoalveolar lavages (BAL)

The thoracic cavity was opened, the trachea was exposed, cannulated, and the lungs were washed with 1ml of ice cold phosphate buffered saline (PBS) by gentle instillation. Cells were washed, resuspended in Dulbecco's minimal essential medium (DMEM Cellgro, Herndon, VA) containing 10 mM *N*-2-hydroxy-ethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES, Sigma-Aldrich), 2 mM $_{L}$-glutamine (Sigma-Aldrich), and 1% MEM nonessential amino acids (100X; Sigma-Aldrich) supplemented with 10% heat-inactivated, endotoxin-low fetal calf serum (Atlas Biologicals, Fort Collins, CO), cytospun onto glass slides, and stained with hematoxylin and eosin.

Single-cell suspension preparation

Lungs from infected and age-matched control animals were perfused through the heart with cold PBS containing 30 U/mL of heparin (Sigma-Aldrich, St Louis, MO). After removal, the lungs were sectioned in ice-cold media using sterile razor blades. Dissected lung tissue was then incubated in DMEM containing collagenase IX (0.7

mg/ml; Sigma-Aldrich) and deoxyribonuclease (30 μg/ml; Sigma-Aldrich) at 37°C for 30 min. A single-cell suspension from digested lung tissue was obtained by passing the organs gently through a 70 μm nylon cell strainer. Red blood cells were lysed using Gey's solution (0.15 M NH_4Cl, 10 mM $KHCO_3$). Cells were resuspended in DMEM with supplements, counted, stained with fluorescent antibodies, and analyzed by flow cytometry.

Flow cytometry

A single cell suspension was prepared as described above and resuspended in deficient RPMI (dRPMI, Irvine Scientific, Santa Ana, CA) containing 0.1% azide. Cells were incubated for at least 1 hour with dRPMI containing azide and stained with specific antibody for 30 min at 4°C in the dark. Cells were stained with antibodies (25 μg/ml) recognizing CD4 (Peridinin chlorophyll protein (PerCP) labeled clone GK1.5), CD8 (Allophycocyanin (APC) labeled clone 53-6.7), and CD69 (fluorescein isothiocyanate (FITC) labeled clone H1.2F3). Control cells labeled with isotype antibodies were also prepared.

For intracellular IFN-γ staining, cells were stimulated with 0.1μg/ml anti-CD3 (clone 145-2C11) and 1μg/ml anti-CD28 (clone 37.51) in the presence of monensin for four hours at 37°C, 5% CO_2. Cells were stained with antibodies for cell surface molecules, as described above, before permeabilization with BD Cytofix/Cytoperm (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. FITC anti-IFN-γ (clone XMG1.2) or IgG1 isotype control antibody was incubated with the cells for

a further 30 min, washed twice, and resuspended in dRPMI with azide before analysis. All antibodies were purchased from BD Pharmingen. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Diego, CA), dual laser, flow cytometer with excitation at 488nm and 633nm, and data were analyzed using CellQuest software (Becton Dickinson, San Diego, CA). Lymphocytes were gated based on their forward and side scatter characteristics. Ten thousand CD4 or CD8 positive lymphocytes were then analyzed.

Histology

At specific time points post infection, the right caudal lung lobe was perfused with 10% formaldehyde in buffered saline. Tissues were sectioned and stained with hematoxylin and eosin. Sections were analyzed by a veterinary pathologist without prior knowledge of the sample identification.

Statistical analysis

Student's *t* test was used for comparisons of means, and values of $p < 0.05$ were considered statistically significant.

Results

Mice lacking CD44 are equally resistant to pulmonary tuberculosis

To determine if mice lacking the CD44 molecule were more susceptible to *M. tuberculosis*, control and CD44-KO mice were exposed to approximately 100 bacteria by aerosol (Fig. 3.1). The bacterial load in the lung and spleen was monitored over 65 days, and it was found that mice lacking the CD44 molecule were able to control the bacterial growth in the lung (Fig. 3.1a) and spleen (Fig. 3.1b) equally well as compared to wild type mice.

Cellular recruitment of activated $CD69^+$ T cells to the lung of infected mice was not impaired in CD44 deficient mice

Activated T cells entering the lung were identified by their expression of the C-type lectin CD69 [231][11]. As shown in Figure 3.2, $CD69^+$, $CD4^+$ and $CD8^+$ T cells accumulated in the lungs of CD44-KO mice in equal amounts to those observed in the wild type mice indicating that lack of the CD44 receptor did not influence the expression of CD69.

To determine if effector T cells, which express high levels of CD44 [106, 232] were altered in the CD44-KO mice, isolated lung cells were triggered ex vivo with anti-CD3 and anti-CD28 antibodies together with an inhibitor of the Golgi apparatus. As shown in Figure 3.3, the number of T cells that stained positive for IFN-γ peaked at 25 days post infection in conjunction with the initial control of the

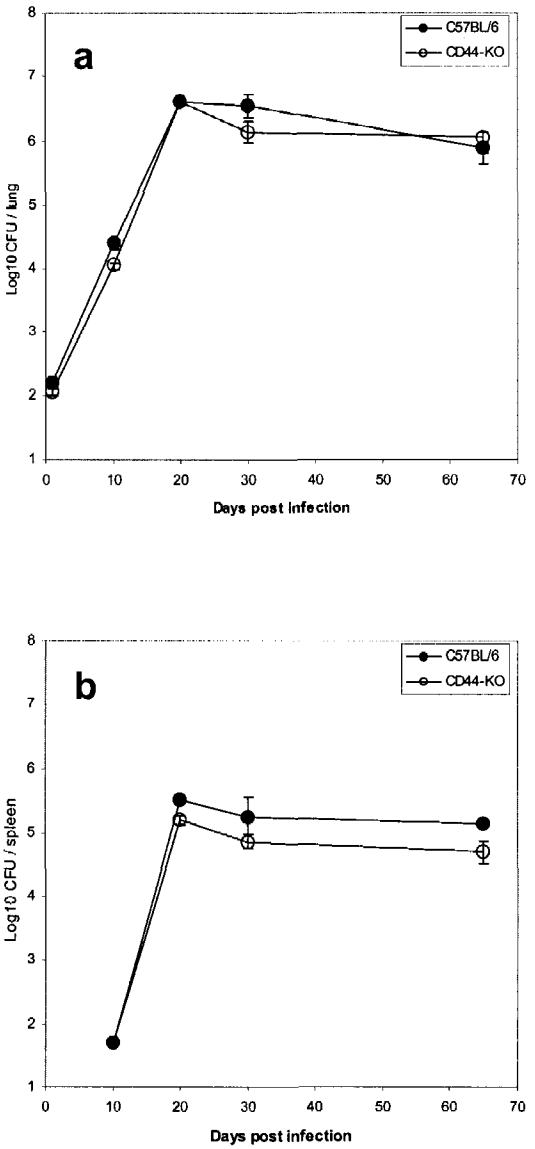

Figure 3.1. Mice lacking the gene for CD44 have similar *M. tuberculosis* bacterial loads in the lung and spleen compared to C57BL/6 mice. Wild type (closed circles) and CD44 gene-disrupted (open circles) mice were infected by the aerogenic route and lungs (a) or spleen (b) were evaluated for bacterial growth over a period of time. Data points represent the mean (± SEM) of values from four mice and are representative of 2 independent experiments.

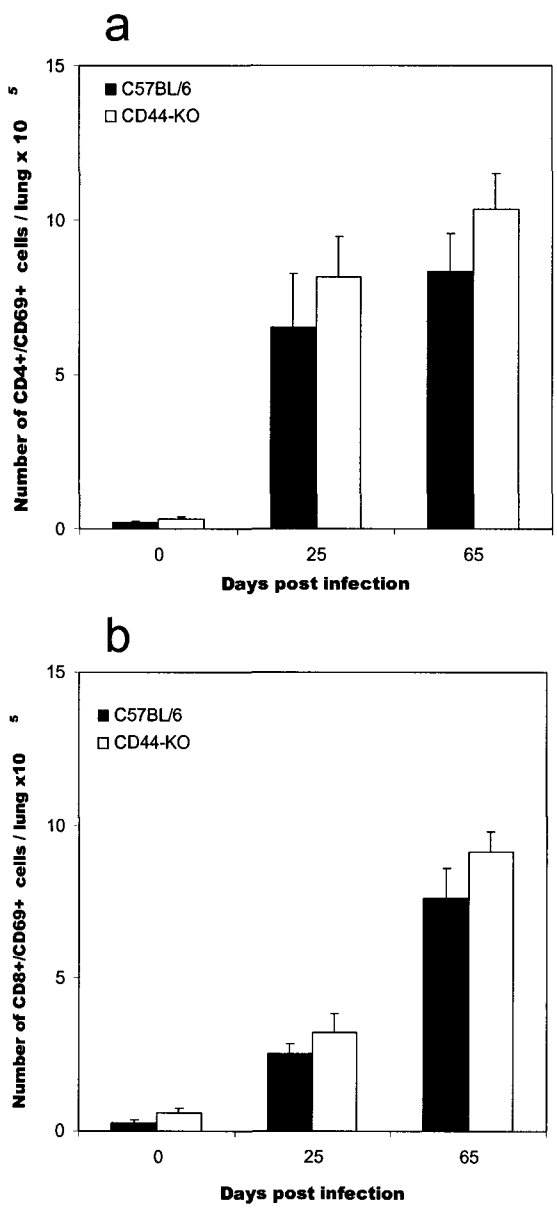

Figure 3.2. Wild type (solid bars) and CD44-KO (open bars) mice are equally able to recruit activated T cells to the lung following aerosol infection. Following infection lung cells were stained for their expression of the early activation marker CD69 and their relative numbers were determined by gating lymphocytes according to their size (FSC) and granularity (SSC) and for staining with anti-CD4 (a) or anti-CD8 (b) fluorescent antibodies. Day 0 represents data from non-infected mice of matched age. Data represent the mean of five mice ± SE and are representative of two independent experiments.

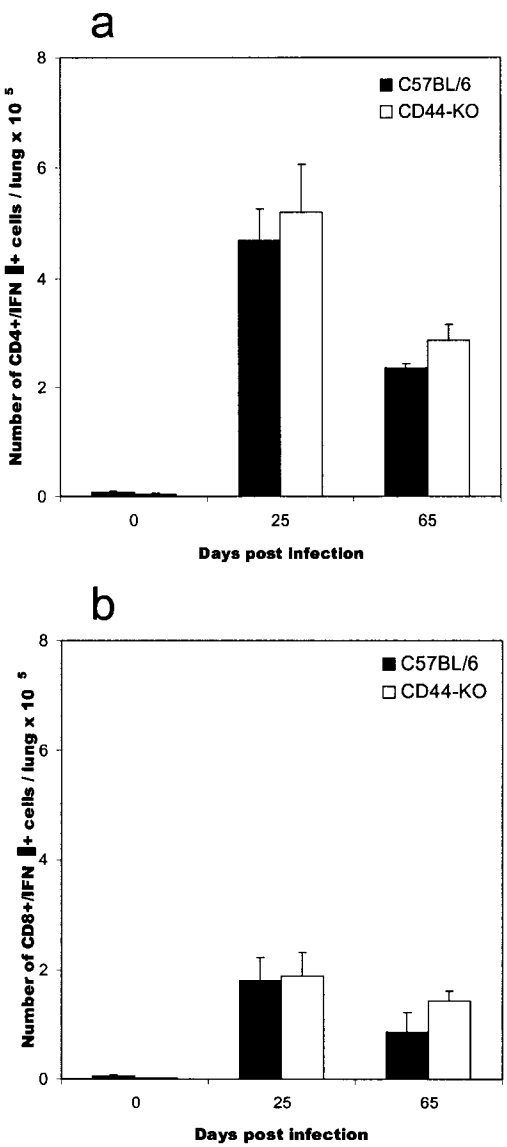

Figure 3.3. Wild type and CD44-KO mice have similar numbers of infiltrating activated T cells into the lung. The number of CD4 (a) and CD8 (b) T lymphocytes that produced IFN-γ in the lungs was determined. Infected and non-infected mice were analyzed during the course of infection for the ability of their cells in the lung to produce IFN-γ. Lung cell preparations from wild type (solid bars) and CD44-KO (open bars) mice were stimulated with anti-CD3 and anti-CD28 and the number of CD4 and CD8 T lymphocytes was determined. Day 0 represents data from non-infected mice of matched age. Data represent the mean of five mice ± SE and are representative of two independent experiments.

bacterial load (Fig. 3.1a). Following this the number of IFN-γ^+ T cells in the lungs decreased through day 65. These data show that the absence of the CD44 receptor did not alter the ability of IFN-γ positive lymphocytes to accumulate in the lung during infection.

Substantially increased neutrophil influx into the alveolar spaces and lung tissues of infected CD44-deficient mice

Representative sections of lung from wild type and CD44-KO mice were evaluated histologically for lesion burden and distribution of inflammatory cell infiltrates. At 10 days post infection there were no visible lesions in either wild type or CD44-KO mice. At day 20 post infection the lesion burden was similar between the two groups, however the distribution of inflammatory cells differed. In the CD44-KO mice the lesions consisted of equal proportions of epithelioid macrophages and lymphocytes; however neutrophils were prominent within alveoli and small conducting airways compared to the wild type controls (Fig. 3.4 and Table 3.1). This cellular distribution was seen cytologically as well, as neutrophils in bronchoalveolar lavage fluid from CD44-KO mice were the predominant cell type compared to wild type mice (Fig. 3.5 and Table 3.2). Lesions in the CD44-KO mice were more loosely organized than those from the wild type group, which had discrete accumulations of perivascular and peribronchiolar aggregates of lymphocytes, some of which involved bronchiolar lumina (Fig. 3.4). Individual necrotic cells were scattered throughout the lesions in equal proportions between the two groups. At day 30 post infection, the lesions were similar in size and cellular make-up but focal lesions in the CD44-KO mice began to coalesce. At day 65 post infection

Figure 3.4. Light photomicrograph of a lung from CD44-KO (a) and wild type (b) mice, after aerosol infection with *M. tuberculosis*. In the CD44 KO mice, neutrophils are the predominant inflammatory cell type in bronchiole (inset) and alveolar lumina (black arrows), with fewer loosely organized peribronchiolar and perivascular lymphocytes 20 days post infection. In wild type mice, lymphocytes are in dense peribronchiolar and perivascular aggregates and occasionally within bronchiolar lumina (inset) 20 days post infection (white arrows).

Table 3.1 Histological evaluation of lung sections stained with hematoxylin and eosin.

