DISSERTATION

ANALYSIS OF T CELL SUBSETS INDUCED IN RESPONSE TO *MYCOBACTERIUM TUBERCULOSIS* INFECTION

Submitted by
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MARCELA I. HENAO-TAMAYO ANALYSIS OF T CELL SUBSETS INDUCED IN RESPONSE TO *MYCOBACTERIUM TUBERCULOSIS* INFECTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ANALYSIS OF T CELL SUBSETS INDUCED IN RESPONSE TO

MYCOBACTERIUM TUBERCULOSIS INFECTION

Tuberculosis, an ancient disease, still kills more people each year than does any other bacterial infection. The global epidemic of tuberculosis (TB) results in eight million new tuberculosis cases per year and two million deaths; 98% of these occur in developing countries. At present the only available vaccine against tuberculosis, *M. bovis* Bacillus Calmette-Guérin (BCG), has proven unreliable and only minimally protects against pulmonary tuberculosis in adults. The reasons why the BCG vaccine is not fully protective are still very unclear, and even though novel vaccines are being developed, there is not a clear understanding of what kind of immune response they should elicit in order to provide maximum protection. The overall aim of these studies, therefore, was to investigate the T cell subsets generated by *M. tuberculosis* [including clinical strains] as well as further analysis of those generated by BCG, in order to explore their role in protection against *M. tuberculosis* infection.

I started my thesis work by studying a strain of *M. tuberculosis* lacking the 19kDa lipoprotein, an important mycobacterial antigen, in order to evaluate if the mutation could alter the capacity of this strain to infect mice *in vivo*. The *M. tuberculosis* 19kDa mutant, the complemented strain and the virulent strain H37Rv were administered to C57BL/6 mice (resistant to the infection) and to IFNγ-KO mice (very susceptible to mycobacterial

infection). The results showed that the expression of the 19kDa lipoprotein is essential for the replication of the organism in the lungs of mice, both normal and immunocompromised, and that without it the bacilli persisted as a low grade chronic infection. In a second set of experiments the 19kDa mutant was used as a vaccine and compared to the gold standard BCG. Interestingly, when resistant C57BL/6 mice were vaccinated with the 19kDa mutant similar resistance to aerosol challenge infection was observed compared to the BCG control.

In an attempt to further understand the type of immune responses necessary to generate long lived protection against *M. tuberculosis* infection, I performed a series of experiments designed to evaluate the types of memory T cells involved in vaccination and challenge infection. C57BL/6 mice were infected by low dose aerosol with *M. tuberculosis*, and compared to animals BCG vaccinated but not challenged.

The rationale for these studies reflected current information that indicates that there are at least two subsets of memory T cells, which are generated to protect the organism against a second infection caused by the same organism. These two subsets have been designated as effector memory cells (T_{EM}) and central memory cells (T_{CM}). T_{EM} are thought to provide a first line of defense and are localized in peripheral tissues, while T_{CM} represent a second line of defense, and are localized in more central lymphoid tissues including the lymph nodes and the spleen.

My studies showed that BCG vaccinated, as well as chronically infected mice, both established significant populations of CD4 and CD8 T cells in the lungs that have characteristics of T_{EM} cells. In contrast, cells with a phenotype characteristic of T_{CM} represented a far smaller population. Based on these findings, our laboratory is now

hypothesizing that the BCG vaccine is a poor inducer of central memory T cells and that defect may be the fundamental reason why the vaccine appears to lose efficacy in children over a period of several years.

I then addressed what might happen to the distribution of these memory T cell subsets if the chronic infection was resolved by chemotherapy. Classical studies in the mouse model have employed this approach to establish a state of memory immunity that makes the animal highly resistant to rechallenge. In my studies this experimental approach indicated that the numbers of central memory cells can be increased and that they respond very quickly in the lungs if the mouse is reinfected. This was then followed by a second wave of T cells expressing an effector memory T cell phenotype, and the combination of these two responses clearly rendered the animal highly resistant, in comparison to control mice undergoing primary infection in which the bacterial load by day 30 was 2-3 log higher. Quite unexpectedly however, in the second month of the reinfection the memory T cell subsets significantly declined in numbers, the lungs become highly consolidated by macrophages, and the animals died. These observations challenge the notion that successful chemotherapy renders the individual highly resistant to secondary infection in the longer term. In this model I thus showed that resistance is potent but it is also transient, and propose that this may potentially be explained by rapid transition of the two main memory T cell subsets into secondary effector cells that are short-lived and rapidly lose their protective capacity.

Clinically, the efficacy of BCG seems to be very poor against newly emerging W-Beijing strains such as the highly virulent strain HN878. In my final piece of work I obtained this strain and characterized it in the mouse model. The results of this study

showed that the strain HN878 is clearly more virulent for mice than other strains of *M. tuberculosis,* growing faster and inducing severe lung damage. After 20 days or so the infection is contained and the lung pathology apparently slows, but animals still die more quickly than other virulent strains. Using flow cytometry I was able to demonstrate that mice infected with HN878 induce a potent TH1 [CD4, IFNγ-secreting] response but this is then followed by the emergence of regulatory T cells expressing a $CD25^{hi}$ Foxp3+ phenotype, with many of these cells also staining positive for IL-10. Whether this is a general characteristic of such highly virulent clinical strains is not known, but if so it could have a serious negative impact on the efficacy of new TB vaccines currently being developed.

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DEDICATION

My thesis is dedicated to my loving parents, Professor Dora H. Tamayo and Professor Hernán Henao, as their work inspired me all my life. Throughout my studies my parents always provided support, encouragement and more importantly love. To my little sister, Natalia Henao-Tamayo, who shared every moment in my childhood, taught me how to love life and most of all was my soul sister in times of need.

My thesis is also dedicated to my husband, Andrés Obregón who has been a great source of motivation, inspiration and passion for family, our children and science. A special dedication goes to my son, Emilio Obregón-Henao whom has enriched my inner being with the joys of motherhood, sleeplessness and endless love....and his precious little brother or sister who wiggles inside me now providing hope for the future.

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Brief history of tuberculosis

Mycobacterium tuberculosis has infected humans for thousands of years. The earliest evidence of tuberculosis (TB) in man and animals is provided by bone finds - mainly fragments of vertebrae - showing the gibbus typical of tuberculous Pott's disease. The oldest examples of spinal TB, in the form of fossil bones, date back to about 8000 BC. A bone from the Neolithic period (ca. 5000 BC), found in the region of Heidelberg, likewise shows evidence of tuberculous changes. Findings in certain Egyptian mummies clearly indicate that spinal caries existed around 2400 BC. The depictions of a gibbus in statuettes of Ancient Egyptian and pre-Columbian origin represents clear evidence of spinal TB. The earliest Chinese writings about lung fever and cough associated with generalized wasting date back to 2700BC, which is evidence for the world wide distribution of the disease since early ages. Lastly, there is the unique bacteriological finding of acid-fast bacilli in smears taken from a psoas abscess in the astonishingly well-preserved mummy of an Inca child from around 700 BC, clearly documenting a case of TB of the lumbar spine (1).

In 460 BC Hipocrates identified "phthisis" (a Greek word meaning consumption) as the most prevalent disease of the time. He also observed that it killed nearly everyone it infected. He even went so far as to warn other doctors not to visit patients in late stages of the disease due to the danger of catching it themselves.

In the 17th century people began to describe and record the exact pathology of the disease. In 1679 Sylvius wrote his Opera Medica in which he described actual tubercles as a characteristic change in the lungs and other parts of the body in consumptive patients. He went on to describe the abscesses and cavities that follow. The infectious nature of the disease also began to appear in writing in the 17th century. Italian medical literature reflected the fact that after death, the patient's possessions still may be contagious and need to be disinfected.

In 1720 Benjamin Marten, an English physician, theorized that TB could be caused by "wonderfully minute living creatures." He also thought that a healthy person could catch TB if they spent a large amount of time in contact with an infected person but the disease was rarely transmitted with short periods of contact. In 1854 Hermann Brehmer introduced the idea that TB was a curable disease. As a patient suffering from TB his doctor advised him to move to a healthier climate. After spending time in the Himalayas he returned home cured. This experience moved him to build the first sanatorium in 1863 in Austria, a place where patients could get plenty of fresh air, and good nutrition.

In 1865 Jean-Antoine Villemin, a French doctor, demonstrated that consumption could be passed from humans to cows to rabbits. With this evidence he postulated that the disease was caused by a specific microorganism and did not arise spontaneously in each affected organism as had been thought.

Mycobacterium tuberculosis was first viewed under the microscope in 1882 when Robert Koch developed a special staining technique that allowed him to see the organism.

By this time sanatoria were widely in use throughout Europe and the United States. Not only did they aid the healing process by providing rest, good nutrition, and a healthy environment, but they also served to isolate the sick from the healthy population. Trudeau demonstrated scientifically in the late 1800s the significance of the diet in the outcome of the disease. He inoculated rabbits with equivalent doses of tubercle bacilli and one group was set with enough food and water, while the other was confined in sunless cages with a poor diet. When the animals were sacrificed, the group with no restrictions showed healing of the disease, while those confined with poor diet all died of tuberculosis (2).

Forlanini, an Italian physician found that lung collapse had a positive effect on the outcome of the disease. For this reason, surgical methods to reduce lung volume and artificial pneumothorax were then developed and put to use. This represents the beginning of active therapy for TB.

In 1895 Wilhelm Konrad bon Rontgen discovered a form of radiation that could be used to view the progress and severity of a patient's disease. The most important advance at this time however was the development of BCG vaccine, which remains the only licensed vaccine today. Albert Calmette, a French bacteriologist, and his assistant and later his colleague, Camille Guérin, a veterinarian, were working at the Pasteur Institute in Lille in 1908. Their work included subculturing virulent strains of the tubercule bacillus and testing different culture media. They noted that a glycerin-bile-potato mixture grew bacilli that seemed less virulent, and continued research to see if repeated subculturing would produce a strain that was attenuated and therefore could be considered for use as a vaccine. By 1919 (over 200 passages) they had a now non-

virulent bacterium that was unable to cause tuberculosis disease in laboratory animals. Two ears later, in 1921, the BCG vaccine was first tested in humans.

In 1943 Selman A. Waksman, who had been working for decades to find an antibiotic that was effective against *Mycobacterium tuberculosis*, was finally successful. Streptomycin purified from *Streptomyces griseus* was first administered to a human on November 20, 1944. The results were very impressive. The disease immediately stopped its progression, the bacteria disappeared from the patient's sputum, and he recovered fully. In the years following, more and more anti-TB drugs were developed. This new drugs were very important in the light that antibiotic resistant mutants quickly began to appear. However, using combination therapies soon solved this problem (2, 3).

The Recent TB Epidemic

The registered number of new cases of TB worldwide roughly correlates with economic conditions: the highest incidences are seen in of Africa, Asia, and Latin America, continents with the lowest Gross National Products. The WHO estimates that eight to nine million people get TB infected every year, of whom 95% live in developing countries. In addition, the WHO reported 9.2 million new cases of TB in 2006, and estimated that around 709 000 (7.7%) were HIV-positive (probably an underestimate). It has also been observed that 3 million people die from TB every year (4).

In industrialized countries, the steady drop in TB incidence began to level off in the mid-1980s and then stagnated or even began to increase. Much of this rise can be at least partially attributed to a high rate of immigration from countries with a high

incidence of TB. It is also difficult to perform epidemiological surveillance and treatment in immigrant communities due to various cultural differences.

A great influence in the rising TB trend is HIV infection. Chances are that only one out of ten immunocompetent people infected with *M tuberculoisis* will fall sick in their lifetimes, but among those with HIV, one in ten per year will develop active TB. In many industrialized countries this is a tragedy for the patients involved, but it these cases make up only a small minority of TB cases. In developing countries, the impact of HIV infection on the TB situation, especially in the 20-35 age group, is worthy of concern (5).

A final factor contributing to the resurgence of TB is the emergence of multi-drug resistance. Resistance to anti-TB drugs arises from selection of naturally occurring mutants during drug therapy. Poor adherence to the therapeutic regimen, improper prescribing by clinicians, and drug interactions or malabsorption can result in partial suppression of bacterial growth and the emergence of resistant organisms. Once this acquired resistance develops, treatment is compromised, further resistance can evolve and resistant organisms can be transmitted to others, leading to primary drug resistance that may fail to respond to standard therapy. Since it is very unlikely that a single bacillus will spontaneously mutate giving rise to resistance to more than one drug, giving multiple effective drugs simultaneously will inhibit the multiplication of these resistant mutants. This is why it is absolutely essential to treat TB patients with the recommended four drug regimen of isoniazid, rifampin, pyrazinamide and ethambutol or streptomycin (6, 7).

Multidrug-resistant tuberculosis is defined as resistance to at least two first line drugs [rifampicin and isoniazid], with or without other drug resistance. Treatment for multidrug-resistant tuberculosis is very lengthy, less effective, costly, and poorly

tolerated. Estimates are that 6-7% of patients with tuberculosis worldwide have multidrug resistant disease, with more than 40% of these patients having been previously treated for tuberculosis (7). Eastern Europe has the highest prevalence: multidrug-resistant tuberculosis is found in about 10% and 40% of new and previously treated patients, respectively (7). Extensively drug-resistant (XDR) tuberculosis is, by definition, resistant to at least rifampicin and isoniazid, plus any fluoroquinolone and at least one injectable second-line agent (capreomycin, amikacin, kanamycin).

The term XDR-TB was first used in March 2006 by the US Centers for Disease Control and Prevention in a report on XDR-TB compiled from laboratory surveillance from a number of countries (8). The recognition that XDR-TB existed caused little notice at that time, but a subsequent outbreak of XDR-TB in HIV-infected people in KwaZulu-Natal, South Africa, first presented at the International AIDS Conference in Toronto in August 2006, definitely caught everyone's attention.

Regarding that outbreak, Gandhi *et al.* (9) reported that of 542 people with TB at the Church of Scotland Hospital in Tugela Ferry in rural Kwazulu-Natal province who had *M. tuberculosis* cultured from their sputum, 221 (41%) had MDR-TB, a level six-fold greater than the already high levels of MDR-TB in the province. More alarmingly, 53 of these 221 (24%) with MDR-TB had an *M. tuberculosis* strain that was also resistant to the two most clinically useful classes of secondline TB drugs, aminoglycosides (amikacin or kanamycin) and fluoroquinolones—that is, XDR-TB. Among the 53 with XDR-TB, 44 were tested for HIV and all were coinfected (median CD4+ T-cell count 63 cells per mm^3). Fifty-two of the 53 died in a median of just 16 days from the time of sputum collection. Molecular typing of the isolates indicated that 85% were clonally related,

implying epidemic transmission of the XDR strains throughout the hospital. Included among the deaths were 15 HIV coinfected individuals receiving antiretroviral therapy, and two HIV coinfected healthcare workers. The startling lethality of XDR-TB in a setting of high HIV prevalence had a shocking effect on clinicians and public health officials. (9). This outbreak emphasizes the need for new antimycobacterial drugs, increased surveillance, and caution in hospitals when nursing patients with suspected multidrug-resistant tuberculosis (10). In the absence of satisfactory practices to ensure adherence to medication, drug resistance will continue to emerge.

Innate Immunity to *Mycobacterium tuberculosis*

Evidence for innate immunity.

Phagocytic cells play a key role in the initiation and direction of adaptive T-cell immunity by presentation of mycobacterial antigens and expression of costimulatory signals and cytokines. In addition, innate defense mechanisms of phagocytic cells are important, as highlighted in Lurie's fundamental studies with resistant and susceptible inbred rabbits (11). Seven days after primary infection through inhalation of tubercle bacilli, the lungs of mycobacterium-susceptible rabbits contained 20- to 30-fold more viable mycobacteria than did the lungs of mycobacterium-resistant rabbits (12). Obviously, this difference during early infection cannot be attributed to T-cell immunity (13, 14) .

M. tuberculosis and complement

Endocytosis of *M. tuberculosis* involves different receptors on the phagocytic cell which either bind to non-opsonized *M. tuberculosis* or recognize opsonins on the surface of *M. tuberculosis*. As an example of the latter mechanism, mycobacteria can invade host macrophages after opsonization with complement factor C3, which is followed by binding and uptake through complement receptor 1 (CR1), CR3, and CR4 (15-17). The relative importance of the various receptors for complement factor C3 is apparent from experiments *in vitro*, in which in the absence of CR3, phagocytosis of *M. tuberculosis* by human macrophages and monocytes is reduced by approximately 70 to 80% (15). For opsonization with C3, the split product C3b is first generated by activation of the complement system. *M. tuberculosis* also utilizes part of the classical pathway of complement activation by direct binding to C2a, even in the absence of C4b; in this way the C3b necessary for binding to CR1 is formed (18). This mechanism facilitates mycobacterial uptake in environments low in opsonins, such as the lung. Nevertheless, non-opsonized *M. tuberculosis* can bind directly to CR3 and CR4 (19). However, the best-characterized receptor for non-opsonin-mediated phagocytosis of *M. tuberculosis* is the macrophage mannose receptor (MR), which recognizes terminal mannose residues on mycobacteria (15). When uptake by CRs and MR is blocked, macrophages may also internalize *M. tuberculosis* through the type A scavenger receptor (20).

M. tuberculosis and Collectins

Enhanced binding of *M. tuberculosis* to epithelial cells or alveolar macrophages may represent a risk factor for developing clinical tuberculosis. Collectins, a structurally

related group of proteins that includes surfactant proteins, mannose-binding lectins (MBLs), and C1q, seem to be important in this respect. Surfactant protein A (Sp-A) facilitates the uptake of *M. tuberculosis* (21), through binding to either the macrophages (22), type II pneumocytes (23), or neutrophils (24). In contrast, another surfactant protein, Sp-D, has been found to block the uptake of pathogenic strains of *M. tuberculosis* in macrophages (25). It may therefore be hypothesized that the relative concentrations of different surfactant proteins correlate with the risk of infection.

Another member of the collectin family, the plasma factor Mannan-binding lectin (MBL), may also be involved in the uptake of mycobacteria by phagocytic cells. MBL recognizes carbohydrate configurations on a wide variety of pathogens (26) and induces phagocytosis directly through a yet-undefined receptor or indirectly by activation of the complement system (26). Genetic polymorphisms of the MBL gene account for significant variability of serum MBL concentrations in different populations. One study has reported elevated concentrations of MBL in the serum of tuberculosis patients, and genetic polymorphisms associated with increased production of MBL have been reported to be a relative disadvantage in mycobacterial infections (27).

M. tuberculosis, Toll like receptors and (NOD)-like receptors

Toll-like receptors (TLRs) are phylogenetically conserved mediators of innate immunity which are essential for microbial recognition on macrophages and DC (28, 29). Members of the TLR family are transmembrane proteins containing repeated leucine-rich motifs in their extracellular domains, similar to other pattern-recognizing proteins of the innate immune system. The cytoplasmic domain of TLR is homologous to the signaling

domain of IL-1 receptor (IL-1R) and links to IRAK (IL-1R-associated kinase), a serine kinase that activates transcription factors like NF-κB to signal the production of cytokines (30).

To date, at least 13 TLRs have been identified in humans and man together; one of the most studied TLRs is TLR2, that recognizes PAMPs (Pathogen-Associated Molecular Patterns) from Gram-positive bacteria, including lipoproteins, lipopeptides, peptidoglycans and lipoteichoic acid, but also lipoarabinomannan from mycobacteria (31), phenol-soluble modulin from *Staphylococcus* (32), zymosan from fungi (33) and glycosylphosphatidylinositol from *Trypanosoma cruzi* (34). It remains unclear how a single receptor can recognize such a broad diversity of stimuli. A possible explanation is the association between TLR1 and TLR6 which were demonstrated to form heterodimeric complexes with TLR2 (35, 36). TLR6 association with TLR2 induced recognition of diacylated lipopeptide (35) but TLR1-TLR2 heterodimer binds preferentially triacylated lipopeptides (36).

TLR4 importance in innate immune response was initially demonstrated in C3H/HeJ and C57BL/ lOScCr mice strains that are resistant to endotoxic shock, due to their possession of a mutation in the *TLR4* gene. This receptor is essential for LPS detection but, like TLR2, TLR4 recognizes other molecules from different origins, including some agonists derived from plants. Furthermore, TLR4 is activated by endogenous ligands, such as heat shock protein 60 (HSP60) (found in *M. tuberculosis*), HSP70, fibronectin, hyaluronic acid, fibrinogen and heparan sulfate. LPS recognition by TLR4 requires the formation of a protein complex containing accessory molecules: LPS is generally bound to LPS-binding protein (LBP) present in the serum, this complex is

firstly recognized by CD14 receptor, strongly expressed in peripheral blood monocytes and macrophages (37). Once bound to CD14, LPS comes in close proximity with TLR4; however the efficient triggering of an inflammatory response requires the expression of the secreted protein MD-2 (37).

Several mycobacterial products have been found to activate mammalian cells in a TLR2-dependent manner. Arabinose-capped lipoarabinomannan (AraLAM), purified from rapidly growing mycobacteria is capable of activating mammalian cells in a TLR2-dependent manner (38). Like Gram-negative bacterial LPS, TLR2 dependent activation of cells by AraLAM was also found to depend on CD14. Subsequent studies have demonstrated that while *M. tuberculosis* does not contain significant amounts of AraLAM, it does possess its own TLR2-agonists. LprG (Rv1411) was found to be a TLR-2 agonist that upon prolonged stimulation through TLR-2, inhibited MHC-II Ag processing (39). A cell-wall-associated protein from *M. tuberculosis*, the 19kDa lipoprotein was found to activate cells in a TLR2-dependent manner (40). In addition to cell associated 19kDa lipoprotein, *M. tuberculosis* has been shown to also secrete another factor that activates cells in a TLR2-dependent manner (41). Investigators saw that culture filtrates of *M. tuberculosis* contained a heat-stable and protease resistant factor (STF). The fraction that had TLR2 stimulating activity had a mass of 6kDa and contained material that comigrated by SDS-PAGE with a small mycobacterial glycolipid, phospatidylinositol dimannoside (PIM). PIM was found to activate cells in a TLR2-dependent manner, similar to STF (42). Together these findings indicate that *M. tuberculosis* is fully capable of expressing distinct factors that can activate or deactivate cells via TLR2.

All TLRs can utilize the adapter protein MyD88 to propagate signals to gene targets and generate a rapid protective response, either by activating NF-κB of via other routes. MyD88 is also used by other inflammatory signaling pathways (e.g., IL-1 and IL-18). However, at least 2 TLRs (TLR3 and TLR4) can use alternative adapters such as Toll/IL-1 receptor resistance (TIR) domain-containing adapter inducing IFN-β (TRIF) and TRIF-related adapter molecule (TRAM; used by TLR4) that can activate responses distinct from those elicited by MyD88.

The role of MyD88 in *M. tuberculosis* infection is controversial, some groups have shown that infected MyD88-/- animals have a greatly enhanced susceptibility to aerosol infection with *M. tuberculosis*, equivalent to that observed with IFNγ-/- deficient mice (43). Also, other groups have seen fatal infection despite adaptive immune response in the absence of MyD88 (44). Another study reported that MyD88-null mice may not always succumb to *M. tuberculosis* infection (45). Collectively, this studies suggest that MyD88 appears to play a major, but still unclear, role in resistance to *M. tuberculosis*.

To complicate the situation even more, it has been difficult to attribute the TLR requirement to the function of a single receptor. Thus, mice deficient in TLR2, TLR4, TLR6, IL-1, or IL-18, although in some cases displaying specific defects in anti-mycobacterial responses, exhibit only minor increases in susceptibility to low dose aerogenic challenge (46-50). For example, *M. tuberculosis*–infected TLR2 -/- mice, have defective granuloma formation, but display only a small elevation in pulmonary bacterial loads late in infection and are able to survive for at least 150 days (50). Interestingly, however, when infected with unconventionally high doses of *M. tuberculosis*, TLR2-/- but not TLR4-/- mice exhibit greatly enhanced susceptibility compared with wild type

animals (46). This loss in resistance is accompanied by alterations in proinflammatory cytokine and nitric oxide synthase 2 expression, as well as, the pulmonary granulomatous response (50). TLR2 also has been shown to play an important role in the regulation of mycobacterial-induced cytokine production by APCs *in vitro* (48, 51).

A recent study showed that DNA harvested from *M. tuberculosis* is a potent stimulus of TLR9-dependent proinflammatory cytokine production by both DC and macrophages and that the *in vitro* responses of these cells to live mycobacteria are also partially dependent on TLR9 (52). In addition their results indicated that TLR9 plays an important role in the regulation of the mycobacteria-induced Th1 responses during *M. tuberculosis* infection *in vivo*. Finally, they demonstrate that mice doubly deficient in TLR9 and TLR2 show enhanced susceptibility to *M. tuberculosis* not observed in mice lacking either TLR2 or TLR9 alone (52). Taken together, these data provide an important example of TLR collaboration in host resistance to infection. Despite these results, previous investigations in which a CpG motif was added to culture filtrate proteins (CFP) of *M. tuberculosis* H37Rv as a vaccine had no effect on the vaccination outcome after the animals were challenged with *M. tuberculosis* (53). They observed that although adding CpG to the vaccines promoted substantially increased IFNγ production by lymph node draining cells, this failed to translate into enhancement of bacterial clearance in the lungs, influx of IFNγ -positive T cells, or changes in histopathology.

Recent studies have revealed a large novel protein family, termed the nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), which have a domain structure similar to the plant cytosolic R protein family that mediates resistance to

phytopathogens. Thus, it was proposed that NLRs regulate innate immunity in response to recognition of bacterial products in the cytosol (54). Two cytoplasmic receptors for viral dsRNA, retinoic acid inducible gene I (RIG-I) and melanona differentiation associated gene 5 (MDA-5), which modulate the anti-viral innate immune response were also identified recently (55, 56). These discoveries suggest that the mammalian cytosol is a rich environment for host–pathogen interactions and is a site of active immune surveillance.

NLRs, like TLRs, sense a variety of characteristic bacterial products, although only some NLRs have known ligands. Each family member contains a leucine-rich repeat region (LRR) required for ligand sensing, a NOD domain (also termed NACHT domain), and signaling units such as the caspase activation and recruitment domain (CARD), pyrin or baculoviral inhibitor of apoptosis repeat (BIR) domains (57). Activation of NLRs by bacterial products can stimulate two major signaling pathways: the nuclear transcription factor (NF)-κB pathway and the inflammasome. NF-κB, a heterodimeric transcription factor, is a key regulator of the pro-inflammatory response, activating genes that encode cytokines and co-stimulatory factors (58). NF-κB is also activated by extracellular binding of microbial ligands to TLRs, thus, although NLRs and TLRs can be triggered independently, the signals from both pathways intersect to use common intermediates such as the inhibitor of κB kinase (IKK) complex . NLR proteins can also activate caspase-1 by way of the inflammasome — a multi-protein complex activated by diverse stimuli (59). A primary function of caspase-1 and the inflammasome is to process the inflammatory cytokines proIL-1β and proIL-18 to their mature and active forms; caspase-1 can also trigger host cell death. TLR signaling induces the expression of proIL-1β.

Thus, cytosolic bacterial detection by NLRs might play a distinct role in amplification of TLR-dependent signaling and modulation of the caspase-1-dependent inflammatory response. TLR and NLR signaling from different cellular compartments could serve to alert the host to immunological signal for danger and help protect the organism from inappropriate immune responses to non-pathogenic bacteria (60).

M. tuberculosis and Nitric Oxide (NO)

The high output expression of nitric oxide (NO) in response to cytokines or to pathogen-derived molecules is an important component in the host defense against intracellular microorganisms as different as *Toxoplasma gondi* , *Leishmania major*, *Listeria monocytogenes*, *Plasmodium* species, Ectromelia virus, Coxsackie B3 virus, *M. leprae*, and *M. tuberculosis* (61-63). NO is formed when the guanidine nitrogen of L-arginine is oxidized by a family of iso-enzymes known as NO synthases (NOSs). Exposure to NO at low concentrations, e.g., <100 ppm, killed more than 99% of *M. tuberculosis* in culture (64). NO and other Reactive Nitrogen Intermediates (RNI) can modify bacterial DNA, protein, and lipids at both the microbial surface and intracellularly. NO can also deaminate as well as directly damage bacterial DNA, generating basic sites and strand breaks. Other potential mechanisms of killing by NO include interaction with accessory protein targets such as iron-sulfur groups, heme group, thiols, aromatic or phenolic residues, tyrosyl radicals, and amines, resulting in enzymatic inactivation or other protein malfunctions (65).

In the murine model of TB, NO plays an important role in killing *M. tuberculosis* by mononuclear phagocytes (61, 66, 67). Intratracheal administration of virulent *M.*

tuberculosis to rats stimulated induced NOS (iNOS) and NO production in alveolar macrophages (68). Moreover, administration of the nitric oxide synthase (NOS) inhibitor L-NG-monomethylarginine (L-NMMA) intraperitoneally attenuated the *M. tuberculosis* induced increase in RNI in lung homogenates and bronchoalveolar fluid. One of the best examples of the protective role of NO in murine TB is demonstrated by the genetically disrupted iNOS mouse strain (iNOS -/-), where infection with *M. tuberculosis* was associated with a significantly higher risk of dissemination and mortality compared with the wild type C57BL/6 mice (67, 69). However when iNOS -/- mice were infected with low numbers of *M. tuberculosis*, and compared with the highly susceptible IFNγ-/- strain, the iNOS -/- mice had significantly prolonged bacterial control(70). This study demonstrated that, even though the iNOS -/- mice eventually die, they are able to control bacterial growth in the lungs of infected animals.

Recent work indicates that preventing reactivation of latent infection appears to be under the control of both RNI and non-RNI pathways (71). In murine models of latent infection, administration of the NOS inhibitor aminoguanidine led to the development of reactivation TB, although a non-RNI pathway also seemed to be involved. In a more recent study, depletion of CD4+ T cells in a mouse model of latent infection showed that although CD4 was required for preventing reactivation disease, it was by an iNOS- and IFNγ –independent antimycobacterial mechanism (71). These findings point out both NO-dependent and NO-independent mechanisms that are functioning to maintain the latent state, although the applicability of these results to humans is uncertain. In humans, a direct role for RNI has been more difficult to demonstrate, although NOS2 has been demonstrated within the granuloma (72).

M. tuberculosis and Macrophages

Alveolar macrophages resident within the lung are considered to be the main cellular host for mycobacteria *in vivo* (73). This is due to fact that *M. tuberculosis* persists in immature phagosomes by preventing their maturation into phagolysosomes and thus evading being killed by lysosomal enzymes. Mycobacteria are taken up via phagocytosis. The type of receptor mediating this event can influence the response generated within the macrophage. The receptor molecules that have been implicated in the uptake of mycobacteria include mannose receptors that bind mannosylated molecules on the bacterial surface, Fc receptors binding opsonized cells, and complement receptors. The use of complement receptor-3 (CR3) by mycobacterial species may be advantageous for the bacterium, as triggering this receptor does not induce the release of potentially cytotoxic reactive oxygen intermediates (74). Binding to the mannose receptor has also been suggested as a possible safe route of entry for mycobacteria that facilitates their intracellular survival (75).

Following uptake into the phagosome and when phago-lysosome fusion occurs successfully the bacteria is destroyed and bacterial antigens are processes and presented to T cells in the context of MHC molecules. The stimulation of T cells in this way activates the adaptive arm of the immune response and induces IFNγ release from CD4+ and CD8+ T cell which are the anti-microbial defense system. However, it has been known for some time that mycobacteria can evade the normal phagosome–lysosome fusion pathway and that this results in persistence of the bacteria within the host. This ensures that the bacteria remain viable but also that bacterial antigens do not become

available for presentation to T cells. Mycobacteria appear to slow the maturation of the phagosome in such a way that they retain early endosomal markers and cannot fuse with the lysosome. There are also increased amounts of host proteins in the mycobacterial phagosome including the host protein tryptophan-aspartate containing coat protein (TACO) which is normally removed from the phagosome just prior to lysosome fusion (76). Mycobacteria appear to increase retention of TACO, which prevents phago-lysosome fusion. These observations indicate that mycobacteria can specifically sequester the phagosome from the normal endosomal pathway as a way to ensure survival.

Sequestration of bacterial antigens from the normal endocytic pathway inside macrophages implies that these cells may not be the major antigen presenting cells for the stimulation of T cells reactive to mycobacteria. Regardless, effects on cytokine synthesis and expression of molecules on the cell surface of macrophages have been reported in some studies due to the interaction of mycobacteria and antigen presenting cells. Macrophages infected with *M. tuberculosis* preferentially secrete pro-inflammatory cytokines including TNFα, IL-1 and IL-6 (77). Infected macrophages are known also to secrete chemokines including IL-8, RANTES and MCP-1 (reviewed by (78)) which would aid the recruitment of lymphocytes to the lung and granuloma formation, thus leading to containment of the mycobacteria. In addition *M. tuberculosis* infected macrophages secrete IL-10, rather than IL-12, which in late infection could suppress Th1 responses (77, 79). IL-10 may also inhibit export of MHC class II molecules to the cell surface, which would down-regulate T cell responses. Reduced MHC class II expression in *M. tuberculosis* infected macrophages has been reported to be linked to prolonged stimulation of TLR2 by mycobacterial lipopeptides (80, 81). A reduced ability of these

cells to signal T lymphocyte activation combined with recruitment of cells to form granulomas may help mycobacterial persistence within the host. However it is known that stimulation of macrophages by other components of the immune response, such as IFNγ or TNFα released by T cells, can enhance macrophage microbicidal activity and this is associated with reduced IL-10 secretion (77, 79).

Alternatively, phagocytosis of particulate antigens (secreted by the mycobacteria) and apoptotic cells by DC and macrophages leads to MHC class-I (MHC-I) antigen processing and presentation through a process termed cross-presentation. Several models have been proposed to account for presentation of these antigens in a MHC-I-dependent manner. Infection with live, but not heat-killed, *M. tuberculosis* facilitates the MHC-I-dependent recognition of soluble OVA in a TAP-dependent manner (82). Similarly, macrophage phagosomes containing live BCG appear to be porous to molecules of 70kDa or less (83). *M. tuberculosis* lipids and lipoproteins, actively trafficking in phagosomally derived vesicles (84), are cross-presented by bystander APC in a TAP independent and proteosome-independent manner (85, 86). This mechanism, of antigen processing, explains why nearly all of the known mycobacterial antigens recognized by CD8 T cells are found in bacterial culture supernatants, many of them known to be actively secreted by the bacterium (87-89).

Another macrophage function recently described, is autophagy. Cells undergo autophagy by sequestering their own cytoplasm into an autophagosome that is the delivered to the lysosome (90). Autophagy has been described as a defense mechanism inhibiting BCG and *M. tuberculosis* survival in infected macrophages. IFNγ induces autophagy and inhibition of autophagy increases the viability of intracellular

mycobacteria in mice and humans (91). Almost certainly new ways in which *M. tubeculosis* interacts with macrophages will be reported in coming years.

M. tuberculosis and Dendritic cells (DC)

Dendritic cells (DC) are cells that are specialized for the presentation of antigen to T cells. They are the most potent of the antigen presenting cells and are central to the initiation of immune responses in naive animals (92, 93). They originate in the bone marrow but there is good evidence that they may be derived from both myeloid and lymphoid precursors.

Different properties have been established that are critical to the function of DC as a crucial antigen-presenting cell population. These include the ability to effectively take up antigen by a number of routes, which may include endocytosis by clathrin-coated caveolae, as well as, macropinocytosis or phagocytosis depending on the maturation stage of the cell. High levels of expression of MHC class II and of a number of co-stimulatory molecules that include CD80, CD86 and CD40 have been shown to contribute to the efficiency of DC as antigen presenting cells (93). In the periphery DC serve as sentinels monitoring the exposure of the body surfaces to antigen. At these sites, DC are considered as immature cells that express low to moderate levels of MHC class II and costimulatory molecules, and which have a high capacity to phagocytose micro-organisms and other particulate antigens (94). As such, these immature DC are efficient in antigen uptake, but are poor stimulators of T lymphocyte responses. These DC are stimulated to migrate away from the body surface by tissue injury, inflammation or

infection (92). This process of migration from the periphery, via the afferent lymphatic vessels to the draining lymph node is associated with functional and phenotypic maturation such that, upon arrival in the lymph node, the DC have acquired the capacity to effectively stimulate naive T lymphocytes. This appears to be related to the up-regulated expression of MHC II, CD80, CD86, CD40 and other surface molecules that are involved in the interaction with, and stimulation of, T lymphocytes by DC (92).

In addition to their capacity as antigen presenting cells, DC can also act as modulators of the immune response and link innate and adaptive immune responses. The innate response of DC to microbial antigens may polarize the T cell response towards Th1 or Th2 phenotypes. This is linked to the capacity of DC to produce the cytokines IL-12 and IFNα (95, 96) that can up-regulate the secretion of IFNγ by T lymphocytes and NK cells thus driving a Th1 biased response. Extensive *in vitro* evidence supports that DC can phagocytose mycobacteria and that DC function and phenotype can be modified following bacterial uptake.

