LOW DOSE BCG VACCINATION IN MICE: IMMUNE RESPONSES AND IMPLICATIONS FOR TUBERCULOSIS CONTROL

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Microbiology and Immunology
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Saskatoon

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DEPARTMENT	Microbiology and Immunology
DEGREE	Doctor of Philosophy

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ABSTRACT

The outcome of an infection is often determined by the qualitative nature of the immune response generated against the infectious agent. Various intracellular pathogens, including those that cause leprosy, tuberculosis, leishmaniasis, and most probably malaria and AIDS appear to require a predominant cell-mediated, Th1, response for effective containment, whereas the generation of a mixed Th1/Th2 or predominantly Th2 response is associated with progressive disease. Therefore, any attempt to develop universally efficacious vaccination against these pathogens must generate an immunological imprint that ensures a strong and stable cell-mediated response upon natural infection with the relevant pathogen. We report here critical tests of a strategy designed to achieve such an imprint using Bacille-Calmette-Guérin (BCG) vaccine. BCG vaccine is an attenuated form of *M. bovis*, the causative agent of tuberculosis in cattle, and is the most widely used vaccine in humans. However, considerable uncertainty still surrounds its efficacy against tuberculosis both in man and animals. As the protective dose is not known, BCG has been given at the maximum tolerable dose. However, recent studies in mice and other animals have shown that the dose of an antigen can be a critical factor in determining the type of immune response generated. I tested the general hypothesis that low dose vaccination would preferentially induce cell-mediated immune response and generate a Th1 imprint that can protect the host against intracellular pathogens in the particular case of mycobacteria. To this end, both adult and newborn mice were vaccinated with different doses of BCG or saline and cell-mediated and humoral immune responses were assessed at different times post-vaccination. Several weeks after vaccination, mice from each group were challenged with a dose of BCG that induces a mixed Th1/Th2 response

in naïve mice, and the T-cell and antibody responses were assessed using ELISPOT and ELISA assays, respectively. The splenic bacterial burden was also determined using colony formation on agar plates.

Our results show that the class of immunity induced by BCG depends on the dose employed for vaccination, independent of the route of administration and the age and strain of mice used. In all cases, lower doses induce an exclusive cell-mediated, Th1, response with no antibody production, while higher doses induce either a mixed Th1/Th2 response or a predominantly Th2, humoral response, with higher titers of both IgG_1 and IgG_{2a} antibodies. Following intravenous high dose BCG challenge, all mice in the vaccinated groups developed a Th1 response associated with a more efficient clearance of BCG from the spleen. The greatest clearance of mycobacteria was generated following vaccination with lower doses, as low as 33 cfu of BCG. In addition, our findings demonstrate that newborn mice are not inherently biased towards generating Th2 responses, but they can generate Th1 responses and Th1 imprints if appropriate vaccination protocols (dose, route and time) are employed. Furthermore, subcutaneous exposure of young mice to environmental mycobacteria can induce a mixed Th1/Th2 response that can abrogate the potential to generate Th1 responses and Th1 imprints upon vaccination with low doses of BCG vaccine. Low dose neonatal BCG vaccination can circumvent the interference caused by “impingement” of environmental mycobacteria on the immune system. Therefore, our observations strongly support a neonatal low dose BCG vaccination strategy to provide universally efficacious protection against infections by pathogenic mycobacteria.

ACKNOWLEDGEMENTS

I sincerely believe that it was a great privilege for me to have the opportunity to join Dr. Bretscher's laboratory and work under his close supervision. I extend my heartfelt thanks to him for providing such a friendly environment for high quality research and training. I am also grateful to him for his great effort not only on my thesis work but also my future career.

My sincere gratitude goes to my Advisory Committee, Dr. Henry Tabel, Dr. Calliopi Havele, Dr Peter Howard, and Dr. Philip Griebel for their technical advice and guidance throughout my study period.

My heartfelt appreciation goes to Guojain Wei for his unreserved technical support and Dr. Brendan O'Connor for doing all the histopathology work. I am also grateful to all the technicians in Dr. Bretsher's laboratory and the staff of the animal care facility for their help in animal handling. I am also thankful to all fellow students in the laboratory for their companionship and scientific discussions.

I owe a great deal of gratitude to the Department of Microbiology and Immunology, the College of Medicine as well as the Vaccine and Infectious Diseases Organization (VIDO) for their kind financial support during the entire study period. This research was funded by a grant from Canadian Institute for Health Research held by Dr. P. Bretscher.

I am very grateful to all who, in one way or another, have contributed to the successful completion of this study.

Last but not least, my special thanks goes to my beloved family, my wife, Rahel, my children, Yohanna and Samuel, for their support, encouragement and the patience they showed to me during the prolonged study period.

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LIST OF ABBREVIATIONS

Ab	Antibody
AFB	Acid Fast Bacilli
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cells
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid
BCG	Bacille-Calmette-Guérin
BCG261	Recombinant BCG that expresses Kanamycin Resistance Gene
BSA	Bovine Serum Albumin
CFA	Complete Freund's Adjuvant
cfu	Colony Forming Units
CO_2	Carbon Dioxide
CTL	Cytotoxic T-Lymphocytes
DTH	Delayed Type Hypersensitivity
ddH_2O	Doubled Distilled Water
DMEM	Dulbecco's Modified Eagles Media
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunospot Assay
FACS	Fluorescence-Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
HBSS	Hank's Balanced Salt Solution
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
hsp60	Heat shock Protein 60
id	Intradermal
ip	Intraperitoneal
iv	Intravenous
IFN-γ	Interferon Gamma
IgG1	Immunoglobulin Gamma-1
IgG2a	Immunoglobulin Gamma-2a
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
IRF-1	Interferon Gamma Regulatory Factor-1
LPS	Lipopolysaccharide
MACS	Magnet Activated Cell Sorting
MHC	Major Histocompatibility Complex
NRAMP	Natural Resistance Associated Macrophage Protein

NO	Nitric Oxide
PBL	Peripheral Blood Leukocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween
pi	Post Infection
PRR	Pattern Recognition Receptor
RBC	Red Blood Cells
RPMI	Roswell Park Memorial Institute
sc	Subcutaneous
SD	Standard Deviation
SE	Standard Error
TB	Tuberculosis
TCR	T-cell Receptor
TGFβ	Tumour Growth Factor Beta
Th	T-Helper Cells
Th1	T-Helper Cell Type 1
Th2	T-Helper Cell Type 2
TH17	T-Helper Cell Type 17
TLR	Toll-like Receptor
TNFα	Tumour Necrosis Factor Alpha
T_{reg}	Regulatory T-cells
UCASC	University Committee for Animal Care and Supply
WHO	World Health Organization

1. General Introduction

Tuberculosis (TB) has been a major health threat to mankind since prehistoric times. The causative agent was isolated and identified more than a century ago, allowing tuberculosis to be among the first diseases against which a vaccine was developed. Nevertheless, tuberculosis still remains the greatest global cause of human morbidity and mortality from a single infectious pathogen. Epidemiological evidence indicates that one third of the world's population is infected with *M. tuberculosis*; annually, 8-10 million people develop clinical disease and of these 2-3 million die (Kaufmann, 2006b). To appreciate the enormity of the problem tuberculosis poses to public health, consider that an active tuberculosis infection is present in 60 million people at any one time with an average of 4 people dying every minute from this disease (Dye et al., 1999).

Tuberculosis is zoonotic, so control of the disease in cattle is important from a public health point of view. The economic impact on the cattle industry is an additional concern; an estimated 50+ million cattle are infected with *M. bovis*, resulting in economic losses of approximately $3 billion per year due to a reduction in milk production, quality of meat, fertility and animal power (Steele, 1995). These facts suggest the urgent need to control the disease both in man and animals. However, diagnosis, treatment and control of tuberculosis still depends on methods developed between the end of the 19th and middle of the 20th century. New cases are still being detected by sputum smear examinations and PPD skin tests developed by Robert Koch 125 years ago. These techniques were novel and a break-through in the effort to control tuberculosis when developed; however, their sensitivity is very poor, detecting only 50% of infected individuals. Differentiating

pathogenic from environmental mycobacteria based on these diagnostic tests is also difficult. Control of the disease also depends on a 90-year-old vaccine, the Bacille-Calmette-Guérin (BCG), developed by Albert Calmette and Camille Guérin at the Pasteur Institute, France. Similar to the detection techniques, this vaccine was a great achievement in its time, but its efficacy remains controversial.

Fortunately, the causative agent of tuberculosis, *M. tuberculosis*, is sensitive to antibiotics. Drugs developed in the 1940s and 1950s can effectively cure the disease. Nevertheless, compliance is poor due to the cumbersome and the long lasting nature of the treatment regimen that discourages some patients from taking the prescription drugs continuously for a year. The high rate of defaulters during this treatment leads to the emergence of drug resistant strains of the bacilli. Multi drug resistant (MDR) strains of *M. tuberculosis* that do not respond to first-line drugs (rifampin and isoniazid) have been reported from several countries (see section 2.4). What is more worrisome is the emergence of extensive drug resistant (XDR) strains that are resistant to both first-line and second-line anti-mycobacterial drugs. Several countries are reporting the occurrence of these XDR strains of *M. tuberculosis* among their TB patients, which is further complicating the problem, particularly in countries where co-infection with HIV is prevalent, resulting in an almost 100% case fatality rate (Gandhi et al., 2006). Unfortunately, we find ourselves today in the same position as Koch and others were more than 100 years ago as far as control of tuberculosis is concerned. We are trying to fight a disease that is threatening to escalate out of control with century-old tools. Therefore, much greater effort and investment in terms of knowledge and finance are

required in order to respond effectively. Rapid, highly sensitive and specific diagnostic techniques, as well as improvements in the efficacy of BCG vaccine or development of a new and universally efficacious tuberculosis vaccine, are urgently needed.

Research on tuberculosis slowed during the mid 20th century, largely due to a sharp decrease of new cases in developed countries, which practically made tuberculosis a disease of the poor. Due to this fact, it seems that very few had interest to work on the disease. Without mentioning the pharmaceutical companies, where profit is the driving force for research and development, the amount of money spent on tuberculosis research by major funding organization was very little, compared to other major infectious diseases. For example, the National Institute of Health (NIH) spent 20-fold less money on tuberculosis research than on HIV in the late 1990s, even though both diseases claim the lives of 2-3 million people every year (Check, 2007). Recently, however, major funding organizations, including the Bill and Melinda Gates Foundation, the Rockefeller Foundation and the NIH, are transforming TB research as it provides a unique opportunity to exploit accumulated knowledge on basic immunology and biology of the pathogen (Kaufmann and Parida, 2007). This knowledge may help in developing new, fast and efficient diagnostic tools as well as in pinpointing the “tricks” the bacterium uses to avoid generating an effective immune response. Clear understanding of the bacterial mechanisms used to evade the host’s immune response will aid the development of new drugs and vaccines or improve the efficacy of BCG as a vaccine.

In any attempt to improve the efficacy of BCG as a vaccine or develop a new one, it is imperative to appreciate the general principles of vaccination. Vaccines are administered to activate natural and protective immunity to a subsequent infection in order to protect the host from the infectious pathogen. Most commercially available vaccines ensure the rapid generation of high levels of antibodies upon subsequent infection by the pathogen. These antibodies are effective in controlling the growth of extracellular bacteria and viruses as well as neutralizing some bacterial toxins. The immunological principle underlying successful vaccination in these cases is the ability of the vaccine to increase the magnitude and speed of the protective antibody response during natural infections. This increase in magnitude and speed of the generated immune response favors the vaccinated individual in the race between the multiplication of the infectious agent leading to pathology and disease, and containment of the pathogen by host immunity. Unfortunately, this strategy does not seem to work against intracellular pathogens that induce chronic infections such as tuberculosis, leprosy, leishmaniasis and others. This may suggest that the magnitude and speed of the immune response generated following infections with chronic intracellular pathogens may not be the only critical factors, but the qualitative nature of the immune response generated is also important in the fight against intracellular pathogens that cause chronic diseases. The qualitative nature of the protective immune response against *M. tuberculosis* can be elucidated by careful analysis of the response generated by healthy contacts, individuals who are able to contain the pathogen, and sick individuals who develop progressive disease following natural exposure to virulent strains of *M. tuberculosis*. Such identification is necessary to provide a rational basis for vaccine design.

M. tuberculosis infects more than 100 million people every year, but only 5-10% of those infected develop disease. The remaining, more than 90%, are individuals able to control infection, though not able to clear it completely, and remain latently infected for the rest of their life (Kaufmann, 2006b). These individuals with latent tuberculosis are commonly known as healthy contacts and demonstrate a protective, Th1, type of immune response. The key for developing a new vaccine or improving the BCG vaccine, therefore, lies in determining the immunological correlates of protection in the healthy contacts and how to ensure the generation of such a response through vaccination. In line with this, clinical observations show that chronic intracellular infections, including tuberculosis, are best contained by an exclusive or predominant cell-mediated, Th1, response. Thus, in addition to the speed and magnitude of the immune response, the Th1/Th2 nature of the response generated against these chronic pathogens is important in ensuring protection. Therefore, vaccination against these chronic infections must generate a Th1 response and Th1 imprint that can guarantee the generation of an effective cell-mediated response during natural infection by the respective pathogens. Having a good understanding of what a protective immune response constitutes, the next step will be to look for a means of vaccination that can ensure the generation of such a response. Previous results from our laboratory in the murine leishmaniasis model have shown that infection with a low dose of *L. major* parasite induces a predominantly cell-mediated, Th1, response that can guarantee protection of the prototype "susceptible" BALB/c mice from a challenge dose that induces progressive disease in naïve mice (Bretscher et al., 1992). Our laboratory has also reported that the Th1/Th2 nature of the BCG specific immune response in adult BALB/c mice is dependent on the dose of BCG used for vaccination (Power et al., 1998):

lower doses induce Th1 type responses; whereas higher doses induce mixed Th1/Th2 responses. As BCG is usually administered neonatlly in humans, it is important to determine if this dose dependence also works in newborn mice. Besides, it will be important to examine if this dose dependence of Th1/Th2 phenotype of the immune response also holds true in different strains of mice as well as for different routes of BCG administration. The principles of Th1 imprinting and the cellular mechanisms associated with it are discussed in detail in section 2.9.

2. Literature Review

The overall purpose of this section is to explain the scientific findings and considerations that led to the hypothesis I attempted to test in my thesis. The aim of my study was to test critical features of a vaccination strategy designed to be effective in a genetically heterogeneous population against intracellular pathogens that cause chronic diseases. These diseases include leprosy, tuberculosis, leishmaniasis and AIDS. However, due to my personal history, I am particularly interested in strategies to prevent/cure tuberculosis, which is the leading cause of human morbidity and mortality from a single infectious pathogen. I feel that the potential significance of my work can be best appreciated by examining tuberculosis as a public heath problem, so I have attempted to provide a broad setting to examine tuberculosis as a public health threat since prehistoric times, and to assess the current global burden as well as the socio-economic impact of the disease. This section will also cover a review of the current literature on the pathogen, pathogenesis of the disease and the host response to the bacilli. The merits and limitations of the control measures used in the past to combat the scourge of tuberculosis will also be discussed. In view of this, I will propose possible interventions methods, vaccination and immunotherapy, that can help in the effort to prevent/cure natural infection with the pathogen that causes tuberculosis. I will also discuss the scientific basis for the proposed objectives of this study.

2.1. History of Tuberculosis

Tuberculosis was the first infectious disease to be recognized in the history of man. Several recorded documents, including the bible, provide a description of a disease that resembles tuberculosis. “The Lord will strike you with consumption, with fever, with inflammation and with severe burning heat” (Deuteronomy, 28:22). These descriptions are more or less identical to the cardinal signs and symptoms of tuberculosis as we define tuberculosis today. The word consumption or phthisis in Greek has been used by several writers since the time of Hippocrates to indicate the wasting nature of the disease, reviewed in (Daniel, 1997). However, the use of written historical documents as evidence of tuberculosis is problematic as the symptoms of tuberculosis can easily be confused with these of other diseases. Therefore, more reliable evidence of tuberculosis in prehistoric times has been obtained from archeological findings. The most compelling evidence has been obtained from bones: a mummy of a five year old girl who died 3000 years ago in Egypt (Zimmerman, 1979), and a 10 year old boy from Peru (Allison et al., 1973). In both cases, the archeologists were able to microscopically detect the acid-fast bacilli in bone samples, confirming tuberculosis in man. However, other reports have shown the occurrence of the *M. tuberculosis* complex group in animals as early as 17,000 years ago (Rothschild et al., 2001). These findings in animals led to the proposal that tuberculosis first occurred in animals as *M. bovis* and began to infect humans when settlements in villages and domestication of animals for farming began several thousand years ago. These circumstances are thought to have led to selective adaptation of the mycobacteria to human hosts and evolution of the bovine tubercle bacilli into *M. tuberculosis*. However, this view has been challenged by reports that indicate the

existence of human tuberculosis long before domestication of animals (Kapur et al., 1994). Furthermore, analysis of the genomic structure of the bacilli indicates that the human specific bacilli, *M. tuberculosis*, did not evolve from *M. bovis* (Brosch et al., 2002). Although one of the oldest known infectious diseases in the history of man, tuberculosis remained very rare due to the scattered living conditions of man in ancient times. Epidemiological studies indicate that tuberculosis requires a social network of at least 180 to 440 persons living in close proximity to become endemic in a community (McGrath, 1988). This observation helps to explain why the disease was not a prominent public health problem until the first tuberculosis epidemics in Europe, which overlapped with the industrial revolution. This historical period saw the development of cities, creating perfect conditions for the person-to-person transmission of the bacteria. During this time, outbreaks of tuberculosis –"the great white plague"– occurred in the large cities of Europe. Almost every European was infected with the bacilli, and one in four deaths was due to tuberculosis infection (Bloom and Murray, 1992). In North America, outbreaks of tuberculosis that occurred in the 18th century were associated with the migration of Europeans into the United States and Canada. Tuberculosis became a public health problem in Asia and sub-Saharan Africa in the 19th and 20th centuries, respectively, following colonization of the continents by European settlers (Daniel et al., 1994).

2.2. Epidemiology and Global Burden of Tuberculosis

Even though tuberculosis has affected man since antiquity, the occurrence of outbreaks is associated with urbanization and crowding of people in cities that create a perfect condition for the person-to-person transmission of the pathogen. An epidemic of

tuberculosis in a community follows a predictable pattern: it first attacks the most vulnerable (susceptible) members of the society, particularly children, and reaches its peak after several years. Infected individuals develop disease and susceptible individuals die, leaving the most resistant individuals, who develop protective immunity, to live and transfer their genetic material to the next generation. When the pool of susceptible individuals is reduced within a community, either due to herd immunity or death, the epidemic starts to decline until a new pool of susceptible individuals emerges in the community and another wave of disease can occur. In between outbreaks, tuberculosis may remain endemic in the area affecting only a few individuals who become susceptible for various reasons including age (young or old), concurrent diseases, and malnutrition. Tuberculosis is a chronic disease, so patients live several years between infection, disease and then death or recovery. Therefore, the time course between epidemics of tuberculosis is very long, counted in centuries, in contrast to other more acute infectious diseases, which may occur every few years (Grigg, 1958). Defining some epidemiological terms at this point will help the reader to understand the words in the right context as the terms are going to be used frequently in this section of the thesis. *Incidence* is defined as number of new cases in a population over a given period of time. *Prevalence* refers to a total number of cases in a population at given point in time. *Mortality rate* refers to the number of deaths due to a given disease in a population at risk during a given period of time, while *case fatality rate* refers to the ratio of the number of deaths caused by a given disease to the number of diagnosed cases of that disease. These rates are expressed per 100,000 persons per year in all TB monitoring and control guidelines published by the World Heath Organization (WHO). Thus, I will follow a similar way of expression whenever I

use these terms. An additional two terms worth mentioning here include: *Risk of infection* that refers to the probability of an individual being infected in a given period of time, and *case detection rate* calculated as the ratio of new smear positive cases to the total number of expected new cases estimated for that country in a given year. These two terms are usually expressed as percentages. WHO has different formulas to calculate each of these rates depending on the nature of the data available.

Epidemics of tuberculosis in Europe reached their peak in the 17th century with an incidence rate of around 1000 cases /100,000 persons/year. Almost one fifth of all deaths in London were due to tuberculosis, which led the writers of the time to describe tuberculosis as the "Captain of all these men of death", reviewed in (Daniel, 1997). Tuberculosis was spread to all corners of the world as a result of emigration of Europeans. Tuberculosis epidemics reached their peak in North America in the 19th century, resulting in around 1600 deaths /100,000 persons per year (Dutt and Stead, 1999). The epidemic of tuberculosis is likely reaching its peak in Latin America today, and is in an early phase in Africa and Asia. The disease has been in a sharp decline in the western world with annual risk of infection dropping from 12% in 1901 to 1.9% in 1950 (Vynnycky and Fine, 1997). However, in the late 1980s the incidence of tuberculosis started to increase at an alarming rate globally, which forced the World Health Organization to declare tuberculosis to be a "global emergency" in 1993 in an attempt to heighten political and public awareness about the gravity of the disease (Glynn, 1998). For example, tuberculosis cases in the United States started to increase in the mid 1980s; and around 52,000 excess cases were reported between 1986 and 1992 when compared to

the number of anticipated cases based on the previous trend (CDC, 1993). According to the WHO annual report, 8.8 million new cases (140 cases/100,000 persons) were reported globally in 2005, resulting in an estimated 1.7 million deaths in the same year (WHO, 2005). In the years 2000-2020, another 200 million people are expected to develop tuberculosis, of which 35 million are expected to die. These numbers clearly show the public health catastrophe that tuberculosis is expected to cause in the foreseeable future. Tuberculosis is a burden not only on public health, but also on the socioeconomics of a society, as it affects individuals between the age of 15 to 45 years at the peak of their productivity (Dye et al., 1999). South East Asia has the highest number of cases every year; however, the per capita incidence is highest in African countries reporting 259-272 cases per 100,000 individuals every year (Corbett et al., 2003).

2.3. Risk Factors Associated with Tuberculosis Infection

Of those infected with *M. tuberculosis*, only 5-10% develop clinical disease. The remaining 90-95% are believed to mount an effective immune response that limits the multiplication of the bacilli and the development of pathology. These facts demonstrate that immunological factors and other circumstances can influence the outcome of infection and I will briefly discuss some of the main factors, which may play a role in the susceptibility/resistant phenotype of the host.

2.3.1. Host Genetics

The average lifetime risk of developing tuberculosis in infected individuals is around 10%. However, a very much higher risk of developing the disease is reported in some individuals among a population existing under similar circumstances (Raviglione et al., 1995). A higher concordance rate of tuberculosis also exists among monozygotic versus dizygotic twins (Kallmann and Reisener, 1943; Comstock, 1978). Furthermore, racial differences in susceptibility upon initial infection have been reported in blacks versus whites from nursing homes in the USA (Stead et al., 1990), and aboriginals as compared to Caucasians in Canada (Hoeppner and Marciniuk, 2000). These observations suggest host genes can affect the outcome of tuberculosis infection. Ample experimental evidence from mouse studies also supports the role of host genetic factors in controlling susceptibility/resistance to several chronic intracellular pathogens, including mycobacteria. Resistance to growth of BCG in mice is under the control of a single, dominant autosomal gene named *Bcg* (Gros et al., 1981). The gene was found to be identical to genes that control innate resistance to infections with *S. typhimurium* (*Ity*) and *L. donovani* (*Lsh*) and mapped to the mouse chromosome 1 region (Skamene et al., 1982). The candidate gene was expressed exclusively in macrophages and subsequently named *Nramp1*, for natural resistance associated macrophage protein 1 (Vidal et al., 1995). However, the role of *Nramp1* gene in controlling mouse resistance to infection with the virulent strain of *M. tuberculosis* has not yet been verified experimentally. The human homologue of *Nramp1* gene, designated *NRAMP1*, has been also cloned, and mapped to chromosome region 2q35 (Cellier et al., 1994). Association of tuberculosis infection with the *NRAMP1* gene was reported in Aboriginal Canadians (Greenwood et

al., 2000) and tuberculosis patients from Gambia (Bellamy et al., 1998), suggesting that the *NRAMP1* gene may also play a role in immunity against mycobacterial infections in man. Furthermore, several studies show the susceptibility/resistance phenotype to tuberculosis is a multi-genetic trait controlled by several genes both within and outside the MHC region (Bellamy, 2003).

Despite variations between studies on the particular alleles involved, depending on the route and dose of infection, the virulence of the bacilli used, as well as the different evaluation criterion used to characterize disease, there is good evidence from mouse studies that MHC genes influence the susceptibility/resistance phenotype to tuberculosis. For example, Brett and colleagues have reported that H-2^k mice died faster and had more bacteria in their lung than H-2^b mice on both B10 and BALB backgrounds (Brett et al., 1992). Similarly, studies on H-2 congenic mouse strains have shown that H-2^k haplotype was able to confer susceptibility on a resistant mouse strain (Medina and North, 1998). In all cases, resistance to infection was associated with high level of *M. tuberculosis* antigen-specific IFN-γ production and better DTH expression, suggesting the importance of a Th1-type response to resistance. Even though it is hard to find direct evidence for how MHC genes play a role in determining the Th1/Th2 nature of an immune response against a given antigen, one can speculate on possible mechanisms, based on the fact that a combination of antigen dose and number of antigen specific CD4+ T-cells present in unprimed animal is believed, at least by some individuals in the field, to determine the Th1/Th2 nature of the immune response generated (Bretscher, 1974). More antigen-mediated CD4+ T-cell interactions are required to generate a response with a substantial

Th2 component than to generate a predominant Th1 response (see section 2.9 for details). In order to properly explain how MHC genes may play a role in determining the Th1/Th2 nature of the immune response, and thus resistance/susceptibility to *M. tuberculosis* infection, one must make some assumptions as to what leads to resistance/susceptibility phenotype. One view I favor is that susceptibility to *M. tuberculosis* infection can be either due to too weak Th1 response or to a response with a significant Th2 component. If susceptibility is due to too weak Th1 response, then we assume that susceptibility is a recessive phenotype, as very weak peptide-MHC interaction in all the alleles is required for the susceptibility phenotype to be expressed. On the other hand, susceptibility due to a response with a significant Th2 component indicates the dominant nature of the phenotype, as strong interaction of peptides with one or few alleles within the MHC can determine susceptibility regardless of the type of interaction that occurs with the remaining alleles. For this discussion, we will assume that susceptibility is due to a response with a significant Th2 component and is of a dominant phenotype. It is also a well-accepted fact that MHC molecules of different haplotypes differ in their ability to bind a given peptide. Therefore, peptides from *M. tuberculosis* proteins could be well presented by H-2 alleles from some mouse strains, and hence favor a response with a significant Th2 component, which will make them to be susceptible to *M. tuberculosis* infections. On the other hand, *M. tuberculosis* derived peptides may not be well presented by H-2 alleles from the resistant mouse strains that lead to the induction of Th1 cells as a result of weak interaction between cooperating CD4+ T-cells. Another possible mechanism as to how MHC genes can determine the Th1/Th2 nature of the immune response to mycobacteria is that resistant strains can express relatively low levels of

MHC-mycobacterial peptide complexes on the surface of their antigen presenting cells, thus leading to less CD4-CD4 T-cell interactions that result in Th1 cell activation, as opposed to the high MHC-peptide complex expression on the surface of APCs in the susceptible mice. Evidence, which supports this hypothesis comes from challenge studies in recombinant mouse strains where lack of the I-E molecule, and thus fewer MHC-peptide complexes on the surface, was reported to increase resistance to *M. tuberculosis* infections (Brett et al., 1992).

The role of MHC on susceptibility/resistant phenotype against *M. tuberculosis* infection has also been reported in humans. An association between alleles on the HLA-DQ locus and pulmonary tuberculosis was reported in Mexican patients (Teran-Escandon et al., 1999). However, the most compelling evidence comes from studies in some Asian populations. Results from different studies, compiled by Hill, have all shown that the HLA-DR2 allele on one of the three class II loci is a susceptibility allele for tuberculosis and leprosy infection, as well as disease progression (Hill, 1998). The same hypothetical mechanism described above for mice may also apply to explain the mechanism as to how this particular allele could contribute to susceptibility. This allele, which seems to be dominant based on the explanation described above, may bind well with peptides derived from mycobacterial protein antigens, resulting in a strong CD4-CD4 T-cell co-operation, and hence Th2 cell activation. As discussed elsewhere in this thesis, the generation of Th2 response following infection with chronic intracellular pathogens is not only ineffective, but can also lead to disease progression by counteracting the effect of Th1 cells. Finally, it is worth mentioning, that the role of MHC genes in the control of

immune responses is not only due to their role in antigen presentation and the activation of *M. tuberculosis* specific T-cells, but also due to the fact that many other important genes that are involved in the immune response, including genes that encode for complement, TNF and major heat shock proteins are mapped into the MHC region (Dunham et al., 1987; Sargent et al., 1989). Genes outside the MHC are also reported to play a role in *M. tuberculosis* immunity. In mice, multiple loci on chromosome 1, 3, and 7 named as *Trl*-1, *Trl*-2 and *Trl*-3, respectively for tuberculosis resistance loci, are reported to greatly influence the pathology and replication rate of the bacilli in the lung (Kramnik et al., 2000; Mitsos et al., 2000; Mitsos et al., 2003). In man, case control studies during an outbreak have identified several other genes, including the Mannose-binding protein, Vitamin-D receptor, IFN-γ and IL-1 receptor genes, that can contribute to risk of developing disease following infection (Marquet and Schurr, 2001).

2.3.2. Socio-economic Factors

Socio-economic factors also contribute to the risk of developing tuberculosis. Urbanization is recognized as having been a major factor for the change of tuberculosis from an endemic to an epidemic disease in Europe. Similarly, overcrowding in settlement areas is believed to be the main reason for the high incident rate of tuberculosis in Canadian Aboriginals in the late 1800s (Hoeppner and Marciniuk, 2000). Poor socio-economic conditions, including low household income, frequently lead to poor hygienic and housing conditions in urban areas and settlements, which will in turn lead to overcrowding that creates a perfect condition for the person-to-person transmission of the disease. Deterioration of health care systems due to economic crises in member states of

the former Soviet Union is also alleged to be partly responsible for the resurgence of tuberculosis in the western world (Shilova, 2001). In addition, homelessness, drug abuse, alcoholism and malnutrition are factors that can facilitate the progression of infection into clinical disease (Brudney and Dobkin, 1991). The interaction between malnutrition and tuberculosis infection is extensively discussed previously (Macallan, 1999). Reports from Latin America and Africa show the association of malnutrition with tuberculosis (Harries et al., 1988; Scalcini et al., 1991). However, whether malnutrition predisposes humans to tuberculosis or whether malnutrition is the consequence of the disease is not clear. On the other hand, sufficient experimental evidence from animals studies exists to show protein deficiency or deficiency of single nutrients, mainly vitamins (A, C, D) and minerals (iron and zinc) can adversely affect cell-mediated immunity (Chandra, 1997). Therefore, it is reasonable to propose that malnutrition can predispose people to tuberculosis as tuberculosis is best contained by cell-mediated immunity. In addition to the high chance of developing tuberculosis, people under poor socio-economic conditions show a high rate of noncompliance during treatment, which may further fuel the scourge of tuberculosis due to the emergence of multi-drug resistant bacilli (Mishra et al., 2005).

2.3.3. Concurrent Diseases

Chronic debilitating diseases such as diabetes mellitus, cancer and immunosuppressive therapy may increase the risk of developing active tuberculosis. Presently, however, HIV infection is the strongest known risk factor for developing active tuberculosis (Styblo 1991; Daley et al., 1992). The risk of developing tuberculosis upon infection of healthy individuals is 0.1-0.2% per year during their life time, assuming that the average life

expectancy is 50 years (Bloom and Murray, 1992; Raviglione et al., 1995), whereas the risk for HIV infected individuals is as high as 10% per year, which is close to 100-fold higher than the risk in HIV negative individuals (Glynn, 1998; Girardi et al., 2000). Therefore, in addition to the poor socio-economic conditions prevailing in developing countries, the HIV/AIDS pandemic has greatly influenced the epidemiology of tuberculosis in South-East Asia and Sub-Saharan Africa. This has also complicated control measures to be employed against tuberculosis in these countries.

2.4. Treatment and Control

Tuberculosis has been the leading cause of death from infectious causes for centuries, with a case fatality rate of 40-60% if untreated (Murray et al., 1990; Bloom and Murray, 1992). In prehistoric times, priests and traditional healers did not even try to heal their patients. Knowing that consumption was incurable, they used to tell their patients “All the gods have no mercy on it”, reviewed in (Daniel, 1997). During the medieval period, newly crowned kings of England and France were believed to have powers to heal by touching those suffering from the disease, which was known at that time as “Kings Evil” (Bloom and Murray, 1992). However, Robert Koch was the first to attempt a scientifically-based therapy in 1890, when he tried to immunize patients with culture filtrates to treat tuberculosis (Burke, 1993). Even though Koch’s culture filtrate was soon proven to have no therapeutic value, it was found to be useful for diagnosis. Austrian physician Clemens von Pirquet showed the allergic reaction to tuberculin could be used to detect individuals who had been exposed to tubercle bacilli, reviewed in (Bothamley and Grange, 1991). Koch’s “magic bullet”, now known as purified protein derivative

(PPD), is widely used in skin testing both in man and animals to assess delayed-type hypersensitivity to mycobacterial antigens.

Despite the absence of modern therapy and control measures, tuberculosis was in sharp decline in both Europe and North America during Koch's era. Many factors probably contributed to this, including development of genetic resistance to tuberculosis due to selection pressure and improved socio-economic conditions. However, the major reduction in the incidence rate is attributed to the introduction of sanatoria and other control measures in Europe by Herman Brehmer in the 1850's and into North America by Edward Livingston Trudeau in 1884, reviewed in (Snider, 1997). Furthermore, a real hope of eradicating tuberculosis arose with the discovery of antibiotics during the late 1940's. Streptomycin was first discovered by Seman Waksman in 1943 and was successfully used to cure a 21 year old woman with advanced tuberculosis in 1944 (Pfuetze et al., 1955). Similarly, the administration of isoniazid showed encouraging results when clinical signs of tuberculosis subsided in 44 treated patients (Selikoff et al., 1952). Since then a combination of different antibiotics has been used to treat tuberculosis patients, with a good chance of recovery. Even though antibiotics provided good reasons for optimism in the control/eradication of tuberculosis, the regular treatment regimen was not able to substantially reduce the global burden of the disease, which led the WHO to introduce DOTS (Direct Observed Therapy, Short course). DOTS is an intensive short-term treatment of patients with multiple anti-mycobacterial drugs, given by or taken in the presence of a health care worker to ensure compliance. The DOTS program is encouraging, with a 70-80% cure rate in some countries. Despite the

introduction of DOTS, and the availability of effective chemotherapy, *M. tuberculosis* is still the number one killer among all infectious pathogens, accounting for about 7% of all deaths. Several factors limit the efficacy of chemotherapy in combating tuberculosis. First and foremost is the lack of access to treatment by most of the victims. One study estimated only one in five tuberculosis patients has access to treatment facilities (Raviglione et al., 1995), possibly due to a lack of financial resources required to access treatment or a lack of public health infrastructure required to detect and treat new tuberculosis cases. The WHO target of reaching a 70% case-detection rate by 2015 does not seem achievable as the rate has already reached a plateau at 40% (Dye et al., 2002). Another important stumbling block in the control of tuberculosis is the emergence of multi-drug resistant (MDR) strains of *M. tuberculosis* that do not respond to several first-line anti mycobacterial drugs. In 2005, more than 10% of new tuberculosis cases globally were resistant to more than one drug (Kaufmann, 2006a). This is a very serious problem in some eastern European countries. For example, as high as 36.9% of new cases in Estonia are resistant to one drug, and 14.1% are resistant to more than one drug. The problem is also severe in some Asian countries such as Iran and China (Espinal et al., 2001). In line with this, it is worth mentioning the emergence of the Beijing strain of virulent *M. tuberculosis*, which has a wide epidemiological distribution particularly in Asian countries (van Soolingen et al., 1995). This strain is prone to the development of resistance to several antibiotics (Anh et al., 2000; Lan et al., 2003). In addition, a recent report from South Africa shows the emergence of *M. tuberculosis* strains resistant to several first-line and second-line anti-mycobacterial drugs. These strains, named as extensive drug resistant (XDR), are highly fatal, particularly in HIV+ tuberculosis

patients where case fatality rates close to 100% were reported (Gandhi et al., 2006). In addition to several other reasons not mentioned here, all these inherent problems of chemotherapy suggest that vaccination would be the ideal means to control the disease. BCG has been used as a vaccine since 1921 and has greatly contributed to the reduction of TB meningitis and miliary tuberculosis in children (Trunz et al., 2006). However, as discussed in detail below, its efficacy against pulmonary tuberculosis is still controversial.

2.5. Etiology of Tuberculosis

In Europe, tuberculosis was believed for many centuries to be a hereditary disease. Even renowned medical professionals did not accept the idea that tuberculosis was an infectious disease until Koch isolated the causative agent and identified it as an infectious bacillus. Inspired by the discovery of Jean-Antoine Villemin, who first showed the infectious nature of tuberculosis by his experiments with rabbits, Robert Koch tried hard to identify the etiology of tuberculosis, reviewed in (Daniel, 1997). He successfully isolated the organism in culture and was able to stain and see the organism under the microscope. He clearly demonstrated in 1882 that it was a rod shaped bacterium and hence named it the “Tubercle Bacillus”.

2.5.1. Classification and Morphology of the Tubercle Bacilli

Today, microbiologists group the bacillus under the genus mycobacterium, which was first introduced by Lehman and Neuman in 1896 (Wayne, 1984). The genus typically

represents slightly curved rods of about 1-10 μm length and 0.2-0.6 μm width and includes a number of pathogenic, opportunistically pathogenic and saprophytic species. Some of the classic species of mycobacteria that cause disease in man and animals include *M. tuberculosis, M. bovis, M. leprae, M. paratuberculosis* and *M. avium*. Mycobacteria in general are classified into two broad groups: rapid growers which form visible colonies in less than 7 days, and slow growers that require more than 7 days, usually weeks, to form visible colonies on solid media. The causative agents of tuberculosis in man and animals are slow growers and are grouped under the *Mycobacterium tuberculosis complex* group. This group includes the human pathogens *M. tuberculosis* and *M. africanum* (native to Africa); the cattle pathogen *M. bovis*, including BCG; and the vole bacillus *M. microti*. Even though humans are naturally susceptible to tuberculosis resulting from infections by the human pathogens and to some extent by the bovine type described above, other species of mycobacteria such as the *M. avium complex* (*M. avium*, *M. intracellulare*, *M. scrofulaceum*, and *M. asiaticum*) can also cause tuberculosis in immunocompromised individuals.

The bacilli have high concentrations of lipids in the cell wall, which is around 20-40% of the dry weight of the bacillus; due to this, some 30% of the bacterial genome is devoted to lipid synthesis and metabolism (Cole et al., 1998). The waxy cell wall is composed of mycolic acids, glycolipids, peptidoglycans and several other proteins, lipids and carbohydrates. These waxy components of the cell wall are thought to be in part responsible for the resistance of the bacilli to host defenses, to inhospitable chemical and physical environments, as well as to regular bacterial staining techniques (Russell 2001).

The bacilli look violet after gram staining and are grouped as gram-positive rods. However, they are better seen after Ziehl-Neelsen staining, which utilizes basic fuchsin that binds to the acidic groups on the unsaturated mycolic acid chains within the bacterial cell wall. Steaming the carbol fuchsin for a few minutes enhances penetration of the dye into the lipid cell wall. Once basic fuchsin has combined with the acid groups in the cell wall, the dye complex is stable and very resistant to de-colorization using mineral acids (HCl in 95% ethanol), hence the term 'acid-alcohol-fast' is used to describe the bacilli (Jenkins et al., 1985).

2.5.2. Growth and Culture Characteristics of Mycobacteria

The genus mycobacteria in general and the *Mycobacterium tuberculosis complex* group in particular are very difficult to grow in ordinary culture media. They need a special medium enriched with serum albumin, glycerol and several other nutrients and trace elements. The most commonly used media are either the whole egg-based (Löwenstein-Jensen and Stone Brink's) medium or agar-based medium such as the modified middlebrook 7H11. Mycobacteria can also grow in a liquid medium such as the middlebrook 7H9 broth, which is egg and agar free but enriched with glycerol, albumin and trace elements. Glycerol may suppress the growth of *M. bovis* and probably BCG, which are found to grow better in the presence of pyruvate instead of glycerol (Grange, 1990). Mycobacteria, classified under the *Mycobacterium tuberculosis complex* group, grow very slow in vitro with a generation time of around 18-24 hours. Visible colonies from clinical specimens are produced after several weeks of incubation. The slow growth of mycobacteria is mainly due to the hydrophobic nature of the cell wall. As a result of

the hydrophobic nature of the cell wall, the bacteria clump together and become less permeable to nutritious products into the cell (Wayne, 1984). Addition of the surfactant Tween 80 to cultures of mycobacteria wets the surface and leads to dispersal of the clumps and more rapid growth of the bacilli. Tubercle bacilli are obligate aerobes but growth is optimal in a humidified incubator in the presence of 5-10% CO_2. The optimal growth temperature is 37^{o}C except for the avian type, *M. avium,* which needs a temperature of 40-42^{o}C. The bacilli are facultative intracellular; however, the causative agent of human leprosy, *M. leprea*, is strictly intracellular and cannot grow in artificial media or culture. Attempts to grow and isolate it from mice and guinea pigs for large-scale studies were not successful due to the low temperature requirements for its optimal growth. The nine band Armadillo, which has a lower body temperature than most mammals, was found to be the perfect animal model to study the course of the disease in susceptible individuals (Kirchheimer, 1975).

2.5.3. Biology of Mycobacteria

The *M. tuberculosis* genome comprises 4,411,529 base pairs and contains around 4000 genes. The majority of these genes are devoted to the production of enzymes and lipids involved in the biosynthesis of its cell wall (Cole et al., 1998). Among the thousands of proteins encoded by these genes, only 52% can be assigned a function, out of which only 367 are unique to the virulent *M. tuberculosis* strain (Camus et al., 2002). Some of these genes are believed to play a role in the unique biology of the bacilli that enables it to survive in one of the most hostile environments of the host's body. During unfavorable conditions, either as a result of lack of oxygen and nutrition or due to the host's immune

response, the bacilli can enter into dormancy by shutting off the transcription of all genes required for growth, while up-regulating several other genes known as the latency genes controlled by DosR, the dormancy regulon (Boshoff and Barry, 2005). Then, when the environment is suitable for the replication of the bacilli, other genes, which encode for resuscitation-promoting factors, are up-regulated. This reactivation of dormant bacilli to replicate and infect nearby macrophages can lead to reactivation disease (Cohen-Gonsaud et al., 2005). Understanding the biology of mycobacteria during the dormant as well as the active replicating stages is important for identifying proteins dominant in each stage, and this in turn may help in designing an effective vaccine against the pathogen in each stage of the disease. In line with this, a strong immune response to a 16-kDa protein antigen, encoded by one of the latency genes is reported in healthy individuals with latent tuberculosis as compared to those with active tuberculosis who strongly respond to the early secretory 6-kDa protein antigen (Demissie et al., 2006).

2.5.4. Diagnosis and Molecular Typing of Mycobacteria

Effective control and prevention of tuberculosis depends heavily on accurate diagnosis and identification of the pathogen. Due to a lack of reliable new techniques, the diagnosis of tuberculosis infection still depends mainly on the 100-year-old techniques of direct microscopy, bacterial culture and the tuberculin skin test. Direct microscopic examination of clinical specimens is an easy, cheap and rapid method; however, it cannot differentiate between the pathogenic and the non-pathogenic species of mycobacteria. Further, the sensitivity of direct microscopy is very low, as it requires 10,000 - 50,000 bacilli per ml of the sputum sample to give a positive diagnosis (Jenkins et al., 1985). Culture

examination of clinical specimens, which can detect very few bacteria in a sample, gives a definitive diagnosis of tuberculosis infection, but requires 4-8 weeks for results to reach the physician. This may be too late for treatment purposes. The skin test is mainly used in cattle, but again the specificity is very low, as it cannot differentiate between pathogenic and non-pathogenic species as well as between infected and BCG vaccinated individuals. The recent development of new techniques based on molecular biology have facilitated the development of rapid means to detect the bacilli directly from clinical specimens, identify the species/strain involved, provide information for the analysis of transmission dynamics, and differentiate between exogenous infection and endogenous reactivation of tuberculosis.

Polymerase Chain Reaction (PCR) based amplification of mycobacterial DNA helps to rapidly detect the pathogen directly in samples both from man (Butcher et al., 1996) and animals (Aranaz et al., 1996), but this may not help in identifying the species/strains involved. Fingerprinting of mycobacterial DNA was developed and standardized in 1993 not only to detect but also identify species/strains of mycobacteria involved in tuberculosis outbreaks (van Embden et al., 1993). This technique utilizes restriction fragment length polymorphism (RFLP) based on the size and number of repetitive DNA elements known as insertion sequences (IS) generated following digestion of the genome with specific restriction endonucleases. The most commonly used insertion sequence is the *IS6110*. It is present in almost all species of the mycobacterium tuberculosis complex group and is known to be absent in other organisms including the non pathogenic environmental mycobacteria (van Soolingen et al., 1991). The number of *IS6110* copies

varies from 0 to 25; the relative number and position of the fragments within the chromosome helps to differentiate between different strains/species of mycobacteria. However, the use of *IS6110* to study the molecular epidemiology of *M. bovis* in cattle is hampered by the fact that the majority of the strains carry only a single copy of *IS6110*, which is usually present at a single chromosomal location (Collins et al., 1993). DNA fingerprinting employing the *IS6110* or other short repetitive DNA elements as probes has been used in the study of global epidemiology and transmission cycles of *M. tuberculosis* (Kiers et al., 1997), as well as in the distinction of exogenous infection and endogenous reactivation of tuberculosis (Small et al., 1993) in man. This technique, by using different genetic markers, is also effectively used to study the molecular epidemiology of *M. bovis* (Romano et al., 1996; Fisanotti et al., 1998), to trace the source of infection in cattle herds (Perumaalla et al., 1996), as well as in the study of the epidemiology and zoonotic importance of *M. bovis* in man and animals (van Soolingen et al., 1994). A serious limitation of DNA fingerprinting is the requirement for culture to generate sufficient bacteria for DNA extraction. Weeks if not months may pass before the result is available in the clinic to identify species/strains involved for treatment purposes and to know whether the bacilli are sensitive to available antibiotics. Recently, several other improved molecular techniques, such as spoligotyping and DNA microarrays have been developed that can simultaneously detect and type members of the *M. tuberculosis* complex group. Such techniques can be used to differentiate *M. bovis* from *M. tuberculosis* infections (Kamerbeek et al., 1997), as well as to identify multi-drug resistant strains of *M. tuberculosis* (Dahle et al., 2003). Spoligotyping has been also used to detect trans-species transmission of *M. bovis* in wild and domestic animals (Aranaz et

al., 1996; Serraino et al., 1999). Spoligotyping is a more rapid, sensitive and reliable technique based on PCR amplification of the mycobacterial DNA, which can be directly applied to samples without the need for culture of bacteria. It is based on the polymorphism of the direct repeat (DR) locus of the mycobacterial DNA that contains multiple 36 base pair repeats (DRs) interspersed by non-repetitive unique spacer sequences ranging from 35 to 41 base pairs in size. These unique spacer sequences can be used for molecular typing of the *M. tuberculosis* complex group by their in vitro hybridization with synthetic spacer oligonucleotides covalently bound onto a nylon membrane in parallel lanes (Groenen et al., 1993). The typing is based on the presence or absence of the unique spacer sequence in the PCR amplified DNA as assessed by hybridization. In addition to this, information obtained from molecular typing of mycobacteria is being used in the development of new diagnostic reagents that can help to differentiate BCG vaccination from *M. tuberculosis* infection. Genetic tools are also widely used to differentiate the genetically close relatives of *M. bovis* and the attenuated form of this mycobacterium, the BCG vaccine, as well as in the formulation of new anti-tuberculosis vaccines (Behr et al., 1999).