	Lesion Extension[a]	Macrophage score [b]	Lymphocyte score [c]	PMN score [d]	Necrosis[e]	Lesion volume[f]
C57BL/6 day 10	nd	0	0	0	0	0
CD44-KO day 10	nd	0	0	0	0	0
C57BL/6 day 20	F	2	2.5	0.5	0	1.5
CD44-KO day 20	F	2	2	1.5	0	2
C57BL/6 day 30	MF	2.5	1.5	1	1	2.5
CD44-KO day 30	MF-C	2.5	1	1.5	1	2.5
C57BL/6 day 65	MF-C	2.5	1.5	1	0	2.5
CD44-KO day 65	MF-C	3.5	2.3	2.5	1	3.5

[a] Lung lesions were scored as not detected (nd), being discrete or focal (F), multiple but discrete lesions (MF), or multiple lesions that had expanded and coalescing with other nearby lesions (MF-C).

[b,c,d] Relative numbers of macrophages, lymphocytes and neutrophils respectively were determined. Scores were qualitatively designated ranging from 0 to 3, with increments of 0.5.

[e] Necrosis was absent in the first 40 days of infection and by day 40 a moderate scattered necrosis within the inflammatory lesions was seen.

[f] Lesion volume was approximately determined as the area of lesions compared to the lung examined.

Figure 3.5. Bronchoalveolar lavages were performed following aerosol infection. A representative slide is shown from wild type (a) and CD44-deficient (b) mice 20 days post infection.

Table 3.2: Cellular composition of the bronchoalveolar lavage fluid from wild type and CD44-KO mice following infection with *M. tuberculosis*.

	Wild type mice			CD44-KO mice		
Days post infection[a]	0	20	65	0	20	65
Cell types[b]						
Macrophages	98.2	74.4	78	93.5	62.7	74.7
Lymphocytes	1	18.3	19.5	4.8	12.5	9
Neutrophils	0.8	7.3	2.5	1.7	24.8	16.3

[a] Day 0 represents uninfected mice. [b] Two hundred cells were counted from each mouse BAL and cell types are expressed as percentages. Data represent mean percentages of 4 mice for each time point and are representative of two independent experiments.

significant histological differences were seen between the two groups. Lesions within the CD44-KO mice were more severe and were predominately composed of epithelioid macrophages with lymphoid aggregates and significant numbers of neutrophils as seen at day 20 (Tables 3.1 and 3.2). Despite the significant lesion burden, necrosis was not a significant feature but was greater than the wild type group. Additionally, inflammatory cell infiltrates extended into the small conducting airway lumina similar to that seen in the lungs at 20 days post infection.

I next examined how γδ T cells increase in the spleen and lung of infected mice following infection. As shown in Figure 3.6, γδ T cells do not increase in the spleen of wild type mice following infection while CD44-KO mice doubled the numbers of γδ T cells present in the spleen by day 65 post infection (Fig. 3.6 a) but remained with similar numbers as spleen from wild type mice. The accumulation of γδ T cells in the lungs of infected mice occurred at a very high rate such that 65 days post infection they were found at ten times higher numbers that in non-infected mice(Fig. 3.6 b). At the days analyzed the lungs of CD44-KO mice always had lower numbers of γδ T cells than controls, although the difference was not statistically significant.

Discussion

The results of this study show that following an aerosol infection of mice with *M. tuberculosis*, the inflammatory response in the lung consisting of the accumulation of macrophages and lymphocytes proceeded normally despite the absence of expression of the CD44 molecule, a major adhesion C-lectin molecule. In such mice lymphocytes were

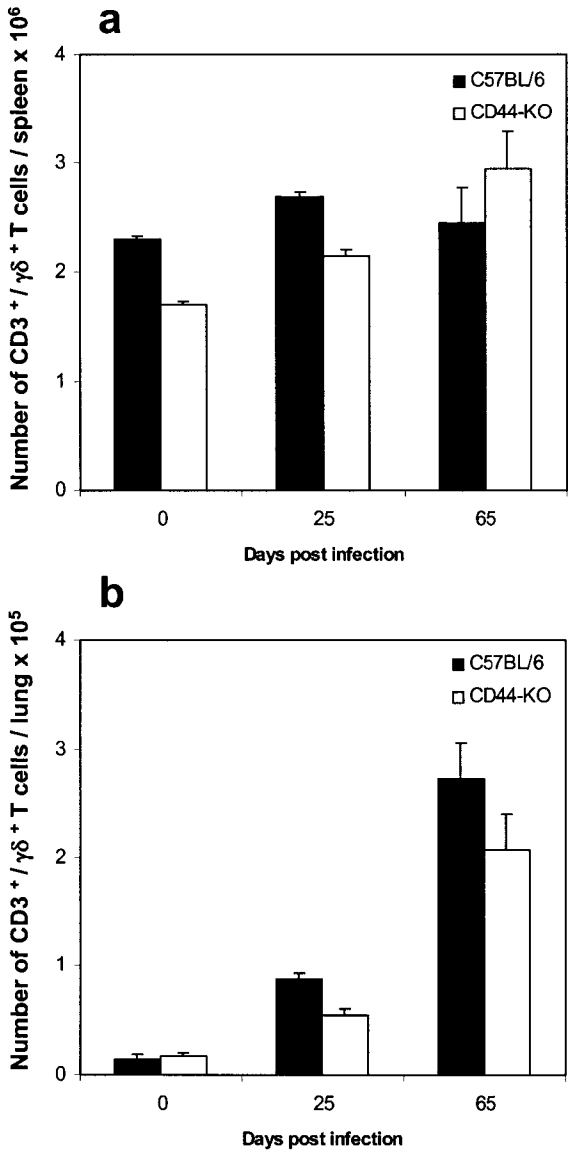

Figure 3.6 γδ T cells in the spleen and lung following infection. The number of γδ T cell was determined in the spleen (a) and lung (b) of wild type and CD44-KO mice following infection by staining cells with anti-CD3 and anti-γδ antibodies and analyzing by flow cytometry. The numbers of γδ T cells in the lungs of naïve mice were also determined and are represented as day 0.

able to migrate to the sites of inflamed tissue, resulting in control and containment of bacterial growth in the gene-disrupted mice at the same levels as the wild type animals. In contrast to the mononuclear cell response, the absence of expression of CD44 resulted in a dramatically increased accumulation of neutrophils into the lungs and alveolar spaces, an event that did not however appear to interfere with the ability of the CD44-deficient mice to control the infection.

These observations are consistent with those of others that CD44 is needed to control inflammation in the lungs, at least in the context of granulocyte influx. It has been shown that during an inflammatory response CD44-KO mice accumulate active forms of hyaluronan, the ligand for CD44, which is induced by the inflammatory cytokines TNF-α and IL-1β [229, 233] but removal of this ligand can be impaired in the absence of CD44 [231]. Additionally, the removal of apoptotic cells from the inflammatory site, a protective mechanism to avoid cell lysis and subsequent release of immunogenic and/or toxic contents, seems to be defective in the absence of CD44. Other factors that may account for the increased inflammatory response in the CD44-KO mice are prolonged inflammatory gene expression as well as reduced levels of active TGF-β [231].

During a low dose aerosol infection with *M. tuberculosis* a mononuclear cell inflammatory response slowly takes place with several stages of pathology observed [77]. However the major difference seen between the wild type and CD44-KO mice in the current study was not within the above cells but consisted instead of a substantially

increased neutrophil influx in the lung parenchyma and alveolar spaces. This excessive number of neutrophils could be explained by an increased influx and/or by a decreased clearance rate. Recent studies have in fact shown that the expression of CD44 on the surface of neutrophils acts as a negative regulator for their migration across endothelial barriers [234], and in the absence of CD44, neutrophils can migrate faster than those in wild type mice [235] resulting in their accumulation in the inflamed tissues. Such an observation would certainly explain my results here.

In this regard, work in my laboratory have shown a pyogenic response in the lungs of mice lacking gamma delta (γδ) T cells, and it has previously been speculated that these cells may play a role in controlling and reducing the influx of neutrophils [131]. It is reasonable to speculate therefore that CD44-KO mice are deficient in promoting the adequate attraction of γδ T cells to the lungs, which in turn contributes to the increase in neutrophilic influx. In fact, my data may support this idea because I have observed a higher percentage of γδ T cells in wild type mice than in CD44-KO mice (Fig. 3.6).

In contrast to my findings, Leemans *et al.*[236] have recently reported that CD44-deficient mice are more susceptible to infection with *M. tuberculosis*, with higher bacterial loads in the lung and liver, and with a concomitant defect of the CD44-KO mice in terms of attracting macrophages and IFN-γ producing T cells. The difference in my study seems to reside in the bacterial dose of infection, in that I used a low dose of about 100 bacteria deposited into the lungs whereas Leemans *et al.* used a dose one thousand times higher than ours delivered intranasally. That report did not identify the actual initial bacterial load obtained by that procedure, but it could be the case that it was higher than

ours. It has been proposed elsewhere [90] that in a low dose infection with *M. tuberculosis* a minimal immune response is generated that is sufficient to control the infection, while if the bacterial load is much higher deficiencies not otherwise interfering with this response now become important. Despite this, however, both studies observed the florid neutrophil influx, and this pyogenic granulomatous response was almost certainly responsible for the eventual mortality observed in the studies by Leemans *et al.*

In summary my observations show that following a low dose aerosol infection with *M. tuberculosis,* a protective immune response proceeds normally in the absence of CD44 expression with trafficking of T cells to the lungs necessary for such protection appearing unaltered. However the absence of CD44 expression resulted in significantly increased neutrophil accumulation in the lungs without compromising the control of bacterial growth.

CHAPTER 4

Cellular aspects of the memory response induced by *Mycobacterium bovis* BCG immunizations in the lungs of mice.

Introduction

Since the first vaccination experiment conducted by Ernest Jenner in 1796, the role of vaccination in controlling infection in humans has increased and thus our quality of life improved considerably. One of the great triumphs of the vaccine history could be pointed to as the global eradication of smallpox in 1977, coincidentally the very same organism with which Jenner performed his vaccine trial. The eradication of diseases is the ultimate goal in vaccine research and for that the understanding of immunological memory to infections is fundamental.

The search for a vaccine against tuberculosis began soon after its casual agent was discovered by Robert Koch in 1882. Koch went back to do research work himself in 1889 after a long time away from the laboratory due to his teaching responsibilities, and in 1890 he implied he had found a cure for tuberculosis [2]. Koch had developed what he called later as tuberculin and this is the observations that led him to believe that he had discovered the cure of tuberculosis in his own words:

"... If a healthy guinea pig is inoculated with a pure culture of tuberculosis bacilli, the wound becomes rapidly healed during a few days. Only after 10 days to 2 weeks, a hard nodule forms, which soon opens, forming an ulcerating spot which persists until the death of the animal. On the other hand, if an animal that is already tuberculous is inoculated, the results are quite different.... In such cases, the small inoculation wound also becomes healed, but no nodule is formed. Instead, a peculiar change takes place at the point of inoculation. The spot becomes hard and dark-colored, and this region gradually spreads to a diameter of 0.5 to 1 centimeter. ....But this remarkable action does

not occur only with living tubercle bacilli. Dead tubercle bacilli also bring about the reaction, whether killed by low temperatures of long duration, by boiling heat, or by certain chemicals....It was then found that if pure cultures of tubercle bacilli were killed, ground up, and suspended in water, they can elicit the reaction....."

Unfortunately soon after its announcement, tuberculin was shown not to have a therapeutic value, but its diagnostic value was soon appreciated such that in 1907 Charles Mantoux continued to develop Koch's work with tuberculin to use in the diagnosis of tuberculosis. The Mantoux test consists of intradermal injection of standardized amounts of tuberculin, known to elicit a cell mediated response seen two to three days later at the site of inoculation. The tuberculin preparation was further improved by Seibert [237] to a purified protein derivative (PPD) secreted from *M. tuberculosis* culture that is used in tuberculin skin testing today. A positive reaction is marked by an erythema and induration of the skin of more than 10 millimeters, and it can represent a past or present exposure to *M. tuberculosis* but it can also be seen in subjects that were vaccinated with BCG.

In 1908 Calmette and Guerin started to perform several passages of virulent *M. bovis* culture *in vitro*, such that in 1920 and after 230 subcultures the strain was shown to be devoid of virulence in animals. In 1921 the attenuated strain known as Bacille Calmette-Guerin (BCG) was administered orally to a child whose mother had died of tuberculosis. The child had no adverse effects from the vaccine and did not develop tuberculosis. After this promising test the BCG vaccine started to be administered in several countries in 1927 and is still used today. Several long term studies of the protection mediated by BCG vaccination have led to the conclusion that BCG varies in its

ability to confer protection in adults enormously, from around 80% protection to no protection at all [238]. The reasons for such variability in protection have been proposed to vary as well, ranging from genetic composition of the population, diet and nutrition status of the host, presence of nontuberculous mycobacteria in the environment, BCG strain used among others [239]. Today after almost 80 years since the development of BCG, we still do not have an alternative to BCG vaccine.

How can BCG vaccination protect from *M. tuberculosis* infection? The mouse model may not be the perfect model to answer this question since BCG immunization can confer only a log of protection but it certainly is useful due to the incredible knowledge of the immune response to *M. tuberculosis* infection in that host as well as to the great resource of biological reagents that can be used.

In order to generate models to study memory responses to *M. tuberculosis* in the mouse researchers have used BCG immunization or *M. tuberculosis* infections followed by a prolonged treatment with a combination of antibiotic drugs prior to challenge/rechallenge [139, 240]. This procedure ensures that an effective immunological response develops and by removing the viable bacteria only long lived memory T cells should survive by the time the animals are challenged with virulent *M. tuberculosis*.

In memory models like those, it has been shown that following challenge both CD4 and CD8 memory T cells respond and probably are involved in the control of infection [241, 242]. Further characterization of the response showed that the cellular response is

characterized by the rapid influx of effector T cells, characterized as $CD44^{hi}/CD62L^{lo}$, capable of producing IL-2 and the important protective IFN-γ cytokine [243].

Recent studies have revealed that memory T cells are a much more heterogeneous population than it was assumed. It was shown by Sallusto *et al.* that human memory T cells can be grouped into central and effector memory T cells based on the expression of CD62L selectin and the CCR7 receptor [140]. This finding brought new insight into how both memory T cells populations could be more efficient in surveying for antigen. Accordingly secondary lymphoid tissues would be surveilled for antigens by central memory T cells, which have the phenotype $CD44^{hi}/CD62L^{hi}/CCR7^{+}$, while effector memory cells would screen for potential pathogenic antigens of the non lymphoid tissues by down regulating the expression of the homing receptors CD62L and CCR7 and localizing in those areas.

Further characterization of memory T cells have been done by the use of tetramers, where one can identify antigen-specific T cells responding to a particular stimulus and characterize the expression of the different molecules believed to be related to memory T cells. In this regard it has been shown that memory T cells, like activated T cells, retain high expression of CD44. In addition CD8 T memory cells have high expression of Ly6C [244, 245] and the common β chain of IL-2 and IL-15 receptors CD122 [246, 247].