The involvement of the DC in the carriage of the bacteria to the lymph node is essential to the initiation of the immune response. Interaction of human DC with *M. tuberculosis* or BCG results in cell maturation and activation that is characterized by changes in cell surface phenotype and cytokine profiles (77, 79). The uptake of mycobacteria by DC triggers their expression of surface molecules that are involved in the interaction with T cells, notably MHC II and the costimulatory molecules CD40 and CD80. Taken together this suggests that infected DC have an augmented capacity to stimulate mycobacteria reactive T cells.

Infection of DC with either *M. tuberculosis* or BCG is associated with increased expression of IL-12, TNFα, IL-1 and IL-6. These cytokines play major roles in protective anti-mycobacterial immune responses. As noted above IL-12 secreted by DC can potentiate IFNγ and TNFα secretion by T cells and this in turn may serve to enhance the anti-microbial activity of macrophages to destroy invading bacilli. In addition to the production of proinflammatory cytokines, mycobacterial infection of DC is also associated with the secretion of IL-10, which may inhibit the cellular response to mycobacteria through the down-regulation of IL-12 secretion (77, 79). This may serve to limit the extent of DC and macrophage activation and thus regulate the potentially damaging immune response that occurs in tissues *in vivo*.

Like non-activated macrophages, DC are reported to provide an environment within which mycobacteria can survive and replicate, although to a low extent (97, 98). It appears that the replication of mycobacteria within DC is not enough to kill the host cell and the outcome of slow replication is reflected by constant availability of antigens for presentation to T cells that will therefore potentiate the immune response. Following activation by IFNγ or TNFα macrophages may kill almost all of the mycobacteria resident within the phagosome. This does not occur in activated DC. Following stimulation with IFNγ DC are able to control the replication of mycobacteria but they are not killed by the DC. Instead they appear to reside in vacuoles separated from the normal recycling pathway (97, 98). DC may, therefore, be a reservoir for mycobacteria *in vivo*, particularly within lymph nodes to which they have migrated following the initial response to mycobacterial infection. The implication of these observations is that uptake

of bacteria by the different antigen presenting cells will influence the outcome of infection.

The study of Macrophage and DC populations in *M. tuberculosis* infection is definitely a complex mater; several techniques have been used to define APC populations entering the airways and lung tissues of infected mice. By the use of cell surface markers, especially CD11b and CD11c, several cell populations can be distinguished, allowing cell sorting and morphological definition. Primary populations of $CD11b^{-}/CD11c^{+/high}$ have been defined as alveolar macrophages, $CD11b^{high}/CD11c^{+/high}$ as dendritic cells, and $CD11b^{+/mid}/CD11c^{+/mid}$ as small macrophages or monocytes (99). This information should facilitate further investigation into both the physiology and contribution of these populations to the host immunity in the lungs.

M. tuberculosis and Neutrophils

In infection, Polymorphonuclear lymphocytes (PMNs), principally neutrophils, are the first phagocytes to arrive from the circulation and attempt to eliminate invading pathogens via oxygen-dependent and oxygen-independent mechanisms. The first mechanism results from the generation of reactive oxygen species (100), whereas the latter mechanism reflects the capacity of PMNs to degranulate and release preformed oxidants and proteolytic enzymes from granules (101). Neutrophil degranulation is meant to target the pathogen; however, these powerful substances may cause the destruction of

neighboring cells and damage of tissue (102). Thus, a strict regulation of neutrophil influx and their turnover in infected tissues is essential for minimizing tissue damage.

PMNs have been implicated in the control of mycobacterial infections (103, 104), but it is not known whether these cells have direct protective functions. Initial *in vitro* studies suggested that human neutrophils are able to kill virulent *M. tuberculosis* (105, 106). The recruitment of PMNs to the lung has been described for acute TB (107) and in experimental animals infected with mycobacteria (108). Human and animal studies indicate that neutrophils, while not very helpful in mycobacterial clearance themselves (109), may play an important role in the transition from innate to adaptive immune responses by producing critical cytokines and chemokines (110, 111). Although this immunomodulatory function has been demonstrated for many intracellular infections, including TB (112, 113), the pleiotrophic nature of mediators leaves unanswered the exact contribution of neutrophils to TB resistance and pathogenesis.

In Guinea pig studies prominent pockets of granulocytes have been described in the lungs of infected animals (114); the researchers hypothesized that these may represent a double-edged sword in that they may be contributing to early protection, as suggested previously, but their accumulation in response to local tissue damage and their own subsequent degranulation may also contribute to the local pathological process. They also demonstrated that the accumulation of granulocytes happens very early during the course of the infection, and they may be the trigger to the initial development of the characteristic central necrotic core.

A recent study assessed *in vivo* and *in vitro* the capacity of neutrophils from I/St (susceptible) and A/Sn (resistant) mice to migrate in response to mycobacteria. They also

evaluated the capacity to engulf and kill the bacilli, to undergo apoptosis, and to produce immune response mediators. Their results suggest that, in highly susceptible I/St mice, neutrophils play the role of a "Trojan horse" for mycobacteria and contribute to the development of severe lung inflammation rather than protection of the host (115).

M. tuberculosis and Natural Killer (NK) cells

NK cells are also effector cells of innate immunity. They are distinct from T and B lymphocytes in that they do not show germ-line receptor rearrangement and their effector functions are governed by combinations of activating and inhibitory receptors. These allow the recognition of altered-self at a cellular, rather than the molecular level of T-cell receptor and immunoglobulin. Upon activation, NK cells can mediate direct cytotoxicity via perforin, or secrete cytokines predominantly of the T helper type 1 (Th1) type. These cells may directly lyse the pathogens or can lyse infected monocytes (116).

In vitro culture with live *M. tuberculosis* led to the expansion of NK cells implicating that they may be important responders to *M. tuberculosis* infection *in vivo* (117). During early infection, NK cells are capable of activating phagocytic cells at the site of infection. Apoptosis is a likely mechanism of NK cytotoxicity. NK cells produce IFN-γ and can lyse mycobacterium pulsed target cells (117).

In a more recent study, the role of NK cells in early innate resistance to infection with *M. tuberculosis* was evaluated in mice, after an aerosol infection. A steady increase in NK, but not NKT, subsets was observed within the lung over the first 3 wk of infection. NK cells increased the expression of cell surface activation markers, were IFNγ positive and labeled positive for intracellular perforin after *in vitro* stimulation. Despite

this, the depletion of NK cells using lytic Abs had no influence on the pulmonary bacterial load. This suggests that in response to an infection with *M. tuberculosis*, lung NK cells can become activated, but are not an essential protective mechanism (118).

Acquired Immunity Against *Mycobacterium tuberculosis* Infection

T CELLS

M. tuberculosis is a classic example of a pathogen for which the protective response relies on cell mediated immunity. This is primarily because the organism lives within cells, usually macrophages; thus T cell effector mechanisms, rather than antibody, are required to control or eliminate the bacteria. The first studies that demonstrated the importance of T cells in the immune response to tuberculosis were done by adoptive transfer, initially transferring a mixture of cells and later transferring more specific populations of cells.

Adoptive immunity to tuberculosis was initially achieved in rabbits (119), guinea pigs (120), and mice (121, 122). With the exception of Sever (122), all these investigators used randomly bred animals in which the inevitable reactions of histocompatibility raised resistance nonspecifically which questioned the significance of the effects observed. In addition, mixed populations of lymphoid cells (lymph node, spleen, or peritoneal exudate cells) were used in all studies.

In 1973, Lefford and Mackaness transferred immunity using lymphocytes obtained from the thoracic duct of female (Lewis x BN)F, rats. Immunity acquired in this

manner was mediated by living donor cells, it was related to the number transferred and the level of resistance in the donors. Moreover, they showed that heat-killed cells or serum from immune donors transmited no protection at all (123).

In the same years, North showed that the generation of immunity against tuberculosis in the mouse is T cell dependent. He made T cell deficient adult mice by thymectomy and irradiation and demonstrated that they failed to develop a mechanism capable of destroying *M. tuberculosis*, but were able to develop immunity if their T cell population was first restored by an infusion of syngeneic thymocytes (124).

Later, Orme and Collins demonstrated that spleen cells taken from mice at the height of the primary immune response to intravenous infection with *M. tuberculosis* possess the capacity to transfer adoptive protection to *M. tuberculosis*-infected recipients, but only if these recipients are first made T cell-deficient, either by thymectomy and gamma irradiation, or by sub-lethal irradiation. They also showed that a similar condition was necessary to demonstrate the adoptive protection of the lungs after exposure to an acute aerosol infection with *M. tuberculosis*. In both infectious models successful adoptive immunotherapy was shown to be mediated by T lymphocytes, which were acquired in the donor animals in response to the immunizing infection (125, 126).

Since the initial observations with T cells, research was focused on the $CD4^+$ T cell response to tuberculosis, but recently there has been increased interest in the roles of $CD8^+$ T cells in the immune response to this pathogen.

M. tuberculosis and CD4 T Cells

M. tuberculosis resides primarily in a vacuole inside the macrophage, and thus, MHC-class-II presentation of mycobacterial antigens to $CD4^+$ T cells is the common outcome of infection. Existing data has demonstrated that growth inhibition of *M. tuberculosis* requires the adaptive acquirement of an *M. tuberculosis*-specific, Th1-type cellular immune response. Abundant experimental evidence supports a central role for the interaction of Th1 CD4+ lymphocytes and macrophages. In this model, *M. tuberculosis*-derived antigens are processed and presented via macrophage-associated Major Histocompatibility Complex (MHC)-II. Antigen recognition by CD4+ T cells then leads to the release of pro-inflammatory cytokines, which in turn limit intracellular *M. tuberculosis* growth.

Numerous studies demonstrate that these cells are the most important in the protective response against *M. tuberculosis*. Murine studies have shown by antibody depletion of $CD4^+$ T cells (127), adoptive transfer (125, 126, 128), or the use of gene-disrupted mice that the $CD4^+$ T cell subset is required for control of infection (129, 130). In mice and humans $CD4^+$ T cells are approximately 2-fold more abundant that are $CD8^+$ T cells at sites of *M. tuberculosis* infection (131, 132). Even more, in humans, the tragedy of HIV has demonstrated that the loss of $CD4^+$ T cells greatly increases susceptibility to both acute and reactivation tuberculosis. Studies indicate that $CD4^+$ T cells from infected subjects produce IFNγ in response to a wide variety of mycobacterial antigens. The primary effector function of $CD4^+$ T cells is believed to be production of IFNγ and TNFα, sufficient to activate macrophages, which can then control or eliminate intracellular organisms (133).

Mice with a targeted disruption of the IFNγ gene are highly susceptible to *M. tuberculosis* infection, fail to produce nitric oxide synthase (NOS), and develop a disseminated form of disease characterized by irregular granulomas and large areas of tissue necrosis (134). Humans with mutations in the IFNγ receptor exhibit increased susceptibility to mycobacterial infections (135), while exogenously administered IFNγ can improve outcome (136, 137). Furthermore, individuals with active TB have a relative deficiency of IFNγ producing T cells (138, 139). TNF receptor knockout or mice depleted of TNFα by monoclonal antibodies are extremely susceptible to *M. tuberculosis*, showing increased bacterial loads, dysregulated granuloma formation, and lower survival (140). Additionally, humans receiving anti-TNFα therapy have increased susceptibility to mycobacterial infection (141).

It was recently demonstrated that not just IFNγ but also IL-17-producing $CD4^+$ T cells are expanded by *M. tuberculosis*-infected DC and are induced *in vivo* during infection. IL-17- producing antigen-specific cells are induced in mice following aerosol infection; these cells and most of the IL-17 response in the lungs depends on the presence of IL-23 (142). In the absence of IL-23, and therefore, in the majority of the IL-17 response in the mouse model, there is a small alteration in the early inflammatory response (142). Whether these cells are protective or damaging we do not know yet. In any case, it was also shown that IFNγ serves to limit the IL-17-producing T cell population and that this counterregulation pathway may be an important factor in limiting mycobacteria-associated immune-mediated pathology (143).

Although IFNγ production by $CD4^+$ T cells is undeniably a very important effector function of this subset, these cells have other roles in controlling *M. tuberculosis*

infection. In MHC class II-/- or CD4-/- mice, levels of IFNγ were severely diminished very early in infection (67, 129, 144). However, by 3 weeks post-infection, IFNγ levels in the lungs appeared to be similar to wild type, with the $CD8^+$ T cell subset contributing substantially to this production. The mice were not rescued by this later IFNγ production and succumbed to the infection. NOS2 expression by macrophages was also delayed in the $CD4^+$ T cell deficient mice but returned to wild-type levels in conjunction with IFNγ expression (130). These results were extended in a chronic model of *M. tuberculosis* infection. Mice infected with a low dose of *M. tuberculosis* were treated with anti-CD4 antibody beginning 6 months post-infection. The $CD4^+$ T cell depletion caused rapid reactivation of the infection, and mice succumbed with high bacterial numbers in the lungs. IFNγ levels overall were similar in the lungs of $CD4^+$ T cell–depleted and control (IgG-treated) mice, due to IFNγ production by CD8 T cells. Amazingly, there was no apparent change in macrophage NOS2 production or activity in the $CD4^+$ T cell–depleted mice (71). This indicated that there are IFNγ and NOS2-independent, $CD4^+$ T cell–dependent mechanisms for control of tuberculosis.

Another role for $CD4^+$ T cells in activating or maturing APCs has been addressed. Interaction of CD40L on CD4 T cells with CD40 on macrophages or dendritic cells results in enhanced antigen presentation and costimulatory activity. This interaction may be important in *M. tuberculosis* infection, although CD40L-/- mice were not more susceptible to acute infection (145). It has been recently demonstrated that CD40, but not CD40L, is required for the optimal priming of T cells and control of aerosol *M. tuberculosis* infection. $CD40^{-/-}$ mice succumbed to low-dose aerosol infection with *M. tuberculosis* due to deficient IL-12 production leading to impaired priming of IFNγ T cell

responses.(146). Furthermore, the importance of $CD4^+$ T cells on priming and maintenance of $CD8^+$ T cell effector and memory functions has been demonstrated in viral models and indirectly in mycobacterial infections (147, 148).

M. tuberculosis and CD8 T cells

For years, many researchers in the tuberculosis field ignored the $CD8^+$ T cell subset. $CD4^+$ T cells recognize antigens presented by major histocompatibility complex (MHC) Class II molecules on antigen presenting cells, such as dendritic cells and macrophages. MHC Class II is loaded with antigenic peptide in a vacuole that is a part of the endocytic system. Conversely, MHC Class I molecules present antigens to $CD8^+$ T cells; MHC Class I molecules are loaded with antigens transported from the cytoplasm into the endoplasmic reticulum, such as viral antigens. Because *M. tuberculosis* lives primarily within a vacuole inside the cell, rather than in the cytoplasm, it seemed improbable that antigens would be effectively presented to $CD8^+$ T cells by MHC Class I molecules.

However, early studies using antibody-mediated T cell subset depletion suggested that $CD8^+$ T cells, in addition to $CD4^+$ T cells, were necessary for the control of *M. tuberculosis* infection (127) and that adoptive transfer of purified immune $CD8^+$ T cells reduced the numbers of *M. tuberculosis* bacteria in the spleens of infected mice, even though with a lower efficiency compared with $CD4^+$ T cells (128). The development of gene-disrupted mice provided stronger data supporting the role of $CD8^+$ T cells in the control of *M. tuberculosis* infection(149).

Mice genetically deficient in β_2-microglobulin (β_2m), which lack functional MHC Class I molecules and consequently $CD8^+$ T cells, were unable to control infection, particularly in the lung, and died early due to tuberculosis (150). It was initially assumed that susceptibility of β_2m-/- mice was due to a drastically reduced total number of peripheral $CD8^+$ T cells, but what is known know is that the defect in β_2m-/- mice compromises other molecules. In addition to the absence of classical MHC Ia molecules, which present peptides to $CD8^+$ T cells, these mice also lack functional CD1 and other nonclassical MHC Ib molecules, which present lipid antigens and N-formylated peptides derived from bacteria. Thus, even though $CD8^+$ T cells were shown to be important for the control of infection, the molecules used to present antigens to the protective T cell subset remained unclear.

The contributions of classical or nonclassical MHC Class I–dependent $CD8^+$ T cell populations in protection against tuberculosis, was confirmed when series of gene-disrupted mouse strains were compared for susceptibility to intravenous *M. tuberculosis* infection, as measured by survival time and bacterial loads. Among the strains tested, the most susceptible mice were the β_2m-/-, followed by TAP1-/- (transporter associated with antigen processing), followed by CD8α-/-, perforin-/-, and CD1d-/- mice (151). The conclusion was that classically restricted (TAP1-dependent) $CD8^+$ T cells contribute to *in vivo* protection against *M. tuberculosis*; however, the role of $CD8^+$ T cells in protective immunity was not limited to perforin-dependent cytotoxicity (151). The β_2m-/- mice developed granulomas that were initially devoid of lymphocytes. As infection progressed, lymphocytes did accumulate, but they failed to infiltrate the macrophage-dominated

lesions, implicating a novel and undefined β_2m-dependent mechanism influencing early lymphocyte accumulation (152).

This confusion has been clarified by recent studies. In one report, mice specifically deficient in classical MHC-Ia molecules were more susceptible to *M. tuberculosis* infection than were wild-type mice, although not as susceptible as ß2m-/-, providing strong evidence for the role of MHC-I classically restricted $CD8^+$ T cells in resistance to *M. tuberculosis* (153). More recently it was demonstrated that apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1. In this latter report, investigators showed how the mycobacteria induced apoptosis in macrophages, causing the release of apoptotic vesicles that carry mycobacterial antigens to uninfected antigen-presenting cells (APCs), facilitating the presentation through MHC-I. Furthermore, they showed that inhibition of apoptosis reduced transfer of antigens to bystander cells and activation of $CD8^+$ T cells (86).

More evidence for the role of CD8+ T cells is supported in studies comparing wild-type and gene-knockout mice deficient in both $CD4^+$ and $CD8^+$ T cells, MHC I (lack $CD8^+$ T cells) or MHC II (lack $CD4^+$ T cells). When these mice were infected via aerosol and monitored for survival and ability to control infection, the results showed that in contrast to $CD4^+$ T cells, $CD8^+$ T cells were dispensable and not essential for the control of infection (144). Mice devoid of $CD4^+$ T cells died earlier from tuberculosis than did $CD8^+$ T cell–deficient mice, but the absence of both $CD4^+$ and $CD8^+$ T cells resulted in even greater susceptibility. The fact that mice devoid of $CD8^+$ T cells still succumb to *M. tuberculosis* infection, despite the development of fully functional $CD4^+$ T cell responses,

argues that $CD8^+$ T cells may play an important role in controlling chronic infection. Furthermore, depletion of $CD8^+$ T cells resulted in reactivation of latent tuberculosis in a murine model, suggesting that this T cell subset may also be essential for controlling latent tuberculosis (154). These results are corroborated by experiments comparing the course of the infection in control and CD8 knockout (CD8-KO) mice(149). Researchers showed that the role of $CD8^+$ T cells was not during the early stage of the infection, when the bacterial load is initially contained, but during the chronic phase of the disease. The gradual loss of resistance in CD8-KO mice was associated with an increased lymphocytic influx of $CD4^+$ T cells that despite the capacity to produce IFNγ, were not sufficient to control the bacterial load.

Other studies demonstrated that old mice display an early transient resistance to *M. tuberculosis* infection in the lung, which is dependent on CD8 T cells and IFNγ production. Following aerosol infection with *M. tuberculosis*, the lungs of old mice contained significantly more IL-12, IL-18, and IFNγ than the lungs of young mice. The investigators concluded that, the increased and early production of Th1 cytokines in the lungs of *M. tuberculosis*-infected old mice, in combination with CD8 T cells that can nonspecifically produce IFNγ, leads to transient control of *M. tuberculosis* growth in the lungs of old mice (155).

$CD8^+$ T cells can function as a source of type-1 cytokines such as IFNγ and TNFα or they may exert their protective effect by killing infected macrophages within the tissues. IFNγ and TNFα are important for the activation of macrophages; both $CD4^+$ and $CD8^+$ T cells have the potential to produce these cytokines at the site of infection (133).

$CD8^+$ T cells can kill infected macrophages via a perforin-mediated mechanism. Perforin, a protein in the granules of $CD8^+$ T cells, forms a pore in the membrane of infected macrophages and allows the entry of toxic proteins, such as granzymes or granulysin, leading to the apoptosis of infected macrophages. Apoptosis (programmed cell death) of macrophages can also be induced by ligation of Fas ligand on activated $CD8^+$ T cells, with Fas on infected macrophages.

Several studies suggest that cytokine secretion may be the major effector function of $CD8^+$ T cells. In experiments using IFNγ-/- mice as $CD8^+$ T cell donors, production of IFNγ was required for $CD8^+$ T cells to exert a modest antimycobacterial effect in $CD4^+$ T cell–deficient mice (129). Moreover, mice with targeted disruptions in the genes for Fas, perforin, or granzyme were no more susceptible to acute infection with *M. tuberculosis* than were their wild-type littermates (156-158). It has also been reported that early in infection, *M. tuberculosis*–specific $CD8^+$ T cells from the lungs expressed perforin *in vivo* and lysed *M. tuberculosis*–infected macrophages in a perforin-dependent and MHC Class I–dependent manner (159). It has been proposed that cytolytic function of $CD8^+$ T cells plays an important role early in infection while the cytokine production role of these cells is more important in chronic stages of the disease (160)(Reviewed in (161)).

Memory T cells

What distinguishes memory T cells from naive and effector T cells is a subject of great importance for immunologists. Recent advances have provided new insights into the differences between naive, effector and memory T cells. Memory $CD4^+$ and $CD8^+$ T cells differ from naive and effector T cells in different ways: - memory cells express distinctive activation markers and intracellular proteins that distinguish them from naive and effector T cells; - memory cells have lower activation thresholds and effector functions different from naive T cells (162); also - memory cells express chemokine and adhesion receptors that allow them to home to infected tissues and organs throughout the body (157).

The generation of memory starts when antigen entering the body is carried in afferent lymphs to draining lymph nodes or taken up by peripheral DC, which are stimulated to migrate to the lymph nodes. There antigen is processed to short peptides and presented by DC on MHC class II and class I molecules to initiate the responses of naive $CD4^+$ and $CD8^+$ T cells (93). Effective immune responses are initiated only in the presence of co-stimulatory molecules present in the surface of DC. Molecules such as CD40, CD80 and CD86, bound to their T-cell counter-receptors, CD154, CD28 and CD152 generating an effective immune response. DC and other cells of the innate immune system also produce stimulatory cytokines and chemokines. The type of signals produced by innate immune cells is determined by PAMPs, which stimulate these cells

via conserved pattern-recognition receptors. The signals generated by these innate cells determines the fate of the newly generated T cells (163).

The magnitude of the initial T-cell response appears to be an important determinant of the duration of memory (164) which is at the same time partly determined by the number and affinity of naive precursors (165). Delivery of a TCR signal with appropriate co-stimuli leads to clonal expansion, but the effects on $CD4^+$ and $CD8^+$ T cells differ. $CD8^+$ T cells stimulated for 24 hours *in vitro* and transferred into antigen-free hosts undergo at least seven divisions and acquire effector and memory function (166). Co-stimuli and cytokines also influence $CD8^+$ proliferation and differentiation (167). By contrast, although a short pulse of antigen can initiate some $CD4^+$ cell division, continuous antigen exposure is necessary for proliferation and differentiation of these cells *in vitro* and *in vivo* (168) . Even under these circumstances, the frequencies of responding $CD4^+$ T cell are lower, and the size of individual $CD4^+$ clones small when compared with those of $CD8^+$ cells (169).

When clonal contraction occurs, generation of T cell memory begins (at least in viral infection models). The peak of the proliferative response to antigen is reached within 1–2 weeks, and there follows a phase of contraction, in which most short-lived antigen-specific effector T cells die by apoptosis. After the loss of these short-lived effectors, a population of longer-lived memory cells remains (170). $CD4^+$ cells have slower kinetics and expand less than $CD8^+$ cells; $CD4^+$ cells show a biphasic pattern of contraction, whereas $CD8^+$ cells show massive and rapid expansion and collapse, followed by life-long preservation of a stable memory population, at least in experimental animals models (169).

Survival of memory T cells requires expression of the antiapoptotic protein B cell leukemia/lymphoma 2 (BCL2) (171, 172) (172). Because, early in the encounter of T

cells with antigen-presenting cells, under the influence of cytokines and chemokines, death receptors and proapoptotic proteins such as CD95 and BCL2-like protein 11 (BIM) are rapidly upregulated, leading to T-cell death.

In addition to signals derived from the innate immune system, it is generally thought that $CD8^+$ T cells require help from $CD4^+$ T cells. Although $CD8^+$ effectors can be produced in a primary response in the absence of $CD4^+$ T cells, memory $CD8^+$ cells generated in their absence make poor responses when re-stimulated and die by activation-induced cell death, mediated via the TNF-related apoptosis-inducing ligand (TRAIL). Exactly what signals $CD4^+$ cells provide for $CD8^+$ memory cells still remains to be fully elucidated (173).

At least two types of memory T cells have been described within the $CD4^+$and $CD8^+$ T cell populations based on their homing characteristics and their effector functions. In humans, T effector-memory (TEM) and T central-memory (TCM) cells differ both functionally and in their migratory properties and can be distinguished based on their CD62L and CCR7 expression (174). TCM cells express CCR7 and CD62L, whereas TEM cells do not express CCR7 or CD62L. In both mice and humans, $CD4^+$ TCM and $CD8^+$ TCM cells reside mainly within lymphoid organs (175, 176). $CD4^+$TCM produce IL-2, IL-10, and divide rapidly upon re-stimulation (175, 176) whereas $CD8^+$ TCM retain their ability to secrete IL-2, but need to be re-primed to regain perforin killing effector function (in viral models) and secrete IFNγ (176). $CD4^+$ TCM T cells are more responsive to TCR stimulation than naive T cells and can also stimulate dendritic cells via CD40 ligand to produce IL-12 (174). In contrast, $CD4^+$ TEM reside in peripheral

tissue to provide protection against reinfection, and upon TCR stimulation, rapidly produce IFNγ or IL-4. $CD8^+$ TEM also reside in peripheral tissue and exhibit immediate cytokine secretion and perforin killing activity (174-176). In summary, TEM cells provide immediate protection against reinfection or reactivation of disease at sites of infection, whereas TCM cells reside primarily in the lymphoid tissue where they can rapidly expand and differentiate to re-supply the effector T cells at peripheral sites.

In humans, differentially spliced isoforms of the leukocyte common Ag (CD45) have historically been used to discriminate between naive (CD45RA) and memory (CD45RO) T cells (177-179). However, more recent longitudinal studies examining $CD8^+$ CTL during the activation, apoptotic and memory phases of acute and chronic Herpes virus infections in humans have suggested that memory cells can also express CD45RA (180, 181). Some of these studies have also shown, that during the activation phase, cycling $CD8^+$ T cells are CD27 positive, whereas terminally differentiated, perforin positive, CTL are CD27 negative (180), suggesting that the loss of CD27 expression may be a marker for effector and effector-memory CTL (182).

Monitoring the generation of TCM and TEM has been possible in the mouse system where the relationship between population of primed T cells and memory subsets has been directly addressed. A study on $CD4^+$ T cells showed that cytokine-secreting cells fail to generate memory, whereas non-polarized cells, with the characteristic of pre-TH1, have the ability to become memory cells with strong TH1 characteristics (183). The precursor to product relationship between TCM and TEM has been analyzed at least in

the $CD8^+$ system by adoptive transfer experiments (184). In this study the authors propose that TEM are present only transiently, and they all convert to TCM by reacquiring CCR7 and CD62L expression. In addition they proposed that the rate of conversion from TEM to TCM is inversely proportional to the strength of stimulation. Lastly, they proposed that TCM convert to TEM upon antigenic re-stimulation but not under steady-state conditions. These findings generated in using a viral infection model led to the suggestion that in mice TCM and TEM do not necessarily represent distinct subsets, and maybe are cells in a linear naive–effector-TEM-TCM differentiation pathway (185). These results contrast with the persistence and stability of both TCM and TEM subsets in humans (186) and with the failure of TEM to reacquire constitutive CCR7 expression *in vitro* (187), suggesting a principal difference between these two species.

IL-7 and IL-15 have been shown to be important for memory cell survival using transgenic and knockout mice, and blocking antibodies. IL-15 is essential for memory $CD8^+$ T-cell survival, and although there is no absolute need for IL-7, it can compensate for the absence of IL-15 (188). In mouse experiments, when $CD8^+$ memory cells are transferred to hosts that are antigen free or lack MHC class I molecules, the number of these cells decline very slowly over time; showing that antigen is not required for maintenance of CD8 memory (189). By contrast, evidence suggests that both TCR signals and IL-7 are required for CD4 memory cell survival (190).

M. tuberculosis and Memory T cells

Much of what is known about memory cells is based on studies using defined antigens or viral infections. What happens in mycobacterial infections where there is extensive inflammation and immune stimulation, and in which infection is often chronic ensuring continued production of antigens, is only just starting to be studied.

It has been assumed that immune memory against tuberculosis is mediated by a very specific subset of T cells. This arose from various early studies in the field, particularly from Orme's laboratory (191) and from that of Andersen (192), which demonstrated that C57BL/6J mice infected with *M. tuberculosis* were found to generate long-lived memory immunity which provided a heightened state of acquired resistance to a secondary infection. Mice heavily infected with the BCG vaccine were able to rapidly generate an acquired cellular immune response to this infection, characterized primarily by the emergence of a splenic T-cell population capable of passively transferring substantial levels of adoptive protection against a challenge infection with *M. tuberculosis* (125). In studies conducted with a mouse model of memory immunity in which mice are rendered immune by a primary infection followed by antibiotic treatment and rest, tuberculosis-specific memory cells were recruited from the recirculating pool, leading to rapidly increasing precursor frequencies in the liver and a simultaneous decrease in the blood (193).

During latency, reactive T cells in the lungs are expected to develop into memory T cells, as observed after clearance of infection by chemotherapy (194). However, in chronic tuberculosis, activated effector T cells are continuously present in the lungs of infected mice (195). This could reflect continuous active activation of naïve T cells and

their differentiation into effector T cells, with no generation of immunological memory, implying that the persisting mycobacteria produce sufficient antigen to continue to drive the effector T cells in the lungs. It has been suggested that in diseases as AIDS (Acquired immune deficiency syndrome), tuberculosis and leprosy the differentiation of the memory T cell population is impaired, because of consistent presence of effector T cells (196).

Regulatory T cells

Regulatory T cells (Tregs) were described for the first time in the early 1970s and were called suppressor cells (197, 198). Unfortunately, this research topic was abandoned in the late 1980s due to difficulties in identifying and isolating the suppressor cells. In 1995, Sakaguchi et al.(199) showed that the interleukin-2 receptor α-chain (CD25) could serve as a phenotypic marker for $CD4^+$ Tregs. These observations led to the revival of the Treg concept, and this research field has evolved rapidly ever since. Currently, various subsets of both $CD25^+$ and $CD25^-$ Tregs populations have been described (200). Different Tregs subsets are now subdivided based on expression of cell surface markers, production of cytokines, and mechanisms of action.

Naturally occurring $CD4^+CD25^+$ Tregs are a T cell population generated in the thymus with immunosuppressive properties that constitute 5–10% of the total peripheral $CD4^+$ T cells (201, 202). Besides the expression of CD25, they constitutively express other several activation markers, such as the glucocorticoid-induced tumor-necrosis

factor (TNF) receptor-related protein (GITR), L-selectin (CD62 ligand (CD62L)), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152). However, it should be noted that none of these markers exclusively identifies Tregs as they can also be expressed on activated T cell subsets and various antigen-presenting cells (APCs). More recent studies have identified the transcription factor forkhead box P3 (FOXP3) as a more exclusive intracellular marker for the identification of Tregs (203, 204). In addition, FOXP3 is also a crucial transcription factor for the development and functionality of $CD4^+CD25^+$ Tregs. Loss of function mutations in FOXP3, both in mice and men, results in the absence of Tregs, leading to a phenotype with severe autoimmune disorders (205), known as scurfy mice and IPEX (immunedysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in men. The important function of FOXP3 was also confirmed by studies showing that ectopic expression of FOXP3 in T cells leads to the generation of cells with a regulatory phenotype and a suppressive function (204, 206). In addition, it was demonstrated that FOXP3 blocks the ability of the Rel-family transcription factors NFAT and NFκB to induce their target genes (207, 208), and as a consequence, it acts as a transcriptional repressor of IL-2 and other cytokine genes (IL-4 and IFN-γ), thereby programming a cell not to exert immune stimulatory functions. Moreover, FOXP3 expression has also been demonstrated in activated T cells in humans (209), presumably acting as a negative feedback in order to control ongoing immune responses.

On the basis of cell surface phenotype or cytokine secretion, Tregs consist of a network of diverse populations, sharing common features of being hyporesponsive to antigen stimulation and possessing immunosuppressive activities. Natural Tregs originate

in the thymus and acquired Tregs (also called antigen-induced or adaptive Tregs) are generated from mature T cells in the periphery. There are evidences supporting both origins. During early life, Hassall's corpuscules, epithelial substructures in the thymus, play an important role in the generation of Tregs (210, 211). Moreover, neonatal mice that have undergone thymectomy spontaneously develop autoimmune diseases (212). Support for the origin of acquired Tregs come the knowledge that while thymic function is largely reduced after puberty in man, Tregs persist throughout life. This implies that (all) Tregs might originate from a pool of self-renewable long-term surviving thymic emigrants. However, Akbar et al. (213) recently showed that the number and function of $CD4^+CD25^+FOXP3^+$ Tregs are maintained in humans even after the age of 70 years. Therefore, they suggested that these cells most probably do not derive from the thymic lineage of Tregs, but they are generated from the peripheral pool of $CD4^+CD45RO^+CD25^-FOXP3^-$ memory T cells.

Furthermore, several other studies also reported the existence of various subsets of antigen-induced or adaptive Tregs. The suppressive function of these induced Tregs is mediated by the production of suppressive cytokines (IL-10 and transforming growth factor-β (TGF-β)). Therefore, the current classification of acquired Tregs is based on expression of different suppressive cytokines. $CD4^+$ regulatory T cells of type 1 (Tr1) express high levels of IL-10 and moderate levels of IL-5, IFN-γ, and TGF-β, and they are negative for IL-2 and IL-4 (214, 215). T helper 3 (Th3) regulatory T cells express high levels of TGF-β (216). Both types of acquired Tregs equally suppress Th1 as well as Th2 mediated immune responses. Tr1 and Th3 have been shown to originate from naive

resting T cells after stimulation with DCs (217), depending on DC type and activation status.

Apart of the existence of $CD4^+$ naturally occurring and acquired Tregs in controlling proper function of the immune system, a $CD8^+$ subset of regulator T cellssuppressor cells have also been described. $CD8^+$ Treg cells are derived from an oligoclonal T cell population, and they lack CD28 and express FOXP3, GITR, CTLA-4, OX-40, and CD62L at the same level as compared to $CD4^+CD25^+$ Tregs (218, 219).

Worth mentioning, is a special population of natural killer (NK) cells and NKT cells with regulatory function has also been described. Their immune suppressive function is mediated by secretion of various cytokines (IL-13, IL-4, IL-10) or by direct cell-cell contact (220).

All Tregs, both naturally occurring and acquired, need T cell receptor (TCR) triggering for their suppressive function. However, once activated, their suppressive activity seems to be antigen-nonspecific (221). So far, the precise mechanism(s) by which Tregs suppress effector T cell activation and/or function remains unclear. Moreover, results from many *in vitro* and *in vivo* studies or studies performed on mice and men are sometimes contradictory.

Several *in vitro* studies have demonstrated that $CD4^+CD25^+$ Tregs suppress proliferation and IFN-γ production by effector T cells through a direct cell-cell contact-dependent stimulation between suppressor and effector cells, possibly mediated by the expression of their cell surface markers GITR and CTLA-4 (222). Ligation of

CD80/CD86 on effector cells may transmit suppressive signals after engagement by cell surface CTLA-4 on suppressor cells, and it results in inhibition of effector T cell function (223). Another mechanism for Tregs to affect effector T cell activation can be established by modulating DC function. Ligation of CD80/CD86 on DCs by CTLA-4 on suppressor cells results in expression and activation of indoleamine 2,3-dioxygenase (IDO) (224) a catabolic enzyme involved in tryptophan degradation.

M. tuberculosis and T Regulatory Cells

In the best-characterized example, Tregs have been shown to be essential for establishing and maintaining persistent infection by *Leishmania major* (225-227); elimination of Tregs in persistently infected mice resulted in the eradication of the parasite from the host (226). In addition, Tregs protect the host during infections by preventing collateral damage to the host's tissue from excessive inflammation induced by the immune response to the pathogen (225). Although Tregs have been studied mostly in respect to persistent viral and parasitic infections (225), their involvement has also been implicated in bacterial infections, including infections caused by *Helicobacter pylori* (228, 229) and *Listeria monocytogenes* (230).

