DISSERTATION

NOVEL VACCINE STRATEGIES AGAINST AEROSOL INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS* IN MICE

Submitted by
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In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, CO
Summer, 2003

UMI Number: 3107102

UMI Microform 3107102

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COLORADO STATE UNIVERSITY

May 6, 2003

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JENNIFER L. TAYLOR ENTITLED NOVEL VACCINE STRATEGIES AGAINST AEROSOL INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS* IN MICE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

NOVEL VACCINE STRATEGIES AGAINST AEROSOL INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS* IN MICE

Tuberculosis, although it is an ancient disease, currently kills more people each year than does any other bacterial disease. Unfortunately, *Mycobacterium bovis* BCG, the only vaccine presently available to fight this pulmonary disease, has proven completely ineffective in certain clinical trials. The overall aim of these studies, therefore, was to investigate the use of novel vaccine strategies against tuberculosis.

Because tuberculosis is primarily a pulmonary disease, a novel vaccine was designed to target the lungs by means of a recombinant adenovirus vector expressing the major mycobacterial antigen, Ag85A, in its native form. E1-deleted adenovirus vectors expressing either Ag85A (Ad85) or the bacterial β-galactosidase (AdZ) were aerogenically administered to C57BL/6 mice. Initial results showed that both the reporter gene encoding β-galactosidase or the Ag85A gene were expressed by the viruses both *in vitro* and in the lungs. While the Ad85 vaccine was able to induce strong antibody responses against the Ag85A protein when delivered by the aerosol route, thereby suggesting that recombinant Ag85A was detected by the adaptive immune response, the vaccine was only able to generate significant protection when it was delivered intranasally to anesthetized mice. In addition, this protection was associated with the production of IFNγ in the spleen and an increase in activated CD8 T cells in the lungs.

In a second set of studies, prime-boost strategies were performed in order to determine if Ad85 was capable of boosting the immunogenicity and protective efficacy of a DNA vaccine expressing the Ag85A protein (DNA85) or that of a subunit vaccine consisting of Ag85A protein emulsified in monophosphoryl lipid A-squalene adjuvant (Ag85A/MPL-SE). Due to the failure of the Ag85A/MPL-SE subunit vaccine and the DNA85 vaccine to confer any degree of protection on their own, however, it was impossible to determine whether Ad85 was capable of boosting the protective efficacy of these two vaccines. Interestingly, data by flow analysis indicated that the Ad85 boost was able to recruit IFNγ-producing CD4 T cells to the lungs following vaccination with DNA85. Despite this cellular recruitment, however, DNA85+Ad85 vaccinated mice were not protected against challenge with *M. tuberculosis.* In contrast, BCG vaccinated mice were protected against tuberculosis, and this protection seemed to be correlated with the recruitment of protective CD4 IFNγ T cells to the lungs following BCG vaccination. Additionally, this recruitment of T cells was perhaps indicative of a protective memory response with the capability of potently and swiftly controlling bacilli growth, since after the tuberculosis challenge, the BCG-vaccinated mice had fewer IFNγ-producing CD4 T cells in the lungs compared to unvaccinated controls and the other test groups, none of which were protected against tuberculosis.

These combined results indicate that the use of Ad85 in prime-boost studies is worthwhile due to the fact that it induced IFNγ, and perhaps more importantly, it was capable of recruiting a significant number of CD4 IFNγ T cells to the lungs when it was administered as a booster vaccine to DNA85. Furthermore, these studies also demonstrate the importance of employing effective adjuvants in the design of protein subunit vaccines, since Ag85A emulsified in dimethyl dioctadecylammonium bromide (DDA) was almost as protective as BCG compared to Ag85A emulsified in MPL-SE, which was not significantly protective. In addition, the failure of the DNA85 vaccine to protect mice against aerosol challenge with *M. tuberculosis* was surprising, since this vaccine has been shown to confer significant levels of protection approaching that of BCG in BALB/c and C57BL/6 mice. The inability of DNA85 to confer protection against *M. tuberculosis* brings into question the usefulness of DNA85 as a realistic vaccine to combat tuberculosis. A successful vaccine against tuberculosis, or any disease for that matter, must provide consistent protection and for practical purposes, it should be easy to administer.

In the final series of experiments, we tested another DNA vaccine, which expresses the hsp60 protein from *M. leprae,* and which has received considerable attention in the last decade due to its ability to significantly protect mice prophylactically and therapeutically against intravenous challenge with *M. tuberculosis.* The final studies presented here, however, show that the hsp60 DNA vaccine failed to protect mice against a more realistic aerosol infection with *M. tuberculosis* in either a prophylactic or therapeutic mode. In fact, the hsp60 DNA vaccine, as well as the DNA85 vaccine, induced severe pulmonary necrosis in the lungs reminiscent of the classical Koch reaction when delivered in a therapeutic mode, especially when given to a susceptible mouse strain. Further studies were performed in gene knockout mice to determine what cell populations might be responsible for these foci of necrosis in the lungs after hsp60 DNA vaccination. Preliminary results suggest that B cells play an important part in perhaps protecting hsp60 DNA immunized mice from the pathological effects caused by an, as of

yet, undetermined source. The results from these studies suggest that although these DNA vaccines seem to be safe when given prophylactically to naïve individuals, when given to individuals who have been previously exposed to tuberculosis, there may be a risk of aggravating *M. tuberculosis*-associated pulmonary lesions or perhaps triggering reactivation of a latent tuberculosis infection.

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ACKNOWLEDGMENTS

I would like to start by thanking my advisor, Dr. Ian Orme, for providing me with the opportunity to work in his laboratory on projects that I believe were worthwhile in the big scheme of things. It was a pleasure and a privilege to work in the laboratory of this world-renowned tuberculosis expert.

A thousand thanks must go to Dr. Joanne Turner, who was an essential mentor to me during the last several years of my graduate work. Thank you for repeatedly reading through much of this dissertation, and for helping me to make it a stronger piece of work. Of course your influence did not begin at the writing stage, as you generously spent much time teaching me how to think about my experiments as well as how to perform them accurately and more efficiently.

I am extremely grateful to my fellow graduate students over the years, especially to Dr. Bridget Vesosky, Dr. Sarah Richart, Dr. Oliver Turner, and Benjamin Espinosa. Our many discussions about immunology, tuberculosis, virology, and life are some of my favorite memories from graduate school. To me, the most important difference between undergraduate and graduate schooling is that in graduate school, students learn more from each other than they do in the classroom.

I would also like to acknowledge Dr. Erica Suchman and Dr. Ralph Smith, the gifted teachers that they are. Thank you for guiding and inspiring me as I begin my teaching career.

DEDICATION

This dissertation is dedicated to my husband, David Taylor.

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LIST OF ABBREVIATIONS

ACK: NH_4Cl, $KHCO_3$
Ad85: Adenovirus expressing Ag85A protein
AdZ: Adenovirus expressing b-galactosidase
ADCC: antibody-dependent cell-mediated cytotoxicity
AFB: acid-fast bacilli
Ag85A: Antigen 85A
APC: antigen presenting cell
APC: allophycocyanin
BALT: bronchus-associated lymphoid tissue
BCG: Bacille Calmette-Guerin
CAR: coxsackie-adenovirus receptor
CD: cluster of differentiation
CFA: complete Freund's adjuvant
CFTR: cystic fibrosis transmembrane conductance regulator
CPE: cytopathic effect
CTL: cytotoxic T lymphocyte
DC: dendritic cell
DDA: Dioctadecylammonium bromide
DMEM: Dulbecco's Modified Eagle Medium
DN: double negative
DNA: deoxyribonucleic acid
DRPMI: Deficient Roswell Park Memorial Institute
DTH: delayed-type hypersensitivity
ELISA: enzyme-linked immunosorbent assay
ESAT: early secreted antigenic target
FBS: fetal bovin serum
FITC: fluorescein isothiocyanate
FPV: fowl pox virus
GKO: IFNγ knockout
GM-CSF: granulocyte-macrophage colony-stimulating factor
HA: hemagglutinin
HCMV: human cytomegalovirus
HIV: human immunodeficiency virus
HRP: horse radish peroxidase
hsp60: heat shock protein (65 kilodalton)
ICAM-1: intercellular adhesion molecules
i.m.: intramuscular
i.v.: intravenous
IFA: incomplete Freund's adjuvant
IFNγ: interferon gamma
IL: interleukin
iNOS: inducible nitric oxide synthase
IP: inducible protein

i.p.: intraperitoneal
KO: knockout
LALT: lymph-associated lymphoid tissue
LAMP-1: lysosome-associated membrane protein-1
LFA-1: lymphocyte function-associated antigen-1
LPS: lipopolysaccharide
M6PR: mannose –6-phosphate receptor
MALT: mucosal-associated lymphoid tissue
ManLAM: mannose-capped lipoarabinomannan
MCP-1: macrophage chemoattractant protein-1
MDR: multi-drug resistant
MHC: major histocompatibility complex
MIP-1: macrophage inflammatory protein
MPL-SE: Monophosphoryl lipid A squalene
NALT: nasal-associated lymphoid tissue
NK: natural killer
NO: nitric oxide
NP: nucleoprotein
NRS: normal rabbit serum
OCT: optimal cutting temperature
PCR: Polymerase Chain Reaction
PE: phycoerythrin
PerCP: peridinin chlorophyll-a protein
PPD: purified protein derivative
RD1: region of difference
ROI: reactive oxygen intermediate
RSV: respiratory syncytial virus
ST-CF: short-term culture filtrate
SV40: Simian virus 40
TCR: T cell receptor
Th1: T-helper 1
Th2: T-helper 2
TNFα: tumor necrosis factor alpha

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CHAPTER 1: LITERATURE REVIEW

M. tuberculosis: The Bacillus and Its Discovery

Evidence dated as far back as 5000 B.C. indicates that humans have been plagued by tuberculosis throughout much of history [47]. However, it was not until late into the 19th century that the causative agent of this disease was discovered by Dr. Robert Koch. Koch is also well known for working out the famous "Koch's Postulates" which still provide the essential experimental foundation for the study of any infectious disease [44]. This meticulous physician of rural Germany, who studied microbial agents such as anthrax almost as a hobby, began his first experiments on tuberculosis in August of 1881. Miraculously, less than a year later on March 24, 1882, he delivered his historic lecture on the discovery of the 'tubercle bacillus' to the Berlin Physiological Society. Friederich Loeffler, who attended the monumental lecture, said this of Koch [44]:

> Koch was by no means a dynamic lecturer who would overwhelm his audience with brilliant words. He spoke slowly and haltingly, but what he said was clear, simple, logically stated - in short, pure unadulterated gold.

Koch's lecture was received by the rest of the world with much exaltation as well, for at the time of Koch's work on the tubercle bacillus, one-seventh of the population died of tuberculosis, and the disease killed at least one-third of the productive middle aged group [163].

The organism that Koch's eyes saw for the first time under his rudimentary microscope is now classified in the Order: Actinomycetales, Family: Mycobacteriaceae, Genus: *Mycobacterium*, and Species: *tuberculosis* [1]. Different species of the genus *Mycobacterium* can be characterized by the rate at which they grow. For instance, species that require over 7 days of incubation at the optimal temperature, 37° C, to produce visible colonies are considered 'slow growers,' such as *M. tuberculosis* and *M. bovis*. Those that require less than 7 days at the optimal temperature to produce visible colonies are considered 'fast growers,' such as *M. fortuitum* and *M. smegmatis* [1].

The *M. tuberculosis* is inclined to grow in "serpentine, cordlike masses," and the off-white to yellow pigmented colonies it produces on most solid media are thick and raised with a wrinkled surface and an irregular thin margin. The bacillus that produces these colonies is a straight or slightly curved rod, which is 0.3-0.6 x 1-4 μm in size and is said to be "acid fast" [1]. The cell wall of the bacillus has a high lipid content due to a group of branched chain hydroxy lipids called mycolic acid to which the property of acid-fastness is attributed [3]. Within one year of Robert Koch's discovery of the tubercle bacillus, Franz Ziehl and Friedrich Neelsen developed the Ziehl-Neelsen method [44] in which heat and phenol are used to drive in basic fuchsin stain. After acid-fast cells have been penetrated with the fuchsin they are not easily decolorized by acid-alcohol, and therefore stay bright red in color when counterstained with methylene blue. Bacteria that are not acid-fast, on the other hand, are decolorized by the acid-alcohol wash step and thus are stained blue by the methylene blue counterstain [3]. In addition to the aforementioned characteristics, *Mycobacteria* are nonmotile, nonsporing, aerobic, and although not readily Gram stained, the bacilli are usually weak Gram Positive [2].

Diagnosis: PPD and DTH

Empty hope resounded throughout the world in 1890 when Robert Koch announced that he had found the cure for tuberculosis in a product he termed "tuberculin"

[44]. Tuberculin, which later became known as "Koch's old tuberculin," was a heat-inactivated concentrate of the constituents present in a broth with glycerol that maintained the growth of *M. tuberculosis* for a period of eight weeks [9]. Although tuberculin was unable to cure tuberculosis, Koch's discovery of this product was a major contribution used in the diagnosis of this disease.

Like many of Koch's other discoveries, tuberculin is still used today in the diagnosis of tuberculosis; however the product has been refined for use in humans and is now called purified protein derivative (PPD). The development of PPD can be attributed to Seibert and Munday, who in 1932 developed a purified version of the tuberculin using a synthetic protein-free medium to support the growth of *M. tuberculosis* [206]. In 1941, Seibert and Glenn made a large, single lot of PPD from a single strain of human *M. tuberculosis* for credit as the International Standard for Purified Protein Derivative of Mammalian Tuberculin. This product is called PPD-S, the standard by which all PPD is produced today [205].

Intradermal injection of PPD induces a classical delayed-type hypersensitivity (DTH) response in individuals vaccinated with BCG or previously exposed to *M. tuberculosis.* The presence of a DTH response can be measured by examining the site of the PPD injection 48 to 72 hours later for the development of a red, indurated lesion. In general, the development of the DTH response requires a number of steps. Upon first contact with BCG or *M. tuberculosis*, specific T helper cells are sensitized and then clonally expand, generating a pool of memory T cells. When mycobacterial antigens are introduced again, this time intradermally (i.e. PPD injection), Langerhan's cells and macrophages at the site of injection secrete chemokines and cytokines that attract sensititzed T cells to the site. The sensitized T cells express upregulated adhesion molecules such as CD44 and LFA-1 allowing them to migrate from the vasculature into the site of injection [91]. In concert with the antigen presenting cells, the sensitized T cells secrete numerous inflammatory cytokines and chemokines that attract neutrophils, T cells, and large amounts of monocytes/macrophages to the site of injection leading to the development of the readable skin lesion [129]. Orme and Cooper speculate that the infiltration of cells to the site of the PPD injection is the same type of reaction that occurs during the formation of lung granulomas during tuberculosis infection, with chemokines as the primary effector molecules in recruiting large numbers of monocytes and macrophages into the site of infection [178].

During the 1960s and 1970s, much debate existed over whether or not the same cells are involved in protective immunity (i.e. protective IFNγ secreting CD4 T cells) and the DTH response [146], [259]. Results driving this dispute indicated that tuberculin hypersensitivity could be induced in the absence of protective immunity, and on the flip side, protective immunity could be generated in the absence of a DTH response [191]. However, this argument was put somewhat to rest in the 1980s when Orme and Collins provided evidence that separate populations of T cells contribute to protection and DTH, which was evident by the expression of the surface marker Ly-2 on protective T cells but not on T cells participating in the DTH response [176]. Moreover, in 1988 Orme showed that mice inoculated with killed mycobacterial vaccines were capable of mounting a DTH response, but failed to generate acquired protection against *M. tuberculosis* infection [172]. In agreement with Orme's findings, Cooper *et al.* demonstrated that mice deficient in IFNγ were able to mount a DTH response suggesting that protective T cells (those that secrete IFNγ) are not required in the DTH response [56].

Treatment: Chemotherapy and Multi-Drug Resistant Tuberculosis

During the late 19th and early 20th century, treatment of tuberculosis mainly consisted of bed rest usually carried out in a sanatorium developed specifically for that purpose. In the 1920s, surgical procedures such as thoracoplasty (removal of ribs to allow for the permanent collapse of the diseased lung) were used to treat tuberculous patients [128]. The chemotherapy era of tuberculosis treatment began in the 1940s with the discovery of para-amino-salicylic acid (PAS) in 1943 by Jorgen Lehmann and the discovery of streptomycin in 1944 by Selman Waksman. Soon after the initial use of these two drugs in humans, it was evident that treatment using one drug at a time resulted in decreased effectiveness due to bacterial resistance. Subsequently, Dunner *et al.* published the first paper demonstrating the effectiveness of combined streptomycin and PAS treatment [34]. Then, in the 1950s a third drug called isoniazid was discovered [195]. The combination of PAS and isoniazid for 18-24 months, complemented with streptomycin in the first two months appeared to be 97 percent successful. However failure to follow this regimen resulted in the development of drug-resistant tuberculosis, usually to both isoniazid and streptomycin [127]. Since then, other major drugs have emerged including rifampin, pyrazinamide, ethambutol and thiacetoazone [162].

Drug treatment regimens for tuberculosis consist of two phases: 1) the intensive phase lasts 1-2 months during which dividing bacilli are killed, and 2) the continuation phase lasts 4-10 months during which dormant bacilli are killed [162]. In the 1970s the effectiveness of short-course chemotherapy (SCC), which lasts 6-8 months and ideally includes rifampin, was demonstrated [66]. In 1993, the World Health Organization recommended a drug regimen consisting of a two-month long intensive phase employing the drugs isoniazid, rifampin, pyrazinamide and ethambutol, and a four-month long continuation phase utilizing isoniazid and rifampin [162]. Due to the extended length and complexity of such a drug regimen, patient compliancy has been poor, resulting in the development of multidrug-resistant strains (MDR-TB) of *M. tuberculosis* [36]. When the drugs are appropriately administered by a health worker, however, patient compliancy is greatly improved. Unfortunately, only 15% of the tuberculosis patients worldwide receive directly observed drug therapy [36].

As mentioned above, the emergence of MDR-TB in industrialized countries essentially began in the 1950s. At that time, MDR tubercle bacilli were resistant to both isoniazid and streptomycin. In the current SCC era, however, MDR-TB bacilli are resistant to both isoniazid and rifampin. This type of MDR-TB is very expensive and difficult to cure, with a cure rate less than 50% [115]. Although most industrialized countries dealt with and reduced the problem of MDR-TB in the 1950s and 1960s, fatal outbreaks of MDR-TB have occurred in the United States even as recently as 1990s [68], [4]. The incidence of MDR-TB remains high in developing countries, and as Kochi *et al.* suggest, the HIV epidemic is likely to increase the risk of developing MDR-TB by overburdening national TB control programs with increased case-loads as well as reducing the effectiveness of the TB drug regimens through compromised immunity [127].

Prevention: BCG

The search for an anti-tuberculosis vaccine was under way almost immediately after Koch's discovery of the tubercle bacillus in 1882. By 1908, in the hopes of finding a vaccine against tuberculosis, Calmette and Guérin began working on the attenuation of a virulent bovine strain of *M. tuberculosis* isolated by Nocard from a cow that had tuberculous mastitis [95]. They subcultured the organism on potato slices that had been soaked in sterile ox bile and 5% glycerol every three weeks for thirteen years. In 1921 and 233 subcultures later, the organism was no longer virulent in animal models and was used to immunize a newborn at high risk of tuberculosis, as his mother had died of the disease and his care-giving grandmother had active tuberculosis [35]. The child lived a tuberculosis-free life. Almost one thousand children were vaccinated that year through 1927, and only 3.9% of those children died of tuberculosis compared to the 32.6% of unvaccinated children who succumbed to the disease [35]. Although the League of Nations recommended the widespread use of BCG as a vaccine in 1928 [35], the safety of this new vaccine was seriously questioned in 1930 when 72 of 251 children vaccinated with BCG developed active tuberculosis. All 72 children died [109]. Investigation into the matter revealed that the BCG vaccine given to the children was accidentally contaminated with *M. tuberculosis* [35]. Today, BCG is considered one of the safer vaccines on the market with infrequent side effects including adenitis and osteitis [109].

This remarkably safe vaccine has never regained virulence, which suggests that deletions in its genome occurred during attenuation. However, while the molecular basis behind the attenuation of BCG is unknown, the invaluable determination of the H37Rv genome sequence by Cole *et al.* has provided insight into this matter [53]. For example, using a DNA microarray constructed from the genomic sequence of H37Rv, Behr and colleagues performed hybridization experiments to compare the genomes of BCG and *M. tuberculosis* H37Rv and found nine regions absent from BCG that were consistently present in *M. tuberculosis* [24]. Further work by Pym *et al.* has shown that while comparative genomics has elucidated a number of deletions in the genome of BCG compared to *M. tuberculosis*, only a single locus called RD1 (region of difference 1) has been deleted from both BCG and *M. microti* (a vaccine similar in protective efficacy to

BCG). Restoring RD1 by gene knock-in resulted in partial reversal of attenuation suggesting that the genes encoded in this region, namely ESAT-6 and CFP10, contribute to the attenuation and safety of the BCG vaccine [187].

Besides the safety of BCG, this vaccine possesses many other advantages [35] including: it is cheap to produce and stable, both essential qualities for use in developing countries; a single immunization provides relatively long-lasting sensitization; it can be given at any age, even at birth; and it produces a visible scar which is useful for epidemiological purposes. Regardless of these many advantages, however, BCG has one fatal flaw: its efficacy is variable [230]. Seventeen controlled trials testing the efficacy of BCG in humans have been conducted worldwide. Some of the trials produced encouraging results with BCG efficacy rates as high as 90%. Other trials were not as promising, especially the trial held in Chingleput, India where there were more cases of pulmonary tuberculosis in the BCG vaccinated group compared to the placebo group [230].

Several different interpretations have been presented to explain the variable efficacy rate of BCG. These include, but are not limited to: 1) methodological differences among the vaccine trials [52], 2) the use of differing strains of BCG, as many daughter strains have arisen from the original strain of BCG now maintained at the Pasteur Institute in France [181], [55], 3) genetic differences of the diverse racial groups tested [215], 4) and finally, differences in the virulence of *M. tuberculosis* strains based on geography. For example, Mitchison reported that in guinea pigs, clinical isolates from the Chingleput area were less virulent than strains from elsewhere [152]. Perhaps the most persuasive argument for BCG's protective variability, however, is based on the hypothesis that infection with environmental or atypical mycobacteria, found in areas such as South India, may interfere with the protective effect of BCG, and that the environmental mycobacteria can provide some degree of protection against tuberculosis [35]. When Palmer and Long exposed guinea pigs to different species of atypical mycobacteria and then measured their abilities to protect against *M. tuberculosis*, they found that although BCG did not add to the protection, different atypical mycobacteria offered varying degrees of protection against the challenge, some offering as much protection as BCG [183]. These results could help to explain the seemingly poor efficacy rate of BCG that was observed in warmer, tropical regions such as South India, southeastern United States, and Puerto Rico where exposure to environmental mycobacteria is prevalent [35]. In places such as these, the protection provided by BCG may have been diluted since its effects were compared to "unvaccinated" people who had already been exposed, or immunized, with environmental mycobacteria [35]. Recently, Brandt *et al.* published work indicating that BCG growth and subsequential protective immunity was inhibited in mice previously exposed to certain environmental strains such as *M. avium* [41].

Regardless of which hypothesis best explains the protective variability of BCG, this truth remains: the development of an improved vaccine is warranted, one that can protect at least as well as BCG against infection with *M. tuberculosis* or against the pathologic effects caused by this disease.

The Immune Response to *M. tuberculosis*

The Macrophage

Tuberculosis is transmitted by the inhalation of droplets of fluid, each containing one to three bacilli [250]. Once the droplet is inhaled, fewer than ten percent of the *M. tuberculosis* bacilli will reach the respiratory bronchioles and alveoli [164]. Bacteria that make it to the deep lung are phagocytosed by alveolar macrophages that may either kill the bacilli or accommodate the intracellular pathogens allowing them to survive and multiply [192].

M. tuberculosis bacilli enter the macrophage by means of conventional phagocytosis involving specific receptor-ligand interactions. Complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18), which are found on the surface of human monocytes and macrophages, have been shown to mediate phagocytosis of *M. tuberculosis* [201]. Besides possessing the ability to bind directly to the complement receptors, *M. tuberculosis* also binds to mannose receptors found on human macrophages [200]. The macrophage mannose receptor participates in the nonopsonic uptake of the bacilli through the recognition of mannose-capped lipoarabinomannan (ManLAM), which is a highly immunogenic, glycolipid molecule associated with the cell wall of pathogenic mycobacteria [200], [110]. Schlesinger's finding that anti-ManLAM monoclonal antibodies reduced the binding of virulent *M. tuberculosis* to macrophages by 49% suggests that ManLAM mediates the binding of the bacillus to the macrophage mannose receptor [202].