There are also functional differences among memory T cells subpopulations as well as compared to both naïve and activated T cells. At the end of the primary response, after the great majority of activated cells die, the frequency of memory T cells are significantly

higher than that of naïve T cells. This is one of the reasons why the secondary immune responses are usually much more intense than the primary response. Memory T cells have a shorter lag time to start proliferation [247], produce cytokines[248], differentiate into cytotoxic T lymphocytes [249], and migrate to the site of infection [250]. The type of cytokine produced by memory cells in response to antigen is also heterogeneous while central memory T cells respond by producing mainly IL-2, effector memory T cells respond by producing IL-4, IL5, or IFN-γ according to their initial differentiation stimuli [140]. While activated T cells are proliferating vigorously, memory cells seem to be in a resting G0/G1 phenotype or proliferating at a very low rate. Recent studies suggest that memory CD8 T cells are in a preactivated state with low expression of the p27Kip1 (a molecule responsible for inducing cell cycle arrest) and high level of expression of the cyclin CDK6 but because the latter are kept in the cytoplasm the cell does not enter the division cycle [251] but could do so more quickly than naïve T cells. Naïve cells conversely remain in a G0/G1 due to high levels of p27Kip1 causing the cyclin CDK6 to be degraded and inducing G0/G1 arrest.

Some of the memory markers described above have not been analyzed in the mouse model of pulmonary tuberculosis. In the present study I analyzed the memory immunity conferred by BCG immunization in light of the new findings regarding memory T cell characterization. The elucidation of memory T cell markers in tuberculosis is a key element to direct the immunological responses in the design of new and more efficient vaccines. I focused my study to the lung tissue since that is the primary organ of infection in our low dose aerosol model.

Material and methods

Mice

Female C57BL/6 mice, 10 – 12 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). CCL2 gene disrupted mice were kindly provided by Dr. Barrett Rollins from the Dana Farber Cancer Institute and bred at the Laboratory Animal Resource facilities at Colorado State University. CCL2-KO mice were backcrossed to C57BL/6 mice for at least 6 generations. All mice were maintained in specific pathogen free conditions and were used between 8 and 10 weeks of age.

Bacterial Strains

M. bovis BCG Pasteur and *M. tuberculosis* strain Erdman, originally obtained from Trudeau Institute (Saranac Lake, NY), were grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and then frozen in aliquots at -70^0C until needed.

BCG immunization and antibiotic treatment

Mice were vaccinated with live *M. bovis* BCG Pasteur subcutaneously, in the scruff of the neck. The vaccine dose was 10^6 bacilli in 200 μL of sterile saline. Thirty days post immunization mice were treated with 100mg/L of isoniazid (ICN Biomedicals, Aurora, OH) in their drinking water to completely eliminate any viable BCG. The drug-containing water was changed weekly for the period of 6 weeks.

Challenge Infections

Two weeks after the end of antibiotic treatment mice were aerogenically challenged with *M. tuberculosis* using a Glas-Col aerosol generator (Glas-Col, Terre Haute, IN) such that 100 bacteria were deposited in the lungs of each animal. The number of viable bacteria in target organs was determined at various time points by plating serial dilutions of partial organ homogenates on nutrient Middlebrook 7H11 agar and counting colonies after 21 days of incubation at 37°C.

Media and reagents

Lung and spleen cell suspensions were cultured in Dulbecco's minimal essential medium (DMEM Cellgro, Herndon, VA) containing 10 mM *N*-2-hydroxy-ethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES, Sigma-Aldrich, St Louis, MO), 2mM L-glutamine (Sigma-Aldrich), and MEM nonessential amino acids (Sigma-Aldrich) supplemented with 10% heat-inactivated, endotoxin-low fetal calf serum (GIBCO BRL; Invitrogen, Carlsbad, CA).

Cell culture

Infected animals were killed by CO_2 asphyxiation and lung, spleen and mediastinal lymph nodes were harvested from infected animals at indicated times after infection. Lungs were perfused through the heart with cold saline containing 30U/mL of heparin. After removal, the lungs were sectioned in ice-cold medium using sterile razor blades. Dissected lung tissue was then incubated in DMEM containing collagenase IX (0.7

mg/ml; Sigma-Aldrich) and deoxyribonuclease (30 μg/ml; Sigma-Aldrich) at 37°C for 30 min. Single-cell suspension from digested lung, spleen or lymph nodes was obtained by passing the organs gently through a 70μm nylon cell strainer. Red blood cells were lysed using Gey's solution (0.15M NH_4CL, 10mM $KHCO_3$).

Flow cytometry

A single cell suspension of either spleen or lung was prepared as described above and ressuspended in deficient RPMI (Irvine Scientific, Santa Ana, CA) containing 0.1% azide. Some cells were stimulated with anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51) and monensin for four hours prior to staining. Cells were incubated for at least 1 hour with dRPMI containing 0.1% azide and stained with specific antibody for 30 min at 4°C and in the dark. Cells were stained with antibodies recognizing CD62L (Fluorescein isothiocyanate (FITC) labeled clone MEL-14), CD11a (FITC labeled clone 2D7), CD62L (Phycoerythrin (PE) labeled clone MEL-14), CD54 (PE labeled clone 3E2), CD122 (PE labeled clone TM-β1), CD27 (PE labeled clone LG.2A10), CD28 (PE labeled clone 37.51), CD4 (Peridinin Chlorophyll-*a* Protein (PerCP) labeled clone GK1.5), CD8 (PerCP labeled clone 53-6.7), CD44 (Allophyocyanin (APC) labeled clone IM7), and/or CD4 (APC labeled clone GK1.5) (all from BD Pharmingen, San Diego, CA). Control cells labeled with isotype antibodies were also prepared. After being stained on their surface, stimulated cells were permeabilized with Perm Fix/Perm Wash (BD Pharmingen) and the presence of intracellular IFN-γ was probed with labeled antibody (FITC-labelled clone XMG1.2). Alternatively intracellular IFN-γ was detected with PE labeled antibody so that Ki67 antibody (FITC labeled clone B56) could be together in the

same panel. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA). All antibodies were purchased from BDPharmingen (San Diego, CA), including appropriate isotype control antibodies that were included throughout the analysis. Cells were collected in a FACSCalibur (Becton Dickinson), dual laser, flow cytometer with excitation at 488nm and 633nm, and analyzed using CellQuest software (Becton Dickinson). One hundred thousands total events were acquired for analysis. Lymphocytes were gated based on their forward and side scatter characteristics.

Staining for Intracellular BrdU

Mice were injected with 100 μL of a 10mg/mL solution of BrdU 12 hours prior to their use in the experiment. After staining the with the appropriate surface markers as described above lung cells were stained for intracellular BrdU using a kit from BD-Pharmingen according to its instructions. Briefly cells were fixed and permeabilized after consecutive incubations with Cytofix/Cytoperm^{TM} and Cytoperm plus buffers and the incorporated BrdU was exposed after DNAse treatment of the cells. Cells were then stained with FITC labeled anti-BrdU (clone B44), washed and analyzed in the FACSCalibur cytometer.

Histology

The posterior lobe of the right lung was perfused with 10% paraformaldehyde in PBS and used for histological analysis. Hematoxylin and eosin staining was performed on sectioned tissues.

Real Time PCR for relative quantification of IFN-γ mRNA

The anterior lobe of the right lung was removed and placed into test tubes with 1 ml of Ultraspec (Biotecx, Friendswood, TX). Samples were homogenized and immediately frozen at -70°C until extraction. Extraction of total RNA was performed by adding 200 µl of chloroform to each sample for organic extraction. The aqueous phase was precipitated first with isopropanol and second by using the combination of 70% ethanol and 0.3M acetic acid. Total RNA was quantified by OD 260nm spectrophotometric readings and 1 µg of total RNA was used for the reverse transcriptase reaction using murine Maloney-leukemia virus reverse transcriptase (Invitrogen Life Technologies, Grand Island, NY) and random primers (Roche Applied Science, Indianapolis, IN). Following the reverse transcription, 175 µl of ultra pure water was added to each sample to yield a final volume of 200 µl. Five microliters of each cDNA sample were used for the real time PCR reaction. TaqMan® Pre-Developed Assay Reagents for Gene Expression (Applied Biosystems, Foster City, CA) were used to detect the messages for 18S Ribosomal and IFN-γ cDNA. The PCR reaction was performed in an ABI Prism 7700 machine (Applied Biosystems, Foster City, CA) and the analysis was done following the manufacturer's directions. Quantification of IFN-γ message was normalized to the 18S ribosomal message by the Delta Delta Ct method and is expressed as the relative amount, assigning the arbitrary value of 1 to the sample that had the least amount of message.

Statistical analysis

Five mice per group of at least two different experiments were used for all studies. Student's *t* test was used for comparisons between the different treatment groups.

Results:

Protection conferred by *M. bovis* BCG vaccination in mice against pulmonary tuberculosis.

In order to determine if BCG immunization would protect mice in my conditions I first immunized C57BL/6 mice with BCG while the control group received PBS alone. After the end of chemotherapy both groups were challenged with low aerosol infection with *M. tuberculosis* and the bacterial load in the lungs was assessed. As shown in Fig. 4.1, BCG immunized mice had an exponential bacterial growth in the lungs with a similar rate as the naïve group in the first 10 days post infection. Around the second week post infection, the bacterial growth in the lungs of BCG immunized mice reached a plateau and remained with a similar load throughout the rest of the experimental period. In contrast non-immunized mice continued to have bacterial growth in the lungs until the third week of infection, after which they remained with a constant bacterial load. The delay in infection control seen in naïve compared to BCG immunized mice resulted in a 10-fold difference of bacterial numbers in the lung and spleen (data not shown) that persisted throughout the experiment duration.

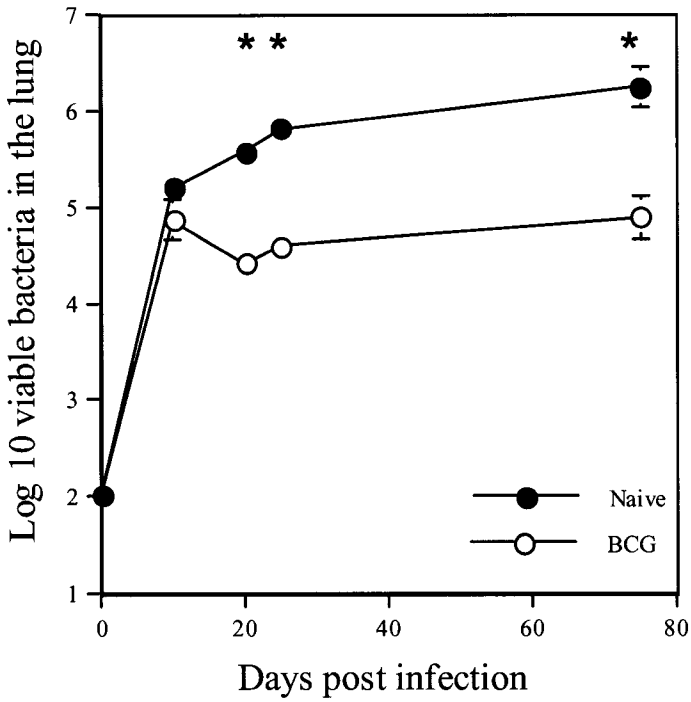

Figure 4.1 BCG immunization confers one log of protection against challenge with *M. tuberculosis* infection. C57BL/6 mice were immunized with BCG and, after isoniazid treatment, challenged with *M. tuberculosis* via aerosol infection. The lung bacterial load of BCG immunized mice (open circles) was determined and compared to non immunized mice (closed circles). Data represent the mean colony forming units from 5 mice at each time point ± SE. *= p value < 0.01.

Analysis of activated lymphocytes present in the lungs following challenge.

In order to characterize the cellular immune response that developed after challenge in BCG immunized and control mice, I used flow cytometry to analyze the trafficking of T lymphocytes into the lungs that is important for the control of infection. As shown in Figure 4.2 activated T cells identified by their expression of high levels of CD44 ($CD44^{hi}$) and low levels of CD62L ($CD62L^{lo}$) could be seen in higher numbers in the lungs of BCG immunized mice at the time of challenge (Fig. 4.2 a and b, day 0). After challenge the numbers of activated T cells increased significantly in both groups but non immunized mice had higher rates of increase such that at 21 days post infection and thereafter naïve mice always had higher numbers of activated T cells in their lungs compared to immunized mice.

Since INF-γ is the major protective cytokine produced by T cells required for the control of infection I next looked at the number of T cells with the ability to express IFN-γ using intracellular staining and flow cytometry. As shown in Fig. 4.2 the pattern is similar to that seen for the $CD44^{hi}/CD62L^{lo}$ activation markers for both CD4 (Fig 4.1 c) and CD8 (Fig 4.2 d) T cells. It is important to note that the difference seen prior to aerosol challenge (Day 0) in the numbers of both CD4 and CD8 T cells that express IFN-γ when stimulated with anti-CD3 and anti-CD28 antibodies is even more significant than the difference seen in the $CD44^{hi}/CD62L^{lo}$ populations. Following challenge, CD4 T cells positive for IFN-γ from immunized mice increased by 10 days post infection while CD4 T cells from non immunized mice showed a lag of 20 days before their numbers increased. The kinetics of T cells capable of expressing IFN-γ seen in both experimental