The role of Tregs in suppressing immunity to *M tuberculosis*, is only beginning to be investigated. In human tuberculosis, three studies have shown an increase in Treg numbers in the blood and at sites of infection during active disease (231-233). In the

murine model, there are two studies that have reached different conclusions about the role of Tregs in tuberculosis. Quinn et al. (234) used anti-CD25 antibodies to reduce the number of Treg cells in mice. Although they observed increased IFN-γ production at early time points after infection, this depletion did not affect the bacterial load. Using a different strategy, Kursar et al. (235) adoptively transferred CD25-negative $CD4^+$ T cells with and without $CD25^+$ T cells into T cell–deficient ($RAG^{-/-}$) recipients before infection with *M tuberculosis*; these investigators did observe a lower bacterial load when the number of Tregs in the recipients was reduced.

In a more recent study Scott-Browne et al. (236) used Foxp3-GFP mice to track the activity of Tregs after aerosol infection with *M tuberculosis*. They reported that during tuberculosis, Tregs proliferate in the pulmonary lymph nodes, change their cell surface phenotype, and accumulate in the pulmonary lymph nodes and lungs at a rate parallel to the accumulation of effector T cells. In the *M tuberculosis*-infected lungs, Tregs accumulate in high numbers in all sites where $CD4^+$ T cells are found, including perivascular/peribronchiolar regions and within lymphoid aggregates of granulomas. To determine the role of Tregs in the immune response to tuberculosis, they generated mixed bone marrow chimeric mice in which all cells capable of expressing Foxp3 expressed Thy1.1. When Tregs were depleted by administration of anti-Thy1.1 before aerosol infection with *M tuberculosis*, they observed ~1 log less of colony-forming units of *M tuberculosis* in the lungs. They concluded that, after aerosol infection, Tregs proliferate and accumulate at sites of infection, and have the capacity to suppress immune responses that contribute to the control of *M tuberculosis*.

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CHAPTER 2:

A mutant of *Mycobacterium tuberculosis* lacking the 19-kilodalton lipoprotein Rv3763 is highly attenuated *in vivo* but retains potent vaccinogenic properties.

The aims of this study were to:

1. Test the virulence of the mutant of *Mycobacterium tuberculosis* lacking the 19-kilodalton lipoprotein Rv3763.
2. Evaluate the immune response elicited by the vaccination with the *Mycobacterium tuberculosis* lacking the 19-kilodalton lipoprotein Rv3763.
3. Compare the immune memory generated by this mutant versus the response generated by Bacillus Calmette Guerin (BCG).

INTRODUCTION

The expression of TH1 immunity to tuberculosis infection has recently been shown to be initially driven by the interaction between lipid containing mycobacterial products and Toll-like receptors [pattern recognition molecules] on the surface of the infecting macrophage [1-6]. A primary example is the 19kDa lipoprotein (Rv3763; LpqH) of *M. tuberculosis,* which interacts with the Toll-like receptor 2 (TLR2) inducing the production of IL-12 p40 when added to macrophages [3], thus initiating the subsequent

production of Interferon gamma (IFNγ) by sensitized T cells. However, the signaling effects of the interaction between the 19kDa lipoprotein and the host cell are complex, and prolonged exposure of cells has negative effects [7-10]. This results in inhibition of the macrophage expression of a subset of IFNγ-induced genes, including CIITA that regulates the expression of MHC class II (MHC-II), interfering with subsequent antigen presentation [9,11].

The development of a mutant of *M. tuberculosis* lacking the 19kDa lipoprotein [Δ19] has allowed investigation of these mechanisms further [12]. Cells infected with this mutant *in vitro* did not show any reduction in surface expression of MHC Class-II, but IL-1β secretion was lower [12]. In the present report, I extended these studies to *in vivo* experiments. These showed that expression of the 19kDa lipoprotein is essential to replication of the organism in the lungs of mice, both normal and immunocompromised, and that without it the bacilli persisted as a low grade chronic infection. Interestingly, despite this, mice inoculated with Δ19 had similar resistance to aerosol challenge infection to that engendered by BCG. Fewer CD4 and CD8, including IFNγ secreting T cells, were needed to control the lung infections compared to controls. Finally, analysis of lung macrophage populations indicated far higher expression of Class-II MHC in Δ19 vaccinated mice compared to control mice.

MATERIALS AND METHODS

Animals

Specific pathogen-free female, 6–8-week-old, C57BL/6 (resistant) and IFNγ knockout (susceptible) mice from Jackson laboratories, Bar Harbor, ME. They were kept under barrier conditions in an ABL-III laboratory and fed sterile water and chow.

Aerosol infection with *M. tuberculosis.*

M. tuberculosis strain H37Rv, and H37Rv-Δ19 (19kDa-/-) and H37Rv-Δ19 complemented were 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. For low-dose aerosol infections, bacterial stocks were diluted in 5 ml of sterile distilled water to $2x10^6$ colony-forming units/ml and placed in a nebulizer attached to an airborne infection system (Glass-Col, Terre Haute, IN). Mice were exposed to 40 min of aerosol where approximately 100 bacteria were deposited in the lungs of each animal. Bacterial load was determined by plating right lobe homogenates onto nutrient 7H11 agar plates supplemented with OADC. Colonies were counted after 21 days of incubation at 37°C. For the vaccination studies, 10^6 CFU of H37Rv-Δ19 or BCG Pasteur were injected subcutaneously. Four weeks afterwards mice were challenged by low-dose aerosol exposure with H37Rv as above.

Lung cell digestion

Mice were euthanized by CO_2 asphyxiation, and the thoracic cavity was opened. The lung was cleared of blood by perfusion through the pulmonary artery with 10 ml of ice cold phosphate-buffered saline (PBS) containing 50 U/ml of heparin (Sigma, St Louis, MO). Lungs were aseptically removed, teased apart and treated with a solution of deoxyribonuclease IV (DNAse) (Sigma Chemical, 30 μg/ml) and collagenase XI (Sigma Chemical, 0.7 mg/ml) for 45 min at 37°C. To obtain a single-cell suspension, the organs were gently passed through cell strainers (Becton Dickinson, Labware, Lincoln Park, NJ). The remaining erythrocytes were lysed with Gey's solution (0.15 m NH4Cl, 10 mm HCO3) and the cells were washed with Dulbecco's modified Eagle's minimal essential medium. Total cell numbers were determined by using a Neubauer chamber.

Histological analysis

The accessory lung lobe from each mouse was fixed with 10% formalin in phosphate buffered saline (PBS). Sections from these murine tissues were stained using haematoxylin and eosin, and by acid-fast staining to detect mycobacteria.

Flow Cytometry for surface markers and intracellular cytokines

For flow cytometry analysis, single-cell suspensions of lung from each mice were re-suspended in PBS (Sigma-Aldrich) containing 0.1% of Sodium Azide (PBS+Na/Az). Cells were incubated in the dark for 25 min at 4°C with specific antibody (directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin-Cholorophyll-protein (PerCP) or allophycocyanin (APC), at 3□g/ml), followed by two

washes in PBS containing 4% sodium azide. Cell surface markers were analyzed with the following specific antibodies: FITC anti-CD44 (clone 7D4), FITC anti-MHC-II (clone AF6-120.1 and 2G9), PE anti-CD25(clone 3C7), PE anti-GR.1 (cloneRB6-8C5), PerCP anti-CD4 (clone RM4-5), PerCP anti-CD8 (clone 53-6.7), PerCP anti-CD11b (clone M1/70), APC antiCD11c (clone HL3) and APC anti-CD62L (clone MEL-14) all antibodies from BD PharMingen. Measurement of intracellular IFN-γ was conducted by pre-incubating lung cells with monensin (3 μM) (Golgi stop, BD PharMingen), anti-CD3 and anti-CD28 (both at 0.2 $\mu g/10^6$ cells) for 4 h at 37°C, 5% CO2. For TNFα staining, lung cells were pre-incubated with monensin (3 μM), and 5 μg/ml of LPS (Sigma-Aldrich, San Louis, MO) for 6 hr at 37°C, 5% CO_2. The cells were then surface stained, washed and then fixed and permeabilized with Perm Fix/Perm Wash (BD Pharmingen). Finally, the cells were stained for intracellular anti-IFN-γ (clone XMG1.2) or anti-TNFα (clone MP6-XT22) or its respective isotype controls (BD Pharmingen) for a further 30 min. All the samples were analyzed on a Becton Dickinson FACScalibur and data were analyzed using CELLQUEST (Becton Dickinson Immunocytometry Systems) or Summit software (DakoCytomation). Cells were gated on lymphocytes or based on characteristic forward and side scatter profiles. Individual cell populations were identified according to their presence of specific fluorescent-labeled antibody. All the analyses were performed with an acquisition of 250000 events.

Statistics

Statistical significance was determined using two-way ANOVA with Bonferroni post tests using GraphPad Prism 4.00 for Windows, GraphPad Software (San Diego CA).

RESULTS

The Δ19 mutant is highly attenuated.

In a first experiment the Δ19 mutant was compared to wild type H37Rv and an H37Rv-Δ19 complemented strain for its ability to grow in normal C57BL/6 mice. As shown in Fig.1a, the Δ19 showed only minimal growth in the C57BL/6 mice whereas H37Rv grew approximately 3 logs, and while the H37Rv-Δ19 complemented strain grew more slowly it reached similar levels by day 40. There was no statistical difference between the H37Rv and the complemented strain at day 40 ($p=0.08$). In mice lacking IFNγ [GKO], H37Rv grew progressively, killing them in 30-40 days, whereas the Δ19 showed only a slight increase over this time (Fig.1b). This was further illustrated by histological examination of the infected lungs [Fig.2]. In mice infected with H37Rv granulomatous inflammation was seen as expected, whereas in the GKO necrotizing lesions consolidated much of the lung tissues. In the mice infected with Δ19, only minimal, scarce, cellular infiltrations were seen, even in the GKO mice.

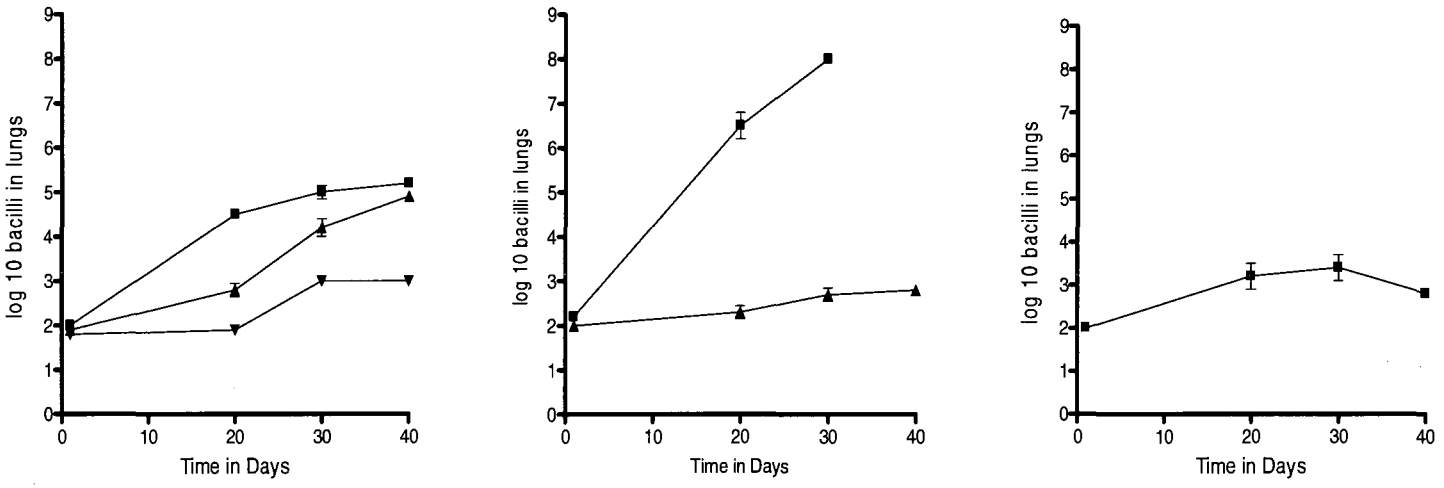

Figure 1. a) Growth of *M. tuberculosis* H37Rv [squares], H37Rv Δ19 complemented [up triangles] and H37Rv Δ19 [down triangles] in C57BL/6 mice. b) Growth of *M. tuberculosis* H37Rv [squares] and H37Rv Δ19 [triangles] in IFNγ gene disrupted mice after low dose aerosol infection. c) Growth of BCG after low dose aerosol in C57BL/6 shown for comparative purposes. The bacterial load was calculated by plating lung homogenates onto 7H11 agar at 37°C and counting colonies 21 days later. Time points were day 1 day 20, day 30, and day 40. The data are expressed as the mean ± the standard error of the mean from five mice per group. The graph is representative of two independent experiments.

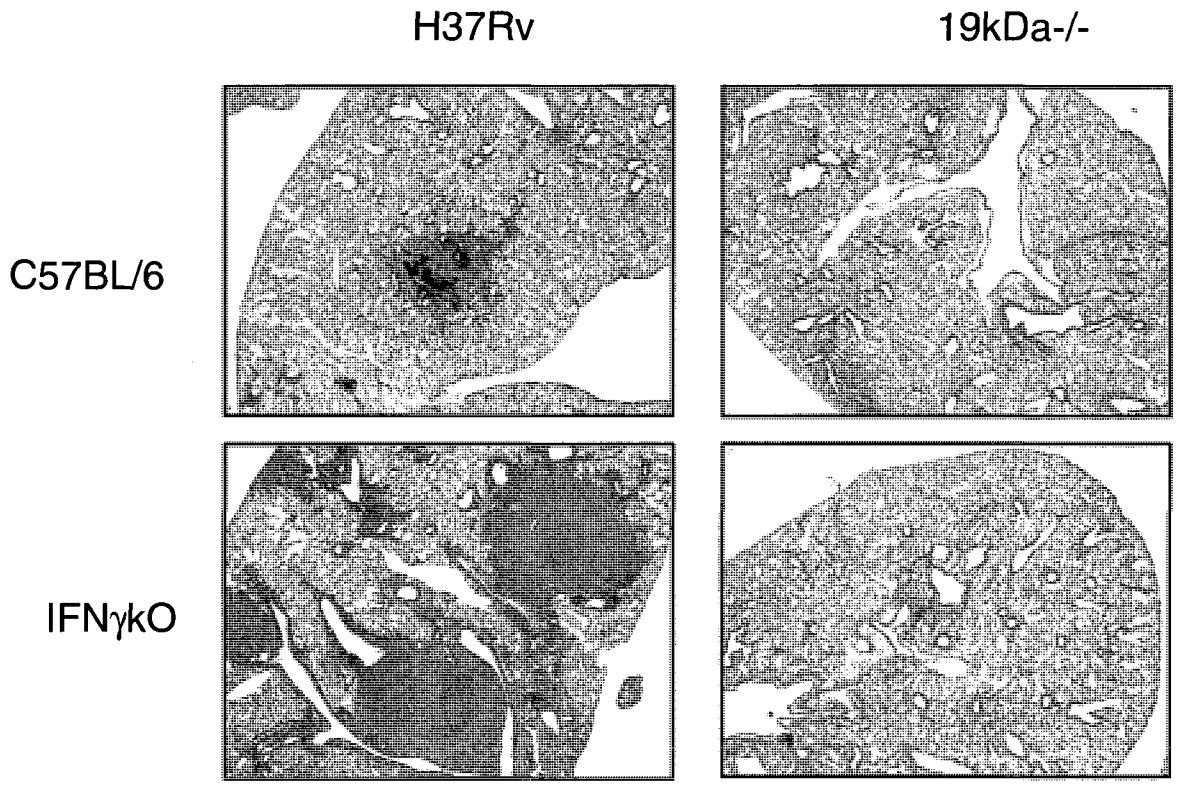

Figure 2. Representative photomicrographs of hematoxylin and eosin stained, formalin fixed lungs from C57BL/6 or IFNγ gene knockout mice infected by aerosol one month earlier.

Mice vaccinated with Δ19 are as well protected as mice vaccinated with BCG.

Given the very poor growth of the Δ19, as well as the negligible inflammatory response to it, it seemed unlikely that this organism was capable of inducing immunity *in vivo*. However, to verify this, mice were vaccinated with 10^6 CFU of Δ19, and compared to BCG vaccinated mice. As shown in Fig.3, the lungs and spleen of the different groups were removed and plated 30 or 120 days later. Surprisingly, vaccination with Δ19 gave similar and sustained protection to that given by BCG.

Given the protection results, the histological data was as expected [Fig.4], but with one major difference. Foamy macrophages are a characteristic of tuberculous granulomas and are interesting because they express dendritic cell markers [13]. These were seen in large numbers in the lungs of the BCG vaccinated mice, but not in the Δ19 vaccinated mice. Instead, in the latter, granulomas were highly lymphocytic.

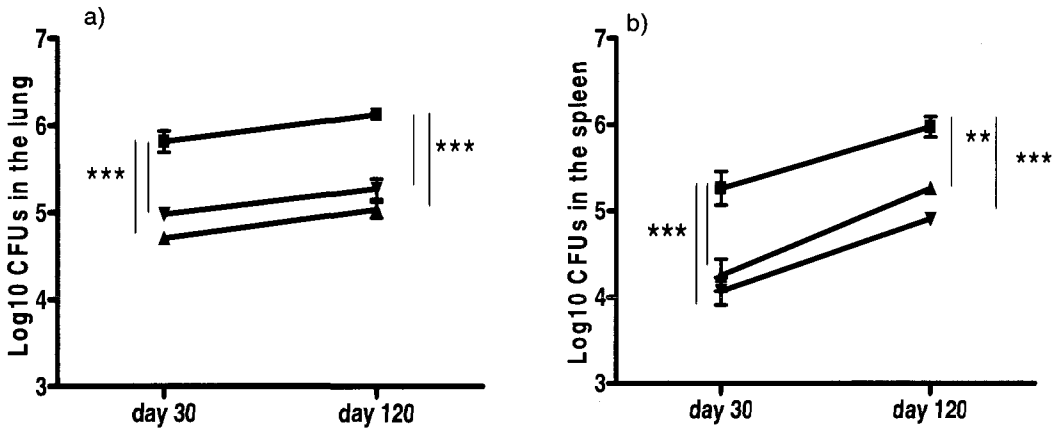

Figure 3. The bacterial load in the lungs [a] and spleens [b] of saline controls [squares], compared to BCG vaccinated [triangles] or Δ19 vaccinated [inverted triagles] mice 30 or 120 days after low dose aerosol challenge. The data are expressed as the mean ± the standard error of the mean from five mice per group. The graph is representative of two independent experiments.

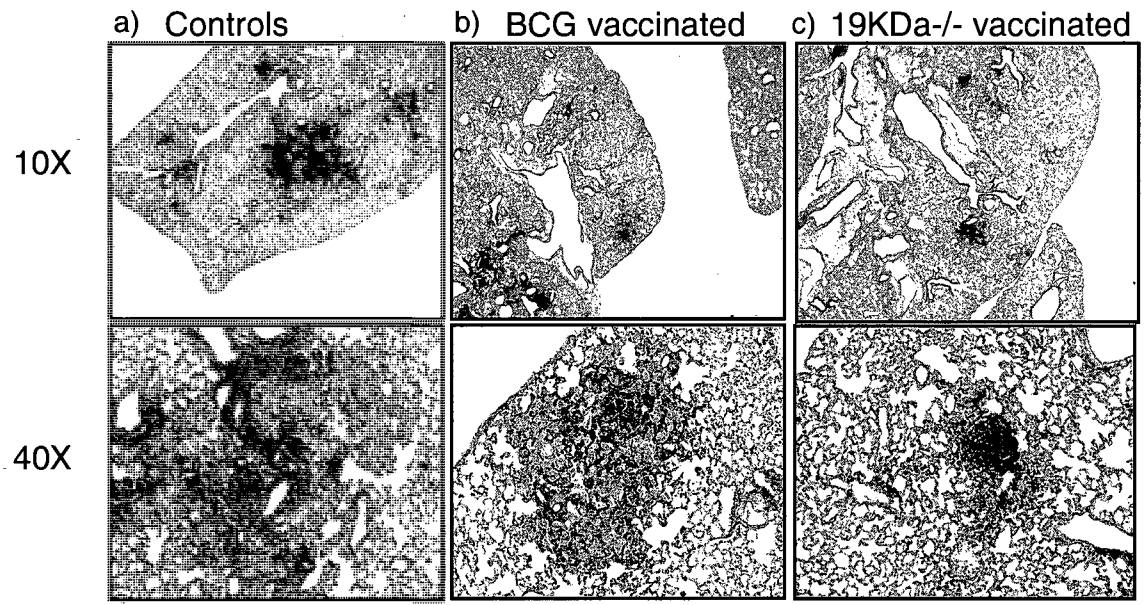

Figure 4. Representative photomicrographs of hematoxylin and eosin stained lungs from C57BL/6 mice inoculated with saline, BCG, or Δ19 (19kDa-/-). Following aerosol challenge the diaphragmatic right lobes were formalin fixed on day 30 of the infection.

The T cell response was similar in the Δ19 and BCG vaccinated mice.

Immune animals need to focus less T cells into infected lesions to control the infection. As shown in Fig.5 this was observed in both groups of vaccinated mice, and applied to both IFNγ-secreting CD4 and CD8 T cells. Further analysis [Fig.6] separating activated [$CD62L^{lo}$] and central memory [$CD62L^{hi}$] CD4 cells showed reductions by day 120 in the former population but not the latter, with significantly higher activated cells remaining in the unprotected controls. Similar observations were made regarding the CD8 T cell response [Fig.7].

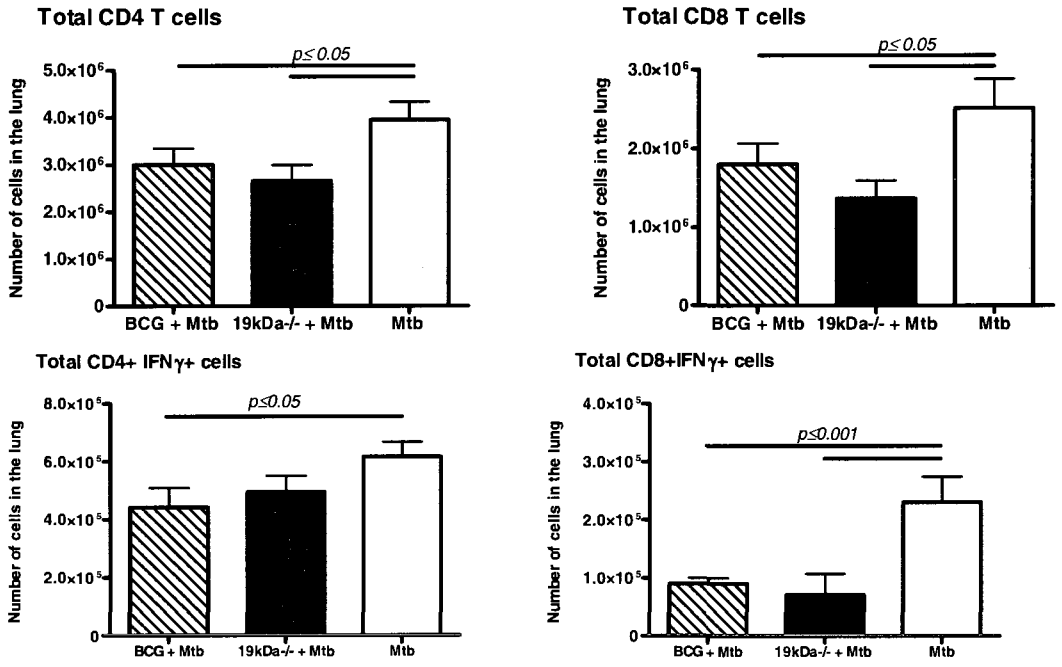

Figure 5. Total CD4 and CD8 T cells numbers, and numbers of IFNγ secreting T cell subsets entering the lungs four weeks after aerosol challenge. The data are expressed as the mean ± the standard error of the mean from five mice per group. Statistical significance between marker expression on lung T cells between the different groups was calculated using two-way ANOVA. Results are representative or two independent experiments.

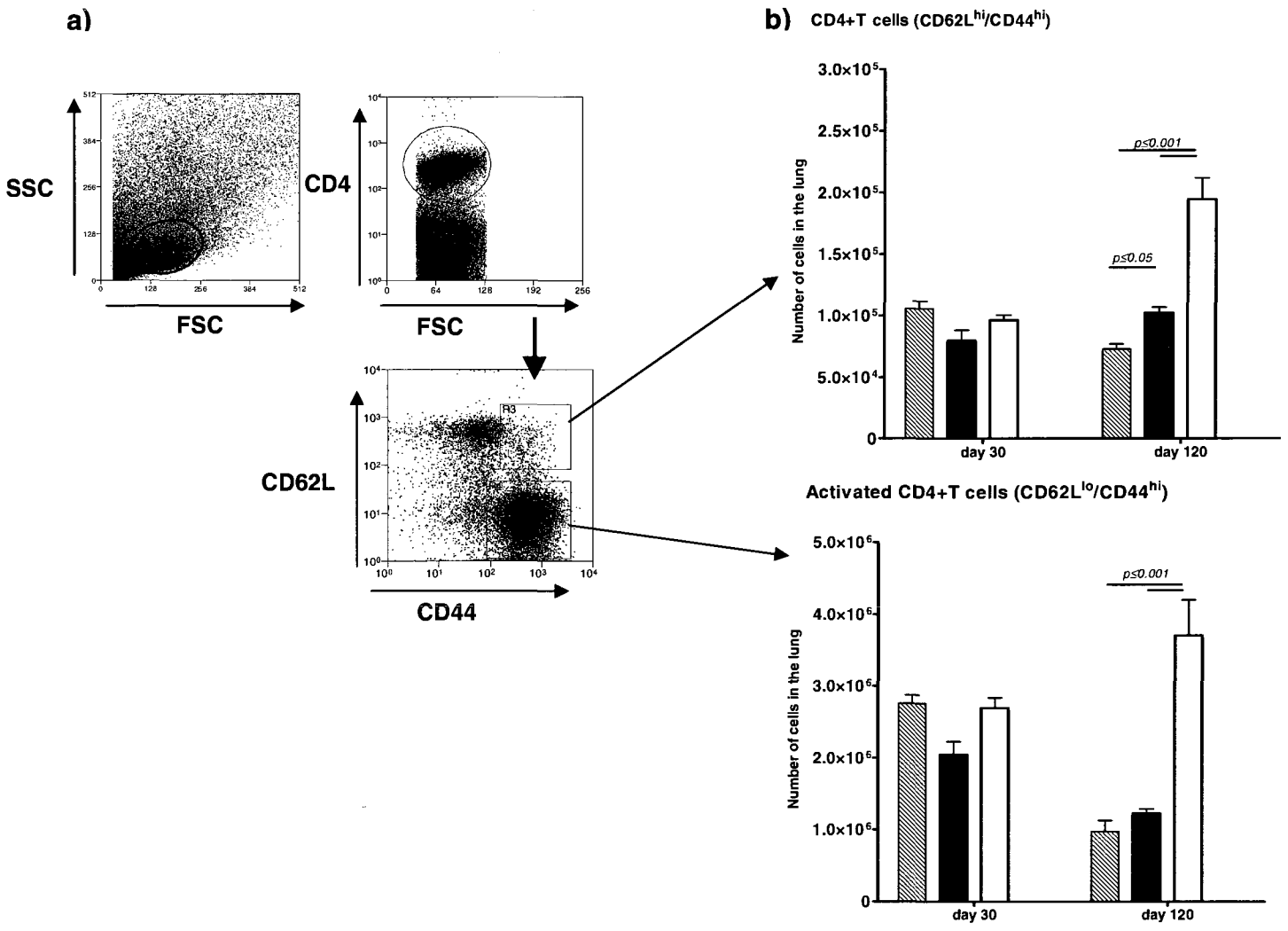

Figure 6. Numbers of CD4 T cell subsets harvested from the lungs 30 or 120 days after aerosol challenge infection. Mice vaccinated with BCG [hatched bars] or with Δ19 [filled] were compared to control mice [open]. After appropriate gating [a] CD4 cells in the lungs were analyzed for [b] $CD62L^{hi}$ central memory cells or $CD62L^{lo}$ activated cells. Significance differences between marker expression was calculated using two-way ANOVA. Results are representative or two independent experiments.

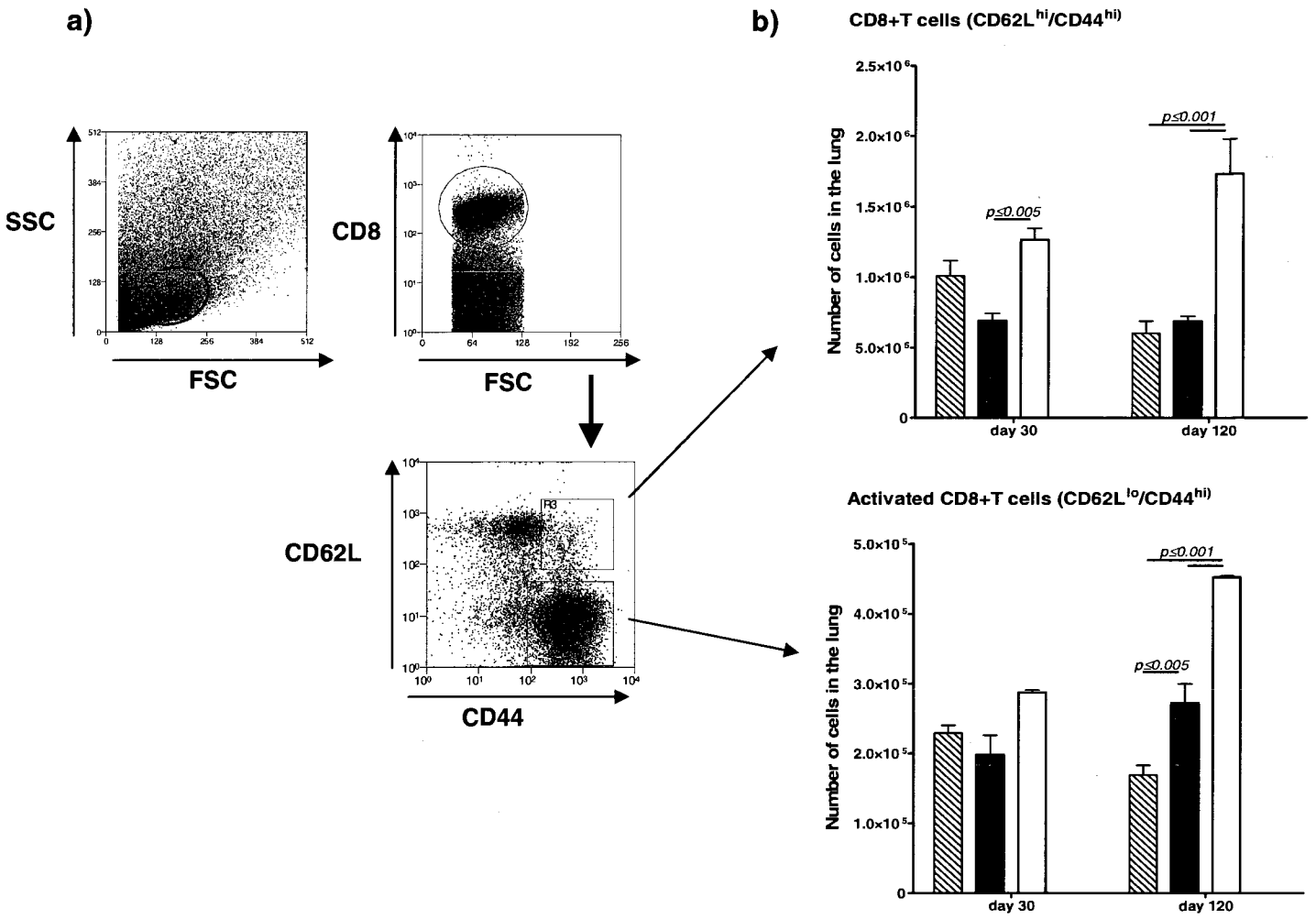

Figure 7. Numbers of CD8 T cell subsets harvested from the lungs 30 or 120 days after aerosol challenge infection. Mice vaccinated with BCG [hatched bars] or with Δ19 [filled] were compared to control mice [open]. After appropriate gating [a] CD8 cells in the lungs were analyzed for [b] $CD62L^{hi}$ central memory cells or $CD62L^{lo}$ activated cells. Significance differences between marker expression was calculated using two-way ANOVA. Results are representative or two independent experiments.

MHC class II expression was higher on macrophages in the vaccinated groups.

I used CD11b and CD11c expression on lung cells to analyze the response of macrophage subsets in vaccinated mice in terms of their ability to express MHC Class-II molecules [Fig.8]. The fluorescence signal from alveolar macrophages in region 3 was modest, but increased in the vaccinated animals. It was also marginally higher in region 5, which contains small macrophages and some monocytes. In contrast, region 4, which is rich in dendritic cells, showed a large increase in MHC expression compared to infected controls.

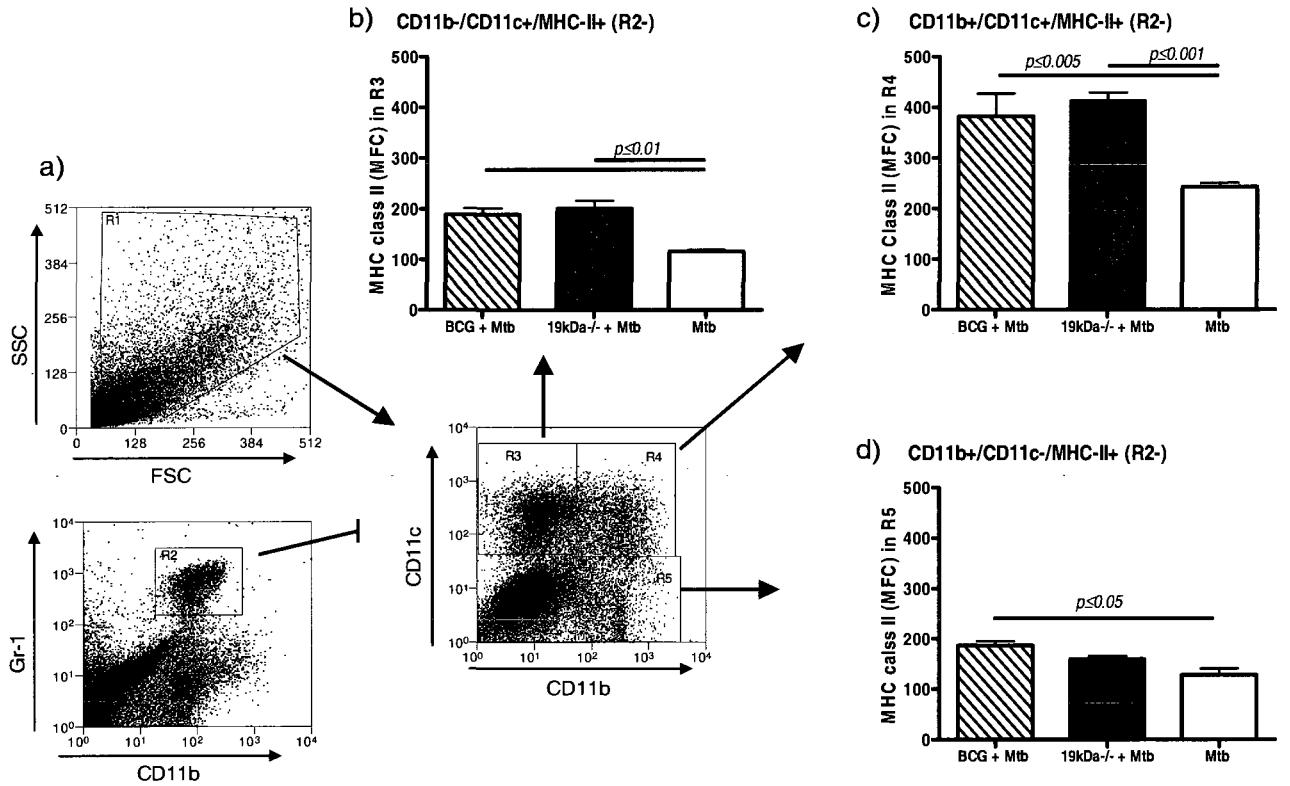

Figure 8. MHC Class-II expression on macrophages subsets in the lungs one month after aerosol challenge infection. Analysis was made based on CD11b and CD11c staining after Gr-1^{hi} $CD11b^{hi}$ granulocytes were gated out. Data shows MFC levels of Class-II expression on region 3 cells [alveolar macrophages], region 4 rich in mature dendritic macrophages, and region 5 [mostly small macrophages and monocytes]. Statistical significance between MFC was calculated using two-way ANOVA. Results are representative or two independent experiments.