Once attachment and phagocytosis of *M. tuberculosis* occurs, the success of this intracellular pathogen depends on its ability to survive and multiply in the normally inhospitable environment of the macrophage. Usually when an antigen is phagocytosed by a macrophage, it enters a membrane-bound structure called a phagosome, which subsequently drops in pH, a process mediated by a proton-ATPase complex associated with the phagosome membrane [174]. Vesicles and lysosomes proceed to fuse with the phagosomal membrane bringing the compartment's pH down below 6.0. At this point, the lysosomal associated membrane protein-1 (Lamp-1) and the mannose-6-phosphate receptor (M6PR) can be detected in the phagosome, thus facilitating the degradation of the phagocytosed antigen [174].

When mycobacteria are phagocytosed, however, a different scenario plays out. In order for mycobacteria to avoid their own deliverance from the phagosome into an acidic lysosomal compartment, they must find a way to alter the phagosome in which they reside. Several different groups have shown that vesicles harboring mycobacteria are less acidic than vesicles empty of bacilli [61], [92], [218], which implies that the mycobacteria somehow interfere with acidification of the phagosome. It has been suggested that mycobacteria may accomplish this by selectively inhibiting the fusion of the phagosome with vesicles containing proton-ATPase, thereby avoiding acidification of the phagosome [218]. When macrophages infected with *M. tuberculosis* were probed with antibodies directed against M6PR, LAMP-1, and proton-ATPase protein, the anti-LAMP-1 antibody labeled all the vacuoles in the macrophage, those with and without mycobacteria. However, neither anti-proton-ATPase antibodies nor anti-M6PR antibodies labeled vacuoles containing mycobacteria [255]. Furthermore, it appears that the high pH of the mycobacterial vacuole reduces the bacteriostatic effect of IFNγ, which would imply another level of protection for the bacilli [17].

IFNγ has been shown to inhibit the growth of *M. tuberculosis* in murine bone marrow-derived macrophages through the induction of reactive nitrogen intermediates (RNI) [79]. Chan *et al.* proposed that RNI, and this includes nitric oxide (NO), are the main effector molecules that activated macrophages use against *M. tuberculosis* [49]. NO and other RNI are derived from L-arginine through an enzymatic pathway controlled by inducible nitric oxide synthase (iNOS), which is activated strongly by tumor necrosis factor-α (TNF) and IFNγ [77]. Evidence that iNOS plays an important role in protection against *M. tuberculosis* infection was obtained using iNOS KO mice infected intravenously with *M. tuberculosis*. Lungs and spleens from these iNOS-deficient mice contained up to three and a half times more viable bacteria than the control mice [148].

The role of reactive oxygen intermediates (ROI) against intracellular mycobacteria is controversial, as results showing oxygen-independent bacteriostatic activity of macrophages infected with BCG have been reported [79]. To the contrary, studies using knockout mice incapable of producing ROI suggest that ROI may play a significant protective role in the lungs against *M. tuberculosis* [5], [60]. Still, other studies indicate that ROI alone may be insufficient in killing *M. tuberculosis*, but ROI and RNI together may enhance macrophage killing of the bacilli [214].

CD4 T Cells

It is presently well known that acquired resistance against *M. tuberculosis* is mediated by cells rather than by humoral immunity [221]. In fact, over the past few decades it has become increasingly clear that Th1-type CD4 T cells are the principal mediators of acquired resistance against tuberculosis infection.

Work done by Suter and Mackaness in the 1950s and 1960s began to elucidate the role of cells in protection against tuberculosis [220], [221], [147], [145], and in 1973, North was able to pinpoint T lymphocytes as the mediators of protection against infection with *M. tuberculosis* [167]. Also in 1973, Lefford demonstrated that adoptive transfer of thoracic-duct lymphocytes in the rat model conferred immunity against tuberculosis [138]. Two years later, Lefford moved to a mouse model in which he showed that when thymus dependent lymphoid cells taken from mice immunized with BCG were injected into mice subjected to sublethal irradiation, adoptive immunity to tuberculosis was transferred [137]. Short of a decade later, Orme and Collins confirmed Lefford's findings when they showed that adoptive transfer of protective immunity against *M. tuberculosis* could be achieved in T cell-deficient mice [175]. Four years later, Orme reported that at least three different protective T cell subsets emerge in mice infected intravenously with *M. tuberculosis,* and that these cells emerge during different phases of the infection. L3T4$^+$ (CD4) T cells were detected by day 5 and peaked between days 20 and 30 after which they continued to protect. Moreover, it was established that the L3T4$^+$ (CD4) T cells offered most of the protection when compared to Lyt-2$^+$ (CD8) T cells. Lyt-2$^+$ (CD8) T cells appeared to offer protection later in the infection, however, that protection was less than half of that offered by the L3T4$^+$ (CD4) T cells [170]. In agreement with Orme's findings, Caruso *et al.* showed that CD4 knockout mice were significantly more susceptible than wildtype mice to *M. tuberculosis* early during the infection [48].

The accepted paradigm of immunity against *M. tuberculosis* is that Th1-type CD4 T cells, recognizing mycobacterial peptides bound to class II MHC, secrete IFNγ which in turn activates macrophages to kill tubercle bacilli as well as recruit monocytes for the initiation of granuloma formation [38], [170]. In accordance with this paradigm, work from the Orme laboratory indicates that the kinetics of emergence and loss of protective T cells that mediate immunity to tuberculosis in mice correlate strongly with the kinetics and emergence of IFNγ [180], [179].

Where mycobacterial sensitization of naïve CD4 T cells occurs is unclear, but bronchial-associated lymphoid tissues are potential sites. In order for this sensitization to occur in mice, antigen must be physically carried to the BALT due to lack of lymphatic drainage to murine alveoli. Gonzalez-Juarrero and Orme hypothesize that immature dendritic cells, which are distributed throughout the alveolar region, are responsible for capturing mycobacterial antigens and carrying them to lymphoid organs where they in their mature form present antigen to naïve CD4 T cells. In support of this hypothesis, dendritic cells isolated from murine lungs were capable of engulfing live *M. tuberculosis*, which led to IL-12 secretion and the stimulation of CD4 T cells to produce IFNγ [90]. The sensitized T cells then circulate and sample mycobacteria-infected lung tissue. It has been hypothesized that once the sensitized CD4 T cells proliferate and secrete cytokines in response to mycobacterial antigen presented by infected macrophages, some remain in the infected area and are continually exposed to antigen. These short-lived cells probably die by apoptosis in order to avoid local tissue damage caused by high concentrations of cytokines like IFNγ, IL-2, and tumor necrosis factor (TNF). Some of the sensitized T cells leave the infected area through the blood or lymph and take on a memory phenotype [177].

Evidence of memory CD4 T cells was first provided by Griffin and Orme who showed that different subsets of phenotypically distinct CD4 T cells arise in mice during infection with *M. tuberculosis*. The expression of two different cell surface antigens, CD44 and CD45RB were used to distinguish four separate subsets of CD4 T cells. Griffin and Orme found that naïve or resting T cells expressed CD44^{lo}/CD45RB^{high}, and of the three remaining subsets (which expressed increased levels of CD44, therefore suggesting an activated state) CD44^{high}/CD45RB^{lo} and CD44^{high}/CD45RB^{neg} were found to be associated with immunological memory [98]. Andersen and Smedegaard, however, showed different results. Using the mouse model of memory developed by Orme, mice were infected with *M. tuberculosis* intravenously and then treated with isoniazid and rifabutin one month after infection. Adoptive transfer studies into nude mice showed that CD44^{lo}/CD45RB^{hi} cells offered the highest level of protection against challenge with

M. tuberculosis suggesting memory cells that have reverted to the naïve phenotype are important in long term protection against *M. tuberculosis* [15].

CD8 T Cells

Although the role of CD4 T cells in tuberculosis immunity is established, evidence has been building over the last decade and a half that CD8 T cells also contribute to protective immunity. Early adoptive transfer studies showed that CD8 T cells offer some protection against *M. tuberculosis*, possibly due to low-level secretion of IFNγ [170]. Studies in which monoclonal antibodies were used to deplete CD8 T cells in mice showed that resistance against *M. tuberculosis* infection was significantly impaired [160].

Convincingly, Flynn's work with β_2-microglobulin knockout (β_2m-KO) mice further suggests that CD8 T cells play a role in protective immunity against tuberculosis, particularly in the lung [82]. Because the formation of functional class I MHC molecules requires the β_2m protein, the lack of this protein prevents the presentation of antigen to CD8 T cells. Therefore, in mice lacking functional class I MHC molecules, CD8 T cells cannot become sensitized by antigen because they recognize antigen only in the context of class I MHC. In Flynn's study, when β_2m-KO mice were challenged with an intravenous sublethal dose of virulent *M. tuberculosis*, most died 6 weeks after the challenge while control mice lived longer than 20 weeks post-infection. Granulomas formed in the lungs of both the β_2m-KO mice and the control mice, but only the lungs of the mutant mice showed an increase in bacterial load as well as caseous necrosis.

Interestingly, a separate study showed that when CD8-KO mice were infected with a low dose aerosol challenge of *M. tuberculosis*, there were no differences in bacterial numbers in the lungs of mutant and wild type mice 50 days after inoculation [71]. These results may coincide with data from Turner *et al.,* which suggest that CD8 T cells play a role in protection against *M. tuberculosis* during the chronic stage of infection by inducing apoptosis through CD95/95L [235].

The manner in which CD8 T cells mediate protection, however, continues to be a point of controversy. It has been suggested that CD8 T cells may contribute to protection against tuberculosis by lysing mycobacteria-infected cells [64]. For instance, as the intra-phagosomal tubercle bacilli divide, damage to the vesicular membrane may allow mycobacterial antigens to escape into the cytoplasm and subsequently enter the class I MHC pathway. Hence, mycobacterial peptides are presented to class I MHC-restricted CD8 T cells which are then sensitized and eventually activated to kill the infected cell. As the target cells are destroyed, infiltrating monocytes, as well as dendritic cells, may then engulf and kill bacilli that have been released into the extracellular space, thus leading to control of the infection [173], [125]. In addition, Stenger *et al.* showed that human CD8 T cells display cytotoxic T lymphocyte (CTL) activity specifically against mycobacteria, and that target cell lysis is followed by inhibition of intracellular *M. tuberculosis* growth [216]. Corroboratively, a recent study by Serbina *et al.* showed that CD8 T cells from the lungs and draining lymph nodes of mice infected by the aerosol with *M. tuberculosis* expressed perforin and specifically lysed infected macrophages *in vitro* in a perforin-dependent manner [209].

In striking contrast to the data presented above in support of a lytic mechanism of protection, results from experiments using perforin and granzyme gene knockout mice showed that upon aerosol infection, both groups of knockout mice behaved the same as control mice [133], [57]. These results suggested to the author that the mechanism by which CD8 T cells are protective in the *M. tuberculosis* infection model is not a lytic one at all, but rather a cytokine-mediated mechanism [57]. It has been established that mycobacterial antigen-specific CD8 T cells from mice and humans secrete IFNγ after restimulation *in vitro* [132], [139], [179]. Although it has been shown that CD8 T cell-secreted IFNγ was essential in mediating protection against *M. tuberculosis* in athymic mice, the presence of these gamma interferon-secreting cells early during the infection had not yet been established [229]. Subsequently, Serbina and Flynn demonstrated that mycobacteria-specific CD8 T cells and CD4 T cells emerged in mice within the first week post-infection with *M. tuberculosis*, and that both cell subsets were primed for IFNγ production. However, CD4 T cells were responsible for most of the IFNγ produced initially in the infection [208].

γδT cells

Greater than 95% of human T cells express the αβ heterodimer T cell receptor (αβ TCR), while a much smaller percentage (2-5%) of T lymphocytes express the γδ TCR [129]. Although important clues regarding the role of γδ T cells in protective immunity against *M. tuberculosis* have been revealed, the exact role of these cells remains undefined.

The connection between γδ T cells and mycobacteria was first suggested in 1989 when an increase in γδ T cells was observed in the draining lymph nodes of mice immunized with heat-killed *M. tuberculosis* in incomplete Freund's adjuvant [117]. Others have confirmed that γδ T cells expand in mouse lymph nodes in response to complete Freund's adjuvant (CFA), however, no memory γδ T cell responses resulted when mice were re-exposed to CFA. In contrast to Janis' finding, γδ T cells appeared to be nonspecifically activated to the oil-based adjuvant rather than to the heat-killed *M. tuberculosis* [97].

Other researchers, trying to identify a role for this T cell subset in tuberculosis infections, have discovered that not only do γδ T cells recognize mycobacterial peptides, but they also recognize nonpeptide ligands [224], [168], [184]. Gamma-delta T cells have also been shown to secrete cytokines such as IFNγ in response to mycobacteria [233], [88], and they seemed to have protective capabilities in the intravenous infection model which utilized mice deficient for TCR-β or TCR-α [131]. In fact, Tsukaguchi *et al.* recently showed that when purified CD4 T cells and γδ T cells from healthy tuberculin donors were stimulated *in vitro* with *M. tuberculosis,* both subsets secreted equal amounts of IFNγ. The authors concluded that since there were half as many IFNγ-secreting γδ T cells as there were CD4 T cells, the γδ T cells were more efficient producers of IFNγ than CD4 T cells [234].

Results from a study that used γδ-KO mice infected with *M. tuberculosis* via the aerosol route, however, suggested that γδ T cells do not play a protective role in the lungs because the bacterial load in the lungs of mutant and wild type mice after aerosol infection were similar. The absence of γδ T cells did, however, result in a pyogranulomatous response suggesting to the authors, therefore, that these cells appear to govern the character of the granulomatous response rather than protect [70]. These data support the hypothesis by Orme and Cooper that γδ T cells probably function as monocyte and T cell recruiters by producing monocyte chemoattractant protein-1 (MCP-1) and other chemokines in order to control efficient granuloma formation while at the same time limiting the infiltration of neutrophils [178].

NK1.1 T cells

In 1987, a population of mouse T cells double-negative (DN) for CD4 and CD8 surface antigens, and which preferentially utilizes the TCR Vβ8 gene family, were discovered [46], [86]. Since then, it has been discovered that a larger subset of DN T lymphocytes exists that express the natural killer (NK) surface receptor NK1.1$^+$, TCR-αβ$^+$, Ly6C and CD44^{hi} [20], [222], [223]. These cells have been shown to secrete cytokines *in vivo* within one hour of TCR engagement with high titers of IL-4 [258]. A smaller population of CD4CD8- T cells expressing αβTCR+, NK1.1+, Ly6C, CD44^{hi} T cells have been shown to secrete not only large amounts of IL-4, but large amounts of IFNγ as well [102], [28], [29], [18].

Perhaps the most unique aspect of this cell is its specificity for CD1 proteins [27]. Five nonpolymorphic genes on chromosome 1 encode for the CD1 family of proteins in humans, including CD1a-e [8], [260]. CD1 surface molecules are expressed on human and murine antigen presenting cells [186], and with resemblance to class I MHC molecules, the CD1 protein associates noncovalently with β2-microglobulin, an association apparently necessary for efficient folding and surface expression of CD1 [21], [219]. With the discovery that mycolic acids, which are a group of branched, long-chain fatty acids unique to mycobacteria, were recognized by a CD1b-restricted *M. tuberculosis*-specific human T cell line, it has been suggested that CD1 molecules are capable of presenting nonpeptide lipid antigens to T cells [23].

With the knowledge that NK1.1 T cells recognize CD1 molecules and that CD1 molecules have evolved to present lipid antigens such as the lipid components from the cell wall of mycobacteria, we can begin to speculate how these cells might fit into the tuberculosis story. Perhaps mycobacterial lipid antigens are presented on CD1 molecules by professional antigen presenting cells to NK1.1$^+$ T cells, which upon activation, release Th1/Th2 regulatory cytokines (i.e. IFNγ/IL-4). Emoto *et al.* recently showed that following infection with *M. bovis* (BCG), the number of liver CD4 NK1$^+$ T cells secreting IL-4 dropped, while the number of IFNγ-secreting CD4 NK1$^+$ T cells increased [72]. Based on these studies, and on the observations under electron microscopy that the morphology and granule content of NK1.1$^+$ T cells resembles something of an intermediate between NK cells and T lymphocytes [247], it seems reasonable to speculate that IFNγ producing CD4 NK1.1$^+$ T cells may be contributors of initial or innate-like resistance against mycobacteria until mainstream CD4 T cells enter the scene and take over the protective, IFNγ secreting role. However, the role of CD1 molecules in this early resistance would probably be minimal since CD1 do not appear to contribute to anti-tuberculosis immunity, at least in the early stages of infection [69].

B Cells

The role of B cells during *M. tuberculosis* infections is currently unclear, although based on serum passive transfer studies, it is the prevailing opinion that antibodies play no role in protection [85]. In one study by Vordermeier *et al.*, B cell knock out (KO) mice had increased numbers of bacteria in the spleen, liver, and lungs 4-6 weeks following i.v. infection with virulent *M. tuberculosis* [243]. These results suggested that B cells play a role in protection against tuberculosis. However, Johnson *et al.* demonstrated that there were no differences in bacterial loads in organs, cytokine mRNA, or organ pathology in B cell KO mice challenged with low dose aerosol of *M. tuberculosis* [118]. A very recent study showed that although wild type mice and B cell KO mice had comparable amounts of bacteria in the lungs after a low dose infection with *M. tuberculosis*, there was less severe pulmonary granuloma formation as well as delayed dissemination of bacteria from the lungs to other organs in the B cell KO mice [40]. These recent results suggest an important role for B cells during the initial development of granulomas and the subsequent dissemination of the bacilli to other organs. Turner *et al.* found similar results including that granuloma integrity was altered in B cell KO mice compared to lesions in wild type mice which contained many B cells [236].

The Protective Role of IFNγ

Acquired resistance to *M. tuberculosis* is mediated primarily by the secretion of IFNγ by T_H1 CD^+4 T cells, which in turn activates macrophages to express mycobacteriostatic mechanisms [170], [179], [165]. Other cells have been shown to secrete IFNγ during mycobacteria infections including NK cells, NK1.1 T cells, CD8 T cells, and γδT cells, but CD4 T cells are the main source of this protective cytokine [30], [170], [29], [88], [233].

Human *in vitro* studies using *M. tuberculosis*-stimulated peripheral blood mononuclear cells from patients with tuberculosis showed a significant decrease in mRNA expression and IFNγ production compared to healthy tuberculin reactors [261], suggesting a protective role for IFNγ against tuberculosis. In addition, patients with defects in the IFNγ receptor gene suffered from infections with usually nonpathogenic mycobacteria, and upon treatment with exogenous IFNγ, they showed improvement [105].

The protective role of IFNγ was convincingly demonstrated *in vivo* using mice with disruptions in the IFNγ gene (GKO). Dalton *et al.* showed that mice deficient for

IFNγ presented a decrease in macrophage antimicrobial products such as RNI and ROI, a decrease in expression of class II MHC molecules, and an increase in their susceptibility to a sublethal dose of *M. bovis* [62]. GKO mice succumbed quickly to both moderate and high doses of intravenous (i.v.) [80] or a low dose aerosol infection of *M. tuberculosis* [56]. In both models of infection, the GKO mice were consumed by lesions filled with acid-fast bacilli (AFB). Such necrotizing granulomas are not generally observed in immunocompetent, wild type mice [56], [80].

The combined results of these GKO studies suggest that IFNγ indeed plays a pivotal role in protection against *M. tuberculosis* infection. As the authors suggest, without IFNγ present in the granuloma, infected and infiltrating macrophages are not activated enough to kill the intracellular pathogen. The macrophages become so heavily burdened with bacteria that they break open, leading to widespread tissue necrosis as was seen in the organs of GKO mice. Furthermore, it seems reasonable that because GKO mice are unable to fully express class II MHC molecules on the surface of macrophages, and therefore are unable to present mycobacterial antigens to CD4 T cells, the induction of protective cell-mediated immunity would necessarily be suppressed.

T_H1 versus T_H2: The Inductive Role of IL-12 in the Protective T_H1 Response

CD4 T helper cells can be separated into three distinct subsets including a T_H1 subset that secretes IFNγ and IL-2 resulting in cell-mediated immune responses; a T_H2 subset that secretes IL-4, IL-5 and IL-10 resulting in humoral immune responses; and a T_H0 subset that secretes both IL-4 and IFNγ [159], [204], [231]. Accumulated evidence indicates that IL-12, which is primarily secreted by activated macrophages, regulates the induction of the T_H1 subset of both murine and human CD4 T helper cells, whereas IL-4, secreted by T helper cells, is the mediating cytokine responsible for the induction of the T_H2 type CD4 T cells [107], [149].

It has now been well established in the murine model that protective immunity to *M. tuberculosis* infection is mediated initially by a wave of T_H1 CD4 T cells that secrete IFNγ, [56], [80], [170], [179], [180], and as Orme suggests, is followed by a second wave of T_H2-like IL-4-secreting CD4 T cells. Although the T_H2 response in tuberculosis infections has not been shown to offer protection, the antibodies generated may be useful in eliminating bacterial debris once the infection has been contained by the initial wave of protective T_H1 helper cells [180]. Huygen *et al.* further confirmed that protection against mycobacteria is conferred by a T_H1-type response when they compared the cytokine profiles generated in BCG-resistant and BCG-susceptible mice [111]. Helper T cells from the spleens of BCG-resistant mice secreted high levels of type-1 cytokines such as IFNγ and IL-2, whereas the susceptible mice secreted IL-4 [111].

The role of IL-12 in inducing this protective T_H1 response to *M. tuberculosis* has been demonstrated in the mouse model. Flynn *et al.* [83] reported that the organs of IL-12-treated mice showed a 50-fold decrease in viable bacilli early in infection compared to the organs of mice treated with PBS, with an increase also in IFNγ expression early during the infection. The results from this study also showed that the granulomas in the lungs of the IL-12-treated mice were small and well defined with very few bacilli compared to the PBS-treated mice [83]. The authors suggest that IL-12 may enhance macrophage activation since IL-12-treated mice were better able to contain and destroy the tubercle bacilli during granuloma formation, and that IL-12 has an early effect on the gene expression of IFNγ which may explain the early decrease in bacilli numbers in the lung. Cooper *et al.* reported similar results in C57BL/6 mice, which showed a decrease in both resistance to infection as well as granuloma integrity after the administration of neutralizing monoclonal anti-IL-12 antibody [59]. The inability of the anti-IL-12 antibody treated mice to form effective granulomas led the authors to hypothesize that IL-12 probably plays an important chemotactic role in T cell recruitment during granuloma formation.

IL-12 KO studies have convincingly confirmed the role of IL-12 in protective tuberculosis immunity. IL-12 is a heterodimeric protein consisting of two subunits called p35 and p40. Cooper *et al.* [58] used IL-12 $p40^{-/-}$ knock out mice, which are unable to express mRNA for the IL-12Rβ2 molecule, to clarify the role of IL-12 in the induction of the protective T_H1 response against *M. tuberculosis*. IL-12 $p40^{-/-}$ mice challenged intravenously with a virulent strain of *M. tuberculosis* were unable to control bacterial growth in all three target organs including the lung, spleen and liver. The expression levels of IFNγ and TNF-α mRNA were delayed and significantly reduced in the IL-12 KO mice compared with the wild-type mice. The authors predicted that the delayed expression of IFNγ and TNF-α most likely resulted in the delay of macrophage activation as was verified by the reduced mRNA expression of iNOS. Also, mice deficient for IL-12 were unable to efficiently recruit lymphocytes to the bacterial lesions within the liver when compared to control mice, thereby lending credence to the chemotactic role of IL-12 in granuloma formation.

Since IL-12 appears to induce T_H1 responses during *M. tuberculosis* infection, the use of this cytokine in vaccines has potential. A successful vaccine against *M. tuberculosis* may require sustained memory or effector T_H1 CD4 T cells. In a very recent study by Seder's group, IL-12 was required to maintain enough type-1, IFNγ producing CD4 T cells *in vivo* to confer long term protection against the intracellular pathogen, *Leishmania major* [217].

The Granulomatous Response

The granulomatous response is somewhat of a mixed blessing because although it is thought to be necessary in the protective immune response against tuberculosis, it is also considered a component in the pathology of tuberculosis. Granuloma formation, and the events leading up to it, has been described in a putative model by Orme and Cooper [178]. Briefly, *M. tuberculosis*-infected macrophages in the lung secrete inflammatory cytokines such as IL-1, IL-6, and TNF-α as well as chemokines including macrophage inflammatory protein-1 (MIP-1). The secretion of these inflammatory cytokines and chemokines leads to the recruitment of monocytes and lymphocytes from the blood, and within 15-20 days, the accumulating cells begin to form a granuloma. The secretion of IL-1 from infected macrophages upregulates the expression of the IL-2 and IL-2 receptors on incoming CD4 T cells leading to clonal expansion of this population, and IL-12 secreted from macrophages leads to the release of IFNγ by the CD4 T cells, which have been sensitized to mycobacterial antigens presented on class II MHC. The release of IFNγ activates bacteriostatic activities of the infected macrophages leading to growth inhibition of the infecting bacilli. Continued accumulation of cells around the infectious foci 'walls off' the infection, thereby preventing additional spread of bacilli. Orme and Cooper speculate that the DTH reaction may be the mechanism responsible for the 'walling off' of the infection and that chemokines, including MCP-1, may be the driving force behind this mechanism, whereas cytokines drive the protective response [178].