2.6. Immunity and Immunopathology

Every year close to 100 million individuals globally are infected with *M. tuberculosis*, but only 5-10% of these develop clinical disease within a couple of years after infection. These data suggest that more than 90% of infected individuals can generate an immune response that can contain but may not eliminate the pathogen (Flynn and Chan, 2001; North and Jung, 2004). Due to this non-sterile immunity, those individuals are believed to

be latently infected and are at risk of developing reactivation disease later in their life (Boshoff and Barry, 2005). Moreover, the disease in susceptible individuals is mainly due to damage caused by the immune response generated against the bacilli rather than by the bacilli itself. Following infection with mycobacteria, the susceptible host may respond with a protective type of response to contain the replication of the pathogen. However, as the mounted Th1-type response is too little to completely inhibit the multiplication of the pathogen, the bacterial population grows progressively and overwhelms the antigen specific immune response generated. As a result of this increase in bacterial load, the host intensifies its Th1-type response to catch-up with the ever-increasing number of bacteria resulting in collateral tissue damage. This indicates that the same immune response involved in protection against tuberculosis could also be responsible for the pathology associated with *M. tuberculosis* infection. This appreciation has led to the use of the common term “double-edge sword” to describe the immune response generated against tuberculosis. Recently, another population of T-cells, the Th17 cells, is reported to play a role in the immunopathology of *M. tuberculosis* infection. Th17 cells produce a pro-inflammatory cytokine, IL-17, which play a role in the pathogenesis of various infectious, autoimmune and inflammatory disease (discussed briefly in section 6.1). As pathology during tuberculosis is mainly due to a profound inflammation, Th17 cells and the proiflammatory cytokine IL-17 may have previousely unappreciated role in TB associated immunopathology. Thus, understanding the pathogenesis of the disease and the nature of the immune responses generated against the bacilli both in the resistant and susceptible individuals will be critical in developing universally efficacious vaccination that can stimulate the generation of protective immunity without inducing pathology.

2.6.1. Pathogenesis of Tuberculosis

Mycobacteria can gain entry to the host body via different routes mainly through inhalation as well as oral infection due to ingestion of contaminated food and water. Following access to the host body the bacteria establish primary infection at the site of entry. Pathogenesis and development of pathological lesions following infection with the virulent strains of *M. tuberculosis* have been discussed in detail in various textbooks and review articles. Thus, I will discuss briefly the pathogenesis of tuberculosis only with respect to the development of pulmonary tuberculosis following aerosol infection. Infection with *M. tuberculosis* occurs usually as a result of the inhalation of 1-5 bacilli in droplet nuclei derived from expelled air arising from a cough of an infected individual. These bacilli will lodge in the lung alveoli and will be ingested by alveolar macrophages. The macrophages will try to kill the bacteria before they establish an infection. However, as alveolar macrophages usually fail to kill the bacilli, blood monocytes are recruited to the site where they differentiate into macrophages and try to fight the bacilli. At this stage the bacilli replicate at an exponential rate, and monocytes and other inflammatory cells will continue to be recruited and accumulate thereby establishing a primary focus of infection. This is a symbiotic stage where the bacilli replicate without severe damage to the lung tissue. The primary focus of infection can be in any part of the lung but, for the infection to progress into clinical disease, the bacilli have to gain access to the most vulnerable part, which is the “apex” or upper part of the lung. This area of the lung provides the bacillus suitable conditions for its rapid replication (Balasubramanian et al., 1994). From the primary focus, the bacilli will reach regional lymph nodes and induce adaptive immunity, leading to lymphadenopathy and formation of the primary complex

known as Ghon's complex, first described by Ghon in 1912, reviewed in (Ulrichs and Kaufmann, 2006). The formation of this primary complex leads to the generation and recruitment of activated antigen-specific T-cells that in turn activate macrophages to kill the bacilli and inhibit their growth. The accumulation of T-cells, macrophages and other inflammatory cells at the site of infection develops into a characteristic microscopic tuberculosis lesion, the "granuloma", which prevents the spread of the bacilli into other organs and localizes the lesion to the site of infection. The granuloma is composed of alveolar macrophages, epithelioid cells, and multinucleated giant cells (Langhans cells) on the periphery and contains a solid necrotic area at the centre. The necrotic area at the center of the granuloma is unfavorable for the replication of the mycobacterium and is full of dead or dormant bacilli located either inside or outside macrophages. The granuloma is also full of infiltrating lymphocytes (T-cells and B-cells) as well as secreted cytokines (Ulrichs and Kaufmann, 2006). During mycobacterial infection, activated endothelium in the lung tissue expresses the VCAM-1 addressin instead of MadCAM-1, which is commonly expressed on mucosal endothelium. Therefore, the lymphocytes that infiltrate the granuloma in the lung are found to express the adhesion molecule $\alpha_4\beta_1$ integrin instead of the $\alpha_4\beta_7$ integrin present on mucosal lymphocytes (Feng et al., 2000). Fibroblasts may also contribute to the formation of the granuloma by forming an encapsulating wall around the granuloma periphery. This tissue wall in turn helps to limit the spread of the bacilli into surrounding tissue. This process of encapsulation is under the control of cytokines, mainly TNF-α secreted by macrophages (Kindler et al., 1989; Saunders et al., 2005). In several animal studies, treatment of latent tuberculosis with anti-TNF-α antibodies resulted in reactivation of disease. The development of fibrosis

around the granuloma and surrounding tissues leads to the formation of macroscopic nodules known as tubercles. The lung tubercles are the hallmark of tuberculosis infection and are used in the diagnosis of human pulmonary tuberculosis when detected by chest x-rays. Activated macrophages will kill most bacilli within the granuloma in the majority of the infected individuals; the granuloma will then regress and eventually calcify. However, a few of the bacilli may remain in a dormant state to be activated later, when the immune response of the host is compromised, resulting in reactivation tuberculosis. In addition to the persistence of dormant mycobacteria inside macrophages within lung lesions, the bacilli may also enter other cells that have a limited antigen-presenting ability, such as endothelial cells or fibroblasts, and become dormant. Such bacilli may avoid immune surveillance, suggesting a new sanctuary for the bacilli during conditions unfavorable for its replication (Cassidy, 2006). This conclusion was supported by the detection of mycobacterial DNA in cells other than macrophages in lung tissues of people from tuberculosis endemic areas who have died of other diseases, and who have no visible lesions of tuberculosis in any organ of their body (Hernandez-Pando et al., 2000). In susceptible individuals, the bacilli are not well contained within the granuloma and so the granuloma will start to increase in size as well as in cellularity, with caseous lesions at the center due to necrosis of cells. At this stage, the granuloma may liquefy and rupture leading to cavitations in the lung. Patients with this type of lung are highly infectious and characteristically have a persistent cough that produces blood-tinged sputum containing numerous bacilli. Rupture of the granuloma also results in spread of the bacilli into other organs through blood/lymph, producing small millet-seed like lesions throughout the body resulting in “Miliary tuberculosis”, which is fatal if not treated.

2.6.2. Protective Immunity to Mycobacterium

Mycobcateria have numerous protein antigens that are highly immunogenic and able to induce both humoral and/or cell mediated immunity (Young et al., 1992). Antibodies seem to play no role in protective immunity against mycobacteria even though hard evidence is difficult to find. Cell-mediated immunity is widely accepted to be effective against chronic intracellular pathogens. To this end, several observations with mice (Huygen et al., 1992; Hernandez-Pando et al., 1996; Azouaou et al., 1997) as well as comparative studies between tuberculosis patients and healthy contacts in man (Sanchez et al., 1994; Surcel et al., 1994; Baliko et al., 1998) support the view that cell-mediated immunity dominated with Th1 cytokines, mainly IFN-γ, is effective in controlling tuberculosis progression. Even though Th1 cytokines are essential for protection their mere presence may not indicate protection, as tuberculosis patients are usually reported to have high levels of both Th1 and Th2 cytokines in their blood. This may suggest that the protective role of Th1 cytokines can be compromised by the presence of cytokines from Th2 cells. Furthermore, the presence of high levels of Th1 cytokines in tuberculosis patients may suggest that effective control of *M. tuberculosis* infection needs more effector cells other than the Th1-cells. It is also possible that cytokines from cells other than Th2 cells, mainly the regulatory T-cells, participate in down-regulating the role of Th1 cells during tuberculosis. Even though the role of Th17 cells in down-regulating Th1 cells is not clear, IL-17 produced by antigen specific Th17 cells may participate in orchestrating the immunopathology during *M. tuberculosis* infection. In this section, I will try to review evidence that show the role of different types of cells and analyze the relative importance of each cell type during mycobacteria infection.

2.6.2.1. T-cells

SCID mice, deficient in both B and T-cells, succumb to mycobacterial infections even including the “non-virulent” BCG strain (Xing et al., 1998). This observation supports the conclusion that T-cells play an important role in protective immunity against mycobacterial infections. The obvious reason is that the organism lives inside cells, mainly in macrophages, and the immune system can only sense what is inside cells when antigens derived from the pathogen are presented on appropriate MHC molecules and recognized by T-cells. The bacilli are taken up by the macrophages through the process of phagocytosis and processed by the exogenous pathway to be presented to CD4+ T-cells via class II MHC. This line of reasoning indicates that CD4+ T-cells recognizing mycobacterial antigens in the context of self-MHC class II are vital in the immune response against tuberculosis. This has been shown in various experiments where increased susceptibility to *M. tuberculosis* as well as to BCG infections was reported in $CD4^{-/-}$ or MHC class $II^{-/-}$ mice (Boom, 1996; Mogues et al., 2001). Similarly, depletion of CD4+ T-cells prior to infection using monoclonal antibodies leads to increased bacterial burden and shortened survival in mice infected with virulent strains of *M. tuberculosis* (Muller et al., 1987). In man, the high susceptibility of HIV/AIDS infected individuals to tuberculosis is considered to be also explicable on the basis of an important role for CD4+ T-cells in protective immunity against mycobacteria. Different CD4+ Th cells have different functions depending on the type of cytokines they produce on stimulation. There are two extreme types of CD4+ Th cells, Th1 and Th2, based on the profiles of cytokines they secrete. Th1 cells need IL-12 for their induction and produce type one cytokines, mainly IL-2 and IFN-γ. Th2 cells require IL-4 for their induction and secrete

type 2 cytokine, such as IL-4, IL-5, IL-10 and IL-13 (Mosmann, 1991). Recently there is compelling evidence in the literature that the main sources of IL-10 are mycobacteria infected macrophages and the T_{reg} cells. The Th1, Th2 cells have also been shown to have different functions: Th1 cells and their cytokines help the activation of cells effective in controlling intracellular pathogens while the Th2 cells and their cytokines are effective against extracellular pathogens, reviewed in (Bretscher et al., 2001). In line with this, protective immunity against mycobacteria is considered to be mediated by Th1 as opposed to Th2 cells. Disruption of genes that code for type one cytokines, such as IFN-γ and IL-12 results in uncontrolled multiplication of mycobacteria in the lung of mice, and eventual death (Cooper et al., 1993; Cooper et al., 1997). Similarly, TNF-α receptor knockout mice or mice depleted of TNF-α by monoclonal antibody have defects in granuloma formation and are extremely susceptible to *M. tuberculosis* infection (Flynn et al., 1995). Furthermore, genetic defect in the IFN-γ receptor (IFN-γR1) gene in man results in high susceptibility to *M. tuberculosis* infection as well as to the normally non-pathogenic BCG vaccine (Jouanguy et al., 1996). On the other hand, recombinant gamma interferon administered via aerosol route together with the same anti-mycobacterial drugs, given previously under the regular treatment regimen, was reported to clear *M. tuberculosis* from the lung of patients infected with multi-drug resistant (MDR) bacilli (Condos et al., 1997). This is thought to be due to the role of rIFN-γ in stimulating the expression of the “signal transducer and activator of transcription” (STAT), and the “interferon regulatory factor” (IRF) genes in alveolar macrophages, which in turn induce the expression of high levels of pro-inflammatory cytokines in the broncho-alveolar fluid of the patients (Condos et al., 2003). From all these data, one can safely suggest that the

primary effector function of CD4+ Th1 cells is their secretion of type one cytokines, which activate macrophages to kill the bacilli. However, activated CD4+ T-cells can also directly kill mycobacteria infected macrophages by cytotoxicity (Boom, 1996). Macrophage activation plays a crucial role in combating infections with *M. tuberculosis*; it leads to the up-regulation of expression of MHC-I and MHC-II molecules as well as the production of reactive nitrogen and oxygen species that are believed to be responsible for the killing of the bacilli within the macrophage. This has been illustrated by the inducible nitric oxide synthase (iNOS) knockout mouse, which exhibits increased susceptibility to infection with the virulent strain of *M. tuberculosis* (MacMicking et al., 1997).

The presence of high levels of IFN-γ in *M. tuberculosis* infected mice and skin test sensitivity of tuberculosis patients, believed to be mediated by Th1 cells, suggest that the mere presence of Th1 cells and their cytokines may not protect the host from tuberculosis. The inability of Th1 cells and their cytokines to protect against tuberculosis in susceptible individuals is believed to be due to the simultaneous generation of Th2 cells and their cytokines that can counteract the effect of Th1 cells on macrophages. This has been substantiated by observations in mice and with human tuberculosis patients (Hernandez-Pando et al., 1996; Baliko et al., 1998; van Crevel et al., 2000). In contrast, an exclusive Th1 response generated upon *M. bovis* infection, following low dose BCG vaccination, is found to be effective in protecting calves from this *M. bovis* infection (Buddle et al., 1995). The implications of these findings are that vaccination should ensure the induction of Th1 cells while suppressing the generation of Th2 cells upon a

subsequent mycobacterial infection in order to be effective in controlling progression of tuberculosis. This is supported by clinical findings in which healthy contacts in tuberculosis endemic areas are found to express high levels of mycobacterial antigen-specific Th1 cytokines and the IL-4 antagonist IL-4δ2 while producing very few or no Th2 cytokines (Demissie et al., 2004). IL-4δ2 is a naturally occurring splice variant of human IL-4, which has a similar structure to IL-4 except a deletion of a few amino acids. It can bind to the alpha chain of the IL-4R as an antagonist blocking the activity of IL-4 (Vasiliev et al., 2003).

Another subset of T-cells that may be involved in immunity against tuberculosis infection is the CD8+ T-cell population. The role of CD8+ T-cells in protecting the host against tuberculosis is unclear, mainly due to conflicting observations from different studies with mice, and due to the fact that mycobacterial antigens are primarily processed via the exogenous antigen-processing pathway and presented on MHC class II to CD4+ T-cells. However, a role for CD8+ T-cells in controlling tuberculosis infection has been shown in β_2 microglobulin KO (β_2 $m^{-/-}$) mice. These mice lack MHC class I expression on the membrane of cells that render them more susceptible to infection due to a lack of CD8+ T-cell activity. Following high dose iv infection with virulent *M. tuberculosis*, the β_2 $m^{-/-}$ mice show increased bacterial burden in all organs examined and quickly succumb to infection (Flynn et al., 1992), suggesting the critical role CD8+ T-cells play during infection with virulent strains of *M. tuberculosis.* The high susceptibility of β_2 $m^{-/-}$ mice in this experiment could not be totally attributed to lack of CD8+ T-cells, as β_2 $m^{-/-}$ mice are deficient not only in CD8+ T-cells but also CD1 restricted NKT cells (Bendelac et al.,

1994). However, the dose and route of infection used in this experiment does not resemble natural infection, which is aerosol with 1-5 bacilli. Another study that employed low dose *M. tuberculosis* aerosol infection found that the effect of β_2 $m^{-/-}$ is not as dramatic as reported previously (Mogues et al., 2001). This study reported that CD8+ T-cells play a marginal role during immunity against natural infections with virulent strains of *M. tuberculosis,* as the consequence of the absence of CD8+ T-cells was very minimal on the survival time of the mice. Furthermore, both the above studies reported that the KO mice were not any different than control mice in controlling BCG infection, suggesting CD8+ T-cells are not important in controlling the non-virulent strains of mycobacteria. This is in agreement with previous studies that reported only a marginal or no role for CD8+ T-cells in controlling BCG infections in mice (Pedrazzini et al., 1987; Ladel et al., 1995; Xing et al., 1998). This might be due to the inability of the non-virulent BCG to escape from the phagolysosome into the cytoplasm, which is probably a requirement for antigens to be presented to CD8+ T-cells on class I MHC molecule. The effector function of activated CD8+ T-cells against the virulent strains of *M. tuberculosis* may depend on their ability to secrete type 1 cytokines, mainly IFN-γ, that can activate macrophages to kill the bacteria. However, the main mechanism by which CD8+ T-cells can protect against tuberculosis infection is their ability to directly kill infected macrophages via two different mechanisms:

(i) Receptor mediated killing, where the FasL is up regulated on cytotoxic CD8+ T-cells (CTL) upon antigen recognition to bind to the Fas molecule, which is constitutively expressed on target cells. This mechanism is good at killing the infected cells by apoptosis, but reports indicate this may not kill the intracellular pathogen, and may thus

contribute to the pathology by further spreading the pathogen if no activated macrophages are available to take up and kill the bacilli (Santucci et al., 2000). (ii) Exocytosis of granules, whereby CTL cells release their granular contents onto target cells to kill both the cell and the pathogen. Perforin is believed to insert itself into the surface of the target cell thereby forming a pore or hole on the surface of the infected cell through which Granzyme B can gain access to induce cell death by activating a cascade of caspases (Cooper et al., 1997). Even though Granzyme B is effective in killing the target cell by apoptosis, which may also lead to the killing of the pathogen, this mechanism is believed to be less effective in killing mycobacteria due to the high lipid content of their cell wall. This cytotoxicity may release viable mycobacteria to the extracellular environment, which, as stated above, will be beneficial only if activated macrophages are present to take up and kill the released bacilli. Granulysin, another protein isolated from the granular content of human CTL cells, interacts very well with lipid membranes and can activate lipid-degrading enzymes on the cell wall of the bacteria. The release of granulycin by CTL may thus result in the direct killing of mycobacteria inside infected macrophage (Stenger 2001).

In summary, a number of studies illustrate that mice depleted of CD4+ T-cells show a higher degree of susceptibility than those lacking CD8+ T-cells, suggesting the primary role of CD4+ T-cells in tuberculosis immunity. However, depletion of both cells further increases susceptibility, indicating that CD8+ T-cells may also play a role in protecting against *M. tuberculosis* infection. On the other hand, the absence of protection in $CD4^{-/-}$ mice with intact CD8+ T-cell population might mean that CD4+ T-cells are very important for the activation and maintenance of CD8+ T-cells during tuberculosis

infection. These observations may suggest a hierarchy that may exist among these T-cells in protecting against *M. tuberculosis* infection, the primary role being played by CD+ T-cells. However, the relative importance of each cell type may vary depending on the tissue involved. For example, CD8+ T-cells must play a great role if the bacilli are inside cells that do not express MHC class II molecules such as endothelial cells fibroblasts and epithelial cells. How the mycobacterial antigens are processed and how the peptides are loaded onto the MHC class I molecule so they can be presented to CTL is yet to be determined. Various studies have tried to address this problem (Grotzke and Lewinsohn, 2005). The most prominent mechanism is referred to as the cytosolic model, according to which microbial products from live mycobacteria form pores on the surface of the phagosome through which protein and lipid antigens and/or the bacilli escape into the cytosol. These proteins or/and bacteria in the cytosol will be processed via the endogenous antigen-processing pathway and presented on MHC class I. This is reported only from macrophages infected with live and virulent bacilli suggesting that it is probably the effect of their virulence factors (Mazzaccaro et al., 1996). On the other hand, Schaible and colleagues suggest mycobacteria or their antigens may not penetrate the phagosomal membrane to access the cytosolic antigen processing pathway, but rather live virulent bacilli induce apoptosis resulting in apoptotic vesicles that contain mycobacterial lipid and protein antigens. These apoptotic blebs will be taken up by bystander APCs, mainly DC, from the extracellular milieu and will be presented on MHC class I and CD1 molecules to CD8+ and CD1 restricted T-cells, respectively, by mechanisms that are as yet unclear (Schaible et al., 2003). These contrasting reports

suggest the various possibilities by which mycobacterial antigens can be processed and presented on MHC class I to activate cytotoxic T-cells.

Unconventional populations of T-cells – the CD1 restricted T-cells and the gamma delta (γδ) T-cells – are also believed to have a role in protective immunity against tuberculosis (Kaufmann, 2002). The CD1 restricted T-cells are phenotypically $CD4^{+}CD8^{-}$ or double negative T-cells bearing the αβ T-cell receptor (Brigl and Brenner 2004), which are most probably the NKT cells. The CD1 restricted T-cells recognize glycolipids abundant on the cell wall of the bacilli, such as mycolic acid and lipoarabinomannon, in the context of the CD1 restriction element. Functionally, they are reported to produce IFN-γ as well as to lyse target cells (Stenger, 2001). However, the pertinence of this is not clear, as macrophages, the main habitat of the bacilli, are believed to be devoid of CD1 molecules on their surface and hence cannot present glycolipid antigens to CD1 restricted T-cells (Naeim, 1996; Kaufmann, 2002). The γδ T-cells directly recognize unusual antigenic elements of the bacilli, such as phospholipids and heat shock proteins, either in the context of CD1 molecule or independent of any restriction element. Due to their ability to recognize antigens independent of MHC class I and II molecules, they are believed to be less specific as compared to the αβ TCR T-cells (Janis et al., 1989). These lymphocytes represent a small proportion of the T-cells in the mouse, on average 5% of those that express CD3. In man, most of the γδ T-cells are CD2+, CD4 and CD8 double negative; however, some activated γδ T-cells may express CD8 marker (Groh et al., 1989). The γδ T-cells are considerably more prominent in cattle, and are CD3+, CD2-, CD4-, CD8-, and express a unique 215 kD MW cell surface antigen called WC1 (Clevers et al., 1990;

Machugh et al., 1997). The level of γδ T-cells is higher in peripheral blood from tuberculosis healthy contacts and tuberculoid leprosy patients as compared to patients with sever pulmonary tuberculosis and lepromatous leprosy (Barnes et al., 1992), leading to the suggestion that these cells may play a role during protective immunity. In addition, the distribution of γδ T-cells in the body and their early appearance during tuberculosis infection in mice (Griffin et al., 1991), in cattle (Pollock et al., 1996; Cassidy et al., 2001), and in man (Barnes et al., 1992) have led to the conclusion that γδ T-cells may play a role as a primary line of defense during mycobacterial infections. The protective role of γδ T-cells, as described above for CD1 restricted T-cells, would likely be due to their ability to secrete type 1 cytokines that can activate macrophages in response to mycobacterial antigens. In addition, CD1 restricted and γδ T-cells can secrete perforin and granulysin that can directly kill the bacilli within the macrophages in a way similar to that mediated by CD8+ T-cells (Kaufmann, 2002). Activated NK cells that secrete high levels of IFN-γ and perforin were also observed to increase in number within the lung in the first three weeks of infection, suggesting these cells may be involved in protecting against tuberculosis. Despite this, depletion of NK cells had no influence on the pulmonary bacterial load, indicating these cells may not be an essential component of the protective mechanism (Junqueira-Kipnis et al., 2003).

Another subset of T-cells gaining attention in the recent literature is the regulatory T-cell subset. The role of regulatory T-cells in ensuring a fine balance between the host and the pathogen, thereby ensuring survival of the pathogen without inducing severe immunopathology to the host during chronic infection, is discussed in detail in a recent

review article (Belkaid and Rouse, 2005). Briefly, regulatory cells limit the magnitude of antigen specific immune responses and result in the failure of the host to clear infections. However, the regulatory cells also help to minimize collateral damage of host tissues, which would otherwise occur as a result of the strong immune response generated against the pathogen. Therefore, the host has to compromise and allow persistent chronic infection in order to avoid tissue damage and to maintain protective immunity against re-infection. In line with this, the level of T-regulatory cells is reported to increase during tuberculosis infection. The finding of increased level of $CD4+CD25^{hi}$ T-cells (Ribeiro-Rodrigues et al., 2006) and the cytokines they secrete, IL-10 and TGF-β (Olobo et al., 2001) during active tuberculosis infection in man, may suggest that these T-regulatory cells inhibit tissue destruction that would be caused by profoundly activated Th1. Paradoxically, suppression of Th1 cells by T-regulatory cells that inhibit the destructive process that leads to pathology, may allow the pathogen to replicate without check, leading to a clinical illness of the host due to a shift on the fine balance in the host-parasite relationship towards the pathogen. Whether this increase in T-regulatory cells during active *M. tuberculosis* infection is a natural host reaction to a very large inflammatory response, or a response to a bacterial product released to evade the immunity generated against the mycobacteria, is not yet clear.

Another subset of T-cells recently reported to play a role in immunity and pathogenesis of mycobacterial infections is the Th17 cells. These cells produce IL-17 under the influence of IL-23 (Harrington et al., 2005). TGFβ in combination with IL-6 is also reported to induce the differentiation of IL-17-secreting Th17 cells from naïve CD4+ T-

cells in mice (Bettelli et al., 2006). Although IL-17 is widely considered to be a product of CD4+ Th17 cells, γδ T-cells were the main source of IL-17 during experimental infection of mice with virulent *M. tuberculosis* (Lockhart et al., 2006), as well as BCG (Umemura et al., 2007). IL-17 is a pro-inflammatory cytokine that stimulates the induction of inflammatory cytokines and chemokines responsible for the recruitment and activation of neutrophils, and was reported to play a role in several autoimmune and inflammatory diseases, reviewed in (Steinman, 2007). Furthermore, reports from infectious mouse model have suggested that Th17 cells may play a role in mediating protection against extracellular bacteria and fungi, including *Candida albicans* (Huang et al., 2004), *Klebsiella pneumoniae* (Happel et al., 2003), and *Bordetella pertussis* (Higgins et al., 2006). On the other hand, the exact role of IL-17 in immunity against intracellular pathogens, including *M. tuberculosis* is not yet clear. However, its pro-inflammatory nature suggests that IL-17 may play a role in pathogenesis and lesion development during mycobacterial infections. Evidence, which supports this view, comes from mouse experimental studies in which IFN-γ deficient mice were reported to have increased numbers of IL-17-producing T-cells that induced tissue damage due to a profound inflammation following BCG infection (Cruz et al., 2006). The immunopathology was not severe in normal mice; furthermore, exogenous IFN-γ was able to reduce the frequency of BCG-specific IL-17-producing T-cells in vitro, suggesting that IFN-γ counter-regulates the secretion of IL-17 to limit tissue damage during mycobacterial infections. In contrast to this, recent studies in mice have shown that IL-17 may play a role in early immunity during *M. tuberculosis* infections by recruiting IFN-γ producing T-cells into the lung and enhancing granuloma formation, as IL-17 deficient mice were

reported to produce less IFN-γ and show impaired granuloma formation (Umemura et al., 2007). These data suggest that IL-17 may play a protective role during the early phase of infection, but can also contribute to the immunopathology during the chronic stage of the disease if not counter-regulated by Th1 cells.

2.6.2.2. Macrophages

It has been repeatedly reported that macrophages activated with IFN-γ and other type 1 cytokines have anti-mycobacterial activity in vitro (Rook et al., 1986). This anti-mycobacterial activity is due to the ability of activated macrophages to produce reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Ding et al., 1988). ROI, generated by the transfer of an electron from NADPH to molecular oxygen in the presence of NADPH-oxidase, may play a role in controlling the multiplication of mycobacteria in macrophages. However, the most potent anti-mycobacterial activity of macrophages is their ability to produce RNI, mainly nitric oxide (NO), from L-arginine via the action of inducible nitric oxide synthase, iNOS2 (Chan et al., 1995). This has been shown experimentally in mice in which deletion of the gene that codes for NOS2 was found to be lethal for mice infected with *M. tuberculosis* (MacMicking et al., 1997), while mice unable to produce ROI were only slightly affected in their ability to control tuberculosis infection (Cooper et al., 2000). The central role of IFN-γ in the activation of macrophages and in the control of infections with *M. tuberculosis* has been shown in vivo both in mouse and human studies. As discussed in section 2.6.2.1, mice with a targeted disruption of IFN-γ genes (Cooper et al., 1993; Flynn et al., 1993) as well as human

individuals genetically deficient in IFN-γ receptor (Jouanguy et al., 1996; Sasaki et al., 2002) were found to be highly susceptible to mycobacterial infection including the non-virulent BCG.

2.6.2.3. Innate Immunity

Mycobacteria can attach themselves to macrophages for phagocytosis by binding to various receptors, including Toll-like receptors on the surface of macrophages. Immediately after ingestion by macrophages, the mycobacteria trigger the transcription of genes that code for various pro-inflammatory cytokines via the NF-κB pathway (Ragno et al., 2001). Engagement of Toll-like receptors, mainly TLR2 and TLR4, on alveolar macrophages is believed to be the mechanism by which mycobacteria induce transcription of pro-inflammatory cytokines during the early stage of infection. Most Toll-like receptors use myeloid differentiation factor 88 (MyD88) as an adaptor molecule to activate the NF-κB pathway and act as a bridge between the innate and the adaptive immune system. This has been shown experimentally in which MyD88-/- mice failed to control *M. tuberculosis* infection and rapidly succumbed to the disease, as compared to control mice, due to failure of both the innate and adaptive immune response (Scanga et al., 2004). On the other hand, there is a recent report, which claims that MyD88 signaling is dispensable in generating an acquired immune response to mycobacteria as there were equal amounts of activated T-cells and cytokines of the adaptive immune response both in Myd88-/- and control mice following infection with virulent *M. tuberculosis* (Fremond et al., 2004). In this case, both the Myd88-/- and control mice were comparable in their ability to resist infection. How can one reconcile these contrasting findings? The findings

from the later group can be explained by the fact that TLRs may use adaptor molecules other than MyD88 to induce signal transduction. For example, TLR4 uses CD14 surface protein as a co-receptor to signal via TIRAP/MyD88 or via TIR domain-containing adapter inducing interferon beta (TRIF), which is attached to TRIF-related adaptor protein (TRAM). TLR3 solely induces signal transduction via TRIF independent of MyD88 (Akira and Takeda, 2004). Furthermore, the commonly accepted concept that innate immunity is a springboard to orchestrate the adaptive immune response is being challenged. Some well-known personalities in the field suggest that innate immunity may not be a prerequisite for the generation of antigen specific adaptive immune response. There are observations from more recent studies, which support this idea. Immunization of MyD88/TRIF double KO mice with protein antigens in the presence of Complete Freund's Adjuvant (CFA) was reported to induce a robust antigen specific, T-cell dependent, antibody response in the absence of Toll-like receptor signaling (Gavin et al., 2006). However, there is ample evidence both from in vitro (Underhill et al., 1999; Thoma-Uszynski et al., 2001) and in vivo (Sugawara et al., 2003) experiments that TLR2 signaling via the MyD88 dependent pathway has an important role in controlling mycobacterial infection. On the other hand, the role of TLR4 signaling during mycobacterial infection is still controversial, as $TLR2^{-/-}$ mice were found to be more susceptible than $TLR4^{-/-}$ mice when exposed to high dose of virulent strains of *M. tuberculosis* under similar conditions (Reiling et al., 2002). In addition, studies with C3H/HeJ mice, which have a single amino acid mutation on TLR4 that make them LPS hypo-responsive, suggests that TLR4 may not play a significant role in tuberculosis immunity, as there was no difference in pathology and immune responses between the

TLR4 deficient C3H/HeJ and control mice, of the same background (C3H), following aerosol exposure to a low dose of *M. tuberculosis* (Kamath et al., 2003; Shim et al., 2003). It is also reported that most mycobacterial cell wall components and culture filtrates are agonists of the TLR2 (Underhill et al., 1999), strengthening the notion that TLR2 is important for signal transduction during mycobacterial infections. However, results from in vitro studies suggest that different mycobacterial components may interact with different members of the toll like receptor family and that both TLR2 and TLR4 can be used for signaling. In a recent study, it was reported that the HSP65 signals exclusively through TLR4 while HSP70 signals through both TLR2 and TLR4 (Bulut et al., 2005). Similarly, soluble heat stable factors from virulent *M. tuberculosis* strains were found to induce TLR2 mediated signaling while a heat labile, cell wall associated mycobacterial factor, mediates TLR4 dependent signaling (Means et al., 1999; Means et al., 2001). In addition, there are reports from in vivo experiments where $TLR4^{-/-}$ (C3H/HeJ) mice were found to succumb to the disease earlier than the corresponding wild type mice following low dose aerosol infection with virulent *M. tuberculosis*. These experiments show that TLR4 signaling can be involved in protecting against tuberculosis in contrast to a previous experiment done under similar conditions, dose and route, of infection (Abel et al., 2002; Branger et al., 2004). These contradictory reports show that our knowledge regarding the signal transduction mechanisms during *M. tuberculosis* infection is far from sufficient and needs further investigation.

The role of other TLRs, other than TLR2 and TLR4, during immune responses against mycobacterial infection does not seem to be clear. As *M. tuberculosis* is intracellular,

TLRs expressed in intracellular organelles may play a role in recognizing mycobacterium specific ligands. One such receptor is TLR9, which recognizes un-methylated CpG motifs in bacterial and viral DNA, and is known to be localized in the endosomes and phogolysosomes of the cell (McCluskie and Krieg, 2006). This implies that mycobacteria, rich in CpG and residing in the phogolysosomes, can be easily recognized by TLR9 for signal transduction. There is a recent report that supports this hypothesis (Bafica et al., 2005). According to this study, $TLR9^{-/-}$ mice displayed defective mycobacterial induced IL-12 and IFN-γ response in vivo, and were found to be more susceptible than wild type mice to low dose aerosol challenge with *M. tuberculosis*. There was also a decreased production of pro-inflammatory cytokines by macrophages and dendritic cells in response to live mycobacteria in vitro. Susceptibility to infection was markedly enhanced in $TLR2/9^{-/-}$ (double knockout) mice when compared to $TLR2^{-/-}$ or $TLR9^{-/-}$ mice, showing that signaling via TLR9 in cooperation with TLR2 was able to induce a better Th1 type response against *M. tuberculosis* infection in vivo. Another intracellular pattern recognition receptor (PRR) that may play a role in immunity against intracellular pathogens is the nucleotide-binding oligomerization domain 2 (NOD2) receptors. The NOD2 receptor is a cytoplasmic protein that can recognize bacterial peptidoglycans from both gram-positive and gram-negative bacteria through interaction with muramyl dipeptide (MDP) (Girardin et al., 2003). *M. tuberculosis*, with a cell wall rich in peptidoglycans, makes NOD2 a highly suitable candidate for the recognition of mycobacteria inside macrophages. Even though NOD2 is located in the cytosol and not inside the phagolysosome, it is possible that mycobacterial antigens escaping into the cytosol can be easily recognized by NOD2 for signal transduction. In line with this, there

is a recent report from an in vitro study showing that mycobacteria can induce signal transduction and transcription of pro-inflammatory cytokines via NOD2. The recognition of *M. tuberculosis* via NOD2 is non-redundant with other TLRs, but synergizes for the induction of pro-inflammatory cytokines (Ferwerda et al., 2005).

2.6.2.4. Immune Evasion Mechanisms in Mycobacteria

There are a lot of review articles in the literature discussing in great detail mechanisms utilized by mycobacteria to evade the host immune response and to induce disease. The aim of this section is not to discuss all the details of immune evasion mechanisms utilized by mycobacteria; however, I will briefly discuss these mechanisms as it might help the reader to understand the host-pathogen relationship, particularly in susceptible individuals. Mycobacteria live within macrophages, which are believed to be able to kill the bacilli if properly activated. To gain entry into host macrophages, mycobacteria use various receptors such as the complement receptors, mannose binding receptors, lectin binding receptors, Fc receptors, scavenger receptors and Toll-like receptors on the surface of macrophages. However, they may use some receptors more frequently than others to avoid the activation of macrophages and thereby to ensure their survival within the macrophages. To this end, the pathogen looks for “surreptitious entry” to gain access to the macrophages by a mechanism that avoids activating the oxidative burst of the host cell. Therefore, it is to their advantage if the mycobacteria gain entry via complement receptors and/or the mannose binding receptors on the surface of macrophages, which are not linked to MyD88. Activation of the NF-κB pathway in the absence of MyD88 is believed to induce transcription of Th2 cytokines (IL-4, IL-10, IL-13) that do not

activate, but inhibit activation of macrophages against mycobacteria (Doherty and Arditi, 2004). It is also reported that MyD88$^{-/-}$ mice failed to make antigen specific IgG_{2a}, while producing high level of IgG_1 and IgE antibodies against the same antigen (Schnare et al., 2001). Furthermore, MyD88/TRIF double KO mice showed higher levels of IgG_1 and IgE antibodies in their pre-immune sera as well as in response to antigens administered with CFA adjuvant, at least in some time points after vaccination, than IgG_{2a} antibodies as compared to wild type mice (Gavin et al., 2006). These observations further strengthen the notion that entry of mycobacteria into macrophages via receptors, which are not attached to the TLR signaling pathway, may induce a Th2 type response, which is not effective in controlling their intracellular replication. Mycobacteria have also developed mechanisms to circumvent the host immune response so that they can live in one of the most hostile organelles of the activated macrophages. They are able to do this by using host molecules to their advantage in addition to proteins and glycolipids of the mycobacterium's cell wall. A host protein molecule known as the tryptophan-aspartate-containing coat protein (TACO) is recruited by pathogenic bacilli and retained in the phagosome thereby inhibiting phagolysosome formation and bacterial killing. This was observed in phagosomes containing live bacilli but not by those containing heat killed bacilli (Ferrari et al., 1999). Similarly, virulent mycobacteria can inhibit phagolysosome fusion by utilizing other host molecules involved in the biosynthesis of molecules required for the biogenesis of the phagolysosome (Flynn and Chan, 2003). In addition to host molecules, microbial proteins, specifically the mycobacterial protein kinase G, *pknG*, secreted only by pathogenic mycobacteria, are also found to inhibit phagolysosome fusion (Nguyen and Pieters, 2005). Glycolipid components of the

mycobacterial cell wall, such as the lipoarabinomanan (LAM) and sulfatides/sulfolipids (ST), are also reported to contribute to the immune-evasion mechanisms of mycobacteria, either by scavenging the ROI produced during the oxidative burst or by inhibiting the generation of the highly lethal RNI compounds (Chan et al., 1989; Chan et al., 1991). Moreover, the sulfatides appear to promote the survival of the bacilli within the macrophages by inhibiting phagolysosome formation (Zhang et al., 1988). On the other hand, LAM can either induce or inhibit activation of macrophages depending on their structural configuration. Mannose-capped Lipoglycans (ManLAM) are believed to be anti-inflammatory as they suppress production of IL-12 and TNF-α and induce IL-10 production independent of the TLR2 and MyD88 pathway (Nigou et al., 2001), whereas those capped with phosphoinositide residues (PILAM) and uncapped LAM are pro-inflammatory as they stimulate secretion of type 1 cytokines via a TLR2 dependent NF-κB pathway (Means et al., 2001). The anti-inflammatory effect of ManLAM is believed to be due to their preferential binding to mannose receptors, the lectin-binding receptors or other macrophages surface receptors that are not linked to MyD88 and hence do not lead to induction of the NF-κB pathway (Nigou et al., 2001; Quesniaux et al., 2004). Besides, the 19kDa mycobacterial lipoprotein is reported to have immunosuppressive effects as it can interfere with antigen processing, presentation and cytokine secretion as well as expression of MHC II by antigen presenting cells (Noss et al., 2001; Flynn and Chan, 2003). It is also reported that mycobacteria produce significant amounts of ammonia that inhibits acidification of lysosomes, which is a requirement for phagolysosome maturation (Armstrong and Hart, 1971; Gordon et al., 1980). The ability of virulent strains of mycobacteria to escape from the phagolysosome into the cytoplasm

may also help the bacilli to avoid the highly acidic environment of the vesicle (Ladel et al., 1995). The mycolic acid of the cell wall can also offer some sort of physical protection and helps the bacterium to be resistant to the proteolytic effect of the acidic phagolysosome (Glickman et al., 2000). Even though virulent strains of mycobacteria are endowed with these all evasion mechanisms, epidemiological data show that 90% of individuals infected with *M. tuberculosis* are able to overcome all these "tricks" by the bacteria and contain disease progression. This may suggest that evasion mechanisms are phenomenon associated with susceptible individuals.

In summary, all the data discussed in this section suggest to me that a good understanding of diseases pathogenesis, the host response to the pathogen, including the type of immune response generated in healthy contacts and tuberculosis patients, are the key for the development of effective intervention methods. Understanding the role of innate immunity in combating the disease in the healthy contacts may also help to reveal the role of innate immunity in the overall fight by the host against the pathogen. Manipulation of innate immune responses may help to contain/decrease the replication of the bacteria during the first few weeks before the host mounts adaptive immunity. This in turn may have an effect on the type of adaptive immunity generated by decreasing the dose of the bacteria and paving the way for the generation of an effective Th1 type response. Similarly, knowledge on how the pathogen dampens the generation of effective immunity or/and evades an otherwise effective host response may help in designing targets for the development of new drugs, immunotherapy or vaccines, as well as to improve the efficacy of the existing one.

2.7. Tuberculosis in Laboratory Animals

Laboratory animals are very important for the study of the host-pathogen relationship during tuberculosis infection, including studies aimed at understanding virulence, pathogenesis and the significance of the host immune response. It was Villemin, a French military surgeon, who first used laboratory animals to prove that tuberculosis is an infectious disease, reviewed in (Daniel, 1997). In his study he infected two rabbits with an extract from material obtained from lesions of tuberculosis patients and observed the rabbits for about four months. The rabbits did not show severe signs of illness, but on postmortem, lesions similar to those in the lungs of human patients were found in the lung of one rabbit. No lesions could be seen in the second rabbit. This difference might have been due to the susceptible and resistant phenotype of the two rabbits to the virulent *M. tuberculosis* infection, as described by Lurie several years later (Lurie et al., 1952). The story might well have been different had he infected guinea pigs as discussed later in this section. Max Bernard Lurie in the early 1900s performed an extensive study to examine the fate of *M. bovis* and *M. tuberculosis* in various organs, namely spleen, liver, bone marrow, kidney and lung (Lurie, 1928), and the histological lesion the bacilli induced in those organs (Lurie, 1932) following low and high dose intravenous injection of the these bacteria. One of his remarkable findings from these studies was that rabbits are relatively resistant to the human form of tuberculosis and behaved in a similar manner upon infection with the less virulent BCG or the virulent *M. tuberculosis*. Following infection with either of these bacilli, the burden of the bacilli increased exponentially in the first few weeks, followed by effective control of bacterial growth in all organs, albeit at different times after infection. However, Lurie showed the same rabbits to be highly

susceptible to the bovine type of tuberculosis, with the development of lesions in the lung similar to those seen in human tuberculosis patients. Rabbits infected with *M. bovis* died within a short period of time due to the unabated replication of the bacilli in the lung and kidney, despite the ability of the rabbits to control growth of the bacilli in liver, spleen and bone marrow. In general, both *M. bovis* and *M. tuberculosis* replicate in rabbits without hindrance for the first few weeks; then, at about 3-4 weeks post-infection *M. tuberculosis* is destroyed in most organs but merely contained in the lung and kidney. However, *M. bovis* still replicates progressively in the lung and kidney, while it is contained in other organs. Another remarkable observation made during this study was that, even though rabbits are more susceptible to bovine tuberculosis than to the human type of tuberculosis, *M. bovis* grows at a much slower rate than *M. tuberculosis* in vivo (Lurie, 1928; Lurie, 1932). Lurie was also a pioneer in describing the role of genetics in susceptibility/resistance of laboratory animals to tuberculosis infection. He categorized rabbits as being of susceptible or resistant phenotype depending on their survival time when infected with *M. bovis* and whether infection resulted in post-mortem lung pathology following infection with *M. tuberculosis* (Lurie et al., 1952). Resistant rabbits infected with *M. tuberculosis* show no signs of tuberculosis lesion in their lung and no bacteria were isolated from their organs, as the animals were able to destroy the bacilli in all organs, including those in the lung, following the generation of an adaptive immune response. In susceptible rabbits, replication of the bacilli is controlled but not completely inhibited in the lung and kidney, resulting in a variable degree of pathology in the lung. All descriptions of tuberculosis in rabbits refer to the disease model in susceptible rabbits.