Discussion

The results of this study show that loss of the ability to express the 19kDa-lipoprotein renders *M. tuberculosis* almost completely unable to replicate *in vivo*. Despite this it still remained immunogenic, generating IFNγ secreting CD4 and CD8 T cells, and protecting mice from low dose aerosol challenge infection to a similar degree to the BCG vaccine. Given that the Δ19 mutant grew perfectly well in cultures, this seems to suggest that the 19kDa-lipoprotein plays an important role in the host cell phagosome, enabling cell division. This mutant thus falls into the category of live attenuated vaccines, several of which have been evaluated in animal models [14].

It was interesting to note that the inflammatory response in the lungs to the Δ19 was minimal, and in fact there is evidence that the 19kDa-lipoprotein is a major mediator of neutrophil activation, characterized by down-regulation of CD62 ligand (L-selectin) and up-regulation of CD35 (CR1) and CD11b/CD18 (CR3, Mac-1). In addition, exposure of neutrophils to MTB 19-kDa lipoprotein enhanced a subsequent oxidative burst [15]. Thus, *M. tuberculosis* 19kDa-lipoprotein produced several changes characteristic of neutrophil priming and activation that are seen with some Pathogen-Associated Molecular Patterns (PAMPs).

Results from the two vaccines tested in parallel were very similar, but one interesting observation was that BCG vaccinated mice had lung granulomas containing large numbers of vacuolated foamy macrophages after challenge infection, whereas the Δ19 vaccinated mice had granulomas that were much more lymphocytic. The foamy cells

are a well known characteristic of tuberculosis granulomas, and are of interest because they express both DEC-205, a dendritic cell marker, as well anti-apoptotic TRAF markers [13]. Why these were not frequent in the granulomas of Δ19 vaccinated mice is unknown.

In this regard, dendritic cells were the population with the highest levels of MHC Class-II expression after challenge. This is in keeping with the hypothesis that both the Δ19 and BCG vaccines enabled the immune mice to more rapidly limit the growth of the challenge infection, thus reducing the levels of the 19kDa lipoprotein produced by the challenge organism and thus facilitating the ability of the host cells to present antigens using Class-II molecules without interference from this antigen.

An alternative approach to try to understand the role of the 19kDa lipoprotein has been to over-express it. It has been known for some time that over-expression in the saprophyte *M. vaccae* interferes with its vaccine properties [17]. When over-expressed in BCG, different results have been reported, with one laboratory showing enhanced IFNγ production but also increased TH2 cytokines, coupled with a failure of the rBCG to protect guinea pigs [18], although more recently it was found that over-expression or deletion of the lipoprotein in BCG did not affect its protective capacity [19].

These results have applications to new tuberculosis vaccine design [14]. In addition to creating considerable attenuation of *M. tuberculosis,* removal of the lipoprotein may also improve vaccinogenic properties in terms of reducing potential interference with antigen processing mechanisms in the host cell.

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CHAPTER 3:

Effector memory T lymphocytes predominate in mice chronically infected with *Mycobacterium tuberculosis*

The aims of this study were to:

1. Study the different populations of memory T cells generated during *M. tuberculosis* infection and the effects of drug treatment.
2. Study the different populations of memory T cells generated after BCG vaccination.
3. Evaluate the potential differences in the memory T cell populations generated by the infection and by the vaccine.
4. Evaluate and compare memory T cells present in the lung, spleen, mediastinal lymph node and bone marrow, after infection or vaccination.

INTRODUCTION

Mycobacterium tuberculosis remains one of the world's most successful bacterial pathogens, with in excess of two million deaths each year and eight million new cases (1). A troublesome aspect of this epidemic is the increasing incidence of isolates that are resistant to multiple drugs, now estimated at approaching half a million new cases each

year (2-4). The seriousness of this situation is amplified by the fact that the existing BCG vaccine is not effective in adults (5-7).

The purpose of vaccination is to establish a long lived state of immunological memory. In the context of vaccination for tuberculosis it has been assumed to date that such a state is fully established, and that it is mediated by a very specific subset of T cells. This arose from various early studies in the field, particularly those by Orme laboratory (8) and that of Andersen (9), which demonstrated that C57BL/6J mice infected with *M. tuberculosis* were found to generate long-lived memory immunity which provided a heightened state of acquired resistance to a secondary infection. Mice heavily infected with the BCG vaccine were able to rapidly generate an acquired cellular immune response to this infection, characterized primarily by the emergence of a splenic T-cell population capable of adoptively transferring substantial levels of protection against a challenge infection with *M. tuberculosis* (10). In studies conducted with a mouse model of memory immunity in which mice are rendered immune by a primary infection followed by antibiotic treatment and rest, tuberculosis-specific memory cells were recruited from the recirculating pool, leading to rapidly increasing precursor frequencies in the liver and a simultaneous decrease in the blood (11).

That the reality is probably more complicated however is provided by current information that indicates that there are at least two subsets of memory T cells, both for CD4 and CD8 cells, based upon the anatomical locations of these subsets, their expression of various cell surface markers, and various functional responses based on

cytokine secretion and rapidity of their response (12-14). A primary distinction is based upon homing receptors that allow entry into lymphoid tissues; hence those that lack or have low expression of CD62L and CCR7 and are found in peripheral tissues where they seem to provide a first line of defense are designated "effector memory" cells or T_{EM}. A second population, high in expression of CD62L and CCR7, is found in lymph nodes and seem to represent a second line of defense, and are designated "central memory" T cells or T_{CM} (15-18).

Much of what is known about these cells however is based upon responses to defined antigens, often as not in TCR transgenic mice, or in mice undergoing acute viral infections. In contrast, what happens in mycobacterial infections where there is extensive inflammation and immune stimulation, and in which the infection is often chronic ensuring continued production of antigens, is only just starting to be studied. In previous work the Orme laboratory found that BCG vaccination of mice established a pool of activated effector ($_{AE}T$) or perhaps effector memory T cells in the lungs for a very long period of time (19), and that similar populations of cells persisted in the lungs of mice chronically infected with *M. tuberculosis* (20). In the present study I revisited this observation and compared this with mice actively infected with *M. tuberculosis*, as well as animals infected and then drug treated to reduce the bacterial load. I show here that both chronically infected mice as well as BCG vaccinated [but not challenged] both generate CD4 and CD8 T cell populations that have the characteristics of T_{EM}. In contrast, cells with a phenotype characteristic of T_{CM} represented a far smaller population

and this occurred primarily in the CD8 subset. In addition, other populations of cells were observed, some of them apparently novel.

MATERIALS AND METHODS

Animals

Specific pathogen-free female, 6–8-week-old, C57BL/6 mice from Jackson laboratories, Bar Harbor, ME. They were kept under barrier conditions in an ABL-III laboratory and fed sterile water and chow. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University.

BCG vaccinations, Aerosol infection with M. tuberculosis and Drug Treatment

M. tuberculosis strain H37Rv, 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. For low-dose aerosol infections, bacterial stocks were diluted in 5 ml of sterile distilled water to 2×10^6 colony-forming units/ml and placed in a nebulizer attached to an airborne infection system (Glass-Col, Terre Haute, IN). Mice were exposed to 40 min of aerosol where approximately 100 bacteria were deposited in the lungs of each animal. Bacterial load was determined by plating right lobe homogenates onto nutrient 7H11 agar plates supplemented with OADC. Colonies were counted after 21 days of incubation at 37°C. A subset of infected mice was treated, from day 40 post infection until the end of the study, with rifampicin (RIF; 15 mg/kg) and isoniazid (INH; 25 mg/kg) in the drinking

water. The control group was treated with the same doses of rifampicin and isoniazid as the infected group for the same period of time. For the vaccination studies, animals were immunized with 10^6 CFU BCG Pasteur subcutaneously.

Preparation of Cells

Mice were euthanized by CO_2 asphyxiation, and the thoracic cavity was opened. The lung was cleared of blood by perfusion through the pulmonary artery with 10 ml of ice cold phosphate-buffered saline (PBS) containing 50 U/ml of heparin (Sigma, St Louis, MO). Lungs were aseptically removed, teased apart and treated with a solution of deoxyribonuclease IV (DNAse) (Sigma Chemical, 30 μg/ml) and collagenase XI (Sigma Chemical, 0.7 mg/ml) for 45 min at 37°C. Spleens and pulmonary lymph nodes (LN) were also harvested. To obtain a single-cell suspension, the organs were gently passed through cell strainers (Becton Dickinson, Lincoln Park, NJ).The remaining erythrocytes were lysed with Gey's solution (0.15 M NH_4Cl, 10 mM $KHCO_3$) and the cells were washed with Dulbecco's modified Eagle's minimal essential medium. Bone marrow (BM) cells were harvested from the femurs of the mice, any possible erythrocyte contamination was lysed with Gey's solution (0.15 m NH_4Cl, 10 mm HCO_3) and the cells were washed with Dulbecco's modified Eagle's minimal essential medium. Total cell numbers were determined by flow cytometry using BD™ Liquid Counting Beads, as described by the manufacturer (BD PharMingen, San Jose, CA USA 95131).

Flow Cytometry for surface markers and intracellular cytokines

For flow cytometry analysis, single-cell suspensions of lung from each mice were re-suspended in PBS (Sigma-Aldrich) containing 0.1% of Sodium Azide (PBS+Na/Az). Cells were incubated in the dark for 25 min at 37°C with predetermined optimal titrations of specific antibody (directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin-cholorophyll-protein (PerCP), allophycocyanin (APC), Pacific Blue, Alexa 700); or after biotin antibody incubations washed and incubated for 25 minutes more with streptavidin Qdot800 (Invitrogen), followed by two washes in PBS containing 4% sodium azide. Cell surface markers were analyzed with the following specific antibodies: FITC anti-CD44 (clone IM7), PE anti-CCR7 (clone 4BR), PerCP anti-CD4 (clone L3T4), PerCP anti-CD8α (clone 53-6.7), APC anti-CD62L (clone MEL-14), Pacific Blue anti-CD127 (cloneA7R34), Pacific Blue anti-IFNγ (clone (XMG1.2), Alexa 700 anti-CD3ε (clone 17A2), Biotin anti-CD45RB (clone C363.16A) all antibodies were purchased from BD PharMingen or eBiosciences (eBioscience, San Diego, CA). Measurement of intracellular IFN-γ was conducted by pre-incubating lung cells with monensin (3 μM) (Golgi Stop, BD PharMingen), anti-CD3 ε and anti-CD28 (both at 0.2 $\mu g/10^6$ cells) for 4 h at 37°C, 5% CO_2. The cells were then surface stained, incubated for 30 minutes at 37°C, washed then fixed and permeabilized with Perm Fix/Perm Wash (BD Pharmingen). Finally, the cells were stained with intracellular anti-IFN-γ (clone XMG1.2) or its respective isotype controls (BD Pharmingen) for a further 30 min. All the samples were analyzed on a Becton Dickinson LSR-II and data were analyzed using FACSDiva v5.0.1 software. Cells were gated on lymphocytes based on characteristic forward and side scatter profiles. Individual cell populations were identified according to

their presence of specific fluorescent-labeled antibodies. All the analyses were performed with a minimum acquisition of 300000 events.

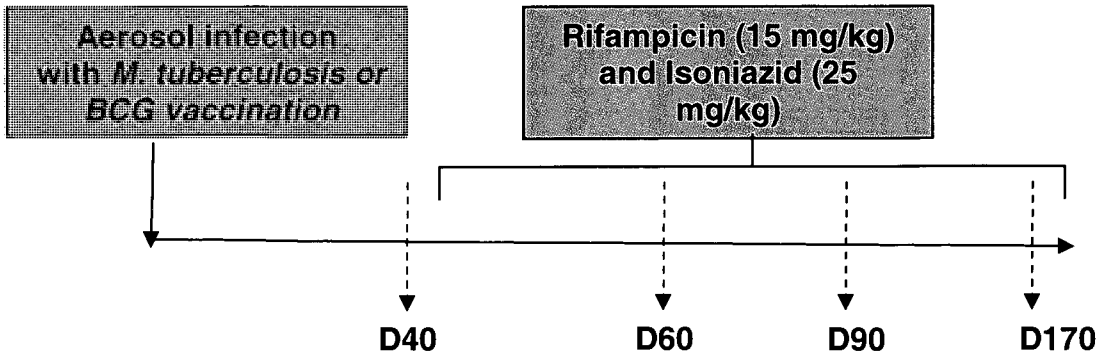
Statistics

Statistical significance was determined using two-way ANOVA with Bonferroni post tests using GraphPad Prism v4.00 for Windows, (GraphPad Software, San Diego CA).

RESULTS

Establishment of memory immune mice.

To establish a state of memory immunity, mice were either vaccinated subcutaneously with them. *M .bovis* BCG Pasteur vaccine, or exposed to a low dose aerosol infection with *M. tuberculosis* H37Rv. Half of these latter animals were then treated with a mixture of rifampacin (15 mg/kg) and isoniazid (25 mg/kg) daily. By day 90, the bacterial load in the lungs of these mice had fallen below levels of detection [Fig.1].



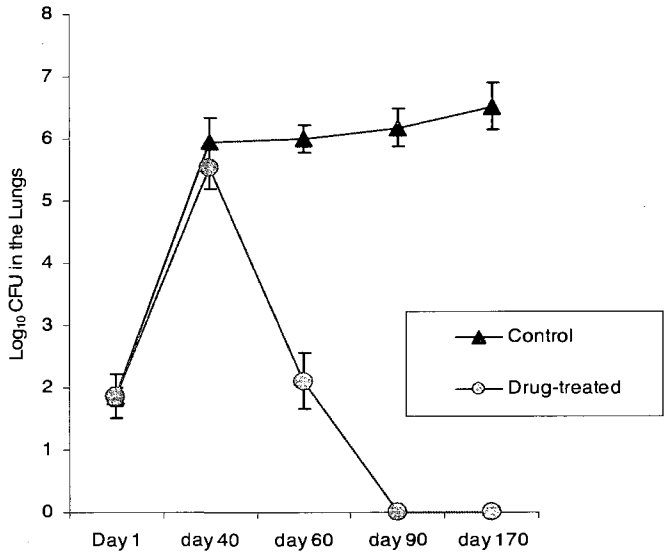
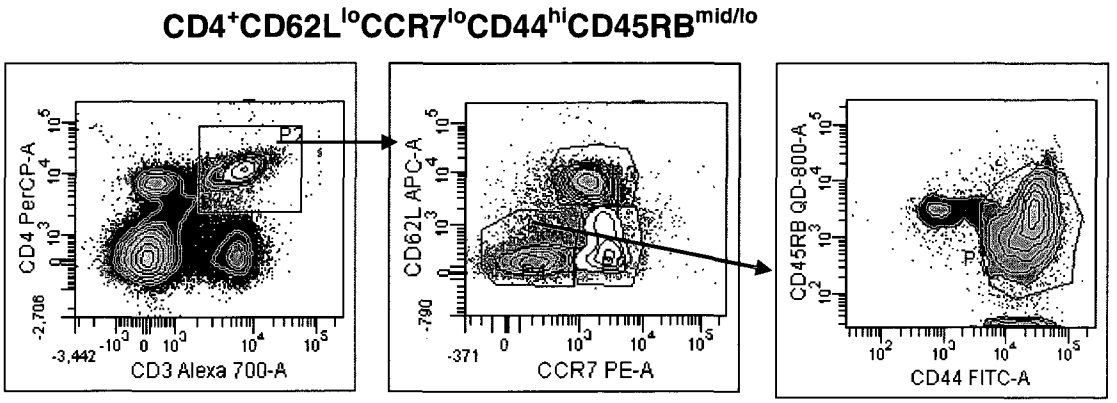


Figure 1. Growth of *M. tuberculosis* H37Rv [triangles] and H37Rv infected and drug treated [circles]. The bacterial load was calculated by plating lung homogenates onto 7H11 agar at 37°C and counting colonies 21 days later. Time points were day 1 day 40, day 60, day 90 and day 170. The data are expressed as the mean ± the standard error of the mean from five mice per group.

Gating and analysis of memory T cell subsets.

To differentiate between T_{EM} and T_{CM} flow cytometry gating was based upon expression of the multiple markers CD62L, CCR7, CD44, CD45RB and CD127, based upon previously published studies (21-23) [Fig.2]. Using this strategy, we then looked at each population in the lungs [target organ] as well as the draining lymph nodes, spleen, and bone marrow over the course of the infections.

A)



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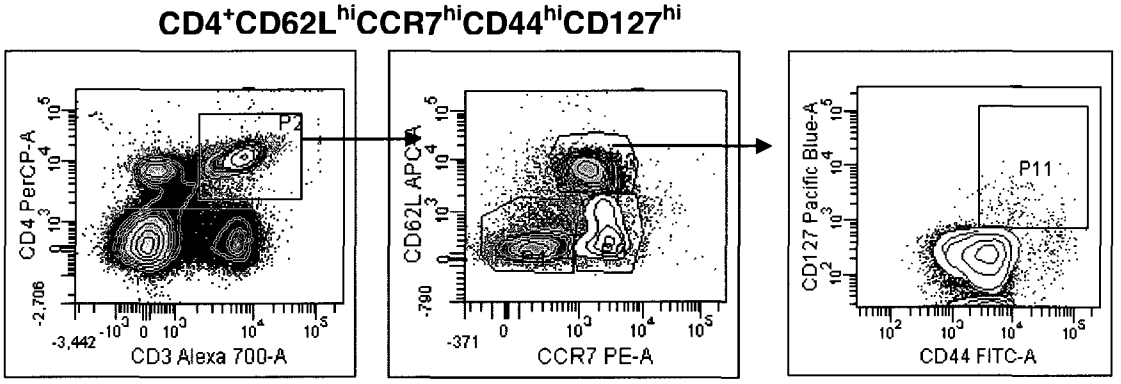

Figure 2. Representative Flow Cytometry Contour plots gating for Effector Memory T cells (T_{EM}) [A] and Central Memory T cells (T_{CM}) [B].

As shown in Fig.3, mice chronically infected with *M. tuberculosis* had approximately ten-fold higher numbers of activated CD4 and CD8 cells expressing a $CD62L^{lo}CCR7^{lo}$ $CD44^{hi}CD45RB^{mid/lo}$ phenotype, which presumably reflected a mixture of $_{AE}T$ and T_{EM}, and which was probably reflected both in the subsequent drop in these numbers in drug-treated mice and in the BCG vaccinated mice. In all three test groups large numbers of these cells could be found in the spleen, including the drug-treated animals, suggesting a reservoir of the T_{EM} subsets. A similar trend to that seen in the lungs was observed in the draining mediastinal lymph nodes, whereas only background levels were seen in the bone marrow.

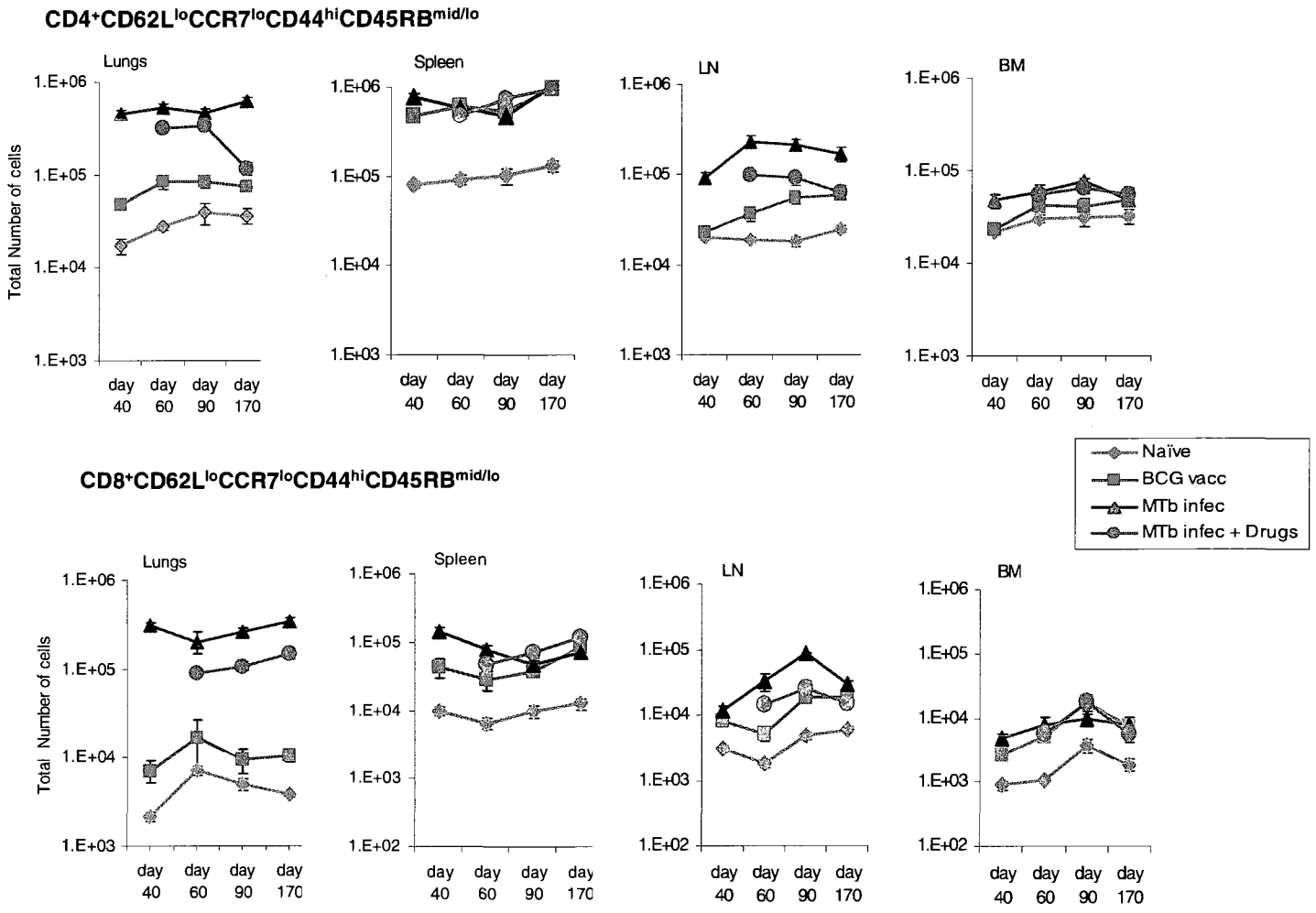

Figure 3. Total CD4+ and CD8+ T_{EM} ($CD62L^{lo}CCR7^{lo}CD44^{hi}CD45RB^{mid/lo}$) positive cells numbers, entering the lungs, spleens, LN and BM at day 40, 60, 90,170 after vaccination or infection with *M. tuberculosis*. The data are expressed as the mean ± the standard error of the mean from five mice per group.

In contrast, much lower numbers of cells expressing the T_{CM} phenotype $CD62L^{hi}CCR7^{hi}CD44^{hi}CD127^{hi}$ were observed in all organs [Fig 4.]. Similar numbers of T_{CM} were seen in the lungs, lymph nodes and spleen, whereas in the bone marrow levels of CD4 subsets were elevated in the BCG and drug-treated groups, as might be anticipated given data suggesting T_{CM} can accumulate here (24-26).

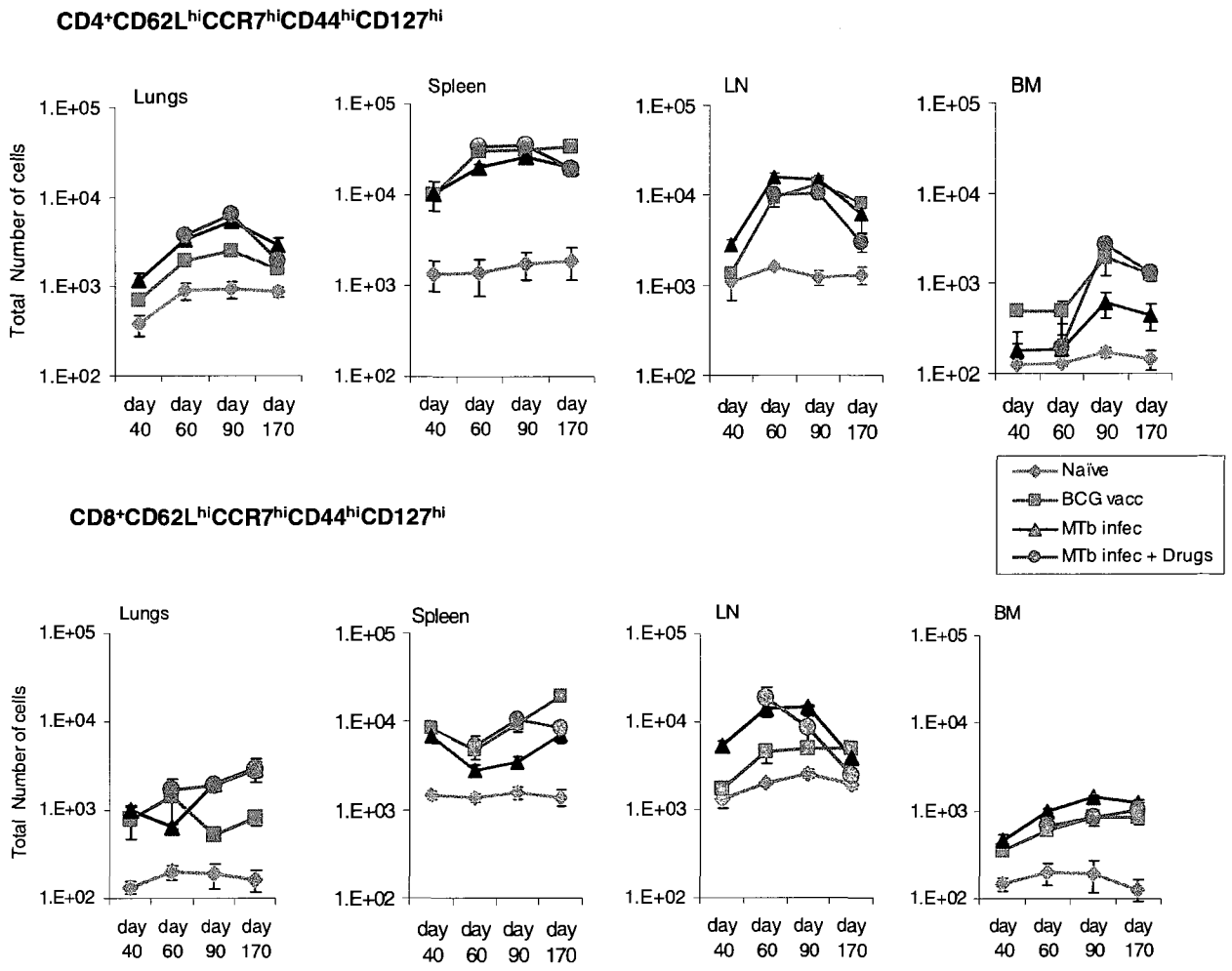

Figure 4. Total CD4+ and CD8+ T_{CM} ($CD62L^{hi}CCR7^{hi}CD44^{hi}CD127^{hi}$) positive cells numbers, entering the lungs, spleens, LN and BM at day 40, 60, 90,170 after vaccination or infection with *M. tuberculosis*. The data are expressed as the mean ± the standard error of the mean from five mice per group.

To be certain the predominant T_{EM} subsets were antigen-specific cells and not just some non-specific polyclonal cellular expansion in response to the presence of the infections, lung cells were also stained with tetramers loaded with a dominant epitope of the major immunogenic protein ESAT-6, or with a major CD8 epitope from the Mtb32 protein [21, 22]. As shown in Fig.2, in chronically infected mice about 3% of CD4 $CD44^{hi}$ cells co-stained for the pMHCII tetramer, whereas staining of cells from the BCG vaccinated mice was background [BCG does not have the ESAT-6 gene]. Similarly, positive staining for the CD8 epitope was about 4% of total CD8 $CD44^{hi}$ cells harvested from the chronically infected animals.

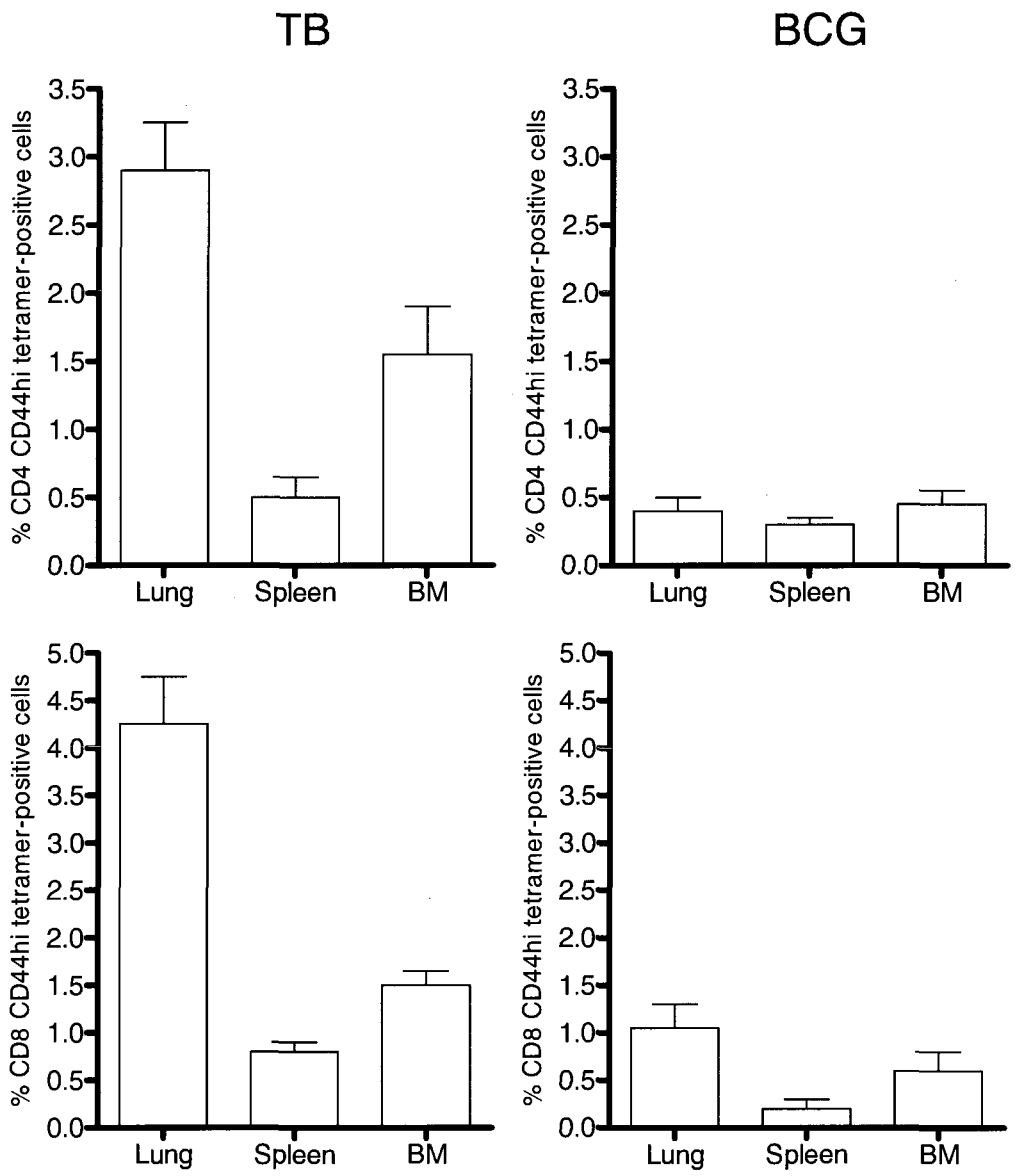

Figure 5. Percentage of CD4+ and CD8+ tetramer positive cells ($CD3^+CD4^+$Tetramer class-II^+CD44^+ or $CD3^+CD8^+$Tetramer class-I^+CD44^+) entering the lungs, spleens, and BM at day 120 after vaccination or infection with *M. tuberculosis*. The data are expressed as the mean ± the standard error of the mean from five mice per group.

Expansion of CD45RB^{hi} subset.

In our earliest studies on this topic, and indeed in the very first application of this technique to tuberculosis infection in mice, we observed a rapid expansion of CD45RB^{hi} cells, which we interpreted at the time as blast cells given their large size (27). In the current study, in which we looked much later on in the infection, T_{EM} cells expressing CD4 and CD45RB^{hi} were only seen in the lymph nodes and bone marrow, and were at background levels [Fig.6]. In contrast, in the case of CD8 cells, these were elevated above background in the lungs, spleen and bone marrow, and in increased numbers in the lymph nodes compared to the other test groups.

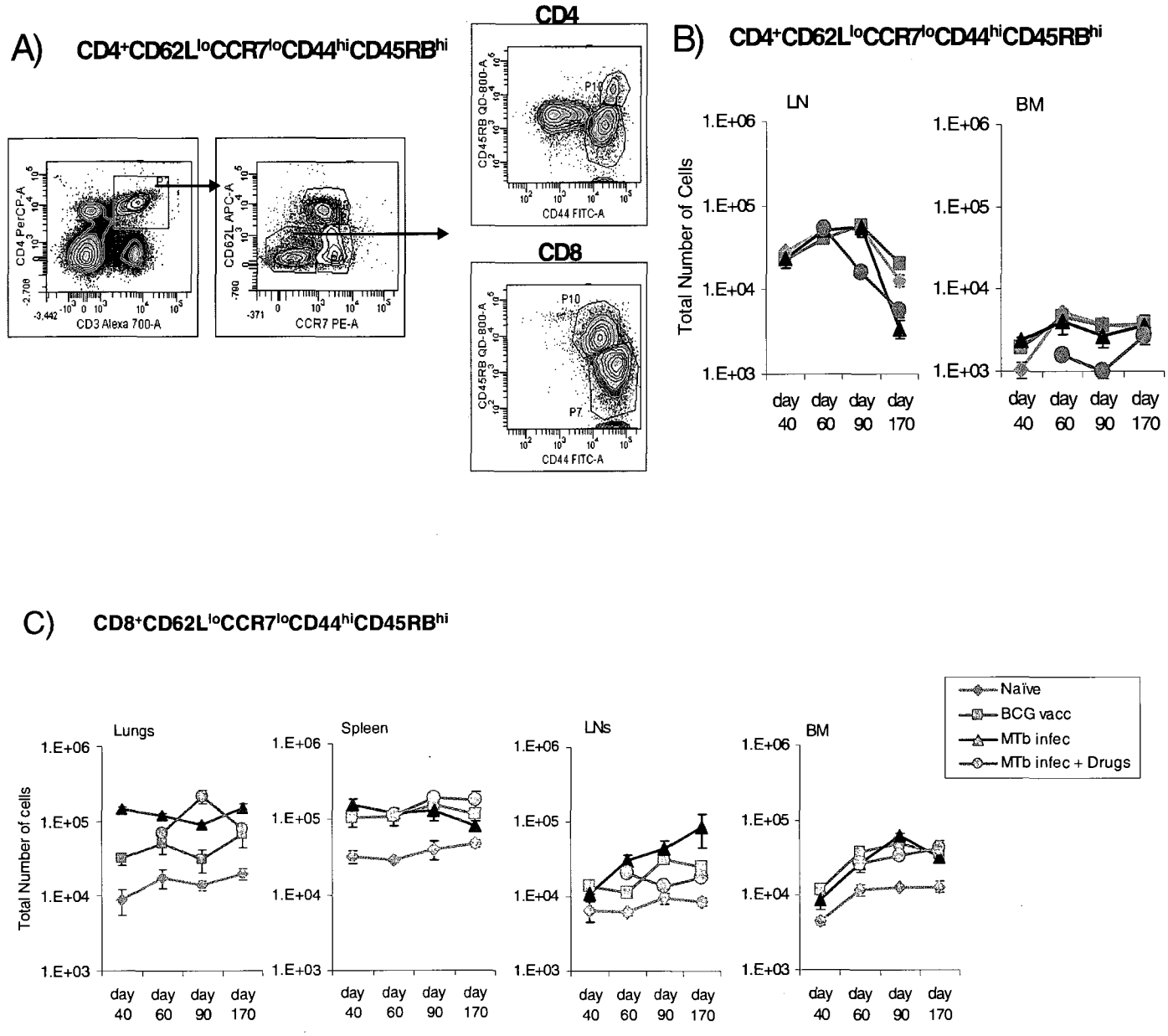

Figure 6. Representative Flow Cytometry Contour plots gating for Effector Memory T cells (T_{EM}) [a]. Total CD4+CD62L^{lo}CCR7^{lo}CD44^{hi}CD45RB^{hi} T cells numbers in LN and BM [b]. Total CD8+CD62L^{lo}CCR7^{lo}CD44^{hi}CD45RB^{hi} T cells numbers in Lung, spleen, LN and BM at day 40, 60, 90,170 after vaccination or infection with *M. tuberculosis* [c]. Statistical significance between marker expression on T cells between the different groups was calculated using two-way ANOVA.

IFNγ-producing T cell subsets.