TNF-α has been shown to play a role in proper granuloma formation in response to mycobacteria. Injection of rabbit anti-TNF antibody into mice infected with BCG results in a drastic suppression of granuloma development [126]. In this study, it was suggested that TNF-α may play two roles in the process of granuloma formation and mycobacterial elimination. First, it may act in an autocrine fashion enhancing the bacteriostatic activities of macrophages in the granuloma, and second, it may amplify or recruit monocytes to the developing granuloma.

TNF-α is known to bind to two different cell surface receptors referred to p55 (TNF-R1) and p75 (TNF-RII) based on their respective molecular weights [33]. Both receptors are present on nearly all cell types excluding erythrocytes and unstimulated lymphocytes, however, most TNF activity takes place through the activation of p55 [33]. Administration of a recombinant adenovirus expressing the p55 receptor to mice infected with a virulent strain of *M. tuberculosis* (H37Rv) resulted in four $\log_{10}$ increase of bacilli in the lungs. The results of this study indicate that blocking the activity of TNF-α changes the immunity and disease progression in the lungs [6].

The use of TNF-α receptor knockout mice has proven useful in further identifying the role of TNF-α in granuloma development. Senaldi *et al.* showed that in p55 (TNF-RI) knockout mice infected with BCG, granulomas were smaller and almost empty of epithelioid cells, which suggests that p55 is essential in recruiting these cells at some point during granuloma formation [207]. This study also found that the administration of soluble p55 (sTNF-RI) inhibited granuloma formation, and is therefore in agreement with the previous study by Adams *et al.* in which antibodies to TNF were used in BCG-infected mice. These results, combined, strongly suggest that p55 is necessary for normal granuloma development.

Flynn and colleagues [81] used two different murine models to examine the role of TNF-α in protection against tuberculosis. In the first model, TNF-α was neutralized *in vivo* by monoclonal antibody. In the second model, mice with a disruption in the gene encoding the p55 gene were used. Results from both models suggest that TNF-α and p55 are required for protection against i.v. *M. tuberculosis* infection as well as for the production of RNI molecules by macrophages. In addition, when mice with a disruption in the TNF gene were infected with a low dose of *M. tuberculosis* via the aerosol route, there was a significant increase in bacteria numbers in the lungs compared to wild type mice [22]. Bean *et al.* also found that infiltrating T lymphocyte organization in TNF-/- mice was restricted to perivascular and peribronchial areas rather than co-located with macrophages in granulomas as they were in wild type mice. The results of this study further confirm that TNF plays an essential role in the local organization of granuloma.

Immunogenic Mycobacterial Antigens

An effective vaccine against tuberculosis should include a mycobacterial component that is capable of inducing strong Th1 CD4 T cell proliferation and IFNγ secretion, the identifying markers of anti-tuberculosis protection. The current belief is that antigens secreted from live *M. tuberculosis* may be the major targets for protective T cell responses in mice and humans. This idea has arisen from studies showing that immunization with live, but not dead, bacteria is necessary for the induction of long-lived specific immunity to *M. tuberculosis.* For instance, Orme immunized mice with either dead or living mycobacterial vaccine preparations and found that while nonspecific acquired resistance and a DTH response were generated by exposure to the dead vaccine preparation, only the living vaccine was capable of generating protective T cells, as was measured by their ability to adoptively protect mice devoid of T cells [172]. The findings of this study led Orme and others to hypothesize that proteins exported or secreted by living bacteria, such as those found in the culture medium *in vitro* (i.e. culture filtrate proteins or CFP), are essential in protection against *M. tuberculosis.*

Andersen *et al.* subsequently compared the proliferative T cell responses in mice infected with live *M. tuberculosis* versus mice injected with killed bacteria. In the mice infected with live bacilli, a ten-fold increase in CD4 T cell proliferation was observed in response to short-term culture filtrate (ST-CF) as well as to a 32-33 kDa cell wall-associated mycobacterial antigen. On the other hand, T cells from mice injected with killed bacteria did not respond to either the ST-CF or the 32-33 kDa protein [13]. These results further confirmed that live, but not dead, bacilli efficiently generate protective responses against *M. tuberculosis,* and they suggested that CD4 T cells directed against antigens secreted by metabolically active bacilli are necessary in mediating resistance to tuberculosis infection. In addition, Hubbard *et al.* demonstrated that subcutaneous injection of CFP into mice was protective against aerogenic challenge with *M. tuberculosis* [108].

Because the goal of any vaccine development is the generation of a long-term protective memory immune response, the ability of live *M. tuberculosis*-secreted proteins to generate long-lasting protective memory CD4 T cells has been analyzed. Orme, using his mouse memory model (described previously in this chapter), provided the first evidence that CD4 T cells mediated memory immunity, and these memory T cells responded in an accelerated manner only upon rechallenge with living *M. tuberculosis*

[171]. Results from this study led Orme to hypothesize that antigens secreted from viable *M. tuberculosis* are presented by infected macrophages to the T cells responsible for mediating anti-tuberculosis memory.

Andersen and Heron investigated the specificity of the protective memory T cell immune response to *M. tuberculosis.* Using Orme's mouse memory model, they showed that memory and activated T cells differ in levels of IFNγ production, proliferation kinetics, and specificity [14]. Active T cells proliferated and produced IFNγ rapidly after stimulation with ST-CFP, whereas proliferation of memory T cells was delayed and IFNγ production was less pronounced. Furthermore, this study showed that when 10 individual fractions of the ST-CF were used to stimulate T cells from memory immune mice (rechallenged mice), the cells proliferated and released IFNγ in response to 9-14 kDa and 25-33 kDa fractions. Interestingly, when the memory immune mice were rechallenged a second time, the cellular response was significantly boosted toward the 3-9 kDa fraction [14].

Antigen 85

The 30-32 kDa antigen (Ag 85 complex) has been purified and well-characterized. The genes that encode the proteins of the Ag85 complex are located at different sites in the mycobacterial genome, and the complex consists of three distinct, cross-reacting proteins ranging in size as follows: Ag85A is 31 kDa, Ag85B is 30 kDa, and Ag85C is 31.5 kDa [251]. The proteins of the Ag85 complex are found abundantly in the culture filtrate of *M. tuberculosis*, and depending on growth conditions, the Ag85 complex has been shown to constitute up to 60 percent of the total culture filtrate protein

[251]. In addition, even though Ag85 is a major secreted constituent of the culture filtrate, this protein complex is also found associated with the mycobacterial cell wall.

Besides possessing the ability to bind T cell fibronectin, thereby diminishing anti-mycobacterial delayed hypersensitivity [89], the Ag85 complex has also been found to play an essential role in cell wall biosynthesis. Belisle *et al.* reasoned that since Ag85 complex homologs are found both in pathogenic and nonpathogenic mycobacterial species, it must serve a more fundamental, physiological role than fibronectin-binding alone. Mycolic acids, which are high-molecular-weight, α-alkyl, β-hydroxy fatty acids, are mostly found in the mycobacterial cell envelope as esters bound to the distal ends of arabinogalactin [43] as shown in Figure 1.1. Belisle's group was able to show that the Ag85 complex is the mycolyltransferase enzyme responsible for mycolic acid deposition leading to the formation of the mycobacterial cell wall [25].

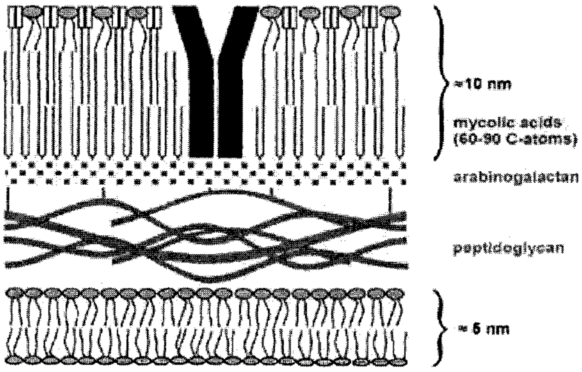

Figure 1.1 Model of the mycobacterial cell envelope. (www.niaid.nih.gov) Mycolic acids are present mainly as bound esters of arabinogalactan, which is a polysaccharide linked to the peptidoglycan layer by phosphodiester bonds. The peptidoglycan-arabinoglactan mycolate forms the cell wall skeleton [43].

Ag85A, also called P32 and MPT44, was cloned and sequenced in 1989 [39]. The DNA sequence of the gene encoding the Ag85A protein contains 1,008 base pairs, and the amino acid sequence corresponds to a 336-residue protein. The DNA sequence of this gene encodes a signal peptide, which is cleaved when the mature protein, composed of 294 amino acids, is secreted from the bacterial cell. This secreted protein has been shown to elicit strong cellular immune responses in mice and humans. For example, Huygen *et al.* showed that Ag85A significantly induced the production of IFNγ by spleen lymphocytes isolated from mice sensitized with BCG [113]. In a human study, Ag85A induced IFNγ secretion as well as the proliferation of peripheral blood mononuclear cells (PBMCs) from patients with positive tuberculin reactivity [113]. It has also been demonstrated that Ag85A induced the proliferation of CD4 and CD8 T cells when PBMCs from BCG-vaccinated individuals were stimulated *in vitro* with Ag85A [213], [161]. In addition, when Munk's group investigated the ability of an expanded T cell line stimulated with Ag85A to lyse *M. bovis*-infected macrophages, they observed significant lysis of the infected macrophages by CTLs. These results suggested to the authors that because Ag85A-specific CTLs recognized infected macrophages, Ag85A must be secreted and introduced into the macrophage's class I MHC processing and presentation pathway [161].

DNA Vaccines

In 1990, Wolff *et al.* firmly established that plasmid DNA injected into mouse skeletal muscle was taken up by the muscle cells and was capable of foreign gene expression *in vivo* [253]. This discovery led researchers to investigate the feasibility of using DNA plasmids encoding immunogenic proteins as vaccines against infectious disease. Ulmer *et al.* were the first to demonstrate that immunization with plasmid DNA encoding the nucleoprotein (NP) for influenza A conferred protection against subsequent intranasal challenge with the influenza A virus in mice [240]. Ulmer's group also showed that the protection against influenza infection in NP DNA-vaccinated mice was due to NP-specific CTLs and not the high-titer antibodies that were also produced. When anti-NP sera obtained from NP DNA-vaccinated mice were passively transferred into naïve mice, no reduction in viral titers in the lungs was observed [240].

The capacity of intramuscularly injected DNA vaccines to generate both humoral and cellular immunity has been demonstrated by others, including Wang *et al.* who showed that both types of responses were generated in mice immunized with DNA encoding the human immunodeficiency virus type-1 envelope protein gp160 [244]. The induction of cellular immunity in humans by a DNA vaccine encoding a malarial protein has also been demonstrated. Twenty individuals received three i.m. injections of the DNA vaccine, each injection given four weeks apart, after which malaria-specific CTLs were observed in the blood of over half the subjects [245].

The success of DNA vaccines depends, in part, on the following: 1) the ability of the DNA vaccine to express the foreign gene in cells in order that the desired immune response might be elicited, and 2) the capacity of the vaccine to induce specific, long-lived host immunity. Seder's group found that DNA encoding the immunodominant LACK protein generated protective T_H1 immunity against infection with *Leishmania major* in mice, with increased levels of the type-1 cytokine, IFNγ [100]. Moreover, Li *et al.* showed that a DNA vector expressing the highly conserved F protein of respiratory syncytial virus (RSV) not only conferred protection in mice against RSV challenge in mice, but that an additional boost of the DNA vaccine could switch an already established anti-RSV T_H2 response to a T_H1 response with increased levels of IFNγ in the lung [141].

The antigen encoded by the DNA vaccine may elicit T cell responses by a number of different mechanisms. First of all, results from various studies indicate that both specific CD8 and CD4 T cells are generated in response to antigens expressed by plasmid DNA, thereby suggesting that the antigen is presented on both class I and II MHC molecules [112], [37]. The first possible mechanism by which the DNA encoded antigen is processed and presented to T cells involves the transfected myocyte. Once the DNA vaccine is injected into the muscle, the plasmid is taken up by myocytes. This uptake may be mediated by DNA receptors on the muscle cell surface [154]. Once inside the cell, the plasmid DNA enters the nucleus where transcription takes place followed by translation in the cytoplasm using host cell translation machinery. The translated protein may be processed in the myocyte and loaded onto class I MHC molecules via the endogenous pathway for presentation to CD8 T cells, however, myocytes may play only a small role in antigen presentation since they express low levels of class I MHC molecules, and they seem to lack the essential costimulatory molecules needed for T cell recognition [130]. The second processing and presentation mechanism involves transfected APCs, such as dendritic cells (DCs). It has been shown that small numbers of DCs (0.4%) were transfected following i.m. DNA injection and that these cells were critical in priming the immune response after DNA vaccination [7]. The third mechanism of antigen presentation involves the idea of 'cross-priming,' which can be defined as "the induction of CD8 T cell responses without *de novo* antigen synthesis within the APCs" [101]. The observation that exogenously derived peptides can be presented by APCs on class I MHC to CD8 T cells suggests that myocytes may actually transfer antigen to APCs which in turn present to CD8 T cells [101]. Cross-priming may also occur when APCs phagocytose and process secreted peptides coming from apoptotic cells [101]. Finally, it is likely that exogenous peptides are presented via the exogenous pathway on class II MHC to naïve CD4 T cells when peptides are secreted from other transfected cells or released from damaged cells and subsequently picked up by APCs sampling the tissue surrounding the injection .

Because protective immunity against *M. tuberculosis* infection requires the induction of T_H1-type CD4 T cells and cytokines, an effective anti-tuberculosis DNA vaccine should necessarily prime this type of response. Several mycobacterial antigens have been employed in anti-tuberculosis DNA vaccines (Table 1.1). A number of these antigens expressed in DNA vaccines have offered some degree of protection against *M. tuberculosis,* however, two DNA vaccine studies in 1996 received significant attention because the protection elicited in both of the studies rivaled the protection conferred by the current vaccine, BCG. Tascon *et al.* used a DNA plasmid encoding the *M. leprae* 65 kDa heat shock protein (hsp60 DNA). Three weeks after the last of a series of DNA injections, mice were challenged with a high-dose parenteral injection of virulent *M. tuberculosis*. The numbers of viable *M. tuberculosis* bacilli in the spleen, liver, and lung of the mice immunized with both these vaccines were approximately the same as in the BCG-vaccinated mice [228]. The same hsp60 DNA vaccine has also more recently been shown to induce a sterilizing effect in the lungs of mice when delivered in a post-exposure mode [144].

The other DNA vaccine study, conducted alongside Tascon's study in1996, introduced a DNA vaccine encoding Ag85A. Ag85A was a logical choice for inclusion in a DNA vaccine because this immunodominant, secreted protein had been shown to induce lymphoproliferation and IFNγ in mice and humans [114], [134]. Huygen *et al.* immunized mice three times at three week intervals with i.m. injections of DNA encoding Ag85A. When stimulated with purified Ag85, spleen cells from mice vaccinated with Ag85A DNA produced increased levels of IL-2 and IFNγ. The protection induced by the Ag85A DNA vaccine against aerosol challenge with virulent *M. tuberculosis* was long lasting, persisting up to 70 days post-vaccination, and was comparable to the protection conferred by BCG under the same experimental conditions [112]. Ag85A DNA vaccines have since been used in a number of studies including a study in the guinea pig model in which Baldwin *et al.* first demonstrated that guinea pigs immunized with the Ag85A DNA vaccine developed similar lung disease to that seen in the BCG immunized guinea pigs [19].

Besides the capacity to induce relevant T_H1 immune responses and protection, a successful anti-tuberculosis DNA vaccine must be capable of inducing protection that is long lasting. Persistence of plasmid DNA and foreign gene expression in mouse skeletal muscle has been shown to last for up to 19 months without integrating into the host's DNA [252]. Additionally, humoral and cell-mediated immune responses to (nucleoprotein) NP have been detected two years after immunization with NP DNA indicating that immunity to influenza after NP DNA vaccination is long lived [239]. Memory cells may be very long lived or they may require persistent exposure to antigen for maintenance of long lived clones [96]. If the latter is accurate, memory cells are probably maintained during persistent infection with *M. tuberculosis* as well as during persistent antigen expression by a DNA vaccine. The generation of long lasting immunity has been demonstrated by use of anti-tuberculosis DNA vaccines (i.e. Ag85A DNA and 65 kDa DNA) [112], [228]. Results obtained by Silva *et al.* [211] suggested that the hsp65 DNA vaccine could generate protective immunity against *M. tuberculosis* 15 months after vaccination. Recipient mice were γ-irradiated followed by an injection of

Table 1.1 Experimental DNA Vaccines against *Mycobacterium tuberculosis*

DNA vaccine	Animal Model	Infection Model	Prophylactic/ Therapeutic	Source of antigen DNA	*Protection	Ref.
Hsp70	BALB/c	i.v./i.p.	Prophylactic	*M. leprae*	Yes	[143]
Hsp65					Yes	
6 kDa					Yes	
Hsp10					No	
36 kDa					No	
Hsp65	BALB/c	i.v.	Therapeutic	*M. leprae*	Yes	[144]
Hsp65	B6	i.n.	Prophylactic	*M. tb*	No	[238]
	G. P.	aerosol			No	
Ag85A	BALB/c	aerosol	Prophylactic	*M. tb*	Yes	[112]
	B6				Yes	
Ag85A	B6	aerosol	Therapeutic	*M. tb*	No	[237]
38 kDa	B6	i.p.	Prophylactic	*M. tb*	Yes	[263]
19 kDa	B6	i.n. BCG	Prophylactic	*M. tb*	No	[75]
AhpC					No	
Mtb39A	B6	Aerosol	Prophylactic	*M. tb*	Yes	[67]
PstS-1	B6	i.v.	Prophylactic	*M. tb*	No	[227]
PstS-2					No	
PstS-3					Yes	
ESAT-6	B6	Aerosol	Prophylactic	*M. tb*	Yes	[121]
MPT-64					Yes	
Ag85B					Yes	
MPT-64+Ag85B					Yes	
MPT-64 + Ag85B + GMC-SF	B6	Aerosol	Prophylactic	*M. tb*	Yes	[122]
ESAT-6, MPT-63, MPT-64, KatG multivalent	B6	Aerosol	Prophylactic	*M. tb*	Yes	[157]
ESAT-6	B6	Aerosol	Prophylactic	*M. tb*	Yes	[157]
KatG					Yes	
MPT-63					Yes	
MPT-64					Yes	
MPT-83					Yes	
a-crystallin					No	
PstS-1					No	
MPT-32					No	
22 kDa	B6	i.v.	Prophylactic	BCG	No	[136]
	C3H				No	
	BALB/c				No	
Mtb8.4	B6	i.v.	Prophylactic	*M. tb*	Yes	[54]
72f	B6	Aerosol	Prophylactic	*M. tb*	Yes	unpubl.
	G. P.				Yes	

*Protection determined as the reduction of *M. tuberculosis* CFU in vaccinated mice compared to unvaccinated mice following TB challenge. G. P. = Guinea Pig, B6 = C57BL/6 mice

T cells from hsp65 DNA or BCG vaccinated mice and an intravenous injection of 1 x 10^5 *M. tuberculosis* bacilli. Results indicated that protective immunity was mediated by $CD44^{hi}/CD4^{-}/CD8$ T cells in both groups of mice, but that the cells from the DNA immunized mice were more protective than those from the BCG immunized mice. Moreover, when cytokine production from lymph node T cells was assessed from both groups of immunized mice, the T cells from the DNA immunized group produced three times as much IFNγ as IL-4 compared to the BCG immunized group which produced mostly IL-4. These trends were consistent up to 15 months [211].

Improvements to the DNA vaccine strategy are currently under investigation. The addition of genes encoding cytokines that promote a T_H1 response would certainly be logical for a DNA vaccine against tuberculosis, however not much information has been published regarding this approach. Seder has proposed that continuous IL-12, with its capacity to push toward T_H1 cellular immune responses, is necessary for long-term protective immunity against *Leishmania major* [99]. Immunization of mice with IL-12 DNA (but not IL-12 protein) plus a leishmanial protein were required for the generation of cellular immunity that could control the infection 12 weeks after vaccination, whereas the protein or IL-12 DNA alone were much less effective at controlling the infection long term [99]. Based on these findings, the incorporation of IL-12 DNA, either encoded in its own plasmid or coupled with Ag85A in the Ag85A DNA vaccine, seems a logical approach to elicit long lasting, protective cellular immunity against tuberculosis infection. The other cytokine gene that has been used in the DNA vaccine strategy against tuberculosis is the gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF). Kamath *et al.* [122] showed that co-immunization with a DNA plasmid encoding MPT-64 or Ag85B and a plasmid encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) did not improve the protective efficacy of the MPT-64 or Ag85B DNA vaccines. However, an increase lymphoproliferation and IFNγ production were observed indicating that the addition of GMC-SF DNA did contribute to immune enhancement [122].

The incorporation of CpG-DNA may also prove effective in the DNA vaccine approach. CpG-motifs consist of an unmethylated CpG dinucleotide with two 5' purines and two 3' pyrimidines on either side. These palindromic sequences have been shown to increase NK cell cytolytic activity as well as stimulate APCs such as dendritic cells and macrophages. Furthermore, CpG motifs, with their ability to stimulate T_H1-associated cytokines such as IFNγ, have important potential usefulness as safe and effective adjuvants in DNA vaccines targeted at *M. tuberculosis* [51]. Seder's group very recently showed that IFNγ production was enhanced in mice vaccinated with BCG plus CpG oligodeoxynucleotides and IL-12 compared to mice vaccinated with BCG alone [87].

The administration of DNA vaccines has also been shown to play a role in directing T helper responses [76], and new technologies concerning DNA vaccine delivery are being rapidly developed. The two common routes of DNA inoculation are 1) epidermal gene gun (gg) bombardment with DNA-coated gold particles and 2) i.m. needle injection of DNA suspended in saline. When BALB/c and C57BL6 mice were immunized with Ag85A DNA using either of these two methods, gene gun immunization elicited T_H2 type antibodies and was not protective against challenge with *M. tuberculosis.* Conversely, i.m. injection induced protective T_H1 immunity against the challenge in both strains of mice with increased levels of IFNγ and IL-2, thus making i.m. inoculation of choice for anti-tuberculosis DNA vaccination [225]. Cutting-edge technologies are currently improving on the delivery of DNA vaccines to cells. One technology uses cationic microparticles coated with DNA to improve the delivery DNA to APCs. With this technique, functional DNA is released over a period of time after i.m. injection, resulting in significantly greater antibody and cellular responses than naked DNA alone [135]. Another DNA vaccine technology in development utilizes electroporation *in vivo* to improve DNA delivery into cells. The electroporation device is hand-held and has disposable electrodes that conform to the skin. Preliminary results indicate that expression of the target gene is increased resulting in a heightened immune response [135]. One more device that has received attention recently is the Biojector, which uses compressed carbon dioxide to deliver DNA vaccines intramuscularly and intradermally. Besides improving the immunogenicity of DNA vaccines delivered with this technique compared to the needle and saline method, the Biojector is strongly preferred over the needle by patients [135].

Subunit Vaccines and Adjuvants

The term adjuvant is derived from *adjuvare* which in Latin means "to help." Because proteins alone are poorly immunogenic, the rational development of a subunit vaccine that is capable of eliciting an adequate immune response often includes an adjuvant. Adjuvants are defined as a group of compounds used to evoke or enhance an immune response. Classically recognized adjuvants include: oil emulsions, saponins, aluminium or calcium salts, non-ionic block polymer surfactants, derivatives of LPS, mycobacteria, and many others [199]. While certain adjuvants are able to direct immune responses that are associated either with a Th1- or Th2-type profile, relatively little is known about the mechanisms of adjuvant action. Table 1.2 lists examples of adjuvants along with their possible immunostimulatory modes of action.

Table 1.2 Examples of adjuvants with the immunological events they induce

Examples of Adjuvants	*Key Immunological Events*
ISCOMS, Quil A, $Al(OH)_3$, Liposomes, Cochleates, Poly(lactic/glycolic acid)	Antigen localization in the lymph node
Oil emulsions, $Al(OH)_3$, gels, polymer microspheres, non-ionic block copolymers	Prolonged antigen presentation
Complement, CpG-rich motifs, LPS (Monophosphoryl lipid A), mycobacteria (muramyl dipeptide), yeast extracts, cholera toxin, ISCOMS	Signaling of Pattern Recognition Receptors (PRR) on innate immune cells
Oil-emulsion surface active agents, $Al(OH)_3$, IFNs, heat shock proteins, hypoxia	Tissue destruction/stress
Cytokines, costimulatory molecules	APC polarization, T and B cell help

Table adapted from Table 1 in [199].