Another animal extensively used in the study of tuberculosis is the guinea pig. It was shown to be highly susceptible to *M. tuberculosis* infections. The growth of the bacilli, as well as the course of the disease, was found to follow the same trend as observed for bovine tuberculosis in rabbits. The bacilli replicate exponentially for the first three weeks and then remain at a stationary level until the end of the experiment or the death of the animal (Smith et al., 1970; Alsaadi and Smith, 1973). The pathology induced in the lung of guinea pigs infected with *M. tuberculosis*, including the histological structure of the granuloma, is more or less identical to the lung lesion observed in susceptible human tuberculosis patients and rabbits infected with *M. bovis* (Smith and Wiegeshaus, 1989). These observations led to the suggestion that guinea pigs are a good model for tuberculosis in man in the same way as rabbits are a good model for bovine tuberculosis. However, the disease in susceptible humans and cattle is mainly confined to the lung, unlike in guinea pigs and rabbits, which show tuberculosis lesions outside the lung, mainly in the spleen of *M. tuberculosis* infected guinea pigs and in the kidney of *M. bovis* infected rabbits. Similarly, many studies during the 1950s analyzed how the course of the disease and the burden of the bacilli varied in various organs of the mouse following intravenous infection with virulent strains of *M. tuberculosis* (Pierce et al., 1953; McCune et al., 1956; Sever and Youmans, 1957). More recently, North and colleagues have investigated the fate of the bacilli and the course of the disease in mice following infection with 10^5 cfu given intravenously or 10^2 cfu administered intranasally. Accordingly mouse strains were categorized as susceptible and resistant based on median survival time after infection with 10^2 cfu of BCG aerosol or 10^5 cfu iv (Medina and North, 1998). One can conclude, from all these mouse studies that the course of the

disease in the lung of resistant mice is more or less identical to the course of the disease in the lung of guinea pigs and susceptible rabbits infected with the virulent strain of *M. tuberculosis*. As summarized by North and Jung in their recent review article, *M. tuberculosis* grows progressively at a linear log scale with a generation time of about 28 hours for the first three weeks in the lung of all three laboratory animal species (guinea pigs, susceptible rabbit strains and resistant mouse strains). At around day 21 post infection, bacterial replication is contained at a stationary level due the generation of an adaptive immune response, which inhibits a further increase in the burden of the bacilli and slows the course of the disease (North and Jung, 2004). Even though replication of the bacilli is stabilized at a stationary level in the lung, the disease in all the laboratory animal species is not resolved and ultimately leads to the death of the animals at different times after infection. In general, lesions in the lung of guinea pigs, susceptible rabbits and resistant mice infected with *M. tuberculosis* displayed pathology more or less similar to that seen in human tuberculosis patients, except for the less organized nature of the granuloma in mice. This indicates that all the laboratory animals can be used equally well as models of tuberculosis in man. However, for logistical reasons, including cost of handling, availability of laboratory reagents, mouse is the preferred model to study human tuberculosis. Although there are similarities in the immune response generated against *M. tuberculosis* in man and mouse, there are still quite considerable differences in the pathology of the disease. The granulomas that develop in resistant mouse strains are not well developed with all the organized structures seen in man but are simply an aggregate of lymphocytes and macrophages distributed among intact air sacs (Boshoff and Barry, 2005). Some even argue that there is no such thing as a granuloma in the lung

of mice and granuloma in mice refers to those formations seen in the spleen and liver of resistant mice. Besides, lesions in organs of susceptible mice are diffused, necrotic, contain few lymphocytes and are dominated by neutrophils, which are more or less similar to tuberculosis lesions in organs of AIDS patients (Chaisson et al., 1987). One important point worth mentioning here, that might contribute to the difference in pathology when comparing *M. tuberculosis* infection in mice and humans, is the dose and route of infection in all mouse studies, which are very different from the natural route and dose of infection that occurs in man. Even though mice are frequently used as an animal model of tuberculosis in man for reasons mentioned above, none of these animal models are able to exhibit the latent form of tuberculosis seen in more than 90% of tuberculosis infected individuals, making these animal models deficient to study all aspects of human tuberculosis. One of the reasons for the failure of these laboratory animals to develop the latent form of the infection could be the high dose of mycobacteria used to challenge the animals in all the studies. If it were not for cost and ethical reasons, the best animal model to study human tuberculosis would be a non-human primate. Infection of these animals with a low number of viable bacteria of *M. tuberculosis* results in a disease course more or less similar to the course observed in man, in the sense that only a small percentage of the infected monkeys develop disease in a short period of time, whilst others develop chronic infection and then succumb to the disease after some time. The granuloma in the lung of chronically infected primates is identical to the granuloma seen in the lung of human tuberculosis patients both in terms of gross lesion and microscopic structures. What is surprising is that more than 40% of the monkeys did not develop disease at all during the study period, and were believed to have the latent form of the

disease as different diagnostic tests have confirmed that they were all infected with the bacilli (Capuano et al., 2003). There is also some evidence that *M. bovis* infection in cattle can develop a form of latency which is similar to the latent type of TB seen in man (Cassidy, 2006).

2.8. The BCG Vaccine

Tuberculosis in man is a curable disease, which can be effectively treated with antibiotics. However, effective antibiotic treatment is undermined by a number of factors. As it is a disease of poverty, only 1 in 5 tuberculosis victims are estimated to have access to treatment (Raviglione et al., 1995). This could be mainly due to individual financial means of the patients, but there is often a failure to detect and hence treat new cases due to poor health delivery systems in most countries. One study estimated the detection rate worldwide to be as low as 27% for the years 1980 to 2000 (Dye et al., 2002). Even for those who have access to chemotherapy, effective treatment requires a long course of antibiotic administration and there is often low compliance. This favors the appearance of multi-drug resistant strains of the bacilli. According to one study, 3.2% of the new tuberculosis cases diagnosed in 2000 were caused by multi-drug resistant bacilli (Dye et al., 2002). The number was even higher in 2005: around 10% of global tuberculosis cases were found to be resistant to more than one drug (Kaufmann, 2006a). All these intrinsic limitations of chemotherapy speak to the fact that vaccination, should this be possible, is a more viable option for controlling tuberculosis, particularly in developing countries, where poor socio-economic conditions and less organized health care systems greatly limit the success of chemotherapy in combating the scourge of tuberculosis. Bacille-

Calmette-Guérin (BCG) is the only registered vaccine against tuberculosis both in man and animals. It is the most widely used vaccine globally; however, there is still controversy as to its efficacy in protecting against the highly contagious form of pulmonary tuberculosis. In this section, I will discuss the history of BCG as a vaccine, some experimental evidence pertinent to its efficacy, as well as some advantages and side effects of the BCG vaccine. This will eventually lead to our hypothesis on how we think the efficacy of the BCG vaccine can be improved.

2.8.1. History of BCG

In addition to his major discovery of the causative agent of tuberculosis, Robert Koch was the first to experimentally prove that immunity exists against tuberculosis. When he inoculated guinea pigs already ill with tuberculosis, with a few mycobacteria, killed or live, or with a culture filtrate, he found that the lesion on the skin becomes dark, necrotic, eventually sloughed off, and this resulted in healing of the wound. This unique type of immune reaction has come to be known as Koch's Phenomenon (Bothamley and Grange, 1991). Koch was trying to develop an immunotherapeutic treatment of tuberculosis by administering the substance in filtrates from mycobacterial culture. He is, therefore, considered to be the pioneer in subunit vaccine development. However, this was a great failure for Koch as the administration of his magic protein failed to treat tuberculosis in man (Kaufmann, 2006a). Despite its failure to cure against tuberculosis, Koch's culture filtrate, now known as PPD for purified protein derivative, was the basis for the development of the skin test that has been used for the last 100 years and is still in use to differentiate between those infected/exposed to *M. tuberculosis* from uninfected healthy

individuals. Knowing of the earlier work of Jenner and Pasteur on vaccination and inspired by the attempt of Koch to cure tuberculosis, Albert Calmette and Camille Guérin started their work on tuberculosis in 1900 in France. The ultimate goal of their research, conducted in the Pasteur Institute, was to develop a vaccine against tuberculosis. It was prudent of them to look for an equivalent of cowpox (Jenner's vaccine for small pox) and they found the etiological agent of tuberculosis in cattle, *M. bovis*, to be the appropriate candidate. They grew the bacilli on culture media containing ox bile and after ~213 passages on this media, for over a period of 13 years, the bacillus was no longer virulent in a variety of experimental animal models. To their surprise, the vaccine was not only safe to use but also was able to protect animals from challenge with virulent *M. tuberculosis* and *M. bovis* (Calmette and Plotz, 1929). After assessing its safety in various laboratory animals, including monkeys, they decided to explore its efficacy in man. It must have been ethically tough to get an appropriate human subject to try their new vaccine. In 1921, they chose a newborn baby whose mother had died of tuberculosis and who was going to be looked after by his grandmother, who also had tuberculosis. The assumption was that the baby would develop tuberculosis and might die; but to the amazement of all he grew up healthy with no tuberculosis, reviewed in (Daniel, 1997). In a short period of time, hundreds of thousands of infants were vaccinated in Europe with no serious side effects. This fact encouraged the then League of Nations to recognize, in 1929, BCG as a safe vaccine for human use following the approval of the vaccine by the microbiologists who were attending an international conference organized by the medical section of the League of Nations and held in Paris, France, in October 1928 (Calmette and Plotz, 1929). However, BCG received only a lukewarm reception in North America,

mainly due to reports of a low rate of protection (14%) in school children in the southern United States (Comstock and Palmer, 1966). It was also clear that the Lübeck catastrophe in Germany, resulting from the injection of children with mislabeled *M. tuberculosis* instead of BCG, had a widely negative impact on the popularity of BCG. Out of the 251 infants vaccinated, 212 developed active tuberculosis and 77 died (Kaufmann, 2006a).

2.8.2. Efficacy of BCG Vaccination

Since its introduction in 1921, BCG has been administered to almost three billion people globally (Kaufmann, 2006a). However, there is still considerable uncertainty as to its efficacy against tuberculosis. Several controlled clinical trials have been conducted in several countries to evaluate the protective efficacy of BCG vaccination. Unfortunately, those trials, as summarized in various review articles (ten Dam, 1984; Fine, 1995) led to disparate conclusions, with the assessed protection rate against pulmonary tuberculosis ranging between 0% to 80%. The last World Health Organization-sponsored trial, called the Madras trial, involving over a quarter million subjects, was carried out between 1968 and 1971 in the Madras province of India. The study led to the conclusion that BCG vaccination had no overall protective effect against tuberculosis (Baily, 1980; ten Dam, 1984). This conclusion has been the cause of considerable pessimism. However, it must be pointed out that some BCG trials in children gave incontrovertible evidence that BCG vaccination was protective with an efficacy of 84% in one case (Hart and Sutherland, 1977). In addition to this, a meta-analysis of all the published literature has shown that BCG vaccination can have an overall protective effect of 50% (Colditz et al., 1994). The variables, which might have contributed to this discrepancy in efficacy of BCG

vaccination, will be discussed in detail in section 2.8.3. Generally, it is agreed that BCG vaccination is effective in protecting children against TB meningitis and disseminated TB with close to 80% efficacy. These facts have led to the inclusion of the BCG vaccine into the Expanded Program on Immunization (EPI) in 1974 and currently around 118 million doses are delivered each year (Fine et al., 1999).

2.8.3. Factors Which May Affect Efficacy of BCG Vaccination

There has been no extensive and systematic research to evaluate how the efficacy of BCG vaccination depends on various parameters of vaccination. All the observations come from different trials, which usually differ in more than one parameter of vaccination. It is clear that there are many variables that differed between each vaccination program, and these variables might have resulted in the disparity in the level of protection achieved in different trials. The most obvious variables are those directly or indirectly associated with the host, the BCG vaccine itself, and the environment under which the vaccination programs were carried.

2.8.3.1. Host Factors

Host genetics plays a role in determining susceptibility/resistance to tuberculosis infection, in part by affecting the nature of the immune response generated against the tubercle bacilli, discussed in detail in section 2.3.1. By the same token, host genetics may also play a role in the type of immune response generated after BCG vaccination. In mice the *Nramp1* gene plays a role in determining the replication rate of the BCG in the

animal's body in the first few weeks after infection before immunity is evident. Mice have been categorized as resistant or susceptible at the level of innate resistance depending on whether their macrophages fail to support or support rapid growth of BCG. In the susceptible strains, BCG replicates at an exponential rate without any opposition from the host for the first few weeks, until it is held in check by adaptive immunity. However, in the resistant mouse strains, replication of the bacilli is much slower and never exceeds the number employed for infection. The BCG burden is reduced in both strains by the generation of an adaptive immune response at around three weeks post-infection (Gros et al., 1981). Even though the effect of the *Nramp1* gene is in the early stage of infection (innate immunity), its effect in controlling bacterial multiplication at the early days of infection may have an effect in determining the nature of the BCG-specific adaptive immune response generated. Many experiments demonstrated that antigen dose is important in determining the Th1/Th2 nature of the ensuing immune response, with lower doses favoring the generation of a predominantly Th1 response. Therefore, mice with *Nramp1* resistant allele will harbor lower levels of BCG than susceptible mice, which will favor the long-term generation of Th1 cells during the generation of an adaptive immune response. Furthermore, there are reports which show differences between blacks and their white counterparts in the level of protection against *M. tuberculosis* following BCG vaccination (Comstock and Palmer, 1966), further suggesting an influence of host genetics on BCG-specific adaptive immune response and hence on the efficacy of BCG vaccination. In addition to host genetics, host age at the time of vaccination and nutritional status of the host may also have an influence on the type of response generated following BCG vaccination. The effect of age on the efficacy

of BCG vaccination can be seen in the context of exposure of the host to cross-reacting environmental antigens that induce an immune response that can easily interfere with the BCG vaccine. This has been mentioned several times in other sections of this thesis, and is briefly discussed in the next section, (section 2.8.3.2). There is well documented evidence from animal studies which shows the effect of nutrition, mainly minerals, vitamins and protein nutrients on cell-mediated immunity (Chandra, 1997). This may suggest that deficiency of these nutrients due to malnutrition of the host can negatively influence the generation of effective cell-mediated immunity against mycobacteria following BCG vaccination. Evidence both from humans studies (Adeiga et al., 1994) and experimental animal studies (McMurray and Yetley, 1982) support the possibility that malnutrition can adversely affect the efficacy of BCG vaccination. Finally, there is a theoretical possibility that maternal antibody may interfere with ability of BCG vaccine to induce effective immunity against tuberculosis; however there is no hard evidence, either from human vaccination studies or animal experimental studies, to support this possibility.

2.8.3.2. The Environment

Exposure of the host to cross-reacting environmental microbes can induce an immune response, which may well interfere with BCG vaccination. In order to discuss this interference in a comprehensible fashion, we will have to define some immunological phenomenon, which may help us to understand the cellular mechanisms that govern this immunological interference. Animals exposed to antigen in a manner that induces humoral immunity are unable to generate a cell-mediated response to the same antigen, a

phenomenon known as humoral immune deviation, as the immune response is already locked into a Th2, humoral mode. Animals immunized in a manner to express a predominant cell-mediated, Th1, response can become unresponsive for the induction of an antibody, Th2, response. The response has become locked into a cell-mediated, Th1 mode. This phenomenon is referred to as cell-mediated immune deviation. We can respectively refer to cell-mediated and humoral immune deviation as due to Th1 and Th2 imprinting. These findings have direct implication for the design of the studies described here as will be discussed in great detail later in section 2.9. In line with this, neonates were found to readily respond immunologically to environmental mycobacteria in the first few weeks of their life, as reported both in man (Marchant et al., 1999) and cattle (Buddle et al., 2002). This exposure may result either in Th2 imprinting, which would inhibit the generation of a protective, Th1, type response upon BCG vaccination, or in weak immunity that is effective against BCG undermining its ability to generate a protective type of response following vaccination. One of the compelling explanations given as contributing to the failure of the Madras trial is the interference or masking of BCG specific immune response by prior infection with environmental mycobacteria (Fine 1995; Smith et al., 2000). There is evidence in the literature, which supports this explanation from human studies in Malawi and Gambia. Vaccination of PPD reactive adults with BCG resulted in very low BCG-specific IFN-γ production (Black et al., 2001), whereas BCG vaccination at birth induced strong BCG- specific IFN-γ response with no or a very low IL-4 response (Vekemans et al., 2001). To minimize the potential interference by environmental mycobacteria on BCG vaccination, it might be sensible to

give the BCG vaccine early, at birth, as is done in most tropical countries, where infection by cross-reacting environmental mycobacterium is high.

2.8.3.3. Factors Associated with the Genotype of the BCG Vaccine

Different vaccine trials often used different strains of BCG for vaccination, which may also contribute to the observed disparity in efficacy of protection. There is experimental evidence that shows different strains of BCG differ in their ability to persist and replicate in vivo, in their ability to stimulate the generation of CD4+ T-cells, as well as in their ability to protect against secondary challenge (Lagranderie et al., 1996). In addition, genotypic differences among different strains of BCG have been demonstrated by DNA fingerprinting using copy number of *IS6110* and presence or absence of the *mpt64* gene as molecular markers (Behr and Small, 1999). Genetic disparity is not only significant among BCG strains, but also extends to differences between BCG and virulent strains of *M. bovis* and *M. tuberculosis*. This may have implications for the efficacy of BCG vaccination, as it is possible that BCG may lack some of the immunodominant antigens expressed in virulent strains of mycobacteria. There is evidence in the literature that supports this argument. Mahairas and colleagues have reported that the BCG strains currently in use lack genetic regions that are present in virulent strains of *M. tuberculosis* and *M. bovis*. The genetic regions which are deleted from BCG, as a result of mutations occurring during the process of attenuation, are designated as regions deleted (RD1, RD2 RD3) (Mahairas et al., 1996). More extensive genomic studies, using microarrays to compare BCG with *M. tuberculosis*, have identified more deleted genes in BCG extending the number of RDs to 16 (Behr et al., 1999). However, the RD1 region is

thought to be the most important, as it is confirmed to be present in all members of the virulent strains of the *M. tuberculosis complex* group but missing from all BCG vaccine strains and other non-virulent species of environmental mycobacteria. Proteins encoded by RD1 genes, mainly the early secretory antigen, ESAT-6 (6-kDa), and a culture filtrate protein CFP-10 (10-kDa), have been considered to be useful candidate antigens for the development of improved vaccines effective against infections with the virulent strains of *M. tuberculosis complex* group. These antigens may also be useful in the development of new diagnostic reagents to detect and differentiate infections with the virulent strains from exposure to the non- virulent species of mycobacteria (Cockle et al., 2002; Mustafa, 2002). In line with this, it has been possible to differentiate *M. bovis* infected from BCG vaccinated cattle using these protein antigens (Vordermeier et al., 2000; Vordermeier et al., 2001). It is also reported that ESAT-6 and CFP-10 are useful antigens to differentiate people with latent or active tuberculosis from those vaccinated with BCG or exposed to environmental mycobacteria (Lalvani et al., 2001). The CFP-10 and ESAT-6 proteins are the immunodominant antigens within virulent species of mycobacteria and were immunologically recognized by more than 70% of human TB patients (Skjot et al., 2000) and by more than 50% of cattle infected with virulent *M. bovis* (Mustafa et al., 2002). In addition to their immunodominance and ability to trigger the generation of immune cells able to mediate protective immunity, the expression of these proteins by virulent species of mycobacteria has led to the suggestion that these proteins may have an important role in virulence (Guinn et al., 2004). Deletion of the RD1 gene region from *M. tuberculosis* resulted in a decrease virulence to the same level as the non-virulent BCG strain, as assessed by infection of mice and human cell lines (Lewis et al., 2003). In addition to

this, ESAT-6 and CFP-10 may participate in the immune-evasion mechanism of mycobacteria by down regulating production of NO and inhibiting the up-regulation of the B7 family co-stimulatory molecules in infected macrophages (Trajkovic et al., 2004).

2.8.3.4. Variables Associated with BCG Vaccination Protocols

The efficacy of BCG vaccination may also vary depending on other factors associated with the vaccination protocols used, such as the route of vaccination and dose of vaccine administered. Originally, Calmette and Guérin administered the vaccine orally but this route of administration was abandoned for various reasons, including the high dose required to induce effective immunity and the reaction in cervical and pharyngeal lymph-nodes (Lotte et al., 1984). Various studies in mice have evaluated different routes of administration (Falero-Diaz et al., 2000; Lagranderie et al., 2000; Chen et al., 2004; Giri et al., 2005). However, for safety reasons, the intradermal route is used today to vaccinate infants immediately after birth with 0.1mg of BCG in 0.05ml volume. The dose is doubled in children and adults. Another factor worth mentioning here is the dose of BCG given. As the relationship between the dose of BCG and the protective effect achieved was not known, the highest tolerable dose of BCG was used in most studies (ten Dam, 1984). However, there is ample evidence in the literature, to be discussed in detail in section 2.9, that the dose of antigen administered determines the type of immune response generated after vaccination. Moreover, the culture methods used for the growth of BCG, the freeze-drying and thawing process of BCG, as well as handling of the vaccine, for example exposure to high temperature and to UV light, may result in

considerable differences in the numbers and proportions of viable to dead organisms per dose of vaccine (Fine et al., 1999).

2.8.4. Additional Merits of BCG Vaccine

Despite its reported low efficacy to protect against pulmonary tuberculosis, the BCG vaccine is the most extensively produced and widely used vaccine today. One of the main reasons for its wide use in infant immunization is its high efficacy against miliary tuberculosis and TB meningitis in children (Colditz et al., 1995). However, there are also other qualities, which other vaccines lack that make BCG a highly popular and widely used vaccine. Firstly, BCG is the safest vaccine, with no or very little adverse reactions, and can be administered safely to neonates. This feature of BCG encouraged some countries in tuberculosis endemic areas to recommend BCG vaccination of HIV positive children with no symptoms of AIDS. In addition, there is good evidence that BCG vaccination can protect with an efficacy as high as 85% against diseases caused by atypical mycobacterium (Romanus et al., 1995) as well as with 20-80% efficacy in protecting against leprosy in Africa (Ponnighaus et al., 1992; Orege et al., 1993). It is also believed that BCG can be used in the treatment of bladder cancer due to its ability to promote the Th1 nature of a response to another antigen when present during the response to this second antigen (Meyer et al., 2002). This property of BCG has encouraged many researches to use it as an adjuvant or as a vaccine delivery vehicle, to induce a Th1 type response against intracellular pathogens. Complete Freund's adjuvant **(**CFA) is a water-in-oil emulsion containing killed mycobacteria and is the most widely used adjuvant in vaccines that are intended to induce strong cell-mediated immunity.

However, due to the severe reaction it causes, it is not licensed for use in human vaccination protocols. Following the successful introduction of foreign DNA and its stable expression in mycobacteria (Jacobs et al., 1987; Snapper et al., 1988), several attempts have been made to use BCG as a recombinant vaccine delivery vehicle (Jacobs et al., 1990; Stover et al., 1991). Accordingly, the gene encoding the leishmania surface protein, gp63, was cloned and expressed in BCG and this recombinant BCG vaccine was able to induce protective immunity against *L. mexicana* (Connell et al., 1993) but not against *L. major* in BALB/c mice. However, another expression system was found to induce protective immune response against *L. major* infections in susceptible BALB/c mice (Abdelhak et al., 1995). Recently, BCG was also used to express genes that code for immunodominant antigens of pathogenic mycobacteria, thereby improving its efficacy in preventing infection by the virulent *M. tuberculosis* strains (Horwitz et al., 2006a; Horwitz et al., 2006b). Finally, BCG is a very cheap and easy to produce, making it easily accessible to people in developing countries, which are the prime targets as far as BCG vaccination and control of tuberculosis is concerned.

2.8.5. Limitations of BCG as Vaccine

Even though the advantages outweigh the disadvantages of BCG vaccination, there are some limitations of the BCG vaccine in addition to its low efficacy against pulmonary tuberculosis. The most obvious one is its interference with the skin test, which makes it very difficult to differentiate between DTH due to *M. tuberculosis* infection or BCG vaccination. This is of particular interest in veterinary medicine in which the skin test is employed for detecting and controlling bovine tuberculosis in the test and slaughter

policy. One can imagine the considerable economical loss that would be incurred from the slaughter of false positive reactors if BCG vaccination of animals were a standard practice in tuberculosis endemic areas. With the advance in knowledge of the genome of different mycobacteria, this problem is being solved by using diagnostic tests that can detect immunity to proteins that are expressed by pathogenic mycobacteria but not by attenuated or non-pathogenic mycobacteria (Kumar et al., 1996; Cockle et al., 2002). Another limitation of BCG is the adverse reactions that BCG can cause under certain circumstances. The most extensive report comes from Lotte and co-workers who have analyzed and classified complications associated with BCG vaccination based on clinical, bacteriological, histological and biological information they obtained from articles published between 1921 and 1982. According to their findings, life threatening complications are very rare, 1-3 per million vaccinated individuals (Lotte et al., 1984). However, mild reaction at the injection site, with or without involvement of regional lymph nodes, is not unusual, particularly when an inexperienced health care worker administers the vaccine. In one study, around 5% of vaccinated individuals complained of side effects following BCG vaccination (Turnbull et al., 2002). A more sever case was reported from Israel when BCG was given by mistake, instead of PPD, during a skin test survey in school children. Adverse reactions at the injection site, with or without involvement of regional lymph nodes, was reported in 50% of the cases (Gross et al., 2004).

Similarly, I have experienced the adverse effect of BCG injection, with abscess formation at the injection site, as a result of the accidental injection of BCG into the wrist of my left

hand while injecting mice in the tail vein. All the signs and nature of the lesion I observed at the injection site were more or less identical to what is described by Gross et al on the school children, except there was no involvement of draining lymph nodes in my case, as diagnosed by a physician. As shown in Figure 1, a papule like lesion started to form on day 3 after injection, with erythema at the injection site (Fig 1A). The lesion was itchy but there was no pain. Between 2-3 weeks post injection (Fig 1B), the lesion started to be painful, with increased swelling and visible necrosis of the skin, which started to slough. Around week 5 after injection, the lesion was big enough (Fig 1C) that I sought medical attention. The lesion was drained by incision, and took a couple of weeks to heal, without any other treatment. However, there was still a visible lesion/scar more than 6 months after the incident (Fig 1D). It is also worth mentioning that the scar left at the injection site that may develop into a keloid, as one of the adverse reactions of BCG vaccination. This scar is also considered advantageous, as one can easily differentiate vaccinated children from those that did not receive the vaccine during childhood immunization programs.

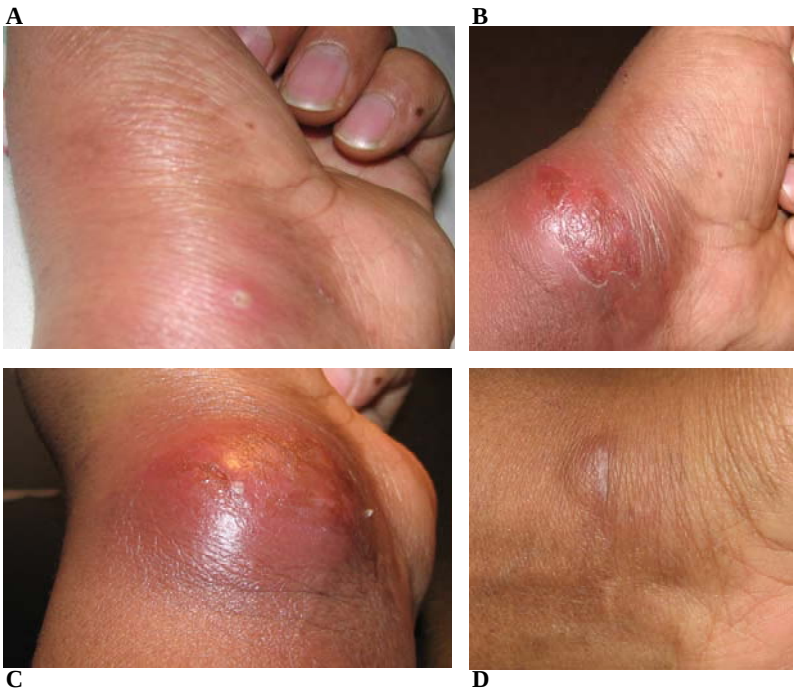

Figure 1. Pictures showing adverse reaction to BCG resulting from accidental injection of BCG during vaccination of mice.

BCG strain Montreal was prepared and re-suspended at $2x10^8$ cfu per ml in saline containing 0.05% Tween 80 to vaccinate mice. While injecting 100 µl of this preparation iv in the tail vein of a mouse the investigator, distracted by a mouse jumping out of the cage, accidentally injected his hand on the wrist. It is not clear how many CFU of BCG were inoculated, but it is easy to estimate close to a million based on the assumption that at least one drop (10 µl) of the preparation had been injected; a microliter of the preparation contained 10^5 cfu of BCG. The pictures show lesion development at 3 days (A), 3 weeks (B), 5 weeks (C) and 6 months (D) post-injection. The lesion completely healed with a scar visible at the injection site as shown in (D). Mycobacteria were grown from samples taken from the lesion and were sensitive to all antibiotics tested.

2.9. Pertinent Observations on the Regulation of the Immune Response: The Basis of Our Working Hypothesis

Our working hypothesis is based on three hypothetical generalizations from the literature, already briefly alluded to but explicitly stated and justified here. I will discuss the evidence supporting these three generalizations one at a time. First of all, it is prudent to assume that any rational approach to vaccination must be based upon knowledge of how the immune response differs in people infected by the pathogen but who remain healthy, presumably due to an effective immune response, and in those who develop the disease, respectively designated as “healthy contacts” and “patients”. There is considerable controversy over what immunological parameters discriminate the immune state of healthy contacts and patients during mycobacterial infections. The difficulty can be illustrated by noting that both healthy contacts and patients usually express delayed type hypersensitivity (DTH) used for diagnosis of *M. tuberculosis* infection. Evidence from other chronic diseases shows that predominant cell-mediated, Th1, responses correlate with protection against intracellular pathogens causing chronic diseases. Therefore, I will discuss evidence from less complex systems before embarking on discussing issues in the context of tuberculosis, which is admittedly complicated.

In the murine model of cutaneous leishmaniasis, it has been repeatedly shown that the nature of the immune response generated following infection with *L. major* parasite determines disease outcome. Resistant mouse strains mount predominantly cell-mediated, Th1, responses with little or no antibody and contain the infection, while susceptible mouse strains which suffer progressive disease, generate Th2 or mixed Th1/Th2 responses with a substantial levels of serum antibodies. Furthermore, it has been shown

that the relative ratio of the amount of *L. major*-specific IgG_1/IgG_{2a} antibodies give a good indication of the relative sizes of the Th2 and Th1 components of the response, as reflected in the number of parasite-specific IL-4 and IFN-γ-producing $CD4^+$ T cells in the spleen (Bretscher, 1992; Menon and Bretscher, 1998). Previously, studies in mice have also clearly shown a correlation between the presence of certain subclasses of antibodies and the Th1/Th2 nature of the associated response to another parasite, *Trichinella spiralis*. In resistant mice a predominant Th1 response with IFN-γ production was associated with high serum levels of antigen-specific IgG_{2a} antibody whereas a Th2 response with IL-4 production was associated with predominant IgG_1 antibody (Pond et al., 1989). Similarly, a correlation between subclasses of *Babesia bigemina*-specific IgG antibodies and antigen specific Th1/Th2 cytokines was reported in cattle (Brown et al., 1999). These results, coming from various animal species infected with different parasites led us to explore the use of the IgG_1/IgG_2 ratio in indirectly assessing the Th1/Th2 phenotype of the response generated following infection with various pathogens in man. The use of serum antibodies as immunological markers to distinguish between the immune states of patients and healthy contacts has a practical significance in humans, as serum is easily accessible and may better reflect the systemic immune response than peripheral blood lymphocytes. The utility of this approach has been demonstrated in human visceral leishmaniasis where a distinction of immune responses between healthy contacts and patients was reported based on the IG_1/IgG_2 ratio of parasite-specific antibodies (Hailu et al., 2001). Disease was associated with a poor expression of DTH and higher titer of antibodies, mainly of the IgG_1 but also the IgG_3 and IgG_4 isotypes, while healthy contacts show antigen specific DTH with only significant levels of IgG_2

serum antibody, indicating a mixed Th1/Th2 and Th1 type of responses, respectively. Furthermore, this study has shown that chemotherapy can modulate the immune response of patients from a non-protective mixed Th1/Th2 type of response to a protective Th1 mode, as the immune responses of treated patients become indistinguishable from that of healthy contacts.

The qualitative nature of the immunity generated against *M. tuberculosis* and *M. leprae* may also determine disease outcome. However, unlike visceral leishmaniasis, there is no a clear distinction between the anti-mycobacteria immune state of all healthy contacts and all patients. In leprosy, patients with tuberculoid form of the disease exhibit a strong *M. leprae*-specific DTH responses that can restrict the growth of bacilli in the lesion site, whereas patients with lepromatous leprosy are characterized by strong humoral, Th2-type, responses with no or little *M. leprae*-specific T cells that mediate DTH. In addition to these two polar groups, leprosy patients can be categorized as borderline, falling somewhere between these two immunologicaly defined polar groups. These borderline leprosy patients show immunological characteristics of the neighbouring polar groups and are further classified as borderline tuberculoid, mid-borderline, or borderline lepromatous (Verhagen et al., 1998). Similarly, observations in the old literature have been interpreted as indicating the existence of spectrum of immune responses during tuberculosis infection. On one end of the spectrum are healthy contacts that express a strong antigen-specific DTH response with no or little serum antibodies. At the other extreme are patients with miliary tuberculosis that generate a predominant Th2 type response associated with high level of serum antibodies and no detectable DTH. The

immune response of the majority of the patients with active pulmonary tuberculosis may lie somewhere between these two polar groups (Bhatnagar et al., 1977; Lenzini et al., 1977). In view of these observations, it seems difficult to correctly discriminate healthy contacts from patients based on the immune parameters at the individual patient level. However, other observations have reported that the immune response in patients has, on average, a significant Th2 component (Sanchez et al., 1994; Surcel et al., 1994). Based on these observations it is safe to state that at the population level, there are statistically significant differences between the immune parameters of patients and healthy contacts, the latter having a predominant Th1 response. Nevertheless, the spread of the spectrum of the immune response in individual patients, anywhere between the two polar groups, may suggest that the immune system can fail in various ways in different tuberculosis patients. For example, an exclusive cell-mediated, Th1 response may be protective, and tuberculosis may arise if either the immunity is of the right kind but not strong enough, or if it is not of the right kind, having a significant Th2 component. There is unpublished data from our laboratory, which support this view. In this study, almost all healthy contacts and around 71% of the patients showed an IgG_1/IgG_2 ratio in the range of 0.001 to 2, while the ratio ranges from 3 to 100 for the remaining 29% patients. Based on these data, it was hypothesized that there may be two distinct types of tuberculosis: Type I tuberculosis is envisage to occur as a result of too weak Th1 response, with the patients showing immune parameters identical to healthy contacts. Patients with type II tuberculosis have a high IgG_1/IgG_2 ratio, reflecting a mixed Th1/Th2 or polarized Th2 response. The identification of immunological parameters that discriminate between the immune response of healthy contacts and tuberculosis patients sets the stage for

considerations of how to guarantee, by means of vaccination, an effective response upon natural infection by the pathogen. Based on the evidence discussed above, we can hypothesise that a strong and exclusive Th1 response is protective against tuberculosis, whereas a weak Th1 response or a response with a significant Th2 component is less protective or insufficient. This viewpoint naturally leads to the question of how to guarantee a strong Th1 response on natural infection. Given the phenomenon of Th1 imprinting, it seems essential to a successful vaccination that we examine how to guarantee a Th1 response upon primary vaccination. We therefore now address what is currently known about the factors/circumstances that determine the Th1/Th2 phenotype of a primary immune response. This will take us to the second hypothetical generalization referred to at the beginning of this section.

The recognition of a tendency for exclusivity between the induction of DTH and IgG antibody came from the finding that different conditions of antigen administration induce different classes of immunity. Guinea pigs immunized to produce only antibody were rendered specifically unresponsive for the induction of DTH, a phenomenon termed as ‘humoral immune deviation’ (Asherson and Stone, 1965). On the other hand, some animals immunized to only express cell-mediated immunity were found to be specifically unresponsive for the induction of antibody (Parish, 1972), a phenomenon that can be referred to as cell-mediated immune deviation. The aim of a series of experiments in the mid 1970s was to explore the cellular basis of the exclusivity of cell-mediated and humoral response. It was shown that the inverse relationship between cell-mediated and humoral immunity is due to the presence of different type of suppressor T-cells, which

need different requirements for their induction. Accordingly, mice immunized to produce only humoral immunity to horse red blood cells (HRBC) were reported to harbor T-cells that suppress the induction of DTH (Ramshaw et al., 1976). Conversely, mice immunized to express potent cell-mediated immunity and rendered unresponsive for the induction of antibody to the same antigen harbor T-cells that specifically inhibit antibody production (Ramshaw et al., 1977). These T-cells were later referred to as TsDTH and TsAb cells, respectively, and differed from each other in the surface markers they express. The former T-cell population are Ly1+ Ly2- (CD4+) cells whereas the latter are reported to be Ly1-Ly2+ (CD8+) T-cells (Ramshaw et al., 1977). They are induced under different conditions and they inhibit the induction of the opposing class of response, leading to the exclusive nature of the DTH and antibody responses. This will again lead to the question of what variables of immunization determine whether TsDTH and hence a humoral response, or TsAb cells and hence a cell-mediated response is generated? Our attempt to address this question, based on experimental evidence from the literature, will lead us to the third generalization. There is ample evidence in the old literature that shows a direct relationship between dose of antigen used for vaccination and the type of immune response generated. It was Salvin who first showed that administration of very low doses of antigen induce an exclusive cell-mediated response, while immunization with higher doses result in a more rapid cell-mediated response that is transient and decays as a strong antibody/humoral response is generated (Salvin, 1958). Several subsequent observations supported the generality of this finding for many different kinds of antigens be they proteins, bacteria, parasites, or particulate antigens in the form of RBC. These studies employed different species, strains and ages of animals as hosts as well as various

routes of immunization, including iv, id, and sc, reviewed in (Bretscher et al., 2001). What can be the potential mechanism underlying this dose dependence? Bretscher proposed a mechanism that may help to explain the differential expression of cell-mediated and humoral immunity following antigen administration (Bretscher, 1974). This hypothesis, known as the "Threshold Hypothesis", is based on some critical scientific assumptions and postulates:

(i) The activation of naïve precursor T helper (pTh) CD4+ cells into effector cells requires antigen-mediated cooperation of CD4+ T-cells.

(ii) More antigen-mediated CD4+ T-cell cooperation is required to generate Th2 cells than Th1 cells.

(iii) Antigen presented on the surface of APC mediates the interaction between these helper CD4+ T-cells.

These three postulates are the basis of the Threshold Hypothesis and based on these, the hypothesis states that less CD4+/CD4+ T-cell interactions (or less help) are required for the induction of Th1 cells than is needed for the generation of Th2 cells. Accordingly, antigens with low foreign sites – either due to small molecular size, or because most epitopes are shared with self, as in skin bearing minor hstocompatibility antigens, for there are only a few Th cells in the periphery – bring very few antigen-specific CD4+ T-cells into the field of cooperation and hence induce the differentiation of Th1 cells from precursor T-helper (pTh) cells. On the other hand, there are many T-helper cells specific for very foreign antigens or for the foreign peptides generated on processing these antigens and the high level of cooperation between these helper CD4+ cells, mediated by optimal doses of antigen, leads to the induction of pTh cells to generate Th2 cells. Once

these pTh cells are differentiated, they start to produce cytokines which amplify their further differentiation while inhibiting both the differentiation of the opposite CD4+ T-cell subsets and most of their important effector functions (Mosmann, 1991). Various in vitro and in vivo experiments conducted over the years in our laboratory support the Threshold Hypothesis and its critical assumptions (Bretscher, 1983a; Bretscher, 1983b; Ismail and Bretscher, 2001; Ismail et al., 2005; McKinstry et al., 2005). The role of antigen in this interaction is to bring the cooperating CD4+ T-cells together so an interaction between CD4+ T-cells will be possible only in the presence of antigen. Non-optimal and low doses of antigen limit the CD4+ T-cell interaction and thus can favor the generation and differentiation of Th1 cells even by a very foreign antigen for which there are many CD4+ T-cells in the periphery. Therefore, a low dose of antigen induces the generation of Th1 cells that play a role in cell-mediated immunity and can hypothetically establish a state of cell-mediated immune deviation, as assessed, for example by a subsequent challenge of the host with a dose that generates a mixed Th1/Th2 or predominant Th2 response in unexposed animal. This phenomenon is commonly described as Th1 imprinting. By the same token, a high dose of antigen induces Th2 cells, which result in an antibody response and can limit the induction of cell-mediated immunity against the respective pathogen upon natural infection (Ogunremi and Bretscher, unpublished data). This phenomenon is referred to as Th2 imprinting. This has practical implications for our low dose BCG vaccination study, as the possibility of Th2 imprinting, due to exposure of the host to cross-reacting environmental mycobacteria, may have a deleterious effect on the ability of the BCG vaccine to generate a Th1 response and Th1 imprint.

In summary, the three hypothetical generalizations referred to are (i) that healthy contacts have a strong, predominant cell-mediated response, whereas patients have either a similar response but too weak to contain the infection, or one with a Th2 component, that also cannot contain the infection; (ii) that the generation of Th1, cell-mediated, and Th2, antibody, responses tend to be exclusive and (iii) that the dose of antigen can determine the Th1/Th2 phenotype of the response, with low dose favoring predominant Th1 responses. Furthermore, as discussed in previous sections, clinical observations have shown that a strong and predominant Th1-type response is protective against chronic intracellular pathogens. Moreover, it would appear from the old literature employing non-living antigens (Parish, 1972) that this type of response can be guaranteed by exposing an animal in a manner that generates an exclusive Th1 response over a period of time. Such an exposure can result in cell-mediated immune deviation, i.e. a state where the animal produces a strong and predominant cell-mediated, Th1-type, response to a challenge that generates an antibody response with at least a substantial Th2 component in naïve mice. One means of establishing cell-mediated immune deviation is to ensure prolonged stimulation with a dose of antigen that is sub-immunogenic for an antibody response and that leads to a substantial cell-mediated response (Parish, 1972).

Experiments in our laboratory have explored whether it was possible to similarly establish a state of cell-mediated immune deviation to a replicating agent by infecting with low number of organisms that were too few to induce specific antibody and Th2 cells. The mouse model of cutaneous leishmaniasis, involving infection with *L. major* parasite, was chosen for the experiments, as it had been so extensively developed.

Infection of BALB/c mice, the susceptible prototype, with very low numbers of *L. major* parasites, 1000 fold lower than the dose that induces progressive disease, is found to induce a stable cell-mediated/Th1 response exclusive of Th2 cell generation and antibody production. Over time these mice become resistant to a high dose challenge that causes progressive infection in naive mice of the same strain (Bretscher et al., 1992; Menon and Bretscher, 1998). It is also reported that BCG doses 100,000 fold lower than previously used provide dramatic protection in calves against a normally lethal *M. bovis* challenge (Buddle et al., 1995). In addition, recent studies in deer (Griffin et al., 1999) show that low doses of BCG induce exclusive cell-mediated, Th1, responses that can protect the host against challenge with virulent mycobacteria. The higher doses used induced mixed Th1/Th2 responses and were inferior in reducing the bacterial burden from the organs of the infected host. In summary, low dose BCG vaccination that induces a Th1 type response and Th1 imprint may constitute effective vaccination against virulent *M. tuberculosis*.

All these facts speak to the possibility that one of the reasons for the failure of BCG vaccination to reliably protect against pulmonary tuberculosis is the high dose of BCG used in all the previous human vaccination trials. In some studies, 1.6 $x10^8$ cfu has been considered the standard dose (Lowry et al., 1998) and 100 fold higher doses were used for oral vaccination of infants (Lagranderie et al., 2000). Cattle have been vaccinated in the past with 50-100 mg of BCG, which is about 10^{10} cfu (Buddle et al., 2003). Therefore, the possibility that vaccination with relatively low doses of BCG provides better protection against tuberculosis than vaccination with the standard dose is

intriguing, particularly in view of the use of the largest acceptable dose of BCG in most if not all previous vaccine trials. Moreover, dramatic protection in calves against bovine tuberculosis has been achieved by lowering the BCG dose to either 10^4 or 10^6 cfu, about a hundred thousand fold lower than the previously employed dose (Buddle et al., 1995). Based on these general facts, we propose here a hypothetical low dose BCG vaccination strategy that can guarantee the correct type of response upon exposure of the host to natural infection by mycobacteria.

I have discussed in detail the variables that may affect the efficacy of BCG vaccination in section 2.8.3; however, there are two important issues, pertinent to our low dose BCG vaccination strategy, which I want to mention here and discuss briefly how these issues will be addressed in this study. Firstly, genetic diversity of the target population, whether in animals or people, can be a stumbling block for the development of a universally efficacious vaccination protocol, as genetically distinct individuals in a population may respond differently to a standard dose of BCG vaccine. A low dose that induces an exclusive cell-mediated, Th1, response in one individual can be high enough to induce a mixed Th1/Th2 or a predominant Th2 response in another individual of the same population. Therefore, the standard dose of BCG vaccine may generate an effective immune response in some individuals and fail to induce a protective response in others. I believe the way out of this impasse may be to use an ultra-low dose vaccination strategy whereby a dose that will not induce any antibody response in any member of the population is employed. This dose will either induce an exclusive cell-mediated response and Th1 imprint or will be too low to induce any kind of response. In the later case, BCG,

a live attenuated vaccine, can easily replicate and reach a threshold level where it can induce a cell-mediated response and Th1 imprint in all individuals. The ultimate goal of this study is to find an effective BCG vaccination strategy in the mouse model that can be applied in the control of tuberculosis in the genetically heterogeneous population of humans and animals. Therefore, it is important to test the low dose BCG vaccination strategy in mice with different genetic backgrounds. The choice of mouse strains will be based on previous reports which classify inbred strains of mice into two broad categories based on their ability to reduce bacterial burden from organs of their body (Gros et al., 1981) and their survival time following exposure to virulent *M. tuberculosis* (Medina and North, 1998). The second problem pertinent to our low dose BCG vaccination strategy is, that exposure of the host to cross-reacting environmental mycobacteria can possibly interfere with the type of immune response generated following vaccination with BCG and prevent Th1 imprinting. This interference could be due to the generation of mixed Th1/Th2 or Th2-type response following exposure to environmental mycobacteria before the host is vaccinated with BCG. Low dose BCG vaccination of individuals with an established Th2-type response, due to prior exposure to environmental mycobacteria, may not induce a Th1-type response and Th1 imprint, as the immune response might have been already locked into a Th2 mode (Asherson and Stone, 1965). It is important to examine whether this problem can be overcome by administering the vaccine immediately after birth, before the newborn animals or humans are exposed to environmental mycobacteria. Another consideration, which may have relevance to our neonatal low dose BCG vaccination strategy, is whether the vaccine is able to protect the two billion people already infected with *M. tuberculosis* against reactivation disease. In

circumstances where vaccination cannot provide the desired level of protection, immunotherapy would be an alternative approach in the fight against chronic disease caused by intracellular pathogens, particularly when there is no effective chemotherapy to cure the disease.

The identification of discriminating immunological parameters between healthy contacts and patients is also essential for the rational design of immunotherapy. The ultimate goal of immunotherapy will be to modify the ongoing immune response from the non-protective Th2 or mixed Th1/Th2 into a protective Th1 mode. In line with this, a number of maneuvers, such as administration of IL-12 (Heinzel et al., 1993; Nabors et al., 1995), treatment with neutralizing anti-IL-4 monoclonal antibody (Sadick et al., 1990; Nabors and Farrell, 1994), or partial depletion of CD4+ T-cells (Titus et al., 1985; Heinzel and Rerko, 1999) have been used to protect susceptible BALB/c mice from infection with the *L. major* parasite. In all cases, treatment was able to help establish a Th1 type response and Th1 imprint – at the expense of Th2 cells – that helps the mice to withstand an otherwise lethal challenge with *L. major* parasite. To me, there seems to be a caveat in all these maneuvers to be used as a model for human chronic disease. All the interventions were done either prior to or immediately after infection, which suggests they influence the decision making process to generate either a Th1 or Th2 type response rather than modifying an on-going response from a mixed Th1/Th2 or predominant Th2 into a Th1 mode and were shown to be ineffective if given a few days later after infection. In man, chronic diseases like tuberculosis and leishmaniasis are diagnosed several months after infection when the response is already established as mixed Th1/Th2 or Th2 type.

However, anti-IL-4 therapy or partial depletion of CD4+ T-cells is reported to cure mice with a stable borderline leishmaniasis associated with mixed Th1/Th2 response several months after infection of BALB/c mice with *L. major* parasite by modulating the response into a Th1 mode (Uzonna and Bretscher, 2001). These reports encouraged me to examine whether the same maneuvers will induce similar outcomes in modifying an ongoing mixed Th1/Th2 response in BCG infected mice into a Th1 type response, and if this switch in response is associated with a more rapid and efficient clearance of the bacilli from the spleen.

2.10. Hypothesis

Ultra-low dose neonatal BCG vaccination will provide universal protection against tuberculosis in a genetically heterogeneous population.

3. Study Objectives

I. To determine if low dose BCG vaccination generates a stable, predominantly cell-mediated/Th1 response against mycobacteria in naïve adult and newborn mice.

II. To determine whether the generation of cell-mediated responses following low dose BCG vaccination results in a Th1 imprint that can persist following high dose BCG iv challenge, and whether such an imprint results in a decreased mycobacterial burden.