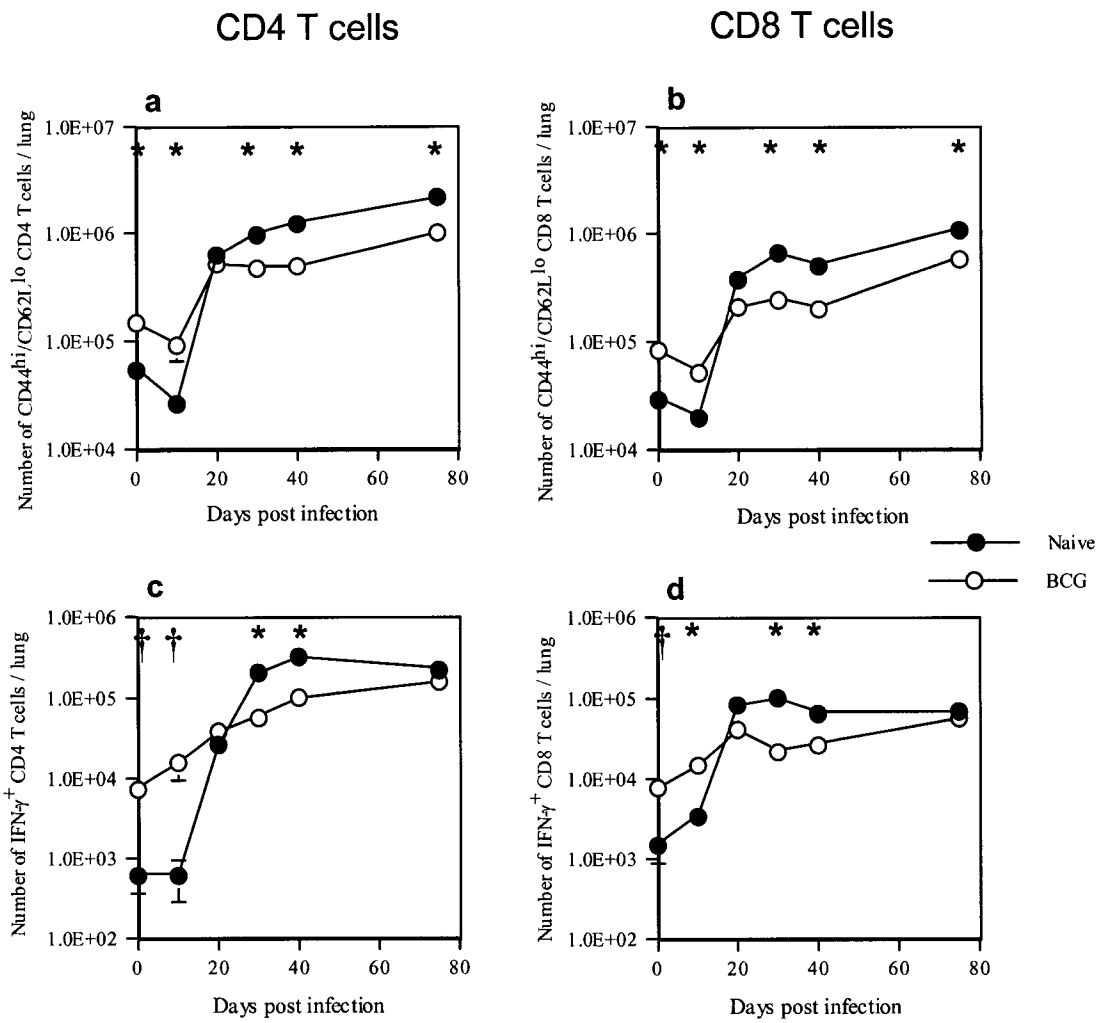

Figure 4.2 Activated T cell trafficking to the lungs of infected mice. The profile of activated CD4 and CD8 T cells expressing $CD44^{hi}/CD62L^{lo}$ was determined by flow cytometry prior to and following mycobacterial challenge. Likewise IFN-γ expressing CD4 (c) and CD8 (d) T cells were determined. Data represent the mean numbers of cells from five mice at each time point ± SE. *=p value < 0.05 † = p value < 0.01.

groups at the first three weeks of infection reflects their bacterial load increase in the lungs during the same period such that immunized mice had a lower rate of increase of both bacteria and IFN-γ producing T cells while naïve mice had the inverse.

Correlation of IFN-γ producing T cells and expression of the activation markers CD44 and CD62L.

In order to characterize the phenotype of IFN-γ secreting T cells with respect to the expression of the activation markers, T cells were first gated according to the expression of CD4 or CD8 marker combined or not with the expression of IFN-γ and the selected subgroup was then analyzed for the expression of the activation markers CD44 and CD62L. A representative panel of dot plots with the pattern of activation markers expression by CD4 T cells is shown in Figure 4.3. When all CD4 T cells are gated (R5 in Fig. 4.3 a) and analyzed for the expression of CD44 and CD62L there are many cells present in all possible combination of the markers used reflecting the presence of a diversity of T cells in terms of their activation status. In particular as the infection progresses I see an increase in the population with a high expression of CD44 and low expression of CD62L selectin. In Figure 4.3 I have depicted a representative dot plot showing the analysis of CD4 T cells from immunized mice 10 days after challenge, when about 50% of the CD4 T cells expressed the $CD44^{hi}/CD62L^{lo}$ phenotype (Fig. 4.3 b). In contrast if I gate only CD4 T cells that are expressing IFN-γ I can see that 100% of them have the activated phenotype $CD44^{hi}/CD62L^{lo}$ (Fig. 4.3 c). The same observation

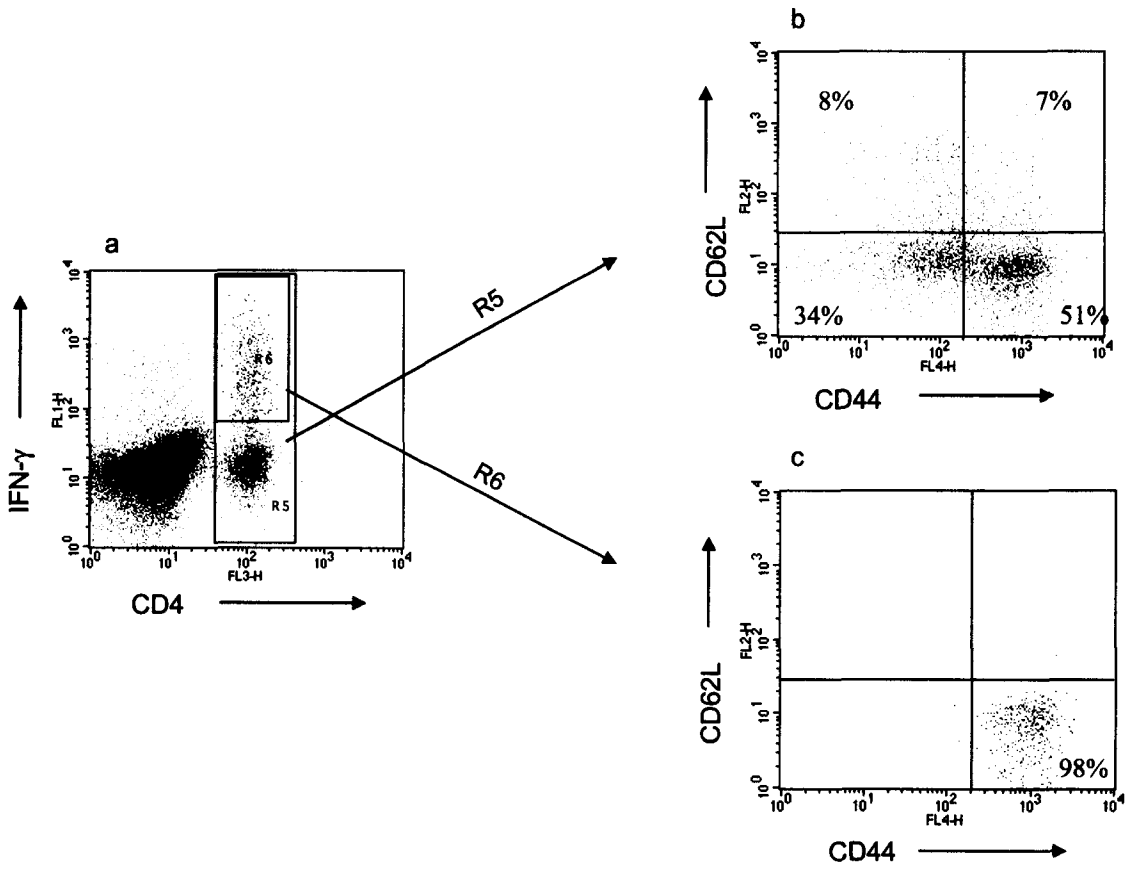

Figure 4.3 Characterization of IFN-γ^+ CD4 T cells according to activation markers. $CD4^+$ T cells from infected mice were gated and their activation pattern in respect to the expression of CD44 and CD62L is shown (b). IFN-γ+ CD4 T cells were gated and the expression of CD44 and CD62L was determined (c). A representative sample from a naive mouse at 10 days post infection is shown.

was seen for CD4 T cells from naïve infected mice as well as for the CD8 T cell populations (data not shown). Taken together these data show that all T cells that produce IFN-γ have the activated phenotype $CD44^{hi}/CD62L^{lo}$.

Adhesion molecules expression by T cells following aerosol challenge.

The capacity of activated T cells to migrate to nonlymphoid tissues is a reflection of up regulation of several classes of integrins. In pulmonary tuberculosis the adequate expression of the adhesion molecules intercellular adhesion molecule 1 (ICAM1, CD54) and CD11a (which when combined with CD18 form the lymphocyte function-associated antigen 1, LFA1 molecule) by activated T cells is directly related to the disease progression[252]. I examined wether BCG immunization resulted in earlier T cell recruitment as a consequence of up regulation of these adhesion molecules. As shown in Figure 4.4 there is a significantly higher percentage of CD4 T cells expressing both CD54 and CD11a (50% of the CD4 T cells Fig. 4.4 b) in BCG immunized mice compared to non immunized mice (10% Fig. 4.4 a). Thus, my data show that the early recruitment of T cells in immunized mice was at least in part due to the up regulation of the adhesion molecules CD54 and CD11a.

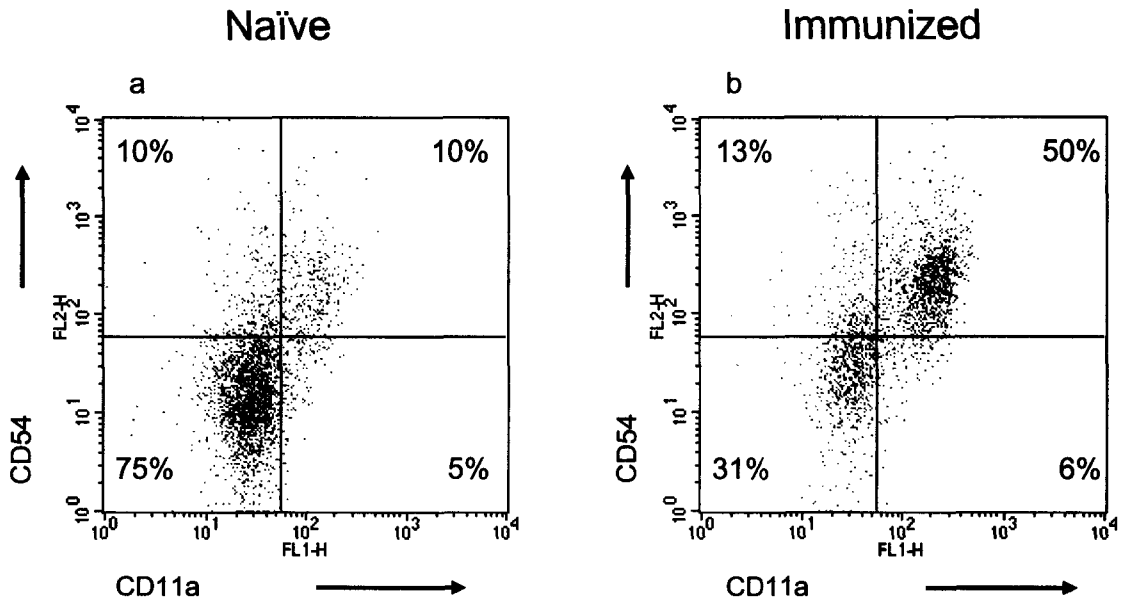

Figure 4.4 Expression of adhesion molecules by CD4 T cells post challenge. $CD4^+$ T cells from infected mice were gated and the expression of CD54 (ICAM1) and CD11a adhesion molecules was determined by flow cytometry. Representative dot plots of non immunized (a) and immunized (b) mice 10 days post challenge is shown.

Proliferation or increased influx of T cells following challenge?

One important question that remains unsolved is whether the increase in T cell numbers seen in the lungs of infected mice can be attributed solely to increased influx from the circulatory system, or if it can be contributed to by a local T cell proliferation. In order to determine the proliferative status of lung T cells, mice received an injection of BrdU 12 hours before the experimental procedures. BrdU positive T cells were recognized by intracellular staining with FITC-conjugated antibodies and analyzed by

flow cytometry. As shown in Figure 4.5 the peak of T cells stained positively for BrdU occured at 3 weeks post infection in both immunized and control groups (Figure 4.5 a and b). I also used a different marker to measure cellular proliferation status which is an antibody specific for the Ki67 protein, an antigen expressed exclusively in proliferating cells. Since staining for Ki67 does not require previous administration of drug to mice, it would provide a clearer snapshot assessment of cells proliferating in the lungs. As shown in Figure 4.3, the pattern of Ki67 expression by both CD4 (Fig. 4.5 c) and CD8 (Fig. 4.5 d) T cells is similar to that seen by BrdU staining assay although it was always at lower levels. In both methods it was seen that at the peak of proliferation, the non-immunized mice had significantly higher levels of T cells in proliferation as compared to the immunized group. Overall these data show that T cell proliferation within the lung tissue of infected mice contributes to the increase seen after infection, but due to the very low levels of Ki67 staining the major contribution is probably due to the cellular influx from the circulation.