Culture filtrate proteins were first used as a subunit vaccine to immunize mice against aerosol challenge with *M. tuberculosis* in 1992 [108]. In this study, the bacterial loads in the lung and spleen were significantly reduced when the CFP was given in Complete Freunds Adjuvant (CFA), even though low levels of protection were seen with the CFA alone. Complete Freunds Adjuvant consists of mineral oil, emulsifier, and heat-killed *M. tuberculosis*, while Incomplete Freunds Adjuvant (IFA) consists of the oil-in-water emulsion only [116]. Other early vaccination studies that employed the CFP subunit vaccine with the addition of different adjuvants, such as IFA, reported levels of protection against *M. tuberculosis* nearly equivalent to BCG, although this protection waned after time compared to that conferred by BCG [193]. Baldwin *et al.* incorporated IL-12 and IL-2 into the CFP subunit vaccine in order to augment TH1 immunity and found that mice were protected against aerosol challenge with *M. tuberculosis* nearly as well as mice vaccinated with BCG. The same vaccine did not decrease the bacterial load in the lungs of guinea pigs, however, when IL-12 was omitted, granuloma formation looked similar to the lesions in the BCG controls [19]. The adjuvant used in this study was monophosphoryl lipid A (MPL), an attenuated version of lipopolysaccharide (LPS). MPL has been shown to induce the production of IL-1, IFNγ, and TNF-α from murine and human macrophages as well as switch T cell responses from Th2 to Th1 in mice, the targeted response against tuberculosis [155].

An interesting study conducted by Andersen's group compared the ability of several different adjuvants to modulate the immune responses to a ST-CFP subunit vaccine in mice. The data showed that both IFA and dimethyl dioctadecyl ammoniumbromide (DDA) were efficient adjuvants for use in a subunit vaccine against tuberculosis as they conferred significant levels of protection in the mouse up to week 27 after intravenous challenge. At least two contradictory results have been found against the use of IFA. First, it is too toxic for use in humans, and second, Roberts *et al.* found that CFP in IFA did not protect animals long term in the study mentioned in the previous paragraph [193]. Andersen suggested that the discrepancies between these two studies could have been due to the fact Roberts used proteins from late culture filtrates, while Andersen used proteins from short-term culture filtrates when larger amounts of critical immunostimulatory proteins such as ESAT-6 may be present [142]. Not as much controversy surrounds the DDA ST-CF vaccine used in this study, first because it had been safely used in human clinical trials, and second, because it had previously been shown to stimulate IFNγ production as well as confer long term protection in the spleens of mice infected intravenously with *M. tuberculosis* [10]. Interestingly, when aluminum hydroxide was used in combination with ST-CF in this study, a Th2 response was induced and was followed by an increase in susceptibility of the mice to tuberculosis infection [142].

Instead of using the entire pool of proteins found in the culture filtrate of *M. tuberculosis*, many researchers have moved to using individual proteins either purified from the culture filtrate or as recombinants. Brandt *et al.* used ESAT-6 in combination with MPL and DDA to immunize mice and reported that this subunit vaccine induced strong, specific T cell responses and that it protected mice against challenge with *M. tuberculosis* as well as BCG [42]. Another study by Coler *et al.* showed that a low-molecular weight protein, also found in the culture filtrate of *M. tuberculosis,* called Mtb 8.4 was also as protective as BCG thirty days following intravenous challenge when mixed with IFA [54].

Proteins that are not found in the culture filtrate of *M. tuberculosis* during optimal growth conditions have also been employed as subunit vaccines. One such example is a subunit vaccine consisting of a mixture of the 65 kDa and 70 kDa heat shock proteins emulsified in MPL [238]. Turner *et al.* found that immunizing guinea pigs with this particular subunit vaccine resulted in a lack of protection against aerosol challenge with *M. tuberculosis* as well as severe lung damage characterized by necrotizing bronchointerstitial pneumonia [238].

The most recent and progressive subunit vaccines contain fusion proteins. Enhanced immunity to several viruses including human papilloma virus, human immunodeficiency virus, and foot-and-mouth disease virus using specific fusion protein subunit vaccines has recently been reported [63], [140], [262]. Fusion proteins to combat bacterial diseases are also being developed. Skeiky *et al.* reported that a fusion protein consisting of three immunodominant leishmanial antigens and formulated in MPL-SE (MPL plus squalene) was more protective against leishmanial infection in mice than the three components each on their own [212]. Protection against tuberculosis using a subunit vaccine based on a fusion protein of Antigen 85B and ESAT-6, both immunodominant, early secreted proteins of *M. tuberculosis,* has also been reported [169]. Table 1.3 summarizes most of the antigens that have been used in subunit vaccines to combat tuberculosis.

Table 1.3 Subunit vaccines against tuberculosis

Antigen	Protection	Animal Model	Reference
CFP	Yes Yes	Mouse Guinea Pig	[10], [193], [182], [106]
30 kDa	Yes	Guinea Pig	[106]
Ag85B	Yes	Mouse, Guinea Pig	[42], [106]
Ag85A	No	Mouse	[226]
ESAT-6	Yes	Mouse	[42]
Ag85B/ESAT-6	Yes	Mouse	[169]
Hsp65	No	Guinea Pig	[238]

Prime-Boost Vaccine Strategy

The generation of unparalleled levels of specific, cellular immunity has very recently been demonstrated by priming with DNA vaccines and boosting with recombinant poxviruses, both of which express the same or similar antigens. This novel prime-boost strategy was first demonstrated in mice primed with DNA expressing the influenza hemagglutinin (HA) protein and boosted two weeks later with a recombinant fowlpoxvirus (FPV) also expressing HA. Robust levels of T_H1-type antibodies (i.e. IgG2a) were generated, which suggests that cell-mediated immunity was induced, and furthermore, the mice were protected against challenge with influenza [189]. Several other studies in murine and nonhuman primate models using similar prime-boost strategies have induced extremely high levels of cell-mediated immunity against intracellular pathogens such as the human immunodeficiency virus and *Plasmodium berghei,* the malarial parasite [189]. The success of the prime-boost strategy appears to depend on two factors: 1) the order in which the DNA vaccine and the recombinant virus are given, and 2) the nature of the booster recombinant virus.

DNA vaccines and non-replicating poxviruses have a number of properties that contribute to the efficacy of the prime-boost strategy. For instance, DNA vaccines efficiently induce CD4 and CD8 T cell responses, they contain CpG motifs which act as adjuvants for T_H1 immunity, and they persistently express the desired antigen resulting in prolonged stimulation of T cells as well as the induction of high-affinity T cells [189]. The non-replicating recombinant poxviruses express and present the vaccine antigen on class I and II MHC, which leads to the expansion of T cell responses induced by the DNA vaccine; they induce heavy production of type I interferons which act as adjuvants for T_H1 responses; and because the poxviruses lack genes that elicit antiviral immunity, the immune response is focused on the vaccine antigen [189].

Adenoviruses have also been used to boost DNA vaccination, although reports of this strategy in the literature are scarce. One study demonstrated that in mice vaccinated with a DNA vaccine encoding a rabies virus antigen followed by an intranasal booster with a replication-defective adenovirus encoding the same antigen, significantly higher amounts of IgG2a antibodies were generated than in mice immunized with the DNA vaccine alone [254]. The increased production of IgG2a antibodies suggest that T_H1 immunity was augmented in this prime-boost vaccination model. Furthermore, this study lends support to recombinant adenoviruses for potential use in an anti-tuberculosis prime-boost vaccine strategy.

Adenovirus

In 1953, Rowe and colleagues isolated the causative agent of acute respiratory disease [197], and three years later, Enders *et al.* proposed that the new respiratory tract viruses be called adenoviruses [73]. Adenoviruses now belong to a family of DNA viruses called Adenoviridae, which can be divided into two genera, *Mastadenovirus* (human) and *Aviadenovirus* (avian). *Mastadenovirus* is further divided into 6 subgroups, A-H, into which 50 serotypes have been assigned. The two most studied serotypes are adenovirus type 2 (Ad2) and adenovirus type 5 (Ad5,) both belonging to subgroup C [248]. Adenovirus gains entry into cells by endocytosis using two receptors, one that allows attachment and the other that mediates internalization. Attachment occurs via the fiber capsid proteins protruding from 12 penton vertices on the virion and a cell receptor, while viral internalization is mediated by interactions between the penton bases and cellular α_v integrins [65], [185], [249], [242]. Once inside the cell, the virion is transported to the nucleus where the viral genome is released into the nucleus where replication occurs.

The nonenveloped, icosahedral nucleocapsid contains a 36-38 kbp linear, double-stranded DNA genome, which can be divided into early (E) and late (L) genes. The early region genes include early region 1A (E1A), E1B, E2, E3, and E4. The first protein expressed is E1A, which, after alternative splicing events occur, yields five proteins responsible for the replication of the adenoviral genome [119]. Expression of structural proteins leads to the formation of the capsid into which the replicated viral genome enters [210]. E3 gene products do not participate in viral replication, but rather these proteins play a critical role in evasion of host immunity. For instance, the gp19K is retained in the endoplasmic reticulum where it forms a complex with class I MHC molecules. Consequently, because cell surface expression of class I MHC is absent, viral peptides are not presented to CTLs, thus preventing lysis by adenovirus-specific CTLs [16], [190].

Recombinant adenoviruses have received much attention over the last two decades as vehicles for expressing foreign proteins *in vivo*. Several features about recombinant adenoviruses make them good vector candidates for use in gene therapy and vaccine strategies including: 1) Ad2 and Ad5 are well characterized and fully sequenced [194], [50]; 2) recombinant adenovirus can be easily constructed using infectious plasmids rather than viral DNA [150]; 3) adenovirus vectors can express high levels of foreign protein [153], [84]; 4) high titers of virus can be obtained easily [93]; 5) the safety of adenovirus has been demonstrated in over one million U.S. military recruits who received live adenovirus vaccines, to combat upper respiratory infections, with very few adverse reactions [151]; 6) recombinant adenoviruses are incredibly stable making them convenient to work with in the laboratory; 7) and they have been shown to induce humoral and cellular immunity, and in some cases protection, in response to the expressed foreign protein in several animal models [120], [78], [166], [84], [45], [246].

Most adenovirus vectors have been derived from Ad2 or Ad5 serotypes, and have deletions in the E1 gene. Deletions in the E1 region render the vector replication-defective, thus preventing, for the most part, virus production and subsequent lysis by CTLs [104]. Furthermore, because the virus vector cannot replicate, immunity focuses on the foreign gene product rather than on virally derived peptides. The recombinant virus can be propagated in 293 cells, which are human embryonic kidney cells that have been transformed with the E1 gene[94]. In this cell culture system, the E1 gene products are supplied in trans by the 293 cell line to the replication-defective virus.

The most common strategy in constructing a recombinant adenovirus uses a shuttle plasmid, which is basically the left end of the virus with the foreign gene of interest or cDNA inserted into the E1 gene deleted region. To increase the coding capacity of the vector, deletions are also made in the E3 region [31]. E1 and E3 deletions combined with the ability of adenoviruses to package a genome that is 5% larger than the wild type genome, means that these vectors can accommodate foreign gene inserts up to 8.3 kb in size [104], [32]. Complete virus particles are generated either by the ligation of the shuttle plasmid with the right end of the viral genome *in vitro*, followed by transfection of the ligated DNA into the 293 packaging cells, or by *in vivo* homologous recombination between the shuttle plasmid and a second plasmid containing the right end of the genome, followed by cotransfection of the DNA into the cell line. The second plasmid contains not only the entire adenovirus genome, but an insertion was also added to the E1 region resulting in a plasmid size exceeding the packaging limits of the viral capsid [150]. This ensures that only the recombinant adenovirus will be packaged and recovered. Although the recombinant adenovirus produced using this system has been immensely successful for gene transfer and expression in mammalian cells, it has faced many problems involving the induction of destructive virus-specific cellular immune responses that lead to a loss in transgene expression, and the development of inflammation [256]. New adenovirus vectors have been designed to overcome these problems in gene therapy and viral-vectored vaccine development. New vector designs include deletions in the pTP gene, the E2, E3, E4 gene regions, and in the case of gutless vectors, deletions in all the protein coding sequences [103], [74], [158], [156], [203], [123].

The use of adenovirus vectors in vaccine development against tuberculosis is a logical choice for two main reasons. First, replication-defective adenoviruses specifically target respiratory epithelium, which would focus the immune response against the encoded tuberculosis transgene to the lung [196]. Katkin *et al.* demonstrated that a replication-defective adenovirus encoding the β-galactosidase reporter gene delivered via the aerosol route to mice was capable of expressing the marker transgene in lung epithelial cells up to 21 days without inducing pulmonary inflammation [124].A large portion of recombinant adenovirus research has concentrated on cystic fibrosis gene therapy. Bellon *et al.* reported that aerosol administration of replication-defective adenoviruses encoding the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis patients has resulted in successful transfer of CFTR gene expression without generating virus-specific antibodies [26]. Furthermore, Scaria *et al.* demonstrated that not only was expression of CFTR possible in airway epithelia, but that this expression was long lasting, up to 70 days [198]. The second reason why adenovirus vectors may be useful in anti-tuberculosis vaccine development is that these vectors have been shown to generate T_H1-dominant responses in the lungs to the transgene product. van Ginkel *et al.* provided evidence that mice generated β-galactosidase-specific CD4 T cells secreting IFNγ and IL-6 in response to a recombinant adenovirus expressing this reporter molecule [241]. These combined results suggest that aerosolized recombinant adenovirus encoding an immunogenic mycobacterial protein may be successfully expressed in the airway epithelial cells and thereby focus anti-tuberculosis immunity in the lung.

Several seemingly contradictory studies exist, however, regarding the stability of the adenovirus vector based on immune responses directed to the vector itself versus the transgene. Adenovirus-specific CD8 and CD4 T cells have been shown to inhibit the extent and duration of transgene expression in the lungs, thus creating barriers for long-term expression of the transgene [257]. However, others have also shown that strong humoral and cellular immune responses directed against foreign transgene-encoded proteins are the major determinants of the stability of gene expression following intramuscular injection of recombinant adenoviruses [232]. In addition, Juillard *et al.* showed that a single intraperitoneal immunization with a replication-defective adenovirus elicited long-term humoral and cellular immune responses against the transgene product, including transgene-specific CTLs and CD4 T helper cells secreting IL-2, but not IL-4, even as late as 7 months after immunization [120]. With transgene expression lasting up to 75 days in myocytes [188], the ability of the transgene to elicit strong, long-lasting cellular immunity suggests that adenovirus vectors delivered to the muscle may prove useful in vaccine development against tuberculosis including prime-boost vaccine strategies.

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CHAPTER 2:

ADENOVIRUS DELIVERY OF ANTIGEN 85A TO MICE AS A POTENTIAL VACCINATION STRATEGY AGAINST *MYCOBACTERIUM TUBERCULOSIS*

The aims of this study were to:

1. develop a recombinant adenovirus capable of expressing Antigen 85A (Ag85A) protein *in vivo*;
2. test the ability of the recombinant virus to induce specific immunity in mice against Ag85A; and
3. determine if the recombinant virus could protect mice against an aerosol challenge with virulent *Mycobacterium tuberculosis.*

INTRODUCTION

Tuberculosis (TB) remains one of the most frequent causes of death in humans from infectious disease worldwide [16]. The need for effective preventative measures is tragically emphasized by the acceleration of this global epidemic by HIV, as well as the appearance of multi-drug resistant (MDR) strains of *M. tuberculosis* [40]. Although the current vaccine, *Mycobacterium bovis* BCG, has been safely administered for 80 years to over three billion people, it possesses two important drawbacks, including that it interferes with the diagnostic PPD test, and that its protective efficacy varies widely from 0-80% [62]. The development, therefore, of a new vaccine offering effective and consistent protection against tuberculosis is a major priority in laboratories worldwide.

Several classical animal studies using antibody-depletion of CD4 T cells [39], adoptive transfer [45], [44], and gene-disrupted mice [4], [8], [19], have confirmed that IFNγ-secreting CD4 T cells are essential for control of the tuberculosis infection. In humans, CD4 T cells play a crucial role in protection as is evident in HIV patients, who have decreased numbers of CD4 T cells, and who are subsequently more susceptible to acute and reactivation tuberculosis [23]. In addition, people with defective IFNγ or IFNγ receptor genes are more susceptible to *M. tuberculosis* infection [47].

Over the past several years it has become increasingly clear that CD8 T cells are also important in immunity to tuberculosis, and they appear to play a strong role in protection during the chronic stage of infection [67], [65]. Studies in mice have shown that CD8 T cells migrate to the lung with similar kinetics to CD4 T cells during infection with *M. tuberculosis*, produce IFNγ, lyse infected macrophages, and in some cases were capable of suppressing intracellular bacilli growth [17], [55], [56], [5]. Furthermore, mice deficient in β2-microglobulin or TAP were significantly more susceptible to infection with *M. tuberculosis* [20], [1].

BCG vaccination has been shown to induce strong CD4 T cell [50] [24] and CD8 T cell [64] [14] responses in humans and mice in terms of IFNγ production and cytotoxcity (CTL). A vaccine superior to BCG, therefore, may require the optimal induction not only of CD4 T cell responses, but also of protective CD8 T cell responses. In order to develop a vaccine that optimally stimulates both CD4 and CD8 T cells, plasmid DNA expressing immunodominant mycobacterial antigens have been of interest in several laboratories, and several DNA vaccines have been nearly as successful as BCG in reducing the bacterial load in the organs of mice [60], [25]. In fact, vaccination with

DNA expressing Ag85A protein was shown to stimulate a broader epitopic repertoire of CD4 and CD8 T cells than that stimulated by infection with *M. tuberculosis* [15]. As CD8 T cells have been recognized as an essential cell population for the clearance of viral infections [30], strategies employing vaccinia viral vectors that express mycobacterial proteins have also been successful at enhancing CD8 and CD4 T cell responses as well as conferring protection against tuberculosis [18], [36], [72].

In recent years, adenovirus vectors have received considerable attention as candidate vaccines against several viral diseases due to their ability to stimulate CD8 and CD4 T cell responses specific for the transgene product [3], [66]. Several other factors render adenovirus a logical choice for consideration as an anti-TB vaccine including the observation that high virus titers can be easily obtained, their genome is well characterized, live adenovirus vaccines have been used previously in humans, techniques for construction are well established, and the virus can infect a variety of mammalian cells including rodent lung epithelial cells [51], [31].

In order to generate and target protective immunity in the lung against aerosol challenge with *M. tuberculosis*, a type 5, replication-defective adenovirus vector was constructed that expresses the mycobacterial protein, Ag85A. Ag85A is a highly secreted member of the Ag85 complex and constitutes a major percentage of the culture filtrate of both *M. bovis* BCG and *M. tuberculosis* [70]. Although the protective antigens for tuberculosis have yet to be precisely defined, it is widely accepted that secreted or cell wall-associated mycobacterial proteins, and not cytoplasmic proteins, are the key antigens recognized by the protective immune response [42].

Furthermore, because sensitization to secreted proteins such as Ag85A occurs very early during the mycobacterial infection [43], recognition of this antigen may represent an important strategy by the host for initial control over bacterial replication, thereby making Ag85A a logical vaccine candidate. Indeed, Ag85A has previously been shown to induce strong proliferative CD4 T cell responses including IFNγ production [26], and it has been shown that human BCG-reactive CD8 T cells react strongly against Ag85A [57]. In addition, the use of the Ag85A gene in a DNA vaccine protocol has proven to be effective against aerosol challenge with *M. tuberculosis* in the mouse model, inducing Th1-type products such as IgG2a, IFNγ, and IL-2 [25].

In these studies, we show that our adenoviral construct expresses Ag85A both *in vitro* and *in vivo*, induced IFNγ production, and when delivered by the intranasal route, conferred significant protection against a low dose aerosol challenge with *M. tuberculosis*.

MATERIALS AND METHODS

Construction of recombinant adenovirus plasmid encoding Ag85A

The Ag85A gene from *M. tuberculosis* was sub-cloned from plasmid p28A (kindly provided by Dr. John Belisle) into the adenovirus left end plasmid, called pAC (kindly provided by Dr. Christine Wilcox). Besides containing the left end sequence of the adenoviral genome, the pAC plasmid also possesses the intermediate early gene promoter from the human cytomegalovirus ($HCMV_{IE}$) to allow for high expression of Ag85A, a Simian Virus 40 (SV40) polyadenylation signal, a pUC19 polylinker site, and an ampicillin resistance gene. The Ag85A gene, along with its mycobacterial signal sequence, was isolated from p28A by digesting the plasmid with *Eco*R1. The DNA fragments were then separated by electrophoresis, and the Ag85A gene (1200 base pairs) was purified from the agarose gel using a QIAEXII Gel Extraction Kit (QIAGEN, Valencia, California). The pAC plasmid was also digested with *Eco*R1 in order to facilitate the cloning of the Ag85A gene into the adenovirus left end plasmid. The 8.8 kbp pAC plasmid representing the left end of the adenovirus genome was then resolved by gel electrophoresis on a 0.8% agarose gel, purified from the agarose as above, and treated with alkaline phosphatase (Invitrogen Corporation, Carlsbad, CA). The treated plasmid was run on an agarose gel again, and extracted as above. Now with compatible sticky ends, an overnight ligation reaction was performed between the Ag85A gene and the linearized pAC plasmid. The next day, the ligation reaction was precipitated, after which electrocompetent *Escherichia coli* cells were transformed by electroporation [35] with the ligated DNA and then plated on ampicillin-containing plates. The next day, bacterial colonies were transferred to broth, cultured, and the DNA was isolated [35]. The purified DNA was digested with *Eco*R1, and the DNA fragments were separated by electrophoresis. Diagnostic restriction enzyme digests using *Hind*III were performed on the newly ligated plasmid, named pAC85, and the new pAC85 plasmid was then amplified in *E. coli* cells and purified using a QIAGEN Maxi Prep kit. AdZ, the control virus used in these studies, expresses the reporter protein β-galactosidase. Its construction was described previously [54].

Production of recombinant adenovirus particles

To produce recombinant adenovirus particles, pAC85 was co-transfected with the pJM17 plasmid [54](kindly provided by Dr. Christine Wilcox), which contains the wild type adenovirus genome plus additional antibiotic resistance genes, into the 293 packaging cell line using Superfect (QIAGEN, Valencia, California). Briefly, 1 X 10^6 293 cells were cultured in a 25 cm^2 flask 24 hours prior to the transfection. The next day, 5 μg each of the pAC85 and pJM17 plasmids were added to a sterile tube along with 135 μl of serum-free, antibiotic-free media and 30 μl of Superfect reagent. The DNA mixture was incubated at room temperature for 10 minutes. Once the media on the 293 cells had been removed, the DNA mixture was added drop wise to the cells, which were incubated overnight at 37°C. The following day, the transfection solution was removed and the cells were maintained on regular media until the monolayer of cells showed complete cytopathic effects (CPE). At that time, the 293 cells and supernatant were harvested, followed by two rounds of plaque purification before the virus was expanded and titrated in 293 cells. Viral titers used in these studies were either $2\text{x}10^8$ pfu/ml or $1\text{x}10^9$ pfu/ml.

Viral infection of Vero cells

Vero cells were infected with the newly constructed adenovius construct expressing Ag85A (Ad85) or AdZ at a multiplicity of infection (MOI) of 10 pfu/cell. Briefly, Vero cells were plated in a 60-millimeter tissue culture dish at $2.5\text{x}10^5$ cells one day prior to infection. The viruses were resuspended at $1\text{x}10^6$ pfu/ml in 5 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS (Invitrogen Corporation, Carlsbad, CA), which was then added to the Vero cells. The infected cells were cultured at 37°C for 48 hours.

SDS PAGE and Western blot analysis

Whole cell extracts were harvested 48 hours post-infection with Ad85 or AdZ, and 10μg of cell extract were loaded onto a 12.5% SDS PAGE gel [34]. Purified recombinant Ag85A protein (0.01-1.0μg) was used as a positive control, and cell lysates from AdZ- and mock-infected cells were used as negative controls. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane [63], blocked with UNI-BLOCK (Analytical Genetic Testing Center, Inc., Denver, CO), and probed with polyclonal anti-Ag85 antibody (Lot `#8/27/97JS;` kindly provided by Dr. John Belisle) at 1:1000 for 1 hour. The blot was washed in a 1:2 mixture of 0.1% PBS-Tween and UNI-BLOCK, and then exposed to a secondary peroxidase-labeled goat anti-rabbit antibody at 1:750 (Vector Laboratories, Inc., Burlingame, CA) for 1 hour. The membrane was incubated for one minute in chemiluminescence reagent (NEN Life Science, Boston, MA) and then exposed to Kodak Elite film for 30 seconds.

Mice

Female C57BL/6 mice, 6-8 weeks old, were purchased from Charles River Laboratories, Worcester, MA. The mice were maintained under specific-pathogen-free conditions in the BL-3 biohazard facility at Colorado State University. They were given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments.

Immunizations

Mice were immunized with Ad85 or AdZ by several different routes throughout the course of this project. Aerosol immunizations were performed in a biosafety cabinet using an aerosol apparatus adapted from Katkin *et al.* [31]. Briefly, mice were placed in a regular Thoren rack cage under a BL2 safety hood. Ten milliliters of viral suspension of either Ad85 or AdZ, both at 2×10^8 pfu/ml, were placed in the cup of a PB raindrop nebulizer and an aerosol mist was generated by dry, compressed air at 8-10 liters per minute. The aerosol was introduced into the cage through corrugated plastic tubing, which was inserted into a fitted hole in the cage cover and was permitted to leave the cage through a second piece of plastic tubing placed in a hole at the opposite end of the cover. The air in the box was completely replaced by the viral aerosol within approximately 3 minutes after the start of the nebulization, at which point a visible mist filled the cage. The aerosol was continued until the viral suspension in the nebulizer ran dry.