III. To examine the low dose vaccination strategy in different strains of mice, to explore whether it is applicable in genetically heterogeneous populations.

IV. To determine whether different methods of immunotherapy to modulate an ongoing mixed Th1/Th2 response into a Th1 mode are effective in the mycobacterial system and to evaluate whether such modulation is associated with a reduction in bacterial burden.

V. To test the hypothesis that incorrect priming, due to pre-exposure to environmental mycobacteria, can negatively affect the efficacy of low dose BCG vaccination and, if so, to explore ways of overcoming this problem.

4. Materials and Methods

4.1. Mice

Adult, female BALB/c mice were obtained from Charles River, Wilmington, Massachusetts and were at least 6 weeks of age when first used. Adult CD1 mice were obtained from Animal Resource Center, University of Saskatchewan and adult CBA mice were obtained from the breeding colony of the animal care facility, College of Medicine. Newborn mice were obtained by mating 10 BALB/c breeding pairs, obtained from the above source, in the College of Medicine animal care facility and newborn mice were used at around 5-7 days of age. Mice were tagged by piercing the ear when necessary to identify individual mice in longitudinal studies. All mice were housed at the College of Medicine animal care facility. Mice were handled and experiments performed in accordance with the guidelines approved by Canadian Council on Animal Care and the University Committee on Animal Care and Supply, UCACS.

4.2. BCG Growth and Enumeration

Mycobacteria, BCG strain Montreal, was kindly provided by Dr. Emil Skamene, of McGill University. BCG stocks were propagated in Dubos broth base (DIFCO, BD Biosciences, Sparks, MD 21152, USA) enriched with Dubos medium albumin supplement containing 5% bovine serum albumin (BSA) in saline. The supplement was added into the Dubos medium to a final concentration of 0.5% and finally Tween-80 was added to a final concentration of 0.05% to avoid clumping of bacteria. The bacteria were

then incubated at 37°C in a humidified incubator with 5% CO_2 for 14-21 days. To quantify the number of mycobacteria growing in the broth media, bacteria were enumerated based on their ability to form colonies as previously described (Gros et al., 1981). Briefly, bacterial stock culture was sonicated for few seconds in a Branson Sonifier (Model 450) to disrupt clumps and bacteria was serially diluted, by 10 fold dilutions in PBS containing 0.05% Tween-80. From each dilution, 5-7 drops of 10 µl each were seeded as a spot on plates containing Dubose-Oleic agar base (DIFCO, BD Biosciences, Sparks, MD 21152, USA) supplemented with 5% BSA in saline and oleic acid at a final concentration of 0.5%. Plates were incubated in a humidified, 5% Co_2 incubator set at 37°C. Colonies in each spot were counted 10-14 days after plating, the average number calculated and the number of bacteria consequently expressed as cfu/ml. Bacterial suspension was then stored in saline-Tween 80 at -70°C until used for injection.

4.3. BCG Vaccination and Challenge

For injection, BCG stock of known cfu content was pelleted by centrifugation at 8000xg for 30 minutes, and subsequently washed three times in saline containing 0.05% Tween-80 by pelleting bacteria at the same speed for 20 minutes. The pellet was re-suspended in a given volume of 0.05%Tween-saline and briefly sonicated for 10 seconds in a Branson Sonifier, Model 450, to disrupt clumps. The homogenous suspension of BCG was serially diluted in PBS to get the appropriate number of bacterial required for injection. Adult mice were vaccinated intravenously (iv) in the tail vein. Newborn mice were vaccinated intradermaly (id) or subcutaneously (sc) on the back around the base of the tail. All BCG challenges were done intravenously.

4.4. BCG Antigen Preparation

Bacteria were grown in Dubos broth base, as described in section 4.2. They were then pelleted by spinning for 30 minutes at 8000xg, re-suspended in saline containing 0.05% Tween-80 and washed two more times in this solution. The bacteria were then re-suspended in 5 ml of ice-cold 0.05% Tween-80-saline and sonicated for 14 cycles of one minute each in a Branson Sonifier, Model 450, according to the manufacturer's instructions to disrupt the mycobacteria. The suspension was in some cases spun for 20 minutes at 8000xg to remove particulate matter and the supernatant collected. The concentration of the protein antigen was determined using the Bicinchronic acid (BCA) protein assay reagent (Pierce, Rockford, Illinois), according to the manufacturer's protocol. Briefly, a BSA standard (2 mg/ml) was serially diluted in the diluent used to prepare the antigen in a low binding ELISA plate. The BCG antigen was also similarly diluted and 10 µl of each dilution from the standard and the unknowns transferred into a Maxisorp ELISA plate. 200 µl of working reagent that contains 50 parts reagent A (Na_2CO_3, $NaHCO_3$ and Sodium tartrate in 0.1N NaOH) and 1 part reagent B (4% Copper sulfate solution) was added to each well and the plate incubated at 37^{o}C for 30 minutes. Plates were read with Emax^{TM} microplate reader (Molecular Devices Corporation, Sunnyvale, CA) wavelength 540–590 nm, the standard curve drawn and the amount of protein in the BCG sample was assessed by extrapolation. Antigens was stored at -70^{o}C until used to stimulate the production of cytokines by spleen cells of BCG immunized mice in the ELISPOT assay (section 4.7) or in the measurement of BCG-specific IgG_1 and IgG_{2a} antibodies in an ELISA assay (section 4.8).

4.5. Media

RPMI 1640 and DMEM media supplemented with L-glutamine, as well as the Leibovitz medium were prepared from powdered stocks (Gibco Laboratories, Grand Island, NY) as per the manufacturer's instructions by dissolving the powder in de-ionized and distilled water ($ddHO_2$). Media were sterilized by filtration through 0.22 μm filters (Millipore, Molsheim, France) and assessed for sterility by overnight incubation at 37^oC.

4.6. Preparation of Single Cell Suspensions

Experimental and control mice were sacrificed by cervical dislocation and their spleens taken aseptically into a test tube containing 10 ml of Leibovitz media. Each spleen was gently disrupted by teasing into small fragments using sterile forceps and/or scissors and by crushing using a blunt glass rod. Single cell suspensions were obtained by passage through a stainless steel wire mesh and then the suspension was put into a 15 ml tube. Suspension were left to stand for around 2 minutes to allow debris to settle and the supernatant collected from each tube leaving around 0.5 ml debris on the bottom. Single cell suspensions were then washed twice in the same media by spinning at 300xg for 10 minutes and finally pellets re-suspended in RPMI 1640 media supplemented with L-glutamine containing, 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.8mM sodium pyruvate, and $5x10^{-5}$M β-mercaptoethanol, here after called as complete RPMI 1640 media. Viable white cells were counted on a haematocytometer employing the trypan blue exclusion technique and re-suspended at 10^7 viable cells/ml of RPMI media. Cells were kept on ice until used.

4.7. ELISPOT Assay to Detect Antigen Specific Cytokine Secreting Single Cells

This assay was used to quantify the number of antigen-specific spleen cells producing IFN-γ or IL-4 and was conducted as described (Power et al., 1999). Briefly 96 well nitrocellulose-bottom culture plates (Whatman-Polyfiltronics, Clifton, New Jersy, USA) were coated with purified 1.25 μg/ml anti-IFN-γ (R4-6A2) or anti-IL-4 (11B11) antibodies (Pharmingen, San Diego, CA) in 1M bicarbonate buffer (pH 9.6) at 100 μl per well and incubated at 4^{o}C overnight. The next morning, plates were rinsed with 150 μl of RPMI 1640 and blocked with 200 μl of complete RPMI 1640 medium for at least two hour prior to addition of cells. Spleen cells from experimental and control mice were prepared as single cell suspensions as described in section 4.6 and re-suspended at 10^7 cells/ml in RPMI 1640 complete medium. Finally, cells were plated in 100 μl RPMI 1640 complete medium at one of the following three densities 10^6, 5 x 10^5, or 2.5 x 10^5 per well. Whenever less than a million sensitized cells per well were used, additional spleen cells from un-immunized mice were added to bring the total number of cells in each well to 10^6. The number of antigen-dependent spots observed was directly proportional to the number of immunized spleen cells plated (Figure 2) and was not affected by the number of naïve spleen cells added to each well. The average number of spots detected with cells from a naïve mouse was around 5-10 for IL-4 and 10-15 for IFN-γ. When peripheral blood lymphocyte (PBL) cells were used, blood was collected by nipping the tail vein of mice after exposure of the mouse to a heat lamp and 10-15 drops of whole blood collected into 2 ml screw cap tubes containing 2 drops of Heparin Sodium Injection B.P anticoagulant (LEO Pharma. inc, Thornhill, Ontario). The whole blood was re-suspended

in 1ml Liebovtiz media, gently layered on top of 0.7 ml ficoll and centrifuged for 20 minutes at 750xg to separate the peripheral blood mononuclear cells (PBMNCs) from dead cells, granulocytes and RBC which pass through the ficoll and pelleted at the bottom of the tube. Finally, the buffy layer was carefully collected from the interface. These cells, were washed 2-3 times in Leibovitz media, re-suspended in 1 ml complete RPMI 1640 media, counted and finally dispensed into a 96 well nitrocellulose plates as described above. The number of peripheral blood mononuclear cells harvested from individual mouse blood was always less than 10^6 per ml when we re-suspend it in one ml complete RPMI 1640 media. Thus, even though the number varies among individual mice, the average number of PBL cells added into each well was close to 10^5. Therefore, spleen cells from naïve mice were added to bring the total number of cell per well to 10^6. Antigen was added to provide a final concentration of 3.33 μg of BCG protein per ml of complete media in a final volume to 200 μl per well. The number of spot-forming cells generated in the presence and absence of antigen by the spleen cells of each experimental mouse was assessed in triplicate. The seeded plates were placed in a 37^{o}C incubator undisturbed for eight hours and then washed thoroughly twice with ddH_2O, waiting for five minutes between each wash, to lyse any remaining cells, and then six times with phosphate buffered saline containing 0.05% Tween-20 (PBST). Biotinylated anti-IFN-γ (XMG1.2) or anti-IL-4 (BVD6-24G2) antibodies (Pharmingen) were added at a concentration of 1.25 μg/ml in 100 μl of PBST and the plates incubated overnight at 4^{o}C. The next morning, plates were again thoroughly washed with PBST and 100 μl of PBST containing 0.2 μg/ml of alkaline phosphatase strepavidin (Jackson Immunoresearch Laboratories Inc., West Grove, PA) added to each well and incubated at room

temperature for 1.5 to 2 hours. The plates were then thoroughly washed with ddH2O and spots developed by addition of a 1 in 50 dilution in 0.1M Tris, 0.1M NaCl, 0.05M $MgCl_2$ buffer, pH 9.5, of 18.75 mg/ml nitro blue tetrazolium chloride (NBT) and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidine in 67% DMSO (v/v), commonly referred to as BCIP/NBT substrate, according to the manufacturers instructions (Boehringer Mannheim, Germany). Spots were counted using a dissecting microscope after the plates had dried and antigen specific spots expressed by subtracting spots in antigen negative wells from antigen positive wells and expressed as mycobacterium-specific spots per million spleen cells. As mentioned in the previous paragraph the number of antigen specific spots per 10^6 spleen cells from naïve mice is around 5-10 spots for IL-4 and 10-15 for IFN-γ.

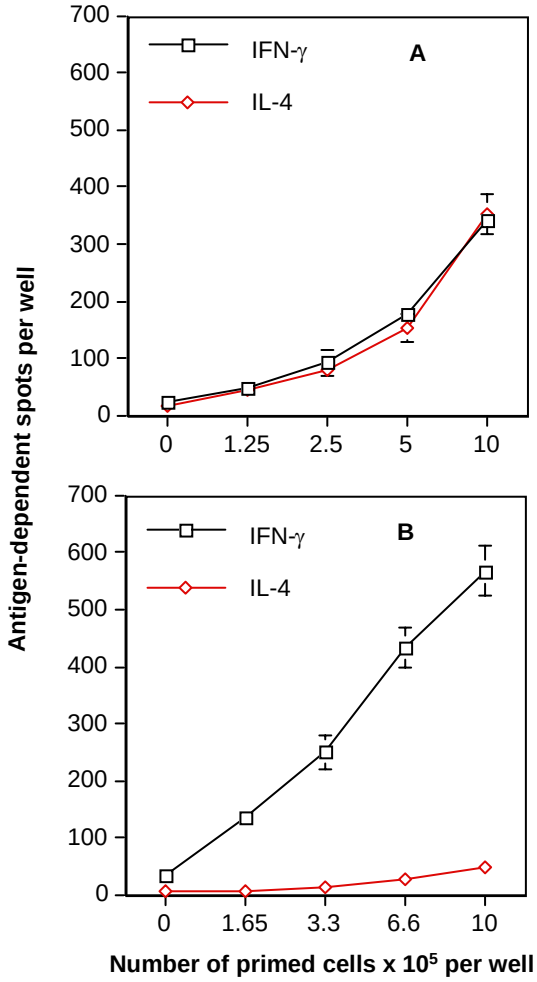

Figure 2. The number of antigen-dependent spots observed in the ELISPOT assay was directly proportional to the number of immunized spleen cells plated.

Spleen cells from BALB/c mice vaccinated with either saline (A) or 10^2 cfu (B) of BCG sc and challenged 12 weeks after vaccination with ~$3.3x10^7$ cfu iv were processed for ELISPOT assay. Pooled splenocytes from 3 mice in each group were plated at different concentrations per well as indicated on the abscissa. Whenever cells less than 10^6 were used, spleen cells from naïve mice were added to bring the total number of cells per well to 10^6. The number of antigen-specific spots is determined by subtracting the number of spots in wells without from the number of spots with antigen. Each point represents the number of antigen-specific spots in 3 assay wells +/- SE.

4.8. Detection of Mycobacteria Specific IgG_1 and IgG_{2a} Antibodies in Serum

Blood was collected into MICROTAINER brand tubes by nipping the tail vein of mice after their exposure to a heat lamp, and serum from individual mice separated by spinning the tubes at 12,000 rpm for 3-5 minutes in a Microspin centrifuge (Sorvall® instruments). Serum was harvested carefully into eppendorf tubes and stored at -70^0C until assayed for antibody content. The IgG_1 and IgG_{2a} serum antibody titers were determined using ELISA as follows. Nunc^{TM} Maxisorp 96 well polystyrene plates were coated with BCG antigen at a concentration of 1 μg/well in 100 μl of 0.1M $NaHCO_3$ and incubated overnight at 4^oC. Plates were washed 2-3 times with PBS containing 0.05% Tween-80 and blocked at 37 ^{0}C for at least one hour with PBS containing 10% calf sera. Serum samples were diluted 1/100 in PBS containing 10% calf serum and then two-fold serial dilutions were prepared in low protein binding dilution plates. From this 100 μl of diluted sera were transferred into the corresponding wells on the Maxisorp ELISA plates. The plates were incubated at 37^{o}C for 2 hours or at 4^oC overnight and washed thoroughly with PBS containing 0.05% Tween 20. Horseradish peroxidase labeled rat anti-mouse monoclonal antibodies against mouse IgG_1 or IgG_{2a} (Southern Biotechnology Associates, Birmingham, AL) were added to the wells at a dilution of 1/5000 in 100 μl per well in assay diluents, 10% calf sera in PBS. Plates were incubated again at 37^{o}C for two hours. After washing, stock ABTS® peroxidase substrate solution (KPL, Gaithersburg, MA) that contains 2.2'-azino-di (3-ethyl-benzthiazoline-6-sulphonate) was added in 100 μl per well to all wells and incubated for 10-20 minutes at room temperature in a dark place. Plates

were read using Emax^{TM} microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 405nm. Readings were corrected for background by subtracting OD values of wells with no serum added (conjugate control) from the corresponding wells of vaccinated and non-vaccinated control mice. The positive cutoff value was then set at 2x the mean plus 2x the standard deviation of the value for pooled non-vaccinated mice sera. The titer was considered to be the reciprocal of the last serum dilution to give a positive result.

4.9. Assessment of Bacterial Burden in the Spleen of BCG Challenged Mice

At different time points after iv challenge of vaccinated or unvaccinated age-matched control (AMC) mice, spleen cells were processed for assessment of bacterial burden (Gros et al., 1981). Briefly, single cell suspensions were prepared from each spleen in saline containing 0.05% Tween-80 and 0.05% bovine albumin, fraction V (Sigma chemicals Co.) and washed 2-3 times by spinning for 20 minutes at 800xg in this solution. Pellets were re-suspended in 5 ml of the same solution and lysed by sonication (Branson Sonifer, Model 450) over a period of 10 seconds, according to the manufacturer's instructions. Then, 5-7, 10 μl drops of each sonicates were placed onto Dubos oleic agar plate and incubated in 5% CO_2 incubator at 37^{o}C for 10-14 days. When bacterial colonies could be enumerated, colonies arising from each drop were counted under a dissecting microscope and results calculated and expressed as cfu per spleen.

4.10. Magnetic Cell Sorting and Flow Cytometric Analysis

Single cell suspension were prepared from BALB/c spleen cells as described in section 4.6 and red blood cells were separated by density gradient separation using ficoll as described in section 4.7. Then, CD4+, CD8+ and Thy1.2+ cells were sorted by magnetic cell sorting with MACS MicroBeads (Miltenyi Biothech GmbH, Germany) as per the manufacturer's instructions. Briefly, cells were washed once in complete RPMI 1640 medium and pellets re-suspended in MACS buffer (5% fetal calf sera in PBS). Cell debris was removed by passing the cell suspension through a 30 μm nylon mesh and cells pelleted by spinning at 300xg for 10 minutes. Pellets were re-suspended at 10^7 cells/ 90 μl of MACS buffer and 10 μl of the respective microbeads were added per 10^7 cells. After thorough mixing, cells were incubated at 4-8^{o}C for 15 minutes and then washed in 3 ml MACS buffer by spinning at 300xg for 10 minutes. Pellets were re-suspended in 500 μl buffer per10^8 cells and applied onto an MS selection column (Miltenyi Biothech GmbH, Germany) placed in a magnetic field and prepared according to the manufacturer's instructions. Unlabeled cells were collected when they passed through the column as a negative fraction and the labeled cells, the positive fraction, were harvested after removing the column from the magnetic field and flushing with an appropriate volume of buffer by firmly applying the plunger into the column. Cells were washed once in complete RMPI 1640 medium, re-suspended in the original (pre-depletion) volume of the same media, counted and adjusted to 10^7 cell/ml by adding APC from naïve mice. Cells were kept on ice until used in the ELISPOT assay and 100 μl of these cells were added per well so that each well of the ELISPOT assay will have equal number (10^6) of spleen cells.

For flow cytometric analysis, a 100 μl aliquot containing around 10^6 cells in complete RPMI media were taken from each test tube into a screw cap 2 ml tube. Tubes were centrifuged at 300xg for 10 minutes, supernatant removed gently and pellets re-suspended in 100 μl FACS buffer (5% FCS in PBS). Cells were stained for expression of CD4(L3/T4, Ly4) and CD8(Ly2) by adding 5 μl of FITC conjugated rat anti mouse CD4 (Clone YTS 191.1.2) and PE conjugated rat anti-mouse CD8 (Clone YTS 169 AG 101HL) antibodies (Cedarlane Laboratories Ltd) to each tube and incubating in the dark for 30-45 minutes at 4-8 ^{O}C. Cells were again washed as above in 1 ml FACS buffer and pellets re-suspended in 1ml buffer and analyzed by flow cytometry using a Beckman Coulter Epics Flow Cytometer and Expo32 v1.2 analysis software (Beckman Coulter, Mississauga, ON, Canada). If flow cytometry was not to be done on the same day, cells were fixed by adding equal volume of 2% formaldehyde in PBS and stored in the dark until next day.

4.11. Anti-IL-4 Antibody Production

The protocol for ascites production was described in detail previously (Coligan et al., 1992). Briefly, adult BALB/c or CD1 mice, age 8-10 weeks, were injected with 0.5 ml of incomplete Freund's adjuvant (IFA) or 0.5 to 1 ml pristane ip to induce nonspecific inflammation. As the hybridoma cells are of rat origin, mice were treated ip with 4 mg/mouse of cyclophosphamide and/or gamma irradiated with ~750 rads to suppress the immune response seven days after pristane or IFA treatment. Then, on the same day or the next morning, the mice were injected, using a 20 or 22 gauge needle, with 0.2 ml saline containing ~10^8 11B11 hybridoma cells (ATCC # HB-188). Starting from day 7

after injection, mice were checked for the development of ascites and ascites fluid was collected into a test tube using an 18-gauge needle. Collected fluid was purified by thiophilic adsorption chromatography using the T-Gel^{TM} purification Kit (Pierce, Rockford, IL, USA) according to the manufacturers protocol and its biological activity was tested using the CT4S (IL-4 dependent cell line) assay as previously described (Hu-Li et al., 1989). Briefly, cells were counted, washed, and re-suspended in RPMI complete medium at a concentration of $1x10^5$ cells/ml. Standard and sample (ascites fluid) to be tested were titrated onto 96-well flat bottom plates at 50 µl/well. The range of the rIL-4 standard was 4 units/ml to 0.5 units/ml without or with 30 µl anti-IL-4 antibodies/well. To the test wells, 50 µl media was added to the first row and the sample (ascites fluid) was serially diluted from 1/25 to 1/3200 in triplicates starting from the second row. Then, 100 units rIL-4/ml was added to all the test wells. Finally, 100 µl culture medium containing the CT4S cells was added to all wells at a concentration of 10^4 cells/well. Plates were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, 0.5 µCi of tritiated thymidine in 50 µl medium was added to each well and plates were again incubated for 4hrs at 37°C. After final incubation, cells were harvested onto a filter paper and placed into scintillation tubes. Finally, each tube was filled with 4 ml of scintillation cocktail and cell proliferation counted using Beckman LS 5000TD liquid scintillation system (Beckman Instruments Inc, Fullerton, CA, USA). The BCA protein assay (Pierce, Rockford, Illinois) was used, as described in section 4.4, to determine the concentration of anti-IL-4 antibody and mice were treated with this antibody as described in section 4.12.

4.12. Treatment of Mice with Anti-IL-4 Antibody

Mice previously vaccinated with a dose of BCG that induced a mixed Th1/Th2 response were divided into two groups and treated ip either with 1 mg/week anti IL-4 antibody for 4 weeks or an equal amount of control antibody. This dose was chosen based on previous results from the mouse model of leishmaniasis in our laboratory (Uzonna and Bretscher, 2001). ELISPOT and ELISA assays were done between 6 to 18 weeks after the last treatment to determine if anti-IL4 antibody treatment is able to alter the immune response from a mixed Th1/Th2, humoral, type of response into a cell-mediated, Th1, response.

4.13. Exposure of Mice to Mycobacterium gordonea

M. gordonea stock was obtained from the TB laboratory of the Royal University Hospital, University of Saskatchewan, Saskatoon. Bacterial stock was propagated in Middlebrook 7H9 broth supplemented with 10% Middlebrook ADC enrichment and the number of bacteria subsequently enumerated by plating on Mycobacteria 7H11 agar supplemented with Middlebrook OADC enrichment (BD Biosciences, Sparks, MD 21152, USA). For infection, bacteria were washed three times in PBS by centrifuging at 8000xg and re-suspended at the required concentration in the same buffer. Mice were exposed orally to *M. gordonea* (~$5x10^6$ cfu/ml) in the drinking water for 2-3 weeks in a attempt to cause immunological priming, as assessed by seroconversion. Our results were not consistent and so we decided to prime mice by exposing them to the same mycobacteria by sc injection with different doses of the bacteria. This was to determine a dose that can induce a mixed Th1/Th2 response as assessed by seroconversion.

4.14. Nitric Oxide Assay

This assay was done to measure the total level of nitric oxide (NO) secreted by activated macrophages. Isolation and culture of mouse macrophages was carried out as described elsewhere (Coligan et al., 1998). Briefly, adult BALB/c mice were injected ip with 1ml of 3% Brewer's thioglycolate medium prepared by the Glassware and Media Preparation Unit of the Department of Vet. Microbiology, University of Saskatchewan. Four days later, mice were killed by cervical dislocation and laid on their back. The abdomen was disinfected with 70% ethanol and a midline incision made with sterile scissors. After reflecting the abdominal skin with forceps, the abdomen was penetrated with a 19G needle attached to a 10 ml syringe that contained 6-8 ml Delbucco's tissue culture medium, DMEM-0%, (DMEM media with no serum but supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.8mM sodium pyruvate, and $5x10^{-5}$ M β-mercaptoethanol). The peritoneum was flushed with the medium and the lavage harvested with the same syringe and needle into a 15 ml centrifuge tube. Cells were washed in the same media by spinning at 300xg for ten minutes, counted and re-suspended in DMEM-10%, (DMEM media supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.8 mM sodium pyruvate, and $5x10^{-5}$ M β-mercaptoethanol) at 10^6 cells/ml. Cells were added into a 24 well polystyrene tissue culture plate (Corning Glass Works, Corning, NY) at 1 ml/per well and incubated in a humidified, 5% CO_2 incubator set at 37°C. After 2-3 hours of incubation, non-adherent cells were removed by careful aspiration and the adherent cells were further incubated for another 48 hours by adding 1 ml DMEM-10% media containing nylon wool-purified T-cells in the presence or absence of BCG antigen.

To separate T-cells using nylon wool columns, spleen cells from mice vaccinated with either low dose or high dose of BCG or saline were prepared into single cell suspension as described above, and then red blood cells were lysed using the hypotonic lysis method (Gordon 2002). Briefly, 9 ml ddH_2O was added into 1 ml cells and lysis stopped after 20 seconds by adding 1 ml of 10x Hank's Balanced Salt solution, HBSS (GIBCO). Cells were washed by centrifuging at 300xg, re-suspended in DMEM-10% at 10^8 cells/ml and purified using nylon wool fiber columns (Polysciences Inc, Warrington, PA) according to the manufacturer's instructions. Cells collected from the column were again washed in DMEM-0% and re-suspended at $5x10^6$ cells/ml in DMEM-10% and added into the wells containing the adherent cells at 1 ml per well. After 48 hours incubation, the supernatant was collected from each well into test tubes and stored at -20 ^oC until the NO level was measured in the Griess reaction using the Parameter^{TM} NO assay kit (R&D Systems, Inc. Minneapolis, USA), as per the manufacturer's protocols.

4.15. Statistical Analysis

Analysis of variance was carried out on the estimated number of antigen-dependent cytokine secreting cells from mice within each vaccination groups using the SAS statistical package PROC GLM (SAS, 1999) and the number of IL-4 and IFN-γ secreting cells as well as their ratios were compared in mice among groups using the Fisher's protected least significant difference (FLSD) test. *P* values <0.05 were considered to be significant.

5. Results

5.1. Low Dose BCG Vaccination in Newborn Mice

5.1.1. Introduction

In humans, vaccination with BCG against tuberculosis is usually done on newborn infants immediately after birth. Moreover, neonatal BCG specific Th1 imprinting may be important in the prevention of Th2 imprinting caused by exposure to environmental mycobacteria, which may interfere with the efficacy of vaccination (Fine, 1995; Black et al., 2001). Studies outlined in the Introduction and carried out with both animals and humans indicate that the neonatal immune system can be easily primed following exposure to environmental mycobacteria. Hence, our objective in this experiment was to determine an appropriate low dose vaccination strategy in newborn mice to establish a Th1 imprint as early as possible and thus prevent possible interference by environmental mycobacteria. Mice, unlike man and cattle, are less immunocompetent at birth. Observations from our laboratory (unpublished data) and from others (Piguet et al., 1981; Ridge et al., 1996) show that the total number of peripheral T-cells (LN plus spleen) at birth are more than 1000 fold lower than in adult mice. Similarly, the number of thymocytes at birth is more than 100 fold lower than in mice 7-14 days after birth (Piguet et al., 1981; Xiao et al., 2003). Therefore, 7-14 day old mice may arguably best represent the immunocompetence of newborn humans (Siegrist, 2000) and mice of about this age can be used as an animal model for our envisaged strategy for tuberculosis vaccination in

humans. In preliminary studies, neonatal BCG vaccination of 2-day-old mice gave results that could not be interpreted due to high variability within mice of the same group (data not presented). This variability could be due to the immune-incompetence of mice at birth, as discussed above, or could be also due to technical problems in vaccine delivery. It was very difficult to reliably inject the required dose of BCG intradermally into 2-day-old mice. Therefore, we decided to use “very young mice” instead of neonates. We use the terms “very young” or “newborn” to refer to mice between the ages of 5 and 7 days.

5.1.2. Results

5.1.2.1. Dose of BCG Administered to Newborn Mice Determines the Th1/Th2 Nature of the Ensuing Immune Response

We wished to determine if the cell-mediated/humoral nature of the immune response to BCG in newborn BALB/c mice was dependent upon the dose of BCG administered. As explained in section 5.1.1, such a study is a required first step in developing a mouse model to examine the potential efficacy for a neonatal low dose BCG vaccination strategy against tuberculosis in humans. We therefore chose to examine the responses of newborn BALB/c mice vaccinated with a range of BCG doses. We chose a highest dose that can induce a mixed Th1/Th2 response in naïve mice on the basis of previous studies from our laboratory in adult mice. We then chose different doses, which are several logs lower than the highest dose to look for the lowest possible dose that can induce an exclusive Th1-type response with no significant Th2 component. Accordingly, mice were vaccinated with 33, 330, 3300, $3.3x10^7$ cfu of BCG or saline sc in a 20 μl volume. Mice

were sacrificed at different times after vaccination and ELISPOT assays were employed to enumerate the number of BCG-specific IFN-γ and IL-4-producing T-cells per million spleen cells (Figure 3). Each sample was analyzed in triplicate and the average of the three wells was used to determine antigen-dependent spots by subtracting spots in antigen negative wells from those counted in antigen positive wells. Figures 4A and B show observations obtained from mice sacrificed 12 and 16 weeks post-vaccination, respectively. Individual rather than pooled responses are shown to demonstrate the consistency of the observed responses. In general, responses much greater than background (seen in age-matched control mice) are generated in mice vaccinated with 33, 330, 3300 and $3.3x10^7$ cfu of BCG. Statistical analysis of the overall result shows that all vaccinated mice have significantly ($P<0.05$) higher numbers of IFN-γ-secreting cells in their spleen than non-vaccinated mice, but no significant differences were observed among vaccinated groups. This reflects the level of IFN-γ production at a relatively long time after vaccination when the Th1-type response is stabilized. We chose to assess the immune response at this time, as we were interested in the long-term response following immunization of newborn mice. The level of the IFN-γ response might well differ significantly between mice given different doses of BCG during the early course of the infection, as the lower doses need time to replicate and induce an immune response comparable to the high dose vaccinated group. On the other hand, no significant numbers of IL-4-secreting cells were found in any of the low dose vaccinated groups (33, 330, 3300 cfu); however, mice in the high dose group ($3.3x10^7$ cfu) displayed a significantly higher ($p<0.05$) number of IL-4-secreting cells than both the control mice and low dose vaccinated groups.

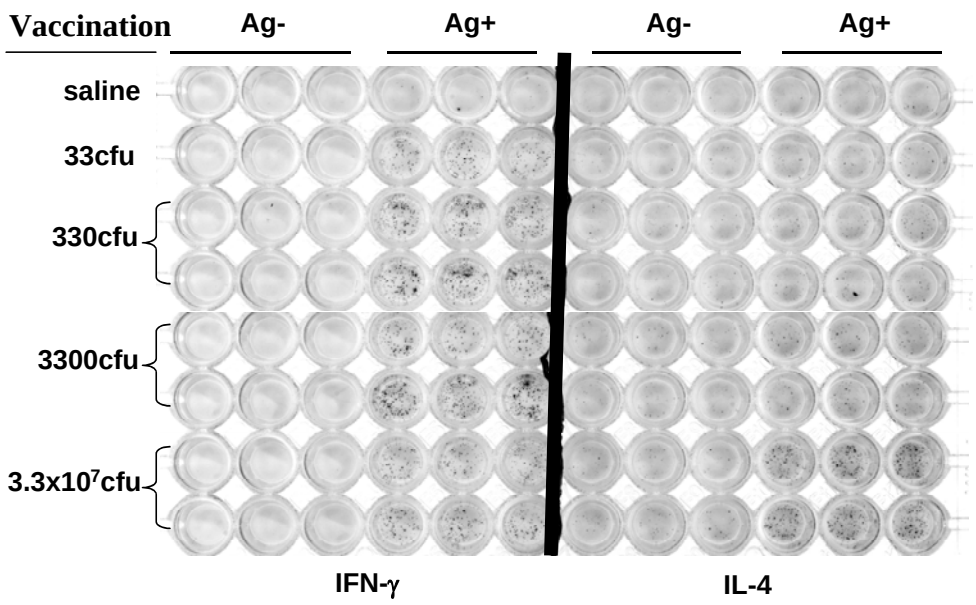

Figure 3. Picture of a 96 well plate showing BCG-specific IFN-γ and IL-4 spots as detected in the ELISPOT assay.

Spleen cells from BALB/c mice vaccinated with different doses of BCG were assessed for the number of antigen-dependent cytokine producing cells by ELISPOT assay. The picture above shows the number of spots seen in mice immunized when very young, with either saline or the indicated number of BCG given sc. Mice were sacrificed around 12 weeks post-vaccination and spleen cells processed for ELISPOT assay. The spots shown in the picture were produced by culturing $5x10^5$ sensitized spleen cells per well.

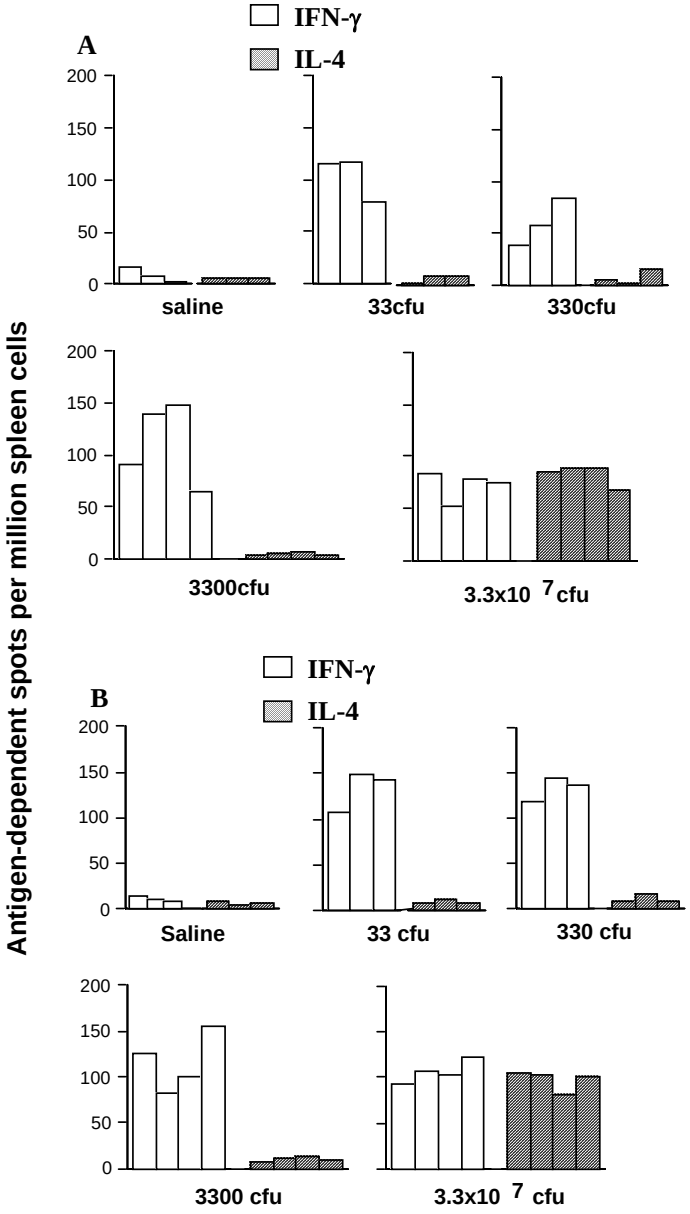

Figure 4. Low doses, but not a high dose of BCG, administered to newborn BALB/c mice generate predominant Th1 responses.

Newborn BALB/c mice were vaccinated with different doses of BCG subcutaneously as shown on the abscissa and were sacrificed at 12 (A) and 16 (B) weeks post-vaccination. Spleen cells were assessed for the number of antigen-dependent cytokine secreting cells. These two sets of observations are representative of more than 4 replicate experiments with 3-5 mice per group in each experiment. Data represent values for individual mice in each group.

In summary, the three lowest doses of BCG employed generated predominantly Th1-type responses with an "indeterminate" value for the ratio of the number of antigen specific IFN-γ to IL-4-producing cells due to an insignificant or barely detectable number of antigen-specific IL-4-producing cells, usually $\leq$ 10 per million spleen cells. The possible detectable numbers of spots were used for analysis purposes. The highest dose of BCG generated a mixed Th1/Th2 response with an IFN-γ/IL-4 ratio less than or close to 1, which is significantly ($p<0.05$) lower than the ratio in the mice belonging to low dose vaccinated groups. Mice were also bled and their peripheral blood leucocytes isolated to assess, by ELISPOT assay, the number of antigen-dependent cytokine producing cells. Serum collected from whole blood was also used to measure, by ELISA, the levels of mycobacterium-specific IgG_1 and IgG_{2a} antibodies. The nature of the response seen in the peripheral blood reflected a trend similar to that observed in the spleen: all low dose vaccinated groups had a high number of IFN-γ-secreting cells but an inconsistent and low number of IL-4 producing cells in their peripheral blood, while the high dose vaccinated group had a substantial number of IL-4-producing cells (Table 1). Again, no significant difference in the level of IFN-γ production was observed among all vaccinated groups, but the level of IL-4 was significantly higher ($p< 0.05$) in the high dose ($3.3x10^7$ cfu) vaccinated group. Our observations in the BCG model as well as results from the leishmania model (unpublished data) show relatively more IL-4-secreting cells are found in the peripheral blood than in the spleen when compared to the number of IFN-γ producing cells. This general finding is reflected in the ratio of IL-4/IFN-γ-producing cells, which is >2 in peripheral blood but <1 in the spleen of the high dose vaccinated group.

Table 1. Low dose BCG vaccination in 5-7 day old BALB/c mice induces a predominantly cell-mediated response with a high level of IFN-γ-secreting cells in the peripheral blood.

Blood taken from the tail vein was processed to isolate peripheral blood mononuclear cells, and the PBMNCs cells were dispensed into ELISPOT plates to determine antigen-specific cytokine secreting cells in blood. Mice in each group were injected with the indicated number of bacteria in cfu, while control mice were injected with an equal volume of saline. Results represent the mean values from 3-4 mice per group with the standard deviation of the mean (SD).

	Antigen-specific cytokine secreting cells per million PBMNCs as detected in the ELISPOT assay				
	IFN-γ		IL-4		IL-4/IFN-γ[c]
BCG dose (cfu)	Mean	SD[b]	Mean	SD[b]	
33	145.0	28.3	24.5	0.7	0.17‡
330	117.5	20.5	66.3	7.1	0.56‡
3300	202.0	12.7	20.0	1.4	0.10‡
$3.3x10^7$	114.5	48.8	231.0	43.8	2.02*
Saline[a]	13.3	NA	18.3	NA	NA

[a] Control mice injected with equal volume of saline
[b] Standard deviation of the mean as calculated in Microsoft Excel, 2003
[c] IL-4 to IFN-γ ratio
NA, Not applicable as pooled blood was used
* Significant difference at 0.05 p value
‡ No significant difference between groups

The qualitative nature of the immune response generated following BCG vaccination of mice was also reflected in the isotype of antibodies produced. Low dose vaccination resulted in either antibody levels below the detection limit when assessed with a serum dilution of 1 in 100 (represented by *** in Figure 5), or predominantly IgG_{2a} antibodies for intermediate doses (data not shown). In contrast, vaccination with the highest dose of BCG induces a mixed IgG_1 and IgG_{2a} antibody response (Figure 5). Our findings agree with a recently published report, which concluded that the BCG antigen-specific antibody response was dose dependent (Horwitz et al., 2006). Extraordinarily few (10 cfu) of BCG was used in this study to vaccinate guinea pigs and only cell-mediated response was induced. In contrast, the highest vaccine dose used in the study induced both cell-mediated and humoral responses, with significant antibody titers.

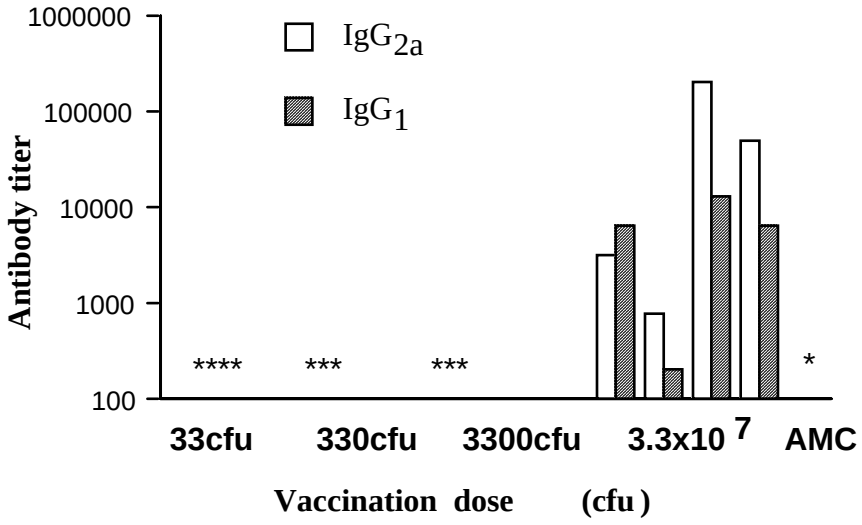

Figure 5. Low dose BCG vaccination in 5-7 day old BALB/c mice induces a predominantly cell-mediated response with no detectable BCG-specific IgG_1 and IgG_{2a} antibodies.

Blood taken from the tail vein was spun at 12000 rpm for 3-5 minutes in a Microspin centrifuge to obtain serum; then assessed by ELISA for BCG-specific IgG_1 and IgG_{2a} antibodies. Numbers in the abscissa represent the dose of BCG vaccine. AMC indicates pooled serum sample from 3 age matched control mice given saline at the time of vaccination. * stands for a titer < 100. Results shown are from one of three replicate experiments with data presented for individual mice in each group (n=3-4 mice).

5.1.2.2. Pre-exposure of Newborn BALB/c Mice to Low Doses of BCG Leads to Th1-Imprinting as Assessed by a High Dose BCG iv Challenge

To examine whether exposure of newborn mice to BCG could result in effective Th1 imprinting, as assessed by high dose BCG challenge, and whether such imprinting resulted in decreased splenic mycobacterial burden following BCG challenge, mice were vaccinated sc with either saline (control group) or different doses of BCG (33, 330, 3300, and $3.3x10^7$ cfu in 20 µl of saline) at 5-7 days after birth. Mice were followed longitudinally for up to a year to monitor the antibody and T-cell responses by ELISA and ELISPOT assays, respectively. As reported in section 5.1.2.1, mice vaccinated with low doses of BCG consistently mounted exclusive Th1-type responses, whereas mice in the high dose vaccinated group ($3.3x10^7$ cfu) generated mixed Th1/Th2 responses. The Th1/Th2 nature of the response in the vaccinated mice was stable for at least 9-12 months (data not shown); a time corresponding to half of the mouse's expected life span. However, the critical question was whether this stable response was associated with a stable Th1 imprint? We wanted to address this issue by high dose challenge of vaccinated mice and assessing the IgG isotypes of the anti-BCG antibody response and the Th1/Th2 nature of the immune response by ELISA and ELISPOT assays, respectively.

Before challenge of vaccinated mice with a high dose of BCG, we wished to determine the highest dose that could be administered without affecting the health of the mice. Several experiments were required to determine an effective challenge dose. We first challenged both vaccinated and naïve mice with ~$3.3x10^7$cfu of BCG iv, but some mice,

particularly in the high dose vaccinated and challenged group demonstrated neurological symptoms, such as loss of balance and head tilting to one side at around six weeks post-challenge. Although ear infection was first suspected, necropsy and histopathological examination performed on representative mice by Dr. O'Connor, (Prairie Diagnostic Service, Western College of Vet. Medicine, University of Saskatchewan) excluded ear infection. This examination revealed severe inflammatory lesions in multiple organs (lung, liver, spleen, kidney, brain, and adrenal glands) with lymphocytic infiltration and a non-suppurative encephalitis in the mid-brain. The lesions were particularly severe in the lung and spleen with severe perivascular and peribronchiolar lymphocytic infiltration and granulomatous inflammation. Numerous acid-fast bacilli were detected inside foamy macrophages in the lung and prominent follicles with similar histopathology were observed in the spleen. Following high dose iv challenge, an increase in the size of the spleen was also observed, with a maximum increase of 2-to-5 fold in the high dose vaccinated and unvaccinated groups when compared to vaccinated but unchallenged mice (Figure 6).

We suspected the gross and microscopic lesions were due to the extremely high challenge dose for various reasons. Since the iv route is more immunogenic than id/sc routes, the challenge dose used here (~$3.3x10^7$ cfu) might be high enough to induce a mixed Th1/Th2 response when given id/sc, but so high that when administered as an intravenous challenge a potential pathogenic capacity of BCG was revealed. The greater effectiveness of antigen given intravenously over that given subcutaneously is not surprising, as in the former case the antigen is expected to reach secondary lymphoid organs more effectively.

In addition, the BCG will likely reach more locations in the body when given iv than sc or id. Our observations are consistent with other reports indicating antigen given iv was more immunogenic than when given sc (Lagrange et al., 1974). Furthermore, re-assessment of the number of bacteria in the challenge dose by plating on agar showed around $1.1x10^8$ cfu, considerably higher than the original estimate of $3.3x10^7$. After a few preliminary challenge experiments, we determined that doses between $2x10^6$ and $1x10^7$ cfu were safe for challenge. All challenges were made iv based on previous protocols employed in *M. tuberculosis* challenge studies. North and Jung have reported that infecting mice with 10^5 cfu virulent *M. tuberculosis* iv can result in similar pathological lesions in the lung and other organs as mice infected with 100 cfu intranasally, which corresponds to the natural mode of infection (North and Jung, 2004). The ultimate goal of our studies was to carry out such *M. tuberculosis* challenges, but these could not be realized due to unavailability of appropriate facilities at our university. The same study by North and Jung has reported that, next to the liver, the bulk of an iv inoculum implants in the spleen. It is, therefore, appropriate to assess the splenic response following iv challenge of mice.