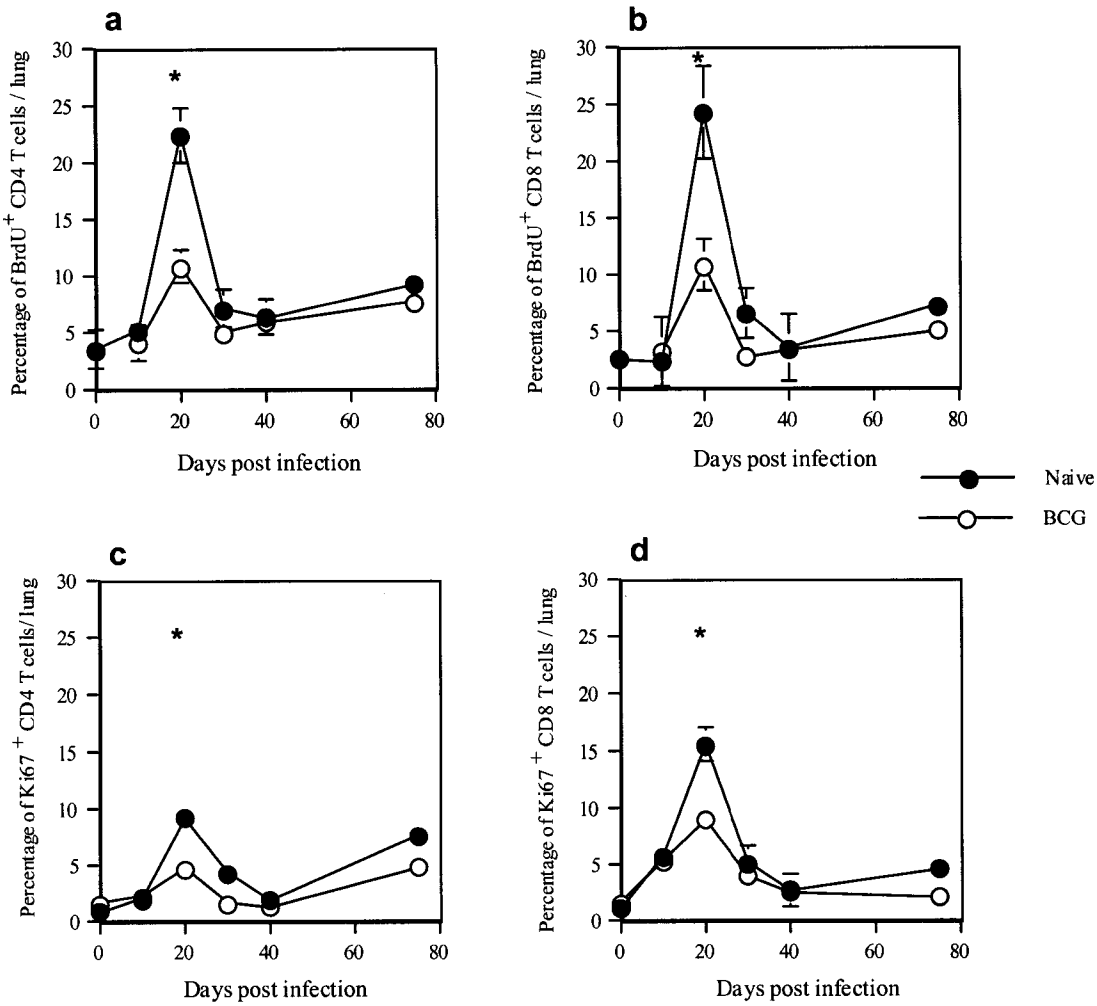

Figure 4.5 Lymphocyte proliferation assessments. At indicated time points lung cells from immunized (open circles) and control mice (closed circles) were analyzed for proliferative status by the BrdU method (a and b) or by analyzing the levels of Ki67 expression (c and d). Data represent mean percentages of 5 mice per group ± SE. * = p value < 0.05.

The methodology used to stain for the Ki67 antigen allowed us to associate the proliferative status of T cells during infection and their ability to produce IFN-γ. As shown in Figure 4.6, 70% of the IFN-γ positive CD4 T cells are negative for Ki67, and similarly about 70% of Ki67 positive CD4 T cells are negative for IFN-γ. T cells can thus be grouped as either being in a proliferative status or cytokine producing status, but not both.

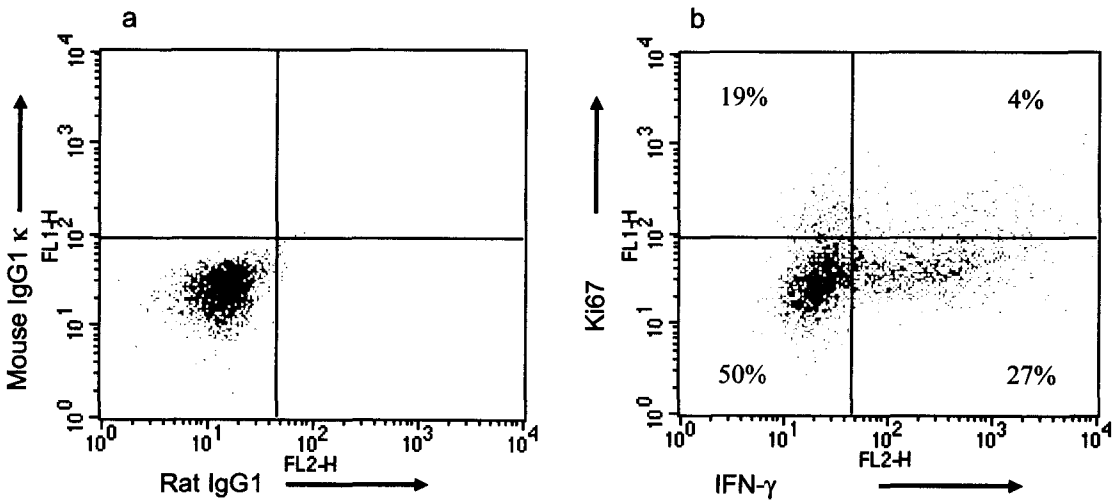

Figure 4.6 Intracellular double staining for Ki67 and IFN-γ. Lung cells from infected mice were stained for the surface marker CD4 and, after permeabilization, they were stained with FITC conjugated anti-Ki67 and PE conjugated anti-IFN-γ (b) or with their respective isotype antibodies (a). Shown are representative dot plots of the one mouse 20 days post infection. Lymphocytes were gated according to their FSC and SSC as well as for expression of CD4.

Histological analysis of the lungs from immunized and naïve mice.

The histological findings from immunized and naïve mice correlate with the findings from flow cytometry analysis. As shown in figure 4.7 at ten days post challenge no major pathological changes could be seen in the lungs of naïve mice (Fig 4.7 a and b) while immunized mice presented a few foci of cellular inflammation (Fig. 4.7 g and h). A higher magnification analysis of the lungs of immunized mice showed that the inflammatory foci were composed of lymphocytes as well as monocytes. At 30 days post infection several inflammation foci were seen (Fig. 4.7 c). Those foci were composed in its majority of monocytes, and a few lymphocytes seen as perivascular cuffs (Fig. 4.7 d). In contrast in the lungs of immunized mice there were fewer and smaller foci of inflammation (Fig. 4.7 i). At higher magnification the high lymphocyte content of the granulomatous lesions can be easily appreciated (Fig. 4.7 j). As infection progresses the granulomas of naïve mice increase in size and at 70 days post infection they occupy very large areas of the lung as the result of coalescent granulomas (Fig 4.7 e). In contrast at 70 days post infection the area occupied by the granulomas of BCG immunized mice does not seem to be much larger than those seen at 30 days (Fig. 4.7 k) and remain as isolated foci of inflammation.

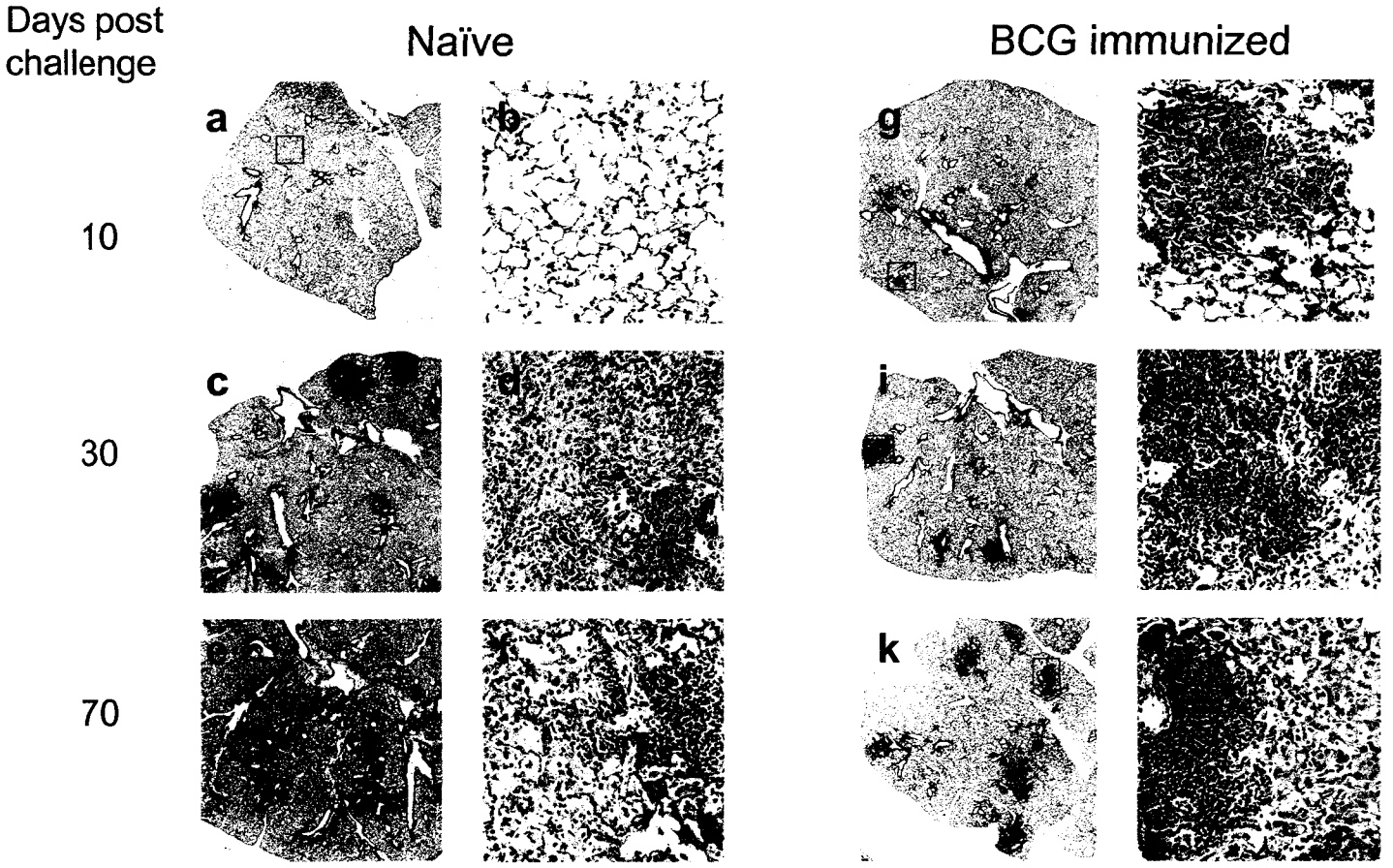

Figure 4. 7 Histological analysis of lungs from naïve (a – f) and immunized mice (g – l) after *M. tuberculosis* aerosol challenge. At indicated time points post infection the right caudal lobe was formalin fixed and sections were stained with hematoxilin and eosin. Low power magnification of representative sections (10x a, c, e, g, i, k) and 40x enlarged resolution of the boxed areas are shown to the left of each image (b, d, f, h, j, l).

Pulmonary characterization of T cells from BCG immunized mice

My data showed that BCG immunization protects mice from a low aerosol challenge with virulent *M. tuberculosis* as expected. The protection was conferred by an early increase influx of activated T cells, capable of producing IFN-γ, to the lungs in the very first few days following challenge. In contrast, a lag period of about three weeks was seen in naïve mice for the appearance of protective T cells. Interestingly BCG immunization alone resulted in the increase in the lung of T cells with phenotype similar to the activated T cells seen during infection with *M. tuberculosis*. Since I had eliminated all viable BCG by treating mice with antibiotics, I reasoned that those T cells might be indeed memory T cells. In order to further characterize the population of T cells present in the lungs of BCG immunized mice I decided to follow the fate of those T cells over a more prolonged period of time after immunization without any challenge. In this regard I followed immunized mice for 170 days after BCG immunization and at 30 day intervals I examined their lungs for the evaluation of their T cell profile. First I analyzed for the presence of T cells with the activation phenotype $CD44^{hi}/CD62^{lo}$, and as shown in Figure 4.8, during the period examined BCG immunized mice remained with significantly higher levels of both CD4 (Fig. 4.8 a) and CD8 (Fig. 4.8 b) activated phenotype T cells in the lungs. The increased presence of cells with an activated phenotype was even more pronounced when I analyzed the capacity of lung T cells to produce IFN-γ when stimulated *in vitro* with anti-CD3 and anti-CD28 antibodies. CD4 IFN-γ positive T cells were found in the lungs from immunized mice at about five to ten times higher numbers than in naïve mice (Fig. 4.8 c). Similarly CD8 T cells capable of producing IFN-γ from immunized mice were seen at about 4 fold increase as compared to naïve mice

throughout the observation period. Hence BCG immunization resulted in the presence of higher numbers of T cells with a phenotype that is important to control *M. tuberculosis* infection for prolonged periods of time.

Expression of memory markers by CD8 T cells in the lungs of immunized mice.

As already stated, immunized mice were devoid of viable bacteria and so I believe that the cells with an activated phenotype found in the lungs of immunized mice are in fact memory cells. There is increasing evidence that a pool of memory T cells are generated following proper antigenic stimulation that acquire an activated/effector phenotype, designated as memory effector T cells. These memory T cells have a phenotype with much similarity to effector/activated T cells such as high levels of CD44 expression, low levels of CD45RB and low levels of the lymph node homing selectin CD62L. Several other memory markers have been reported to be associated with memory CD8 T cells and I wanted to know if some of those markers were associated with the memory T cell population induced by immunization with BCG. Accordingly I analyzed the expression of the markers CD122 (common β chain of IL-2 and IL-15 receptors) and CD27 on CD8 T cells as they have been reported to be a marker for memory CD8 T cells [246, 253-255]. As shown in Figure 4.9 the expression of CD122 by CD8 T cells is strongly associated with the high expression of the CD44 molecule (Fig. 4.9 a), but when I looked to see if the lungs of BCG immunized mice presented higher levels of $CD44^{hi}/CD122^{+}$ CD8 T cells I saw no increase relative to non immunized mice. Surprisingly the immunized mice always presented lower numbers of CD8 T cells with this phenotype (Fig. 4.9 c). The expression of the marker CD27 by CD8 T cells was seen in both $CD44^{hi}$

and $CD44^{lo}$ expressing cells (Fig. 4.9 b), and like expression of CD122, immunized mice showed lower levels of CD8 T cells expressing the phenotype $CD44^{hi}/CD27^{+}$ (Fig. 4.9 d) compared to naïve mice.

One of the characteristics of memory T cells is their lower requirement of co-stimulatory signals to respond once they encounter an antigen in the context of MHC as compared to naïve T cells [256-258]. This lower threshold is reflected by a diminished expression of the co-stimulatory molecule CD28 on T cells. I then asked wether memory T cells in the lungs of BCG immunized mice indeed down regulate the expression of the CD28 co-stimulatory molecule on their surface. Firstly looking at naïve T cells among $CD4/CD44^{hi}$ that presented low expression of CD62L, the great majority of CD4 T cells (86%) expressed CD28 (Fig. 4.10 b, naïve $CD4/CD44^{hi}/CD62L^{lo}$), but during infection the percentage of CD4 T cells expressing CD28 decreased to 62% (Fig. 4.10 b, infected $CD4/CD44^{hi}/CD62L^{lo}$). In the lungs of immunized mice a pattern of CD28 expression similar to the infected group was seen (Fig. 4.10 b, $CD4/CD44^{hi}/CD62L^{lo}$ memory) supporting the idea that T cells present in the lungs of immune mice are memory T cells which down regulate the expression of CD28 molecule. There was no change in the pattern of CD28 expression among $CD4/CD44^{hi}/CD62L^{hi}$ T cells in the lungs, indicating that this phenotype of cells is not affected during infection or in immunized mice (Fig. 4.10 b).