For experiments during which the mice were awake for intranasal immunizations, mice were inoculated with 25 μl of Ad85 or AdZ (both at $2\text{x}10^8$ pfu/ml) in each nostril with a needle-free syringe. During the experiments in which the mice were anesthetized for intranasal immunizations, a 1 inch by 1 inch paper towel square, soaked with 50 μl of metophane, was sealed in a small semi-air-tight jar for 45 seconds. Mice were individually added to the jar for no more than three minutes or until a slower breath rate by the mouse could be visually detected. Twenty microliters of Ad85 or AdZ (both at $1\text{x}10^9$ pfu/ml) were gently placed under one nostril using a pipette tip. For intramuscular immunizations, mice received $2\text{x}10^7$ pfu in 50 μl of Ad85 or AdZ in one quadriceps muscle using a 30.5 gauge needle. Mice vaccinated with BCG received 10^6 cfu/200 μl in the scruff of the neck using a 26.5 gauge needle.

Detection of anti-Ag85A antibodies

Levels of anti-Ag85A or anti-β-galactosidase antibodies were determined by ELISA of individual serum samples taken from the tail veins of five mice 10, 20, and 30 days after aerosol immunization with Ad85 or AdZ respectively. A 96-well plate was coated with native Ag85 complex protein (kindly provided by Dr. John Belisle) at 500 ng/well or with β-galactosidase at 50 ng/well. Two-fold dilutions of the sera starting at 1:50 were added to the coated wells, followed by the addition of a horseradish peroxidase-conjugated, polyvalent secondary antibody (1:1000) specific for mouse IgM, IgA, IgG1, or IgG2a antibodies (The Binding Site, Birmingham, England).

Bone marrow-derived macrophages and dendritic cells (DC)

Naïve mice were euthanized in a CO_2 chamber and their femurs and tibia bones were dissected out. The ends of the bones were trimmed, and using a 26.5 gauge needle, the bone marrow was flushed out with cold complete DMEM supplemented with 10% FBS. For bone marrow-derived macrophage cultures, cells were resuspended at a concentration of 2×10^6 cells/ml and plated on a 96-well plate at 200 μl per well. The media was changed after 48 hours and again after 5 days. On day 8, the cells were pulsed overnight with 1 μg/well of Ag85 complex protein or media alone.

For bone marrow-derived DC cultures, the cells were resuspended at a concentration of 2×10^5 cells/ml in complete DMEM supplemented with 10% FBS and 2 μg/ml of GMC-SF, and then plated at 1 ml/well on a 24 well plate. The medium was changed after 48 hours, and the cells were used for immunohistochemistry after 7 days of culture at 37°.

IFNγ production *in vitro*

Spleens were removed from euthanized mice and passed through a cell strainer. Single cell suspensions were prepared at 1×10^6 cells/ml in complete DMEM supplemented with 10% FBS and added at 100 μl/well to bone marrow macrophages pulsed with antigen the previous day. Recombinant IL-2 (0.1ng/ml) was added to the cultures at 2 μl IL-2 per 1 ml of cells. The cells were cultured for 72 hours, after which they were placed in a -80°C freezer until a IFNγ ELISA assay was performed. Briefly, the thawed supernatants were added to 96 well plates coated with IFNγ capture antibody and blocked with a 1% BSA solution. After one hour of incubation at 37°C and washing, biotin-conjugated IFNγ detection antibody (Pharmingen, San Diego, CA) was added followed by streptavidin-horse radish peroxidase (DAKO Corp., Carpinteria, CA). The reaction was developed with TMB substrate (Zymed Laboratories, Inc, San Francisco, CA).

Flow Cytometry

Lung cells were isolated from each individual mouse and incubated with monoclonal antibodies labeled with fluorescein isothiocyanate [FITC], phycoerythrin [PE], peridinin chlorophyll-a protein [PerCP], or allophycocyanin [APC] at 25 μg/ml at 4°C for 30 minutes in the dark, after which the cells were washed twice in DRPMI (Irvine Scientific, Santa Ana, CA) containing sodium azide (Sigma). Cell surface markers analyzed were PE-labeled anti-CD3 (145-2C11); PerCP-labeled anti-CD4 (L3T4 clone RM4-5); and APC-labeled anti-CD8 (53-6.7). All antibodies were purchased from PharMingen, San Diego, California. Some cells were prepared for IFNγ intracellular staining by incubation at 37°C with anti-CD3 (0.1 μg/ml), anti-CD28 (clone 37.51; 1 μg/ml), and monensin (Fix/Perm kit, PharMingen) for 4 hours. Cells were stained with PerCP-anti-CD4 or APC-anti-CD8 before the permeabilization step according to the kit instructions. FITC-isotype control antibody (rat IgG1) and FITC-anti-IFNγ (XMG1.2) were incubated with the surface-stained cells for 30 minutes, and the cells were washed twice and resuspended in DRPMI prior to analysis. Acquisition was performed on a FACscalibur (Becton-Dickinson, Mountain View, CA), and data was analyzed using CellQuest software (Becton-Dickinson). Cells were gated on lymphocytes by forward and side scatter according to their characteristic scatter profile. The percentage of each cell population within the lymphocyte gate was determined using dot plots and drawing quadrants on each plot to distinguish the specifically labeled cell subsets. Absolute numbers for each given cell subset were calculated from the flow cytometery data and total cell counts on the hemacytometer.

Immunohistochemistry

Vero cells were plated in a 24 well plate at 2.5 X 10^4 cells/well 24 hours prior to infection. The next day, the cells were infected at an MOI of 10 with Ad85 or AdZ. After 48 hours, the cells were fixed with cold methanol, and polyclonal anti-Ag85 antibody or normal rabbit serum (NRS) was added to the appropriate wells at 1:400. Goat anti-rabbit IgG linked to peroxidase (Vector Laboratories, Inc., Burlingame, CA) was used at 1:200 to detect the presence of antibody-bound Ag85A.

After 7 days of incubation, bone marrow-derived DC [22] were infected with either Ad85 or AdEGFP (expresses enhanced green fluorescent protein), both at an MOI of 10. The Ad85-infected DC were fixed in cold methanol 48 hours after infection and staining as above was performed. The AdEGFP-infected DC were fixed in cold methanol and viewed directly under a fluorescent microscope.

Slide sections prepared from frozen lung tissue taken from mice 72 hours after aerosol immunization with AdZ were stained with Xgal substrate according to the kit directions (Biogenex, San Ramon, CA), and then counter-stained with Nuclear Fast Red. Seventy-two hours after immunization by aerosol with Ad85, lung tissue was fixed in 10% formalin, blocked in paraffin, and sectioned onto slides. The sections were subjected to an antigen retrieval system (Biogenex, San Ramon, CA) and then stained with rabbit anti-Ag85 serum followed by secondary anti-rabbit IgG antibody conjugated to peroxidase (Caltag Laboratories, Burlingame, CA). The reaction was developed with AEC (Immunogenex, San Ramon, CA), and the cells were counterstained with Meyer's Hematoxilyn (Biogenex, San Ramon, CA).

Animal challenge studies.

Thirty days following the last immunization in each experiment, mice were challenged by low-dose aerosol exposure with *M. tuberculosis* strain H37Rv using a Glas-Col (Terre Haute, Ind.) aerosol generator calibrated to deliver 50-100 bacteria into the lungs. Bacterial counts in the lungs were determined by plating serial dilutions of individual whole-organ homogenates on nutrient 7H11 agar and counting colony-forming units (cfu) following 3 weeks incubation at 37°C.

RESULTS

Production of Ad85

A recombinant adenovirus was constructed to encode Ag85A with its bacterial signal sequence. Flanked on either side with *Eco*R1 restriction sites, the Ag85A gene along with its mycobacterial signal sequence, was isolated from p28A by digesting the plasmid with *Eco*R1 (Figure 2.1). After ligation of the 1.2 Kbp fragment with the adenovirus left-end plasmid, pAC, an *Eco*R1 digestion was run in order to confirm that the Ag85A gene had been cloned into the adenoviral left end plasmid. The restriction digest yielded two bands of the expected sizes: one at 8.8 kbp for the pAC plasmid, and one at 1.2 kbp for the Ag85A gene (Figure 2.2). A diagnostic digestion using *Hind*III was performed on pAC85 to confirm the correct orientation of the Ag85A gene under the CMV_{IE} promoter (Figure 2.3). Recombinant adenovirus was produced in 293 cells by co-transfection with pAC85 and pJM17 (the entire adenovirus genome) between which occurred an homologous recombination event resulting in the packaging of recombinant adenovirus particles (Figure 2.4).

In vitro expression of Ag85A

In order to test the ability of the recombinant adenovirus to express Ag85A protein *in vitro*, Vero cells were infected and expression was analyzed by Western blot analysis using a polyclonal anti-Ag85 antibody. A protein of approximately 35 kDa was expressed by the Ad85-infected Vero cells, while no protein bands were detected in uninfected cells or in cells infected with AdZ (Figure 2.5). The Ag85A protein expressed in the Ad85-infected cells migrated on the gel at a slightly higher molecular weight than the purified Ag85A standards, possibly due to post-translational modifications put forth by the eukaryotic cells such as glycosylation, as has been reported in studies involving the expression of recombinant Ag85A in DNA-transfected RD cells [25].

In vivo expression of Ag85A and β-galactosidase

Immunohistochemistry was performed on lung tissue from mice that were aerogenically infected with Ad85 or AdZ to determine whether the adenovirus constructs were capable of expressing Ag85A or β-galactosidase, respectively, *in vivo.* As shown in Figure 2.6, Ad85 was capable of expressing Ag85A protein *in vivo,* with the recombinant protein concentrated mostly in the vascular endothelium. Likewise, AdZ expressed β-galactosidase *in vivo*, however, the reporter protein was localized solely to the airway epithelium. No recombinant protein was detected in lung sections from naïve mice, and normal rabbit serum did not stain Ag85A protein in lungs taken from Ad85-infected mice.

Humoral immune responses

In order to determine if the Ag85A protein expressed by the recombinant adenovirus was detected by the immune system, individual sera from vaccinated mice

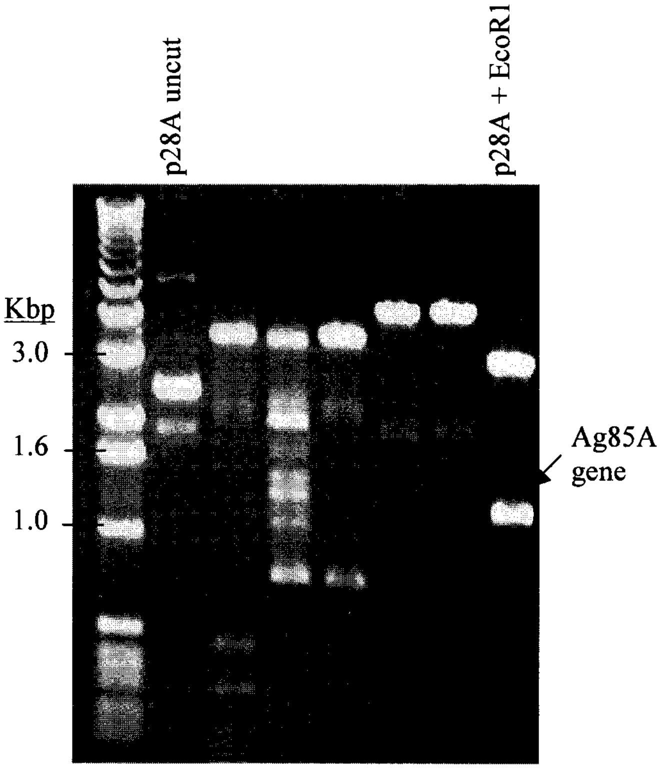

Figure 2.1 The Ag85A gene was isolated from p28A.
The Ag85A gene was removed from p28A by digesting with EcoRI. The 1.2 Kbp fragment represents the Ag85A gene, and the 2950 Kbp fragment represents the linearized p28A plasmid. The 1.2 Kbp band was cut out of the 0.8% agarose gel and prepared for ligation into pAC.

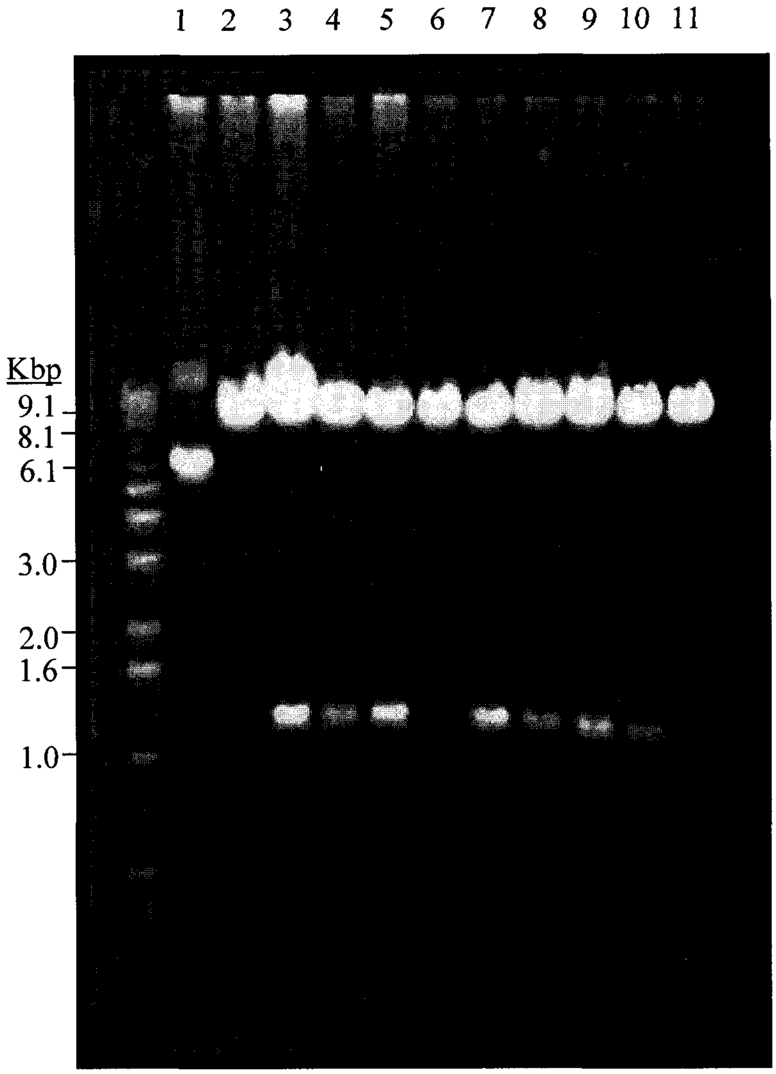

Figure 2.2 The Ag85A gene was successfully cloned into the adenovirus left end plasmid, pAC.

The Ag85A gene was cloned into the EcoR1 site in the polylinker region on the adenovirus left end plasmid, pAC. A restriction digest was performed using EcoR1 in order to confirm the presence of the 1.2 Kbp gene in the plasmid. The DNA fragments were then separated by electrophoresis on a 0.8% agarose gel and visualized under ultraviolet light after staining the gel with ethidium bromide. The pAC plasmid and the Ag85A DNA fragments migrated on the gel to their expected sizes 8.8 Kbp and 1.2 Kbp respectively. A linear, 1 Kbp DNA ladder was run simultaneously to verify the sizes. Lane 1 = uncut pAC. The pAC DNA in Lanes 2-11 were cut with EcoR1. Lane 2 = empty pAC. DNA in all lanes except 6 and 11 contain the Ag85A gene.

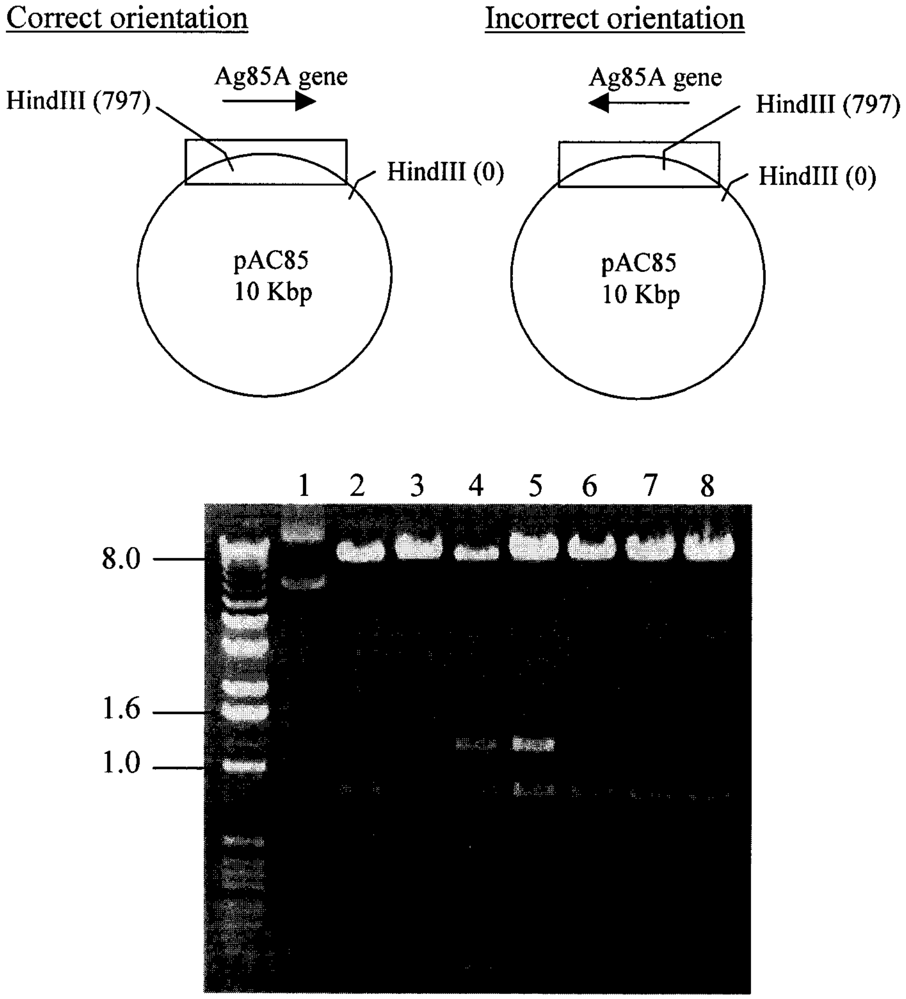

Figure 2.3 Correct orientation of Ag85A in pAC85 verified.
A diagnostic restriction enzyme digest using HindIII was performed on pAC85 in order to verify that the Ag85A gene was in the correct orientation. HindIII digestion of the correct plasmids yielded bands of the expected sizes, approximately 9200 bp and 797 bp as seen in lanes 2, 6, 7, and 8 of the 0.8% agarose gel.

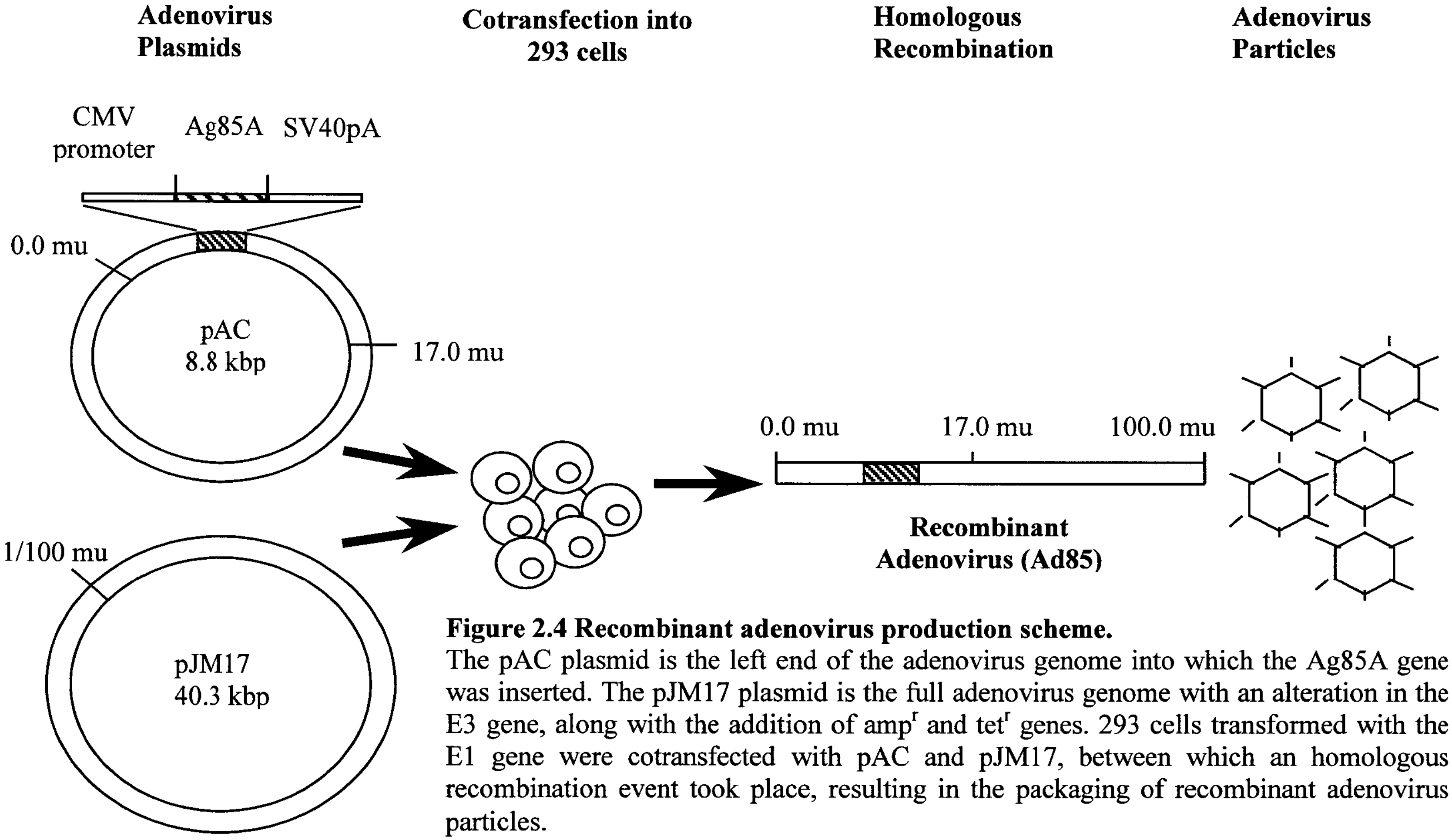

Figure 2.4 Recombinant adenovirus production scheme.
The pAC plasmid is the left end of the adenovirus genome into which the Ag85A gene was inserted. The pJM17 plasmid is the full adenovirus genome with an alteration in the E3 gene, along with the addition of amp^r and tet^r genes. 293 cells transformed with the E1 gene were cotransfected with pAC and pJM17, between which an homologous recombination event took place, resulting in the packaging of recombinant adenovirus particles.

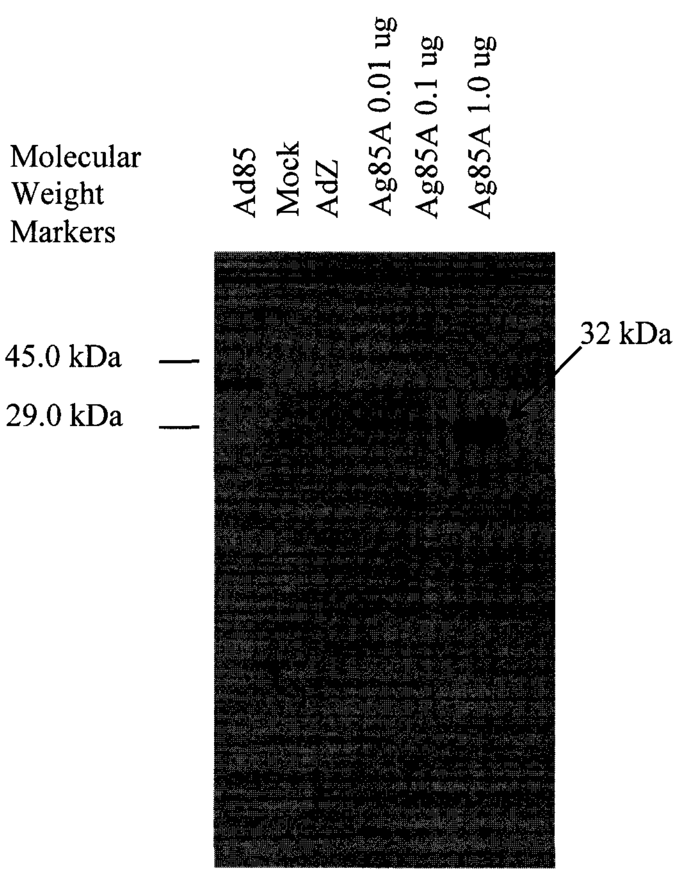

Figure 2.5 Adenovirus vector expressed Ag85A *in vitro.*
Vero cells were infected with adenovirus over-expressing Ag85A. Purified recombinant Ag85A protein was used as a positive control. Lysates from AdZ and mock-infected cells were used as negative controls. Expression of Ag85A was analyzed by Western blot using a polyclonal anti-Ag85A antibody.

were collected. Antibodies reactive against Ag85A or β-galactosidase were measured in sera from mice immunized by aerosol with Ad85 or AdZ respectively, increasing in titer over time (Figure 2.7A, B). No Ag85A-specific antibodies were detected in the sera from naïve mice. The anti-Ag85A sera showed statistically significant increases in IgG1, IgG2a and IgA isotypes from day 10 to day 30 after aerosol infection with Ad85, with a predominance of IgG1 by day 30 (Figure 2.7A). The anti-β-galactosidase sera showed a statistically significant increase in IgG2a antibody from day 10 to day 30 after immunization with aerosolized AdZ (Figure2.7B). Following aerosol challenge with *M. tuberculosis*, antibody profiles in pooled sera from Ad85-vaccinated mice switched from a predominately IgG1 response to a predominately IgG2a response against the Ag85A protein (Figure 2.7C).