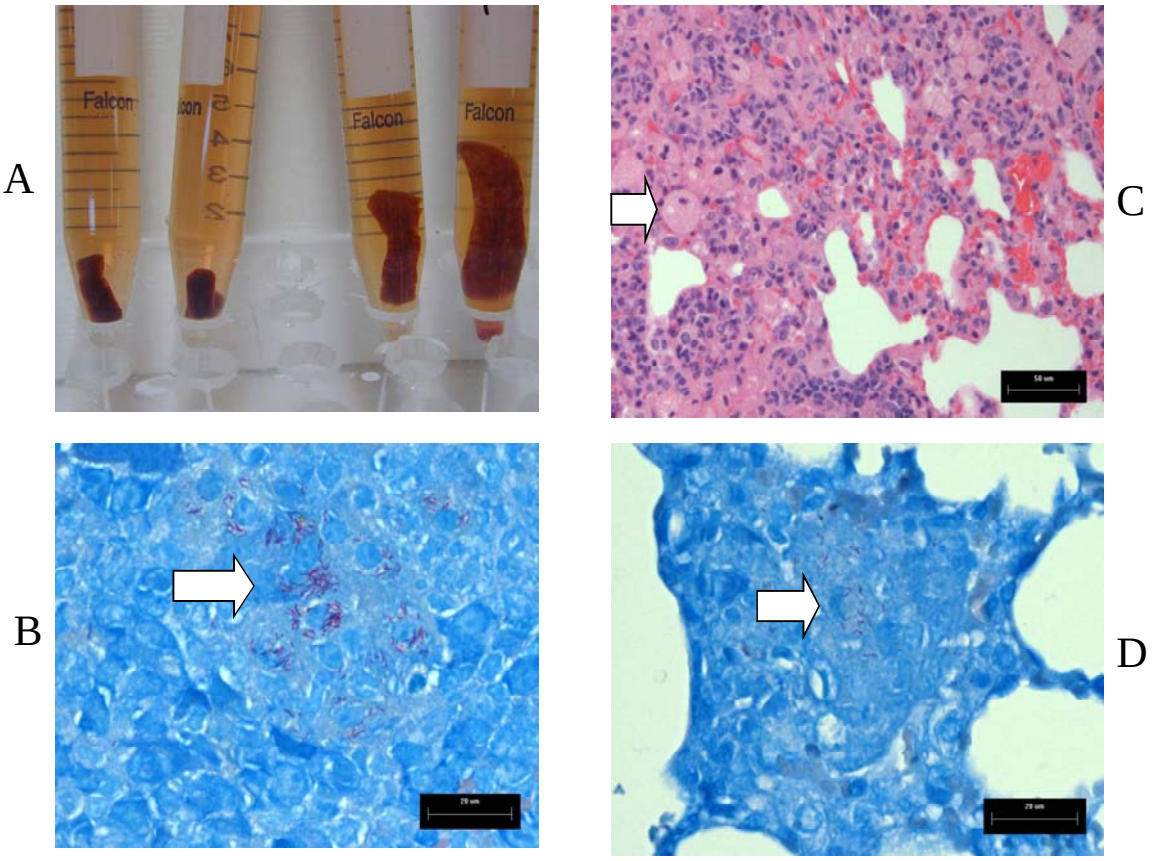

Figure 6. A very high dose of BCG ($1.1x10^8$ cfu) given iv following sc vaccination with different doses of BCG induces pathological lesions in the spleen and lung of BALB/c mice.

Gross and histopathology studies of spleen and lung were conducted on BALB/c mice vaccinated with different doses of BCG sc and challenged iv with ~1.1×10^8 cfu of BCG 12 weeks post-vaccination. Mice were sacrificed between 6 and 9 weeks following high dose iv challenge. A) Gross size of spleen from vaccinated but unchallenged mice (first two tubes) as well as in mice vaccinated with 3.3×10^7 cfu of BCG and then challenged with a high dose BCG (last two tubes). B) Acid-fast bacilli inside a macrophage from spleen lesions. C) Lymphocyte infiltration with giant foamy macrophages in the lung. D) Acid-fast bacilli in one of these macrophages from the lung lesion.

We tried different doses to find a dose of BCG that can induce a mixed Th1/Th2 response in naïve mice, and with little or no complication in vaccinated mice when given intravenously. By using a dose of BCG that induces a mixed Th1/Th2 response in naïve mice as a challenge dose, we are trying to mimic the type of response observed in human tuberculosis patients with a significant Th2 component. Accordingly, doses between $2x10^6$ and $1x10^7$ cfu were found to be safe for our purpose and naïve mice as well as mice vaccinated with different doses of BCG were challenged with a dose of BCG within this range, injected iv in 250 µl of saline. Each group contained 9-12 mice and mice in each group were challenged between 3 to 6 months after vaccination. Three to five mice from each group were bled for assessment of the BCG-specific antibody response by ELISA and sacrificed for an assessment of the Th1/Th2 nature of the splenic immune response by ELISPOT, and splenic bacterial burden by colony formation, at a time between 12 and 18 weeks post-challenge. ELISA results indicated that all challenged mice had high titers of both BCG-specific IgG_1 and IgG_{2a} antibodies (Figure 7). On average, the ratio of IgG_{2a} to IgG_1 was between 5 and 15 in the low dose vaccinated group, between 2 to 4 in the high dose vaccinated group, and around 1 for the non-vaccinated but high dose challenged group. Similarly, results from the ELISPOT assay revealed that low dose BCG (33, 330, 3300 cfu) vaccinated mice mounted a Th1-type response upon high dose iv challenge, with IFN-γ to IL-4 ratio ranging between 15 to 60. In contrast, the high dose ($3,3x10^7$ cfu) vaccinated and non-vaccinated group when challenged iv with a high dose of BCG generated mixed Th1/Th2 responses, with IFN-γ to IL-4 ratios of around 1 and < 1, respectively (Figure 8). No significant differences were found in the level of IFN-γ secreting cells when comparing among groups.

Furthermore, the level of IL-4 was not different among mice from the low dose (33, 330, 3300 cfu) vaccinated groups. Accordingly, the IFN-γ to IL-4 ratios of the low dose vaccinated groups were not statistically different. Similarly, there was no significant difference in IL-4 levels between the high dose ($3.3x10^7$ cfu) vaccinated and non-vaccinated groups after challenge. However, a significantly higher ($p<0.05$) level of IL-4 in the high dose vaccinated and non-vaccinated mice after high dose iv challenge was noted when compared to the low dose vaccinated groups. Accordingly, the IFN-γ to IL-4 ratios from all low dose vaccinated groups were significantly different ($p<0.05$) than the high dose vaccinated and non-vaccinated groups. These results confirm that low dose BCG vaccination in the newborn can induce the appropriate type of response, with a subsequent lock of the response into a Th1 mode as seen upon high dose iv challenge. It is tempting, when comparing Figures 4 and 8, to summarize these observations by saying that long term infection of newborn BALB/c mice with 33, 330 and 3300 cfu led to Th1 responses and Th1 imprints, and with $3.3x10^7$ to balanced Th1/Th2 responses and Th1/Th2 imprints.

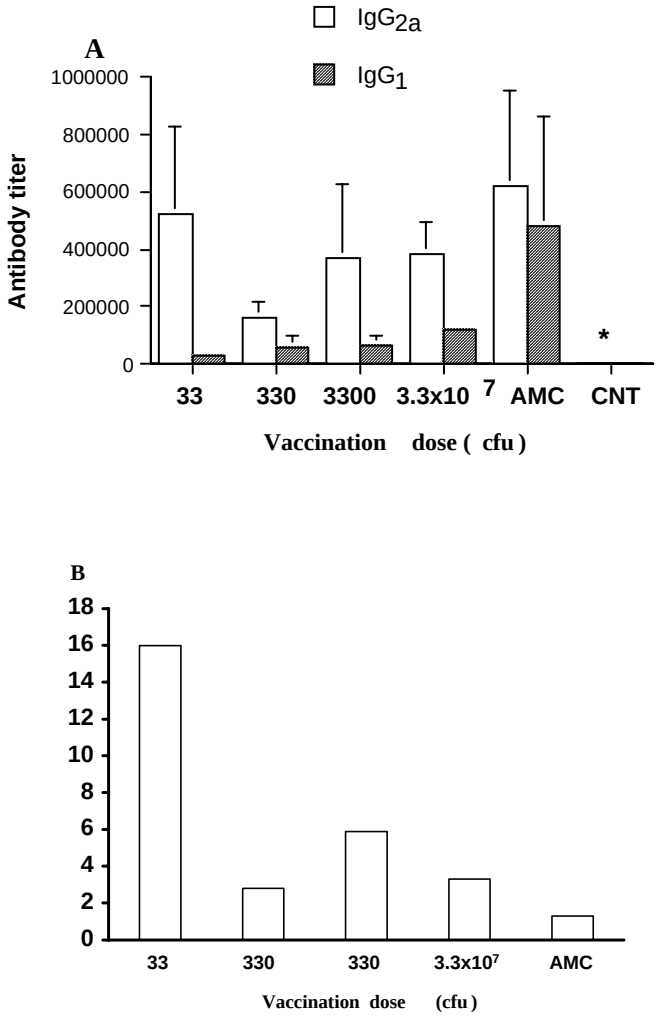

Figure 7. The BCG-specific IgG_{2a} to IgG_1 ratio following iv challenge with high dose BCG was higher in the sera of mice vaccinated with low dose of BCG than mice vaccinated with a high dose of BCG or naïve mice.

Newborn BALB/c mice vaccinated with different doses of BCG were challenged iv with $6x10^6$ cfu of BCG at 16 weeks post-vaccination. A) Titers of BCG-specific IgG_1 and IgG_{2a} antibodies present in serum at 12 weeks post-challenge were assessed in ELISA. The ratio of IgG_{2a} to IgG_1 antibodies for each group is also shown (B). These data represent mean antibody titers of 3-5 mice per group with SD and is representative of one of at least three replicate experiments. AMC (age matched control) refers to the unvaccinated but challenged group. CNT (control) refers to pooled sera from unvaccinated and unchallenged mice. * represents titers <100

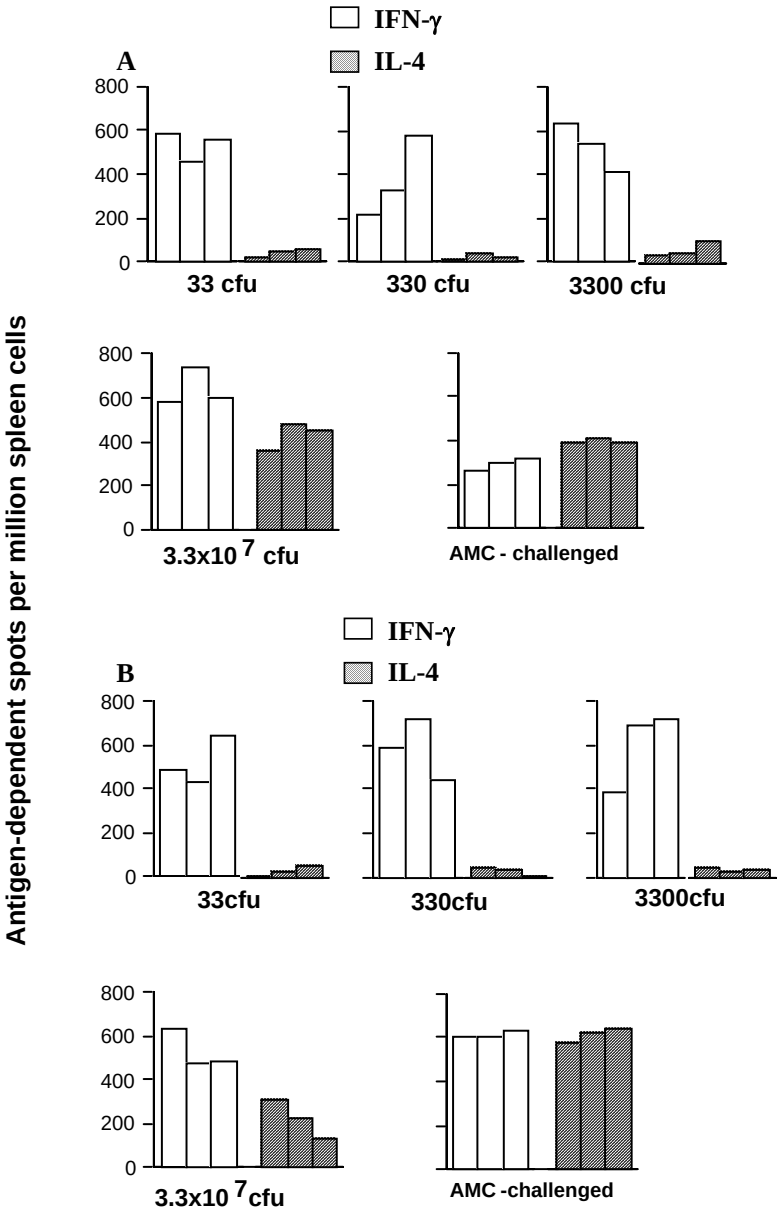

Figure 8. Low dose BCG vaccination induces Th1 type response and Th1-imprinting in newborn (5-7 days old) BALB/c mice.

Newborn BALB/c mice were vaccinated with different doses of BCG, and were challenged iv 16 weeks post-vaccination with ~$6x10^6$ cfu of BCG. BCG-specific IFN-γ and IL-4-producing T-cells present in the spleen were measured by the ELISPOT assay at (A) 12 and (B) 18 weeks post-challenge. AMC (age matched control) refers to naïve mice challenged with high dose of BCG. The results shown are from one of three replicate experiments with data presented from individual mice within each group (n= 3).

We also determined if the Th1 imprint seen in the low dose vaccinated mice was accompanied by a more efficient clearance of BCG and hence a lower bacterial burden in the spleen. Spleens were removed from each mouse at the indicated time points following BCG challenge and processed to generate single cell suspensions at 10^7 cells/ml. Half the spleen was used for ELISPOT assay and the remaining half processed for assessing bacterial burden as described in the Materials and Methods section (see also Figure 9).

Figure 10 summarizes our findings from four independent experiments determining how the mycobacterial burden in the spleen, following an intravenous challenge of BALB/c mice with a high dose of BCG, depends on the dose of BCG vaccine given to them sc as newborn mice. The scale for the bacterial burden is different for the different experiments; however, in all cases greater bacterial burdens were seen in mice given higher doses of BCG challenge. The exact conditions of these experiments are slightly different, as described in the legend. However, the overall pattern is very clear. Vaccination with the lowest dose (33 cfu) is the most consistent and reliable means of reducing the mycobacterial burden following the high dose BCG challenge, with a reduction ranging from around 50 to about 200 fold. In general, no statistically significant difference was observed among the low dose vaccinated groups. However, non-vaccinated and high dose vaccinated mice were significantly different ($p<0.05$); both these groups were also significantly different than the entire low dose vaccinated groups. In some experiments, no difference was found between high dose vaccinated mice and low dose vaccinated mice in the efficacy of clearing mycobacteria from the spleen (Figure 10C and D). The reason for this could be due to the technical difficulty of trying

to deliver $3.3x10^7$ cfu of BCG in a very small inoculum (10-20 μl) administered sc to newborn mice. An assessment of the bacterial suspension after vaccination led on one occasion to an estimate of only 10^6 cfu of BCG in each aliquot of the high dose, which is 10-fold lower than the intended dose. Such a dose generates a mixed Th1/Th2 response with relatively higher levels of IFN-γ than IL-4 (data not shown). Even after high dose challenge there is a tendency for mice vaccinated with a high dose of BCG to show a slight bending of response generated to the high dose challenge towards the Th1 pole. It is interesting to examine the situation represented in Figure 8A that comes from the same experiment that provided the observations recorded in Figure 10B. Vaccination of newborn mice with high dose of BCG resulted in a slight but significant bending of the response to the Th1 pole, a bending associated with about a 5-fold drop in mycobacterial burden. The greater the modulation of the immune response towards the Th1 pole, caused by vaccination of mice with lower doses of BCG, the greater are the reductions in the mycobacterial burden.

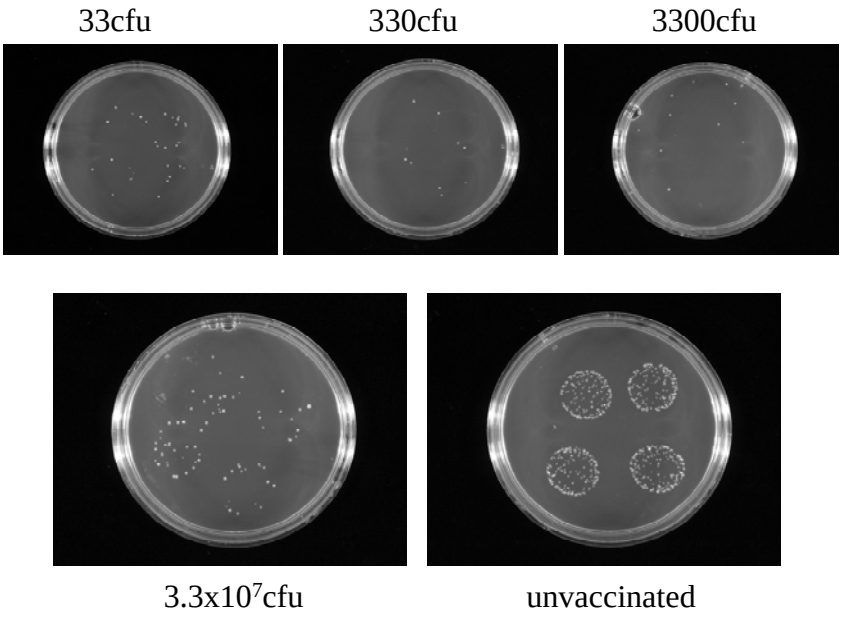

Figure 9. Pictures of plates used to measure mycobacterial burden in the spleen of BALB/c mice following high dose BCG challenge.

Spleen cells were pooled from 3 mice in each group vaccinated with different doses of BCG and challenged iv with a high dose of BCG. Mice vaccinated with low doses of BCG had a lower bacterial burden than non-vaccinated mice or those vaccinated with a high dose of BCG following a high dose BCG iv challenge. Numbers represent dose of BCG used to vaccinate newborn mice sc before high dose iv challenge. Result represents one of more than 4 replicate experiments.

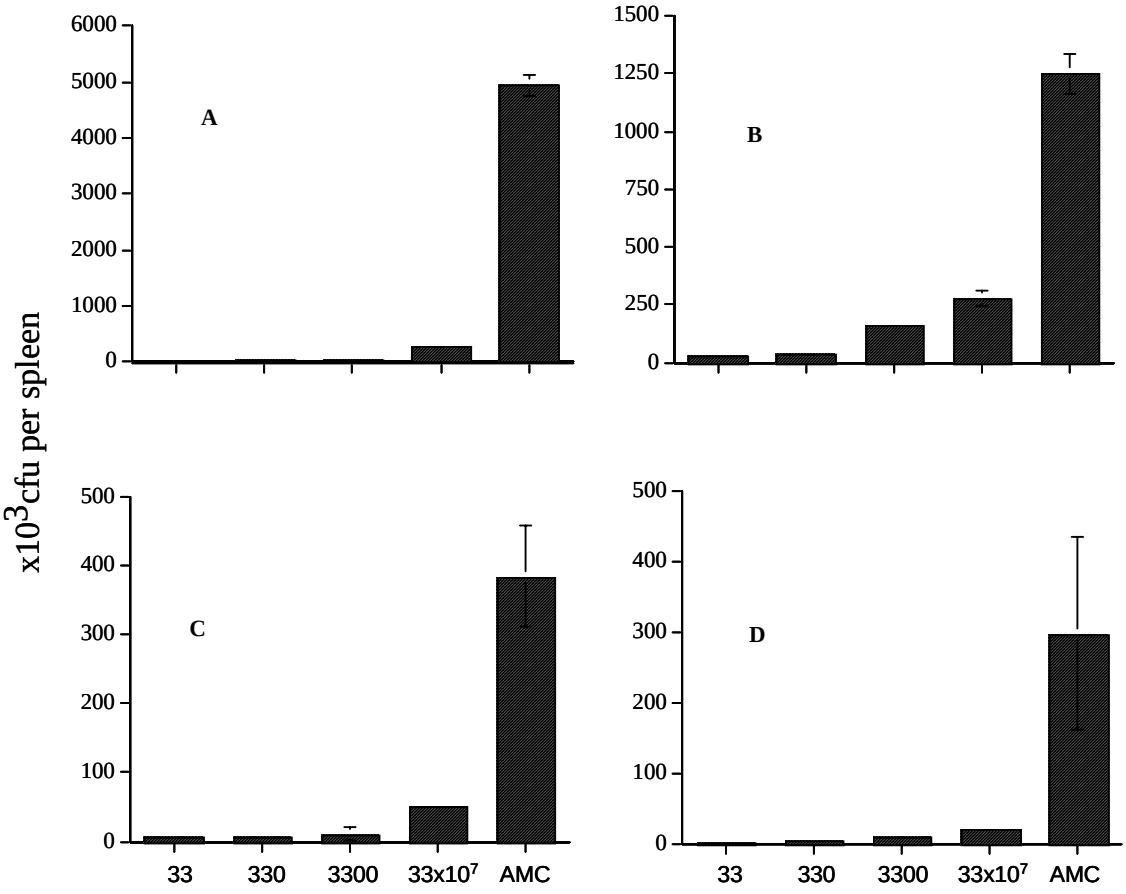

Figure 10. Th1 imprinting as a result of vaccination of newborn BALB/c mice with low doses of BCG was associated with more efficient BCG clearance from the spleen following iv challenge of mice with high dose of BCG.

BALB/c mice were vaccinated with different doses of BCG and challenged iv with ~$3.3x10^7$ (A), ~$6x10^6$ (B) and $2x10^6$ (C and D) cfu of BCG at 12 weeks post-vaccination. Mice were then sacrificed between 12 and 18 weeks after challenge and spleen cells processed for assessment of bacterial burden. Results shown represent mean of 4-6 mice per group with error bars (SD). AMC (age matched control) refers to naïve mice challenged with high dose of BCG.

Table 2. Results of statistical analysis for data shown on Figure 10A-D

BALB/c mice vaccinated and challenged as described in the legend for Figure 10 were sacrificed at (A) 14 weeks, (B) 12 weeks, (C) 16 weeks, and (D) 18 weeks post-challenge. Spleens were removed from each mouse and processed for assessment of bacterial burden. Data presented show average bacterial burden $x10^3$ cfu per spleen of 4-6 mice in each group. Statistical analysis was done to compare bacterial burden among groups following high dose BCG iv challenge. Groups with the same p value letters indicate no statistical difference in bacterial burden at p=0.05.

BCG vaccine dose (cfu)	A‡		B‡		C‡		D‡	
	Mean	p* value	Mean	p* value	Mean	p* value	Mean	p* value
33	32.8	a	26.8	a	7.7	a	2.7	a
330	47.0	a	39.2	a	7.8	a	6.0	a
3300	58.7	a	163.4	b	11.2	a	9.7	a
$3.3 x10^7$	285.4	b	275.2	c	52.1	a	22	a
AMC	4936.7	c	1248.4	d	384.2	b	297.7	b

‡ Stands for the four different experiments described in Figure 10

*Vaccination groups with the same letter (p value) indicates no significant difference in bacterial burden at p=0.05

5.1.3. Discussion

The major aim of this study was to develop a mouse model of human neonatal BCG vaccination. Such vaccination is most often administered intradermally. Reliable intradermal delivery of BCG to neonatal mice was difficult, so we adopted subcutaneous administration. We made this compromise under the premise that the Th1/Th2 phenotype of the immune response in adult mice depends on the dose of BCG independently of the route of administration (Power et al., 1998). We also initially attempted to vaccinate mice within two days of birth and obtained relatively inconsistent immune responses. For reasons discussed in section 5.1.1, we chose to inject mice at the age of 5-7 days, and obtained very consistent responses.

The immune response in neonates is generally believed to be intrinsically biased towards Th2-type responses. Antigen administration during the neonatal period is also thought to often result in antibody-dominated responses later in life, contributing to the poor capacity of neonates to mount an effective cell-mediated response against intracellular pathogens (Barrios et al., 1996; Adkins and Du, 1998; Adkins, 2000; Siegrist, 2000). However, evidence indicates that neonates can generate effective cell-mediated immunity (CTL) that can clear viral infections (Sarzotti et al., 1996), supporting the possibility that whether antigens generate specific Th1 or Th2 cells in neonates depends on the dose and mode of vaccination (Forsthuber et al., 1996; Sarzotti et al., 1996). Our observations reinforce conclusions made by Forsthuber and Sarzotti, which indicate that neonatal mice can produce cell-mediated and antibody responses upon exposure to low and higher doses

of antigen, respectively. We employed the ratio of the number of BCG-specific IFN-γ to IL-4-producing cells per million spleen cells in vaccinated mice as a measure of the relative magnitude of the Th1 and Th2 components of the immune response. This ratio is referred to as the index of the Th1/Th2 phenotype of the response. A high Th1/Th2 index thus corresponds to a predominantly Th1 response, and a low index, below 1, to a predominantly Th2 response. This ratio was > 10 with spleen cells derived from mice vaccinated 12 weeks previously with lower doses of BCG, whereas the corresponding ratio was around 1 for mice vaccinated with $3.3x10^7$ BCG. Goriely and colleagues suggested that the Th1 response in human neonates may be defective as cord blood derived dendritic cells are unable to produce the pro-Th1 cytokine, IL-12, in response to stimulation with LPS or CD40L (Goriely et al., 2001). In contrast, and in agreement with our results, BCG vaccination of infants at birth induced a potent Th1 response with levels of IFN-γ similar to that achieved with vaccination of adults (Marchant et al., 1999; Vekemans et al., 2001), suggesting our findings are pertinent to vaccination strategies of human neonates.

We also demonstrated that the generation of a Th1 response by low dose BCG vaccination of newborn mice led not only to a predominantly Th1 response, but also to a modulation of the Th1/Th2 phenotype of the response towards the Th1 pole following a high dose BCG iv challenge. This dose generated a mixed Th1/Th2 response in adult naïve mice. Moreover, this Th1 biased response was associated with a decreased mycobacterial burden in the spleen. The observations of Figures 8 and 10 clearly indicate that the greater the modulation of the response to the Th1 pole, the greater was the

decrease in mycobacterial burden. Furthermore, the most consistent and greatest Th1 bias and bacterial clearance was achieved by vaccination of newborn mice with the lowest dose of BCG (33 cfu) injected sc. This has been supported by statistical analysis as described in the corresponding text for each figure and also shown in Table 2. Our finding that robust Th1 responses and efficient Th1 imprinting can be achieved by administering as low a dose of BCG as 33 cfu to newborn BALB/c mice is in agreement with a very recent finding in which 10 cfu of live rBCG vaccine induced strong protective immunity against tuberculosis in guinea pigs (Horwitz et al., 2006a). Our results, therefore, support the possibility that an ultra-low dose BCG vaccination strategy in neonates may be used to achieve Th1 imprinting in a genetically diverse population. Ultra-low dose of BCG is defined as a dose of BCG that initially induces either no immune response at all or induces only a cell-mediated immune response in every individual of a heterogeneous population. In the former case, as BCG is a live replicating organism, we expect it to replicate and reach the threshold level over a certain period of time and induce a cell-mediated response in all individuals. These observations may have implications for a vaccination strategy in humans, a strategy that can induce protective immunity against infections with *M. tuberculosis* in all individuals.

5.2. BCG Vaccination Studies in Adult Mice

5.2.1. Introduction

The aim of this study was to determine if our neonatal low dose BCG vaccination strategy was also effective in adult mice and if the results obtained after sc vaccination were consistent for other routes of BCG administration. The rationale for this study was that our planned study with virulent *M. tuberculosis* would be done with adult mice and all challenges with the virulent strain would be best done intravenously. Previous studies demonstrated that iv challenge with 10^5 cfu of a virulent strain of *M. tuberculosis* was equivalent in inducing disease to aerosol challenge with 10^2 cfu (North and Jung, 2004). In addition, we wanted to determine if our low dose BCG vaccination strategy would work on a genetically heterogeneous population, as the ultimate goal of such studies is to establish a universally efficacious vaccination protocol that can be applied to humans. The dose of BCG that is low enough to induce a Th1-type response in one individual may be high enough to induce a mixed Th1/Th2 response in another individual, as the genetic diversity of the host may have an impact on the outcome of our vaccination protocol. We hypothesized that giving an ultra-low dose of BCG that would not induce a mixed Th1/Th2 type response in any individual of the population could solve this problem. This dose will either induce an exclusively Th1-type response initially or be too low to induce a detectable immune response. In the latter case, as BCG is a slow replicating live vaccine, it should in time reach levels sufficient to induce a Th1-type response.

5.2.2. Results

5.2.2.1. Low Dose BCG Vaccination Induces Th1 Type Responses and Th1 Imprints in Adult BALB/c Mice Independently of Whether BCG is Administered Intravenously or Subcutaneously

In an attempt to examine the type of response generated following subcutaneous and intravenous vaccination in adult mice, 6-8 week old BALB/c mice were vaccinated sc and iv with different doses of BCG, ranging from 10^2 to 10^8 cfu. To determine the type of the primary response generated, mice were bled and sacrificed at different times starting from week 9 post-vaccination, and antibody and T-cell responses were measured in ELISA and ELISPOT assays, respectively. Similar to our findings in the newborn mice, as well as previous report from studies in adult mice (Power et al., 1998), mice given relatively lower doses of BCG either iv or sc generated a predominantly cell-mediated response, with IFN-γ-secreting cells dominant over IL-4-secreting cells. In contrast, mice given higher doses (>10^6 cfu) of BCG mounted a mixed Th1/Th2 response (Figure 11). As clearly shown in Figures 11 and 13, spleen cells from all vaccinated mice produced significantly higher ($p<0.05$) levels of IFN-γ than the non-vaccinated group, with no significant difference among groups at 12 and 24 weeks post-vaccination. However, the level of IL-4 differs significantly among groups. Mice given less than 10^6 cfu of BCG did not produced detectable levels of IL-4. In contrast, the spleen cells of mice vaccinated with BCG doses higher than 10^6 cfu had significantly higher ($p<0.05$) levels of IL-4 when compared to low dose vaccinated mice. Consequently, the IFN-γ to IL-4 ratio was significantly lower in the high dose vaccinated (~1) than the low dose vaccinated group (usually > 10).

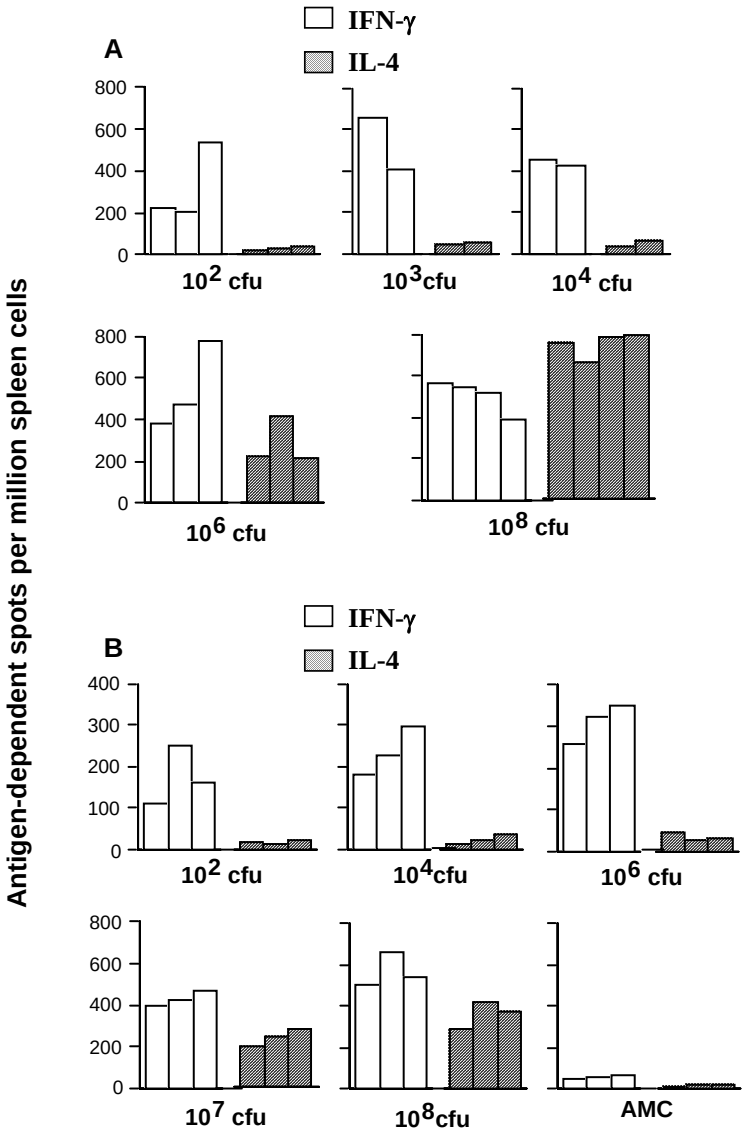

Figure 11. Low dose BCG vaccination induces a predominantly Th1-type response in adult BALB/c mice independently of whether the vaccine is administered by the intravenous or subcutaneous route.

Adult BALB/c mice were vaccinated with different doses of BCG, shown along the abscissa, via the intravenous (A) or subcutaneous (B) routes. Mice were sacrificed 12 weeks post-vaccination and their spleen cells assessed for BCG-specific cytokine producing T-cells. Results shown are one of three replicate experiments, each with 2-4 mice per group. Data presented are for individual mice within each group. AMC (age matched control) refers to naive control mice.

A comparison of Figures 11A and B suggests that the transition number – the number of BCG cfu above which a mixed Th1/Th2 response was induced in naïve mice – is 10 fold higher for sc vaccination as compared to iv vaccination on the same strain of mice. As discussed above, this may not be surprising as the iv route of antigen administration was more immunogenic than sc immunization (Lagrange et al., 1974). Even though the transition numbers seems to vary between iv and sc routes of BCG administration, in both cases relatively lower doses induced Th1-type responses, while higher doses induced mixed Th1/Th2 responses. Figure 13 represents ELISPOT results 24 weeks after sc vaccination of mice and confirms that the response generated after low dose vaccination remained stable for as long as 6 months, which is almost 1/3 of the life span of the mouse. Again, the Th1/Th2 index (IFN-γ to IL-4 ratio) is significantly higher ($p< 0.05$) in the low dose vaccinated groups when compared to mice vaccinated with high dose of BCG. The index ranges between 6 and 17 in mice vaccinated with 10^6 and 10^2 cfu of BCG, respectively. In contrast, the index was close to 1 in mice vaccinated with10^8 cfu, and between 1 and 2 in mice vaccinated with 10^7 cfu of BCG sc. As discussed in our neonatal vaccination studies, this stable cytokine level probably only occurs several weeks post-vaccination, as the lower doses probably require time to replicate and induce response comparable in magnitude to that induced by higher doses of BCG. As assessed by ELISA, the low dose vaccinated groups produced either no detectable antibody response or an IgG_{2a} antibody response, which was dominant over the IgG_1 antibody response. A mixed Th1/Th2 response associated with higher levels of both IgG_1 and IgG_{2a} antibodies was seen in the high dose vaccinated mice (Figure 12). The ELISA results presented here came from mice vaccinated subcutaneously, but similar results

were also obtained when mice were vaccinated intravenously (data not shown). The only observed difference following iv vaccination was that mice started to seroconvert at a lower dose of BCG, between 10^4 and 10^5 cfu, versus to between 10^5 and 10^6 following subcutaneously vaccination.

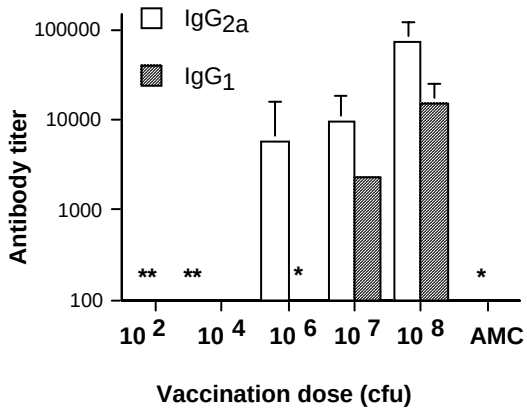

Figure 12. Relatively lower doses of BCG induced immune responses with no detectable level of antibody or with a relatively higher IgG_{2a} than IgG_1 antibody production as assessed by the ELISA assay.

Blood was taken from the tail vein of BALB/c mice vaccinated subcutaneously with different doses of BCG, and serum was assessed by ELISA 12 weeks after vaccination for the level of BCG-specific IgG_1 and IgG_{2a} antibody titers. Results represent mean antibody titer of 3-5 mice per group and are from one of three replicate experiments. Numbers in the abscissa represent dose of BCG vaccine in cfu. AMC (age matched control) refers to pooled sera from control mice injected with saline only. * indicate titers below detection level.

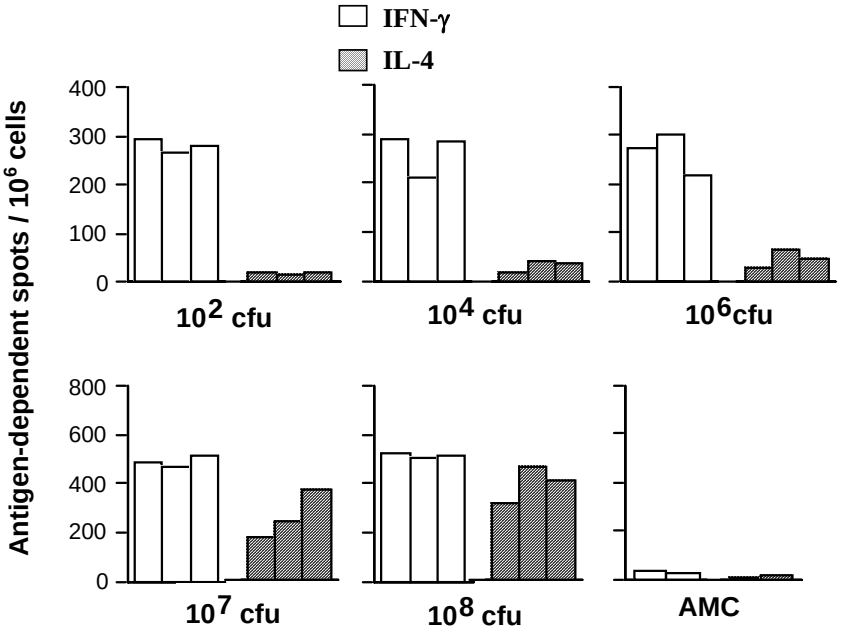

Figure 13. The Th1/Th2 nature of the anti-BCG response remains stable six months after BCG vaccination.

Adult BALB/c mice were vaccinated subcutaneously with different doses of BCG, as shown on the abscissa. Mice were sacrificed 24 weeks post-vaccination. BCG antigen-specific cytokine production by spleen cells was assessed in the ELISPOT assay. Results represent one of two replicate experiments with 3-5 mice per group. Data presented are values for individual mice in each group (n=3 per group). AMC (age matched control) refers to non-vaccinated control mice.

To determine if the cell-mediated response generated following low dose BCG vaccination in adult BALB/c mice was associated with a Th1 imprint, mice vaccinated iv and sc with different doses of BCG were challenged iv, between 12 to 16 weeks post-vaccination, with a high dose of BCG, which induced a mixed Th1/Th2 response in naïve mice. Representative mice from each group were sacrificed between 12 to 24 weeks after challenge. Spleen cells were used for ELISPOT assay to determine the type of response generated and for the assessment of bacterial burden in the spleen. We wished to determine whether low but not high dose BCG vaccination had generated a Th1 imprint as assessed by a high dose BCG iv challenge and examine if this Th1 imprinting is associated with more efficient clearance of mycobacteria from the spleens of mice. Vaccination of mice with doses $<10^6$ cfu for iv vaccinated mice (Figure 14) and $\leq 10^6$ for sc vaccinated mice (Figure 15) abrogated the generation of Th2 cells following BCG iv challenge, whereas vaccination with saline or higher doses of BCG could not prevent the generation of Th2 cells following high dose BCG iv challenge. This clearly shows that, similar to the situations in newborn mice, low dose BCG vaccination generated a Th1-type response and a Th1 imprint that predisposed adult mice to a Th1 biased response following exposure to a high dose challenge. This may imply that low dose BCG vaccination is more efficacious in generating effective immune response against mycobacterial infections than the standared dose of BCG commonly used to vaccinate humans and animals against *M. tuberculosis*.

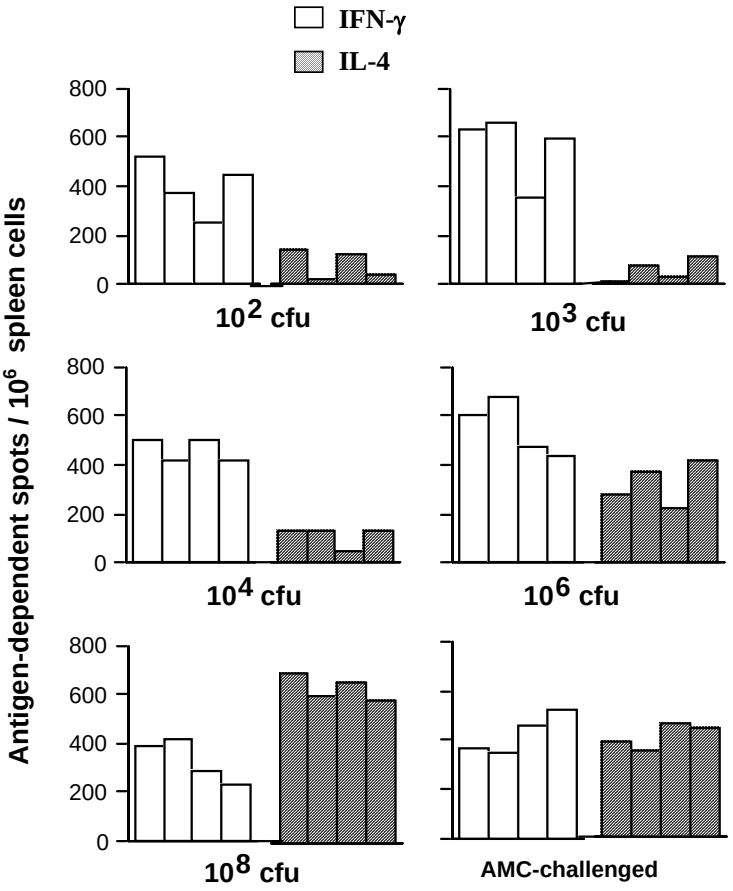

Figure 14. Evaluation of how vaccination of adult mice with different doses of BCG affects the response to a subsequent high dose iv challenge with BCG.

Adult BALB/c mice were vaccinated iv with different doses of BCG and challenged iv with ~$5x10^6$ cfu of BCG 16 weeks post-vaccination. Mice were sacrificed 12 weeks post-challenge and spleen cells processed to assess the level of antigen-specific cytokine producing T-cells in the ELISPOT assay. Results shown are representative of one of three replicate experiments and data presented are values for individual mice in each group with 4 mice per group. Numbers in the abscissa represents dose of BCG vaccine in cfu. AMC (age matched control) refers to naive control mice.

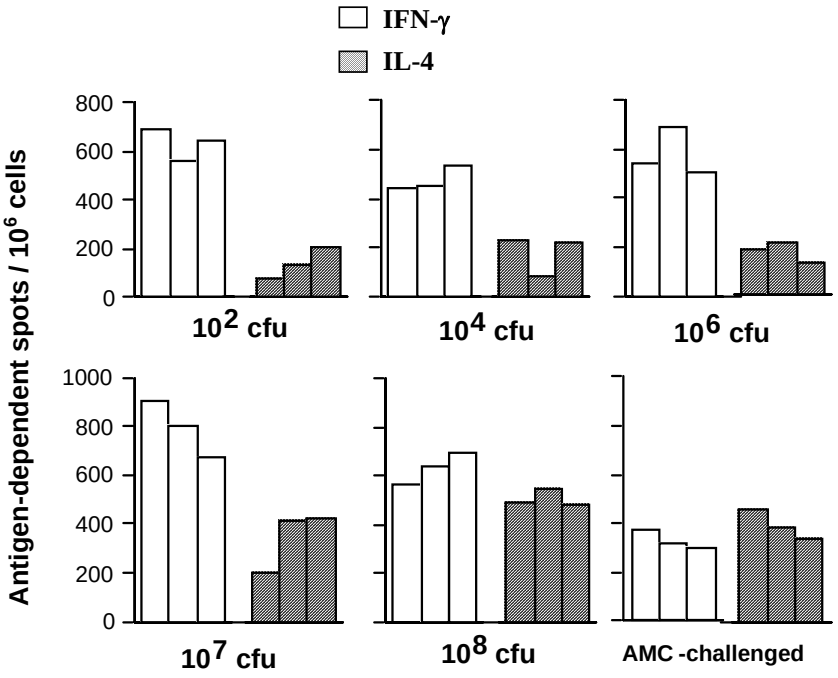

Figure 15. Low dose BCG vaccination of adult BALB/c mice induces a Th1 imprint as assessed by a high dose intravenous BCG challenge.

Adult BALB/c mice were vaccinated sc with different doses of BCG and challenged iv, 16 weeks after vaccination, with ~$1.1x10^7$ cfu of BCG. Mice were sacrificed 12 weeks post-challenge and spleen cells processed for ELISPOT assay to enumerate the type of antigen-specific cytokine producing cells generated. Results shown are representative of one of three replicate experiments and data represent values for individual mice with 3 mice per group. Numbers in the abscissa indicate dose of BCG used for vaccination. AMC (age matched control) refers to naïve mice challenged with a high dose BCG.

The Th1 and mixed Th1/Th2 imprinting, respectively following vaccination of adult mice with low and high doses of BCG leads to a stable Th1 and mixed Th1/Th2 responses, with a similar responses generated at 12 weeks (Figure 14) and 20 weeks (Figure 16) post-challenge for iv vaccinated mice, as well as 12 weeks (Figure 15) and 24 weeks (Figure 17) post-challenge for sc vaccinated mice. Even though there were some variations in the level of IFN-γ produced by spleen cells of mice in different vaccination groups, particularly at 12 weeks post-challenge, the over all picture is that there was no statistically significant difference at the p value of 0.05 in the levels of IFN-γ among groups. Similarly there was no significant difference in the level of IL-4 produced by spleen cells from mice vaccinated with $<10^6$ cfu of BCG for iv and $\leq 10^6$ cfu for sc vaccinated mice. In contrast, the level of IL-4 was significantly higher ($p<0.05$) in all mice vaccinated with higher doses of BCG as well as in naïve mice following high dose BCG iv challenge. This is reflected in a lower IFN-γ to IL-4 ratio observed following high dose BCG challenge in mice vaccinated with higher doses of BCG as well as in naïve mice than in mice vaccinated with lower doses, independently of whether BCG was administered by the sc or iv route. Table 3 shows the results of a statistical analysis done on data obtained 20 weeks post-challenge for iv vaccinated mice and 24 weeks after challenge for sc vaccinated mice.