To analyze which CD4 or CD8 T cells were producing IFNγ we started by gating on CD44 and CD62L [Fig.6]. Populations representing $_{AE}$T or T_{EM} $CD62L^{lo}CD44^{hi}$ cells were the biggest producers of IFNγ, as might be anticipated. We then analyzed each subset in terms of their kinetics. Results for the $CD62L^{lo}CD44^{hi}$ subsets are shown in Fig.7. Numbers of these cells were the highest in the *M. tuberculosis*-infected groups, regardless of reduction of the bacterial load by drug treatment, and a steady increase was seen in the BCG vaccinated mice. In the spleen, lymph nodes and bone marrow drug therapy caused a drop in such cells, with the exception of CD8 cells in the lymph nodes and bone marrow.

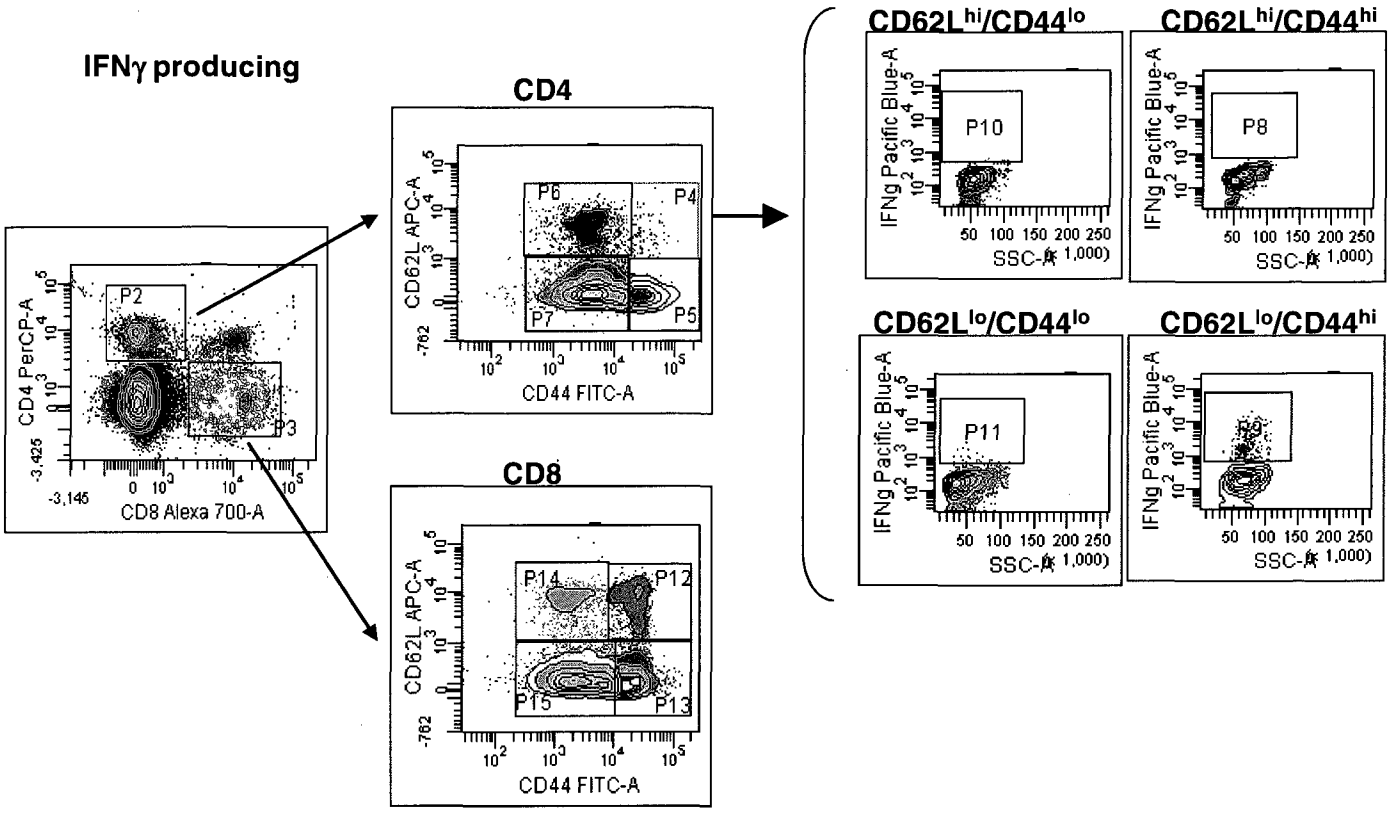

Figure 7. Representative Flow Cytometry Contour plots gating for IFNγ producing CD4+ and CD8+ T cells according to CD62L and CD44 expression.

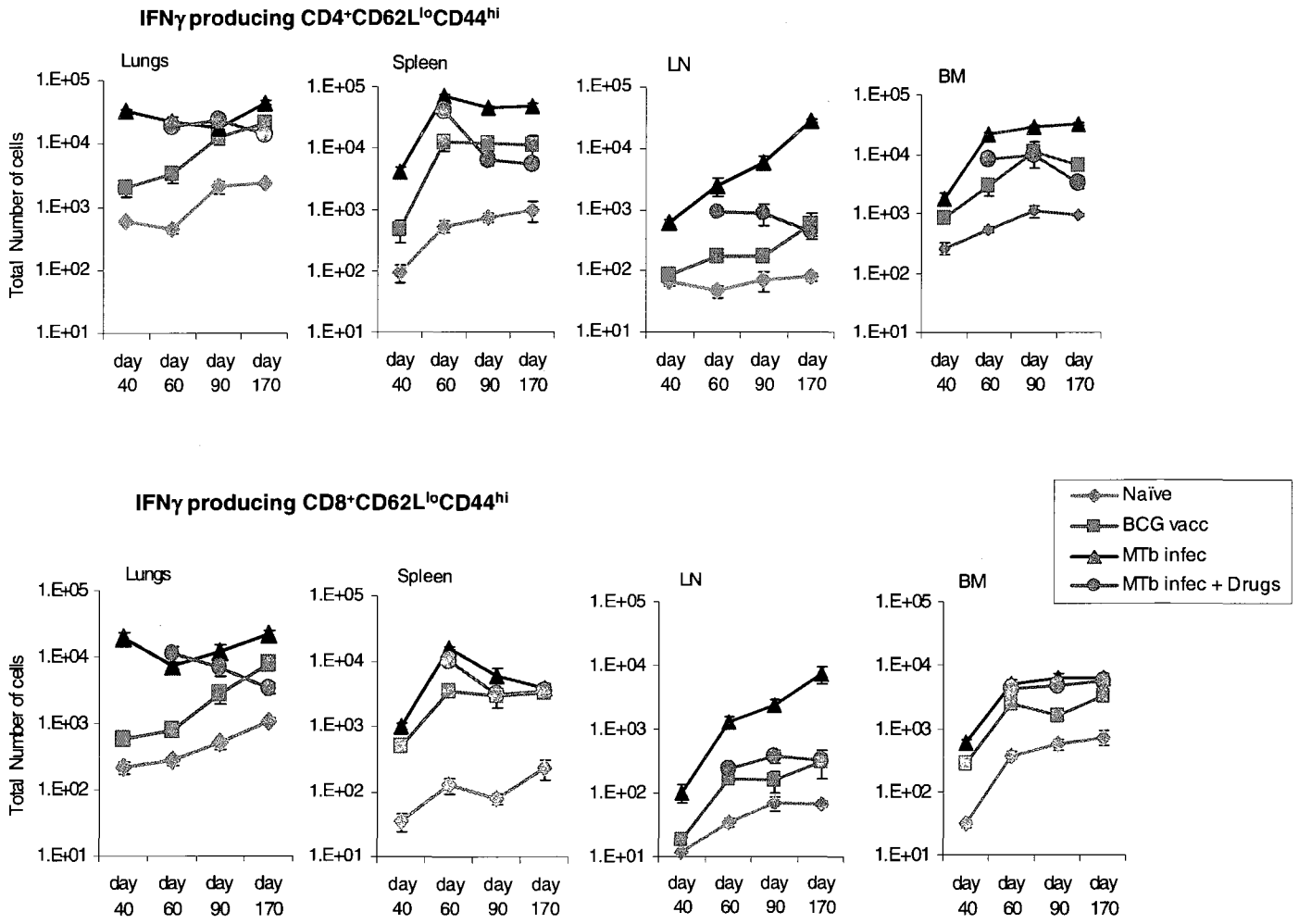

Figure 8. Total CD4+ and CD8+ IFNγ producing CD62L^{lo}CD44^{hi} cells numbers, entering the lungs, spleens, LN and BM at day 40, 60, 90,170 after vaccination or infection with *M. tuberculosis*. The data are expressed as the mean ± the standard error of the mean from five mice per group.

In the case of IFNγ-positive $CD62L^{hi}$ $CD44^{hi}$cells, these were elevated in the lungs, but could only be detected in very low numbers in the other organs [Fig.8]. In the case of $CD62L^{hi}CD44^{lo}$gated IFNγ-positive cells only low numbers of cells could be found, and in most cases numbers were similar to uninfected age-matched controls [Fig.9].

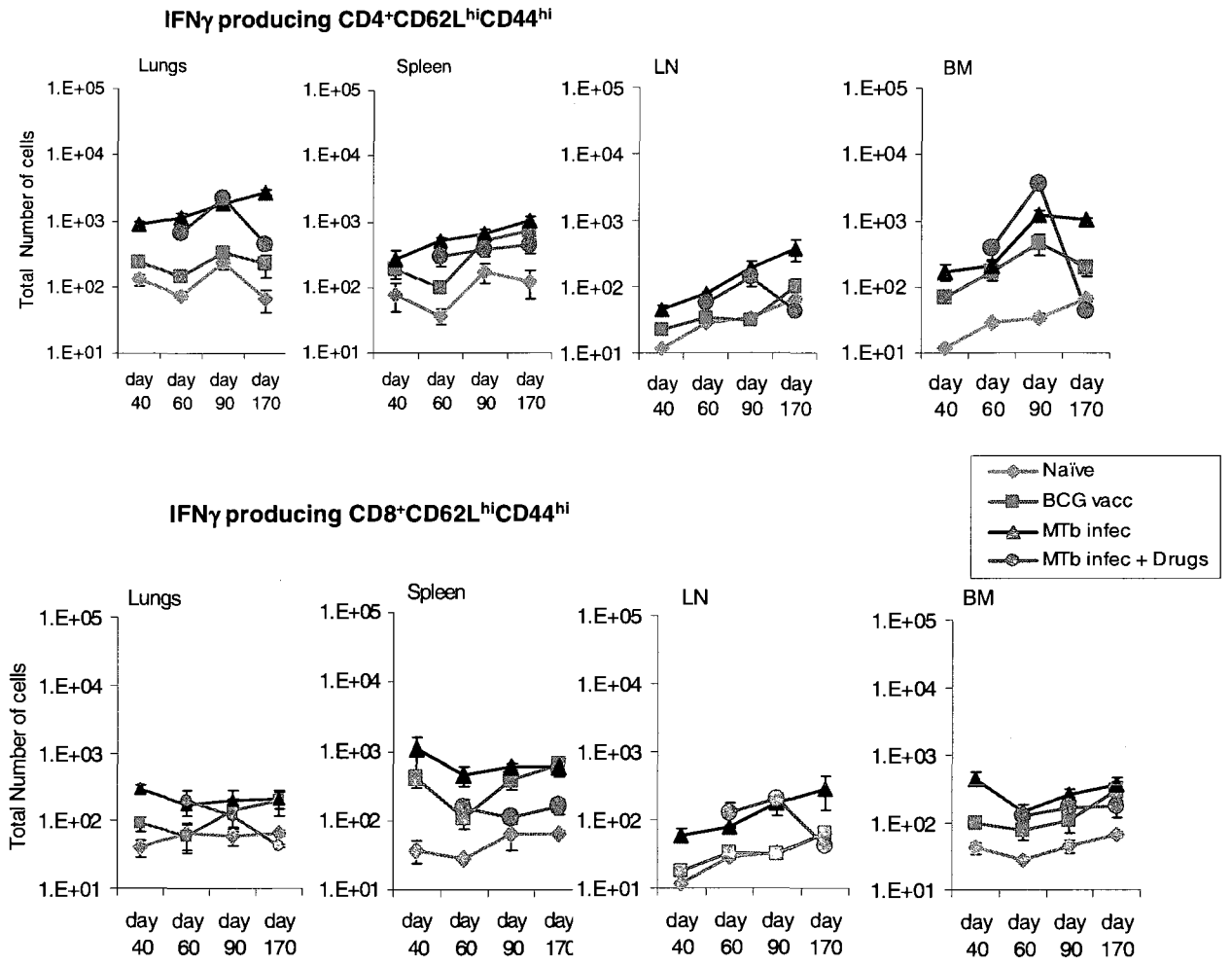

Figure 9. Total CD4+ and CD8+ IFNγ producing CD62L^{hi}CD44^{hi} cells numbers, entering the lungs, spleens, LN and BM at day 40, 60, 90,170 after vaccination or infection with *M. tuberculosis*. The data are expressed as the mean ± the standard error of the mean from five mice per group.

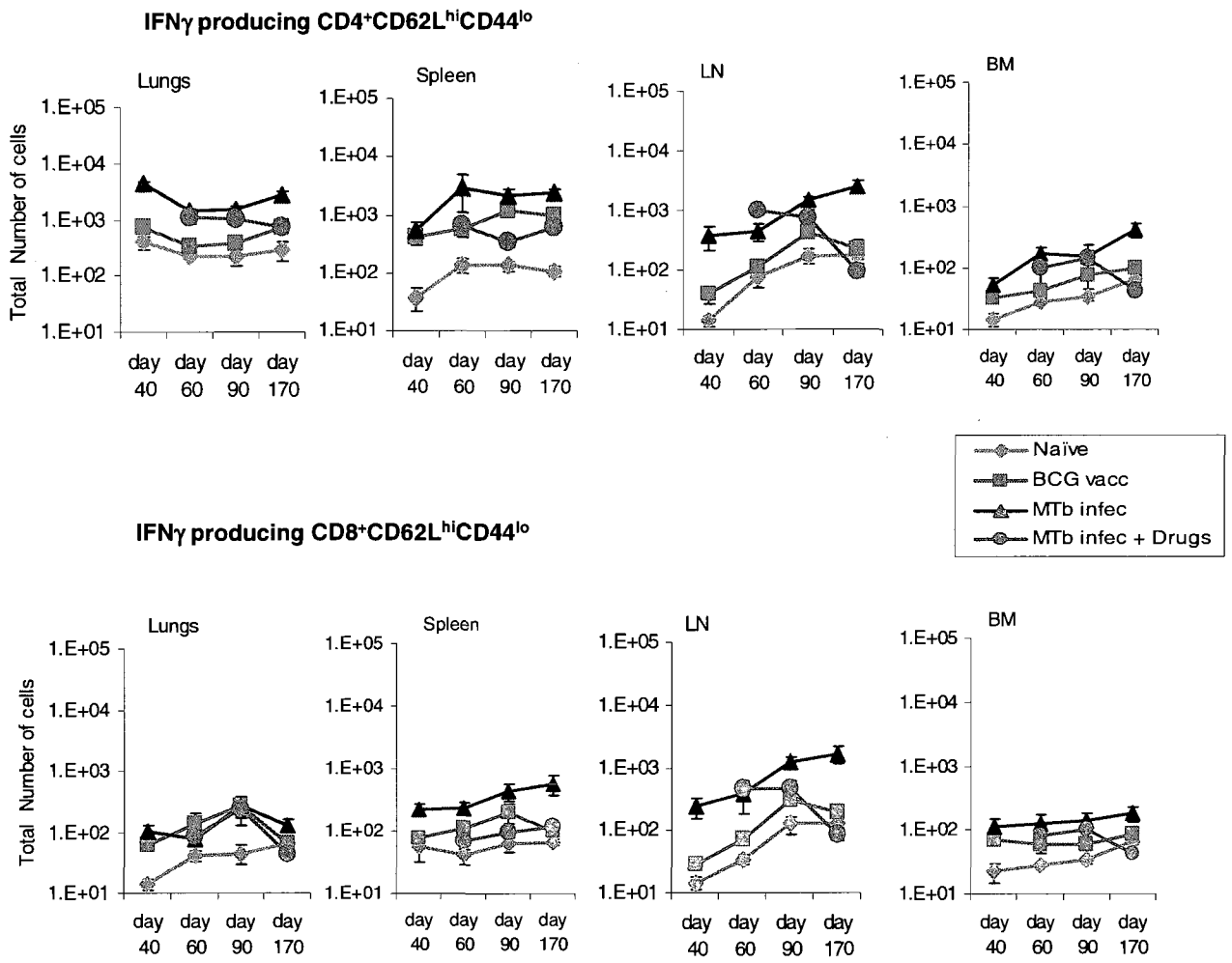

Figure 10. Total CD4+ and CD8+ IFNγ producing CD62L^{hi}CD44^{lo}ells numbers, entering the lungs, spleens, LN and BM at day 40, 60, 90,170 after vaccination or infection with *M. tuberculosis*. The data are expressed as the mean ± the standard error of the mean from five mice per group.

Expansion of CD62L^{lo}CCR7^{hi} T cells in the organs of infected animals.

During our initial gating procedures on cells from infected mice we consistently saw a population of CD62L^{lo} cells that had high expression of the CCR7 chemokine receptor [Fig.10a]. It has been reported that following antigen stimulation T_{EM} transiently up-regulate CCR7 (28), and that CD62L is rapidly shed after TCR triggering or following lymph node migration (29). So we decided to look at their expression of CD45RB as well as CD127 as the signaling through the IL-7R appears to promote memory T cell survival by inducing expression of anti-apoptotic members of the Bcl-2 protein family (30).

When analyzed in the context of CD45RB^{lo} expression [Fig.10b] these were consistent with a mixture of $_{AE}$T and T_{EM}, as suggested by a drop in mice given drug-treatment. Lower numbers were seen after CD127 gating, but again an accumulation was seen in the spleen suggesting establishment of a reservoir of these cells [Fig.10c].

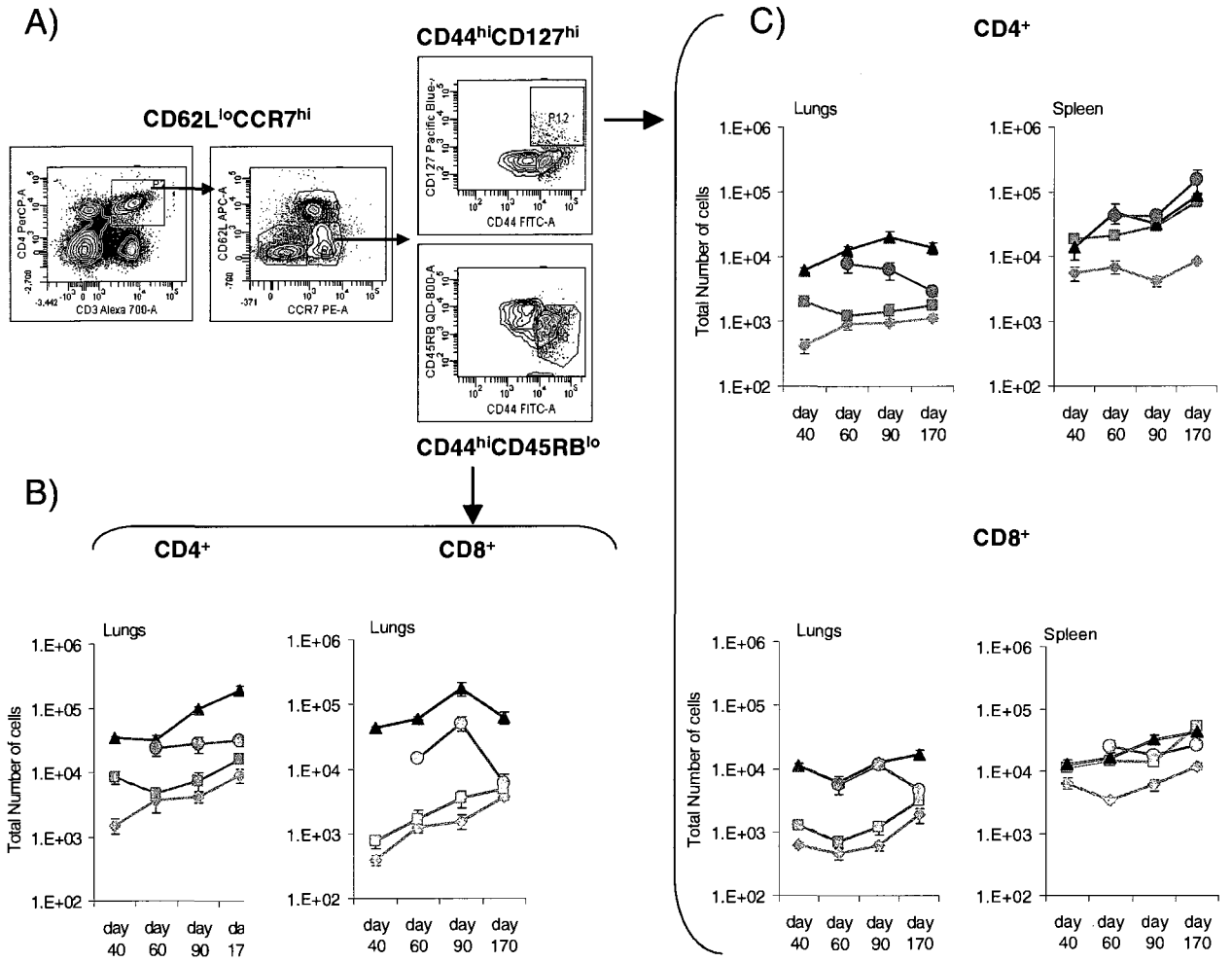

Figure 11. Representative Flow Cytometry Contour plots gating for $CD62L^{lo}CCR7^{hi}$ cells either $CD44^{hi}CD127^{hi}$ (top) or $CD44^{hi}CD45RB^{hi}$ (bottom) [a]. Total $CD4+CD62L^{lo}CCR7^{hi}CD44^{hi}CD45RB^{hi}$ T cells vs $CD8+CD62L^{lo}CCR7^{hi}CD44^{hi}CD45RB^{hi}$ numbers in the lungs [b]. Total CD4+ or $CD8+/CD62L^{lo}CCR7^{hi}CD44^{hi}CC127^{hi}$ T cells numbers in Lung and spleens at day 40, 60, 90,170 after vaccination or infection with *M. tuberculosis* [c]. Statistical significance between marker expression on T cells between the different groups was calculated using two-way ANOVA.

DISCUSSION

The results of this study show that the immune response mediated by CD4 and CD8 T cells both in actively infected and in BCG vaccinated mice can be divided into further subsets, some of which clearly have characteristics of T_{EM} and T_{CM} populations. Moreover, these subsets are widely distributed in both lung tissues and in lymphoid tissues. These subsets are not static, change with time, or can be manipulated by destruction of the *M. tuberculosis* bacillus by chemotherapy.

The primary conclusions that can be drawn from these simple descriptive studies are two-fold. The first is that the memory T cell response to chronic mycobacterial infection or to vaccination is dominated by a T_{EM} response approximately 100-fold larger than a T_{CM} response. Secondly, there appear to be additional T cell subsets involved that have not previously been recognized as being of any importance, and which do not exist in antimicrobial models in which antibody production is the key outcome.

Since BCG does not reach the lungs after subcutaneous injection (31, 32), the increase in $CD62L^{lo}CCR7^{lo}CD44^{hi}CD45RB^{mid/lo}$ CD4 and CD8 T cells in this organ up to 170 days later almost certainly can be regarded as a true T_{EM} population. In contrast cells with this phenotype in the *M. tuberculosis* infected mice probably represents a mixture of $_{AE}T$ as well as T_{EM}. In both cases these not only represent the major IFNγ+ population, but also a major reservoir of such cells in both the spleen and lymph nodes. Indeed, the fact that drug treatment of infected mice did not reduce the numbers of T_{EM} in

the spleen further suggests that this organ is a primary reservoir of these cells. In contrast there were far fewer $CD44^{hi}CD62L^{hi}CCR7^{hi}CD127^{hi}$ cells that could be found, representing T_{CM}. A great proportion were of the CD8 phenotype, an observation similar to that of Kamath et al (33) who found a preferential expansion of cells with this phenotype in drug treated mice that were subsequently re-infected. I did not re-challenge the mice in these studies, and moreover I suspect that my much lower estimate of T_{CM} numbers compared to the Kamath et al. study reflects my more stringent specific gating and phenotypic definition of this subset. In this study CD8 T_{CM} primarily accumulated in the spleen and lymph nodes, but could also be found in the lungs, and in the bone marrow, a known reservoir of T_{CM} (24-26).

The present data also suggest that additional cell subsets may exist. I found a population of CD8 cells that had the phenotype of T_{EM} but also had high expression of CD45RB. A previous graduate student in our laboratory had earlier noted such events in CD4 cells and showed that they were large in size (27), and hence I speculate that the cells observed here, although low in number, might represent blasting CD8 T_{EM} or CD8 cells transitioning from T_{EM} to $_{AE}T$ in response to the chronic infection and hence chronic antigen stimulation.

As noted above, when injected subcutaneously BCG drains into the regional lymph nodes where it grows for a brief while. Some bacilli reach the spleen in low numbers but there is no evidence they ever get any further. Despite this I found here a sustained increase in various T cell subsets in the lungs compared to that in age-matched

uninfected controls, consistent with earlier observations (19). My thesis work thus indicates that subcutaneous BCG inoculation establishes a persistent, apparently long lived population of T cells that is widely distributed in tissues including the lungs and the bone marrow, as well as lymphoid tissues such as the spleen and the mediastinal lymph nodes. Part of these is presumably within the recirculating lymphocyte pool, but it also appears that they may represent a reservoir of potentially reactive cells ready to respond to active infection.

It has always been assumed that the BCG vaccine establishes a state of memory immunity, and this is consistent with the accelerated response and quicker containment of *M. tuberculosis* infection previously demonstrated (7, 34). However this earlier more simple view of events is complicated by the newer concept that in mouse, memory itself may consist of the gradual differentiation of activated effector T cells to T_{EM} and then possibly to T_{CM}. In fact, the actual lineage relationship between these populations is still unclear. Moreover, the current ground rules on which these memory T cell populations are defined do not necessarily exist in the context of live mycobacterial infections. Indeed, it has been suggested (35) that true memory immunity to mycobacteria cannot be achieved because the organism cannot be fully eradicated by host immunity. The present data seems to suggest that BCG is adept at generating T_{EM} cells but far less so in its ability to establish T_{CM}. A similar situation seems to exist in terms of active tuberculosis infection, although here it is possible that both memory T cell populations may be transitioning to a $_{AE}T$ phenotype due to the continuing chronic infection, which could explain the changes then seen if the infection is reduced by chemotherapy.

This inability to establish a significant CD4 or CD8 T_{CM} population may underlie the inability of the BCG vaccine to protect adult humans from tuberculosis (5). It could be that the T_{EM} that are generated as the primary memory pool are just used up or exhausted by exposure to the pathogen in endemic areas, or that they simply experience an age-related loss in activity over the 10-15 year span between neonatal BCG vaccination and the adult onset of active tuberculosis [in fact, in this regard whether such cells are lost over a long period of time has never been addressed even in the acute viral or transgenic mouse models of memory immunity]. If this is the case then it could thus provide a marker with which to guide rational vaccine design, and could be applied to looking at which strategies, such as BCG prime boost, can strengthen the longevity of the specific T_{EM} response, or more effectively drive a potential T_{EM} to T_{CM} transition pathway in order to establish a better and longer lived recall response.

It may also be important to rethink ways in which vaccines are measured. To date, vaccine efficacy is usually selected on the basis of whether it can generate an IFNγ response, and then tested in short term assays in which the challenge is given 4-6 weeks after immunization. Given the obvious complexity of the memory response this may be misleading. First, such short term assays almost certainly do not give any pertinent information as to whether a T_{CM} population can be generated by a given vaccine, and this may be critical to whether a new vaccine or prime boost strategy could establish memory immunity capable of lasting decades or more, which is what is obviously needed. Moreover, IFNγ is considered another key read-out, and this obviously differs between

$_{AE}T$, T_{EM}, and T_{CM} populations, both in quantity and kinetics. Indeed, both earlier studies in the Orme laboratory (19) and those of others (23) seem to be indicating that in the context of memory immunity at least this is not a reliable marker, and a recent review on this topic reaches a similar conclusion (36).

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CHAPTER 4:

Loss of memory T cell populations in a mouse model of exogenous reinfection.

The aims of this study were to:

1. Generate a model to study post-primary tuberculosis in mice using a low dose aerosol as the rout of infection of *M. tuberculosis.*
2. Study the lung pathology generated after secondary aerosol infection with *M. tuberculosis* when BCG vaccination was administered before the first aerosol infection.
3. Evaluate the lung pathology generated after secondary aerosol infection with *M. tuberculosis* when chemotherapy was administered after the first aerosol infection.
4. Evaluate and compare the immune response generated in post-primary tuberculosis after BCG vaccination or chemotherapy.

INTRODUCTION

The global epidemic of tuberculosis (TB) results in eight million new tuberculosis cases per year with an annual projected increased rate of 3% (1). About 2 million people in the world die of tuberculosis every year and 98% of the mortality of cases occur in developing countries (1). At present the only available vaccine against tuberculosis

readily utilized in these countries, *M. bovis* Bacillus Calmette-Guérin (BCG), has proven unreliable to fully protect against pulmonary tuberculosis in adults (2, 3).

Secondary infection with *M. tuberculosis* can occur in three ways. First, as relapsing disease in people given chemotherapy in which treatment was not completely successful in eradicating persisting organisms. A second form is disease arising from an infection that was previously latent or dormant, which can be reactivated by a variety of conditions including co-infection with HIV. The third means is the acquisition of a second new infection, via exogenous reinfection [WHO report 2006] (4-6).

While for a long time the latter occurrence was considered uncommon, the development of new genotyping methods has allowed the direct demonstration that secondary infections can be exogenously acquired at a far greater incidence than previously thought (5, 7, 8). In fact, these genetic methods have thus allowed the distinction to be made between relapse and exogenous reinfection that would otherwise be clinically indistinguishable. As a result, there is now a growing appreciation that exogenous reinfection is a significant source of disease (1, 9).

This however raises a significant paradox. If an individual becomes infected and is subsequently treated successfully with chemotherapy, if he/she is otherwise immunocompetent then they should have generated a state of immunological memory that would make them highly resistant to secondary infection. This of course is predicted by classical studies in animal models in which mice that are infected and then given

chemotherapy develop memory T cells that render them highly resistant to secondary infection (10-12).

In the present chapter I have made various observations that may help resolve this paradox. I have previously demonstrated that mice chronically infected with *M. tuberculosis* retain various populations of T cells in their lungs. These appear to be a mixture of memory T cells and activated [presumably secondary] effector cells, but within the memory population in the previous chapter I showed that >95% of these have an effector memory T cell phenotype. In contrast numbers of T cells with a central memory T cell phenotype are rare, although larger numbers can be found in an apparent reservoir in the spleen. I show in the current chapter that numbers of these cells can be increased if the mouse is given chemotherapy to remove live bacteria, and if the animal is then reinfected this population of central memory cells responds very rapidly. This is followed by a second wave of T cells arising from the effector memory subset, and the combination of these two responses renders the animal highly resistant, with the lung bacterial load ~3 log lower than in control mice undergoing primary infection. Quite unexpectedly however, in the second month of the reinfection the memory T cell subsets decline in numbers, the lungs become highly consolidated by macrophages, and the animals die.

These novel observations challenge the notion that successful chemotherapy renders the individual highly resistant to secondary infection. In the model here at least, resistance is potent but it is also transient, and seems to involve rapid transition of the two

main memory T cell subsets into secondary effectors that are short-lived and rapidly lose their protective capacity. Such events may provide an immunological basis for events in humans presenting with exogenous reinfection.

MATERIALS AND METHODS

Animals

Specific-pathogen-free female C57BL/6 mice, 6 to 8 weeks old, were purchased from the Jackson Laboratories, Bar Harbor, Maine. Mice were maintained in the biosafety level 3 biohazard facility at Colorado State University, and were given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments. The specific pathogen-free nature of the mouse colonies was demonstrated by testing sentinel animals. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University.

Experimental Design

Groups of mice were challenged by a primary low-dose aerosol exposure (1) with *M. tuberculosis* strain Erdman using a Glas-Col (Terre Haute, Inc.) aerosol generator calibrated to deliver 50-100 bacteria into the lungs. Groups of mice were then treated or not treated 30 days post primary challenge with *M. tuberculosis* with chemotherapy of rifampicin (100 mg/L) and isoniazide (200 mg/L) in the drinking water and lasted a duration of 3 months. Some drug-treated mice were then immunosuppressed with dexamethasone (120 μg of dexamethasone was injected intramuscularly every other day

for 14 days). Animals were then rested for 2 weeks prior to a secondary *M. tuberculosis* reinfecction. Bacterial counts in the lungs (n=5) at each time point of the study were determined by plating serial dilutions of homogenates of lungs on nutrient 7H11 agar and counting colony-forming units after 3 weeks incubation at 37°C. Lungs from other mice (n=5) in the same groups were harvested for histological analysis and lung cell suspensions for flow cytometry after primary infections on days 30, 130, 140, 160, and 190. The results shown in this study are representative of two experiments.

Histological analysis

The accessory lung lobe from each mouse was fixed with 10% formalin in phosphate buffered saline (PBS). Sections from these tissues were stained using haematoxylin and eosin and acid fast bacilli.

Cell preparation

Single cell suspensions were prepared as described before (13-15). Briefly, the lungs were perfused with a solution containing PBS and heparin (50 U/ml; Sigma-Aldrich, St. Louis, MO) through the pulmonary artery and aseptically removed from the pulmonary cavity, placed in media and dissected. The dissected lung tissue was incubated with complete DMEM (cDMEM media) containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 μg/ml; Sigma-Aldrich) for 30 minutes at 37°C. The digested lungs were further disrupted by gently pushing the tissue through a cell strainer (BD Biosciences, Lincoln Park, NJ). Red blood cells were lysed with ACK

buffer, washed and resuspended in cDMEM. Total cell numbers per lung were determined using a haemocytometer.

Cells suspension from each individual mouse was incubated with MAbs labelled with Fluorescein isothiocyanate [FITC], Phycoerythrin [PE], Peridinin chlorophyll-a protein [PerCP], or Allophycocyanin [APC] at 4°C for 30 minutes in the dark. After washing the cells with deficient RPMI (dRPMI; Irvine Scientific, Santa Ana, CA) containing 0.1% sodium azide (Sigma-Aldrich) the cells were incubated with MAbs. MAbs against CD4+ (clone RM4-5, rat IgG2a,k), NK-1.1 (clone PK136, ms $IgG_{2a,k}$), CD8 (clone 53-6.7, rat $IgG_{2a,k}$), CD3 (clone 145-2C11, Ar Ham $IgG_{1,k}$), γδ TCR+ (Clone GL#, Ar Ham $IgG_{2,k}$), CD62L (L-selectin, clone MEL-14, rat $IgG_{2a,k}$) , CD44 (clone IM7, rat $IgG_{2b,k}$) markers and rat IgG_{2a}, rat IgG_{2b}, rat IgG1, mouse IgG_{2a}, and hamster IgG were used in this study. These MAbs were purchased from BD PharMingen (San Diego, CA), Serotec Inc (Releigh, NC) or eBioscience (San Diego, CA) as direct conjugates to FITC, PE, PerCP, PerCP-Cy5.5 or APC. Data acquisition and analysis were done using a FACscalibur (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences, San Jose, CA) respectively. Compensation of the spectral overlap for each fluorochrome was done using CD4 antigen from cells gated in the FSC^{low} / SSC^{low} region. T cells were gated according to their sizes and granularity defined in the FSC and SSC plot. All analyses were performed with an acquisition of at least 100,000 total events.

Intracellular cytokine staining

Cells were first stained for cell surface markers as indicated above and thereafter the same cells suspensions were prepared for intracellular staining. Cell membranes were permeabilized according to the kit instructions (Fix/Perm kit, BD PharMingen, San Diego, CA). Antibodies against IFN-γ (XMG1.2, rat igG1, eBiosciences, San Diego, Ca), and Granzyme B (clone GB12, ms IgG1, Caltag Laboratories) were incubated with the appropriate surface-stained cells for 30 minutes, and the cells were washed twice and resuspended in dRPMI prior to analysis.

Statistical analysis

Statistical significance was determined using two-way ANOVA with Bonferroni post tests using GraphPad Prism v4.00 for Windows, (GraphPad Software, San Diego CA).

RESULTS

Impact of chemotherapy and post-primary tuberculosis on bacterial loads

Chemotherapy was started on day 30 post-primary challenge and lasted a duration of 3 months, animals were rested for 2 weeks prior to a secondary challenge (Figure 1 A, B). After chemotherapy *M. tuberculosis* bacilli in the lungs was undetectable, compared to untreated mice. Mice infected and treated with chemotherapy were further treated with the immunosuppressive steroid, dexamethasone to determine the amount of latent persistent bacilli, which was Log10 of 2.68 colony forming units. Individual groups were evaluated for bacterial loads and animal survival (Figure 1 A, B).