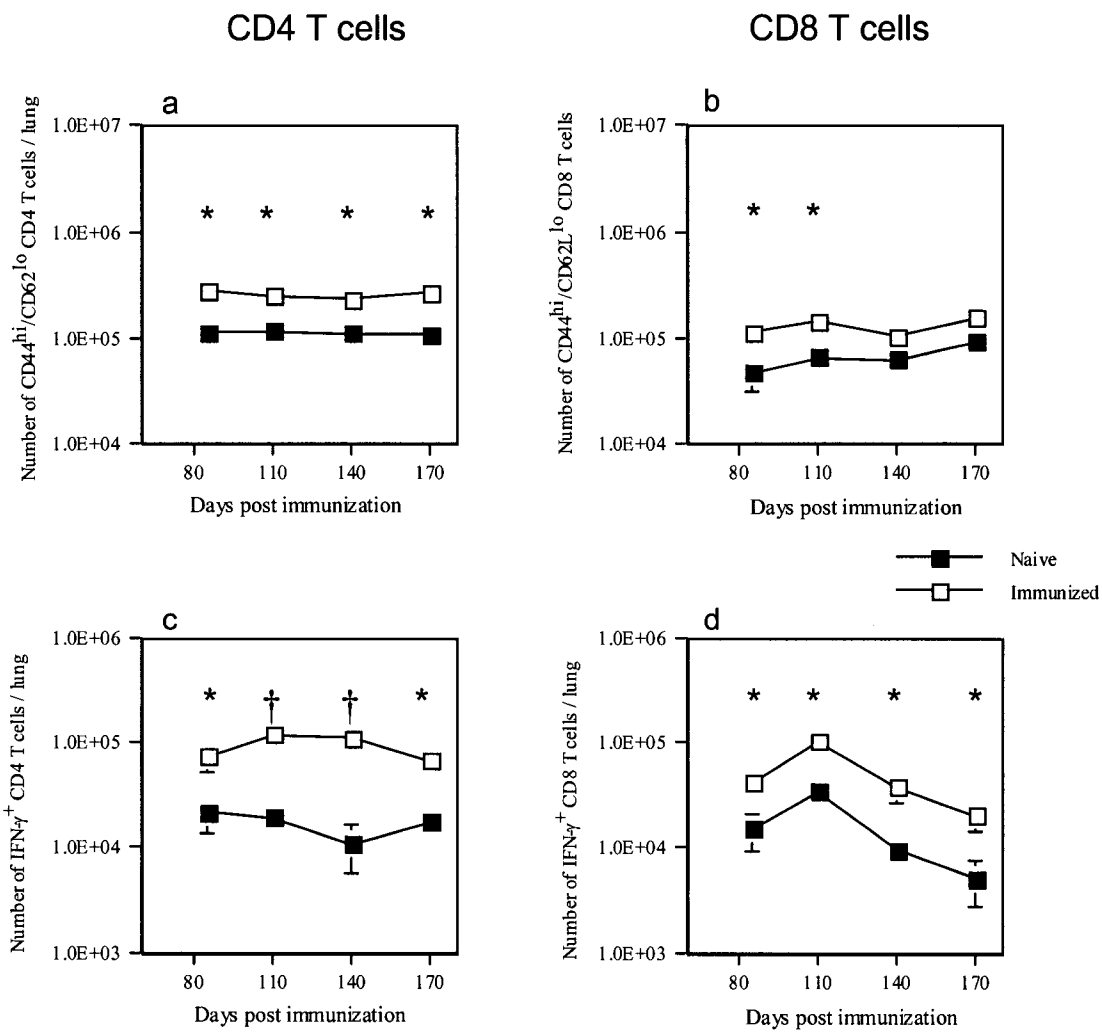

Figure 4.8 Analysis of activation markers in BCG immunized mice. Lungs of unvaccinated and BCG vaccinated mice were analyzed at 30 days intervals starting 15 days after the end of INH treatment. Lung cells were stained for the activation markers CD44 and CD62L and the number of CD4 (a) and CD8 (b) activated T cells with the phenotype $CD44^{hi}/CD62L^{lo}$ was determined. Likewise the number of CD4 (c) and CD8 (d) secreting IFN-g was determined. * = p value < 0.05, † = p value < 0.01 .

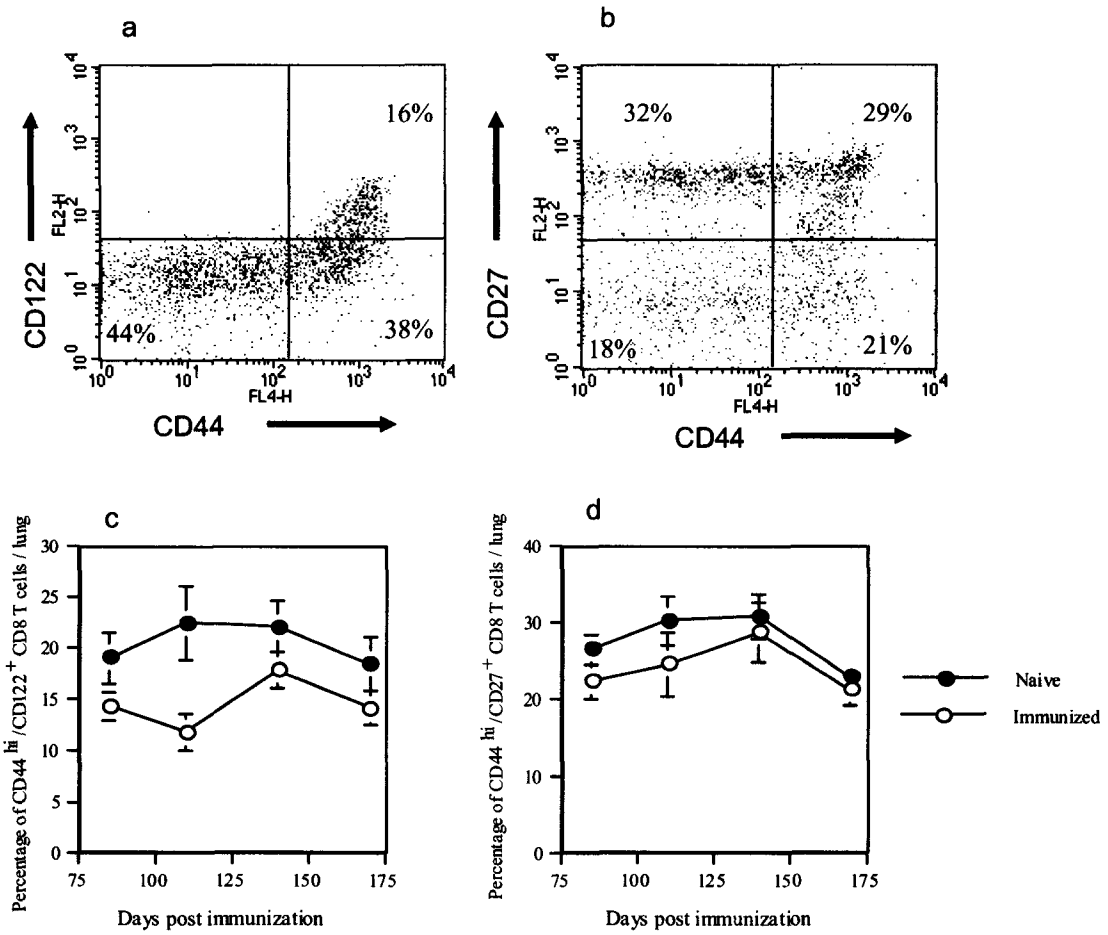

Figure 4.9 Analysis of memory markers of CD8 T cells in BCG immunized mice. Lung cells from BCG and naïve mice were analyzed for the expression of CD122 and CD27 markers in conjunction with CD44 on CD8 T cells. Following BCG immunization and drug treatment lungs from BCG vaccinated and control mice were stained for expression of CD44, CD122 and CD27 markers. Representative dot plot of the profiles of CD122 (a) or CD27 (b) and CD44 expression within CD8 T cells are shown (a). The percentages of $CD44^{hi}/CD122^{+}$ CD8 T cells were determined (c) as well as $CD44^{hi}/CD27^{+}$ (d).

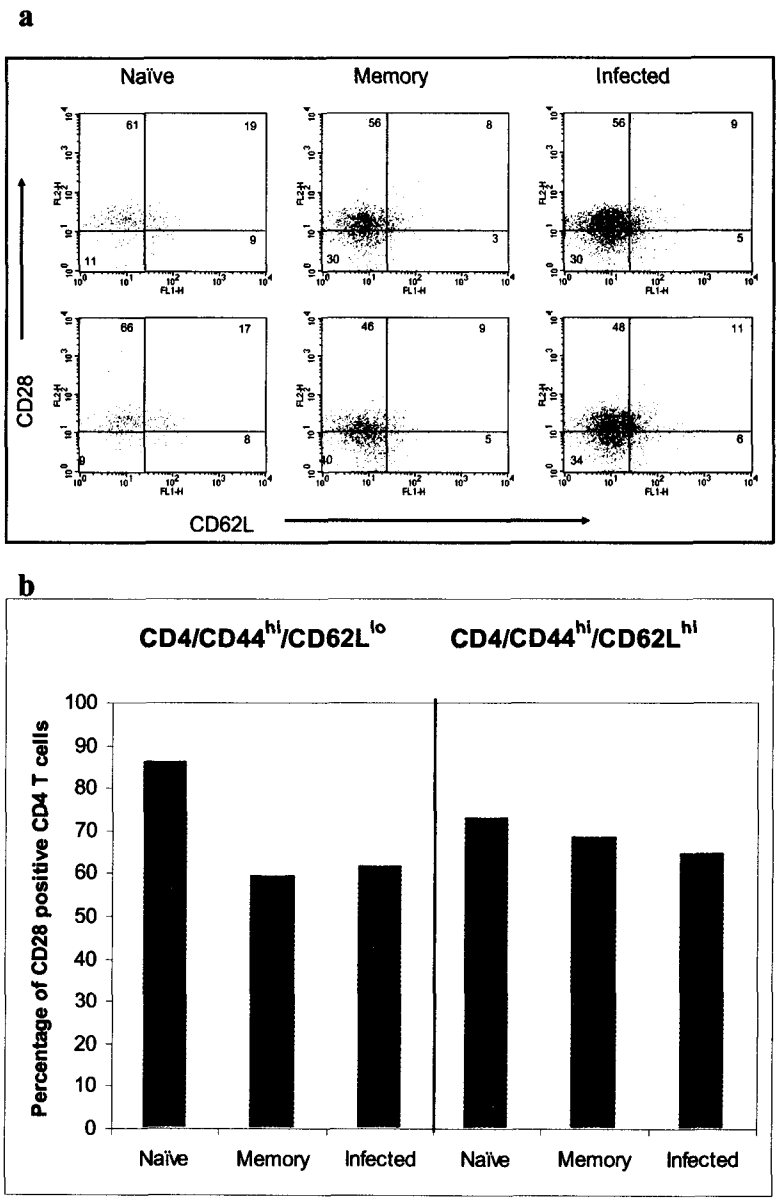

Figure 4.10 Expression of CD28 and CD62L molecules among CD4/CD44^{hi} T cell populations. Lung T cells from naïve, BCG immunized (memory), and *M. tuberculosis* infected mice lungs were labeled for the expression of CD4, CD44, CD62L, and CD28. Shown are representative dot plots from 2 mice per group of lung profiles of CD44^{hi} CD4 T cells from naïve, BCG immunized mice 140 days post vaccination, and infected mice at 150 days post infection (a). The numbers in each quadrant represent the relative percentages among CD4/CD44^{hi} T cells. The percentage of CD28 positive cells was calculated among CD4/CD44^{hi}/CD62L^{hi} cells and plotted on graph b. Likewise the percentage for CD28 positive cells among CD4/CD44^{hi}/CD62L^{lo} was calculated.

Cytokine mRNA levels in the lungs of immunized mice.

Although effector T cells that are actively responding to antigen share many surface marker expression similarities to memory effector cells, one fundamental difference is that while the former are producing and secreting cytokines, the latter does not. I have shown that some of the T cell population present in the lungs of immunized mice produce IFN-γ when stimulated *in vitro* with purified antibodies, but that does not tell us whether those cells are actively producing the cytokine *in vivo*. To address this question, real time PCR was performed to determine the levels of mRNA levels for IFN-γ in naïve non-infected, BCG immunized non-infected, and *M. tuberculosis* infected mice. Single amplification curves from immunized and infected samples are shown to illustrate that even though samples were normalized after amplification by the 18S Ct values, there were equal amounts of RNA recoveries from those samples (Fig. 4.11 a). As shown in Figure 4.11 cells from immunized mice had levels of IFN-γ message very similar to naïve mice while *M. tuberculosis* mice have levels 50 times higher (Fig. 4.11 c). These findings further support my idea that the T cells found in the lungs of BCG immunized mice are indeed true memory effector cells.