Cell-mediated immune response

In order to determine if the Ad85 vaccine induced an Ag85A-specific cell-mediated immune response when delivered intranasally, spleen cells from vaccinated mice were restimulated with bone marrow-derived macrophages pulsed with Ag85A protein *in vitro*. ELISA analysis of the supernatants from the stimulated cell cultures showed that Ad85 induced more IFNγ than mice vaccinated with AdZ or saline (Figure 2.8C).

Flow cytometry analysis was also performed to assess whether intranasal immunization with Ad85 would affect the amount of IFNγ produced in the lungs of vaccinated mice before challenge with *M. tuberculosis*. Cells isolated from the lungs of mice 2 weeks after the last vaccination were stained with fluorescently labeled anti-bodies against surface markers CD4 or CD8, and against intracellular IFNγ, followed

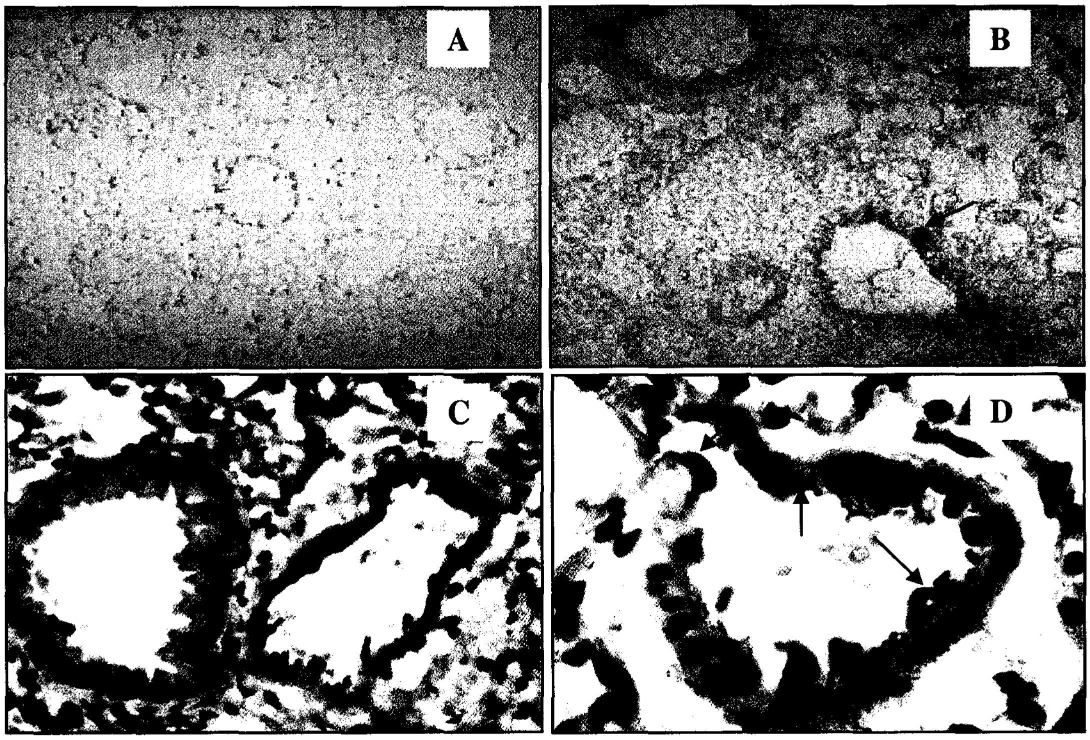

Figure 2.6 Ad85 expressed Ag85A protein in mouse lung tissue.
C57BL/6 mice were immunized with AdZ or Ad85 by the aerosol route. AdZ-infected lung tissue was collected from the mice 72 hours post-vaccination, placed in O.C.T., and snap frozen in liquid nitrogen. Sections 10 microns thick were then stained with X-gal substrate to detect the production of β-galactosidase, which appears blue upon staining. Here, β-galactosidase was expressed in the airway epithelial cells by the virus vector. Slides in A and B are at 200X magnification and counter-stained with Nuclear Fast Red. **(A)** Uninfected lung tissue, **(B)** AdZ-infected lung tissue. Paraffin blocks were prepared from Ad85-infected lung tissue that was collected 72 hours post-vaccination and then fixed in 10% formalin. Tissue sections 10 microns thick were stained with rabbit anti-Ag85 serum followed by secondary antibody conjugated to HRP. The reaction was developed with AEC, and the cells were counterstained with Meyer's Hematoxilyn. **(C)** Uninfected lung tissue at 40X **(D)** Ad85-infected lung tissue at 100X

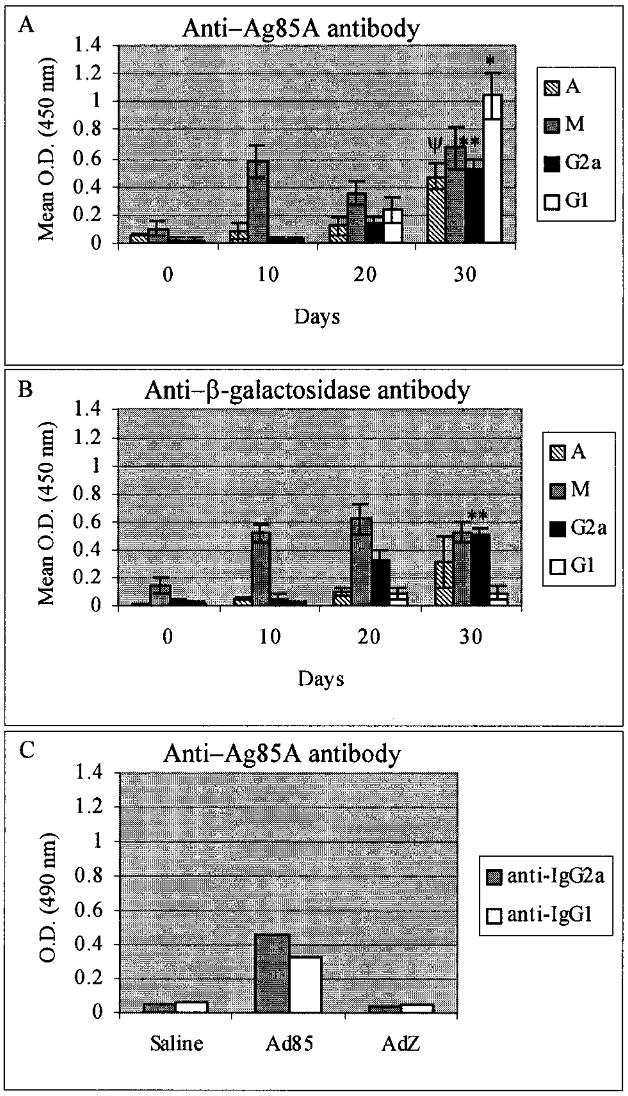

Figure 2.7 Ad85 induced an IgG1-type antibody response against Ag85A protein *in vivo*. Sera were collected from the tail veins of mice 10, 20 and 30 days after aerosol infection by (A) Ad85 or (B) AdZ. The sera were tested by ELISA for the production of IgA, IgM, IgG2a, and IgG1 anti-Ag85A antibodies in (A) or anti-β-galatosidase antibodies in (B). In (C), ELISA plates were coated with Ag85 protein, and sera were pooled from mice that were immunized i.n. with Ad85 or AdZ and subsequently challenged with *M. tuberculosis* (n=5).

**p < 0.001 versus Day 10 IgG2a, *p < 0.01 versus Day 10 IgG1, and $^{\Psi}$p < 0.01 versus Day 10 IgA. p values were calculated using a two-tailed Student's *t*-Test. Error bars represent +/- SEM where n=5 in (A) and n=4 in (B).

followed by flow cytometry analysis. Figure 2.8A shows that Ad85 vaccination increased the percentage and total number of CD8 IFNγ T cells in the lungs compared to the unvaccinated and the AdZ controls, although the increase was not statistically significant. BCG induced a slightly higher percentage of CD8 IFNγ T cells, however, the absolute number of this subset was equivalent to that which was induced by the Ad85 vaccine (Figure 2.8B). While BCG induced a significantly higher percentage and number of CD4 IFNγ T cells (data not shown), there were no differences in the percentage this T cell subset between the unvaccinated and Ad85-vaccinated groups (Figure 2.8A).

Protective efficacy in mice

To measure Ad85 vaccine efficacy, mice were immunized with recombinant virus by several routes including intranasally, intramuscularly, and by aerosol. Mice were subsequently challenged with aerosolized *M. tuberculosis* H37Rv, and mycobacteria were quantified in the lungs. In each separate experiment, BCG was administered to mice as a positive control, and AdZ and saline were used as negative controls. As shown in Figure 2.9B, statistically significant protection (p=0.04 by Student's t-Test) against challenge was observed only when the Ad85 vaccine was administered intranasally to anesthetized mice. In Figure 2.9C, unanesthetized mice were given Ad85 intranasally three times at three week intervals, and although the protection was not statistically significant (p=0.06), the bacterial load in the lungs was decreased compared to the saline control group. Likewise, when mice were immunized by the aerosol route, a trend of protection resulted (Figure 2.9A, p=0.09). Ad85, however, did not confer protection in the lungs of mice vaccinated by the intramuscular route (Figure 2.9D p=0.96).

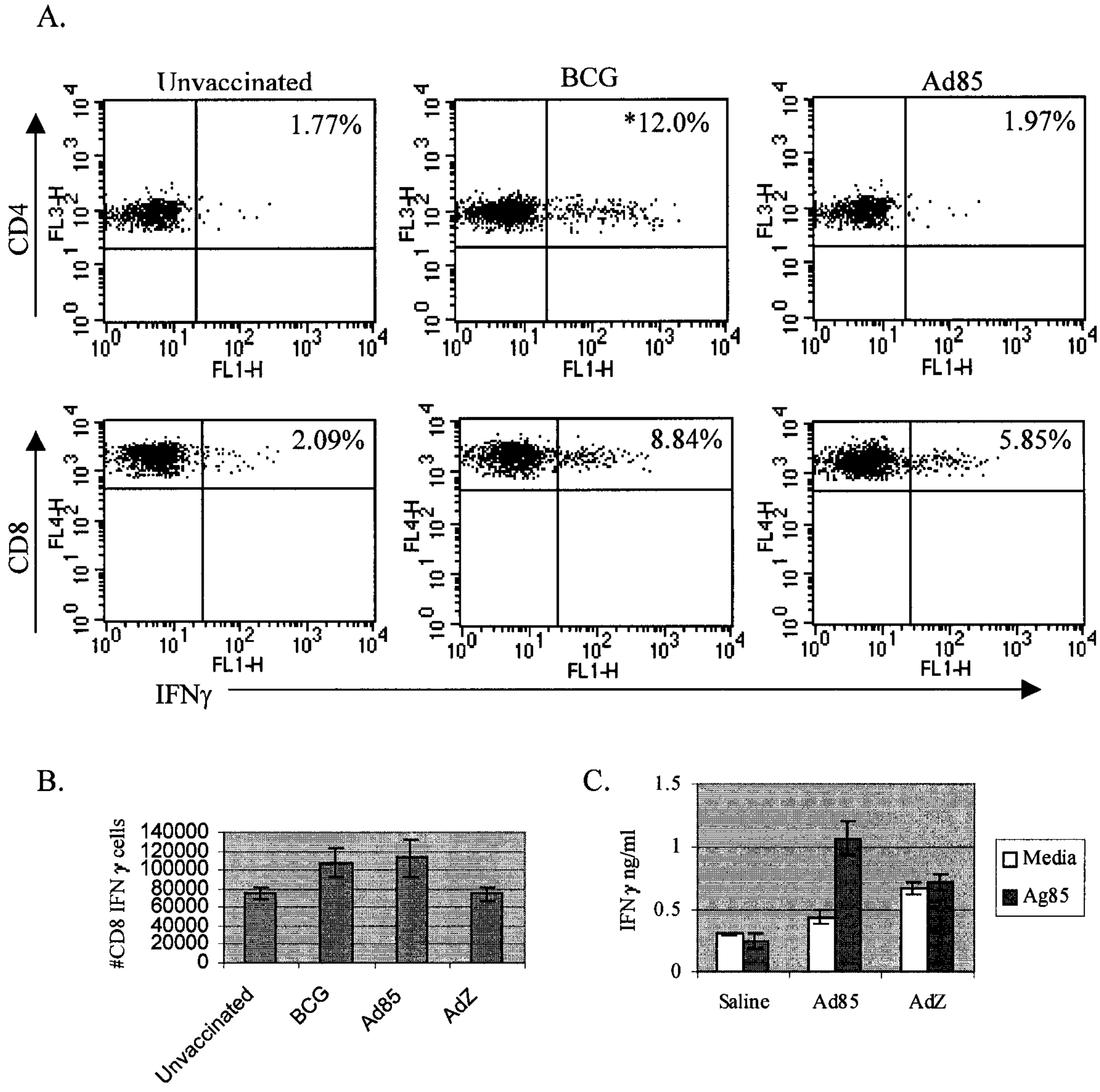

Figure 2.8 Ad85 vaccination induced IFNγ T cell responses. Anesthetized mice were immunized twice with Ad85 i.n. 3 weeks apart, or once with s.c. BCG. Lungs cells from individual mice were isolated, stained for CD8, permeabilized, and then stained for intracellular IFNγ. Cells were gated on CD8 T cells. (A) Representative dot plots depicting the mean percentages of CD4 and CD8 T cells producing IFNγ. (B) Data are expressed as the absolute number of CD8 that produce IFNγ. These data are based on whole lung samples. (C) Spleen cells were isolated, cultured with macrophages pulsed with Ag85 protein or with media alone for 72 hours. Supernatants were frozen until an IFNγ ELISA was performed. Naïve mice or AdZ-vaccinated mice were used as negative controls.
Error bars represent +/- SEM where n=4.

*p value < 0.05 versus the saline group using a two-tailed Student's *t*-Test.

Ad85 infects dendritic cells *in vitro*

To determine if AdEGFP, an adenovirus construct similar to Ad85 but expressing green fluorescent protein, was capable of infecting dendritic cells, bone-marrow derived dendritic cells were infected with the virus and subsequently viewed under a fluorescent microscope. Figure 2.10A shows that AdEGFP successfully infected dendritic cells and was able to express recombinant green fluorescent protein in these cells. In order to test the ability of Ad85 to infect dendritic cells and express Ag85A therein, bone marrow-derived dendritic cells were transduced with Ad85. Anti-Ag85 antibody positively stained Ad85-infected dendritic cells (Figure 2.10B), while no staining was visible with the isotype control antibody (Figure 2.10C).

DISCUSSION

The data presented here indicate that the aims of this study were met. First, we developed an adenoviral vector that expressed the major mycobacterial protein, Ag85A. Figures 2.1, 2.2, and 2.3 show that a recombinant adenovirus containing the mycobacterial Ag85A gene was successfully cloned, and the expression of Ag85A both *in vitro* and *in vivo* (Figures 2.5 and 2.6D, respectively) indicate that the Ad85 construct was capable of expressing the Ag85A gene in eukaryotic cells after immunization by the aerosol route. Second, strong B and T cell responses were induced by the Ad85 vaccine when it was delivered intranasally, thereby indicating that the adaptive immune response was induced by this vaccine strategy. For instance, spleen cells from mice that were vaccinated with intranasal Ad85 produced IFNγ after *in vitro* stimulation with Ag85A protein, and we detected an increase in CD8 T cells capable of producing IFNγ in the lungs after Ad85-immunization. Third, the Ad85 vaccine conferred significant protection in mice that were

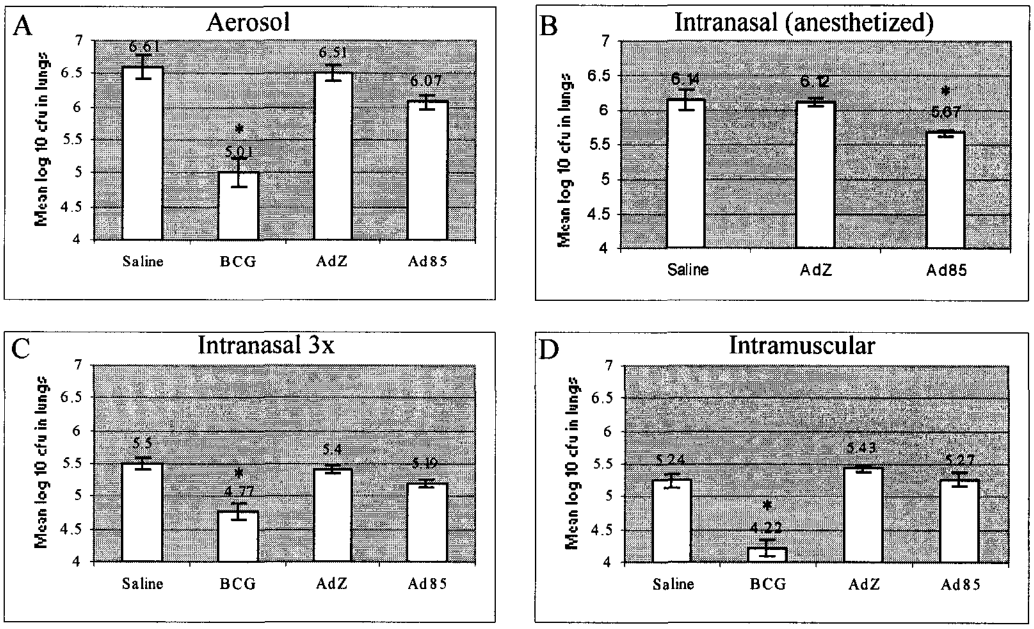

Figure 2.9 Ad85 delivered intranasally significantly protected mice against *M. tuberculosis*. Ad85 was delivered by three different routes in four separate experiments, and upon aerosol challenge with *M. tuberculosis* H37Rv, only the anesthetized mice that received Ad85 intranasally were significantly protected. (A) Mice were subjected to aerosolized Ad85 at 2.0 X 10^8 pfu/ml for 30 minutes. (B) Anesthetized mice were inoculated intranasally with Ad85 at 1.0 X 10^9 pfu/ml. (C) Mice were given Ad85 at 2.0 X 10^8 pfu/ml intranasally, three times at three week intervals. (D) Mice were given one dose of intramuscular Ad85 at 2.0 X 10^7 pfu.

*p value < 0.05 versus the saline group using a two-tailed Student's *t*-Test. Error bars represent +/- SEM where n=5.

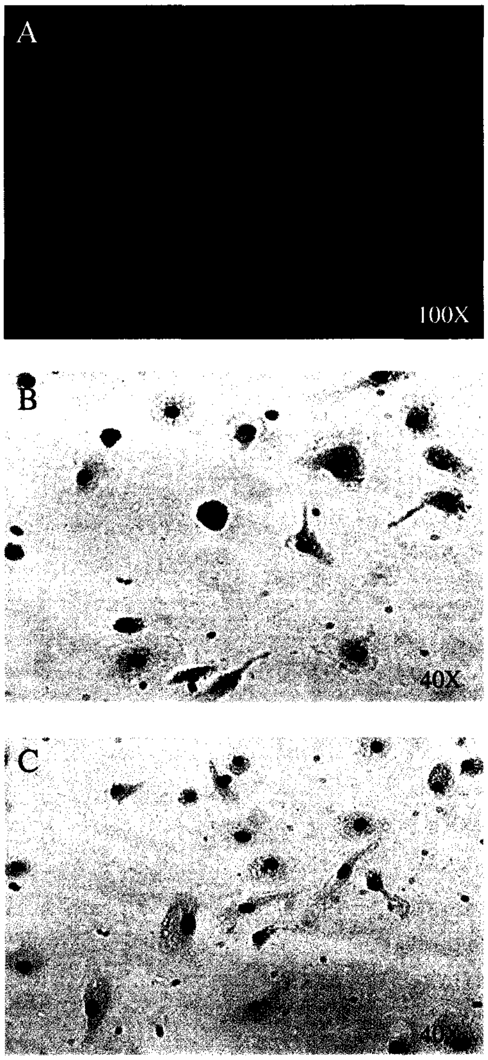

Figure 2.10 Ad85 can infect dendritic cells *in vitro*. In A) bone marrow-derived DC were infected with AdEGFP and visualized under a fluorescent microscope 48 hours post-infection. In B) and C), bone marrow-derived DC were infected with Ad85 and then fixed in cold methanol 48 hours post-infection. Immunohistochemistry was performed to determine the ability of the Ad85 virus to infect DC and to express recombinant Ag85A protein in vitro. In B) the cells were stained with a polyclonal antibody against Ag85A followed by secondary staining with a goat anti-rabbit IgG antibody conjugated to HRP. In C) the cells were stained with normal rabbit serum followed by the same secondary antibody in B). The reactions were developed with AEC substrate.

immunized by the intranasal route followed by aerosol infection with *M. tuberculosis* H37Rv, while the intramusclar and aerosol routes of immunization were not as effective. The overall results from this study indicate that intranasal delivery of Ad85 may be a promising strategy in the development of an effective vaccine against tuberculosis.

An effective vaccine against tuberculosis must be one that sensitizes not only memory CD4 T cells that can produce IFNγ [46], [8], but also memory CD8 T that can make IFNγ and/or express lytic functions, as research over the past decade strongly suggests that CD8 T cells also play an important role in controlling tuberculosis infection [41], [59]. While the generation of specific, memory CD4 and CD8 T cells was not dissected out in this study, we looked at whether the Ad85 vaccine induced T cell responses based on 1) antibody isotype profiles, 2) IFNγ production, and 3) protection conferred after aerosol with *M. tuberculosis*.

In order to successfully protect against tuberculosis infection, we hypothesized that Ad85 would necessarily have to prime specific CD4 T cells, and in order for this to happen, we proposed that the aerosolized Ad85 would infect lung epithelial cells, in which copious amounts of Ag85A protein would be produced under the CMV promoter. Speculation arises at this point because we were uncertain as to whether the bacterial signal sequence could traffic the Ag85A protein out of the eukaryotic cell. If secretion from the lung epithelial cells were possible, it is reasonable that resident lung dendritic cells would phagocytose the extracellular protein, migrate to regional lymph nodes, and present epitopes of the Ag85A protein on class II MHC molecules to specific, naïve CD4 Th1-type cells. The fact that the Ad85 vaccine generated strong antibody responses in Figure 2.7A lends support to the idea that secreted Ag85A protein should have been available for phagocytosis by lung dendritic cells. In addition, some recombinant protein would presumably remain within the cytoplasm, and could then enter the endogenous pathway for presentation on class I MHC molecules to CD8 T cells. Following initial sensitization, Ag85A-specific CD4 and CD8 T cells would proliferate as effector cells, and some would differentiate into long-term memory cells [2].

The production of Ag85-specific antibodies indicates that Ag85A was detected by the acquired immune system, with isotype analysis demonstrating the production of IgA, IgG2a, and IgG1 anti-Ag85A antibodies increasing in titer over time (Figure 2.7A). While the induction of antibodies was not our intended goal, as it is controversial as to whether B cells play a significant role in protection against tuberculosis [28], isotype analysis of the antibodies generated by vaccination with Ad85 can offer some insight as to whether a Th1- or Th2-dominate response was induced. For instance, Th2-type immunity is often associated with the release of IL-4, which induces class switching in B cells from IgM to IgG1 antibody [37], [32]. On the other hand, IFNγ has been shown to stimulate class switching from IgM to IgG2a, which is therefore associated with a Th1-type response [58]. The predominate production of IgG1 anti-Ag85A antibodies here implies that Ad85 immunization induced a dominate Th2-type response in the mice. In further support of this, IgA production is more often associated with Th2-type immunity than Th1-type immunity [33], and as Figure 2.7A shows, Ag85A-specific IgA was elevated in Ad85-immunized mice. Interestingly, sera taken from Ad85-immunized mice that were subsequently infected with *M. tuberculosis,* showed a predominant IgG2a anti-Ag85A response compared to IgG1, indicating that the TB infection induced a switch in immunity from a Th2- to a Th1-type response. In contrast, the control virus, AdZ, induced predominantly more IgG2a against β-galactosidase compared to IgG1 before challenge with *M. tuberculosis*, suggesting that a stronger Th1-type response was induced by AdZ (Figure 2.7B).