After a secondary 2 challenge, 30 days later bacterial loads began to rise, peaking at 190 days post-primary infection. Mice receiving a primary challenge and chemotherapy resulted in bacterial loads significantly reduced when compared to mice receiving a secondary 2 challenge. Animals receiving a primary challenge and then a subsequent second challenge resulted in 100 percent mortality 225 days after primary infection (Figure 1B). In addition, 75 percent of animals receiving a primary challenge followed by chemotherapy and a secondary challenge died 225 days after primary infection (Figure 1B). Interestingly, mice receiving a primary challenge followed by chemotherapy and a secondary challenge at 190 days showed bacterial loads equal to that of a single challenge.

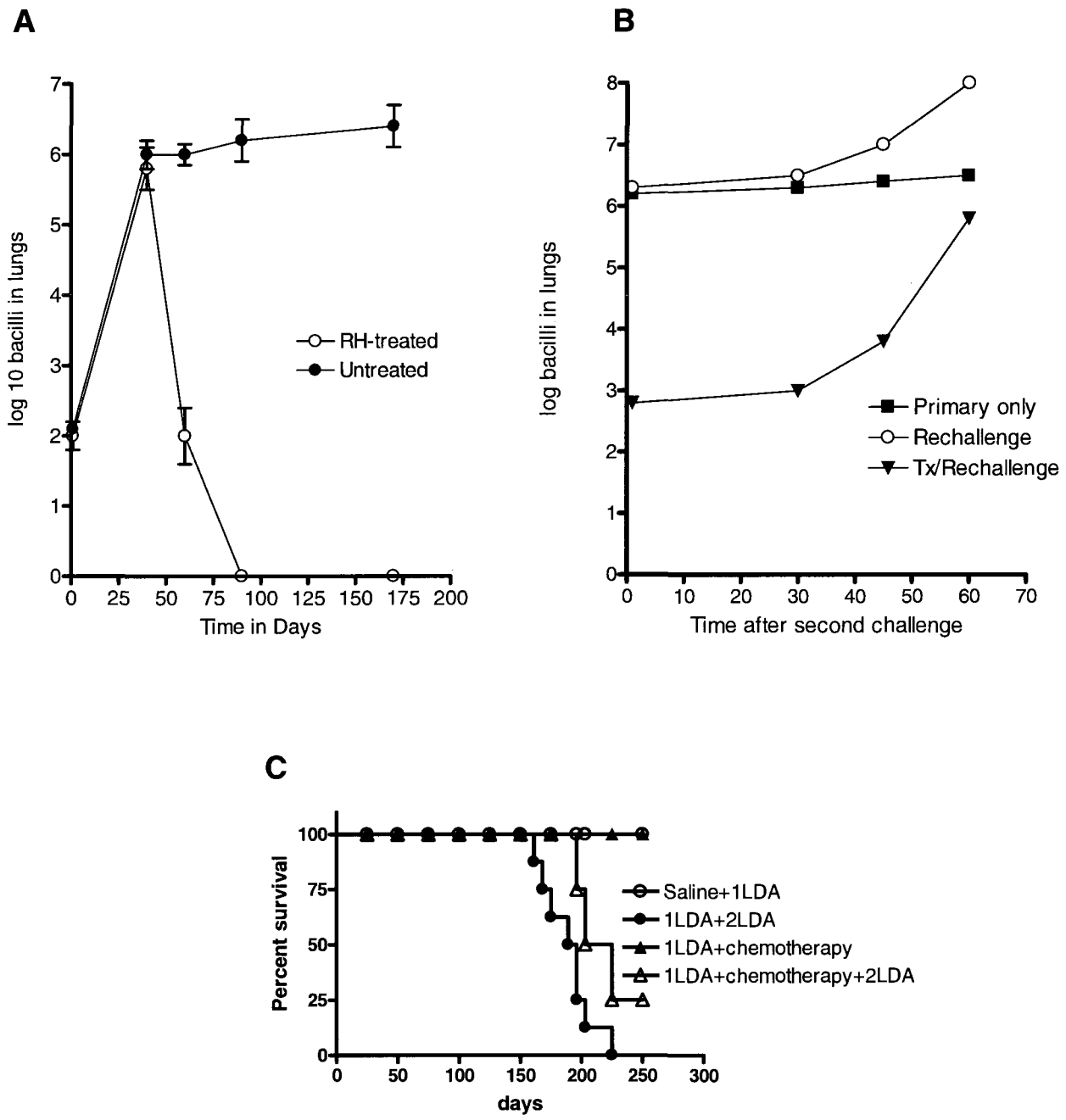

Figure 1. Impact of chemotherapy on bacterial loads and animal survival in post-primary tuberculosis. Panel A shows bacterial loads in groups of mice which were infected with a primary challenge (closed circle) and then 30 days later chemotherapy [INH, RIF] was started and lasted a duration of 3 months (open circle). Panel B shows animals that were submitted to a secondary challenge after a primary challenge (open circle), animals that had a primary challenge were treated with chemotherapy rested for 2

weeks prior to secondary *M. tuberculosis* challenge (closed triangle), and animals that had only the first challenge (closed squares). Panel C shows the percentage survival of mice infected with a primary challenge (open circle) and followed by a secondary challenge (closed circle) and animals infected with a primary challenge infection and then 30 days later chemotherapy [INH, RIF] was started and lasted a duration of 3 months (closed triangle), animals rested for 2 weeks prior to secondary *M. tuberculosis* challenge (open triangle). Results are expressed as the average Log_{10} cfu of 5 mice (± SE). Student t-test test, A, **p=0.03, *p=0.01; C, **p=0.03, *p=0.02.

Immunopathology of post-primary tuberculosis.

Lung pathology from lung sections was evaluated at various timepoints after primary and secondary challenge. Sections were stained for hematoxylin and eosin and for acid fast bacilli (AFB) staining (Figure 2 A) as previously described (13). Primary infection with one challenge of *M. tuberculosis* (Figure 2 A, A-D) depicts well organized lung granulomas from C56BL/6 mice consisting of dendritic cells, macrophages and lymphocytes developed through a total of 190 days post-infection. It is evident in these lung sections that during late chronic infection the structure of the granuloma consists of a smaller lymphocytic center with foamy cells surrounding the center, with undetectable numbers of bacilli (Figure 2B, A) compared to those animals receiving a secondary challenge which show large numbers of acid fast staining bacilli (Figure 2B, B).

Animals receiving a primary challenge and then a secondary challenge showed worsened lung pathology at 30 days post secondary challenge (Figure 2A, E-G) evident by increased thickening of the parenchyma wall, increased lymphocytic influx and reduce air space. In addition during late chronic infection the structure of the granuloma consisted of a larger lymphocytic center with increased numbers of foamy cells surrounding the center, with large numbers of bacilli (Figure 2A, F). Furthermore during late chronic infection lung pathology consisted of multiple coalescing granulomas with multiple centers and severally compromised airways (Figure 2A, G). Eventually animals receiving a secondary challenge demonstrated reduced survival at 30 days post secondary challenge.

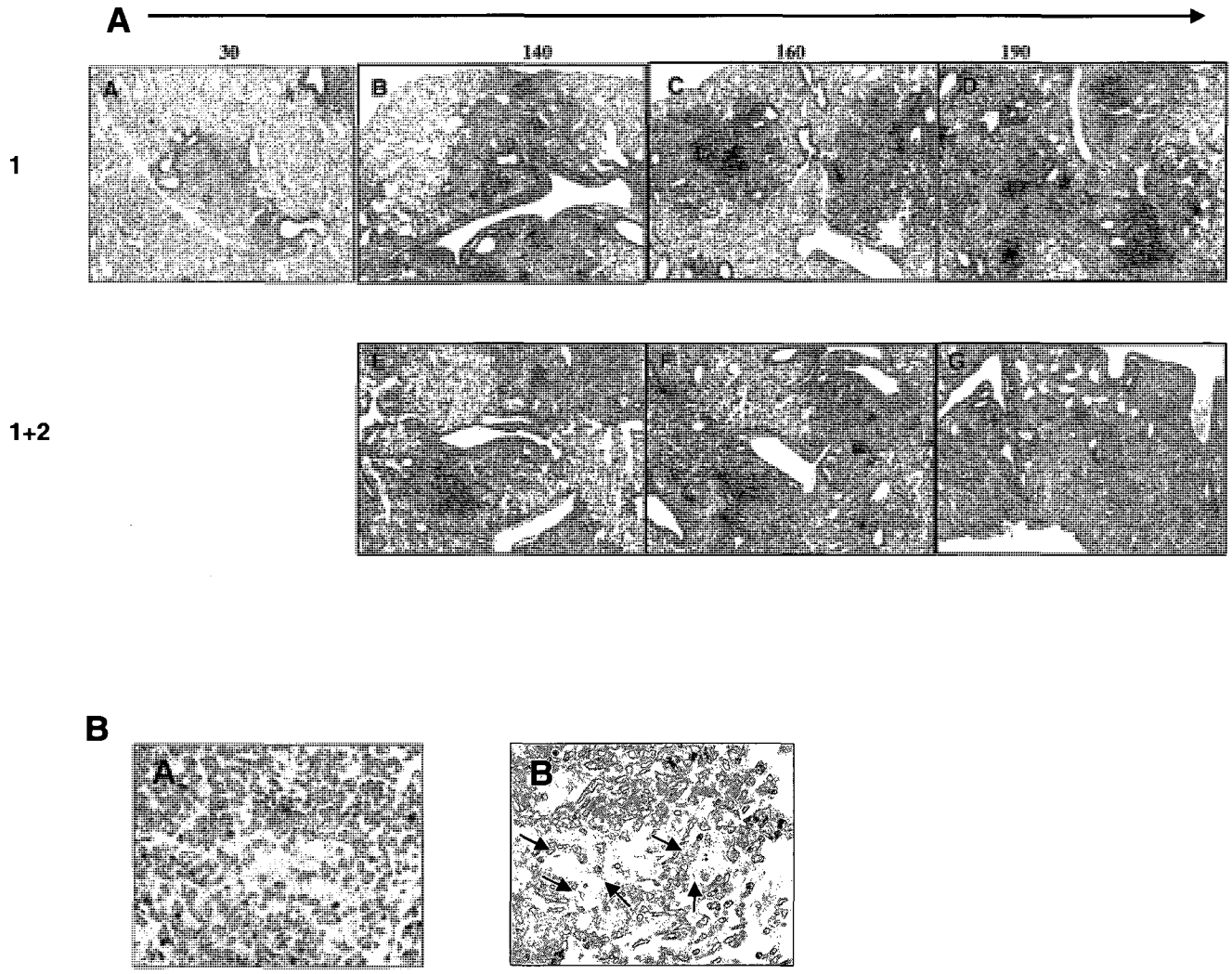

Figure 2. Lung pathology in post-primary tuberculosis.

Sections from paraffin embedded lungs from mice receiving primary *M. tuberculosis* challenge and a secondary challenge with *M. tuberculosis* were stained with haematoxylin and eosin (H&E) and acid fast bacilli staining (AFB). Animals receiving a primary challenge showed organized lung lesions from C56BL/6 mice consisting of characteristic foamy macrophages surrounding the caseous center of the granuloma (A, A-D). Animals receiving a primary challenge and then a secondary challenge showed worsened lung pathology at 30 days post secondary challenge (A, E-G) evident by increased thickening of the parenchymal wall, increased lymphocytic influx and reduce air space. In post-primary tuberculosis, during late chronic infection, the structure of the

granuloma consists of a larger caseous center with increased numbers of foamy cells surrounding the caseuos center, with large numbers of bacilli (B, B) compared to a primary challenge infection (B, A). A, Hematoxylin and eosin and B, acid fast bacilli. A, 10x total magnification. B, 100x total magnification.

Immunopathology in chemotherapy treated and untreated post-primary tuberculosis.

Lung pathology from lung sections at various timepoints after primary challenge, chemotherapy and secondary challenge were stained for hematoxylin and eosin and for acid fast bacilli (AFB) staining (Figure 3). Primary challenge infection and treatment with chemotherapy resulted in smaller and more compact lung granulomas from C56BL/6 mice consisting of both dendritic cells, macrophages devoid of bacilli and lymphocytes developed through a total of 190 days postinfection (Figure 3 A-D). Animals receiving a primary challenge, chemotherapy and then a secondary challenge showed worsened lung lesions (Figure3 E-H), with large numbers of foamy cells surrounding the caseuos center harboring bacilli, compared to those animals receiving a primary challenge. In addition, animals receiving a primary challenge, chemotherapy followed by a secondary challenge had reduced survival compared to animals vaccinated with BCG and receiving a primary and secondary challenge.

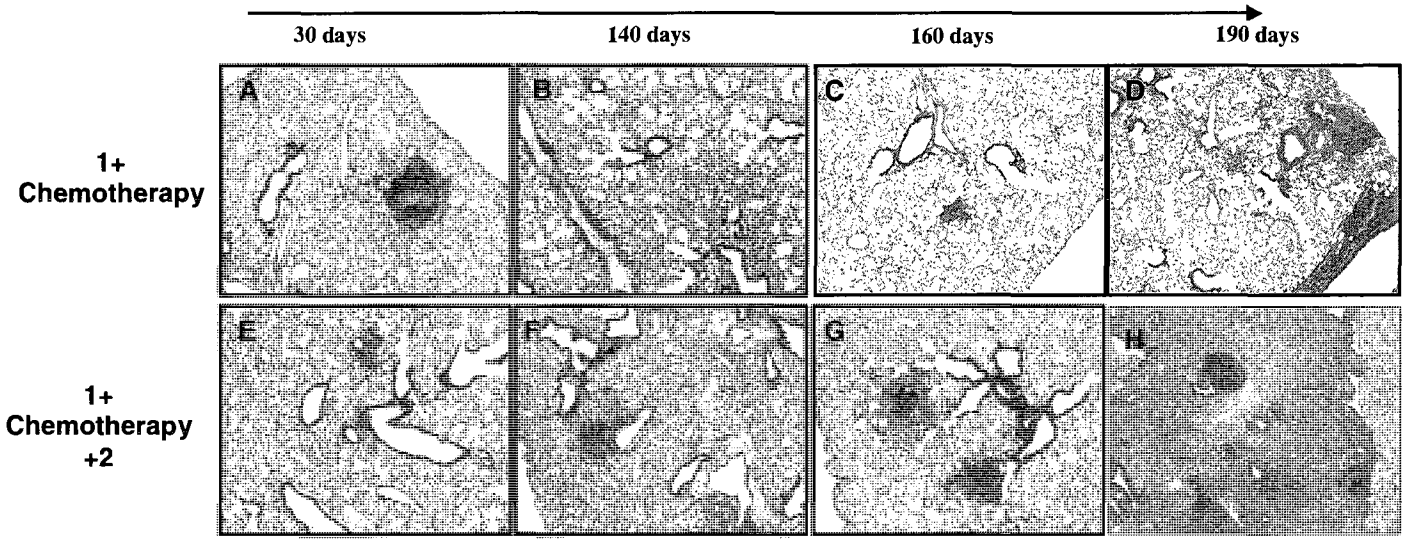

Figure 3. Impact of chemotherapeutic treatment on the lung pathology in post-primary tuberculosis. Sections from paraffin embedded lungs from mice receiving a primary *M. tuberculosis* challenge , and then 30 days later chemotherapy [INH, RIF] and a secondary challenge with *M. tuberculosis* were stained with haematoxylin and eosin (H&E). Panel A-D, shows mice infected with a 1 and then 30 days later treated with chemotherapy [INH, RIF] lung pathology shows minimal granulomas with small aggregates of lymphocytes with small number of foamy cells, reduced parenchymal wall thickening which all indicate better lung pathology compared to the animals with primary challenge, chemotherapeutically treated and a secondary challenge (E-H). Hematoxylin and eosin. A, 10x total magnification.

CD4+ memory and effector immune responses in chemotherapy and post-primary tuberculosis.

In order to follow the immune response during post-primary tuberculosis groups of mice were challenged by a primary challenge followed by a secondarychallenge, and at various timepoints the number of cells expressing CD4 memory markers defined as $CD44^{high}$, $CD62L^{high}$ and IFN-γ+ and effector markers defined as $CD44^{high}$, $CD62L^{low}$ and IFN-γ+ were monitored over the course of the infection. The progression of a murine primary challenge infection has been previously reported during early (16) and chronic *M. tuberculosis* infection (17) and confirms the present results of a predominate CD4+ effector immune response which increases during infection coincident with an increasing memory phenotype (Figure 4 A).

After 133 days of the primary challenge a second challenge was completed (Figure 4 C) and the CD4+ memory and effector cells were assayed. Interestingly, upon re-infection (Figure 4 C) I observed a significantly increased (Student t-test test, p=0.002) peak in the numbers of CD4+ memory cells compared to that present with a single challenge (Figure 4 A). More importantly, for 30 days after re-infection this memory phenotype predominated (Figure 4C) coincident with stable bacterial loads (Figure 1 A) compared to a single challenge infection. Notable, 30 days after re-infection (secondary challenge) Figure 4 C) CD4+ memory cell numbers decline as CD4+ effector cell numbers remained increased. A single challenge (Figure 4 A) results in increasing numbers of memory and effector cells (Figure 4 A) while 30 days after re-infection

(secondary challenge) the numbers of memory cells began to decline and at 60 days after re-infection effector cell numbers drop most likely due to a yet unknown immunosuppressive event.

As shown in Figure 4 B, the total number of effector CD4+ T cells in the lungs after primary infection challenge and chemotherapy gradually increased from day 130 until peaking around day 160 and thereafter plateau. The number of memory CD4+ cells coming into the lungs after primary challenge infection and chemotherapy gradually increase during infection although in reduced numbers compared to the effector cells.

Comparing the number of memory CD4+ T cells in the lung after chemotherapy and a secondary challenge (Figure 4 D) an increase in memory cells was evident which then declined after 30 days of infection, during a time in which effector immunity increased. However, after chemotherapy and a secondary challenge the effector cell numbers remain increased for 60 days coincident with bacterial control (Figure 1A) and thereafter decline as bacterial numbers increase and eventual death resulted.

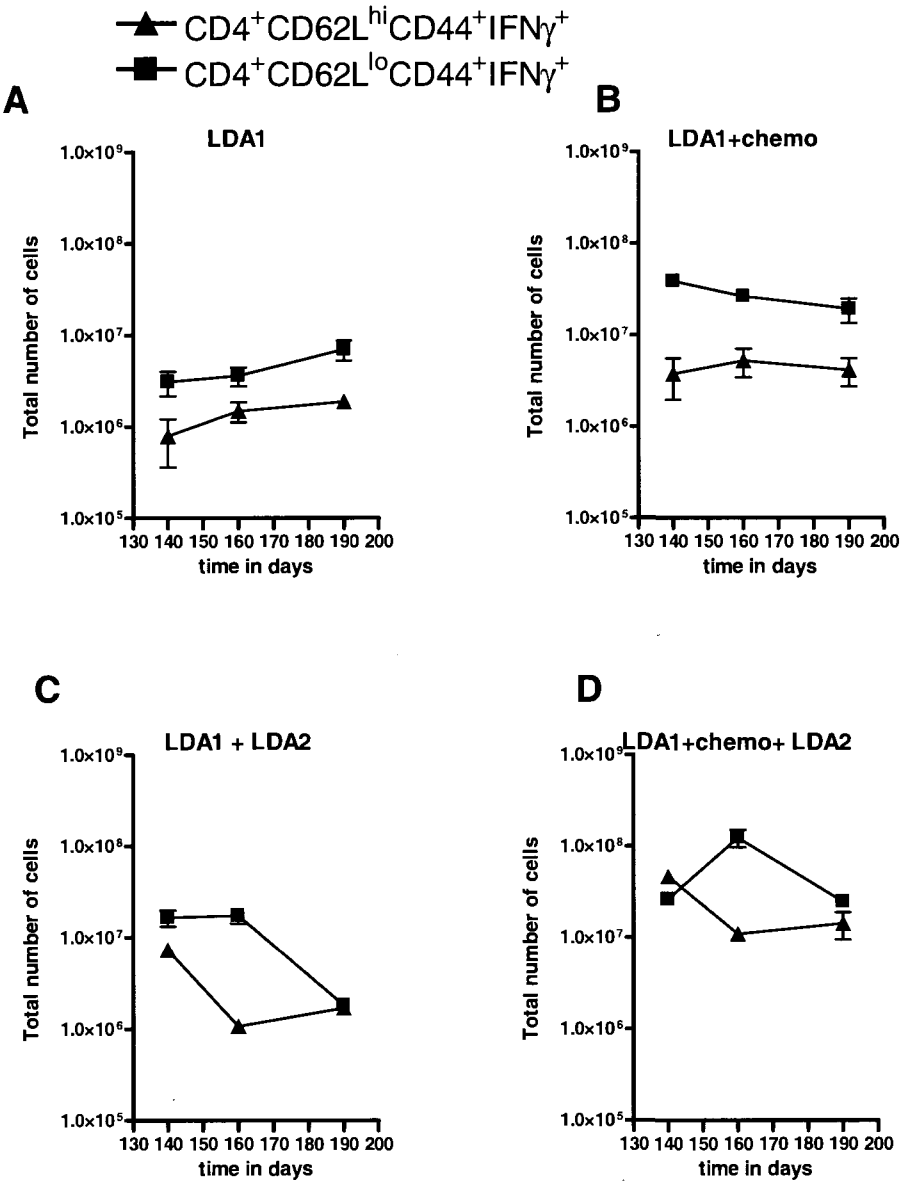
CD4+CD62Lhi CD44+IFNγ+
CD4+CD62Llo CD44+IFNγ+
A
LDA1
B
LDA1+chemo
C
LDA1 + LDA2
D
LDA1+chemo+ LDA2
Total number of cells
1.0x10^9
1.0x10^8
1.0x10^7
1.0x10^6
1.0x10^5
130 140 150 160 170 180 190 200
time in days

Figure 4. Impact of chemotherapy on CD4+ memory and effector cell in post-primary tuberculosis. Lung cells obtained from animals challenged with *M. tuberculosis* (A) and a subsequent challenge (C) and animals challenged with a primary challenge and then 30 days later chemotherapy [INH, RIF] was started and lasted a duration of 3 months (B) and then subsequent re-infection with *M.tuberculosis* (D) were assayed by flow cytometry at day 30, 140, 160 and 190 post secondary challenge for numbers of memory $CD4^{+}CD62L^{hi}CD44^{+}IFN\gamma^{+}$ (triangle) and effector $CD4^{+}CD62L^{hi}CD44^{+}IFN\gamma^{+}$ (square) cells. Results are expressed as the average (n=5) of the memory $CD4^{+}CD62L^{hi}CD44^{+}IFN\gamma^{+}$ and effector $CD4^{+}CD62L^{hi}CD44^{+}IFN\gamma^{+}$ cells in each group expressed as mean (± SE).

CD8+ memory and effector immune responses in chemotherapy and post-primary effector tuberculosis.

Mice were challenged by a primary infection followed by a secondary challenge, and at various timepoints the number of cells expressing CD8+ memory and effector cells were monitored over the course of the infection. The progression of primary infection (Figure 5 A) has been previously reported (16, 17) demonstrating a predominant CD8+ effector immunity which increases during infection coincident with an increasing memory phenotype.

The comparative analysis of CD8+ memory and effector cells from the lungs of animals receiving a primary challenge and re-infection (secondary challenge) (Figure 5C) showed the numbers of CD8+ memory and effector cells in the lungs after a secondary challenge is significantly increased (Students t test, $p=0.003$) and remained elevated for 30 days and thereafter declined coincident with increasing bacterial loads and subsequent death.

In order to follow the immune response during chemotherapy and post-primary tuberculosis groups of mice started chemotherapy on day 30 post-primary challenge which lasted a duration of 3 months and animals were rested for 2 weeks prior to secondary challenge and at various timepoints the number of cells expressing CD8+ memory and effector cell were monitored over the course of the infection.

As shown in Figure 5 B, the total number of effector and memory CD8+ cells in the lungs after primary infection and chemotherapy gradually increased peaking around day 140 and thereafter declining.

Comparing the number of CD8+ effector and memory cells in the lung after chemotherapy and a primary challenge compared to that with a primary and a subsequent second challenge we found an increase in CD8+ memory and effectors cells (Figure 5D). However, after a secondary challenge the effector cell numbers gradually began to decline during chronic infection.

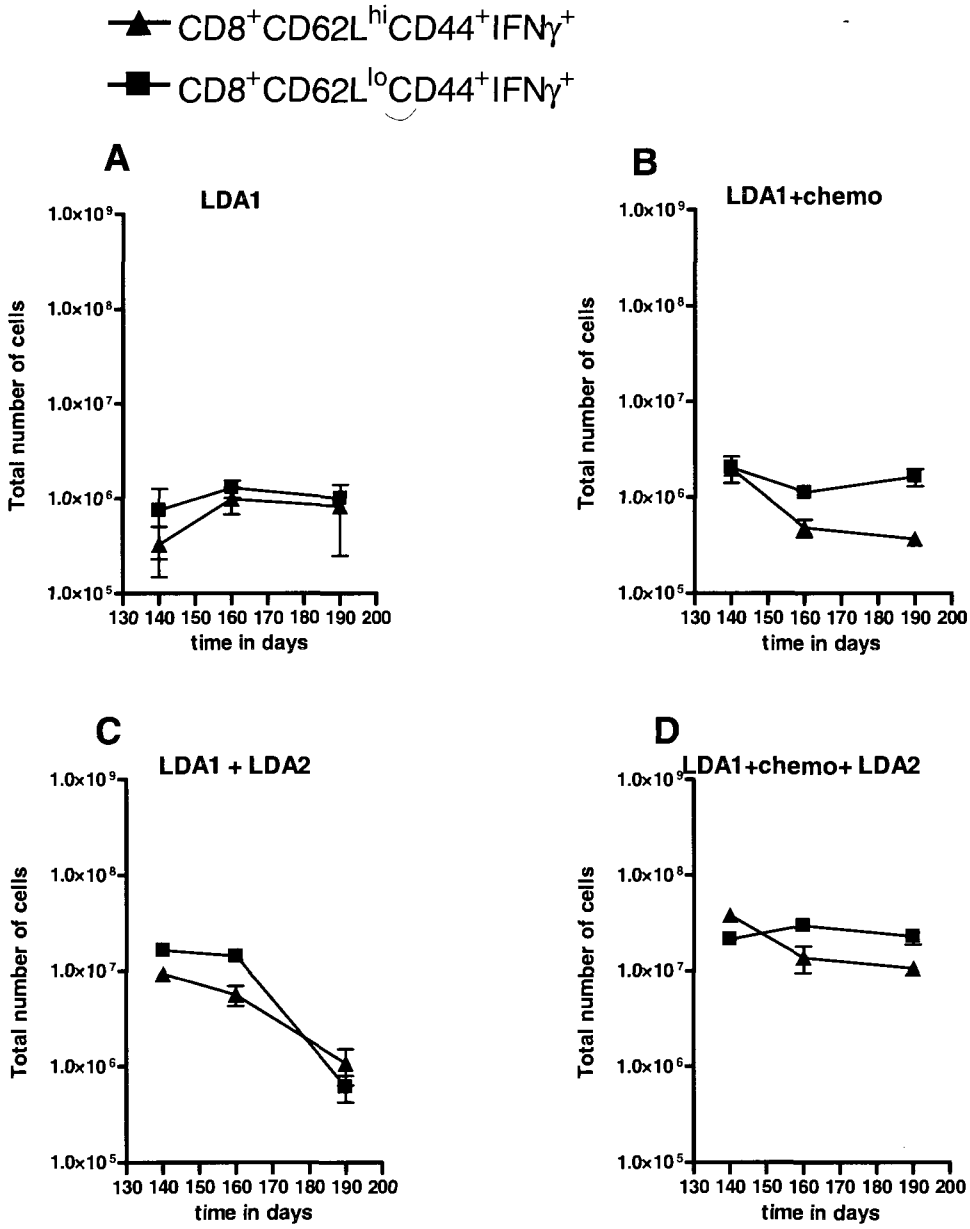
CD8+CD62Lhi CD44+IFNγ+
CD8+CD62Llo CD44+IFNγ+
A
LDA1
B
LDA1+chemo
C
LDA1 + LDA2
D
LDA1+chemo+ LDA2
Total number of cells
1.0x10^9
1.0x10^8
1.0x10^7
1.0x10^6
1.0x10^5
130 140 150 160 170 180 190 200
time in days

Figure 5. Impact of chemotherapy on CD8+ memory and effector cell in post-primary tuberculosis. Lung cells obtained from animals challenged with *M.tuberculosis* (A) and a subsequent infection (C) and animals challenged with a primary challenge and then 30 days later chemotherapy [INH, RIF] was started and lasted a duration of 3 months (B) and then subsequent re-infection with *M. tuberculosis* (D) were assayed by flow cytometry at day 30, 140, 160 and 190 post secondary challenge for numbers of memory $CD8^{+}CD62L^{hi}CD44^{+}IFN\gamma^{+}$ (open triangle) and effector $CD8^{+}CD62L^{hi}CD44^{+}IFN\gamma^{+}$ (open square) cells. Results are expressed as the average (n=5) of the memory $CD8^{+}CD62L^{hi}CD44^{+}IFN\gamma^{+}$ and effector $CD8^{+}CD62L^{hi}CD44^{+}IFN\gamma^{+}$ cells in each group expressed as mean (± SE).

Impact of chemotherapy on alveolar macrophage and dendritic cell numbers in post-primary tuberculosis.

In order to follow the immune response during primary and post-primary tuberculosis groups of mice were infected with a primary challenge followed by a subsequent challenge. Additional groups of mice received chemotherapy on day 30 post-primary challenge which lasted a duration of 3 months and animals were rested for 2 weeks prior to secondary challenge. These groups of mice were then assayed at various timepoints for the number of alveolar macrophages ($CD11b^{neg}/CD11c^{hi}$) and dendritic cells ($CD11b^{+}/CD11c^{hi}$) over the course of the infection. The progression of primary infection (Figure 6 A, B) and accumulation of macrophages and dendritic cells has been previously reported by the Orme laboratory (16-18) demonstrating predominant foamy cells expressing dendritic cell markers.

The comparative analysis of macrophages and dendritic cells from the lungs of animals receiving a primary challenge and a subsequent challenge (Figure 6 A, B) showed the numbers of macrophages and dendritic cells in the lungs after a secondary challenge significantly increases (Students t test, $p=0.004$) for 30 days and thereafter declined coincident with increasing bacterial loads and subsequent death.

Comparing the numbers of macrophages and dendritic cells in the lung after a single challenge and chemotherapy (Figure 6 A, B) we observed a dramatic increase in these cell numbers when compared to the groups of animals receiving a 1 and no chemotherapy or a 1 and a subsequent 2. Comparing the numbers of macrophages and

dendritic cells in the lung after a primary 1, chemotherapy and a secondary 2 challenge (Figure 6 A, B) we found the highest numbers of macrophages and dendritic cells in the lungs compared to all the other groups assayed.

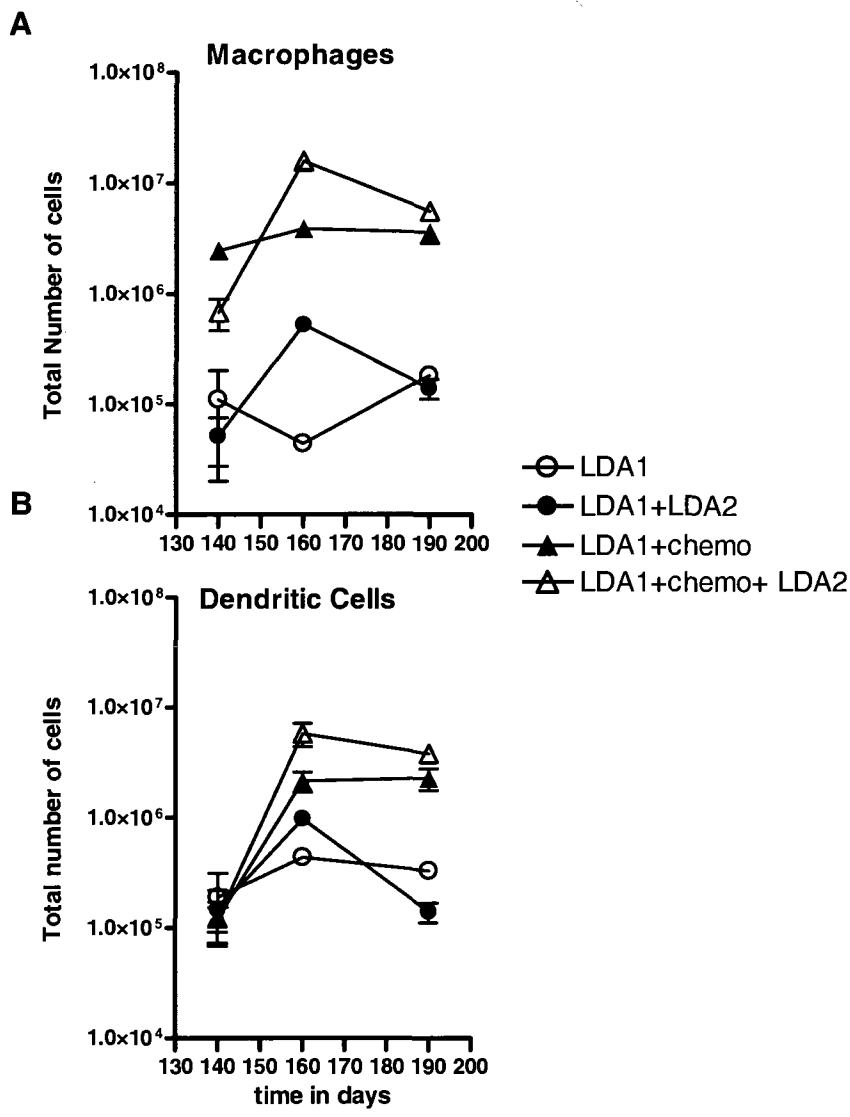

Figure 6. Impact of chemotherapy on dendritic and alveolar macrophage cell numbers in post-primary tuberculosis. Panel A and B show the dendritic and alveolar macrophage lung cells obtained from animals challenged with a 1 (open circle) and a subsequent 2 (closed circle) and animals challenged with a primary 1 and then 30 days later chemotherapy [INH, RIF] was started and lasted a duration of 3 months (closed

triangle) and then subsequent re-infection with 2 (open triangle). All cell numbers were assayed by flow cytometry at day 30, 140, 160 and 190 post secondary challenge for numbers of alveolar macrophages ($CD11b^{neg}/CD11c^{hi}$) and dendritic cells ($CD11b^{+}/CD11c^{hi}$). Results are expressed as the average (n=5) of the numbers of alveolar macrophages ($CD11b^{neg}/CD11c^{hi}$) and dendritic cells ($CD11b^{+}/CD11c^{hi}$) in each group expressed as mean (± SE).

DISCUSSION

In this study, the question of whether clearance of the bacilli from a primary aerosol infection with *M .tuberculosis* (1) by chemotherapy induces protective immunity against a subsequent aerosol infection with *M. tuberculosis* (2) in the murine model was addressed.

One major finding in this study was that in the mouse model a primary *M. tuberculosis* infection (1) does not protect against a secondary tuberculosis (2) infection. After challenge with a secondary 2, bacterial loads were contained for 30 days, however thereafter steadily increased into chronic disease. Animals with post-primary tuberculosis showed increased lung pathology and resulted in 100% mortality after 225 days compared to those animal only receiving a primary 1. Interestingly, upon re-infection with 2 animals with post-primary tuberculosis showed increased CD4+ and CD8+ memory and effector cells which declined after 30 days coincident with the increase in bacterial loads and eventual death compared to a primary 1 infection. These results suggest that long term protective pulmonary immunity is not achieved by a primary natural infection.

The loss of CD4+ and CD8+ memory and effector cells will require further investigation. However, the decline in CD4+ and CD8+ memory and effector cells appears to represent a true effect and not a dilution by other cell types entering the granulomas. The decline was rapid and may have resulted from cell necrosis or perhaps apoptosis (19) . Another possibility is that the T cells entering the lungs are either short-

lived or not replenished due to progressive destruction of peripheral lymphoid tissues in the chronic infection (20). One possibility to consider, based on the results shown in the next chapter, were a rapid fall-off in Th1 CD4 T cell activity in the lungs of mice infected with strain HN878 was observed, is the emergence of regulatory T cells (14).

Another important finding in this study was that clearance of the bacilli from a primary aerosol infection with *M. tuberculosis* by chemotherapy did not induce protective immunity against a subsequent aerosol infection with *M. tuberculosis*. The impact of chemotherapy after a secondary 2 challenge resulted in increased memory cell numbers which declined while CD4 effector cells increased mimicking the immune response seen in viral infections after the antigen has been cleared and re-infection occurs (21). However and contrary to what is seen in the viral model the decline in memory cells occurred 30 days after the second 2 coinciding with increased bacterial numbers and 75% mortality after 225 days.