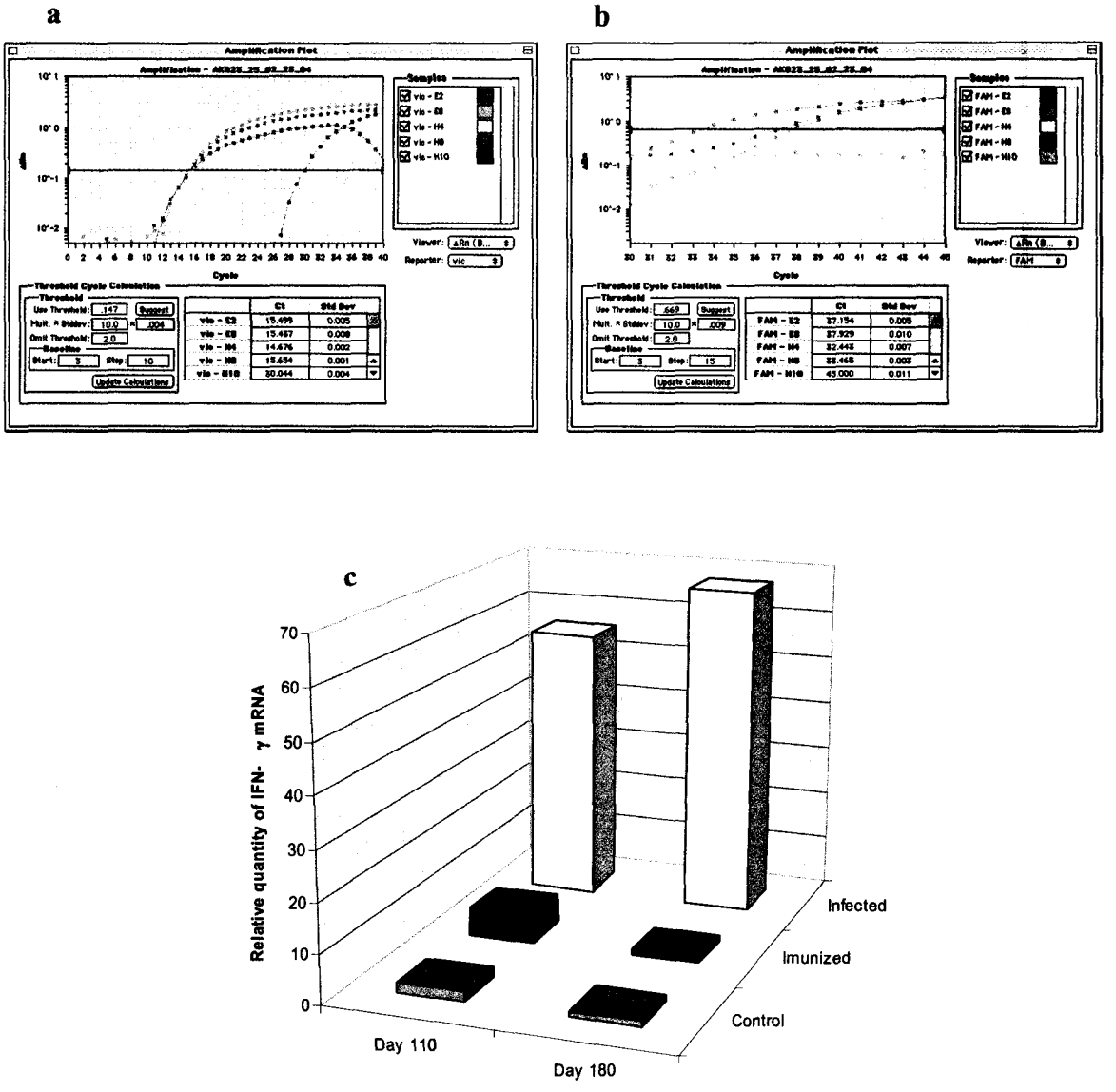

Figure 4.11 Gamma interferon production mRNA levels in the lungs of naïve uninfected, BCG immunized and *M. tuberculosis* infected mice. At designated time points post immunization or infection total RNA was extracted and the levels of mRNA messages for IFN-γ were determined by real time PCR. Snapshots of the amplification curves of the housekeeping gene 18S ribosomal RNA from BCG immunized and *M. tuberculosis* challenged mice are shown (a). The amplification curves for the IFN-γ message from the same samples are shown for comparison (b). Within the snapshots samples E2 and E8 represent amplification from BCG immunized tissues while samples H8 and H10 are from *M. tuberculosis* infected tissues. After normalizing the amount of RNA input to the reaction the IFN-γ levels were determined by the Delta Delta Ct method (c).

BCG immunization can rescue defect of lymphocyte recruitment in CCL2-KO mice in the early stages of infection.

Finally I addressed the question whether the induction of memory to BCG could overcome the deficiency seen in CCL2-KO mice (reported in chapter 2) of attracting IFN-γ secreting T cells to the lung, by infecting CCL2-KO and C57BL/6 mice that were either immunized or not with BCG and determined the bacterial load in the lungs at different time points. As shown in Figure 3.12 immunized CCL2-KO mice were able to control infection at the early stages of infection similarly to wild type mice, while non-immunized mice had much higher bacterial loads than control group mice (Fig. 3.12 a).

I looked at the number of activated T cells that were present in the lungs of challenged mice that were capable of producing IFN-γ and as shown in Figure 3.12 panel b, the immunization resulted in a higher number of CD4 T cells capable of producing IFN-γ prior to infection in both strains indicating that memory cells can mobilize to the lungs efficiently in the absence of the CCL2 chemokine.

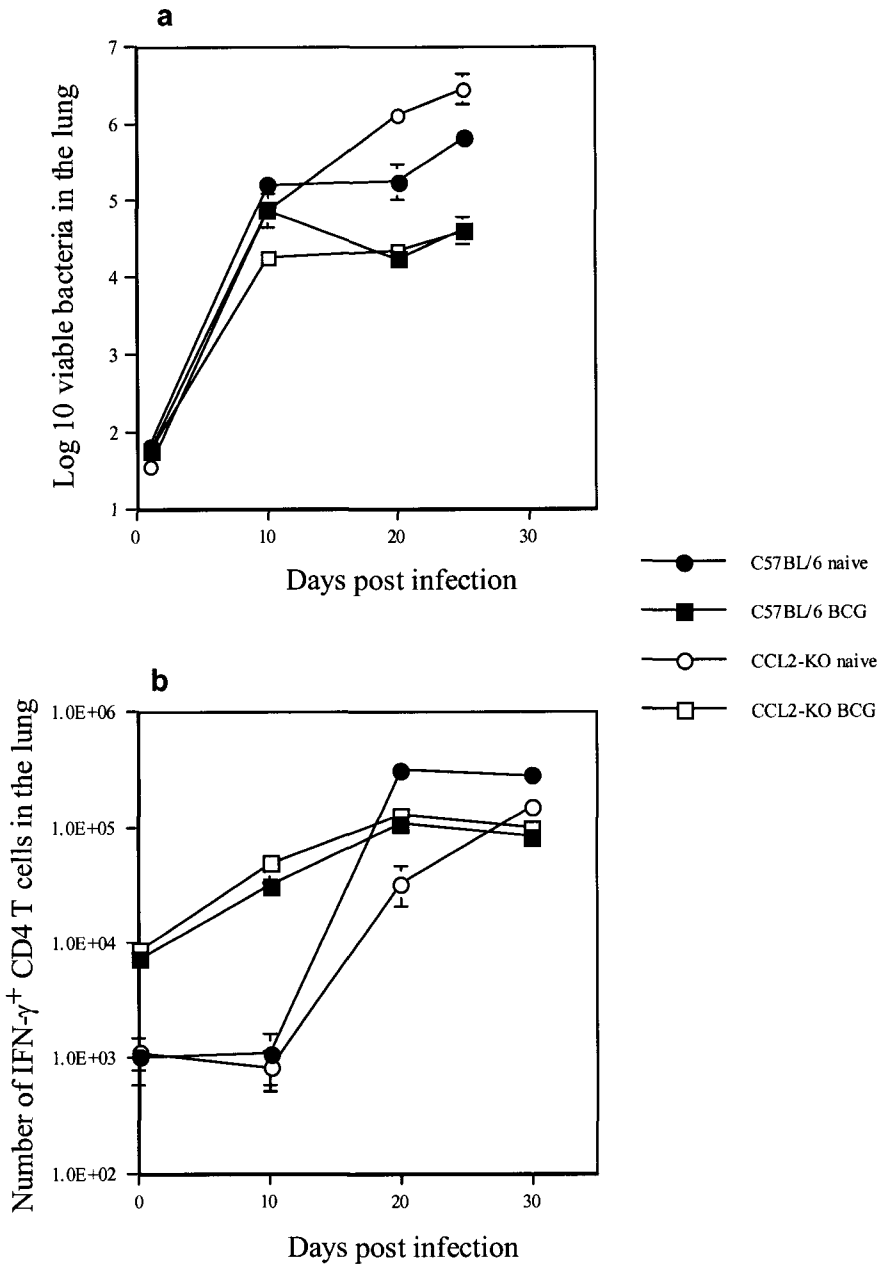

Figure 4.12 CCL2-KO (open symbols) and C57BL/6 (closed symbols) mice, naïve (circles) or previously immunized (squares), where infected with *M. tuberculosis* and the number of viable bacteria was determined (a). The number of CD4 T cells capable of producing IFN-γ was also determined. Data represent one of two independent experiments with similar results.

Discussion

The present work shows that BCG immunization induces the generation of memory T cells that remain present in the lungs of immunized mice for prolonged times in the absence of viable bacteria. Those T cells have high levels of expression of CD44 and low level of expression of CD62L and CD45RB, similar to the phenotype of activated T cells. Unlike activated T cells they are not constitutively producing cytokines but quickly do so when stimulated with antigen. In my studies antigen stimulation was done by *in vitro* crosslinking the T cell receptors with purified antibodies against CD3 and CD28 which is well correlated to the response one would get with antigen specific stimulation.

Treatment of immunized mice with antibiotics prior to initial analyses was important to assure that no viable bacteria from the immunization could remain as a potential active infection and hence a strong source of antigen that could be generating activated T cells able to migrate to the lungs of immunized mice. I checked for the presence of bacteria by plating lung and spleen homogenates from immunized mice in agar 7H11 after the end of antibiotic treatment. No viable bacterium was detected in those organs throughout the period of these experiments (data not shown).

Immunized mice have in their lungs a much higher level of T cells with the activated phenotype $CD44^{hi}/CD62L^{lo}$ as well as higher level of T cells that produce IFN-γ when stimulated *in vitro* with purified antibodies. The important role of these cells in producing IFN-γ and consequently protecting mice from a challenge infection with *M.*

tuberculosis has already been shown [259]. I have further evaluated this observation by analyzing more carefully the cellular composition of immunized mice without being challenged with *M. tuberculosis*. Immunized mice retained increased numbers of T cells with the activated phenotype throughout the period of this study (170 days post immunization). I have demonstrated that it is within the $CD44^{hi}/CD62L^{lo}$ population of T cells that reside the subpopulation that respond by producing the protective IFN-γ cytokine. I think that it is this initial higher accumulation of cells that promptly respond to infection producing the important protective cytokine, although I cannot rule out the contribution of memory T cells localized in the lymph nodes and spleen (central memory T cells) that may also respond when they encounter their cognate antigen.

Following challenge with virulent *M. tuberculosis*, immunized mice respond faster by increasing their numbers of activated T cells and producing IFN-γ in the first days, while naïve mice have a lag period of two weeks until they reach levels similar to immunized mice. Because of the delay seen in naïve mice the virulent bacteria remain unchecked in the lungs and continue to proliferate until about 21 days post infection when the infection is controlled. Consequently immunized mice can restrain the bacterial growth in their lungs at earlier stages of the infection resulting in a ten fold lower bacterial load in their lungs. After the critical period of 21 days when naïve mice start to control infection, although at higher numbers than immunized mice, I see an inversion in terms of activated T cell numbers where naïve mice then had higher numbers of $CD44^{hi}/CD62L^{lo}$ T cells as well as IFN-γ^{+} T cells as compared to immunized mice. I think that the higher bacterial load in naïve challenged mice seen after 21 days post infection reflects in a higher degree of inflammation in the lungs with high levels of

cytokine and chemokine production that result in the accumulation of higher numbers of activated T cells.

I wanted to determine if the increase in activated T cells following challenge was due to T cells that were actively proliferating in the lungs or if they are a result of influx from the circulation. The histological data of lungs from mice show that at about 3 weeks post infection a major influx of lymphocytes is seen that coincides with the control of infection. This influx is seen as lymphocytic perivascular cuffs in the lungs of both naïve and immunized mice indicating that the increase of lymphocytes was contributed by blood extravasation. To investigate if other mechanisms of lymphocyte proliferation were going on in the lungs of challenged mice I performed two distinct techniques based on flow cytometry and compared the findings with the histological observations. The first technique consisted of BrdU incorporation by cells that are actively dividing. I administered BrdU by injection rather than providing it in the water because the latter requires prolonged time of treatment and would increase the chances of BrdU incorporation in cells in other places of the body with sufficient time to migrate to the lungs. By doing so I observed that the peak of T cell proliferation was seen at 3 weeks post infection. At this time naïve mice had significant higher numbers of BrdU positive T cells in the lungs than immunized mice, which contributed to the inversion regarding the numbers of activated T cells. That is, naïve challenged mice had higher number of activated T cells after three weeks compared to immunized mice. Although injecting BrdU would seem to be better than administering it in water, it still required a certain amount of time to allow for BrdU incorporation and the number of BrdU positive cells seen in the lung could still be a consequence of cells that acquired BrdU in a location

elsewhere. For this reason I decided to look at the presence of the nuclear protein Ki67 which is only present when cells are cycling [260]. By performing intracellular staining I could have a real time determination of the cells that were actively dividing in the lungs. My data have shown that the kinetic profile of the number of CD4 and CD8 T cells expressing Ki67 was very similar to that seen by the BrdU method. This gave us confidence that Ki67 staining can be used in place of BrdU. When compared to BrdU the overall numbers of positive cells seen in the staining with Ki67 were lower, which means that the cells that were positive by the BrdU method only had their division taken place elsewhere and then migrated to the lung. The amount of T cells that are undergoing division in the lungs following challenge starts at very low levels and peaked at 3 weeks post infection where about 10-15 % of the T cells are proliferating in the lungs based on Ki67 staining. At the peak of proliferation naïve mice showed a significant higher number of proliferative activity which probably contributed to the increased numbers of activated T cells seen in that group after this time point. So there may be an important proliferative activity within the lungs of challenged mice that contributes to increase of activated cells seen in addition to the influx of cells from the blood circulation, especially at about 20 days post infection.

During tuberculosis infection activated T cells migrate to the lungs by preferentially expressing the adhesion molecules CD11a and CD54. The failure to do so may result in increased susceptibility to tuberculosis, as seen in the susceptible mouse strains CBA/J and DBA/2 compared to the resistant C57Bl/6 [261]. The expression of these same adhesion molecules seems to also be important to assure effector memory T cells home to non lymphoid tissues, in my case the lungs. During pulmonary tuberculosis, T cells may

mobilize to the lungs using other adhesion molecules. VCAM1 and VLA4 were seen to be up regulated [262] during pulmonary tuberculosis infection. It would be interesting to see if memory T cells in the lungs of immune mice are also using this pair of adhesion molecules.

There is significant evidence that IL-15 is important for the maintenance of subsets of CD8 memory cells even in the absence of antigen [263] and these memory cells are able to respond to this cytokine by maintaining high levels of expression of the CD122 molecule [254]. I have analyzed the expression of CD122 following challenge as well in immunized mice and although the expression of CD122 was seen mainly in activated/memory phenotype cells with high expression of CD44, I did not see higher numbers of $CD44^{hi}/CD122^{+}$ CD8 T cells in those mice. One of the possible reasons for this discrepancy in my observations may be that during BCG immunization as well as *M. tuberculosis* infection there is not a significant increase in this subset of CD8 T cells, or if there is for some unknown reason they do not migrate to the lung. A second explanation for not seeing an increase in the $CD44^{hi}/CD122^{+}$ CD8 T cells may be in my inability to look at antigen specific cells since I did not use tetramer staining of memory cells as most of the studies that characterized those cells used. Finally it has been proposed that the importance of CD8 T cells in response to pulmonary tuberculosis occurs in the chronic stages of infection [110, 117], but my studies did not look into chronically infected mice. It could be that the generation of memory CD8 T cells is a late event and that it had not yet taken place by in the time frame of my analyses.