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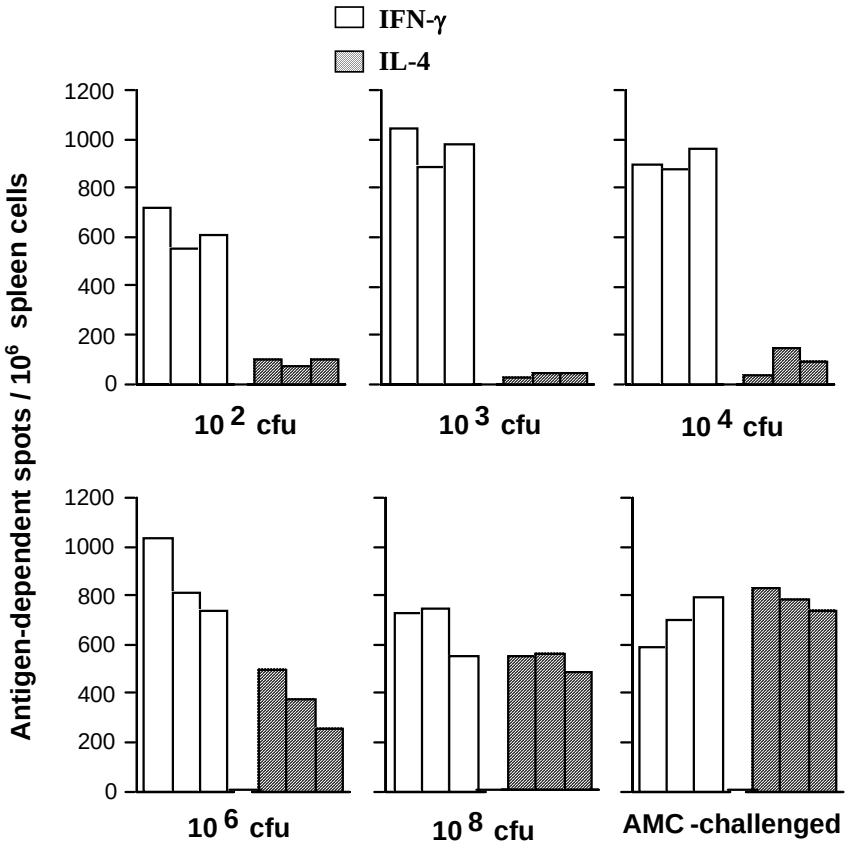

Figure 16. The Th1 imprinting induced by low dose BCG iv vaccination ensures stable, long-term Th1 responses following high dose BCG iv challenge in adult mice.

Adult BALB/c mice vaccinated with different doses of BCG iv were challenged with ~5x10^6 cfu of BCG 16 weeks post-vaccination. Mice were sacrificed 20 weeks post-challenge and splenic antigen-specific T-cell responses assessed by the ELISPOT assay. Results shown represent one of three replicate experiments with 3 mice per group and data presented are for individual mice in each group. Numbers on the abscissa indicate dose of BCG in cfu used for vaccination. AMC-challenged (age matched control) refers to naive mice challenged iv with the high dose of BCG.

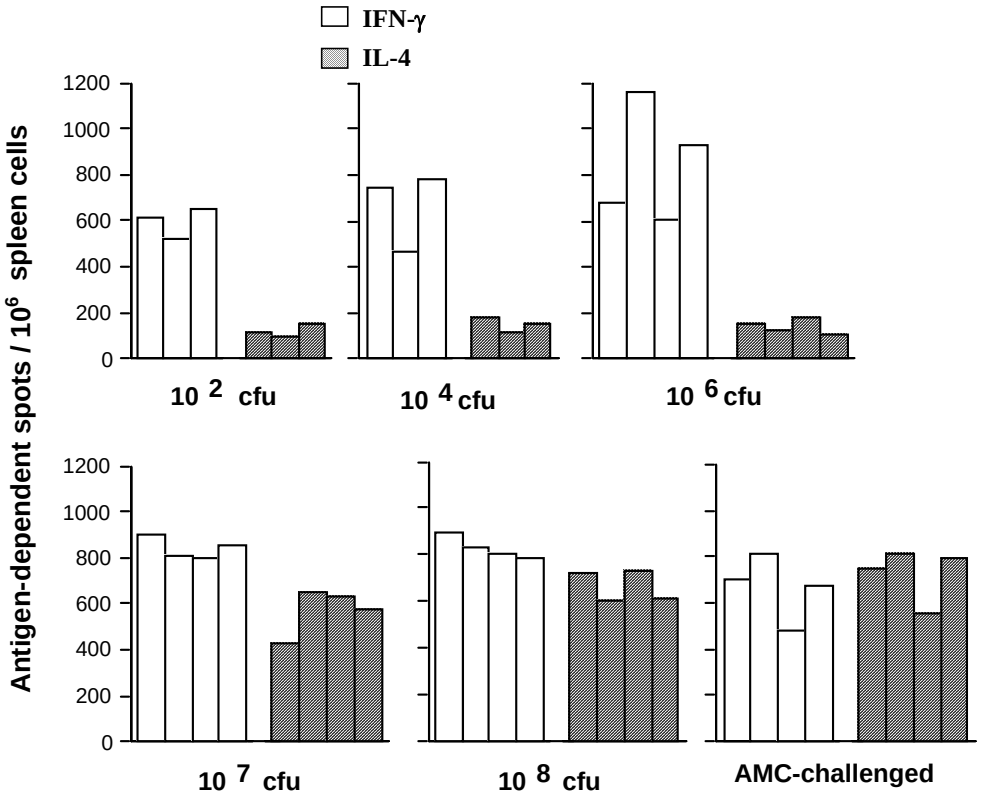

Figure 17. The Th1 imprinting induced by low dose sc BCG vaccination of adult BALB/c mice ensures stable, long-term Th1 responses following high dose BCG iv challenge.

Adult BALB/c mice vaccinated subcutaneously with different doses of BCG were challenged intravenously, 16 weeks later, with ~$1.1x10^7$ cfu of BCG. Mice were sacrificed 24 weeks post-challenge and spleen cells used in the ELISPOT assay to assess antigen-specific responses. Representative results from one of three replicate experiments are shown. Data presented are values for individual mice in each group with 3-4 mice per group. Numbers on the abscissa represent the dose of BCG used for vaccination. AMC-challenged (age matched control) refers to naïve mice that were challenged iv with the high dose of BCG.

Table 3. Results of a statistical analysis on cytokine levels produced by spleen cells from adult mice vaccinated with different doses of BCG and challenged iv with a high dose BCG.

Mice vaccinated with different doses of BCG and challenged with high doses of BCG as described in the legend for Figures 16 and 17, were sacrificed at 20 weeks post-challenge for iv vaccinated mice and 24 weeks for sc vaccinated mice. Spleens were removed from each mouse and antigen-specific cytokine producing cells enumerated by the ELISPOT assay. Data presented show average number of cytokine producing cells per million spleen cells for 3-4 mice per group following high dose iv challenge. The Th1/Th2 index is presented as the ratio of the number of IFN-γ to IL-4 producing cells. Statistical analysis was done to compare the number of cytokine producing cells as well as the Th1/Th2 index among groups. Groups with the same letter along each column indicate no statistical difference.

Vaccination groups***	Subcutaneously vaccinated mice*			Intravenously vaccinated mice**		
	Mean value		Ratio	Mean value		Ratio
	IFN-γ	IL-4	IFN-γ/ IL-4	IFN-γ	IL-4	IFN-γ/ IL-4
Group 1	594^{a}	120^{a}	5^{a}	624^{a}	88^{a}	8^{a}
Group 2	664^{b}	146^{a}	4.6^{a}	970^{b}	38^{a}	26^{b}
Group 3	846^{b}	140^{a}	6^{a}	913^{b}	91^{a}	14^{a}
Group 4	841^{b}	566^{b}	1.5^{b}	861^{bc}	377^{b}	2^{c}
Group 5	827^{b}	674^{bc}	1.2^{b}	675^{a}	531^{c}	1^{c}
Group 6	663^{b}	727^{c}	0.9^{b}	691^{ac}	782^{d}	0.89^{c}

*Assay done 24 weeks post-challenge

**Assay done 20 weeks post-challenge

*** Refers to vaccination dose of BCG as described, respectively, in Figures 16 and 17 for iv and sc vaccinated mice

The nature of the response after challenge was also reflected in the IgG isotypes of BCG specific antibodies (Figure 18A). Vaccination with doses of BCG less than or equal to 10^6 cfu resulted in significantly higher ($p<0.05$) levels of IgG_{2a} than IgG_1 antibodies following high dose BCG iv challenge, indicating a Th1 biased response. On the other hand higher doses of BCG vaccination generated a mixed Th1/Th2 response with comparable levels of IgG_{2a} and IgG_1 antibodies following high dose BCG iv challenge. Vaccination with a low dose of BCG clearly favored a type of response, on challenge, that can more efficiently clear BCG from the spleen than in naïve mice or mice vaccinated with a high dose of BCG (Figure 18B). All mice vaccinated iv with doses less than or equal to 10^6 cfu of BCG were able to substantially reduce the bacterial load in their spleen after high dose challenge, with no statistically significant difference between groups. However, mice vaccinated with the highest dose of BCG (10^8 cfu) and naïve mice had significantly higher ($p<0.05$) levels of bacterial load in their spleen after high dose challenge than mice vaccinated with less or equal to 10^6 cfu. Similar findings were also observed in mice vaccinated sc (data not shown). Therefore, from these observations we conclude that Th1 imprinting was essential for immune-mediated protection against BCG infection, and vaccination of mice with relatively low doses of BCG, independently of the route of BCG administration, can best generate this type of imprint.

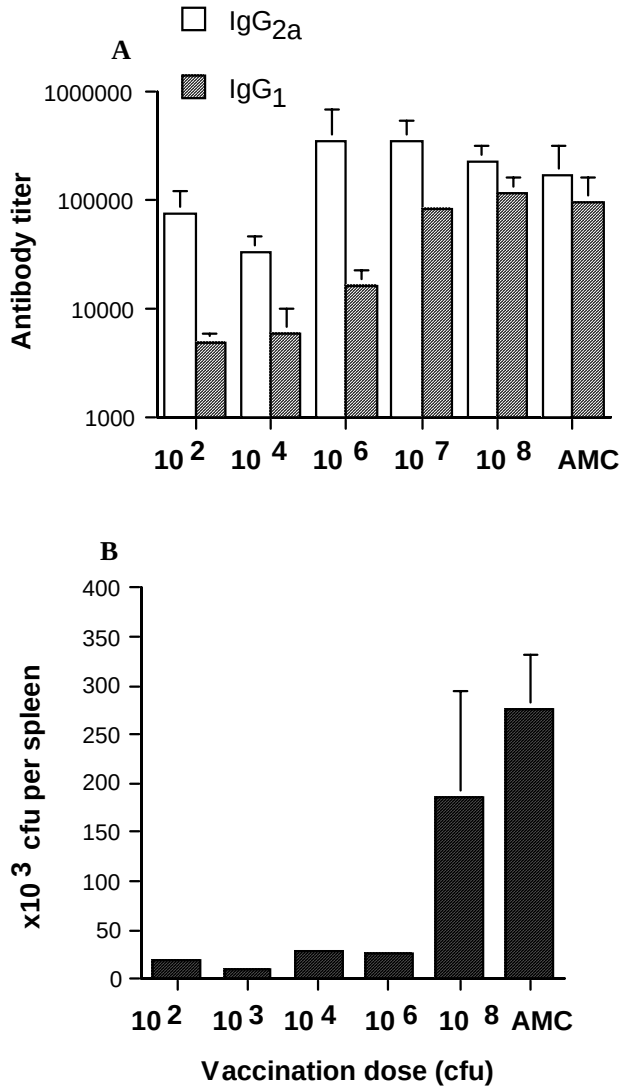

Figure 18. Low dose BCG vaccinated adult BALB/c mice have higher levels of IgG_{2a} than IgG_1 antibodies in their sera and more effectively reduced the bacterial burden in the spleen than high dose vaccinated or naive mice following high dose iv challenge.

Adult BALB/c mice vaccinated subcutaneously (A) or intravenously (B) with different doses of BCG were challenged iv with ~$1.1x10^7$ cfu and $5x10^6$ cfu of BCG, respectively. Mice were bled to determine the isotypes of antigen-specific IgG antibodies in serum samples (A) and sacrificed to assess the splenic bacterial burden (B) 12 weeks after challenge. Result show mean and SD of values for 3-5 mice per group and are representatives of at least two replicate experiments each. AMC (age matched control refers to naive control mice challenged iv with a high dose of BCG.

5.2.2.2. Low Dose BCG Vaccination Induces Th1 Type Responses and Th1 Imprints in Genetically Heterogeneous Mice

The objective of this study was to explore if low dose BCG vaccination will induce predominantly cell-mediated immunity and Th1 imprints in a genetically heterogeneous population. To address this objective we used three different strains of mice: CBA ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), and CD1. The first two mouse strains were chosen based on a previous report, which claimed that these two strains of mice differ considerably in their susceptibility/resistant phenotype to *M. tuberculosis* challenge (Medina and North 1998). We wanted to compare the efficacy of BCG vaccination in these two different strains of mice. The CD1 mice are an out-bred strain of mice derived from Swiss mice, and are believed to be genetically heterogeneous. These mice may represent the genetic heterogeneousity of humans and animals. All mice were injected intravenously with different doses of BCG (10^2 to 10^8 cfu). Starting from nine weeks after vaccination, cell-mediated and antibody responses were evaluated using ELISPOT and ELISA assays, respectively. Results in BALB/C mice were similar to results obtained in our previous studies presented in section 5.2.2.1. The value of the transition number (t_n), defined as the dose of BCG below which, when given to mice, induces predominantly cell-mediated response was determined to be between 10^5 and 10^6 cfu for adult BALB/c vaccinated intravenously. All mice vaccinated with doses $\leq 10^4$ cfu responded with an exclusively cell-mediated response with either no detectable antibody production or only a low titer of IgG_{2a}. These responses were consistent with a predominantly Th1 type response. On the other hand, doses of BCG between 10^5 and 10^6 cfu induced BCG-specific antibody

responses dominated by IgG_{2a} isotype (IgG_{2a} /IgG_1 ratio ~4), and doses $\geq 10^7$ cfu of BCG induced higher titers of both IgG_1 and IgG_{2a} antibodies with a ratio close to 1 (Figure 19A). The ratio of BCG specific IFN-γ to IL-4-producing T-cells for the low dose vaccinated group ($<10^6$ cfu) was large but not ascertainable as the number of IL-4-producing cells is usually insignificant. However, significant numbers of IL-4-producing T-cells were detected following injection with BCG doses $\geq 10^6$ cfu, with the IFN-γ/IL-4 ratio approaching 1 at a dose of 10^6 cfu (Figure 19B). Statistical analysis of the number of BCG specific IFN-γ and IL-4-producing T-cells was similar to those described in section 5.2.2.1 for adult BALB/c mice vaccinated iv. The value of t_n for BCG given iv was found to be 100 fold higher in CBA mice than BALB/c mice. All doses $< 10^8$ cfu induced exclusively cell-mediated immune responses with higher levels of BCG-specific IFN-γ and few or no detectable IL-4-producing T-cells. In contrast the highest BCG dose used (10^8 cfu) induced a mixed Th1/Th2 response, with comparable levels of antigen-specific IFN-γ and IL-4 cytokine producing T-cells (Fig 20B). Determining the exact ratio of IFN-γ to IL-4-producing T-cells was some times difficult due to indeterminate levels of IL-4 secreting cells in some of the low dose vaccinated CBA mice, but in general it was ≥ 10 for mice vaccinated with BCG doses $\leq 10^6$, around 5 for a dose of 10^7, and significantly lower ($p< 0.05$) with a ratio approaching 1 for the highest dose (10^8 cfu). All doses $\leq 10^6$ cfu induced undetectable antibody, as assessed by ELISA, indicating an exclusively cell-mediated immune response, while a dose of 10^7 cfu induced either predominantly IgG_{2a} antibodies or undetectable BCG-specific antibody levels. The highest dose used, 10^8 cfu, induced a mixed Th1/Th2 type of response with comparable titers of IgG_{2a} to IgG_1 antibodies (Figure 20A).

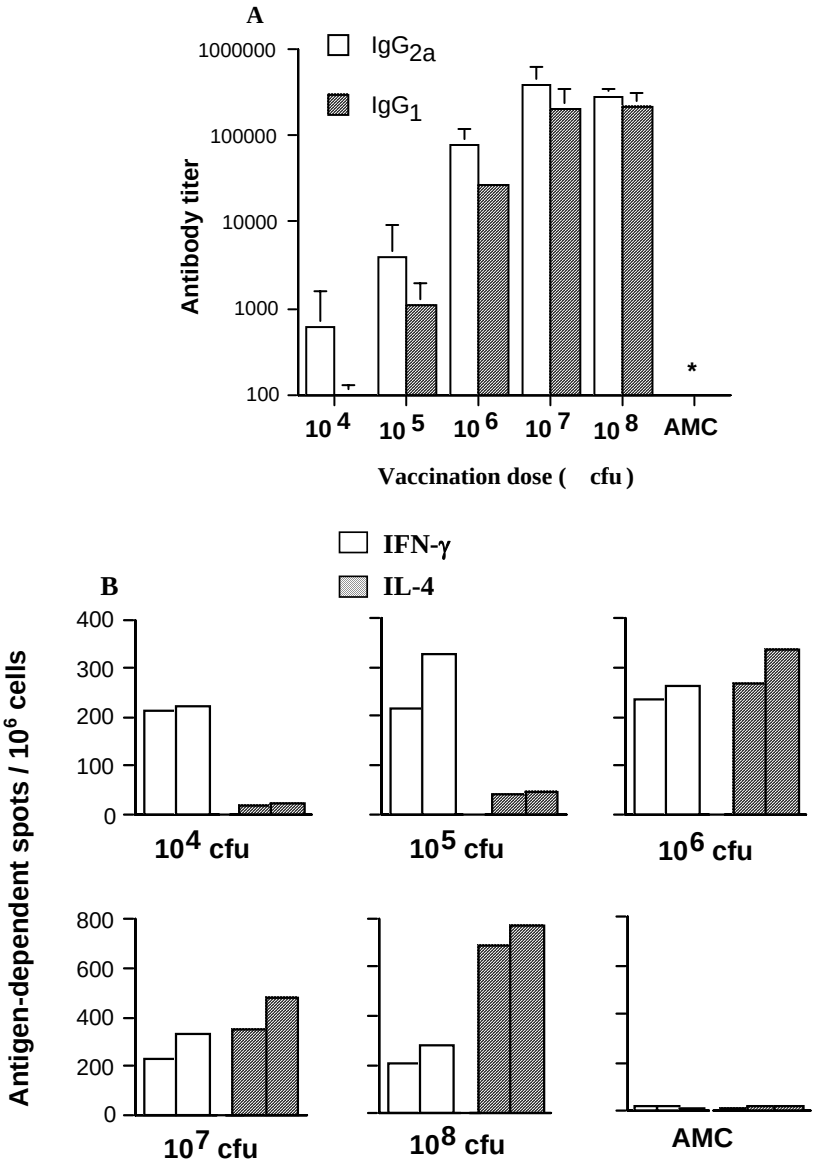

Figure 19. The Th1/Th2 nature of the splenic immune response, as well as the isotypes of BCG-specific IgG antibodies produced in adult BALB/c mice depend on the dose of BCG employed for immunization.

Adult BALB/c mice were vaccinated intravenously with different doses of BCG or saline. Mice were bled and antibody titers assayed with ELISA at 9 weeks post-vaccination (A). Mice were also sacrificed at 12 weeks post-vaccination to determine antigen-dependent immune responses by ELISPOT assay (B). ELISA results represent mean antibody titer of 4 mice per group with SD. Results shown are representative of at least three replicate experiments. Numbers in the abscissa represent dose of BCG vaccine in cfu. AMC (age matched control) refers to naive control mice.

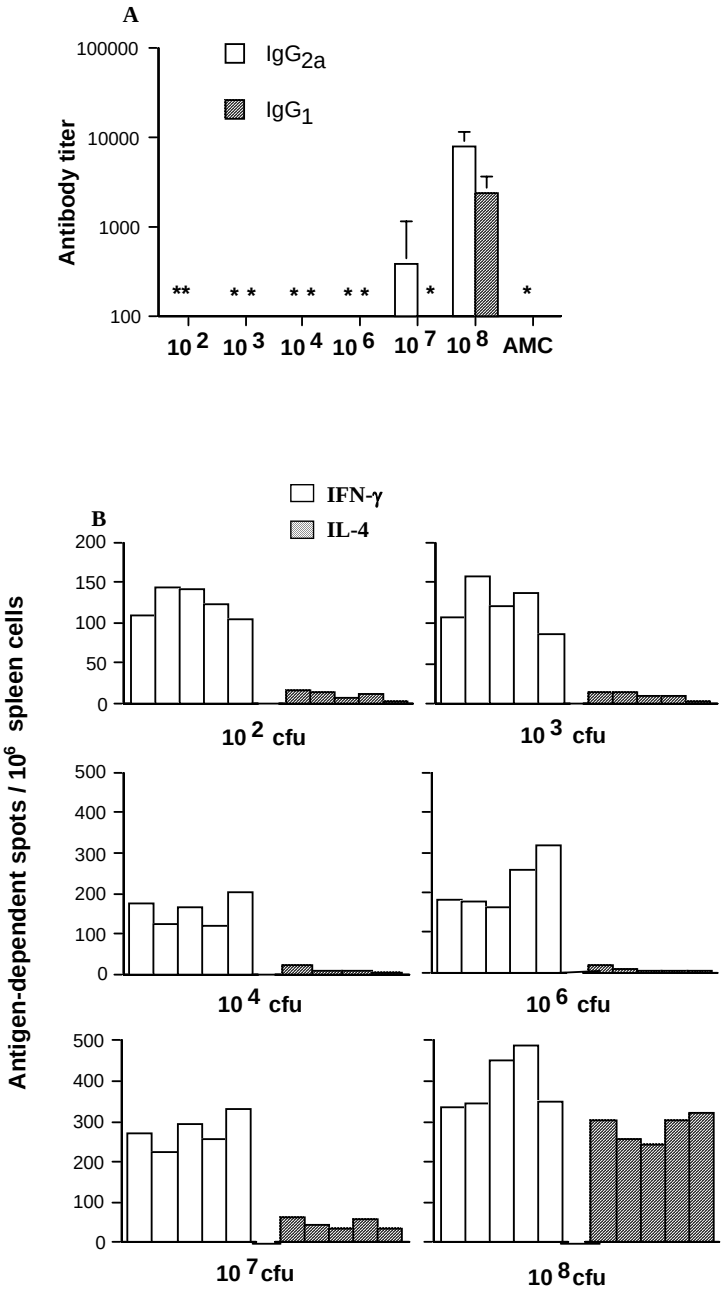

Figure 20. The Th1/Th2 nature of the splenic immune response and the IgG isotypes of serum antibodies in adult CBA mice depend on the dose of BCG used for vaccination.

Adult CBA mice vaccinated iv with different doses of BCG were bled to determine antigen-specific serum antibody levels (A) and sacrificed to assess the levels of antigen-specific cytokine producing T-cells in the spleen (B), 12 and 16 weeks post-vaccination, respectively. ELISA results represent average antibody titer of 3-5 mice/group and *stands for titers below detection level. Results are representative of three replicate experiments. Numbers in the abscissa represent the dose of BCG vaccine. AMC (age matched control) refers to naive control mice.

Observations made with CD1 mice were contrary to our expectations. As CD1 mice are said to be out-bred and believed to be genetically heterogeneous, we expected the transition number, t_n, might vary by ten-fold or more between individual mice within a group. However, results indicate that the CD1 mice behave roughly like a homogenous population with a similar t_n of close to 10^6 cfu among individual mice (Fig. 21A and B). Data from 9 and 16 weeks post-vaccination demonstrate that the response was stable over time except that the t_n appears to shift towards 10^7 cfu at later time period after vaccination. However, results from other similar experiments and data from post-challenge experiments (Figure 26) show that the t_n in CD1 mice is ~10^6 cfu. Therefore, similar to adult BALB/c mice vaccinated iv, all CD1 mice that received a dose of BCG $\leq 10^6$ cfu demonstrated a predominantly Th1-type response with high levels of BCG-specific IFN-γ and no or few detectable IL-4-producing T-cells. In contrast, higher BCG doses induced significantly higher ($p<0.05$) numbers of IL-4-producing cells, with an IFN-γ/IL-4 ratio of close to 1, representing a mixed Th1/Th2 response. Data from all these studies can be summarized as supporting the concept that relatively low doses of BCG vaccination can efficiently induce predominantly Th1-type responses in all mice strains following iv or sc routes of vaccine administration. Furthermore, the values of t_n can vary by up to 100-fold among different mouse strains and by up to 10-fold between sc and iv routes of vaccination.

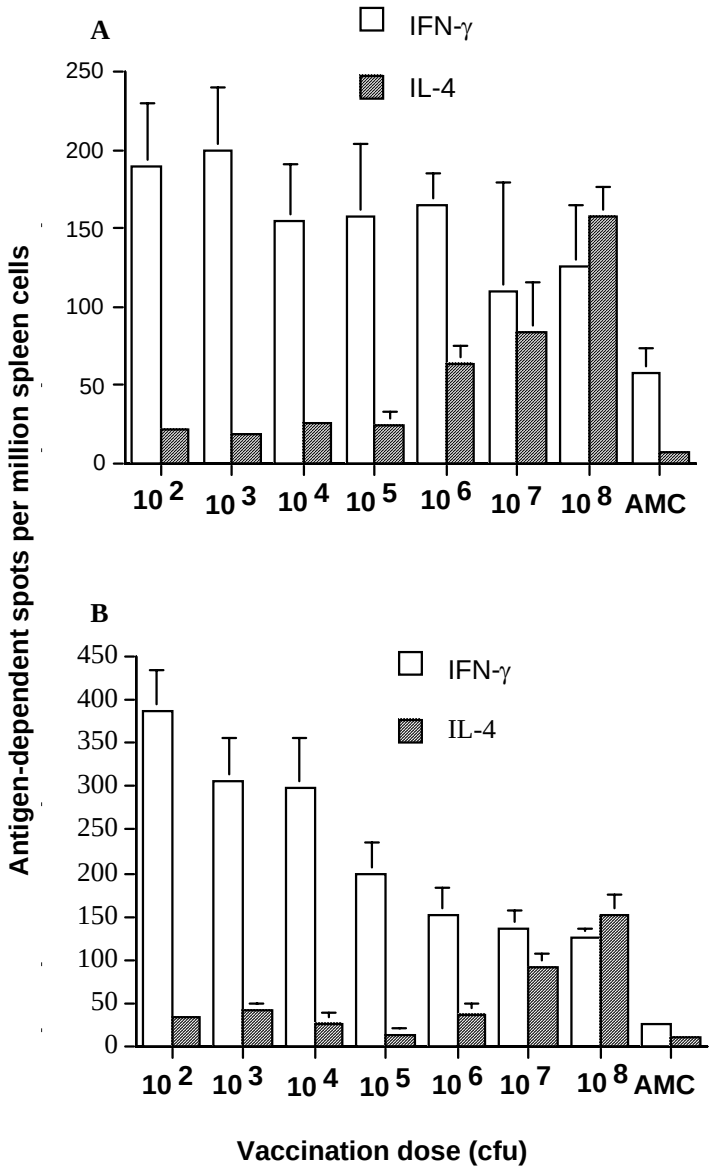

Figure 21. Enumeration of BCG-specific IFN-γ and IL-4-producing cells in the spleen of adult CD1 mice vaccinated with different doses of BCG.

Adult CD1 mice were vaccinated iv with different dose of BCG and levels of antigen-specific cytokine producing T-cells in the spleen assessed in the ELISPOT assay 9 weeks (A) and 16 weeks (B) post-vaccination. Data presented are average $\pm$ SD of values of 4 mice per group. Results shown represent two of the four replicate experiments done at different time points after immunization. Numbers in the abscissa indicate doses of BCG vaccine in cfu. AMC (age matched control) refers to naïve control mice.

The next step in testing the validity of our strategy was to challenge a subset of mice from each group with a high dose of BCG iv to determine if the immune response was locked into a Th1 mode in mice vaccinated with low doses of BCG. To this end mice vaccinated with different doses of BCG were injected iv at 12-16 weeks post-vaccination with a BCG dose that induced a mixed Th1/Th2 type response in naïve mice. Based on our previous results, we chose to use ~$5x10^6$ cfu in CD1 and BALB/c mice and ~$2x10^8$ cfu in CBA mice as our challenge doses.

Similar to previous findings, the immune response in all low dose vaccinated adult BALB/c mice was found to be locked into a Th1-type following a high dose BCG iv challenge. The mean values for antigen-specific cytokine producing cells, as well as the ratio of the number of IFN-γ to IL-4-secreting T-cells (Table 4), demonstrate that vaccination with low doses of BCG resulted in a Th1 imprint. In Figure 22, the data for individual mice are presented to demonstrate the consistency of the responses. In all cases, vaccination with BCG doses $\geq 10^6$ cfu resulted in a mixed Th1/Th2 response with comparable levels of IFN-γ and IL-4 cytokine producing cells following high dose iv challenge. However, BCG challenge of mice vaccinated with doses of BCG less than 10^6 cfu induced a predominantly Th1-type response with high numbers of IFN-γ and significantly lower numbers of IL-4-producing cells. These data confirm that the immune responses are locked into a Th1 mode. This deviation of the response was also reflected in the isotype of BCG-specific antibodies detected in serum (Figure 23A), and was significant with respect to the protection offered, as indicated by the level of bacteria isolated from the spleen of the mice after high dose BCG challenge (Figure 23B and C).

Assessment of bacterial burden shows mice vaccinated with doses $<10^6$ were able to more effectively reduce the bacterial load in spleens when compared with the naive group. At 10^6 cfu, the transition number, mice had significantly higher ($p<0.05$) bacterial burden in the spleens than in the spleens of mice vaccinated with lower doses at 12 weeks post-challenge (Figure 23B), but this difference was not significant at 18 weeks post-challenge (Figure 23C). This may suggest that immune mediated BCG clearance is influenced by BCG vaccine dose only during the early period post-challenge. However, as shown in several other experiments (Figure 18B), higher doses (10^7 and 10^8 cfu) had shown significantly higher bacterial burden in the spleen when compared to the low dose vaccinated groups at all time points post-challenge. One such data is shown in Figure 18B.

Table 4. BCG-specific IFN-γ and IL-4 cytokine production by spleen cells isolated from mice vaccinated with different doses of BCG and challenged iv with a high dose of BCG.

Adult BALB/c mice were vaccinated iv with different doses of BCG and then challenged intravenously with ~$5x10^6$ cfu at 12 weeks post-vaccination. Four mice from each group were sacrificed at 12 weeks post-challenge and splenic immune response assessed by ELISPOT as described previously. Mean numbers of antigen-specific cytokine producing spleen cells with standard deviation and the ratio of the numbers of IFN-γ and IL-4-producing cells are presented. Statistical analysis was done to compare the level of cytokines as well as the Th1/Th2 index (IFN-γ/IL-4 ratio) among groups. The same letters along each column indicate no statistical difference among groups and two letters in one group show an overlap.

	Antigen-specific cytokine secreting cells per million spleen cells as detected in the ELISPOT assay				
	IFN-γ		IL-4		IFN-γ/IL-4*
BCG dose (cfu)	Mean	SD	Mean	SD	Ratio
10^4	593.3^{ab}	152.6	72.7^{a}	18.2	8.2^{a}
10^5	543.7^{ab}	172.9	132.3^{a}	68.9	4.1^{b}
10^6	478.7^{b}	20.6	290.7^{b}	17.4	1.6^{c}
10^7	655.8^{a}	17.8	605.8^{c}	88.2	1.1^{c}
10^8	607.5^{ab}	88.9	756.3^{d}	96.5	0.8^{c}
AMC	488.5^{b}	82.0	494.0^{e}	30.7	1.0^{c}

AMC refers to naive mice challenged iv with a high dose of BCG
SD stands for standard deviation of the mean as calculated in Microsoft Excel, 2003
* refers to ratio of IFN-γ to IL-4-producing cells

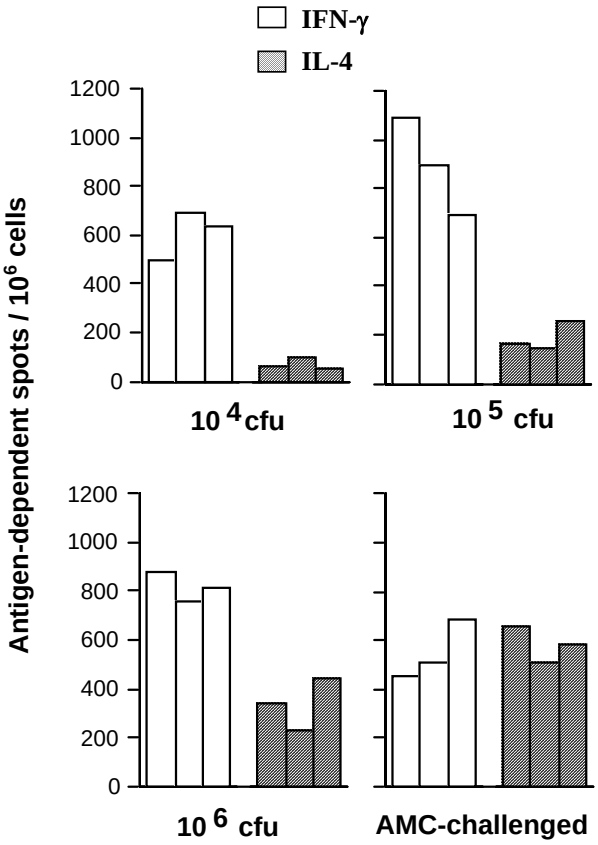

Figure 22. Low dose BCG vaccination predisposes adult BALB/c mice to a predominantly Th1 response following high dose BCG iv challenge.

Adult BALB/c mice were vaccinated iv with different doses of BCG and then challenged intravenously with ~5x10^6 cfu of BCG at 12 weeks post-vaccination. Three mice from each group were sacrificed around 18 weeks post-challenge to assess, in the ELISPOT assay, the type of antigen-specific response generated by spleen cells. Data represent values of individual mice in each group and are from one of at least two replicate experiments. Numbers in the abscissa represent dose of BCG used for vaccination. AMC (age matched control) refers to naïve control mice.

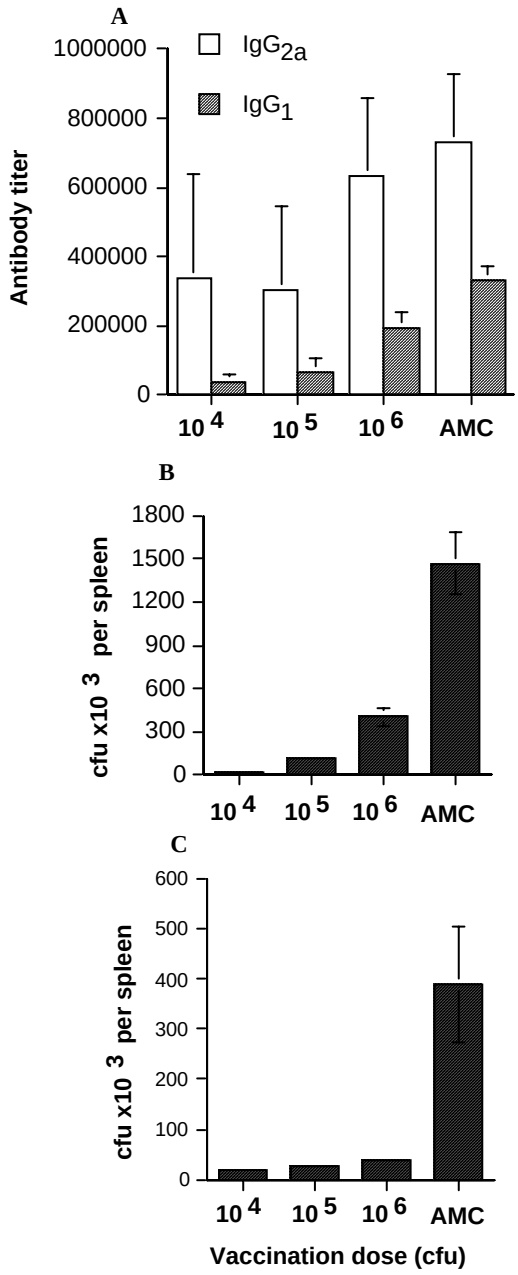

Figure 23. Antibody levels and bacterial burden assessment in adult BALB/c mice vaccinated with different doses of BCG and subsequently challenged with a high dose of BCG.

BALB/c mice vaccinated iv with different doses of BCG were challenged iv with $5x10^6$ cfu 12 weeks post-vaccination. Mice were bled 18 weeks after challenge to determine the isotypes of BCG-specific serum antibodies (A), and sacrificed 12 weeks (B) or 18 weeks (C) post-challenge to assess the splenic bacterial burden. Data represent average antibody titer of 3-5 mice per group (A) or average numbers of bacteria (cfu) per spleen of 5 mice per group (B and C) with error bars (SD). Results shown are from one of at least 2 replicate experiments each. AMC refers to naive control mice.

Figure 24 presents the immune responses generated in CBA mice at 12 weeks post-challenge. The BCG challenge was given iv at 16 weeks following iv vaccination of mice with different doses of BCG or an equal volume of saline. The responses in CBA mice were similar to those observed in BALB/c mice, except the t_n in CBA mice was between 10^7 and 10^8 cfu. Thus the t_n for CBA mice is 100 fold higher than BALB/c. In general, the amount of IFN-γ produced following high dose BCG iv challenge was not significantly different among all groups. However, the number of antigen-specific IL-4-producing spleen cells in the spleen from the high dose ($\geq 10^7$ cfu) vaccinated and unvaccinated groups are significantly ($p<0.05$) higher than in the low dose vaccinated groups. Accordingly, the ratios of antigen-specific IFN-γ to IL-4-producing spleen cells in the high dose vaccinated and naive mice, following high dose challenge, were both significantly lower than the low dose vaccinated groups. Table 5 shows results of statistical analysis on data obtained 18 weeks after high dose BCG challenge. Even though there is some overlap at 10^7 cfu, which is the t_n, it is clear that all groups of mice vaccinated with low dose of BCG had significantly ($p< 0.05$) lower numbers of IL-4-secreting cells in the spleen than mice in the high dose vaccinated groups. Therefore, we can conclude that CBA mice, vaccinated with BCG doses $>10^7$ cfu respond with mixed Th1/Th2 responses, while vaccination with doses $\leq 10^7$ induced primarily Th1 responses and Th1 imprints. For doses close to the t_n (10^7 cfu), the lock into a Th1 mode became clear at a later time after challenge (Figure 25B). The Th1 imprint generated following low dose BCG vaccination in CBA mice was also associated with high titers of IgG_{2a} antibody following high dose BCG iv challenge, while mice vaccinated with high dose or naïve mice produced higher levels of both IgG_1 and IgG_{2a} antibodies (Figure 25A).

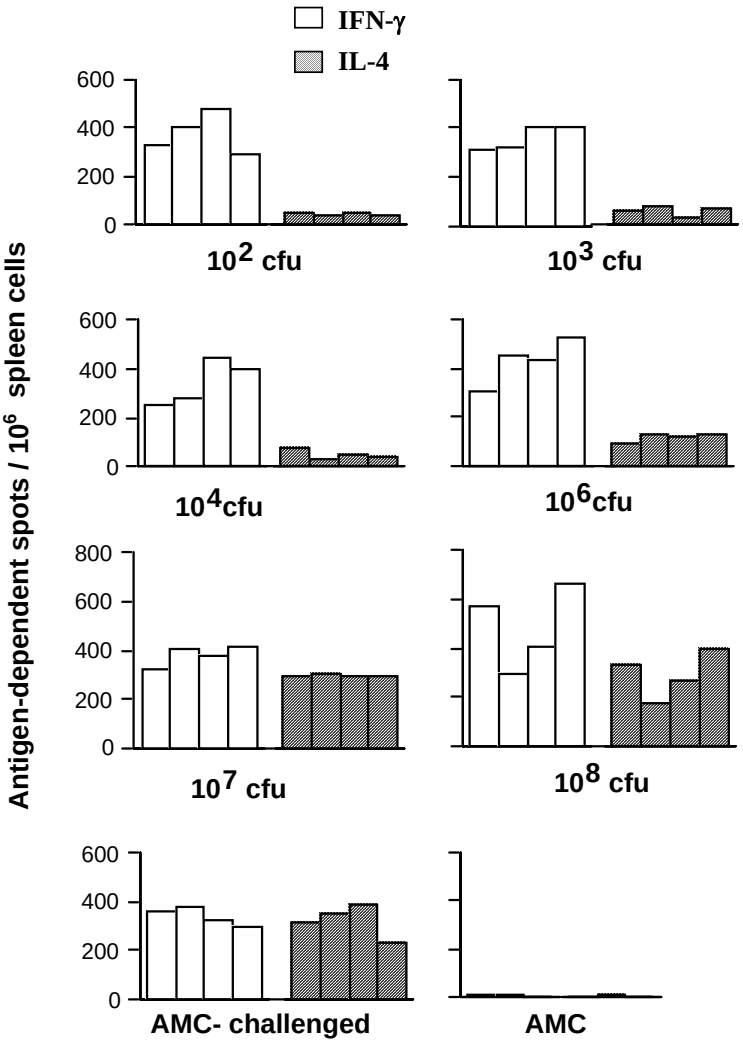

Figure 24. BCG-specific IFN-γ and IL-4 production by spleen cells from adult CBA mice immunized with different doses of BCG and subsequently challenged iv with a high dose of BCG.

Adult CBA mice vaccinated iv with different doses of BCG were challenged iv 16 weeks post-vaccination with $2x10^8$ cfu of BCG and sacrificed 12 weeks post-challenge to assess the immune response generated by spleen cells. Results shown represent one of two replicate experiments with 4 mice per group and data represent values of individual mice in each group. Numbers along the abscissa refer to dose of BCG vaccine. AMC (age matched control) stands for naïve control mice.

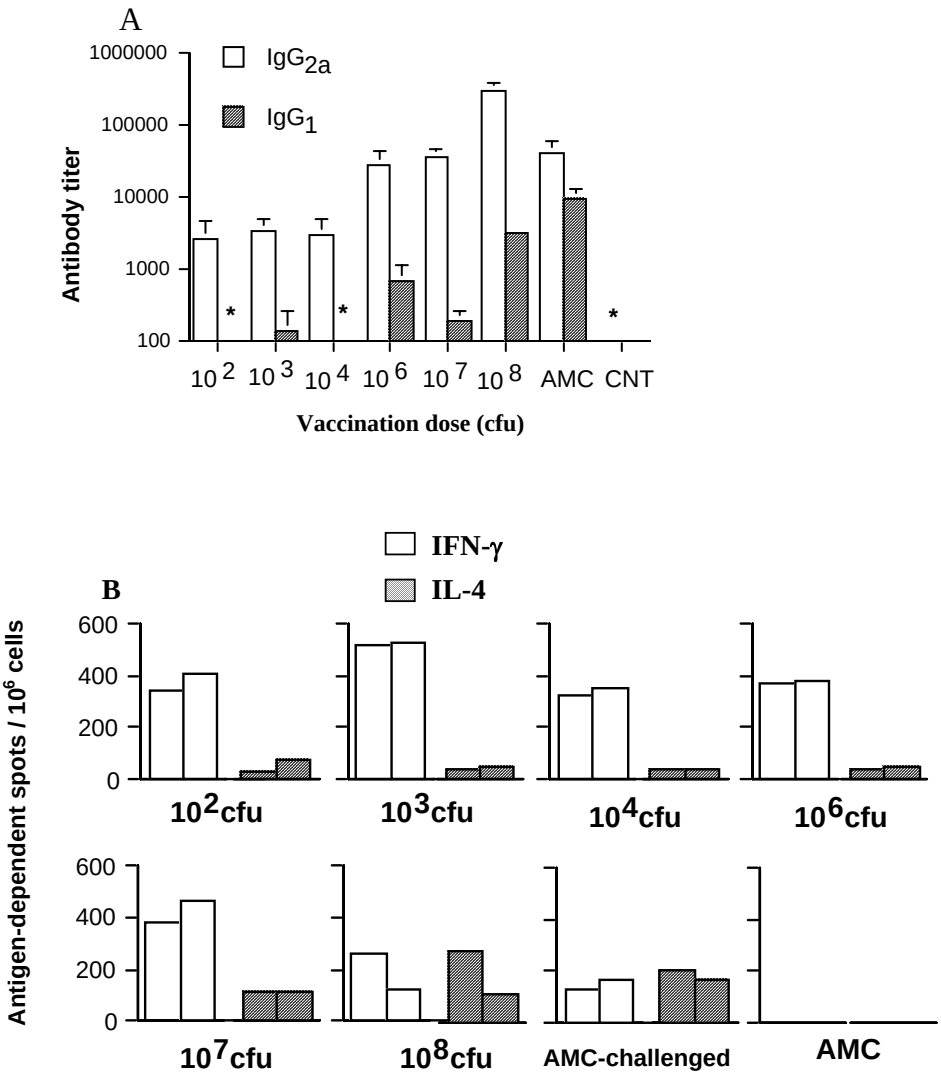

Figure 25. The Th1 imprinting induced by low dose BCG vaccination ensures stable, long-term Th1 responses following high dose BCG iv challenge in adult CBA mice.

Adult CBA mice vaccinated and challenged as described in the legend of Figure 24 were bled at 18 weeks post-challenge to assess BCG-specific serum antibody levels in ELISA (A). Mice were also sacrificed 24 weeks post-challenge and their splenic immune response assessed by ELISPOT assay (B). ELISA data represent average antibody tiers of 4-6 mice per group $\pm$ SD and ELISPOT data are values from individual mice in each group. Numbers in the abscissa refer to dose of BCG vaccination. AMC (age matched control) refers to naive control mice. CNT stands for a naïve control mice used as source of APC.

Table 5. Results of a statistical analysis on the number of cytokine producing spleen cells from adult CBA mice vaccinated with different doses of BCG following high dose BCG iv challenge.

Adult CBA mice vaccinated with different doses of BCG iv and challenged with $2x10^8$ cfu of BCG were sacrificed at 24 weeks post-challenge. Spleens were removed from each mouse and processed for ELISPOT assay as described in Figure 25. Data presented show average cytokine levels secreted by spleen cells following high dose iv challenge. The Th1/Th2 index is presented as IFN-γ to IL-4 ratio and statistical analysis was done to compare the level of cytokines as well as the Th1/Th2 index among groups. The same letters along each column indicate no statistical difference among groups and two letters in one group show an overlap.

BCG vaccine dose (cfu)	Average number of cytokine secreting spleen cells		
	IFN-γ	IL-4	IFN-γ/IL-4
10^2	372^a	54^a	8.3^a
10^3	523^b	36^a	14.5^b
10^4	338^a	33^a	10.2^a
10^6	372^a	42^a	8.9^a
10^7	426^{ab}	93^{ab}	4.6^{ac}
10^8	192^c	191^b	1.1^c
AMC	151^c	190^b	0.8^c

Another important question addressed here was whether the transition number (t_n) for the BCG dose, and hence the type of immune response generated, was affected by changing the route of antigen administration in CBA mice. To this end, adult CBA mice were vaccinated subcutaneously with different doses of BCG and the immune responses assessed following both primary vaccination and a subsequent high dose BCG intravenous challenge. As shown in Figures 26B, BCG doses $<10^8$ cfu given sc induced primarily a Th1-type response with exclusive IFN-γ production by spleen cells. There was variation in the number of IFN-γ-producing splenocytes among vaccination groups but all vaccinated mice respond with significantly higher levels of IFN-γ than naïve mice. In contrast, there was no significant difference ($p<0.05$) in the level of IL-4-secreting cells in mice vaccinated with lower dose of BCG and naïve mice. However, mice vaccinated with the highest BCG dose ($2x10^8$ cfu) had significantly higher ($p<0.05$) numbers of IL-4-secreting splenocytes when compared to mice in all the low dose vaccination groups and naive mice. Consequently the IFN-γ/IL-4 ratio is significantly higher in the low dose vaccinated groups when compared to mice in the high dose vaccinated group, which is close to 1. There was no detectable BCG-specific antibody in the sera from all the low dose BCG vaccinated mice. In contrast, the high dose BCG vaccinated mice had high titers of both IgG_1 and IgG_{2a} antibodies (Figure 26A).