Surprisingly, chemotherapy generated a robust influx of macrophages and dendritic cells to the lungs of treated animals. This cellular influx of cells to the lungs could occur as a response to the large quantity of antigen being released by rapidly dying bacilli, which could disrupt the initial immune granuloma restriction generated to control the bacilli. Paradoxical worsening of TB after initiation of anti-tuberculosis treatment has been reported in about 2-23% of treated patients without HIV infection (22). Paradoxical reactions, in humans, are though to be due to intensification of cell-mediated immune responses following anti-tuberculosis treatment. In patients with active tuberculosis,

proinflammatory and immunosuppressive immune mechanisms are present concomitantly, the balance of which might be altered during early stages of anti-tuberculosis treatment (23, 24). Our results agree with the theory of paradoxical reactions in humans as we saw increased influx of macrophages and dendritic cells as well as a modest increase in memory and effector CD4+ T cells, in mice treated with chemotherapy after primary aerosol infection.

It is necessary to underlie the importance of the results shown in this study, because very little is known about the immune mechanisms generated in response to anti-tuberculosis chemotherapy. Based in results of studies generated using viral infections as a model, it has been thought that anti-tuberculosis treatment could generate an immune memory population capable of protecting the host against a secondary infection, but according to the current results this does not seem to be the case.

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CHAPTER 5:

Regulatory T cell subsets in mice infected with *Mycobacterium tuberculosis*

The aims of this study were to:

1. Compare survival and lung bacterial loads in the lungs of C57BL/6 and IFN-αβ receptor KO mice infected with virulent strains of *M. tuberculosis*
2. Compare the immune cell response induced by the infection of mice with different strains of *M. tuberculosis*
3. Elucidate the immune mechanism causing the high virulence and pathology of mice infected with the strain HN878 when compared to mice infected with the strain H37Rv

INTRODUCTION

It is becoming apparent that a significant percentage of new clinical isolates of *Mycobacterium tuberculosis* are of high virulence (1, 2). As an example, initial studies by Manca *et al* demonstrated that the HN878 strain is of unusually high virulence, wherein mice failed to induce a strong TH1 T cell and cytokine response early in infection and died more rapidly in comparative survival studies (3, 4). This "hypervirulence" was hypothesized to be associated with induction of increased production of Type-I interferon (4), and further work has subsequently shown that HN878 expresses a TH2-cytokine

inducing biologically active phenolic glycolipid antigen in its cell wall, removal of which ablates its lethality (5).

The role of Type-I interferons in the protective immune response to *M. tuberculosis* infection is not well understood. More recent reports from Manca *et al* demonstrated that wildtype HN878-infected mice treated with anti-IFN-α/β antibody results in higher TH1 cytokines and increased survival of animals without concomitant differences in bacterial growth (3, 4). Taken together, these studies suggested that the increased Type-I interferons during infection with HN878 may be deleterious for survival of *M. tuberculosis*-infected mice which in turn might be associated with reduced TH1 immunity (3, 4).

Cell mediated immunity (CMI) is responsible for control of tuberculosis (6, 7). After pulmonary infection of the mouse with *M. tuberculosis*, alveolar macrophages and dendritic cells phagocytose the bacilli and dendritic cells carry antigens to draining lymph nodes where recognition by T cells generate CMI (6-9). These antigen-presenting cells (APCs) secrete IL-12 which is central to the generation of antigen-specific lymphocytes that are able to produce IFN-γ (10-13). Emergence of a population of protective CD4+ T cells that secrete cytokines in the lungs, results in local activation of macrophages and the recruitment of monocytes to initiate granuloma formation. Additional influx of monocytes, macrophages, B cells and neutrophils at the site of mycobacterial replication (14-17) helps contain multiplication and further dissemination of the bacteria to other cells.

Although there is an initial presence of a strong TH1 antigen specific response in mice, which leads to the control of *M. tuberculosis* growth, these responses may also increase inflammation and lung immunopathology. In the latter event immunosuppressive cytokines such as TGFβ, IL-4, IL-10 (9, 18, 19) which are generally considered to increase the susceptibility to intracellular pathogens, may be necessary with a bacterial strain of higher virulence to attenuate the potentially tissue-damaging inflammatory response (18-20).

An example, of reduction of inflammatory tissue damage is the development of a CD4 T regulatory population, T regulatory cells (Tregs). Tregs consist of a network of populations, sharing common features of being hyporesponsive to antigen stimulation and possessing immunosuppressive activities (21, 22). Natural Tregs originate in the thymus and acquired Tregs (also called antigen-induced or adaptive Tregs) are generated from mature T cells in the periphery. Tregs are characterized by the expression of CD25, they may also constitutively express other several activation markers, such as the glucocorticoid-induced tumor-necrosis factor (TNF) receptor-related protein (GITR), L-selectin (CD62 ligand (CD62L), CD223 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152). Recent studies have identified the transcription factor forkhead box P3 (FOXP3) as a more exclusive intracellular marker for the identification of Tregs.

In this study I looked at the colony forming units, pathology and immune responses generated by the infection with the highly virulent strain HN878, compared to H37Rv, CSU123, CSU93 and Erdman-KO1 *M. tuberculosis* strains. Initially I used C57BL/6 animals and IFN-αβ receptor knockout mice to determine the role played by the Type-I interferons in the protection or susceptibility to the infection. Finally, different

immune cells were evaluated, by flow cytometry, in an attempt to find which type of cells were important in the generation and the control of the pathology seen in mice infected with *M. tuberculosis* strain HN878.

MATERIALS AND METHODS

Mice

Specific-pathogen-free female C57BL/6 mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. Mice lacking the IFN-αβ receptor on a 129 background were bred in house at Colorado State University from breeders kindly provided by Dr. P. Marrack (National Jewish Medical and Research Center, Denver, CO). Mice were used at approximately 6 to 8 weeks of age. Mice were maintained in the biosafety level 3 biohazard facility at Colorado State University, and were given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments. The specific pathogen-free nature of the mouse colonies was demonstrated by testing sentinel animals. All experimental protocols were approved by the Animal Care and Use Committee of Colorado State University.

Aerosol infection with *M. tuberculosis.*

Mice were challenged by low-dose aerosol exposure with *M. tuberculosis* using a Glas-Col (Terre Haute, Inc.) aerosol generator calibrated to deliver 50-100 bacteria into the lungs. Information regarding preparation of bacterial stocks and growth characteristics of the various bacterial strains utilized were as described previously (1, 2).

Strain H37Rv was originally obtained from the Trudeau Institute collection, Saranac Lake NY; HN878 was kindly provided by Dr Barry Kreiswirth [PHRI, New Jersey]; CSU 123 was provided by Dr. Ray Cho [Soeul, South Korea]; CDC1551 [CSU 93] was provided by Dr Thomas Shinnick [CDC Atlanta]; and Erdman-KO1 was obtained from Mycos Research, Loveland CO with permission from the Aeras Foundation.

Bacterial counts in the lung (n=5) were determined by plating serial dilutions of organ homogenates on nutrient 7H11 agar and counting colony-forming units after 3 weeks incubation at 37°C. Lungs from mice (n=5) in the same groups were harvested for histological analysis, and lung cell suspensions were used for flow cytometric analysis at indicated time points. In survival studies animals showing substantial weight loss with no evidence of weight rebound were euthanized. The results shown in the survival studies are based upon 10 mice per group.

Histological analysis

The accessory lung lobe from each mouse was fixed with 10% formalin in phosphate buffered saline (PBS). Sections from these murine tissues were stained using haematoxylin and eosin, and by acid-fast staining to detect mycobacteria.

Lung cell digestion

To prepare single cell suspensions the lungs were perfused with a solution containing PBS and heparin (50 U/ml; Sigma-Aldrich, St. Louis, MO) through the

pulmonary artery and aseptically removed from the pulmonary cavity, placed in media and dissected. The dissected lung tissue was incubated with complete DMEM (cDMEM media) containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 μg/ml; Sigma-Aldrich) for 30 minutes at 37°C. The digested lungs were further disrupted by gently pushing the tissue through a cell strainer (BD Biosciences, Lincoln Park, NJ). Red blood cells were lysed with ACK buffer, washed and resuspended in cDMEM. Total cell numbers per lung were determined using a haemocytometer.

Flow Cytometry for surface markers and intracellular cytokines

Cells suspensions from each individual mouse were incubated with mAbs labeled with Fluorescein isothiocyanate [FITC], Phycoerythrin [PE], Peridinin chlorophyll-a protein [PerCP], or Allophycocyanin [APC] at 4°C for 30 minutes in the dark. After washing the cells with deficient RPMI (dRPMI; Irvine Scientific, Santa Ana, CA) containing 0.1% sodium azide (Sigma-Aldrich) the cells were incubated with mAbs. mAbs against mAbs against CD4+ (clone RM4-5, rat IgG2a,k), NK-1.1 (clone PK136, ms $IgG_{2a,k}$), CD8 (clone 53-6.7, rat $IgG_{2a,k}$), CD3 (clone 145-2C11, Ar Ham $IgG_{1,k}$), CD11c (clone HL3, hamster IgG1), CD11b (Mac-1, clone M1/70, rat IgG2a), Ly-6C/Gr1 (RB6-8C5, rat IgG_{2b}), CD45R/B220 (RA3-6B2, rat $IgG_{2a,k}$), CD25 (3C7,Rat TgM), CD223 (C9B7W, Rat TgG1), GITR (DTA-1, Rat TgG2b), markers and rat IgG2a, rat IgG2b, rat IgG1, mouse IgG2a, and hamster IgG were used in this study. These mAbs were purchased from BD PharMingen (San Diego, CA), Serotec Inc (Raleigh, NC) or eBioscience (San Diego, CA) as direct conjugates to FITC, PE, PerCP, PerCP-Cy5.5,

pacific blue, pacific orange or APC. Data acquisition and analysis were done using a FACscalibur (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences, San Jose, CA) respectively or samples were examined with a LSRII flow cytometer (BD Biosciences, Mountain View, CA), and data were analyzed using the FACSDiva software (BD Biosciences) . Compensation of the spectral overlap for each fluorochrome was done using CD4 or CD11b antigens from cells gated in the FSC^{low} verses SSC^{low} or $FSC^{mid/high}$ verses $SSC^{mid/high}$ region respectively. Analyses were performed with an acquisition of at least T cells 100,000 or 500,000 total events and a minimum of 10,000 CD11c positive events.

Intracytoplasmic cytokine staining

Cells were first stained for cell surface markers as indicated above and thereafter the same cells suspensions were prepared for intracellular staining. Staining for markers of the Foxp3, IFN-γ, IL-10, granzyme B family was performed using an intracellular staining technique. Cell membranes were permeabilized according to the kit instructions (Fix/Perm kit, BD PharMingen, San Diego, CA). Antibodies against Foxp3 (FJK-16s, Rat IgG2a), IFN-γ (XMG1.2, rat IgG1), eBiosciences, San Diego, CA), IL-10 (JES5-16E3, Rat TgG2b) were incubated with the appropriate surface-stained cells for 30 minutes, and the cells were washed twice and resuspended in dRPMI prior to analysis.

Inflammatory cytokine measurement

A Cytometric Bead Array kit (CBA; BD Biosciences, San Jose, CA) was used to measure inflammatory cytokines of lung cell suspensions incubated for 72 hours at 37°C

with CFP at 2 μg/ml and then frozen at -80°C. After thawing the supernatants, the CBA mouse inflammatory cytokine assay procedure was performed according to kit instructions. Assays were completed with duplicate samples and results are expressed as a mean of 2 experiments. Values are represented by the mean cytokine pg/ml minus the non-infected media control. The beads were analyzed on a Becton Dickinson FACscalibur flow cytometer.

Statistical analysis

Data is presented using the mean values (n=5) using replicated samples and duplicate or triplicate assays. The parametric Student t-test was used to assess statistical significance between groups of data.

RESULTS

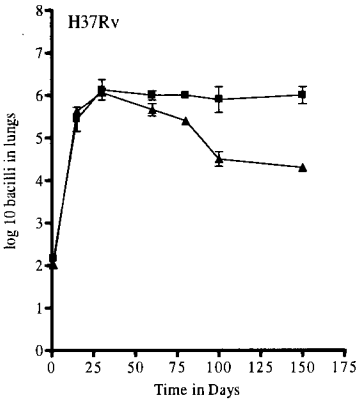
Comparison of survival and lung bacterial loads in the lungs of C57BL6 and IFN-αβ receptor KO mice infected with virulent strains of *M. tuberculosis.*

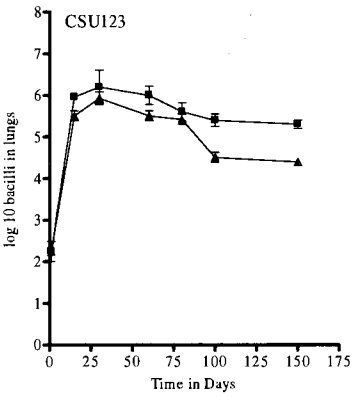
In a first study we compared the growth of each of the five test strains in the lungs of mice exposed to low dose aerosol infection (Fig.1A). In the cases of H37Rv, CSU123, and Erdman-K01, all showed evidence of slowing when reaching approximately one million bacteria in the lungs, whereas levels of CSU93 continued to rise, peaking at 10^7 after day 50. In contrast, HN878 grew faster than the others, reaching a lung bacterial load of 10^7 by as early as day 15 before showing evidence of containment.

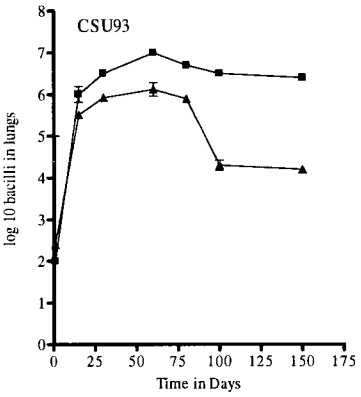
A second study comparing the survival times of mice infected with the panel of laboratory and clinical strains were consistent with the above data and confirmed earlier reports (4, 5) illustrating the much higher virulence of the HN878 strain (Fig.1B).

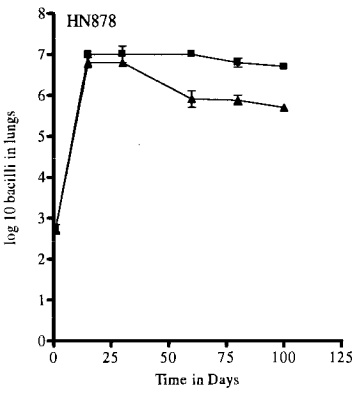
These studies also addressed the previous hypothesis (4) that this virulence reflected the production of type-I interferon by comparing the growth of HN878 in control and IFN-αβ receptor gene disrupted [KO] mice. We found this to be the case, with better control of the bacterial load in mice unable to make type-I interferon (Fig.1A). However, this was not restricted to HN878, but was a common observation with all five strains tested. Moreover, whereas an earlier report (4) implied that these cytokines had an early influence, the data obtained from the IFN-αβ-KO mice showed that the inhibitory effects of these cytokines was predominantly expressed during the chronic stage of the disease process.

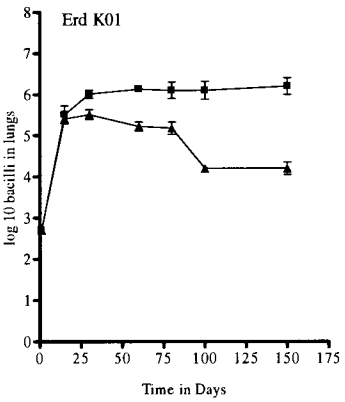
A


H37Rv
log 10 bacilli in lungs
Time in Days


CSU123
log 10 bacilli in lungs
Time in Days


CSU93
log 10 bacilli in lungs
Time in Days


HN878
log 10 bacilli in lungs
Time in Days


Erd K01
log 10 bacilli in lungs
Time in Days

B

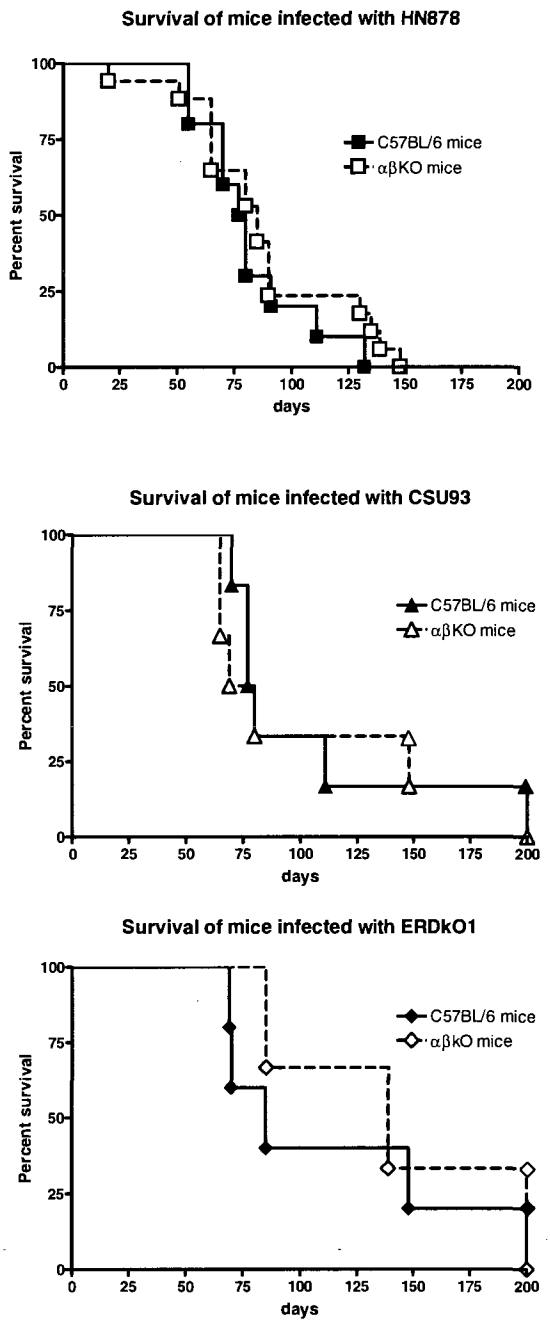


Figure 1. Decreased bacterial growth during chronic infection in the lungs of IFN-αβ receptor KO mice. Bacterial counts in the lungs (A) in C57BL6 (solid squares) and IFN-αβ receptor KO (solid triangles) mice infected with a low dose of *M. tuberculosis* strain H37Rv, CSU 123, CSU 93, HN878 or ERD-KO1. Results are expressed as the mean of the bacterial load in each group expressed as

Log_{10} CFU (± SEM, n=5). Panel B shows the survival of C57BL6 (solid symbols) and IFN-αβ receptor KO (open symbols) mice infected with *M. tuberculosis* CSU 93, HN878 and ERD-KO1. Survival results are from 10 mice per group.

Differences in the granulomatous response in the lungs.

We then examined the lung pathology in the HN878 mice and compared this to the other infections. Lesions were ranked in order of severity from 1 to 5 and scored for granuloma formation, alveolitis, perivasculitis, and peribroncholitis. This was added for a total score. As can be seen in Fig.2 the rapid growth of HN878 was associated with substantial lung damage, whereas the pathology scores rose much more slowly in the other infections. Interestingly, lung damage in the HN878 infected mice clearly slowed after day 30.

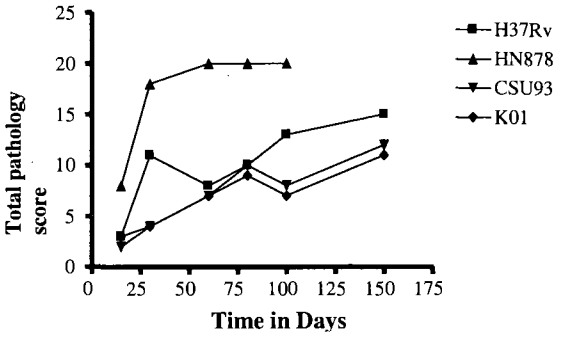


Figure 2. Increased total pathology scores in C57BL/6 mice infected with *M. tuberculosis* strain HN878. Sections of formalin-fixed and paraffin embedded lung tissue from mice infected with *M. tuberculosis* strains H37Rv (square), HN878 (triangle), CSU 93 (down triangle) and ERD-KO1 (triangle) were stained with haematoxylin and eosin (H&E) and then added to give the total lesion score. Lesions were ranked in order of severity from 1 to 5 and scored for granuloma formation, alveolitis, perivasculitis, and peribroncholitis and the total scores calculated.

This is also illustrated in Fig.3, which shows the appearance of the lung tissues in mice infected with HN878 compared to animals infected with H37Rv or CSU93. Examination of the lungs of C57BL/6 mice 15 days after being given a low-dose aerosol infection showed small aggregates of cells (Fig.3A). By day 30 moderately sized lesions were present which consisted predominantly of macrophages and large aggregates of lymphocytes were seen within the epithelioid macrophage fields. The development of organized multifocal granulomas containing lymphocytes and macrophages progressed through day 100 (Fig.3C). Infections with CSU123 and Erdman-K01 showed similar pathology (data not shown).

The development of pathology in the lungs of mice given CSU93 was similar but more exaggerated. During early infection lesions tended to be larger than those seen with H37Rv and again consisted of aggregates of lymphocytes and some macrophages (Fig.3D). Evidence for worsened lung pathology was apparent by day 30 (Fig.3E) characterized by increased numbers of lymphocytes and highly vacuolated cells infiltrating into the lung tissues resulting in large lesions. By day 100 however, the frequency of lesions and lung consolidation had appeared to stabilize (Fig.3F).

Marked differences in pathology were seen in mice infected with *M. tuberculosis* HN878. Early in infection moderately sized lesions were already developing, denoted by increased lymphocytic and phagocytic cellular infiltration, and thickening of the parenchymal walls indicative of inflammation in excess to the other strains tested (Fig.3G). These lesions continued to increase in size as the infection progressed (Fig.3H),

characterized by coalescing inflammatory granulomas that were larger than those seen in the other groups. Most lesions contained large numbers of highly vacuolated "foamy" macrophages containing large numbers of acid fast bacilli. By day 100 most of the lung tissues were consolidated, with some lesions showing evidence of necrosis (Fig.3I).

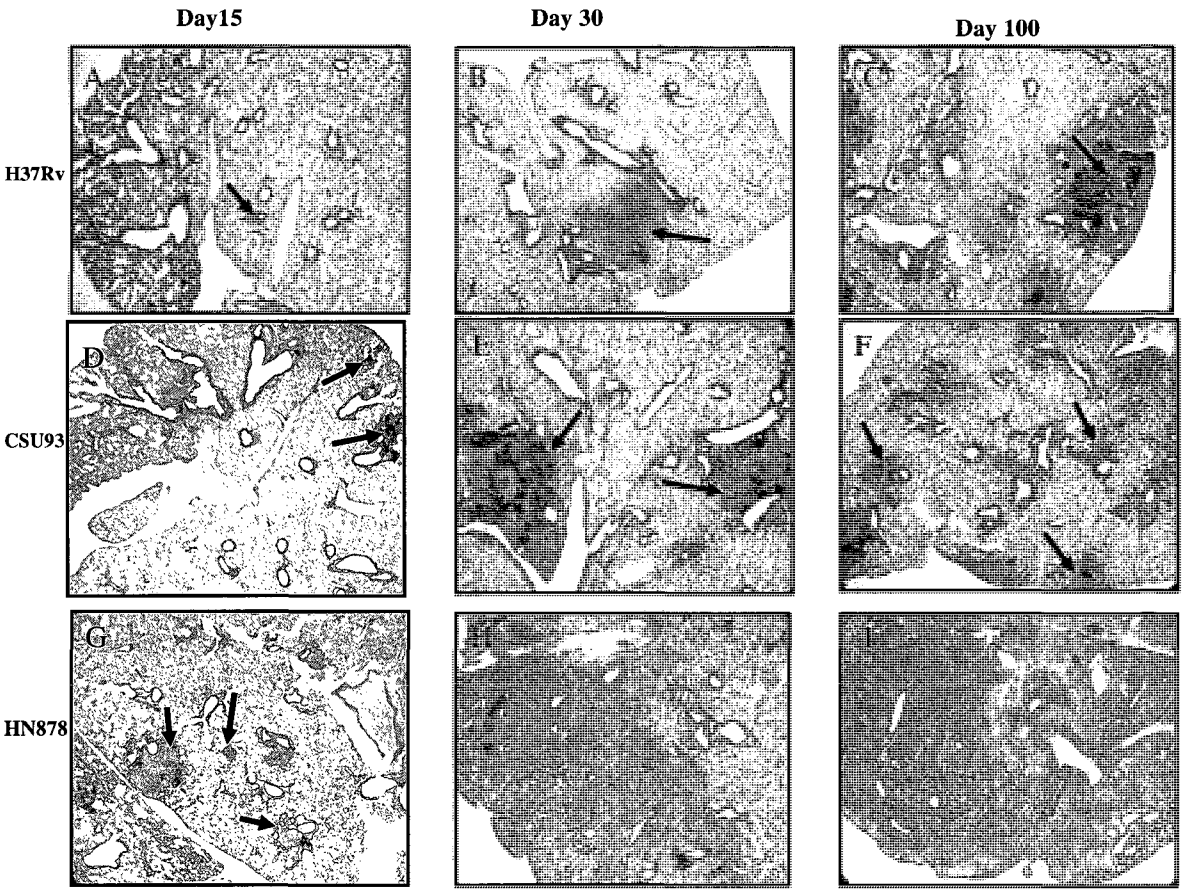


Figure 3. Increased lung pathology in C57BL/6 mice infected with *M. tuberculosis* strains HN878. Sections of formalin-fixed and paraffin embedded lung tissue from mice infected with *M. tuberculosis* strains H37Rv (A, B, C), CSU 93 (D, E, F) and HN878 (G, H, I) on days 15, 30 and 100 post *M. tuberculosis* challenge were compared. Lesions (arrows) were more numerous and larger in *M. tuberculosis* HN878 (G, H and I) infected mice than in *M. tuberculosis* H37Rv (A, B and C) eventually resulting in complete lung consolidation (I). *M. tuberculosis* CSU 93 (D, E and F) induced smaller lesions in terms of size compared to *M. tuberculosis* H37Rv infection but increased numbers. Total magnification A-I=10x.

Differences in the influx of $CD4^+$ and $CD8^+$ T cells in the lungs of infected mice.

Given the clear differences seen in cell influx indicated by the histological analysis, we conducted a comparative flow cytometric analysis of $CD4^+$ and $CD8^+$ T cell populations in lung cell digests from each group of infected mice. T cells were gated with a primary gate on viable FSC^{low} versus SSC^{low} lymphocytes and then on $CD3^+$ T cells, and analyzed for changes in the total percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells over the course of infection.

These results are shown in Fig.4. In mice infected with HN878, there were rapid influxes of both CD4 and CD8 cells (Fig.4 A, B), reaching higher levels on day 15 of the infection compared to the other four strains. This stabilized by day 30 and then declined at a similar rate in all groups. These observations were consistent with histological data from day 15, in which much larger lymphocytic lesions were observed in the animals infected with HN878).

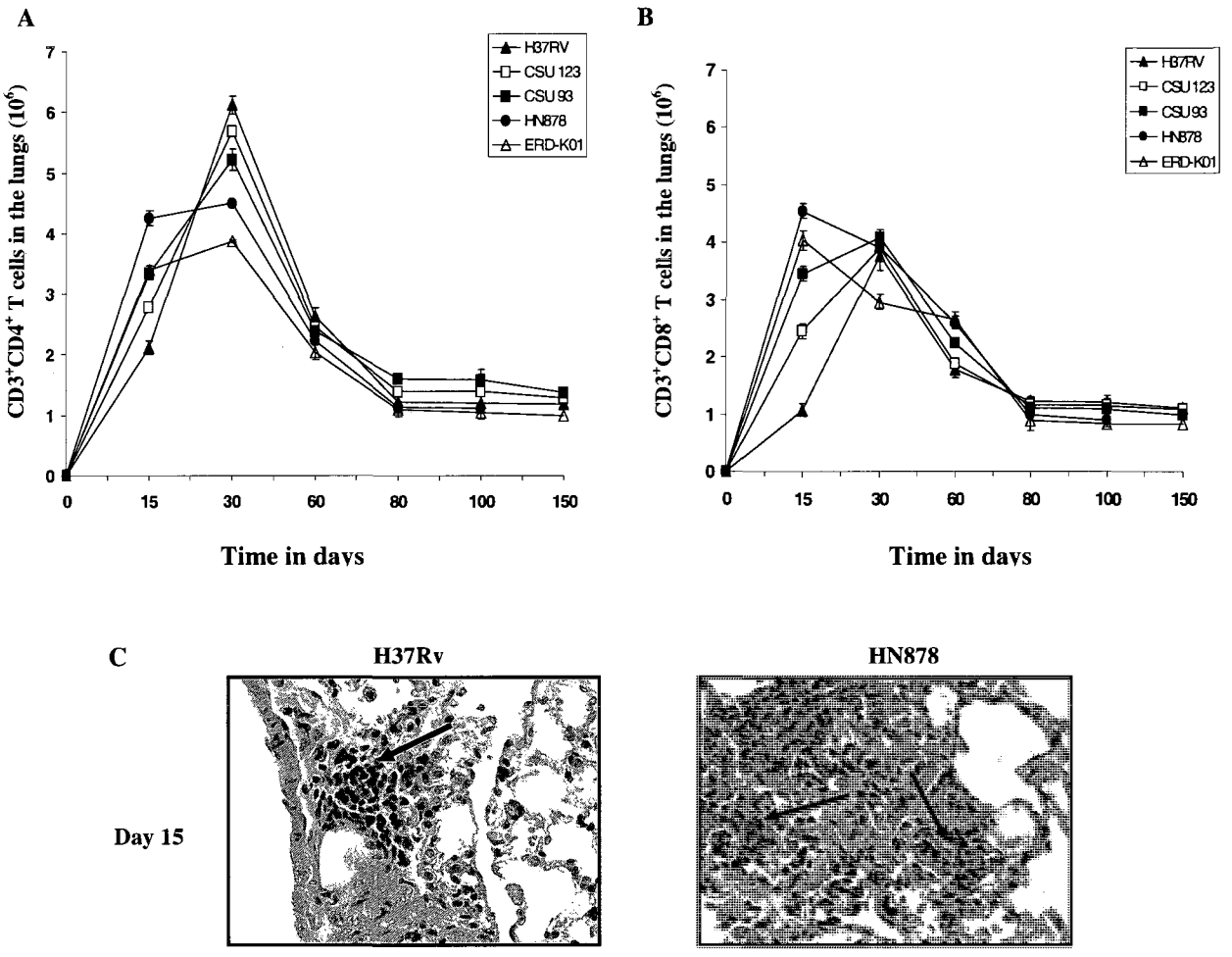


Figure 4. Early infiltration of $CD4^+$ and $CD8^+$ T cells in the lungs of mice infected with *M. tuberculosis* strains HN878. Lung cells obtained from C57BL/6 mice infected with a low dose of *M. tuberculosis* strains H37Rv (solid triangle), CSU 123 (open triangle), CSU 93 (solid square), HN878 (solid circle) and ERD-KO1 (open triangle) were assayed by flow cytometry. Panel A shows the total number of cells expressing $CD3^+CD4^+$ surface markers and panel B shows $CD3^+CD8^+$ surface markers in the lungs of mice infected with the various strains of *M. tuberculosis*. Results are expressed as the average number of (± SEM, n=5) $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells in the lungs. As shown in panel C, mice infected with *M. tuberculosis* HN878 demonstrated a substantial increase in lymphocytes accumulating in lesions (right panel, arrows) compared with *M. tuberculosis* H37Rv infection (arrow, left panel).

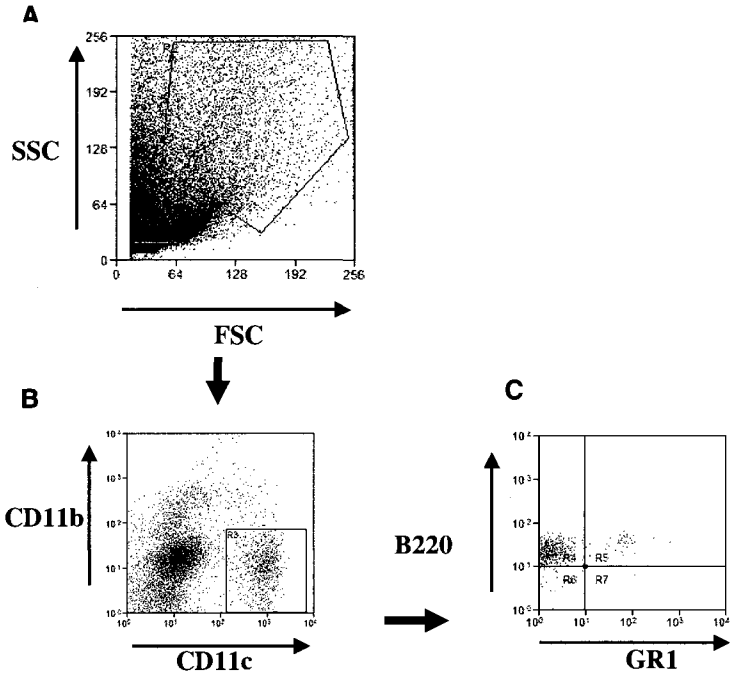
Differences in the influx of dendritic and plasmacytoid dendritic cells in the lungs of infected mice.

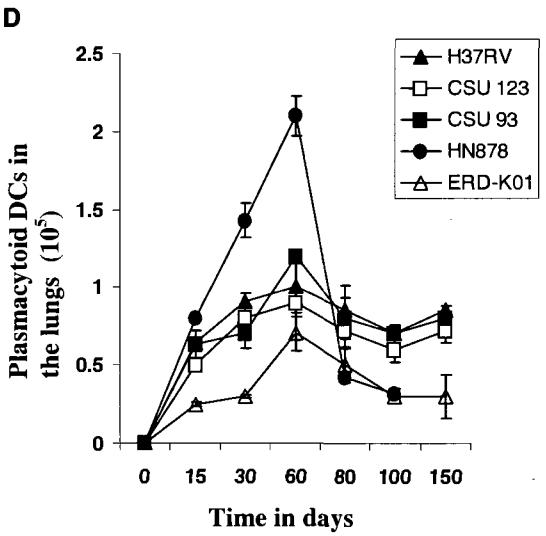
Plasmacytoid dendritic cells (pDCs) and dendritic cells (DCs) are major producers of Type-I interferons which are involved in inflammation, immunoregulation and T-cell responses during bacterial and viral infections (43-45). Given earlier reports implicating increased Type-I IFN in mice infected with *M. tuberculosis* HN878 we decided to monitor the numbers of pDCs and DCs infiltrating the lungs over the course of the disease.

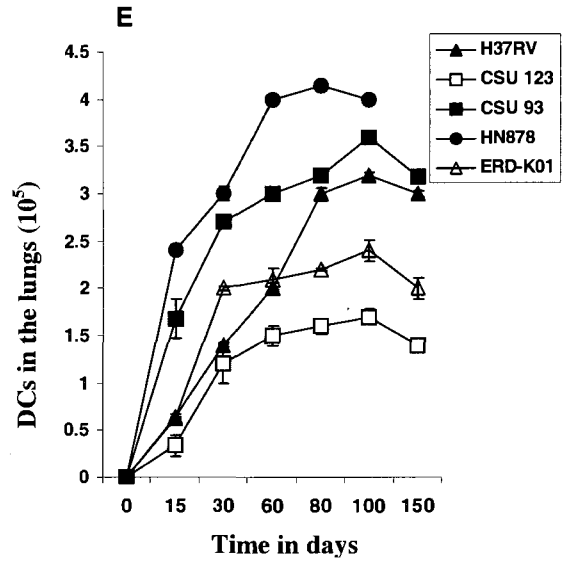
The numbers of $CD11b^{neg}CDllc^{+}B220^{neg}Gr1^{neg}$ DCs and $CD11b^{neg}CDllc^{+}B220^{+}Gr1^{+}$ pDCs was determined by primarily gating on FSC^{high} versus SSC^{high} granulocytes (Fig.5A) and then detecting DCs in the $CD11c^{+}$ region containing $CD11b^{neg}$ $CD11c^{+}$ cells (Fig.5B) which also were $B220^{neg}GR1^{neg}$ and thereafter pDCs were present in the region containing $CD11b^{neg}CD11c^{+}GR1^{+}$ $Gr1^{+}$ cells (Fig.5C).

As shown in Fig.5D, the total numbers of pDCs in the lungs obtained from *M. tuberculosis* HN878 infected mice increased in much larger numbers from day 15 to day 60 of the infection compared to the other groups. Similarly, total numbers of DCs in the lungs of these mice steady increased with time at a higher rate than seen in the other strains (Fig.5E). In previous studies we have found evidence of DC markers on highly vacuolated foamy macrophages in granulomatous lesions (46), and so it was interesting to note in the current study that many more cells of this type appeared early in lesions in

lungs of HN878 infected mice compared to H37Rv infection (Fig.5F) and that these contained large numbers of acid fast positive bacteria (Fig. 5F).