Although Ad85 induced a predominant Th2-type antibody profile after vaccination, the presence of IgG2a in the sera of Ad85-immunized mice and the predominant presence of IgG2a in the sera of Ad85-immunized mice after TB challenge, suggests that Ad85 induced a mixed Th1/Th2 phenotype, and was therefore perhaps capable of inducing IFNγ. In support of this, spleen cells isolated from Ad85-immunized mice produced IFNγ when re-stimulated with soluble Ag85 protein *in vitro* (Figure 2.8C), suggesting that Ag85A-specific CD4 T cells had been stimulated. However, IFNγ responses by CD4 T cells were not detected in the lungs of Ad85-immunized mice two weeks after immunization compared to BCG-vaccinated mice (Figure 2.8A, B). This suggests that protective CD4 T cells may not have migrated to the lungs early during infection or that the frequency of Ag85A-specific CD4 T cells was not sufficient to confer a protective response in the lungs. However, Figure 2.8 (A, B) shows that there was an increase, although not statistically significant, in the number and percentage of CD8 IFNγ T cells in the Ad85-immunized mice compared to the unvaccinated group, indicating that Ad85 was capable of activating CD8 T cells in the lung. Whether this CD8 T cell response was Ag85A-specific could be determined by performing a cytotoxicity assay.

While the Ad85 vaccine was able to stimulate antibody and T cell responses, this immunogenicity must translate into protection, and the Ad85 vaccine, though it elicited statistically significant protection in the mice, was capable of reducing the bacterial load in the lungs by only 0.5 $\log_{10}$ compared to unvaccinated controls (Figure 2.9B). BCG, on the other hand, normally confers approximately 1 $\log_{10}$ of protection in mice against TB challenge compared to unvaccinated controls (Figures 2.9A, C, and D) and is known as the gold standard in vaccine development. A new vaccine, therefore, must elicit protection that surpasses this gold standard.

A number of explanations may account for the partial protection elicited by Ad85. First, perhaps Ad85 initially generated a stronger Th2-type response, which has been associated with the production of cytokines such as IL-4 [61], IL-5 [53], IL-10 [32], and IL-13 [13], all of which have been associated with the down-regulation of Th1-type immunity. IL-10 for instance, has been shown to inhibit the expression of IL-12, a cytokine necessary for differentiation of Th1 cells. It has also been reported that IL-10 can inhibit the expression of other inflammatory cytokines such as TNF-α, as well as down-regulate costimulatory molecules and class II MHC on APCs [38]. If, therefore, Ad85 initially induced a skewed Th2-type response in the lungs, and IL-10 were in fact generated, presentation of Ag85A peptides by dendritic cells to CD4 T cells capable of producing IFNγ may have been reduced. Furthermore, the production of IL-10 has also been shown to prevent the migration of dendritic cells to lymph nodes, which in the case of this vaccination strategy, could have resulted in fewer Ag85A-specific CD4 T cells clones that were capable of producing IFNγ and trafficking back to the lung [10]. In contrast, BCG has been shown to stimulate strong IFNγ responses early after vaccination [52], thereby favoring the activation and development of Th1-type cells, which then may participate in the suppression of Th2-type cells via the production of IFNγ [12] and subsequently protect against TB.

Along these same lines, data from several studies have suggested that mucosal tissues, such as the pulmonary and gastrointestinal tracts, favor the induction of Th2-dominated responses unless a strong proinflammatory signal is present such as that from infection with live *M. tuberculosis* [7], [29], [6]. Perhaps the Ag85A-specific T cells capable of producing IFNγ in the spleen were suppressed by Th2-type cytokine signals present in the lung environment, or perhaps Ad85 did not induce a strong enough pro-Th1 signal to overcome the dominant Th2-type environment in the lung.

Because conflicting reports exist as to whether B cells play a significant role in protecting mice against TB challenge [68], [28], and because Ad85 induced a significant antibody response, we must consider that antibodies may have played a role in the partial protection conferred by the intranasal delivery of Ad85. Daffe and Etienne showed that Ag85 is present in the capsule of the tubercle bacilli, therefore, it seems plausible that Ag85-specific immunoglobulins could have mediated ADCC, as well as the classical complement pathway, thereby playing a role in the early control of the TB infection [9]. Another way that Ag85A-specific antibody may have played a role in protection was by the induction of IgA. In this study we saw a significant increase in anti-Ag85A IgA over time (Figure 2.7A) in the serum. This leaves open the question whether anti-Ag85A IgA may have played a role in generating mucosal immunity against TB in the lungs. IgA has been shown to exert mucosal immunity against infectious agents, including intransally-delivered adenoviral recombinants [71], by a number of ways such as through the inhibition of bacterial adherence as well as by neutralizing viruses inside infected epithelial cells [48].

The other possibility is that Ag85A-specific antibody played no significant role in conferring protection upon Ad85-immunized mice, and that T cells were the mediators of protection. The Ag85A-specific response exerted by CD4 T cells, however, may have been inhibited somewhat due to the fact that anti-Ag85A antibodies were produced. Although the production of Ag85A-specific antibody suggests that recombinant Ag85A was most likely secreted, and therefore should have been available to lung dendritic cells as was mentioned above, these anti-Ag85A antibodies may have blocked the presentation of Ag85A peptides by dendritic cells to CD4 T cells. The CD4 T cells, therefore, may have not been able to exert as strong an IFNγ response had the antibody production been absent. Interestingly, Ag85A-specific antibodies were undetectable in BCG-vaccinated mice before and after TB infection (data not shown), suggesting that antibodies against Ag85A, when present, may have some sort of deleterious effect in regard to protection against TB. Others have also reported that BCG does not elicit significant antibody production against Ag85A [70].

Because the amount of protection that was generated by the Ad85 vaccine was considerably lower than what is usually generated by BCG, and since viral infections generally elicit class I MHC-restricted CD8 T cell responses, it seems reasonable to speculate that CD8 T cells, rather than CD4 T cells, may have been the protective cell population induced by this vaccine strategy. Furthermore, the fact that Ad85 induced an increased number of CD8 IFNγ T cells, but not CD4 IFNγ T cells, in the lung after vaccination indicates that CD8 T cells, more so than CD4 T cells, played a role in protection in the lungs. In this regard, it is known that virally infected dendritic cells can present viral antigens on class I MHC to CD8 T cells and activate them directly [27].

Figure 2.10 shows that both AdGFP and Ad85 were capable of infecting bone marrow-derived dendritic cells *in vitro*. It is reasonable, therefore, in the context of this immunization strategy to imagine that Ad85-infected lung dendritic cells could potentially present Ag85 epitopes to naïve CD8 T cells in the draining lymph nodes following intranasal administration of the vaccine. These newly activated CD8 T cells may then proliferate and migrate to the lung where they would encounter more Ag85A presented by infected airway epithelial cells and infected lung dendritic cells. After further expansion, some of these cells may then proceed to express memory phenotypes, so that upon challenge with *M. tuberculosis*, they would be recruited back to the lung to put forth their protective effect.

The route of immunization seemed to have a profound effect on the protective efficacy of the Ad85 vaccine. For example, in Figure 2.9D, intramuscular delivery of Ad85 conferred no protection on the mice whatsoever. Perhaps this was due to a lack of viral entry receptors on muscle cells, although recombinant adenoviruses have been shown to infect muscle cells and even protect mice against *Leishmania* infection [49], [21]. The cellular adenoviral entry molecule, called CAR (coxsackie-adenovirus receptor), is mainly located on nonciliated cells of the upper respiratory tract, therefore it is not surprising that intranasal delivery of the virus seemed to work the best (Figure 2.9B and C). Another possible reason that Ad85 was not as effective via the intramuscular route compared to the intranasal and respiratory routes could be that the vaccine was not immunogenic in the muscle, perhaps because this tissue is not as equipped or efficient as mucosal tissues in presenting antigen to T cells. For instance, the mucosal environment of the respiratory tract provides ready access to lymph tissue such as the MALT, BALT, LALT, and NALT, thereby allowing for efficient antigen presentation by APCs to T cells [11].

While Ad85 elicited some degree of protection when it was delivered either by the aerosol route or by the intranasal route, intranasal immunization only provided statistically significant protection ($p = 0.04$) when the mice were anesthetized. Anesthetized mice demonstrated a visibly decreased breath rate, presumably allowing for better uptake of the virus by cells of the nasopharynx. When the vaccine was administered intranasally to mice that were awake, swallowing may have led to oral rather than respiratory delivery. Although delivery by the aerosol apparatus was less efficient at generating protection, some protection was evident by this route. This lesser extent of protection could have been due to lower expression of CAR in the lower respiratory tract, which has been shown to be harder to infect [69]. This is not to say that adenovirus is unable to infect airway epithelial cells in the lung, because several studies, including this one, have shown that it is possible (Figure 2.4) [31]. However, in order to infect a higher number of cells in the lung, thereby increasing the odds that the target antigen will induce an adequate immune response, the starting viral inoculum may need to be much higher than what was used in the aerosol here (2×10^8 pfu/ml). Using an almost identical aerosol apparatus as the one used here, Katkin *et al.* were able to uniformly infect almost all the cells in the airway epithelium of the lung, however, their starting virus inoculum was 1.6×10^{10} pfu/ml, almost 100 times more concentrated than the Ad85 used in our study [31]. Quantitative polymerase chain reaction (PCR) could be conducted in order to test the level of infectivity by Ad85 in this study.

All in all, the fact that aerosol delivery, either by introducing the Ad85 virus directly into the lungs of mice or by the intranasal route, conferred some degree of protection against aerosol challenge with *M. tuberculosis* suggests that the cells responsible for eliciting that protection were perhaps specific effector or memory cells proficiently expressing adhesion molecules that would allow them to traffic back to the lungs after tubercle infection where Ag85A presentation by infected macrophages would be occurring. In support of this theory, it has been proposed that memory T cells exhibit a preference for migration to the tissue in which initial T cell priming occurred, here that tissue being the lungs or the lymph nodes draining the lungs [2].

A number of questions still need to be answered regarding the protective efficacy of the Ad85 vaccine when it is delivered intranasally to anesthetized mice. First, does the vaccine elicit specific CD4 and CD8 T cell responses such as IFNγ production in the lungs? This could be accomplished by isolating CD4 and CD8 T cells from the lungs of Ad85-immunized mice about 14 days after vaccination, stimulating the cells *in vitro* with Ag85 protein-pulsed APCs, and using flow cytometry to stain for activation markers and intracellular IFNγ. Staining at a much later time point could be done in order detect specific T cells expressing a memory phenotype. It would also be interesting to determine if dendritic cells isolated from the lungs and draining lymph nodes of Ad85-immunized mice are able to present Ag85A peptides to Ag85A-specific CD4 and CD8 T cell clones.

In summary, this vaccine strategy was successful in that significant protection was generated in mice against a low dose aerosol challenge with *M. tuberculosis*, however, the protection was not as high as that which is generally elicited by the current BCG vaccine. In order to improve the protective efficacy of the Ad85 vaccine, further studies could be conducted that use this recombinant virus expressing Ag85A perhaps as well as a cytokine such as IL-12 in order to skew initial priming toward a Th1-type response in the lungs. Additionally, the fact that BCG induces immune responses against several different protective antigens, it may be useful to develop an adenovirus that encodes several mycobacterial antigens. The large cloning capacity of adenovirus may lend itself very well to a strategy such as this. Ad85 may also prove useful in a prime-boost vaccination strategy against tuberculosis, as other viral vectors expressing secreted antigens of *M. tuberculosis* have been shown to boost specific CD4 and CD8 T cell responses that were initially primed by DNA vaccination [18], [36].

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CHAPTER 3:

THE USE OF RECOMBINANT ADENOVIRUS IN PRIME-BOOST VACCINATION STRATEGIES AGAINST *MYCOBACTERIUM TUBERCULOSIS* IN MICE

The aim of this study was to evaluate the immunogenicity and protective efficacy of several heterologous prime-boost vaccine strategies against aerosol infection with *M. tuberculosis* including boosting plasmid DNA with a recombinant virus, BCG, or a subunit vaccine.

INTRODUCTION

Tuberculosis remains a major health problem world wide, killing approximately 2 million people every year [8]. The only vaccine presently available to counteract this disease is the attenuated strain of *Mycobacterium bovis* called *M. bovis* BCG. Controversy, however, surrounds the protective efficacy of BCG, particularly against pulmonary tuberculosis in young adults [1]. The global AIDS epidemic and the emergence of multi-drug resistant strains of TB emphasize the need for the development of a better vaccine in order to effectively prevent and control this highly infectious, global disease.

While CD4 T cells essentially mediate protective immunity against tuberculosis [5], [19], CD8 T cells have also been shown to play a role in resistance against *M.*

tuberculosis infection [24]. It is reasonable, therefore, that vaccination strategies stimulating both CD4 and CD8 T cell subsets should lead to protection against challenge with the tubercle bacilli. Generally speaking, soluble protein-based vaccines induce mostly CD4 T cell responses [9], viral vaccines induce strong CD8 T cell responses [2], and DNA vaccines induce strong CD4 and CD8 T cells responses [7], [14]. In addition, all three modes of vaccination have been shown to generate antibody responses [25], [2], [16]. While a number of DNA and subunit vaccines have been shown to confer significant levels of protection against tuberculosis, the level of protection conferred by BCG has yet to be surpassed by homologous vaccination [12], [26], [18].

Over the last several years, heterologous prime-boost immunization strategies have been successfully developed with the purpose of improving upon the immunogenicity and efficacy of "dead" vaccines such as subunit and DNA vaccines against tuberculosis and other diseases. One such prime-boost regimen consists of priming with DNA and boosting with recombinant vaccinia virus in order to augment immunity against malaria, HIV and tuberculosis [21], [13], [15]. McShane *et al.* showed that priming mice with DNA encoding ESAT-6 and MPT63 followed by a boost with vaccinia virus Ankara expressing the same fusion protein was also successful in enhancing CD4 T cell responses and protection against tuberculosis infection [15]. Another approach has been to prime with DNA expressing Ag85A and boost with Ag85A protein. This prime-boost regimen also improved the immunogenicity and protective efficacy of the DNA vaccine encoding Ag85A with protection levels rivaling that of BCG [25]. In a third prime-boost approach, Feng *et al.* successfully improved upon the protection conferred by BCG by first vaccinating mice with DNA expressing Ag85B followed by a booster with BCG [9].

In the previous chapter, we showed that intranasal immunization with adenovirus expressing Ag85A (Ad85) protected mice against aerosol infection with virulent *M. tuberculosis.* However, the reduction in the bacterial load in the lungs was lower than that conferred by BCG immunization. The work presented in this chapter was designed to develop an immunization strategy that is more effective than current vaccines. We hypothesized that delivery of Ag85A in either a DNA or protein-based vaccine followed by a boost with either Ad85 or BCG may enhance protection against aerosol infection with *M. tuberculosis.*

MATERIALS AND METHODS

Mice

Female C57BL/6 mice, 6-8 weeks old, were purchased from Charles River Laboratories, Worcester, MA. The mice were maintained under specific-pathogen-free conditions in the BL-3 biohazard facility at Colorado State University. They were given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments.

Plasmid construct

As previously described, the Ag85A gene from *M. tuberculosis* was amplified from plasmid p85A.tub by PCR using primers that contained *Bgl*II restriction enzyme sites [3], [12]. The Ag85A DNA was digested with *Bgl*II, run on a 1% agarose gel, and extracted for insertion into the *Bgl*II site of the V1Jns.tPA vector [17], [22]. In the present study, the V1Jns.tPA plasmid (4.9 kbp) expressing the Ag85A gene (1.2 kbp) is termed DNA85 (kindly provided by Dr. Kris Huygen).

Plasmid verification

The DNA85 plasmid was digested with *Bgl*II, run by electrophoresis on a 0.8% agarose gel containing ethidium bromide, and viewed under a UV light source.

Immunizations

For DNA immunizations, mice were injected intramuscularly twice (three weeks apart) in both quadriceps with 50 μg DNA85 (100 μg total/mouse). Subunit vaccinations consisted of 10 μg/mouse recombinant Ag85A protein emulsified in MPL-SE (Corixa Corporation, Seattle, WA; 25 μg/mouse) or 100 μg/mouse Ag85A protein in dimethyl dioctadecylammonium bromide (DDA) (Sigma; 250 μg/mouse). Mice vaccinated with subcutaneous BCG received 10^6 cfu/200 μl in the scruff of the neck using a 26.5 gauge needle. Intranasal immunizations were carried out by anesthetizing mice using a 1 inch by 1 inch paper towel square soaked with 50 μl of metophane and sealing it into a small semi-air-tight jar for 45 seconds. Mice were individually added to the jar for no more than three minutes or until a slower breath rate by the mouse could be visually detected. A volume of 20 μl of Ad85 or AdZ (both at $1x10^9$ pfu/ml) was then gently placed under one nostril using a pipette tip. Unvaccinated mice were included as a negative control.

Isolation of Cells for Flow Cytometry

Single cell suspensions were prepared from the spleens and lungs of euthanized mice. Spleens were harvested and placed in 5ml cold, complete DMEM consisting of Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO), 1% HEPES buffer (Sigma, St. Louis, MO), 1% L-glutamine (200nM; Sigma), 1% penicillin-streptomycin (Sigma), and 2% modified Eagle medium-nonessential amino acids (100X; Sigma). The spleens were individually passed through a cell strainer, centrifuged at 200*g*, and red blood cells were lysed using ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$). The cells were centrifuged again and resuspended in 2ml of complete DMEM. The spleen cells were counted using a hemocytometer and the absolute number of cells per spleen was calculated. The lung was cleared of blood by perfusing 10 ml of 1X phosphate buffered saline (PBS) containing heparin (50 U/ml; Sigma) through the pulmonary artery. The lungs were removed from the pulmonary cavity, placed in cold DMEM, and then cut into small pieces with sterile razor blades. The dissected lung tissue was then incubated in a final volume of 2ml DMEM containing collagenase XI (0.7 mg/ml; Sigma) and type IV bovine pancreatic DNase (30 μg/ml; Sigma) for 1 hour at 37°C. Enzyme action was stopped by adding 10 ml of complete DMEM medium to the tissue. The digested lungs were further disrupted by gently pushing the tissue through a cell strainer after which the cells were centrifuged at 200 *g*. Red blood cells were lysed with ACK buffer, resuspended in 2ml of complete DMEM, and counted using a hemacytometer. The total number of cells per lung was then calculated.

Flow Cytometry

Spleen and lung cells were isolated from each individual mouse and incubated with monoclonal antibodies labeled with fluorescein isothiocyanate [FITC], phycoerythrin [PE], peridinin chlorophyll-a protein [PerCP], or allophycocyanin [APC] at 25 μg/ml at 4°C for 30 minutes in the dark, after which the cells were washed twice in DRPMI (Irvine Scientific, Santa Ana, CA) containing 0.1% sodium azide (Sigma). Cell surface markers analyzed were PE-labeled anti-CD3 (145-2C11); PerCP-labeled anti-CD4 (L3T4 clone RM4-5); and APC-labeled anti-CD8 (53-6.7). All antibodies were purchased from PharMingen, San Diego, California. Some cells were prepared for IFNγ intracellular staining by incubation at 37°C with anti-CD3 (0.1 μg/ml), anti-CD28 (clone 37.51; 1 μg/ml), and monensin (Fix/Perm kit, PharMingen) for 4 hours. Cells were stained with PerCP-anti-CD4 or APC-anti-CD8 before the permeabilization step according to the kit instructions. FITC-isotype control antibody (rat IgG1) and FITC-anti-IFNγ (XMG1.2) were incubated with the surface-stained cells for 30 minutes, and the cells were washed twice and resuspended in DRPMI prior to analysis. Acquisition was performed on a FACscalibur (Becton-Dickinson, Mountain View, CA), and data were analyzed using CellQuest software (Becton-Dickinson). Cells were gated on lymphocytes by forward and side scatter according to their characteristic scatter profile. The percentage of each cell population within the lymphocyte gate was determined using dot plots and drawing quadrants on each plot to distinguish the specifically labeled cell subsets. Absolute numbers for each given cell subset were calculated from the flow cytometery data and total cell counts on the hemacytometer.

Bone marrow-derived macrophages

Naïve mice were euthanized and their femurs and tibia bones were dissected out. The ends of the bones were trimmed, and the bone marrow was flushed out with cold complete DMEM supplemented with 10% FBS using a 26.5 gauge needle. Cells were resuspended at a concentration of 2×10^6 cells/ml and plated on a 96-well plate at 200 μl per well. The media was changed after 48 hours and again after 5 days. On day 8, the cells were pulsed overnight with 1 μg/well of Ag85 complex protein or media alone.

IFNγ production *in vitro*

Spleens were aseptically removed from euthanized mice and passed through a cell strainer. The cells were then centrifuged at 200 X *g*, and red blood cells were lysed using ACK lysis buffer. The cells were centrifuged again, after which single cell suspensions were prepared at 1 X 10^6 cells/ml in complete DMEM and added at 100 μl/well on a 96-well plate. For some cultures, antigen was added directly to the spleen cells at 20 μg/ml in 100 μl/well. For other cultures, 100 μl of the spleen cells (1X10^6 cells/ml) were added to bone-marrow derived macrophage cultures that had been pulsed overnight with antigen. Recombinant IL-2 (R&D Systems, St. Paul, MN) was added to the cultures at 10 units per well. The cells were cultured for 72 hours, and then frozen back at -80°C until a IFN-γ ELISA assay was performed. Briefly, the thawed supernatants were added to 96 well plates coated with IFN-g capture antibody and blocked with a 1% BSA solution. After one hour of incubation at 37°C and washing, biotin-conjugated IFN-g detection antibody (Pharmingen, San Diego, CA) was added, followed by streptavidin-horseradish peroxidase (DAKO Corp., Carpinteria, CA). The reaction was developed with TMB substrate (Zymed Laboratories, Inc, San Francisco, CA).

Detection of anti-Ag85A antibodies

Levels of anti-Ag85A antibody were determined by ELISA of individual serum samples taken from the tail veins of five mice per group 15 or 25 days after the last vaccination. A 96-well plate was coated with recombinant Ag85 complex protein (kindly provided by Dr. John Belisle) at 500 ng/well. Two-fold dilutions of the sera starting at 1:50 were added to the coated wells, followed by the addition of a horseradish peroxidase-linked, polyvalent secondary antibody (1:1000) specific for mouse IgG1 or IgG2a antibodies (The Binding Site, Birmingham, England).

Animal challenge studies.

Thirty to forty days following the last immunization in each experiment, mice were challenged by low-dose aerosol exposure with *M. tuberculosis* strain H37Rv using a Glas-Col (Terre Haute, IN) aerosol generator calibrated to deliver 50-100 bacteria into the lungs. Bacterial counts in the lungs were determined by plating serial dilutions of individual whole-organ homogenates on nutrient 7H11 agar and counting colony-forming units (cfu) following 3 weeks incubation at 37°C.

RESULTS

Prime-boost vaccination schedules

Three separate prime-boost experiments were conducted according to Table 3.1. In experiment #1, we tested whether we could enhance the protective efficacy of plasmid DNA expressing Ag85A by boosting it with either Ad85 or recombinant Ag85A protein in DDA adjuvant (Ag85A/DDA). The immunogenicity of these vaccines was not investigated in experiment #1. In experiment #2, we essentially repeated experiment #1, except that the Ag85A/DDA boost was replaced by a BCG boost. In addition, we examined the ability of these prime-boost strategies to enhance not only the protective efficacy, but also the immunogenicity, of the DNA85 vaccines. In experiment #3 the ability of Ad85 to enhance the immunogenicity and protective efficacy of recombinant Ag85A in MPL-SE (Ag85A/MPL-SE) adjuvant was investigated.

Experiment #1

Group	Day 0	Day 21	Day 42	Day 72	Day 102
1	Unvaccinated	-----	-----	LDA *M. tuberculosis*	Bacilli viability assay
2	-----	-----	BCG s.c.		
3	DNA85 i.m.	DNA85 i.m.	DNA85 i.m.		
4	DNA85 i.m.	DNA85 i.m.	Ad85 i.n.		
5	DNA85 i.m.	DNA85 i.m.	AdZ i.n.		
6	-----	-----	Ad85 i.n.		
7	-----	-----	AdZ i.n.		
8	DNA85 i.m.	DNA85 i.m.	Ag85A/DDA s.c.		
9	-----	-----	Ag85A/DDA s.c.		

Experiment #2

Group	Day 0	Day 21	Day 42	Day 56	Day 67	Day 72	Day 102
1	Unvaccinated	-----	-----	IFNγ ELISA Flow Cytometry	Collected Sera	LDA *M. tuberculosis*	Bacilli viability assay Flow cytometry
2	-----	-----	BCG s.c.				
3	DNA85 i.m.	DNA85 i.m.	BCG s.c.				
4	DNA85 i.m.	DNA85 i.m.	-----				
5	DNA85 i.m.	DNA85 i.m.	Ad85 i.n.				
6	DNA85 i.m.	DNA85 i.m.	AdZ i.n.				
7	-----	-----	Ad85 i.n.				
8	-----	-----	AdZ i.n.				