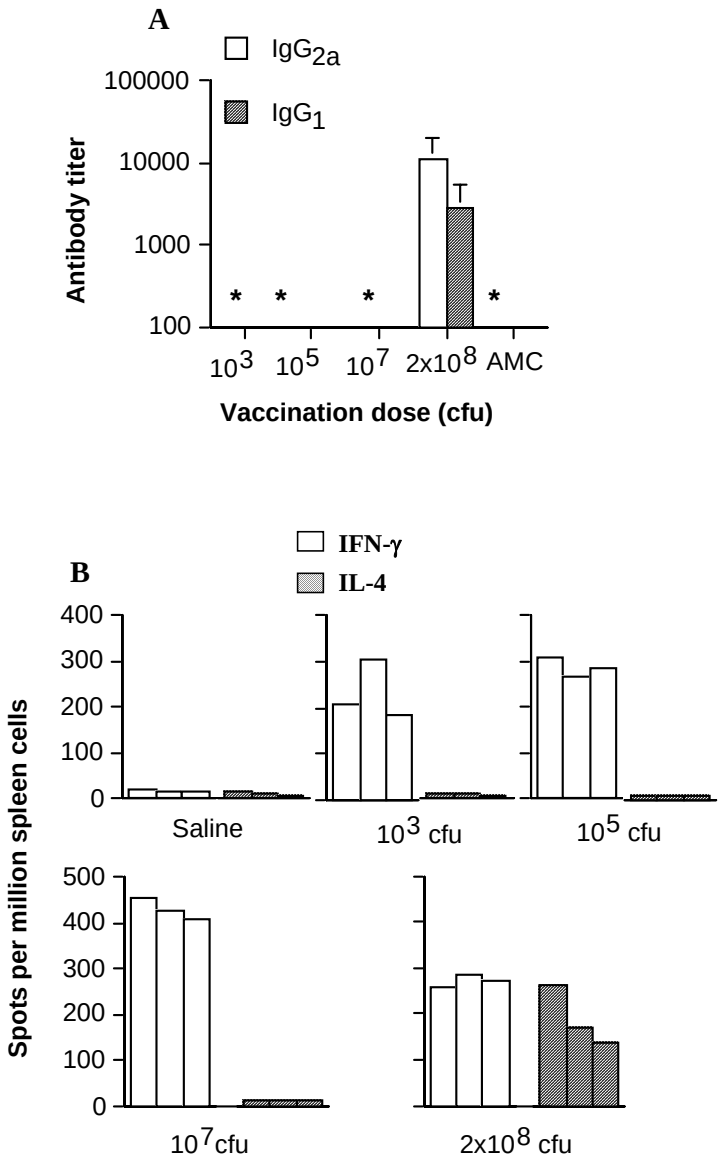

Figure 26. Low dose sc vaccination with BCG induces an exclusively Th1 response with undetectable levels of serum antibodies in adult CBA mice.

Adult CBA mice were vaccinated sc with different doses of BCG and bled at 20 weeks post-vaccination to determine the levels of BCG-specific IgG_1 and IgG_{2a} antibody titers (A). Mice were also sacrificed at 24 weeks post-vaccination to assess the type of BCG-specific immune response generated by spleen cells (B). Antibody levels represent mean titer of 3 mice per group with error bars (SD); * denotes titers below detection level. ELISPOT data represent values for individual mice in each group (n=3). Results shown are representative of three replicate experiments. Numbers on the abscissa represent the dose of BCG vaccine. AMC (age matched control mice) refers to naive control mice.

Following high dose challenge, adult CBA mice vaccinated sc with BCG doses $\leq 10^7$ cfu still generated a predominantly Th1 response, whereas mice vaccinated with the highest dose ($2x10^8$ cfu) or naïve mice generated a mixed Th1/Th2 response (Table 6). There is no significance difference in the amount of IL-4 produced among mice vaccinated with dose of BCG $\leq 10^7$ cfu after high dose BCG iv challenge. However, the high dose vaccinated group and naive mice had significantly higher ($p<0.05$) numbers of IL-4-producing spleen cells than the other groups. Consequently, mice vaccinated with BCG doses $\leq 10^7$ cfu had significantly higher IFN-γ/IL-4 ratios when compared to mice vaccinated with the highest dose ($2x10^8$ cfu) or naïve mice following high dose BCG iv challenge. Again, the IgG isotype of the anti-BCG antibody response correlated well with the type of the T-cell response in the spleen (Figure 27). Mice vaccinated with doses of BCG $<10^7$ cfu induce no detectable levels of IgG antibodies suggesting an exclusively cell-mediated immune response, while 10^7 cfu of BCG induced only the IgG_{2a} isotype. In contrast, mice vaccinated with the highest dose and naïve mice had higher titers of both IgG_1 and IgG_{2a} antibodies following high dose BCG iv challenge suggesting a mixed Th1/Th2 response. These observations support the conclusion that the route of BCG administration did not alter the Th1/Th2 nature of BCG-specific immune response. This is consistent with observation made with adult BALB/c mice. Echoing the results following intravenous administration, vaccination of mice with BCG by subcutaneous administration caused a Th1 imprint in CBA mice in a dose dependent manner, as assessed by a high dose BCG iv challenge. In addition, comparing Figures 24 and Table 6, it appears that the t_n is 10 fold higher in sc vaccinated than iv vaccinated CBA mice, which parallels findings in BALB/c mice.

Table 6. Th1 imprinting in adult CBA mice following sc vaccination with low doses of BCG and high dose BCG iv challenge.

Adult CBA mice vaccinated sc with different doses of BCG were challenged iv with $2x10^8$ cfu of BCG at 16 weeks post-vaccination. BCG-specific immune responses generated by spleen cells were assessed by ELISPOT assay 12 weeks post-challenge. Results show mean number $\pm$ SD of cytokine producing cells per million spleen cells from 3 mice per group and the ratio of the numbers of IFN-γ to IL-4-secreting cells for each group. Results of statistical analysis are also presented. The same letters in each column show no statistical difference among groups at a p level of 0.05. Data are from one of two similar experiments.

	Antigen-specific cytokine secreting cells per million spleen cells as detected in the ELISPOT assay				
	IFN-γ		IL-4		IFN-γ/IL-4
BCG dose (cfu)	Mean	SD	Mean	SD	Ratio
10^3	900.0[a]	117.5	59.7[a]	9.9	15.1[a]
10^5	770.3[a]	93.3	49.0[a]	3.0	15.7[a]
10^7	1017.0[b]	78.0	75.0[a]	25.5	13.6[a]
$2x10^8$	410.0[c]	16.6	339.3[b]	28.9	1.2[b]
AMC	308.0[c]	36.8	348.0[b]	37.6	0.9[b]

AMC stands for naïve control mice challenged iv with high dose of BCG
SD represents standard deviation of the mean

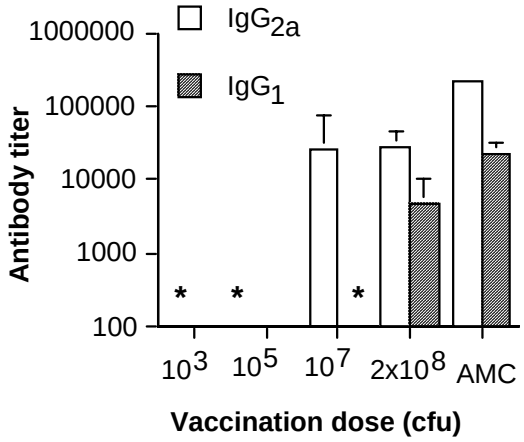

Figure 27. Vaccination of adult CBA mice with lower doses of BCG induced a Th1 imprint as assessed by the amount and isotype of IgG antibodies produced following a high dose BCG iv challenge.

Adult CBA mice vaccinated sc with different doses of BCG were challenged iv with $2x10^8$ cfu of BCG at 16 weeks post-vaccination. Mice were bled 12 weeks post-challenge to determine the amount and isotype of BCG-specific IgG antibodies in serum. Data shown represent mean titer of 3-5 mice per group and * denotes titers below the detection level. Results shown came from one of at least three replicate experiments. Numbers on the abscissa represent dose of BCG vaccine. AMC (age matched control) refers to naive control mice.

Experiments with CD1 mice yielded results nearly identical to results from BALB/c mice both during primary exposure to BCG (Figure 19 and 21) and after high dose iv challenge (Table 4 and Figure 28). Similar to data from adult BALB/c mice, there was no statistical difference in the number of BCG-specific IFN-γ-secreting splenocytes among all groups. However, the number of IL-4-secreting splenocytes was significantly higher ($p<0.05$) in mice vaccinated with doses of BCG $\geq 10^6$ cfu. Consequently the IFN-γ/IL-4 ratio is significantly lower ($p<0.05$) in all mice vaccinated with doses of BCG $\geq 10^6$ cfu. Results from CD1 mice were contrary to our expectations in that CD1 mice, claimed to be genetically heterogeneous, would display a more heterogeneous response. Thus, we did not pursue further experiments with CD1 mice, as they did not prove to be a useful model to assess the validity of a general vaccination strategy in a heterogeneous population. Nonetheless, our hypothesis that ultra-low dose of BCG, as low as 33 cfu as shown in the newborn mice, can induce a protective immune response against mycobacterial infections, is still compelling since all the lower doses used in this study, in all the three different strains of mice, induced Th1-type responses and Th1 imprints following a high dose BCG iv challenge.

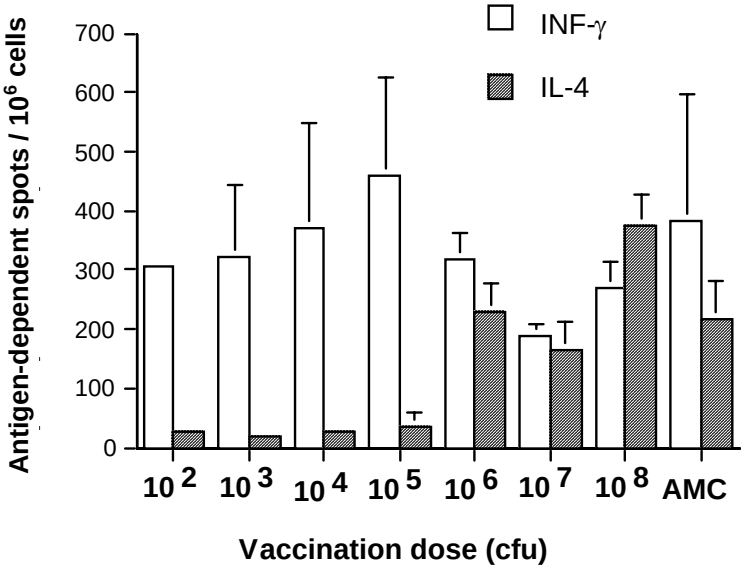

Figure 28. Low dose BCG vaccination induces Th1 imprinting in CD1 mice.

Adult CD1 mice were vaccinated iv with different doses of BCG and then challenged intravenously 12 weeks post-vaccination with ~$5x10^6$ cfu of BCG iv. Four mice from each group were sacrificed 12 weeks post-challenge to assess the BCG-specific immune response generated by spleen cells. Results represent one of at least two similar experiments and show the average number of cytokine producing cells per group with error bars (SD). Numbers on the abscissa are dose of BCG vaccine in cfu. AMC (age matched control) refers to naive control mice.

5.2.3. Discussion

In agreement with a previous finding in adult mice (Power et al., 1998), we show here that the dose of BCG given to adult BALB/c mice determined the Th1/Th2 phenotype of the response, with relatively lower doses inducing predominantly Th1-type responses and higher doses inducing mixed Th1/Th2 responses. This pattern was seen independently of whether BCG is given iv or sc. We have also shown that a similar relationship holds true between the dose of BCG and the Th1/Th2 phenotype of the ensuing response in newborn BALB/c mice when BCG was administered sc (section 5.1.2.2.). The main difference was that higher doses of BCG ($>3.3x10^7$ cfu) were required to induce a significant Th2 component to the immune response when BCG was given to newborn than to adult mice, where a mixed Th1/Th2 response began to appear at doses $\geq 10^6$ cfu of BCG injected sc. This is surprising in view of the generally accepted concept that the neonatal immune system is inherently biased to mount antibody, Th2, responses (Barrios et al., 1996; Adkins, 2000). Similarly, the vaccination dose of BCG given to adult CBA mice determined the Th1/Th2 nature of the ensuing immune response independently of whether BCG is administered by the iv or sc route of vaccination. However, the transition number, t_n, was 100-fold different between BALB/c and CBA mice when BCG was given iv. In agreement with this, the transition number for another intracellular pathogen, the *L. major* parasite, was found to vary by almost a million fold among different strains of mice (Menon and Bretscher, 1998). Additionally, the t_n seems to vary by about 10-fold between sc and iv routes of vaccination both in BALB/c and CBA mice; this is not surprising due to reasons discussed in section 5.1.2.2.

Our finding that low doses of BCG induce Th1 biased responses in adult mice is in agreement with a recent report of a study in guinea pigs (Horwitz et al., 2006). In this study, extraordinarily small amounts (10 cfu) of BCG vaccine induced an exclusively cell-mediated response with no detectable levels of antibody, which protected the host against lethal challenge with *M. tuberculosis* given by the aerosol route of infection. This is in direct agreement with our finding that the lowest dose BCG vaccine (33 cfu) was the most consistent and effective in terms of inducing an exclusively Th1 response and reducing the bacterial burden from the spleen after a subsequent high dose BCG iv challenge. Horwitz et al also reported that, despite the presence of high antibody titers, the highest dose used (10^6 cfu) was able to induce cell-mediated immunity to the same level as that of the lower doses used, and was as efficient as the lower doses in protecting the host against challenge with virulent *M. tuberculosis*. Based on these results, the authors conclude that the BCG-specific humoral response was dose-dependent, but the cell-mediated response was dose-independent. All the doses they used induced a cell-mediated response of comparable magnitude, as assessed by BCG-specific proliferation and expression of DTH, as well as equal efficacy in reducing the bacterial burden from lung and spleen of guinea pigs following aerosol challenge with *M. tuberculosis*. While the results reported by Horwitz and colleagues, as well as our findings, are fundamentally the same, their conclusion implies that dose of antigen may not have an effect on the efficacy of BCG vaccination, as both their lowest and highest doses reduced bacterial burden with comparable efficacy. We contend this conclusion is misleading for several reasons. First, we believe the highest dose they used (10^6 cfu) was not high enough to induce a Th2 biased response. In our studies, equal doses were found to induce pure Th1

responses in CBA mice and a mixed Th1/Th2 response, with a bias towards the Th1 pole in BALB/c as well as in CD1 mice. Therefore, the highest dose they used might have induced mixed Th1/Th2 response with a bias towards the Th1 pole similar to our findings in BALB/c and CD1 mice or a predominantly Th1 response as in CBA mice. Second, their conclusion that cell-mediated immunity was dose-independent was based on lymphocyte proliferative response and DTH assays, which cannot distinguish between Th1 and mixed Th1/Th2 responses. However, the presence of high levels of BCG specific IgG antibodies they reported may be an indication of the presence of Th2 helper cells. This suggests that the immune response generated following vaccination with 10^6 cfu may have a Th2 component, differentiating it from the response of the low dose vaccinated group, which was exclusively a Th1 response. Thus, concluding the type of cell-mediated response generated following BCG vaccination was dose-independent is questionable, as we have shown in our studies that doses higher than $\geq 10^7$ and $\geq 10^8$ in adult BALB/c and CBA mice, respectively, induce qualitatively different immune responses than those generated following immunization with lower doses. The responses generated following relatively higher doses of BCG in each mouse strains had a significant Th2 component, which we think may counteract the effect of a protective Th1 response. Third, as discussed in the Introduction, the standard dose of BCG ($>10^8$ cfu) administered to school children showed variable efficacy ranging from no effect at all in India to 80% efficacy in England. Therefore, these results may suggest that the guinea pig model in this study is not an appropriate model for determining why BCG failed to protect against tuberculosis in tropical areas like India and Africa, but presents an appropriate model for the English trial. There can be several factors to explain the failure

of BCG on these areas, but we believe that one of the most important variables was the dose of BCG used during these vaccination trials. The reason why high dose of BCG was effective in England and not in India or Africa could be due to the fact that there is genetic variation in susceptibility/resistance to tuberculosis between these different populations used in the studies. Tuberculosis is an old disease in Europe when compared to Asia or Africa and hence the population may have developed better resistance to tuberculosis than the population in areas where tuberculosis became a public health problem in the late 19^{th} and early 20^{th} century, following colonization of these areas by European settlers. As a result the transition number (t_n) for *M. tuberculosis* and the BCG vaccine may be higher in the European population, resulting in a better efficacy of high dose BCG vaccination as reported in England. This is consistent with our finding that the t_n can vary by several folds between different mouse strains. These comments are clearly of a speculative nature, but it is this kind of consideration that might explain the diversity of results from BCG trails and the dramatic results of Buddle and colleagues (Buddle et al., 1995) on the efficacy of low dose BCG vaccination in cattle.

We have also shown the Th1 responses generated following vaccination with relatively low doses BCG are associated with a Th1 imprint in both adult and newborn BALB/c mice, as demonstrated by the more efficient clearance of BCG from the spleen following high dose iv challenge of low dose vaccinated mice. Based on the present results, one can conclude that low dose BCG vaccination induced a predominantly Th1-type response and Th1 imprinting in mice independent of, to the limited extent we examined, the age or strain of mice used, as well as the route of antigen administration. Similarly, $5x10^8$ cfu of

BCG vaccine was reported to be inferior as compared to lower ($5x10^4$ cfu) and intermediate ($5x10^7$ cfu) doses in protecting deer against challenge with *M. bovis* independent of the route of administration (Griffin et al., 1999). In their recent review article, Griffin and colleagues have discussed in detail the summary of results they obtained from several vaccination studies in deer (Griffin et al., 2001). Based on these results, they conclude that BCG doses in the range of 10^4 to 10^7 cfu were able to induce responses dominated with type 1 cytokines that provided better protection against infection with *M. bovis*. In contrast, the high dose (10^8 cfu) induced type 2 cytokines dominated with high level of IL-4 and strong antibody responses, which was less efficient in protecting deer against *M. bovis* infection. The response generated following vaccination with high dose BCG seems to be a mixed Th1/Th2 response as they reported both strong DHT responses and high antibody titers in those animals. In another study, low dose BCG vaccination ($6x10^4$ and $6x10^6$ cfu), which was 10,000 to 100,000 lower than the commonly used dose (10^{10} cfu), induced immune response with higher levels of mycobacterial antigen-specific IFN-γ and IL-2 cytokines that protected calves from lethal *M. bovis* challenge (Buddle et al., 1995). These observations, made in various animal species including mice, guinea pigs, deer and cattle, imply that low dose BCG vaccination is sufficient to generate Th1 type responses and Th1 imprints that can effectively control infection with pathogenic mycobacteria. The consistency of this response among species argues strongly that this may also be applicable to human vaccination protocols against *M. tuberculosis* infections.

5.3. Isolation and Identification of T-cell Subsets Responsible for the Secretion of BCG-Specific Cytokines

5.3.1. Introduction

Knowledge of the nature of the primary immune response generated following infection with chronic intracellular pathogens, such as mycobacteria, and identifying the T-cell subsets responsible for protection, is very important in designing an improved anti-tuberculosis vaccine. Evidence in the literature, discussed in detail in section 2.6, has unequivocally shown that anti-tuberculosis immunity is mediated by cytokines, mainly IFN-γ from antigen-specific CD4+ Th1 cells and CD8+ cytotoxic T-cells. On the other hand, cytokines from antigen specific CD4+ Th2 cells, mainly IL-4 may play a role in the failure of immunity to tuberculosis infection (Sanchez et al., 1994; Surcel et al., 1994; Baliko et al., 1998). We therefore wished to determine the subset of lymphocytes responsible for the production of cytokines (IFN-γ and IL-4) during BCG infection. To this end, BALB/c mice vaccinated sc with low and high doses of BCG were subsequently challenged intravenously with a high dose of BCG and sacrificed between 12 to 16 weeks post-challenge. Single cell suspensions of spleen cells were depleted of particular T-cell subsets by antibody dependent complement mediated lysis. In addition, positive and negative selection using magnet assisted cell sorting (MACS) was used to obtain specific cell populations. Positive and negative fractions isolated from the spleen cells using the MACS beads, as well as the population recovered following complement mediated lysis, were washed and re-suspended in the original (pre-depletion) volume in complete RPMI

media. Cells were counted and whenever the number is less than 10^6 per 100 μl, the amount dispensed per well, feeder cells from naïve mice were added so that each well in the ELISPOT assay would have 10^6 cells in 100 μl media. A 100 μl aliquot, containing around 10^6 cells, was also taken from each tube and processed for flow cytometric analysis to determine the level of expression of the respective markers in comparison to stained normal spleen cells.

5.3.2. Results

Our results clearly show that purifying T-cell subsets using MACS is an efficient method of sorting T-cell subsets when compared to the complement dependent depletion of a particular subset of T-cells (data not shown). In addition, cells treated by complement-mediated lysis appear unhealthy under the microscope, which may have a detrimental effect on the survival of cells and their ability to produce cytokines in the ELISPOT assay. For this reason, all our remaining cell-sorting experiments were carried out using the MACS procedure despite the greater cost of this method. Figure 29 shows FACS analysis of cell surface markers following MACS depletion of a given T-cell subset from the spleen cells of BCG infected mice. Both the negative and positive fractions obtained from MACS were processed for ELISPOT assay as described in Materials and Methods. Results from ELISPOT assays show both IFN-γ and IL-4 cytokines produced during BCG infection come from lymphocytes that express the surface markers of Thy1.2 and CD4, which confirms that the source of BCG-specific IFN-γ and IL-4 cytokines are CD4+ T-cell subsets or cells dependent on CD4+ T cells.

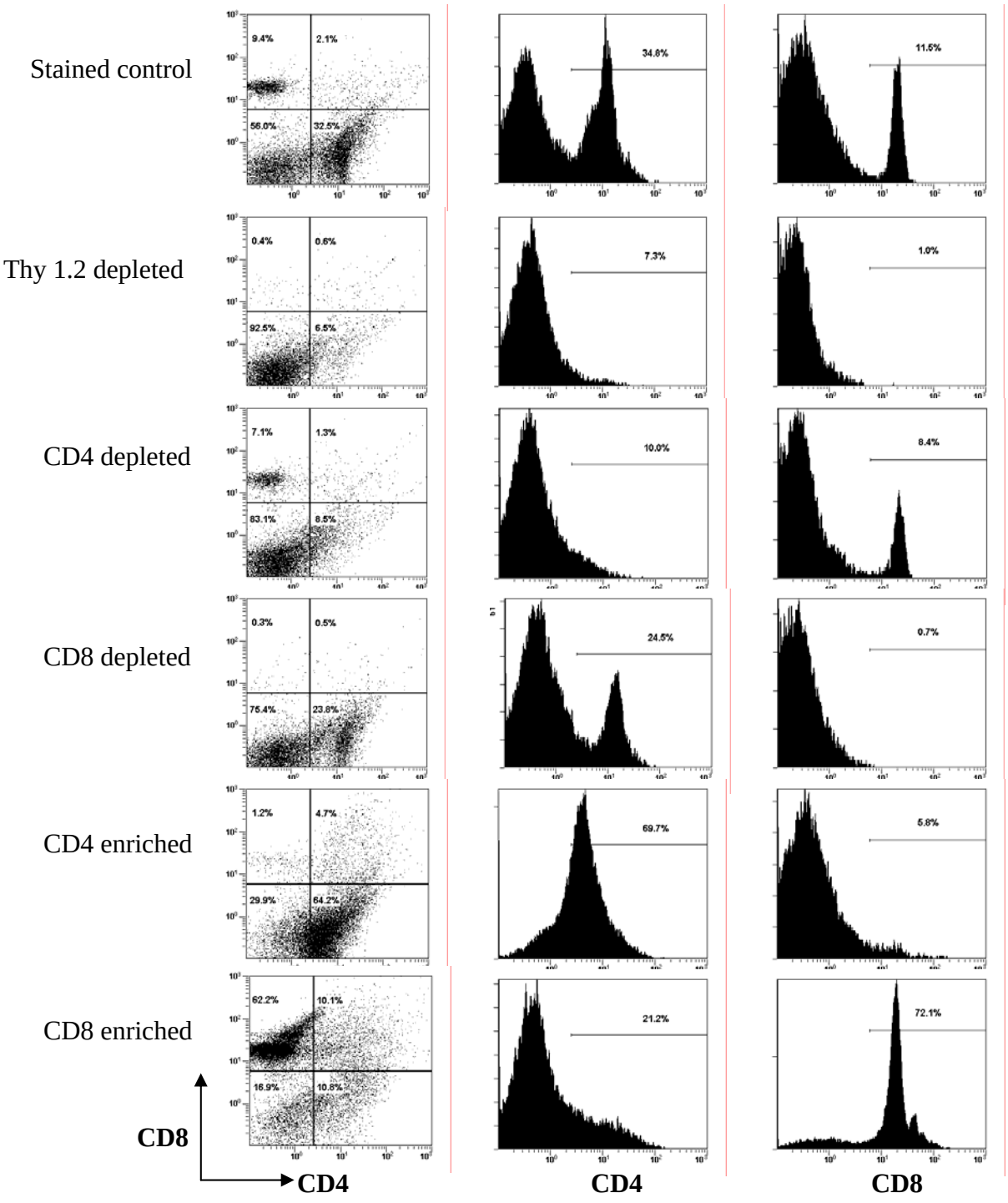

Figure 29. Expression of markers on MACS depleted or enriched cells as compared to normal spleen cells.

Adult BALB/c mice vaccinated with 10^7 cfu of BCG subcutaneously and then challenged intravenously with $5x10^6$ cfu of BCG were sacrificed 12 weeks post-challenge. Pooled spleen cells from 3 mice were sorted using MACS beads and samples taken for FACS analysis as described in Materials and Methods. Expression of Thy1.2, CD4 and CD8 markers are shown in comparison to un-depleted stained spleen cells.

Figure 30 is a picture of an ELISPOT plate illustrating the number of IFN-γ and IL-4 spots per million spleen cells plated. Even though counting the number of spots in each well was difficult, comparable numbers of spots can easily be seen both in the un-depleted and CD8 depleted spleen cells, suggesting few, if any, of the cytokines are secreted by CD8 cells (compare row 3 with row 4). However, Thy1.2 and CD4 depleted cells secrete almost no IFN-γ and very little IL-4 as compared to un-depleted (control) spleen cells, which indicates that the source of BCG-specific IFN-γ and the majority of the BCG-specific IL-4 cytokines in the spleen are Th1.2+CD4+ cells. Similarly, Table 7 clearly shows >90% of the BCG-antigen dependent IFN-γ-secreting cells, as well as 70-80% of the IL-4-producing cells were Thy1.2+ CD4+ cells. The level of cytokines produced by CD8 depleted spleen cells was not significantly different than the control group. However, depletion of Thy1.2+ or CD4+ T-cells reduced the levels of both IFN-γ and IL-4 cytokines.

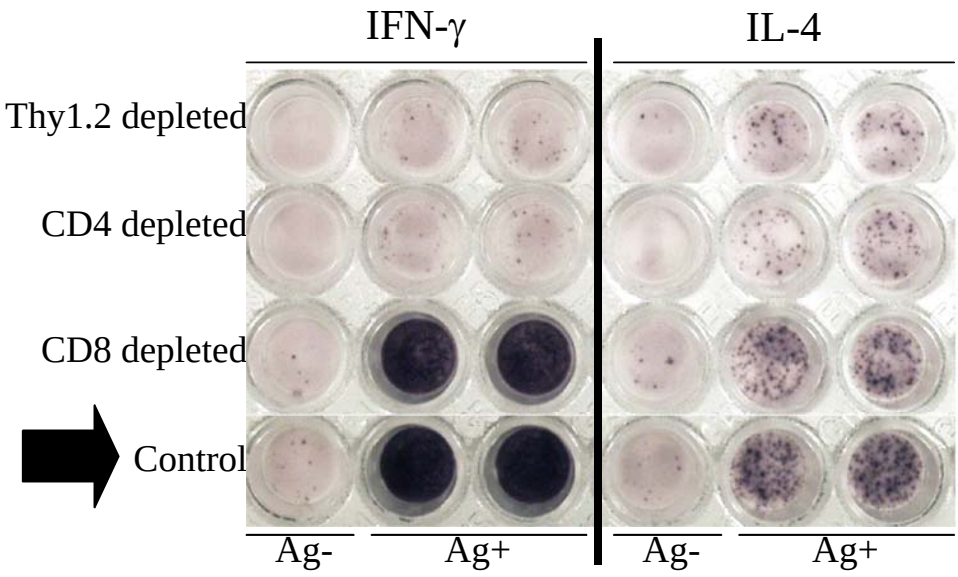

Figure 30. A picture of an ELISPOT plate with spots formed by antigen-specific cytokine secreting T-cell subsets of spleen cells sorted using magnetic beads.

Pooled spleen cells from 3 BALB/c mice vaccinated sc with 10^7 cfu of BCG and challenged iv with a high dose BCG were sorted using MACS. Cells depleted of specific T-cell subsets were used in the ELISPOT assay to determine the source of cytokines during BCG infection. Numerous antigen-specific IFN-γ and IL-4 spots were observed in wells with un-depleted spleen cells as well as CD8 depleted cells. In contrast, almost no IFN-γ spots and only a few IL-4 spots were observed in wells with Thy1.2 depleted and CD4 depleted spleen cells.

Table 7. BCG-specific IFN-γ and IL-4 cytokine production by T-cell subsets of the spleen following MACS depletion.

Spleen cells from 3 BLAB/c mice vaccinated with 10^7 cfu of BCG sc and challenged with high dose of BCG iv were pooled together and sorted into specific subsets using MACS. Both the positive and negative fractions were re-suspended in the original volume and 100 µl aliquots added per well in the ELISPOT assay to assess the level BCG-specific cytokines produced. Feeder cells from naïve mice were added to bring the total number of cells per well to 10^6. Results represent the average of three different ELISPOT assays.

	Antigen-specific cytokine secreting cells per million spleen cells as detected in the ELISPOT assay			
	IFN-γ		IL-4	
Treatment group	Mean	SD[b]	Mean	SD[b]
Control cells[a]	1308	334	551	91
CD8+ cells depleted	1285	305	453	61
CD4+ cells depleted	56	27	155	33
Thy1.2+ cells depleted	43	14	96	11
Thy1.2+	1396	173	349	60
CD4+	1199	168	221	149
CD8+	89	61	25	16
APC[c]	29	14	22	6

[a] Undepleted control cells
[b] Standard deviation of the mean as calculated in Microsoft Excel, 2003
[c] Antigen presenting cells from naive mice

5.3.3. Discussion

The results from the depletion experiments clearly show that the majority of the BCG-specific IFN-γ-producing cells, detected by the ELSPOT assay and present in immune spleen, are CD4+ T-cells. This suggests that BCG vaccination, if administered under optimal conditions, can induce mycobacterial-specific CD4+ T-cells capable of secreting cytokines responsible for the activation of macrophages. Activated macrophages in turn are responsible for the destruction of the mycobacteria. Our findings are in agreement with previous reports where in vivo depletion of αβ T-cells or MHC class $II^{-/-}$ was associated with progressive disease and rapid death of tuberculosis infected mice. In contrast, normal mice were able to contain infection at a stationary level from day 20, associated with increased levels of IFN-γ in the lung, resulting in a relatively longer survival time (Mogues et al., 2001). Observations in human HIV patients have also shown that CD4+ T-cells secreting IFN-γ are very important in maintaining *M. tuberculosis* infection in a latent state and prevent reactivation disease (Scanga et al., 2000). These observations are clear evidence in support of the hypothesis that CD4+ cells and their cytokines are very important in controlling tuberculosis disease progression in both humans and animals. Our results also show that BCG-specific IL-4-producing cells, present in immune spleen, are mainly CD4+ cells, suggesting that the same populations of T-cells (probably a different subset) are responsible for the down regulation of the protective immune response in mice vaccinated with high dose of BCG. This is consistent with our hypothesis that a low dose BCG vaccine induces Th1 cells, which secrete IFN-γ that activates macrophages, whereas a high dose of BCG induces mixed Th1/Th2 cells with a substantial level of IL-4 that may counteract the effect of Th1 cells.

5.4. Examining whether Anti-IL-4 Therapy can Modifying an Ongoing, Mixed Th1/Th2, anti-BCG Response into a Th1-type response

5.4.1. Introduction

In situations where no effective vaccine is available that can prevent infection, or no appropriate chemotherapy exists to control disease, immunotherapy may become a feasible approach in fighting chronic diseases. Evidence in the literature and results from our laboratory indicate treatment with appropriate cytokines or anti-cytokine antibodies can redirect the immune response from a non-protective response to one that can control infection. Partial depletion of CD4+ T-cells, administration of pro-Th1 cytokines, such as rIL-12 or neutralizing Th2 cytokines, such as IL-4 prior to and/or during the time of infection with *L. major*, resulted in a Th1 response that protected BALB/c mice from a high dose challenge (Titus et al., 1985; Sadick et al., 1990; Heinzel et al., 1993; Nabors et al., 1995; Heinzel and Rerko, 1999). Interestingly enough, anti-IL-4 therapy also cured mice with a stable "border-line leishmaniasis" associated with a mixed Th1/Th2 response several months post-infection (Uzonna and Bretscher, 2001). These results prompted us to explore whether treatment of BCG infected mice, with a stable mixed Th1/Th2 anti-BCG response, using anti-IL-4 or anti-CD4 antibody, could switch the BCG specific response into a Th1 mode. If this could be achieved, we would examine whether such a modulation led to increased clearance of the bacteria as reflected in a reduced bacterial burden in the spleen. The specific objective of this study was to determine if anti-IL-4 treatment could shift an ongoing mixed Th1/Th2 anti-BCG response into an exclusive

Th1 mode in BCG infected mice. To determine the minimum dose of anti-IL-4 antibody that produced the desired effect, BALB/c mice vaccinated with 10^7 and 10^8 cfu BCG were divided into three groups. Starting at week 12 post-vaccination, groups were treated intraperitoneally (ip) with ascites fluid that contained either 1 mg/mouse/wk (G1) or 0.25 mg/mouse/wk (G2) of anti-IL-4 monoclonal antibody (11B11), or 1 mg/mouse/week of isotype-matched control antibody (G3), for four weeks. We could not ascertain any effect of these treatments on the Th1/Th2 nature of the response in two different experiments; a third experiment was conducted to specifically address variables we suspected might be responsible for the failure of anti-IL-4 treatment to have an effect. In the third experiment, the dose of BCG used to create a mixed Th1/Th2 response was $6x10^6$ cfu, and the biological activity of the ascites fluid in terms of ability to neutralize IL4, was determined using the CT4S assay as described in section 4.11. In addition, CBA mice, with a predominant Th1-type response, but also with a readily detectable level of BCG-specific IL-4-producing cells, were also used as a positive control, to help determine if the lack of effect observed in the previous experiments was due to an excessive polarization of the response towards the Th2 pole. Evidence in the leishmania system showed that highly polarized Th2 responses could not be easily modulated to the Th1 mode by anti-IL-4 treatment. The treatment groups in BALB/c mice were: 1 mg/week (G1), 0.5 mg twice a week (G2), 1 mg/week isotype control antibody (G3). Parallel experiments were conducted with CBA mice, but with only two groups: G1 receiving 1 mg/week anti-IL-4 monoclonal antibody (11B11), and G2 receiving an equal amount of control antibody. All treatments were given ip for 4 consecutive weeks.

5.4.2. Results

Adult BALB/c and CBA mice were injected with $6x10^6$ and $5x10^7$ cfu of BCG iv, respectively. Three mice from each group were sacrificed 12 weeks post-vaccination to determine the type of response generated. As shown in Table 8, BALB/c mice mounted a mixed Th1/Th2 response with IFN-γ/IL-4 and IgG_{2a}/IgG_1 ratios close to 1, with no significant difference in the level of cytokines and antibodies in each mouse. In contrast, CBA mice generated a predominantly Th1 response with significantly higher levels of IFN-γ-secreting cells associated with higher BCG-specific IgG_{2a} than IgG_1 antibody titers. However, the spleen cells of CBA mice also had a considerable number of IL-4-secreting T-cells. This allowed us to determine whether anti-IL-4 treatment could eliminate or reduce the frequency of these cells and modulate the BCG-specific immune response into an exclusively Th1 mode. Mice were treated with ascites fluid containing anti-IL-4 antibody as described above. Starting from week 6 after the last treatment, 3 mice per group were bled to assess antibody titers and isotypes in serum and then sacrificed to assess the Th1/Th2 nature of the BCG-specific cells present in the spleen. Figure 31A shows results from both BALB/c and CBA mice after anti-IL-4 treatment. These results clearly show that anti-IL-4 treatment did not significantly alter the mixed Th1/Th2 response in BALB/c mice. Similarly, treatment did not have a significant effect on reducing the frequency of IL-4-secreting cells in CBA mice. We conclude that our anti-IL-4 treatment protocol does not significantly modulate a mixed Th1/Th2 response towards the Th1 pole during BCG infection. Parallel results were also obtained when the isotype of BCG-specific IgG antibodies was assessed by ELISA 18 weeks after the last treatment of BALB/c mice, with no detectable effect observed (Figure 31B).

Table 8. Effect of anti-IL4 antibody treatment in modulating an ongoing mixed Th1/Th2 anti-BCG response into a Th1 mode, as assessed by determining BCG-specific IL-4 and IFN-γ-producing spleen cells and levels of BCG-specific IgG_{2a} and IgG_1 serum antibodies.

Adult BALB/c and CBA mice were injected iv with a dose of BCG that induced a mixed Th1/Th2 response. 12 weeks after infection, representative mice from each strain were bled to determine the isotype of BCG-specific IgG antibodies and sacrificed to assess the nature of antigen-specific cytokine response by splenocytes. Results are from one of two replicate experiments.

	Cytokine secreting cells per million spleen cells as detected in ELISPOT			Log_{10} titer of Serum Antibodies as detected in the ELISA assay		
	IFN-γ	IL-4	IFN-γ/IL-4[b]	IgG_{2a}	IgG_1	IgG_{2a}/IgG_1[c]
BALB/c						
[a] M1	446	502	0.88	5.9	5.6	1.1
M2	480	470	1.02	5.3	5.3	1.0
M3	397	508	0.78	5.7	5.5	1.0
APC[d]	47	9	NA			
CBA						
M1	387	96	4.03	4.4	3.2	1.4
M2	406	93	4.37	4.7	2.6	1.8
APC[e]	35	19	NA			

[a] M refers to mouse 1, 2, 3
[b] Ratio of IFN-γ to IL-4
[c] Ratio of IgG_{2a} to IgG_1
[d] Spleen cells taken from naïve BALB/c mice
[e] Spleen cells taken from naïve CBA mice
NA; not applicable

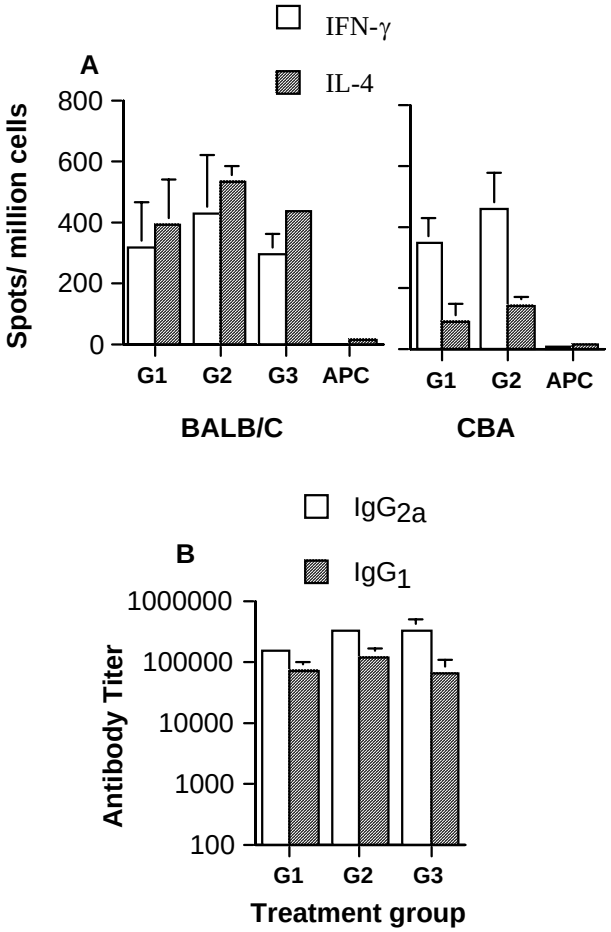

Figure 31. Treatment with anti IL-4 antibody does not modulate BCG-specific immune response in either BALB/c or CBA mice.

Mice were injected iv with a dose of BCG that can induce a mixed Th1/Th2 response. Starting from week 12 post-infection, mice were treated with anti-IL-4 antibody for 4 consecutive weeks as described in the text. Starting from week 6 after the last anti-IL-4 treatment, the isotypes of the anti-BCG IgG antibodies and the nature of the splenic cytokine response were assessed. (A) Shows the results of treatment on the splenic anti-BCG response at 12 weeks post-treatment and (B) is the isotypes of anti-BCG serum antibodies 18 weeks post last treatment of the BALB/c mice. Results shown are representative of three replicate experiments carried out at weeks 6, 12 and 18 after the last anti-IL-4 treatment. Labeling in the abscissa represents treatment groups. In BALB/c mice, G1 refers to administration of (1 mg Ab/week), G2 (0.5 mg twice a week), G3 (1 mg/week control antibody). In CBA mice, G1 is (1 mg/week), G2 (1 mg/week control antibody). APC stands for spleen cells from naïve mice used as feeder cells in the ELISPOT assay.

5.4.3. Discussion

Contrary to our hypothesis, anti-IL-4 therapy did not modulate an ongoing mixed Th1/Th2 anti-BCG response towards the Th1 pole in BALB/c mice. This was not due to the pro-IL-4 nature of the BALB/c mice, as a similar lack of effect was seen with CBA mice. This may suggest that the fine balance between Th1 and Th2 cells is differently regulated in mycobacterial and *L. major* infections. A possible mechanism could be via IL-10, as it was shown in vitro that IL-10 inhibits the activation of macrophages to destroy intracellular pathogens. IL-10 inhibits macrophage activation either by suppressing the transcription of pro-inflammatory cytokines (mainly IL-1, TNFα, IFN-γ) and/or by down-regulating surface molecules such as the B7.1 and B7.2 co-stimulatory as well as MHC class II molecules (Moore et al., 2001). It also suppresses the production of reactive oxygen and nitrogen intermediates in activated macrophages (Redpath et al., 2001). Thus, IL-10 is sometimes known as a macrophage deactivation factor; mycobacteria may cause IL-10 production in order to ensure their survival within the macrophages. In line with this, the excessive level of IL-10 seen in active tuberculosis patients, as compared to healthy contacts, is believed to be the cause for the depressed mycobacterial antigen-specific immunity during pulmonary tuberculosis infections (Hirsch et al., 1999) and for the immunosuppression exhibited during trypanosome infections (Uzonna et al., 1998). A recent study reports patients with active tuberculosis have higher levels of IL-10 and CD4+CD25+ T-cells in their PBMC than PPD positive healthy contacts, suggesting a possible role of the T_{reg} cells and their primary cytokines, IL-10, in the regulation or suppression of immune response during tuberculosis infection (Ribeiro-Rodrigues et al., 2006). Therefore, inhibiting the effect of IL-10 either by

neutralizing the cytokine using monoclonal antibodies or by blocking its effect using anti IL-10R monoclonal antibody is worth considering. However, in direct contrast to previous findings that report a direct role for IL-10 in suppressing mouse immunity against *M. avium* (Denis and Ghadirian, 1993) and BCG (Murray et al., 1997) infections, a recent study by North and colleagues reported increased IL-10 expression is not responsible for the failure of Th1-type response to resolve airborne infection with virulent *M. tuberculosis* in mice (Jung et al., 2003). Therefore, the idea of treating mice with a mixed Th1/Th2 response following BCG infection, using anti-IL-10 or anti-IL-10R monoclonal antibody would be interesting in view of these conflicting reports. Anti-IL-4R therapy is also worth investigating, as BALB/c mice deficient in IL-4R are more effective in controlling leishmaniasis than mice that lack the IL-4 cytokine (Noben-Trauth et al., 1999). In addition, healthy contacts in tuberculosis endemic areas are found to express high levels of type one cytokines and a considerable amount of the IL-4 antagonist, IL-4δ2, that functionally competes with IL-4 by binding to the α chain of the IL-4R (Demissie et al., 2004). These results suggest inhibiting the function of IL-4 by blocking its receptor with anti IL-4R monoclonal antibody may more effectively down-regulate the effect of type two cytokines than directly neutralizing the cytokines with anti-cytokine monoclonal antibody. It would also be interesting to try partial depletion of CD4+ cells as this was found to be effective in modulating the immune response from mixed Th1/Th2 into a Th1 polarized response in the leishmania model (Uzonna and Bretscher, 2001).

5.5. Modeling in Mice the Effect of Environmental Mycobacteria on BCG Vaccination

5.5.1. Introduction

Reasons for the low efficacy of BCG vaccination and/or variation in its efficacy between different vaccination trials have been debated for a long time. One compelling explanation is the interference or masking of a BCG specific immune response due to prior exposure to environmental mycobacteria. Such interference might explain the reduced efficacy of BCG vaccination in tropical countries, such as India and Africa, where exposure to environmental mycobacteria is more common when compared to temperate regions, for example England, where efficacy rates as high as 80% were reported (Fine, 1995).

The effect of environmental mycobacteria may well be of a different nature in different people exposed to different species of environmental mycobacteria. Reports from decades ago (Palmer and Long, 1966; Edwards et al., 1982) indicate exposure to environmental mycobacteria can induce partial protection against tuberculosis infection. Therefore, the observed lower efficacy of vaccination due to prior exposure to environmental mycobacteria could be due to the induction of a protected state by cross-reacting antigens prior to BCG vaccination. The potential effect of BCG vaccination in naïve individuals would therefore be masked, which would hinder differentiation of the response when comparing vaccinated and placebo groups, as both groups will have a similar state of protection (Edwards et al., 1982). Immune responses due to

environmental mycobacteria may also interfere with the ability of BCG to generate a protected state. Prior exposure to environmental mycobacteria can induce an immune response which may prematurely eliminate the BCG vaccine from the host before it induces a sustainable immune response against *M. tuberculosis* (Andersen and Doherty, 2005). Experimental observations support this hypothesis in the sense that environmental mycobacteria, cross-reactive with BCG, induced a transient immune response too weak to protect against infection by *M. tuberculosis*, but strong enough to block replication of the BCG vaccine and hence abrogating its capacity to induce protective immunity against tuberculosis infection (Brandt et al., 2002). On the other hand, depending on the timing or dose of exposure, oral exposure of mice to *M. vaccae* was found to either enhance, mask, or interfere with a subsequent immune response generated following BCG vaccination (Brown et al., 1985).