A
SSC
FSC
B
CD11b
CD11c
B220
C
GR1
R5
R6
R7


D
Plasmacytoid DCs in the lungs (10^5)
Time in days
H37RV
CSU 123
CSU 93
HN878
ERD-K01



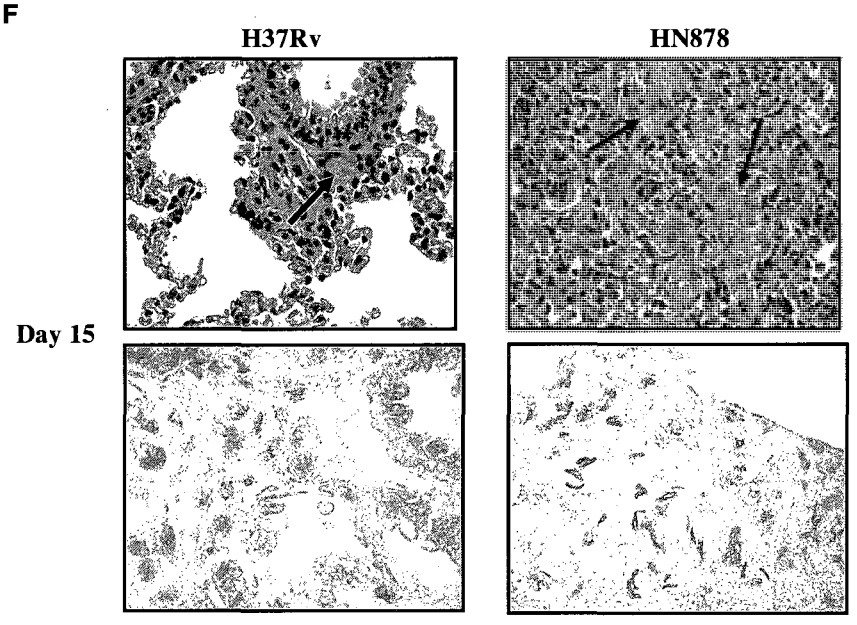


Figure 5. Early infiltration of dendritic and plasmacytoid dendritic cells in the lungs of mice infected with *M. tuberculosis* strains HN878 and CSU 93. Panel A shows a representative dot plot of lung cells from a representative mouse primarily gated on FSC^{high} versus SSC^{high} granulocytes (A) and then on DCs by gating on the region containing $CD11b^{neg}CD11c^{+}GR1^{neg}$ $CD11b^{neg}$ cells (B) and

on pDCs by gating on the region $CD11b^{neg}CD11c^{+}B220^{+}$ $Gr1^{+}$ cells (C). Panel D and E shows the lung cells obtained from C57BL/6 mice infected with a low dose of *M. tuberculosis* strains H37Rv (solid triangle), CSU 123 (open triangle), CSU 93 (solid square), HN878 (solid circle) and ERD-KO1 (open triangle) were assayed by flow cytometry for the total number of pDCs (D) and DCs (E). Results are expressed as the average number of (± SEM, n=5) pCDs or DCs in the lungs. Panel F shows that mice infected with *M. tuberculosis* HN878 demonstrated large numbers of highly vacuolated foamy cells (upper right, arrows) containing large numbers of acid fast positive staining bacilli (lower, right, arrows) compared with *M. tuberculosis* H37Rv. (upper left, and lower left). Haematoxylin and eosin staining (upper panels); acid fast positive staining (lower panels). Total magnification= F, upper panels=20x; F, lower panels 100x.

Differential production of IFN-γ, TNF-α, MCP-1, IL-10 and IL-6 cytokines by cells from infected lungs.

As shown in Fig.6A we were able to demonstrate that T cells harvested from the lungs of all infected groups and stimulated *in vitro* with culture filtrate proteins were able to produce IFN-γ, thus questioning earlier reports that HN878 infected mice could not generate a TH1 response. Further analysis of supernatants also revealed similar amounts of TNF-α, MCP-1, and IL-6 (Fig.6B-E). Interestingly, and consistent with further results described below, IL-10 production was considerably elevated in the HN878 group after day 30.

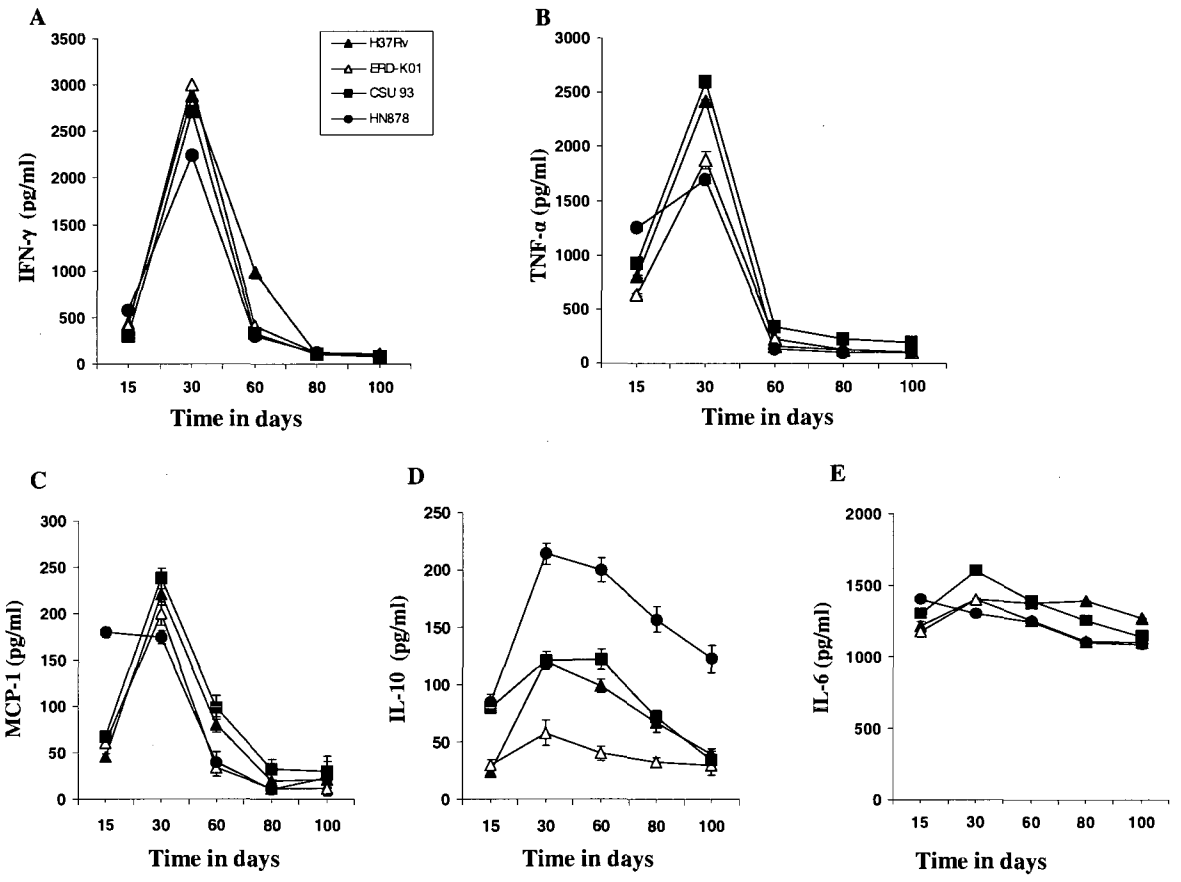


Figure 6. Differential production of IFN-γ, TNF-α, MCP-1, IL-10 and IL-6 cytokines by lung cells from C57BL/6 mice exposed to various virulent strains of *M. tuberculosis*. Inflammatory cytokine responses in the lung after cells were isolated from mice infected with a low dose of *M. tuberculosis* strains H37Rv (solid triangle), ERD-KO1 (open triangle), CSU 93 (solid square) and HN878 (solid circle) were incubated with CFP at 37°C and frozen after 72 hours. Levels of IFN-γ (A), TNF-α (B), MCP-1 (C), IL-10 (D) and IL-6 (E) were measured using a inflammatory cytometric bead array. Results are expressed as the mean values in pg/ml (± SEM, n=5) in the lungs.

Characterization of $CD4^+$ and $CD8^+$ cells in the lungs of C57BL/6 mice exposed to M. tuberculosis H37Rv or HN878 reveals the appearance of regulatory T cells in the latter.

It was interesting to note that despite the rapid growth of the HN878 strain it was rapidly controlled by host immunity and quickly showed evidence of entering a chronic state. An initial rapid worsening of lung pathology also began to apparently stabilize, and these mice somewhat paradoxically showed evidence of increased IL-10 production. To try to understand these early events we performed studies in which we reexamined the early T cell response. Figure 7 shows a representative dot plot of lung cells from a representative mouse after 21 days of infection with H37Rv or HN878 primarily gated on viable FSC^{low} versus SSC^{low} lymphocytes and then on $CD3^+$ T cells (Fig. 7A) and then gated on $CD4^+IFN\text{-}\gamma^+$ cells (Fig. 7B). Fig.7C shows the bacterial load in the lungs over the first 21 days and the numbers of IFN-γ-secreting CD4 or CD8 found in the lungs at that time. Fig.7D and 7E shows increased numbers of IFN-γ-secreting CD4 or CD8 T cells in the HN878 mice but also evidence of a drop at day 21. We found evidence that initially HN878 induces a strong TH1 response characterized by significant IFN-γ production which by day 14 begins to decline.

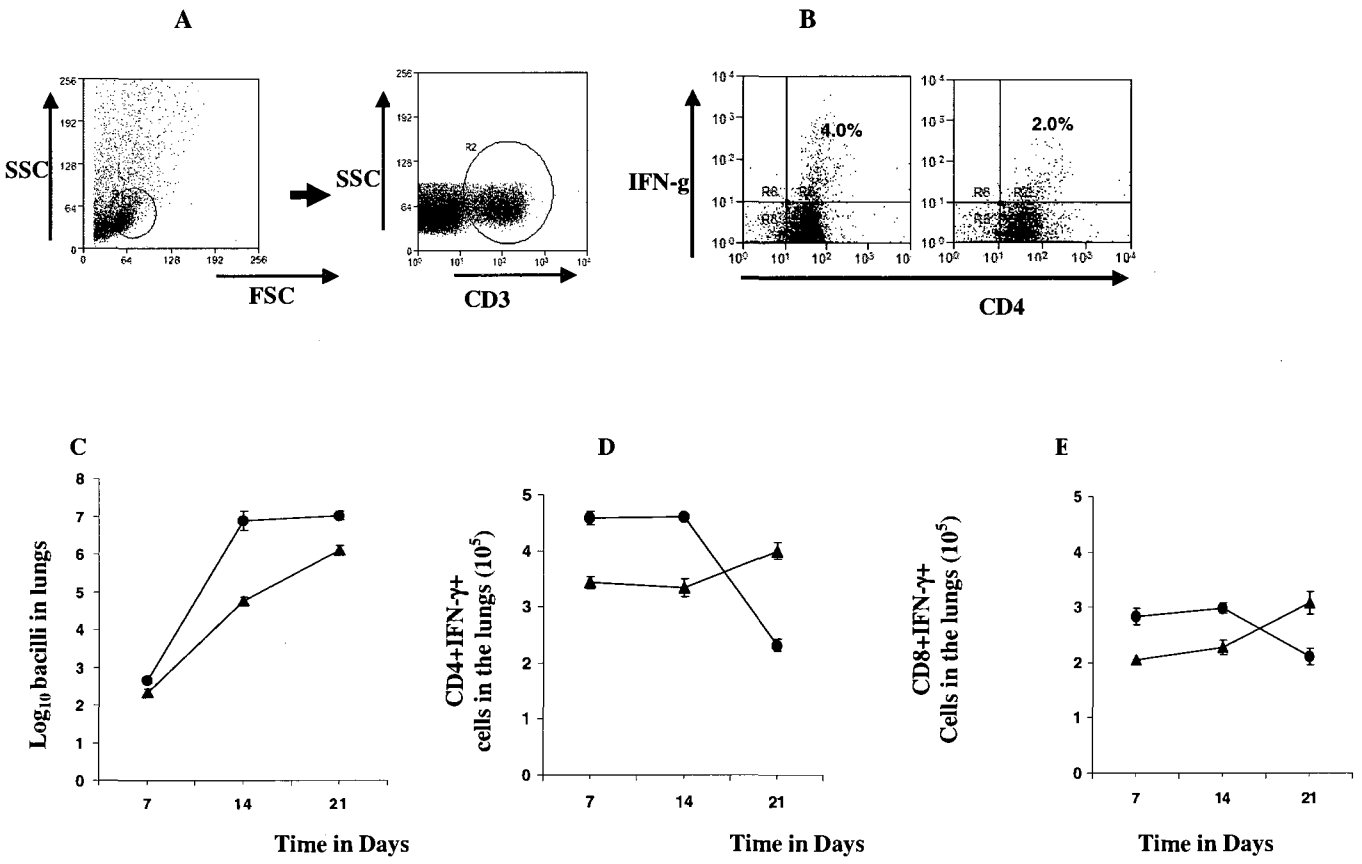


Figure 7. Early increased numbers of CD4$^+$IFN-γ$^+$ and CD8$^+$IFN-γ$^+$ cells in HN878-infected mice. Mice were infected with a low dose of *M. tuberculosis* strains H37Rv (solid triangle) or HN878 (solid circle) and then were assayed for bacterial loads (C) or by flow cytometry (A, B, D, E) on days 7, 14 and 21. Panel A shows a representative dot plot of lung cells from a representative mouse after 21 days of infection with H37Rv or HN878 primarily gated on viable FSC^{low} versus SSC^{low} lymphocytes (A) and then on CD3$^+$ T cells (A) and then gated on CD4+IFN-gamma+ cells (B). Panel C shows increased bacterial counts in the lungs of mice infected with HN878 compared to H37Rv (± SEM, n=5). Panel D shows increased numbers of CD4$^+$ cells expressing IFN-γ and panel E shows increased CD8$^+$cells expressing IFN-γ in the lungs of mice infected with HN878 compared to H37Rv. Results are expressed as the mean number of CD4$^+$IFN-γ$^+$, CD8$^+$IFN-γ$^+$, (± SEM, n=5) T cells in the lungs.

In view of this we performed a much more comprehensive examination of the phenotypes of the CD4 and CD8 cells present in the lungs using multi-parameter flow cytometry. The numbers of T cells were gated with a primary gate on viable FSC^{low} versus SSC^{low} lymphocytes (Fig. 8A) and then on $CD4^+$ T cells (Fig. 8B) co-expressing CD25 and Foxp3 (Fig. 8C) and CD223 and IL-10 (Fig. 8D). Changes in the total percentage of these cells were monitored over the course of infection. To our surprise we not only found evidence of CD4 cells expressing the regulatory T cell markers CD25, FoxP3, and CD223 in the lungs, but also evidence of a rapid expansion of these cells by day 21 (Fig. 8E). Moreover, many of these cells were also positive for intracellular IL-10 (Fig. 8F). Very low numbers of such cells were seen in the H37Rv infected mice but with no evidence of any expansion (in previous studies we monitored mice infected with H37Rv for over 300 days and found no evidence for any expansion of CD4 cells with a regulatory T cell phenotype).

To further validate the presence of immunosuppressive T regulatory cells during infection with the HN878 strain we evaluated expression of the mouse glucocorticoid-induced TNF-related receptor (GITR). GITR interaction with its ligand augments T cell activation, proliferation, IL-2, IFN-γ, IL-4 cytokine secretion and abrogates regulatory T cell suppression (47-49). C57BL/6 mice infected with HN878 demonstrated increased numbers of $CD4^+CD25^+GITR^+$ T cells in the lungs during the early course of infection compared to *M. tuberculosis* H37Rv infected mice (Fig. 8G). However, C57BL/6 mice infected with *M. tuberculosis* H37Rv showed a substantial increase in $CD4^+CD25^+GITR^+$ T cells in the lungs after 21 days of the infection. By this time this population was

declining in the HN878 infected mice indicative of reduced $CD4^+$ T cell proliferation and activation. GITR plays a key role in regulating the immunosuppressive function mediated by regulatory T cells. Therefore the increased expression of GITR by *M. tuberculosis* H37Rv infected mice indicates a state of T cell activation and prevents the development of immunosuppressive T cells. In contrast, decreased expression of GITR by *M. tuberculosis* HN878 infected mice indicates removal of GITR signaling which leads to the development of immunosuppressive T cells.

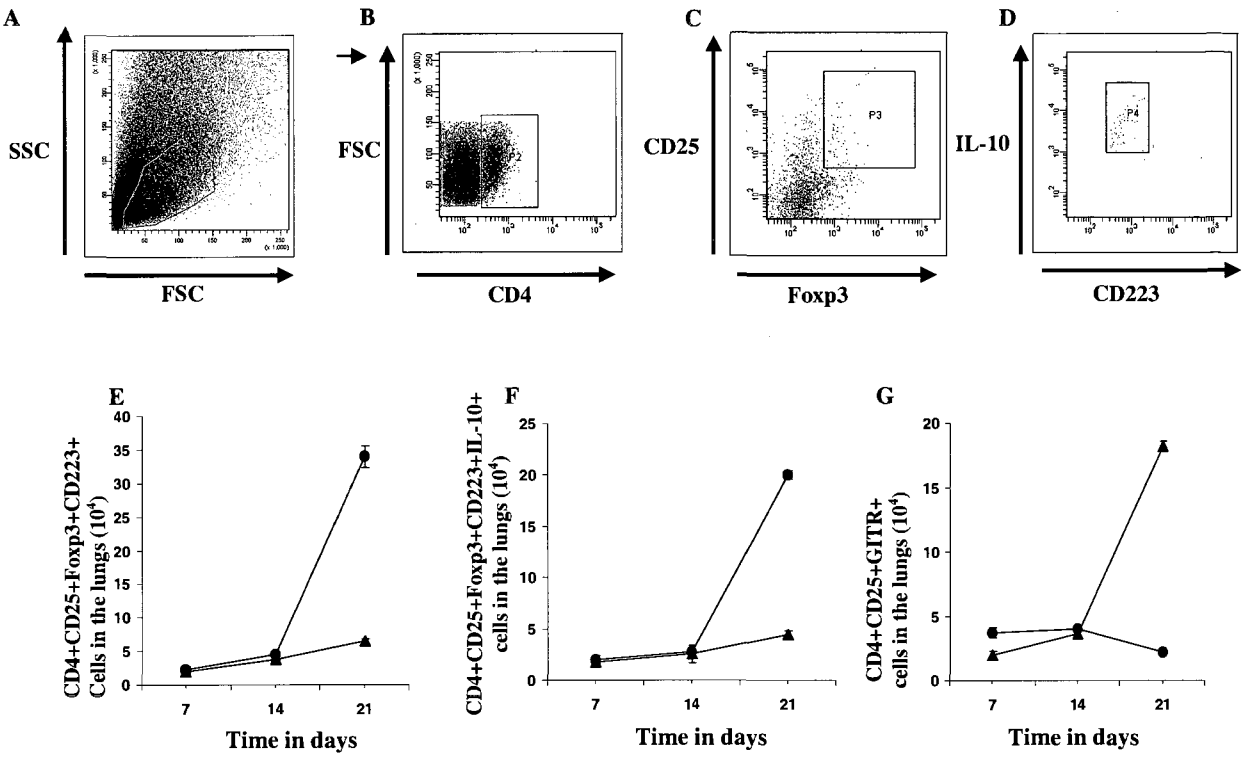


Figure 8. T cell activation decreased with increased numbers of $CD4^+CD25^+Foxp3^+CD223^+IL\text{-}10^+$ cells in HN878-infected mice. Lung cells obtained from C57BL/6 mice infected with a low dose of *M. tuberculosis* strains H37Rv (solid triangle), or HN878 (solid circle) were assayed by multi-parametric flow cytometry on days 7, 14 and 21. Panel A shows a representative dot plot of lung cells from a representative mouse primarily gated on viable FSC^{low} versus SSC^{low} lymphocytes (A, red) and then on $CD4^+$ T cells (B, blue) co-expressing CD25 and Foxp3 (C, green) and CD223 and IL-10 (D, pink). Mice infected with HN878 showed a substantial increase in $CD4^+CD25^+Foxp3^+CD223^+$ (E) and $CD4^+CD25^+Foxp3^+CD223^+IL\text{-}10^+$ (F) regulatory T cells in the lungs at 21 days of infection compared with H37Rv infected mice. Panel G shows that mice infected with H37Rv had a significant increase in $CD4^+CD25^+GITR^+$ T cells in the lungs at 21 days of infection while negligible numbers were seen in HN878 infected mice. Results are expressed as the mean number of $CD4^+CD25^+Foxp3^+CD223^+$,

$CD4^+CD25^+Foxp3^+CD223^+IL\text{-}10^+$ or $CD4^+CD25^+GITR^+$ (± SEM, n=5) T cells in the lungs.

Discussion

The results of this study shows that the strain HN878 is clearly more virulent for mice than other strains of *M. tuberculosis*, growing much faster in the lungs and inducing severe lung damage and consolidation. After 20-30 days or so the infection is contained and the lung pathology apparently slows, but animals still die more quickly than other virulent strains. The reason for this paradox can hypothetically be explained by the observation that the HN878 infection rapidly induces progressive lung pathology during a time when the protective TH1 response declines which was associated with the appearance of a regulatory T cell response. In this study I show that these fore mentioned events occurred during the same time frame and demonstrate a causal link between the lung pathology and the appearance of a regulatory T cell population during HN878 infection.

In this regard, I found evidence that initially HN878 induces a strong TH1 response, contrary to the hypothesis of Manca and colleagues (4, 5) who have argued that the hypervirulence of HN878 reflects its ability to avoid a TH1 response, an ability ascribed to its possession of a cell wall polyketide synthase-derived phenolic glycolipid that induces cytokines known to block TH1 responses (5). However, in the current work HN878 induced a potent TH1 response characterized by significant IFN-γ and TNF-α production; this peaked by day 14 and then slowly declined. It is possible that differences in the current results and the earlier reports from Manca and colleagues (4, 5) could be due to differences in the experimental methods utilized as I tracked cellular kinetics by

flow cytometry whereas they employed RT-PCR for cytokine mRNA in the animals lungs. Moreover, the present study carefully examined the lung pathology in the mice infected with HN878 strain whereas their studies provided no pathological analysis (4, 5).

Their early studies also found evidence for an increased production of Type-I IFN and hypothesized that this interfered with the ability of the animal to control the infection (4). To test this hypothesis I compared normal and IFN-αβ receptor knockout mice, reasoning that inability to respond to these cytokines would improve protection. This indeed seemed to be the case, but in contrast to the earlier studies my data showed that this applied as well into the chronic phase of the disease.

Instead, I showed that a steady increase in both plasmacytoid dendritic cells and dendritic cells was observed in the lungs of all the infection groups, and these cells are known to be rich sources of Type-I IFN (43, 50). The apparent lack of TH1 immunity during HN878 infection in earlier reports by Manca and colleges was associated with increases in IFN-α but changes in the levels of IFN-β were not evaluated. This is important, because other reports have shown that infection of C57BL/6 mice with *Listeria* induces IFN-β expression and suppresses the accumulation of $CD11b^+$ macrophages secreting TNF-α cells at the sites of infection (51). In contrast IFN-αβ receptor KO mice were more resistant to *Listeria* infection and had greater numbers of $CD11b^+$ cells producing TNF-α and IL-12p70 cells at the site of infection (51). In the present study plasmacytoid dendritic cells and dendritic cells were very prominent in the early response to HN878, and were found to be harboring large numbers of acid fast

bacilli. In addition foamy cells, which express the dendritic cell marker DEC-205 were also prominent; these cells down-regulate molecules involved in antigen presentation and may potentially provide safe niches for *M. tuberculosis* survival (46).

In the present evaluation of the early T cell response in the HN878 infected mice, Iobserved that both CD4 and CD8 cells secreting IFN-γ were rapidly induced, but these began to decline at 21 days, while at the same time a rapid increase in IL-10^{+} CD4 cells was observed. These latter cells stained positive for CD25, CD223 and FoxP3 which are all known markers of immunosuppressive regulatory T cells (27, 28). A recent report showed co-transferred $CD4^{+}CD25^{+}$ T regulatory cells from naïve C57BL/6 mice into C57BL/6J-$Rag1^{-/-}$ mice infected with *M. tuberculosis* H37Rv resulted in suppression of protective immunity which was not associated with IFN-γ, inducible NO synthase nor increased IL-10 (52). The current study evaluated the T regulatory cell response throughout the course of the *M. tuberculosis* H37Rv infection and supports the notion that the presence of Treg cells was not associated with inhibition of the early acquired immune response by reduced IFN-γ. It is probable that differences in the virulence and kinetics of the immunopathology induced by these two bacterial strains are associated with the degree to which T regulatory cells are "turned on".

In addition I evaluated the expression of GITR on T regulatory cells by *M. tuberculosis* HN878 infected mice which was down regulated by day 21 and associated with the appearance of immunosuppressive regulatory T cells. (47-49). GITR is expressed at low levels on responder T cells and is up-regulated in Treg cells and in activated T cells (47). Activation of GITR enhances co-stimulation of responder T cells

and suppressor activity is abrogated, resulting in enhanced immune responses (47-49). It is plausible that the reduced expression of GITR in *M. tuberculosis* HN878 infected mice evident by day 21 which coincided with reduced IFN-γ represents lack of GITR signaling and less potent T cell activation. In contrast *M. tuberculosis* H37Rv showed increased GITR expression which was associated with increased IFN-γ expression (47).

Whether the speed of the emerging TH1 response was the stimulus for the induction of the regulatory T cell subset is unknown, but it was clearly much faster than that seen in the other *M. tuberculosis* infections, and indeed we have yet to detect this latter subset in these other cases [even high dose intravenous models]. A second possibility is that regulatory T cells responded to the acute lung damage caused by the HN878 infection, consistent with the Orme laboratory previous observation that IL-10, a product of regulatory T cells as well as macrophages, may play a role in maintaining the stability of chronic lung disease in the face of increasing necrosis (19). [A similar observation has been made in the case of *Bordetella pertussis* infection (53)]. Moreover there is evidence that patients with active tuberculosis have raised levels of circulating regulatory T cells (22, 54). More importantly these tuberculosis patients demonstrated T regulatory cells were expanded at sites of active tuberculosis disease in parallel with high local concentrations of *M. tuberculosis*-specific TH1-type IFN-γ secreting T cells and in areas of immunopathology (22). Future studies are warranted which could perhaps involve sorting these T regulatory cells for adoptive cell transfer to show a causal link between the aggressive TH1 response and the appearance of immunosuppressive T regulatory cells during HN878 infection.

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CONCLUDING REMARKS

The fact that *M. tuberculosis* is still killing 8 million people per year even though it has infected humans for thousands of years seems hard to believe. In recent years the control of tuberculosis has been complicated by the appearance of the HIV infection, with significant aggravating factors as the emergence of multi-drug and extensively drug-resistant strains of *M. tuberculosis*. Due to the aforementioned factors, the best strategy would be to generate an immune response capable of controlling the infection before it has caused disease in the organism. In turn this would reduce the possibility of the bacillus being transmitted to another person. Unfortunately the only approved vaccine against tuberculosis, BCG, does not provide consistent protection to all individuals, and when it does, its protective efficacy wanes in the adult population. Even when it does work, it limits disease but does not prevent infection.

In order to generate an efficacious vaccine, the immune mechanisms generated by the infection as well as the protective mechanism elicited by BCG need to be better understood. It is accepted that cellular immunity is key to control *M. tuberculosis* infection, and the generation of cellular immune memory is the goal, in order to control the disease. In my thesis work I centered my studies on the type of memory T cells and regulatory T cells generated in response to *M. tuberculosis* infection, and to the BCG vaccine.

Initially I evaluated the infective capacity of a strain of *M. tuberculosis* lacking the 19kDa lipoprotein in resistant and susceptible animals, and found that the 19kDa mutant was almost completely unable to replicate *in vivo.* Despite this, when used as a vaccine, it still remained immunogenic, generating IFNγ secreting CD4 and CD8 T cells, and protecting mice from low dose aerosol challenge infection to a similar degree to the BCG vaccine. It was interesting to learn, though, that the type of cells found in the lung lesions from the two groups of vaccinated animal differed substantially. The BCG vaccinated mice had lung granulomas containing large numbers of vacuolated foamy macrophages, while the animals vaccinated with the 19kDa mutant had granulomas that were much more lymphocytic. According to these histological results, one would think that the 19kDa mutant would be a better vaccine than the BCG, since earlier work here at CSU has suggested that the foamy cells are reservoirs for the remaining bacteria, but when flow cytometry was performed no differences in activated, memory or IFNγ producing cells were found. This could mean several things; first, that while different attenuated mycobacterial strains generate similar immune responses capable of a partial protection against *M. tuberculosis* infection, none of them are able to generate a complete protection against the disease. It could also imply that we do not know exactly what kind of immune response, mostly in the context of flow cytometry markers, is telling us if a particular vaccine is efficacious. Finally, it could be hypothesized that a complete protection form *M. tuberculosis* infection is impossible to generate.

Much of what is known about memory T cells is based upon responses to defined antigens, or in mice undergoing acute viral infections. In contrast, what happens in

mycobacterial infections where there is extensive inflammation and immune stimulation, and in which the infection is often chronic ensuring continued production of antigens, is just starting to be studied. There are at least two subsets of memory T cells, both for CD4 and CD8 cells. T effector memory cells (TEM) provide immediate protection against reinfection or reactivation of disease at sites of infection, whereas central memory T cells (TCM) reside primarily in the lymphoid tissue where they can rapidly expand and differentiate to replenish the effector T cells at the peripheral sites.

In the present work I studied the type of memory T cells in BCG vaccinated animals and compared it with mice actively infected with *M. tuberculosis*, as well as animals infected and then drug treated to reduce the bacterial load. The hypothesis was that increased numbers of central memory T cells would be present in BCG vaccinated mice and in infected animals following chemotherapeutical treatment. I was anticipating these events because reactive T cells in the lungs are expected to develop into memory T cells, which are crucial in protecting the animal in the event of a reinfection.

When BCG is injected subcutaneously it drains to the regional lymph nodes where it grows for a brief period. Some bacilli reach the spleen in low numbers, but there is no evidence they ever get any further. Despite this I showed here a sustained increase in various T cell subsets in the lungs compared to that in age matched uninfected controls. This indicates that subcutaneous inoculation establishes a persistent, apparently long lived population of T cells that is distributed in tissues including the lungs and bone marrow, as well as lymph tissues. Some of these cells, at least, may represent a reservoir of potentially reactive T cells ready to respond to active infection.

The primary conclusion drawn from these studies is that memory T cell response in chronic mycobacterial infection or vaccination is dominated by a TEM response. The present data shows that BCG is very effective in generating TEM cells but far less effective in its ability to generate TCM cells. The inability to establish a significant TCM population could be the reason why BCG fails to protect adult humans from tuberculosis. TEM cells that are generated as the primary memory pool could be "used up" by exposure to the pathogen in endemic areas, and the far lower numbers of TCM that were generated are not sufficient to replenish in time the effector memory pool necessary to control the infection.

Alternatively, *M. tuberculosis* infection could be generating a functional impairment of T cells which is characteristic of many chronic mouse and human infections. Previous studies in our laboratory analyzed CD8 T cells generated in response to *M. tuberculosis* infection in mice, and showed increased expression of the exhaustion marker PD-1 during chronic infection. These would also explain why there are far less activated and IFNγ producing cells in the lungs of infected animals, than total CD4 and CD8 T cells.

In any case, the results presented here could be used as a marker with which to guide rational vaccine design, and could be applied to look at which strategies can increase the longevity of TEM response, inhibit or reverse the exhaustion generated in these cells, or more effectively induce a substantial population of TCM cells.

I show in the current work that numbers of central memory cells can be increased if the mouse is given chemotherapy to remove live bacteria, and then is reinfected by an

aerosol challenge. Secondary infection with *M. tuberculosis* occurs in people in whom chemotherapy is not fully successful, in reactivation of latent disease, or by exogenous reinfection.

The increase in central memory T cells was followed by a second wave of effector memory cells, and the combination of these two subsets made the animals highly resistant to the second infection. Quite surprisingly however, all these memory T cell subsets decline in the second month of reinfection, which resulted in increased bacterial numbers in the lungs, increased pathology and finally death of the animals.

These unexpected finding could be partly explained by the paradoxical reaction seen when the infected animals were treated with chemotherapy. Chemotherapy, without rechallenge, generated a robust influx of cells of all types, mainly macrophages and dendritic cells to the lungs. It is possible therefore that the intensity of the cell mediated immune response could generate an imbalance between the proinflamatory and immunosuppressive mechanisms necessary to control tissue damage. This imbalance could account for the increase in lung pathology I observed, making the animals unable to control the second infection beyond an initial transient period of time.

These novel observations challenged the notion that successful chemotherapy renders the individual highly resistant to secondary infection. In the model presented here, resistance is potent but is transient, and thus insufficient to protect the animal to the secondary challenge.

All the findings discussed above were further worsened in studies in which I examined newly emerging W-Beijing strains such as the highly virulent strain HN878. Clinically, the efficacy of BCG seems to be very poor against these strains, for as yet

unknown reasons. For my final piece of work therefore I used the mouse model to characterize the immune response generated in response to infection with HN878.

The results of my studies showed that the strain HN878 is clearly more virulent for mice than other strains of *M. tuberculosis,* growing faster and inducing severe lung damage. Despite this, when I used flow cytometry to evaluate the type of cells generated in response to the infection, I observed that mice infected with HN878 had a potent TH1 [CD4, IFNγ-secreting] response. I then observed however that the early T cell response in the HN878 infected mice declined at day 21, when a rapid increase in IL-10 positive CD4 cells was observed. I suspect what might be happening here was a compensation of the inflammatory mechanisms induced by the bacterial infection by the generation of immunosuppressive mechanism. These IL-10 positive CD4 T cells also stained positive for CD25 and Foxp3, which are all well known markers of regulatory T cells, consistent with my hypothesis. Whether the speed of the emerging TH1 response was the stimulus for the induction of the regulatory T cell is unknown. Alternatively the inflammation per se might be the stimulus. In the latter case, Th17 cells may have an important responsibility, as they have been recently demonstrated to have a leading role in the early inflammatory response generated against *M. tuberculosis* infection.

Do these observations have any clinical relevance? It is tempting to speculate that the usage of the BCG vaccine might be responsible for "selecting" the widespread transmission of the W-Beijing strains. These strains predominate in regions of the world [such as Hong Kong] where BCG has been widely used, but are less prevalent in areas [such as Vietnam and Ethiopia] where it is not. It is pure speculation at this point, but it is possible that protective immunity induced by BCG is overcome by regulatory T cell

activity induced by W-Beijing strains. A further speculation is that TEM induced by BCG are particularly sensitive to down-regulation by regulatory T cells.

In summary, the results presented here suggest that it may be important to rethink ways in which vaccines are measured, as well as how to evaluate new chemotherapeutic compounds generated to treat tuberculosis. To date, vaccine efficacy is usually selected on the basis of whether it can generate a potent TH1 response with good IFNγ levels. These immune response generated by the vaccine is then followed in a short term assay in which the challenge is given 4-6 weeks after immunization. First, such short term assays almost certainly do not give any pertinent information as to whether a TCM population can be generated by a given vaccine. This may be critical to whether a new vaccine or prime boost strategy could establish memory immunity capable of lasting decades or more, which is what is obviously needed.

Additionally, the generation of a very potent TH1 response could be detrimental to the individual if it induces a dampening regulatory T cell response [making the assumption that the latter are directly induced by the former, which may not be the case]. In this context the evaluation of both inflammatory and immunosuppressive mechanisms should be evaluated when the response to vaccines or new forms of chemotherapy are being investigated.

It is important to emphasize that according to the results presented in this thesis we may have to seriously rethink the way we are studying the generation of immune memory and the type of cells that we should look at. While we can use the viral models as an initial guide to study memory immunity, we may have to throw away the "rule

book" since memory in the context of chronic disease or a persisting BCG vaccine does not appear to follow these rules.

Future experiments should be centered in the generation of vaccines capable of generating long-lasting central memory T cells. Paralleled with this, immune mechanisms able to reactivate exhausted effector T cells could be researched; with the goal being to create an immune response capable of better controlling the infection. Future studies should also be done to better understand the balance between the inflammatory response and potentially immunosuppressive mechanisms triggered by *M. tuberculosis* infection.

Finally, the utilization of more virulent, clinically relevant strains could and should be used to evaluate new regimens of chemotherapy and vaccination. To date, it is not even known if the BCG vaccine can protect against these strains. Furthermore, it would be crucial to evaluate if other highly virulent strains are capable of inducing a potent regulatory T cell response that would dampen completely the TH1 response and thus potentially interfere with vaccines. If this is the case, the generation of an effective vaccine against *M. tuberculosis* will be far more complicated and may be even impossible.