As already mentioned, effector memory T cells have the property to rapidly respond to antigenic stimulation by producing cytokines so it is difficult to affirm that T cells that are IFN-γ positive by intracellular staining in my study are memory cells or if they are effector T cells. In my model I measured the levels of IFN-γ message by real time PCR and I could show that indeed there is no production of such cytokine in immunized mice, with similar levels to the levels seen in resting cells from naïve mice. In contrast lungs from infected mice had much higher levels of mRNA specific for IFN-γ. This observation strongly supports the hypothesis that the higher levels of IFN-γ positive cells seen by intracellular staining in the immunized mice were truly memory cells with a phenotype that matches the description of effector memory T cells that were able to produce the cytokine upon *in vitro* stimulation, while IFN-γ positive cells seen in the lungs of infected mice were T cells actively producing this cytokine *in vivo*.

Several reports strongly support the idea that a subpopulation of memory T cells reside in nonlymphoid tissues [264, 265], including the lungs, and that those memory cells have the effector memory T cell phenotype. In a recent study [266] that looked for the generation of memory T cell in the lungs of mice chronically infected with *M. tuberculosis,* it was found that only effector phenotype T cells were seen and according to that report memory T cells were not being generated. Due to the chronic characteristic of *M. tuberculosis* infection in mice, it is likely that in the lungs there is a constant antigenic stimulation resulting in the persistence of activated T cells throughout the course of infection. In order to become memory cells, T cells that are actively responding to antigen must have their stimulation ceased and/or decreased to basal levels which probably does not happen during pulmonary tuberculosis infection. On the other hand it

is possible that activated T cells migrate from the lungs to places where bacterial antigen is low and thus the conditions for them to become memory in those places are adequate. Having said that, I think that *M. tuberculosis* infection is likely to generate memory T cells as does BCG immunization, but the memory T cells that will remain in the lungs will have a phenotype similar to the phenotype of activated T cells, which indeed was observed in that report. Unfortunately that report did not address the question of the cytokine production status of the cells in situ. However, I would anticipate that during chronic pulmonary infection, aside from the generation of memory T cells there still is a large amount of viable bacteria and thus antigen stimulation of T cells, causing them to remain in an activated status. Thus memory effector T cells and effector T cells would not be discernable in the infected lung. Memory T cells with high levels of expression of CD62L is characteristic of central memory T cells and Junqueira-Kipnis *et al.* could not find any significant increase of this population, probably because it would be in the lymph nodes and spleen. I have tried in my analyses to look for the presence and/or increase of that population in those organs but I could not find any discernible results. Additionally within the CD4/CD44^{hi}/CD62L^{hi} population I could not correlate the expression of the costimulatory molecule CD28 when comparing naïve, immunized and infected mice. The use of tetramers will be of invaluable help to stain antigen specific cells for the *M. tuberculosis* or BCG infection to try to identify and characterize central memory T cells in lymphoid tissues.

Taking the above considerations together it is reasonable to say that although BCG immunization induces a consistent protective memory T cell population, it is also possible that the persistence of effector T cells during the course of pulmonary

tuberculosis infection may also be important and even necessary to keep the pathogens in check. Future studies using adoptive transfers of memory T cell populations would be useful to address the role the different T cells populations in the control of *M. tuberculosis* infection.

Lastly I addressed the question of whether memory T cells generated by BCG immunization could overcome the deficit of CCL2 chemokine following *M. tuberculosis* challenge. My data clearly show that memory CCL2-KO mice respond normally to infection and control infection in similar levels as immunized wild type mice. The numbers of activated T cells that accumulated in the infected lungs of challenged mice were also similar between the two immunized groups. The ability of memory CCL2-KO memory mice to control infection may be simply to the presence of higher numbers of T cells in the lungs, implying that the recall response can be achieved singly by those cells. Alternatively, CCL2-KO immunized mice could promote a quick attraction of activated T cells to the lungs following challenge and that would be done by the rapid production of compensatory chemokines. If compensatory chemokines are playing a role in the compensation mechanism of protection, it implies that memory T cells can produce those chemokines more efficiently and for such the chemokine expression should be determined.

In summary I have shown that BCG vaccination induces a stable population of T cells in the lung that contribute to the protection against *M. tuberculosis* infection. Those cells have on their surface many of the same markers seen in activated T cells. Markers used to characterize memory CD8 T cells such as CD122 and CD27 were not associated

with the memory cells produced by BCG immunization. The memory T cell population observed in this study fit the characterization of effector memory T cells which are characterized by high level of expression of CD44 and low level of expression of CD62L and CD45RB. Those memory T cells are not actively producing cytokines but may quickly do so when presented by its specific antigen and probably require low levels of costimulatory signals to do so.

CONCLUDING REMARKS

Tuberculosis is one of the greatest infectious disease threats for humans, with billions of infected people and millions of deaths annually. Although the world has experienced a significant decline after the initial use of antibiotic drug treatment in the 1950's, the disease bounced back due to the advent of AIDS in the 1970's such that in 1993 the World Health Organization considered it as an emerging infectious disease. The appropriate control of tuberculosis requires a great effort that many developing countries cannot achieve. For example, the treatment against tuberculosis requires six months of antibiotic treatment that must not be interrupted by the patient and it is the responsibility of the public health system of each country to make sure that the regimen is followed to completion. The failure to complete the chemotherapy will result in patients with latent tuberculosis that may reactivate and be an extra source of disease spread, and in the worst case scenario, a source of multidrug-resistant (MDR) mycobacteria. In fact MDR is now one of the great threats in developed countries where tuberculosis is controlled to very low levels. Indeed our attention must be turned to developing countries because that is where 95% of the 40 million HIV infected individuals are, as well as more than 80% of

tuberculosis infected people. Today it is estimated that between 15 and 20 percent of tuberculosis cases are attributable to HIV, meaning that tuberculosis control is tightly associated with HIV infection. It has been more than a century since Robert Koch discovered the casual agent of tuberculosis, and since that discovery the world has started to search ways to treat and or prevent this disease. Although great progress has been achieved, when I look at the epidemiologic data I realize that the fight will be long before it is finished.

Tuberculosis treatment and/or prevention are the ultimate goal of research but many other important applications have been drawn from the thousands of tuberculosis research reports. Tuberculosis is a disease model for intracellular pathogens and much of the knowledge on how these organisms cause pathogenicity as well as how the host responds to them is the result of such studies. Although intracellular pathogens share many common mechanisms of pathogenicity, each microorganism also has its individual characteristics, reflecting its particular physiology.

Tuberculosis has its name due to the clinical findings of lung tubercles in patients with pulmonary tuberculosis. Those tubercles are indeed the macroscopic manifestation of the granuloma formation, which is the hallmark of tuberculosis. The formation of granuloma in pulmonary tuberculosis is a consequence of the host response induced by the presence of the pathogen. In humans and guinea pigs "true" classical granuloma are formed and are characterized by a center of caseous necrosis that can be calcified or not, surrounded by a layer of macrophages, which in turn are surrounded by a layer of lymphocytes with fibrotic tissues surrounding the entire lesion. The mouse model does

not produce the classical model of granuloma where the concentric organization of different cell types is not seen. Additionally necrosis is not evident until very late stages of infection. While human and guinea pigs are considered more susceptible to infection than mice, it is reasonable to correlate differences in type of granuloma formation with differences in disease progression. It is important to determine if the organization of granuloma is the result of the effects of the acquired immune system or just an intrinsic property of the host or the organ. If I would consider that humans and mice can generate T lymphocytes in response to *M. tuberculosis* infection, with similar properties with respect to capability to produce sufficient amounts of IFN-γ and to lyse infected cells, then I could argue that the difference between the two species relies on the host properties to direct lymphocytes and macrophages to the site of infection. In this regard much is known about chemokine and cytokine secretion profiles in the mouse model following infection, and while such studies are not possible in humans, the guinea pig model will provide clues to answer this question. On the other hand it could be the case where the mouse can have a more robust acquired immune response and control infection more efficiently, but comparisons like these are difficult to make.

The granuloma is an important entity in tuberculosis as it is required to contain infection spread and reactivation. On the other hand the relative rigidness of the granuloma makes it difficult for protective lymphocytes, as well as activated macrophages, to infiltrate the core of the lesion where the bacteria and arrested macrophages are. In this regard the observation that mouse granulomas have more lymphocytes interspersed with macrophages than human and guinea pig granulomas could indicate that mice have more efficient activation of macrophages and thus control

infection more efficiently. It is of great importance to understand the factors governing the formation of granuloma and as already mentioned chemokines and cytokines are key players to this process. Identifying important chemokines in the attraction of different cell populations at different phases of the granuloma formation can provide important information for the design of new vaccines and treatments of active and latent tuberculosis.

I hypothesized that the CC chemokine CCL2 would be important in the early stages of granuloma formation based on clinical and experimental findings that this chemokine is produced at early phases of infection in the mouse lungs. As well, it is one of the major chemokines produced by active pulmonary tuberculosis patients. I tested my hypothesis by using mice that had its CCL2 gene disrupted genetically. In fact CCL2 chemokine was shown to be important in the early stages of granuloma formation such that, in its absence, important protective T cells could not be attracted to the inflammatory region. This finding corroborates the idea that if the cellular quality of the granuloma precursor is not right, there could be deleterious consequences like the increased bacterial load shown in this study. Chemokines are highly redundant in that different chemokines can bind to the same receptor and provide the same function. The increased susceptibility seen in CCL2-KO mice was transient, reflecting this redundancy, so during infection there is probably a sequence of events in chemokine production. Regarding those chemokines with homologous function to CCL2 I could say that initially CCL2 is the major source of lymphocyte attraction with protective capabilities, but as infection progresses other CCL2 homolog probably are. In the case of CCL2-KO mice, other homologs compensate for its absence and effectively attract protective T cells to maintain the infection at levels similar

to wild type mice. The observation that CCL2 is required early during infection can provide grounds for the development of new therapies based on chemokines.

The cellular response requires the orchestrated movement of cells from the circulatory system to the inflamed tissue and, as already mentioned, the chemokine and cytokine networks play a major role, but the adequate expression of cell surface markers on the cells required to mount the response is also a relevant requirement. Circulating leukocytes on the circulation that are needed at infection sites must express the appropriate molecules so they can extravasate into and migrate through the lung parenchyma. Naïve lymphocytes enter in contact with mycobacterial antigens most probably in the draining lymph nodes of the lungs following an aerosol infection. They are presented to antigen most probably by dendritic cells that have migrated from the lungs, and during this period became activated by upregulating costimulatory molecules and producing differentiating cytokines such as IL-12. In the lymph nodes T cells become activated by interacting with dendritic cells and induced to become Th1 T cells by IL-12 stimulation. Activated T cells up regulate the expression of CD44 molecules and chemokine receptors such as CCR2 and CCR5, and down regulate expression of CD62L, CCR7 and CD45RB molecules and leave the lymph node to enter the circulatory system. The expression of specific surface molecules on leukocytes is necessary for their extravasation from circulation and migration in the tissue toward the source infection. I have hypothesized that like chemokines the adequate expression of surface molecules on important T cells are required to have the proper response to tuberculosis infection. I was particularly interested in CD44 as this molecule is highly upregulated in all activated lymphocytes entering the lungs. During leukocyte movement in tissues the surface

glycoprotein CD44 is thought to interact with interstitial proteins, hyaluronic acid being the most important, and result in adherence of the cell to the tissue. To my surprise, the absence of CD44 expression on leukocytes did not interfere with control of tuberculosis infection, indicating that the migratory properties of the protective lymphocytes remained intact. Unexpectedly I found that CD44 deficiency resulted in an intense influx of polymorphonuclear cells that usually are not seen in the granulomatous response of mice until the chronic stages of infection. Despite the presence of those cells, the development of a protective response was not compromised. The presence of excessive polymorphonuclear cells may result is usually associated with tissue necrosis in granulomatous response to *M. tuberculosis* infection. During the period of my studies, I did see an increase in the necrotic lesions seen in the pulmonary granulomas from CD44-KO mice. It needs to be further investigated to determine if a low dose aerosol infection of CD44-KO mice could result in worsening of infection during the chronic stages of infection resulting in increase in bacterial numbers and/or decrease of life span of infected animals.

Many aspects of cellular trafficking during *M. tuberculosis* also applies to the trafficking of memory T cells and it would be valuable to have more insights in the role of chemokines and other adhesion molecules during a recall response. I first approached a more basic question on the nature of the memory response generated by BCG vaccination. The induction of memory T lymphocytes after vaccination with *M. bovis* BCG in mice is a phenomenon very well accepted, but there are many aspects of the induced protection that needs clarification. Understanding the characteristics of memory T cell populations, as well as the mechanisms by which they protect mice from virulent

M. tuberculosis challenge, is of great importance for designing new vaccines. In this regard the presence of memory T cells with an effector phenotype in the lungs of immunized mice is of great significance. I need to further investigate wether this cell population alone is sufficient to confer protection, and to that end I am planning to perform adoptive transfer studies of different T cell populations and challenge the receptive mice with *M. tuberculosis*. If indeed the memory effector T cells are proven to be important in protection, I can anticipate that designing vaccines that could induce the generation of higher numbers of memory effector cells in the lungs would be more protective. BCG vaccination in humans has long been questioned regarding its protection against pulmonary tuberculosis. It would be interesting to study if the vaccination in humans induces the generation of memory cells that localize in the lungs and if any correlation could be made between the generation of memory T cells and protection. The other class of memory T cells, central memory T cells could also be important for protection. These cells remain in the lymph nodes and in my studies I could not determine if BCG immunization induced an increase in this population. It is most likely that BCG immunization does induce such an increase but they were not detected in the context of spleen lymphocytes. By doing adoptive transfer studies, I could also address the question whether they are important for protection as well as whether there is correlation between the generation of memory T cells in tuberculosis infection, that is if effector memory T cells can be precursors of central memory T cells or vice versa. Another important question that remains unanswered is how memory status is maintained in BCG immunized mice. I have eliminated the presence of any viable *M. bovis* BCG by treating mice with antibiotics but it is known that follicular dendritic cells in the lymph

nodes retain antigens for long periods and could be a source for stimulating memory T cells. Again by performing adoptive transfer studies I can address the question if antigen is required to maintain the different memory populations or if their maintenance is cytokine driven.

In summary I have shown that there are chemokines hierarchies following *M. tuberculosis* infection, and this could be further exploited to target vaccines and even chemotherapies to improve tuberculosis prevention and treatment respectively. If I can further determine the type of memory cell population that provides the best protection against infection I can also apply this knowledge in the study of new vaccine candidates and see if they can promote the generation of the desired memory cell population, thus providing insights to which candidates would work best.

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