The aim of our present study is to see if exposure of mice to *M. gordonea* (saprophytic mycobacterium frequently isolated from tap water), in a way that can induce a mixed Th1/Th2 response, will abrogate the generation of Th1 responses and Th1 imprints normally generated upon low dose BCG vaccination. We anticipated this prior mycobacterium exposure would interfere with the efficacy of BCG vaccination. We will also examine if early vaccination of mice, before exposure to environmental mycobacteria, can avoid this potential interference. These experiments test critical aspects of the neonatal low dose BCG vaccination strategy against tuberculosis.

5.5.2. Results

5.5.2.1. Determining the Dose of *M. gordonea* that Induces a Mixed Th1/Th2 Response, as Measured Against BCG Antigens, in BALB/c Mice

As we had neonatally vaccinated and age matched control BALB/c mice at our disposal, we exposed these groups to 10^6 cfu *M. gordonea* bacilli per ml of drinking water for 4 weeks. We chose the oral route of exposure to mimic the natural route of infection with this environmental mycobacterium (commonly called tap water bacilli). We changed the water every week based on preliminary results indicating that the bacteria could survive in water at room temperature for at least 15 days. To roughly estimate the bacterial dose taken by each mouse, water consumption data was collected for four weeks and found to be between 3 and 4 ml per mouse/day. We, therefore, estimate each mouse was getting ~$3x10^6$ cfu every day, which we considered might be a dose sufficient to seroconvert naïve mice and induce a mixed Th1/Th2 response. However, 12 weeks after the last challenge, no mice from the unvaccinated or low dose vaccinated group serconverted as assessed by ELISA. All mice had mounted a Th1-type response as determined in the ELISPOT assay (data not presented). We therefore chose another route of challenge to determine the dose of *M. gordonea* that could induce a mixed type of response in naïve mice. To this end, we injected mice (5-7 mice/group) subcutaneously at the base of the tail with different doses (10^5 to 10^9 cfu) of live *M. gordonea.* Serum antibody levels were assessed in ELISA at ~14 weeks post-infection. The lower doses used (10^5 and 10^6 cfu) induced only IgG_{2a} antibodies, while all higher doses induced both IgG_1 and IgG_{2a}

isotype antibodies with IgG_1/IgG_{2a} ratio of <1 for 10^7 cfu, ~1 for 10^8 cfu and >1 for 10^9 cfu. Similarly, the Th1/Th2 nature of the response as assessed by ELISPOT assay revealed that doses $\leq 10^7$ cfu induced predominantly Th1 responses, while doses $\geq 10^8$ induced mixed Th1/Th2 responses with an increasing bias towards Th2 as the doses increased (Table 9). No significant difference in the number of IFN-γ-producing spleen cells was found between mice belonging to the different groups. However, the number of IL-4-producing cells was significantly higher for mice immunized with doses $\geq 10^8$ cfu than for mice immunized with lower doses. Therefore, based on these results, a dose $\geq 10^8$ cfu of *M. gordonea* was chosen as the dose that would induce a mixed Th1/Th2 response for the subsequent experiments.

Table 9. Determination of a dose of *M. gordonea* that induces a mixed Th1/Th2 response in naïve mice.

Adult BALB/c mice were injected subcutaneously at the base of the tail with different doses of live *M. gordonea* and were bled and sacrificed between 12-16 weeks post-infection. BCG-specific cytokine secreting spleen cells and serum antibody titers were determined by the ELISPOT and ELISA assays, respectively. Numbers under the Group heading refer to the number, in cfu, of *M. gordonea* injected. Data shown are an average ± SD of values from 5-7 mice per group, and are from one of two replicate experiments.

	Cytokine secreting cells/ 10^6 cells					Serum antibodies titers				
	IFN-γ		IL-4		Ratio[d]	IgG_{2a}		IgG_1		Ratio[d]
Group	Mean	SD[c]	Mean	SDc		Mean	SD[c]	Mean	SD[c]	
10^5	190.3	140	28.3	24	6.8	949	607	***	NA	__
10^6	199	63	64.3	38	3.1	1166	1435	***	NA	__
10^7	226	44	71	15	3.2	975	1725	327	281	3
*10^8	195.3	26	183	27	1.1	8138	6034	4287	2733	1.9
10^9	232	43	218	28	1.1	6453	4762	13163	7741	0.5
AMC[a]	72.7	34	19	4.6	NA	461	88	109	95	NA
CNT[b]	15	NA	17	NA	0.9	***	NA	***	NA	__

[a]AMC refers to age matched control mice vaccinated with an equal volume of saline.
[b]CNT refers to naïve control mice used as source of APC in the ELISPOT assay.
[c]Standard deviation of the mean as calculated in Microsoft Excel, 2003
[d]IFN-γ to IL-4 and IgG_{2a} to IgG_1 ratio
NA applies when value is not applicable.
*** titer less than 100.
* Dose of *M. Gordonea* used for subsequent experiments

5.5.2.2. Pre-exposure to *M. gordonea* Interferes with the Type of Immune Response Generated Following BCG Vaccination

We wanted to test the hypothesis that priming by environmental mycobacterium can abrogate the ability of low dose BCG vaccination to generate a predominantly Th1 response and Th1 imprint. We also wanted to examine whether neonatal low dose BCG vaccination of mice, before exposure to environmental mycobacteria, could prevent the interference normally caused by the environmental mycobacterium. Young mice, around 4 weeks of age, were divided into seven different groups (12 mice/group) and each group was treated as shown in Table 10. Mycobacterial doses used for exposure, vaccination and challenge were based on results from earlier experiments. To differentiate between mycobacteria used for vaccination (BCG) or for exposure (*M. gordonea*) from that used for challenge, mice were challenged with BCG expressing a vector with a kanamycin resistant gene, pMV261 (Connell et al., 1993). Thus, all bacterial burden assessments were carried out on agar plates without or with 20 μg/ml kanamycin. A preliminary experiment demonstrated that mice vaccinated intravenously with 10^7 cfu BCG pMV261 still expressed the antibiotic resistant gene for as long as 12 weeks post-vaccination (data not shown). Unfortunately our attempt to grow bacteria from spleen cells of challenged mice on agar plates with kanamycin was not successful for reasons that we could not explain. In one experiment there was no growth at all in most of the plates both from vaccinated and non vaccinated mice. On another experiment, there was growth but the high variation between mice of the same group made it difficult to make comparison among groups. Therefore, bacterial burden assessment results are not presented.

Table 10. Treatment groups and plan of exposure for *M. gordonea* sensitization experiments.

Young BALB/c mice were obtained immediately after weaning (3 weeks) and divided into seven groups with 12 mice in each group. Each group was vaccinated sc with BCG and exposed sc to live *M. gordonea* as shown, then challenged intravenously with a high dose of BCG.

Age**	4 weeks	13 weeks	22 weeks	34weeks
Expos* Group	1st exposure (sc)	2nd exposure (sc)	3rd challenge (iv)	Test to be done
G1	Low (10^4cfu) BCG	High (10^8cfu) *M.gordonea*	High ($2x10^6$cfu) BCG	**Bleed**= for Ab test **Sacrifice**=to assess response & burden
G2	Low (10^4cfu) BCG	None	High ($2x10^6$cfu) BCG	„ „
G3	None	High (10^8cfu) *M.gordonea*	High ($2x10^6$cfu) BCG	„ „
G4	High (10^8cfu) *M.gordonea*	Low (10^4cfu) BCG	High ($2x10^6$cfu) BCG	„ „
G5	None	Low (10^4cfu) BCG	High ($2x10^6$cfu) BCG	„ „
G6	None	None	High ($2x10^6$cfu) BCG	„ „
G7	None	None	None	„ „

*Stands for exposure or/and vaccination of mice with *M. gordonea* or/and BCG
**Refers to age of mice at each treatment/exposure

The observations presented on Figures 32A and B show that early exposure of mice to cross-reacting environmental mycobacteria, *M. gordonea*, interferes with low dose BCG vaccination and can alter the nature of the immune response generated following low dose BCG vaccination, compare Groups 4 and 5. Furthermore, vaccinating mice early in life, before they are exposed to environmental mycobacteria, can abrogate the negative impact of *M. gordonea* on the type of immune response generated following low dose BCG vaccination, see Groups 1 and 2. When challenged with high dose of BCG intravenously, Group 1, which had been exposed to a high dose of *M. gordonea* following low dose BCG vaccination, showed a similar response to Group 2, which received only the low dose BCG vaccine. This indicates that the Th1 imprint generated following low dose BCG vaccination was not altered by exposure to a high dose of environmental mycobacteria. In contrast, mice in Group 4, which were vaccinated with a low dose of BCG following exposure to a high dose of *M. gordonea*, generated a mixed Th1/Th2 response, indistinguishable from the responses of mice in Group 3, which received only a high dose of *M. gordonea*. This again suggests that pre-exposure to an environmental mycobacteria can abrogate the generation of the desired type of response achieved in naïve mice upon low dose BCG vaccination. Results from two different experiments performed 4 and 6 months after challenge with BCG expressing a vector with kanamycine resistant gene (pMV261) are presented to show the consistent nature of our observations. Statistical analysis of cytokine secreting cells revealed that the frequency of BCG-specific IFN-γ-secreting cells was similar in all groups. In contrast, the IL-4 levels differ significantly, which altered the IFN-γ/IL-4 ratio among groups. Table 11 summarizes the results of a statistical analysis demonstrating no difference

between groups 1, 2 and 5, as well as, between groups 3, 4 and 6. However the difference in the level of IL-4 and hence the IFN-γ/IL-4 ratio was significant ($p<0.05$) between these groups. An independent study carried out a few months later gave identical results to those obtained from the first study (Figure 33A and B), further strengthening evidence that pre-exposure to environmental mycobacterium can potentially interfere with low dose BCG vaccination. This in turn suggests the importance of vaccinating early in life with a low dose of BCG in order to avoid possible interference with Th1 imprinting that may result from pre-exposure to environmental mycobacteria.

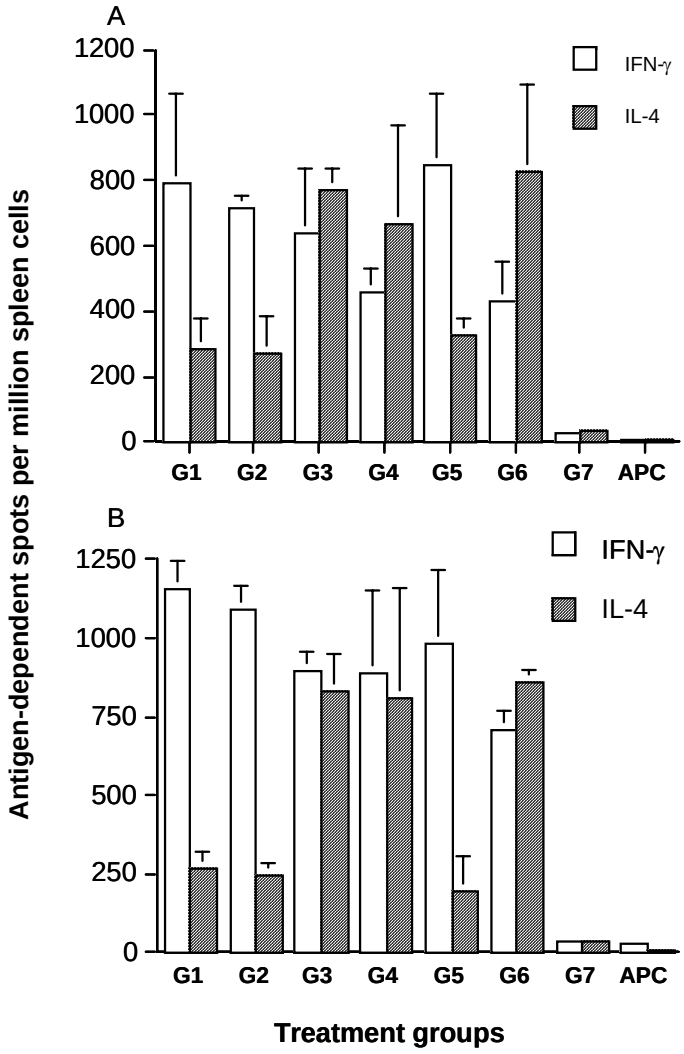

Figure 32. Pre-exposure of mice to environmental mycobacteria, *M. gordonea,* alters the immune response to low dose BCG vaccination in mice.

Young BALB/c mice, 3-4 weeks of age, were divided into seven different groups of 12 mice each and treated as described in Table 10. At around 6 months of age, all mice in groups 1-6 were challenged with a high dose of BCG expressing kanamycin resistant gene intravenously. Results show BCG-specific IFN-γ and IL-4-secreting cells in the spleen 16 weeks (A) and 24 weeks (B) after challenge with the high dose of BCG261. Labels in abscissa represent treatment groups as shown on Table 10. APC (antigen presenting cells) stands for spleen cells from a naïve mouse. Data are average of values from 4 mice per group with error bars (SD) and represents one of two replicate experiments conducted at each time point.

Table 11. Statistical analysis of the results shown in Figure 32.

Young BALB/c mice treated as described in the legend for Table 10 were sacrificed for ELISPOT assay to determine the levels of BCG-specific cytokines produced. Levels of BCG-specific IL-4 and IFN-γ as well as ratio of both cytokines were compared statistically as described on the Materials and Methods section. Numbers refer to BCG-specific spots per million spleen cells.

		IFN-γ		IL-4		IFN-γ/IL-4	
Group	N**	mean	t – group*	mean	t – group*	mean	t – group*
G1	4	797	A	286.8	A	3.08	A
G2	4	716.8	A	270.8	A	3.18	A
G3	4	644.5	AB	771.3	B	0.88	B
G4	4	459	B	668.5	B	0.8	B
G5	4	851.3	A	327.8	A	2.7	A
G6	4	434.3	B	828.8	B	0.55	B
AMC	4	31.5	NA	35.5	NA	1.5	NA

* t-group with the same letter indicates no significant difference in values of the indicated cytokines between the groups, while different letters indicate significant difference ($p<0.05$) between groups. t-group with more than one letter indicates overlap.
** Number of mice per group
NA= not applicable

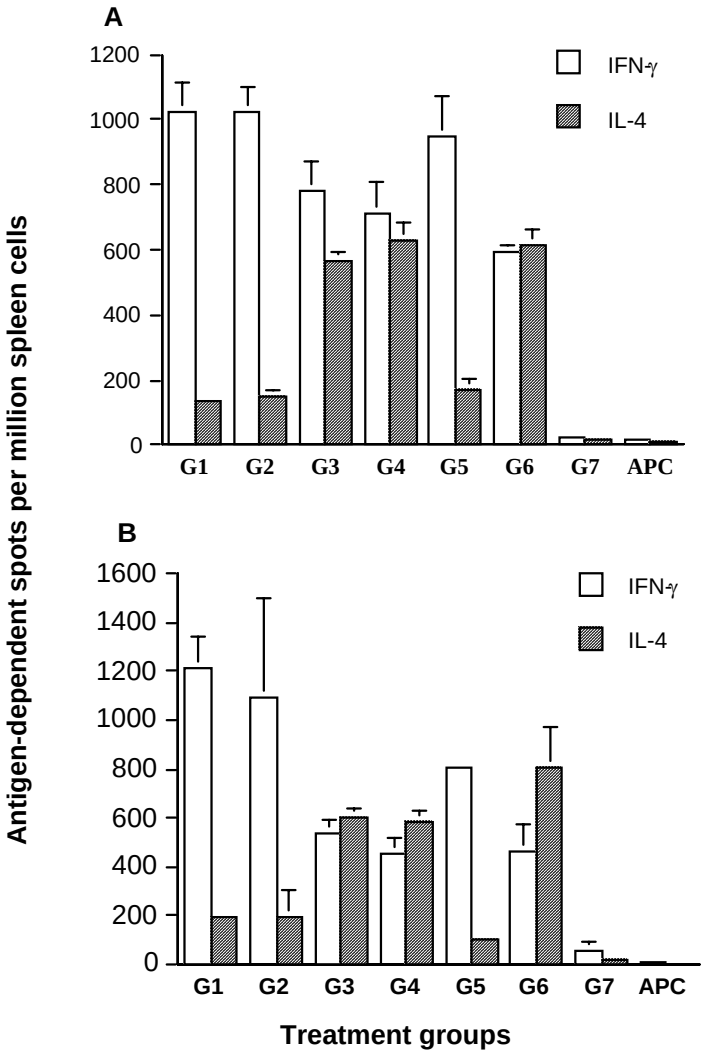

Figure 33. Exposure of mice to environmental mycobacteria, *M. gordonea*, alters the immune response to low dose BCG vaccination in mice.

Young BALB/c mice, 3-4 weeks of age, were divided into seven different groups of 12 mice and treated as described in Table 10. At around 6 months of age all mice in groups 1-6 were challenged with a high dose of BCG expressing kanamycin resistant gene intravenously. Results show BCG-specific IFN-γ and IL-4-secreting cells in the spleen 20 weeks (A) and 30 weeks (B) after challenge with the high dose of BCG261. Labels in abscissa represent treatment groups as shown on Table 10. APC stands for spleen cells from a naïve mouse. Data presented are average of values from 4 mice in each group with error bars (SD) and represent one of two replicate experiments at each time point.

5.5.3. Discussion

The impact of exposure to environmental mycobacterium on the host response to BCG vaccine remains controversial. The fact that BCG can give up to 80% protection in places with few or no environmental mycobacteria (Hart and Sutherland, 1977) and little or no protection in tropical countries where there is a plethora of environmental mycobacteria (Baily, 1980), suggests prior exposure to environmental mycobacteria may alter the protective efficacy of BCG vaccination.

As discussed in the Introduction, neonates can be exposed to environmental mycobacteria within a few weeks after birth. Depending on the type of environmental mycobacterium present, their frequency in the environment, as well as the level of exposure of the host to one or a mixture of mycobacteria, newborns may generate either a Th1-type response or a mixed Th1/Th2 response, and BCG vaccination can enhance each of these responses (Rook, 1981; Standford, 1981). Therefore, BCG vaccination can either increase the Th1-type response and hence enhance protection, or increase the non-protective mixed Th1/Th2 response, leading to interference and failure of protection. Rook and Stanford claim different species of environmental mycobacteria have an inherent capacity to induce either one of these two types of responses. Furthermore, they suggested that pre-exposure to a given species that can induce one type of response can inhibit the subsequent induction of the other response by another species of mycobacteria (Rook, 1981; Standford, 1981). On the other hand, the same species of environmental mycobacterium that Rook and Stanford used was reported to induce either of these two

types of responses depending on the dose of exposure, suggesting that the dose is rather important in determining the type of response generated following exposure to any cross-reacting environmental mycobacteria. High doses of environmental mycobacterium induce a mixed Th1/Th2 response while lower doses of the same species induce Th1-type response (Hernandez-Pando et al., 1997). In our studies, prior injection of mice with high dose of *M. gordonea* induces a mixed Th1/Th2 response and abrogated the generation of a predominantly Th1-type response following low dose BCG vaccination, which induced exclusive cell-mediated response in naïve mice. A similar finding was reported from cattle vaccination studies in which pre-exposure of calves to environmental mycobacterium reduced the protective efficacy of a subsequent BCG vaccine (Buddle et al., 2002). Furthermore, our observation shows the generation of the *M. gordonea* specific mixed Th1/Th2 response can be inhibited by early vaccination of mice with low doses of BCG, which induced a Th1-type response. This finding is also in agreement with a previous report in which the non-protective response, usually induced after infections of mice with *M. kansai*, was completely inhibited as a result of pre-exposure of mice to a cross-reacting bacteria that induces the protective type of response (Rook, 1981). These findings support our hypothesis that neonatal vaccination of humans with appropriate doses of BCG, before exposure to cross-reacting pathogens, can induce a protective immune response against tuberculosis infections. These observations lend a support to our strategy of low dose BCG vaccination of newborns in an attempt to generate Th1 imprint before significant and detrimental exposure to environmental mycobacteria. The Th1 imprinting following low dose vaccination in our first study on environmental mycobacteria, see Figure 32, seems to be rather weak when compared to our previous

findings both in newborn and adult mice. This could be due to the very high dose of BCG used for challenge, which was determined to be 10-fold higher than intended. When corrected in our second study, Th1 responses and imprints were similar to our previous findings. Overall, our results show pre-exposure of mice to a high dose of environmental mycobacteria abrogates the efficacy of the low dose BCG vaccination strategy to induce a Th1 imprint. Moreover, we confirmed here that early vaccination of mice before exposure to environmental mycobacteria may circumvent the deleterious effect of exposure to environmental mycobacteria

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6. General discussion

6.1. Dose of Antigen Determines the Th1/ Th2 Nature of the Immune Response Generated Following BCG Vaccination

The studies described here test in both adult and newborn mice two propositions central to a vaccination strategy against tuberculosis. They support the hypothesis that BCG dose is crucial in determining the Th1/Th2 nature of the immune response, with relatively lower doses favoring an exclusive cell-mediated, Th1 response, independently of the route of antigen administration or strain of mice used. They also support the hypothesis that the generation of a relatively exclusive cell-mediated, Th1, response results in the generation of a Th1 imprint, such that the immune response to a subsequent BCG iv challenge remained biased towards a cell-mediated, Th1-type response; administration of higher doses of BCG did not produce such an imprint. This inclination of the immune response to the cell-mediated, Th1 pole, was also associated with more efficient clearance of mycobacteria from the spleen following a high dose BCG iv challenge. In addition, our observations from vaccination of newborn BALB/c mice with low dose BCG show the immune response in newborn mice is not inherently biased towards a Th2 response; newborn mice can produce a predominantly Th1 response if an appropriate vaccination protocol is applied. Our observations support the general conclusion of an earlier study in which neonatal mice produced cell-mediated and antibody responses, respectively, upon infection with low and higher doses of a retrovirus (Sarzotti et al., 1996). Similar observations were also reported from human studies in which BCG vaccination of neonates induced a Th1 response against tuberculosis of similar magnitude seen in adults

(Marchant et al., 1999; Vekemans et al., 2001; Davids et al., 2006). In line with this, we found that robust Th1 responses and most efficient Th1 imprinting can be achieved in newborn mice by administering lower doses of BCG (33, 330 and 3300 cfu). We also found that relatively low doses always induce a Th1-type response in all strains of adult mice used independently of the route of administration. In all cases, the lowest doses of BCG vaccine used were as efficient as the other doses in the low dose category, both in inducing a Th1-type response and generating Th1 imprints, as well as ensuring clearance of BCG from the spleen following high dose iv BCG challenge. Even though a broad range of doses within the low dose category show similar efficacy, we strongly argue for the use of the lowest possible dose in a genetically heterogeneous population, as the higher doses in this category may induce Th2 biased response in some individuals, and fail to protect the host against natural infections with virulent *M. tuberculosis*. Therefore, our findings lend a support to the proposition that an ultra-low dose vaccination strategy to achieve Th1 imprinting in a genetically diverse population is realizable (Bretscher et al., 2001). In addition to our findings, a recent report from studies in guinea pigs supports our ultra-low dose vaccination strategy (Horwitz et al., 2006b).

Our observations naturally lead to the question of why the dose of BCG determines the Th1/Th2 phenotype of the ensuing response. This dose dependence has been observed with many antigens administered to different species by different routes. How this dose-dependence might be accounted for in terms of the requirements for inducing the generation of Th1 and Th2 cells has been discussed in detail in this thesis (see section 2.9). However, there are two considerations that make the relevance of our studies to

achieving efficacious vaccination in mice against *M. tuberculosis* uncertain. The first question is whether intradermal and/or subcutaneous vaccination can protect against infection with *M. tuberculosis* that usually occurs through inhalation of the pathogen, the usual route of infection in man. The second point worthy of consideration is whether the "protection" achieved against BCG is also applicable to *M. tuberculosis*. Observations suggest, for example, that mycobacterium specific CD8+ T-cells play some role in protection against the virulent *M. tuberculosis* but not in the clearance of BCG (Flynn et al., 1992; Xing et al., 1998; Mogues et al., 2001). This could be due to the presence of some virulence factors in *M. tuberculosis* that can lyse and form pores on the phagolysosome membrane thereby facilitating the release of mycobacterial proteins into the cytosol or allowing the escape of the pathogen into the cytoplasm. This in turn facilitates the processing and presentation of antigens via the MHC class I pathway and activates CD8+ T-cells (Mazzaccaro et al., 1996). These virulence factors are the immunodominant antigens expressed by virulent strains of *M. tuberculosis* and *M. bovis*, but the genes that encode these proteins are deleted in BCG. Therefore, the potential efficacy of BCG vaccination against tuberculosis might be compromised. However, immunization with the immunodominant protein antigens or a DNA vaccine that encodes these proteins did not generate any protection which was superior to BCG (Britton and Palendira, 2003). Nonetheless, reports still claim the efficacy of BCG vaccination can be improved by expressing genes that encode the virulence factors, ESAT-6 and CFP-10 proteins, in BCG (Pym et al., 2003). Both of these questions can be decisively addressed by determining whether low dose BCG vaccination given via the systemic route will protect mice against an intrapulmonary challenge with virulent strains of *M. tuberculosis*.

However, the very high degree of protection provided to humans by intradermal BCG vaccination, as seen in some trials, suggests that the issues surrounding these two kinds of considerations do not provide over-riding impediments to the achievement of effective BCG vaccination against tuberculosis.

Another important consideration is the duration of immunity induced following neonatal BCG vaccination. Previous studies indicate immunity against tuberculosis, generated following BCG vaccination, is on average not more than 15 years (Sterne et al., 1998). This may suggest that our neonatal low dose BCG vaccination strategy, if found to be effective against *M. tuberculosis* infection, may lose its efficacy in humans during adolescence and thus may not provide protection against adult pulmonary tuberculosis. One way of overcoming this problem would be a heterologous prime-boost vaccination strategy whereby neonates receive a low dose BCG pre-exposure vaccine and then are boosted as adults with another vaccine that contain antigens predominantly expressed in virulent strains of mycobacteria. Viral based recombinant live vaccines are good candidates for this approach. One such vaccine – the MVA-85A, which expresses the strong immunogenic antigen (Ag85A) from the virulent *M. tuberculosis* strain in a replication deficient vaccinia virus Ankara – is found to be effective as a booster vaccine against tuberculosis in BCG vaccinated animal models (Goonetilleke et al., 2003) and is also safe to use in man (McShane et al., 2004). Adenoviruses, with a natural tropism to the respiratory tract and good Th1 adjuvant properties, are also good candidates for the development of recombinant vaccines for a prime-boost vaccination strategy (Wang et al., 2004). Despite promising results from a prime-boost vaccination protocol using

rBCG, boosting the cell-mediated response generated following vaccination with a second dose of the same BCG strain is controversial. While results from human studies show no increase in level of protection (Fine, 1995), studies in calves indicate reduced levels of protection (Buddle et al., 2003). In contrast, BCG vaccination studies in deer show two subsequent low dose vaccinations given at an interval of 8 weeks induce a better Th1 response and better protection against infection with *M. bovis* than a single low dose BCG vaccination (Griffin et al., 1999). Due to these conflicting reports, a prime-boost vaccination protocol using the same strains of BCG is not recommended.

The correlates of a protective immune response against *M. tuberculosis* are disputed, particularly in conditions where both sick and healthy individuals display mycobacterial-specific DTH reactivity, proposed to be protective, as assessed by a positive PPD-skin test. Sometimes people with active tuberculosis can express stronger skin sensitivity than infected healthy contacts. Such findings have generated the suggestion that this immune state does not correlate with the expression of protective immunity (Comstock, 1988). However, recent observations in ill and healthy individuals who are both skin test positive indicate both groups have similar numbers of mycobacterial-specific cells able to produce IFN-γ upon appropriate stimulation in their peripheral blood lymphocyte (PBL) cells, but the PBL of tuberculosis patients appear to have a higher number of mycobacterial-specific IL-4-producing cells (Surcel et al., 1994; van Crevel et al., 2000). Similar observations from other tuberculosis patients and healthy contacts (controls) support the view that a cell-mediated, Th1-type response is protective, since all the healthy controls show high tuberculin reactivity, an increased proliferation index, and

higher levels of IL-2 and IFN-γ secretion (Sanchez et al., 1994). In contrast, tuberculosis patients exhibited low reactivity to tuberculin and had high percentage of IL-4 and IL-10 producing cells in the peripheral blood associated with higher titers of IgG antibodies in their sera (Baliko et al., 1998). This suggests that the immune response of tuberculosis patients is biased towards Th2, which may contribute to disease progression by counteracting the effect of Th1 cells. The possible mechanism by which Th2 cells can be so detrimental to the health of the individual during chronic intracellular infections can be explained by the fact that pathogens like *M. tuberculosis* and *L. major* are obligate intracellular parasites that productively infect macrophages. Th1 cells (CD4+) as well as mycobacterial-specific CD8+ T cells, which produce IFN-γ and other Th1-associated cytokines, are responsible for activating macrophages in a variety of ways to become bacteriocidal or to limit mycobacterial replication (Kaufmann, 1988; Bloom et al., 1994; Scanga et al., 2001). The delivery of cytokines such as IL-4 and IL-10 by Th2 cells and possibly regulatory T-cells may counteract the activation signals delivered to the macrophage (Lehn et al., 1989; Hernandez-Pando et al., 1996; Redpath et al., 2001). However, recent reports suggest Th2 cells or their cytokines may not be responsible for the failure of immunity in tuberculosis infected mice (Jung et al., 2002; Jung et al., 2003). Therefore, the above presumption, however plausible, will only become convincing when it is demonstrated that ensuring a greater Th1-like component of the anti-mycobacterial immune response, at the expense of the Th2 component, prevents tuberculosis or results in a cure of this disease. We believe the experimental system developed here for low dose vaccination should be useful in testing this possibility in the mouse model.

A recent study attempted to explore whether secondary anti-mycobacterial Th1 responses were more effective than primary Th1 responses in protecting mice against a lethal challenge of *M. tuberculosis* (Jung et al., 2005). This study involved infecting mice with a normally lethal dose of *M. tuberculosis,* followed by antibiotic treatment from day 30 to day 100 post-infection, at which time mycobacteria could no longer be detected in the spleen. These primed as well as control mice were then given a lethal challenge (200 cfu) of virulent *M. tuberculosis* via the airborne route. The priming had a small effect on the size and kinetics of the ensuing Th1 response, and led to only a 10-fold reduction in mycobacterial burden. The authors concluded that a secondary immune response to *M. tuberculosis* infection to be neither qualitatively nor quantitatively superior to the primary response. This inference, as a general conclusion, is questionable for several reasons:

(i) This pessimistic conclusion was based on results from one trial that used only one mode of vaccination (dose, route, strain of *M. tuberculosis* and strain of mice). This group of investigators did not consider that the Th1/Th2 nature of the immune response can be affected by various variables, as discussed in section 2.8.3 of this thesis.

(ii) As few as 1-3 bacilli can induce diseases in susceptible individuals. In their experiment, 100 cfu of a virulent strain of *M. tuberculosis* (H37Rv) was used as a vaccine to prime the mice. This dose, according to their previous work (North and Jung 2004), is equivalent to 10^5 *M. tuberculosis* given intravenously. This is a very high dose that may induce the undesired type of immune response, leading to chronic disease upon future challenge. Our studies on BALB/c mice using BCG show doses as low as 33 cfu can reliably induce cell-mediated response and a Th1 imprint; a dose of 10^5 cfu of BCG given iv is a critical dose above which responses with a substantial Th2 component are

generated, likely rendering the mice susceptible to a subsequent challenge with virulent *M. tuberculosis*. Besides, considering the high in vivo replication rate of virulent strains of *M. tuberculosis* as compared to non-virulent BCG (North and Izzo, 1993), the 100 cfu (equivalent to 10^5 cfu given i.v.) of *M. tuberculosis* used to vaccinate the mice will be several hundred folds higher at week 3 post-vaccination when the adaptive immune response starts to become apparent. This extremely high dose may not generate predominantly cell-mediated responses. Importantly, the authors did not look at the type of response generated after vaccination. If the immune response generated after the primary challenge (vaccination) was a mixed Th1/Th2 or predominantly Th2 response, the secondary response would not be expected to be any more protective than the primary response in a naive mouse upon high dose challenge. According to our results from BCG vaccination, we might even expect the secondary response to be worse than the response in the naïve mice, at least as far as a protective Th1 dominated response is concerned.

(iii) As stated in point (ii) above, only a few bacilli of virulent *M. tuberculosis* are required to induce disease. The dose the authors used to challenge the mice (200 cfu) is arguably so high that any beneficial response induced by the vaccine (primary exposure) would have been minimal. In our studies, we observed that low dose BCG vaccination can induce predominantly cell-mediated response and hence Th1 imprint as assessed by a challenge of $2x10^6$ cfu iv; however, this Th1 imprint was broken with a massive challenge of 10^8 cfu iv. Thus, Th1-imprints are not absolute and their stability depends upon the nature of the challenge.

(iv) The authors looked at the generation of Th1 cells and their production of cytokines both in the vaccinated and non-vaccinated mice after a virulent challenge, and reported a relatively rapid and heighten Th1 type response in the vaccinated group. This response was associated with a reduced (~10-fold) bacterial burden in the lung, and was a good indication of an effect of vaccination, even though not to the extent of their expectation. However, they did not consider the role of Th2 cells and their cytokines, which are very important, since cytokines from Th2 cells, as previously discussed, are reported to have a down-regulatory function on the effect of Th1 cells. While they might have dismissed the influence of Th2 cells and their cytokines based on their previous reports which conclude no negative influence of Th2 cells (Jung et al., 2002) or their cytokines (Jung et al., 2003) on the protective ability of Th1 cells against tuberculosis, unequivocally accepting this line of reasoning is difficult for various reasons. First, several reports both from mouse and human studies indicate the role of Th2 cytokines on the severity of tuberculosis infection (Hernandez-Pando et al., 1996; Baliko et al., 1998; Seah et al., 2000; van Crevel et al., 2000). Second, the study was carried out employing Th2 cell/cytokine gene knock out mice with a genetic background the authors characterized as an *M. tuberculosis* resistant phenotype. Deleting Th2 cells or their cytokines in resistant strains of mice may not further increase their resistance to infection with *M. tuberculosis,* but may affect the immune response to *M. tuberculosis* in susceptible mice. This deletion can considerably increase the generation of Th1 cells and hence increase the resistance of susceptible mice, provided that deletion of these genes does not have any other unwanted effect on the mouse. Ample evidence in the literature indicates that partial depletion of CD4+ cells or neutralization of their cytokines and/or blocking their cytokine receptors by treating with

appropriate monoclonal antibodies in the leishmania system can render susceptible mice resistant (see 5.4.1). Thus, the 10-fold decrease in bacterial burden demonstrated in their study and reports from human and cattle studies on the efficacy of BCG vaccination are reasons to be optimistic that vaccination against tuberculosis under optimal conditions can generate a protective response.

In summary, type 1 cytokines, such as IFN-γ secreted mainly by CD4+ Th1 cells, are protective against mycobacterial infection, whereas type 2 cytokines, mainly IL-4, IL-10, secreted by Th2 cells, can dampen the anti-mycobacterial response. Our results and several other reports indicate that low doses and high doses of antigen can induce the generation of CD4+ T-cells producing these two groups of cytokines, respectively. In our studies, doses around the transition number (t_n) were found to induce a mixed Th1/Th2 response with an increasing bias towards the Th1 pole as the dose is lowered in all mouse strains tested. In some of our experiments mixed Th1/Th2 response with a bias towards the Th1 pole, generated following vaccination with BCG numbers close to the t_n, was found to be more efficient than the Th2 biased response seen in naïve mice or mice vaccinated with doses much higher than the t_n in clearing the bacteria from the spleen following high dose BCG iv challenge. This may seem to contradict our hypothesis that cytokines from Th2 cells may dampen the protective effect of Th1 cells. We argue against this for two possible reasons. Firstly, the mixed Th1/Th2 response observed in most of our experiments was biased towards Th1 pole with more IFN-γ than IL-4-secreting cells in the spleen as well as more IgG_{2a} than IgG_1 antibodies in the serum. This might have contributed to the efficient clearance of BCG from the spleen, which was not

observed in mice with more Th2 biased response. Secondly, a mixed Th1/Th2 response may be better than predominantly Th2 response in controlling the replication of the non-virulent BCG. However, this might not be true in controlling the virulent *M. tuberculosis*, and thus the protection against BCG challenge observed in mice with mixed Th1/Th2 primary response may not work against challenges with virulent strains of mycobacteria.

6.2. Source of Cytokines during BCG Infection

Our results clearly show that the majority of the cytokines produced by BCG specific cells during BCG infection are secreted by Thy1.2+CD4+ cells. Our findings are in agreement with previous reports both from human and animal studies (section 2.6.2.1) that show depletion of CD4+ cells or their cytokines ultimately increases susceptibility to tuberculosis or increases the chances of reactivation disease from latent infection. Spleen cells depleted of Thy1.2+ or CD4+ cells secreted very little IFN-γ but still produce ~30% of the total IL-4 detected in ELISPOT assays (see Figure 30; Table 7). This could be due to the presence of cells that express Th1.2 (7.3%) and CD4 (10%) markers at very low levels even after depletion of cells expressing these markers (Figure 29). Therefore, cells that express low levels of Th1.2 and CD4 markers after depletion might be responsible for the presence of few IL-4 spots in Thy1.2 and CD4 depleted spleen cells. Furthermore, there is evidence in the literature that cells other than T-cells can also produce IL-4. For example, mast cells are capable of interacting with mycobacteria, whose secreted antigens can directly bind to CD48 (a mannose rich, GPI-anchored protein) on the lipid raft of the mast cells (Munoz et al., 2003). As a result of this interaction, mast cells respond by releasing the pre-stored mediators and de novo synthesized cytokines. This

may suggest mast cells are capable of secreting mycobacterial specific IL-4 that may play a role in the activation of BCG specific Th2 responses. However, as IL-4 is produced only in high dose BCG vaccinated mice in all our experiments, and since it could not be detected in the low dose vaccinated group or in normal mice, the possibility that IL-4 could have been produced by mast cells in response to BCG antigen stimulation is remote. On the other hand, it is also possible that high dose of BCG induces the production of very low level of BCG-specific IgE antibody, which in turn induces mast cell de-granulation and release of IL-4 from mast cells contributing to the presence of IL-4-secreting cells in the Th1.2 and CD4 depleted population.

The mechanism by which Th1 cells constitute protective immunity against tuberculosis infection is believed to be related to their ability to secrete high level of IFN-γ, which in turn activates macrophages to secrete reactive oxygen and nitrogen intermediates that destroy or inhibit multiplication of mycobacteria in the animal body. Reactive nitrogen intermediates (RNI) - nitrite (NO_2^-) and nitrate (NO_3^-) - are the most potent anti-microbial compounds. Measuring the level of total nitric oxide (NO) produced upon stimulation with BCG antigens of spleen cells obtained from mice vaccinated with high and low dose of BCG could be informative, as low dose BCG primed cells might produce more NO than cells from high dose primed mice. To this end, mouse peritoneal macrophages were cultured with nylon wool purified T-cells from low and high dose BCG vaccinated mice and the level of NO measured using the Griess reaction as described previously (Stuehr and Marletta, 1987; Ding et al., 1988). Despite repeated experiments, our findings were not conclusive and thus not included in this thesis.

6.3. Environmental Mycobacterium can interfere with the Protective Efficacy of BCG Vaccination

BCG vaccination in humans is usually given neonatally within a few days of birth. A logical reason for this could be to minimize the effect of priming by environmental mycobacteria, which might prevent Th1 imprinting by causing a mixed Th1/Th2 or Th2 imprint. There are reports in the literature claiming that neonates can be easily primed by environmental mycobacteria in the first few weeks of their life and that this pre-sensitization can induce an immune response, which either masks or interferes with the efficacy of BCG vaccination (Palmer and Long, 1966; Edwards et al., 1982). Evidence supporting this explanation has been recently reported in human studies, in which BCG vaccination in PPD positive adults resulted in a very low BCG specific IFN-γ production (Black et al., 2001). Similar results were also reported from cattle, where calves which were PPD test positive before vaccination with BCG were found to be less protected than the PPD negative counterparts when challenged with virulent *M. bovis* (Buddle et al., 2002). Our findings that mice pre-sensitized with a high dose of cross-reacting environmental bacilli, *M. gordonea*, were resistant to Th1 imprinting by low dose BCG vaccination as assessed by a subsequent high dose BCG challenge, support these prior observations. The exact mechanism of interference is not well understood, but it could be due to either: (i) inappropriate bias of the immune response as a result of priming by a high dose of the environmental mycobacterium, leading to the generation of Th2 or mixed Th1/Th2 type response upon BCG vaccination (Hernandez-Pando et al., 1997); or (ii) cross-reactive immune responses to BCG generated after exposure to the

environmental mycobacteria, which interferes with the replication of the BCG vaccine. Rapid clearance of BCG due to this cross-reacting immune response could inhibit the generation of protective immune response and memory T-cells against a subsequent infection with *M. tuberculosis* (Brandt et al., 2002). Therefore, in both cases, vaccination may not have any beneficial effect. The generation of the non-protective type response following exposure to environmental mycobacteria can inhibit the generation of protective immunity upon BCG vaccination by swaying the response to BCG into a Th2 or mixed Th1/Th2 mode, and the protective/Th1-type response can be too weak to prevent infection by a virulent strain of *M. tuberculosis* yet strong enough to block replication of the BCG vaccine before it induces any sort of immune response. Our results make it plausible that neonatal imprinting can minimize undesirable Th2 imprinting by environmental mycobacteria. It is, therefore, imperative to consider the age at which vaccination is carried out, in addition to the dose of the antigen, in order to minimize the interfering effect of environmental mycobacteria on a potential effective strategy of vaccination. Findings in calves (Hope et al., 2005) and newborn humans (Vekemans et al., 2001) support our proposition that neonatal vaccination with BCG is able to induce a high level of IFN-γ and low level of IL-4, signifying Th1 and Th2 type responses, respectively. High efficacy of BCG vaccination (as high as 83%) in children against the most severe forms of tuberculosis, particularly TB meningitis and disseminated tuberculosis, is reported from a meta-analysis of data coming from several vaccination trials (Colditz et al., 1995). Such high efficacies may be partly explained by the fact that BCG was usually administered neonatally, before exposure of the host to environmental mycobacteria. The high efficacy of BCG vaccination against TB

meningitis and military tuberculosis could be also explained based on fact that these two forms of tuberculosis are usually associated with highly polarized Th2 type responses, and the mixed Th1/Th2 response induced by the standard dose of BCG vaccine may be effective in protecting against diseases due to Th2 response but not against pulmonary tuberculosis, which is associated with a mixed Th1/Th2 type response.

7. Conclusion

One of the success stories of medicine in the last century is the development of effective vaccines against acute bacterial and viral infections that can induce rapid and heightened immunity upon exposure of the host to natural infection. However, there is no universally efficacious vaccination strategy against chronic diseases caused by slow growing intracellular pathogens such as mycobacteria, responsible for leprosy and tuberculosis. In this thesis, I have tried to identify and address four issues/problems, which I believe are impediments in the effort to develop a universally efficacious vaccination strategy against these chronic intracellular pathogens in general and *M. tuberculosis* in particular.

First and foremost, identification of the immune correlates of protection is a pre-requisite in any effort to develop an effective vaccine against a given pathogen. Even though we could not conduct the envisaged *M. tuberculosis* challenge experiments for logistical reasons, our findings in both very young and adult mice from BCG vaccination and challenge studies have shown that protection is correlated with a predominantly cell-mediated, Th1-type, response. The second problem is how to guarantee Th1-type response upon natural infection of the host by *M. tuberculosis*? The results presented in this thesis clearly demonstrate that vaccination dose is an important factor that determines the Th1/Th2 nature of the immune response to BCG. Relatively low doses always induce Th1-type responses and Th1 imprints that can efficiently clear BCG from the spleen of mice as assessed by high dose BCG iv challenge. Thirdly, our experiments in different strains of mice illustrate that an ultra-low dose of BCG vaccine can be employed to induce a Th1-type response in all individuals of a genetically divers population,

addressing the problem of finding a standard vaccination protocol in humans and animals, which are genetically heterogeneous. Finally, we have shown that neonatal BCG vaccination can overcome the problem of immune interference to BCG vaccine due to pre-exposure of the host to environmental mycobacteria. Our hope is that the models reported here for BCG induced Th1-imprinting will allow us to examine in the future whether Th1-imprinting is effective in modulating responses against the virulent species of mycobacteria, and to determine whether this strategy of vaccination will protect mice against infections with virulent *M. tuberculosis*. Our observations on mycobacterial burden assessment strongly support the notion that BCG is best contained by a Th1 response and not by a Th2 biased mixed Th1/Th2 response or predominant Th2 responses, and that in all cases relatively lower doses of BCG vaccine generate the most effective response against a subsequent BCG challenge. Whether similar findings would have been made if the challenges were with virulent species of mycobacteria is not clear. As discussed elsewhere in this thesis, studies in cattle and deer support the suggestion that low dose but not high dose BCG vaccination can protect against tuberculosis caused by virulent *M. bovis*. These studies give reason for optimism that a low dose BCG vaccination strategy might contribute towards the global effort of preventing tuberculosis in man. Some might argue against this optimistic vision due to the genetic difference between *M. tuberculosis* and BCG that may compromise the efficacy of BCG vaccination against infections with virulent strains of mycobacteria. Two options may solve this problem: (i) developing a new attenuated vaccine from *M. tuberculosis* by deleting some of the genes responsible for its virulence, or (ii) using the immunodominant antigens or DNA that encodes these proteins as subunit vaccines. However, the usefulness and

practicality of both these options is questionable, for the following reasons: (i) Investing in the development of a vaccine by attenuating the virulent strains of *M. tuberculosis* may not be prudent as the safety in immunocopromised individuals will be questionable; and (ii) The subunit vaccines need an adjuvant safe for human use that can guarantee a Th1 type response. The only adjuvant licensed for human use is alum, which favors the generation of Th2 responses. Furthermore, the efficacies of subunit vaccines have not been found to be better than BCG, as discussed previously.

Therefore, I believe that the best option to control infection by virulent species of mycobacteria is low dose BCG vaccination. If necessary, its efficacy and durability of protection can be improved by expression of the genes missing from BCG and by the use of a prime-boost vaccination protocol, respectively. Also worth considering in the search for effective control against tuberculosis is the development of immunotherapeutic vaccines that can prevent the progression into active disease in the >2 billion people already infected with tuberculosis. The use of proteins expressed by *M. tuberculosis* strains during the chronic phase of infection as candidate antigens in the development of therapeutic (post-exposure) vaccine is a promising area of research to prevent reactivation disease in individuals with latent tuberculosis infection. One study estimates a post-exposure vaccine with 50% efficacy could reduce the global burden of tuberculosis by up to 25% in 15 years (Lietman and Blower, 2000). In summary, we recommend vaccination of neonates with low doses of BCG, as low as 10^3 cfu in humans and 10^4 cfu in cattle, to induce an exclusive cell-mediated response and a Th1 imprint that can guarantee protection against natural infection with virulent mycobacteria. Vaccination of

human neonates with low dose of BCG vaccine followed by booster immunization with recombinant post-exposure vaccine can most likely substantially reduce the burden of tuberculosis in developing countries by preventing both primary childhood tuberculosis and pulmonary tuberculosis in adults, as well as preventing reactivation tuberculosis in immuno-compromised individuals who are already latently infected with *M. tuberculosis*. Therefore, since vaccine failure could be due to the way in which the vaccine is used rather than any inherent deficiency of the vaccine per se, improving protocols for BCG administration should be further explored before replacing BCG with a new-generation vaccine.

8. References

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