Table 3.1 (continued)

Experiment #3

Group	Day 0	Day 21	Day 80	Day 100	Day 115	Day 125	Day 140	Day 170
1	Unvaccinated	-----	-----	-----	IFNγ ELISA Flow Cytometry	Collected Sera	LDA *M. tuberculosis*	Bacilli viability assay Flow cytometry
2	BCG s.c.	-----	-----	-----				
3	Ag85A/MPL-SE s.c.	Ag85A/MPL-SE s.c.	Ad85 i.n.	Ad85 i.n.				
4	Ag85A/MPL-SE s.c.	Ag85A/MPL-SE s.c	AdZ i.n.	AdZ i.n.				
5	Ag85A/MPL-SE s.c.	Ag85A/MPL-SE s.c.	-----	-----				
6	-----	-----	Ad85 i.n.	Ad85 i.n.				
7	-----	-----	AdZ i.n.	AdZ i.n.				
8	MPL-SE s.c.	MPL-SE s.c.	Ad85 i.n.	Ad85 i.n.				

Table 3.1 Prime-boost vaccination schedules. In experiment #1, mice were immunized intramuscularly twice with DNA expressing Ag85A, and subsequently boosted intranasally with Ad85 or s.c. Ag85A/DDA. Controls included BCG alone, DNA85 alone, DNA85 boosted by AdZ, Ad85 or AdZ alone, and no vaccination. In experiment #2, mice were vaccinated with DNA85 twice followed by either Ad85 or BCG. In experiment #3, mice were immunized twice subcutaneously with recombinant Ag85A protein emulsified in MPL-SE, and later boosted intranasally with Ad85. Controls included BCG alone, Ag85A/MPL-SE boosted by AdZ, Ag85A/MPL-SE alone, Ad85 or AdZ alone, MPL-SE boosted by Ad85, and no vaccination. LDA = low dose aerosol with *M. tuberculosis* H37Rv.

Plasmid verification

To verify that the DNA85 plasmid contained the gene encoding for Ag85A protein, the plasmid was digested with restriction enzyme *Bgl*II. The agarose gel in Figure 3.1 shows that the restriction digest yielded two bands of the expected sizes with one at 1.2 kbp (Ag85A gene) and one at 4.943 kbp (V1Jns.tPA plasmid DNA).

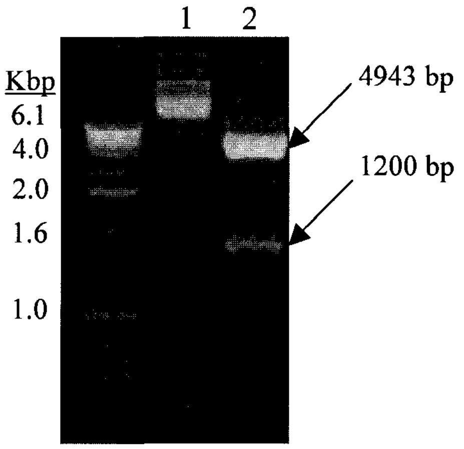


Figure 3.1 DNA85 plasmid encodes the Ag85A gene. The Ag85A gene was removed from the V1Jns.tPA-85A by digesting the plasmid with restriction enzyme BglII and running the digested DNA on an 0.8% agarose gel containing ethidium bromide. The restriction digest yielded bands of the expected sizes in Lane 2 with the Ag85A gene running at 1.2 kbp and the linearized V1Jns.tPA plasmid DNA running at 4943 bp. The uncut V1Jns.tPA-85A plasmid band ran at approximately 6100 bp in Lane 1.

Vaccine-induced IFNγ responses

In Experiment #2 (Table 3.1), we investigated whether a prime-boost strategy using DNA85 vaccine boosted with Ad85 (DNA85+Ad85) or BCG (DNA85+BCG) could induce IFNγ-production *specifically* against Ag85A protein. Spleen cells were isolated from mice two weeks after the last vaccination, and were re-stimulated directly with recombinant Ag85A protein *in vitro*, however there were technical difficulties with the assay and the supernatants from the stimulated cell cultures, even positive controls, gave no signal when analyzed by ELISA. The negative data are not shown. Conversely, in Experiment #3 (Table 3.1), ELISA analysis of supernatants from spleen cell cultures that were isolated 2 weeks after the last vaccination and that were overlaid onto bone-marrow derived macrophages pulsed with Ag85A protein, showed that a significant amount of IFNγ was produced by the spleen cells from mice that were immunized twice with intranasal Ad85 (Figure 3.2).

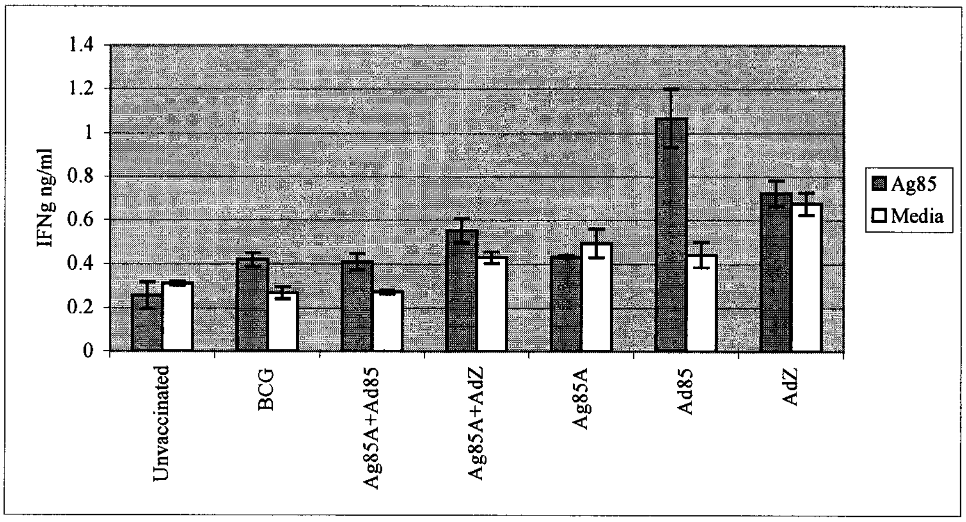
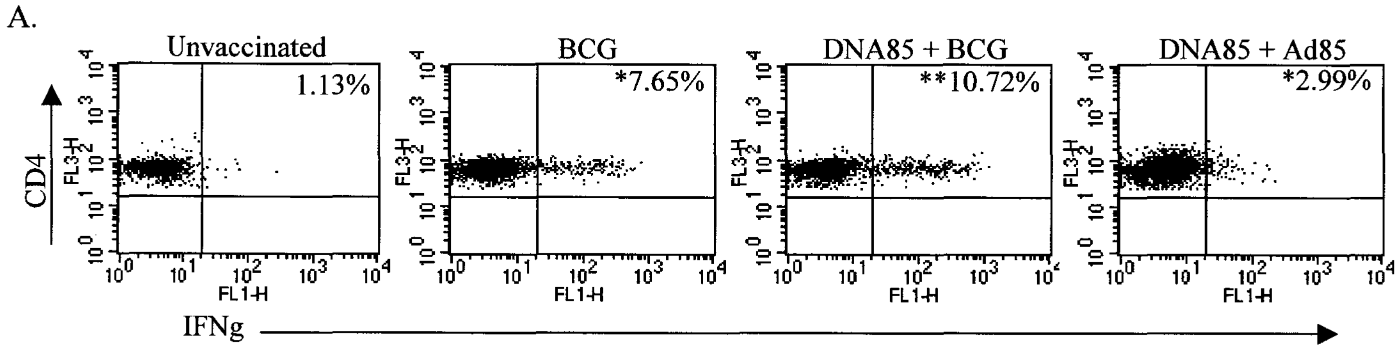


Figure 3.2 IFN-g produced by Ag85-stimulated spleen cells from immunized mice.

Mice were immunized twice subcutaneously 3 weeks apart. Two months later, the mice were anesthetized and immunized twice with Ad85 i. n. three weeks apart. Two weeks after the last vaccination, mice were sacrificed and spleen cells were isolated and cultured with macrophages pulsed with Ag85 protein or with media alone for 72 hours. Supernatants were frozen until an IFN-g ELISA was performed.

Flow cytometry analysis was also performed to assess whether either of the prime-boost vaccine strategies in experiments #2 and #3 (Table 3.1) would affect the amount of IFNγ produced in the lungs or spleens of vaccinated mice before challenge with *M. tuberculosis*. In experiment #2, cells were isolated from the lungs of mice 2 weeks after the last vaccination, and were stained with fluorescently labeled antibodies against surface markers CD4 and CD8, or against intracellular IFNγ, followed by flow cytometry analysis. The prime-boost strategy in experiment #2 showed statistically significant increases in the percentage (Figure 3.3A) and total number (Figure 3.3B) of CD4 IFNγ T cells in the lungs of mice vaccinated with DNA85+Ad85 compared to the unvaccinated group, with highly significant increases of this cell population in the DNA85+BCG group. The percentage of CD4 IFNγ T cells was also significantly higher in the BCG control lungs. No differences in CD8 IFNγ T cells were observed among the groups (data not shown).

In experiment #3, cells isolated from the spleens or lungs of mice 2 weeks after the last vaccination were stained as above. Table 3.2 shows that the prime-boost vaccination employing Ag85A/MPL-SE+Ad85 did not increase the percentage or total number of CD4 IFNγ T cells in the spleens or the lungs compared to the unvaccinated control. BCG, however, induced a significantly higher percentage and absolute number of CD4 IFNγ T cells in the lung compared to the unvaccinated controls. While the values were not statistically significant, the same trend was observed in the spleen with a higher percentage and absolute number of CD4 IFNγ T cells in the BCG vaccinated group compared to the unvaccinated mice (p=0.06). There were no significant differences in CD8 IFNγ T cells among the groups in the lung or spleen.



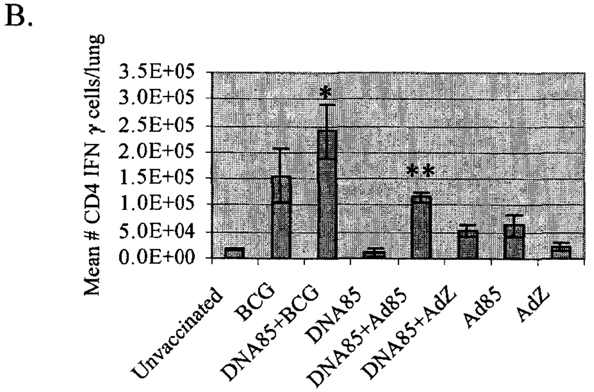


Figure 3.3 IFNγ response by CD4 T cells in the lungs of vaccinated mice before TB challenge (Experiment #2). Mice were immunized twice with DNA85 i. m. followed by BCG s. c. or Ad85 i. n. at 3-week intervals. Control mice were vaccinated according to the immunization schedule in Table 3.1. In A and B, lungs cells from each mouse were isolated, stained for CD4, permeabilized, and then stained for intracellular IFNγ. Cells were gated on CD4 T cells. (A) Representative dot plots depicting the mean percentages of CD4 T cells producing IFNγ. (B) Data are expressed as the absolute number of CD4 that produce IFNγ. These data are based on the right cranial, middle, and caudal lobes from each mouse lung.

*$p<0.05$ and **$p<0.01$ compared to the unvaccinated mice based on a two-tailed Students' *t*-Test. Error bars represent +/- SEM where n=4.

Spleen	% CD4+ IFNγ+ T cells	Total # CD4+ IFNγ+ T cells (x10^5)	% CD8+ IFNγ+ T cells	Total # CD8+ IFNγ+ T cells (x10^5)
Unvaccinated	0.43	2.3	1.77	5.7
BCG	2.86	15	3.65	9.8
Ag85A/MPL-SE +Ad85	0.39	1.9	2.1	6.5
Ag85A/MPL-SE+AdZ	0.61	1.4	2.51	3.8
Ag85A/MPL-SE	0.52	2.2	3.11	7.1
Ad85	0.43	1.4	3.36	7.0
AdZ	0.65	2.1	3.26	4.8
MPL-SE+Ad85	0.54	1.4	3.31	5.2
Lung				
Unvaccinated	1.95	0.4	3.48	0.8
BCG	9.83*	2.4**	6.05	1.1
Ag85A/MPL-SE +Ad85	0.96	0.5	2.44	1.1
Ag85A/MPL-SE+AdZ	0.99	0.5	1.65	0.7
Ag85A/MPL-SE	2.82	0.5	4.20	0.6
Ad85	1.97	0.6	5.12	1.1
AdZ	1.35	0.6	3.70	0.7
MPL-SE+Ad85	1.36	0.4	1.93	0.5

Table 3.2 IFNγ response by CD4 T cells in the lungs of mice vaccinated with Ag85+Ad85 before TB challenge

Mice were immunized twice subcutaneously 3 weeks apart with Ag85A protein emulsified in MPL-SE adjuvant. Two months later, mice were immunized twice with intranasal Ad85 or AdZ three weeks apart. Spleen and lungs cells from each mouse were isolated, stained for CD4 or CD8, permeabilized, and then stained for intracellular IFNγ. Cells were either gated on CD4 or CD8 T cells. These data are based on approximately half of the spleen or the entire lung organ.

*$p<0.05$ and **$p<0.01$ compared to the unvaccinated mice based on a two-tailed Students' *t*-Test. Error bars represent +/- SEM where n=4.

Humoral immune response to the vaccines

In order to assess the levels and istoypes of Ag85A-specific antibodies induced by the prime-boost vaccination strategies using DNA85+Ad85 or DNA85+BCG in experiment #2 and Ag85A/MPL-SE +Ad85 in experiment #3, sera were collected and pooled from vaccinated mice 25 days after the last vaccination. Antibodies specific for Ag85A were measured in sera by ELISA from mice immunized with DNA85+Ad85 or DNA85+BCG (Figure 3.4A) or Ag85A+Ad85 (Figure 3.4B). In Figure 3.4A, DNA85 vaccination alone induced equivalent titers of IgG2a and IgG1 antibody against Ag85. The level of IgG2a anti-Ag85 antibody increased when either the BCG or the Ad85 was added as a booster. No Ag85-specific antibody was detected in mice that were vaccinated with BCG alone.

The data in Figure 3.4B show that although a mixed IgG2a/IgG1 antibody profile was induced by the vaccines employing the Ag85A protein in MPL-SE in Experiment #3, the IgG1 response was predominant. Interestingly, an increased amount of IgG2a antibody was generated by the mice immunized with Ag85A/MPL-SE+Ad85 compared to that observed in mice vaccinated with Ag85A or Ad85 alone. Again, BCG did not induce Ag85-specific antibody.

IFNγ production in the lungs after TB challenge

To determine the levels of IFNγ-production by CD4 and CD8 T cells in the lungs of immunized mice that were subsequently challenged with aerosolized *M. tuberculosis*, isolated lung cells were stained with fluorescently labeled antibodies against CD3, CD4, CD8, and intracellular IFNγ. Flow cytometry analysis of the lung cells from experiment #2 showed that there were significantly fewer CD4 IFNγ T cells in the lungs of BCG and

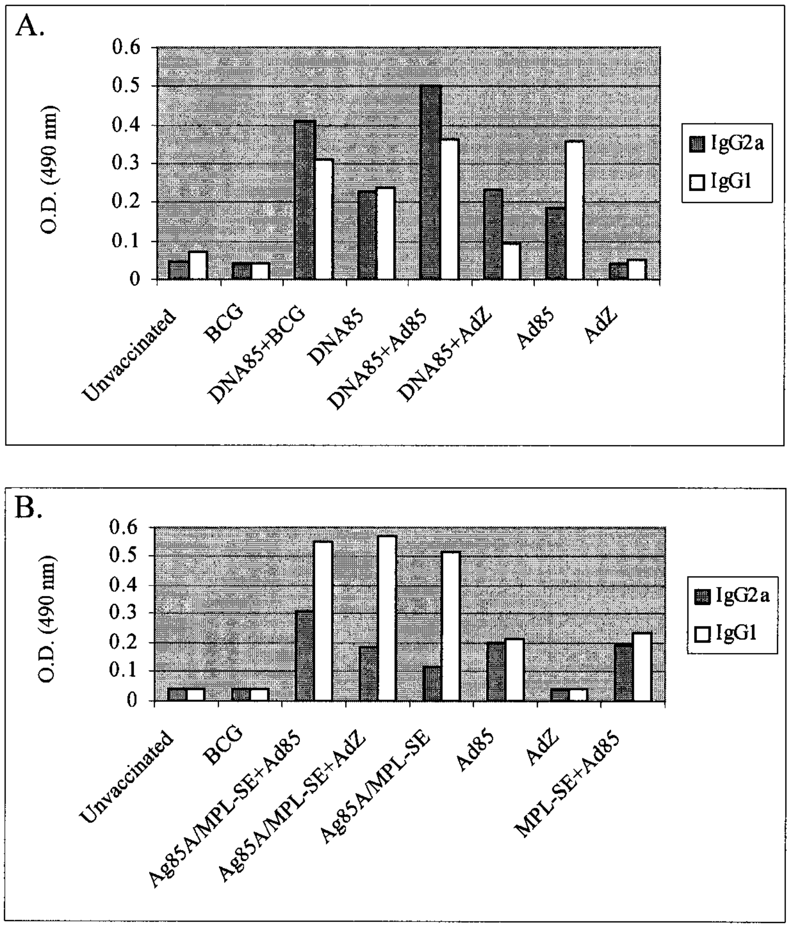


Figure 3.4 Mixed antibody responses to prime-boost vaccinations
Sera were collected from the tail veins of mice 25 days after the last vaccination with either DNA85+Ad85 in (A) or Ag85A/MPL-SE+Ad85 in (B). The sera were pooled and tested by ELISA for the production of IgG2a or IgG1 anti-Ag85A antibodies. (n=5)

DNA85+BCG vaccinated mice compared to the unvaccinated controls (Figure 3.5A). The data also showed that although the number of CD4 IFNγ T cells in the lungs of the DNA85+Ad85 and unvaccinated mice was similar, there were significantly more CD4 T cells in the lungs of mice vaccinated with DNA85+Ad85 (Figure 3.5B). The CD8 T cell population showed the same trends (Figure 3.6).

Flow cytometry analysis of the lungs cells from Experiment #3 also showed that there were significantly fewer CD4 IFNγ T cells in the lungs of BCG-vaccinated mice compared to the unvaccinated controls (Figure 3.7). The same trend was evident with the CD8 IFNγ T cells.

Protective efficacy in mice

To measure the protective efficacy of DNA85+Ad85, DNA85+BCG, DNA85+Ag85/DDA, and Ag85A/MPL-SE+Ad85, mice were immunized according to Table 3.1. Mice were subsequently challenged with aerosolized *M. tuberculosis* H37Rv, and bacilli were quantified in the lungs. In all three experiments, BCG was administered to mice as a positive control. Negative controls in all three experiments included no vaccination, vaccination with DNA85 alone, Ag85A/DDA alone, Ag85/MPL-SE alone, AdZ as a booster or alone, or vaccination with MPL-SE boosted by Ad85.

The protection data for experiment #1 showed that BCG alone, Ag85A/DDA alone, and DNA85+Ag85A/DDA elicited significant protection against tuberculosis (Table 3.3). In experiment #2, BCG and DNA85+BCG conferred significant protection against challenge, while no reduction in bacterial numbers was observed in the lung from mice that were vaccinated with DNA85+Ad85 (Table 3.3).

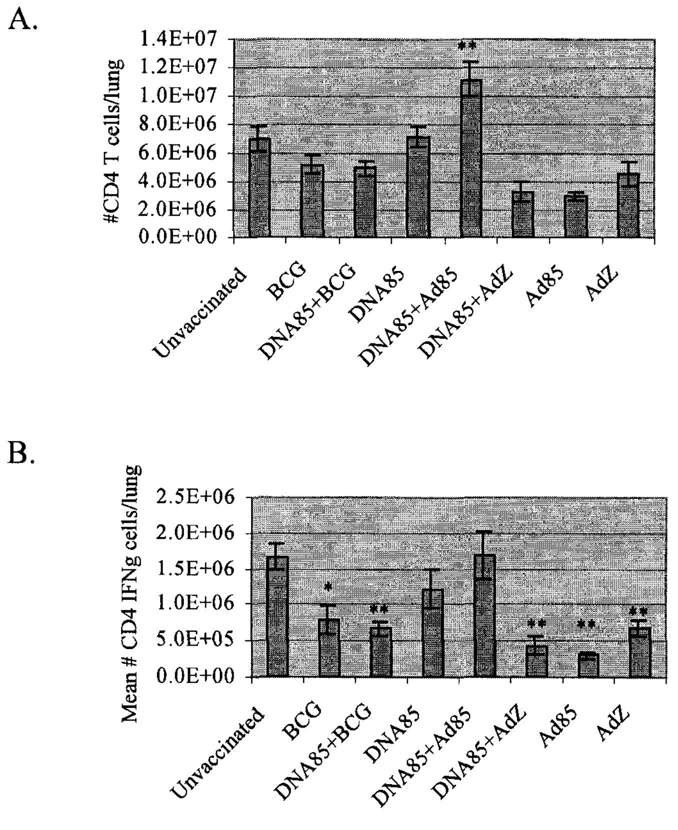


Figure 3.5 IFNg responses in the lungs of vaccinated mice following challenge with *M. tuberculosis*. Mice were vaccinated at three-week intervals with DNA85 i.m. two times followed by one dose of Ad85 or AdZ i. n., BCG s. c. As controls, mice were vaccinated once with BCG s. c., Ad85 or AdZ i.n., or twice with i.m. DNA85. Each group was exposed to a low dose aerosol of *M. tuberculosis* thirty days after the last vaccination, and sacrificed thirty days post-infection. Lungs from each mouse were cells were isolated, stained for CD4, permeabilized, and then stained for intracellular IFNg. Cells were gated on CD4 T cells. In A, data are expressed as the absolute numbers of CD4 T cells in the lungs. In B, data are expressed as the absolute numbers of CD4 T cells that produce IFNg. These data are based on cell counts from the 3 right lobes of the lung.

** $p<0.01$ compared to unvaccinated mice
* $p<0.05$ compared to unvaccinated mice

Error bars represent +/- SEM where n=5. All p values are based on a two-tailed Student's *t*-Test.

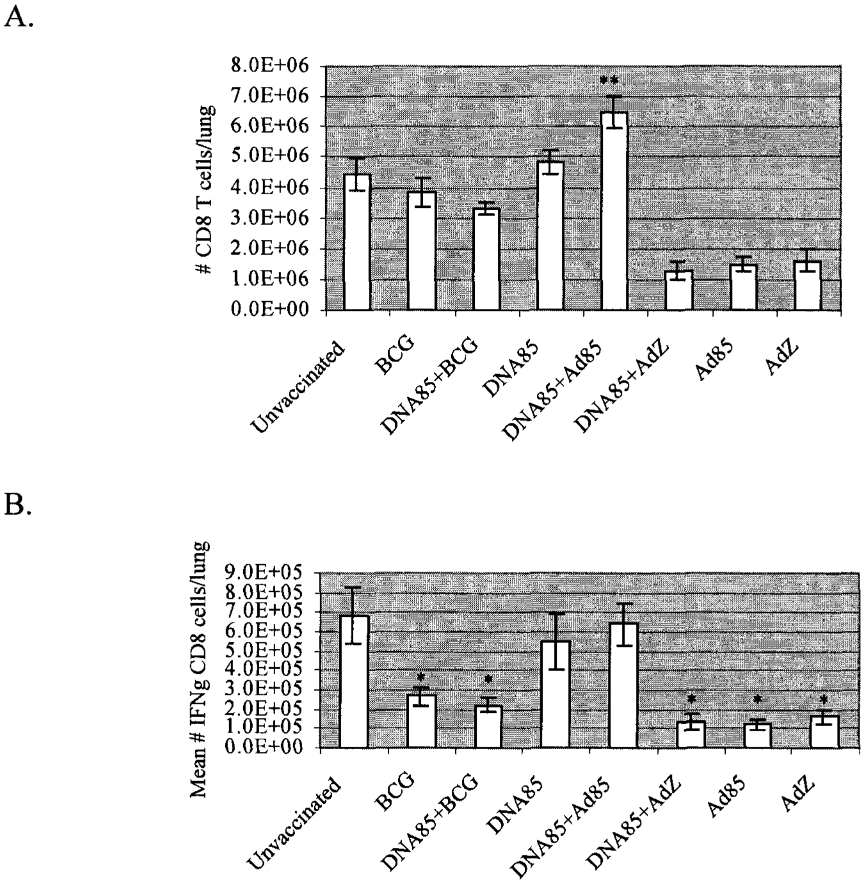


Figure 3.6 IFNg responses in the lungs of vaccinated mice following challenge with *M. tuberculosis*. Mice were vaccinated at three-week intervals with DNA85 i.m. two times followed by one dose of Ad85 or AdZ i. n., BCG s. c. As controls, mice were vaccinated once with BCG s. c., Ad85 or AdZ i.n., or twice with i.m. DNA85. Each group was exposed to a low dose aerosol of *M. tuberculosis* thirty days after the last vaccination, and sacrificed thirty days post-infection. Lungs from each mouse were cells were isolated, stained for CD8, permeabilized, and then stained for intracellular IFNg. Cells were gated on CD8 T cells. In A, data are expressed as the absolute numbers of CD8 T cells in the lungs. In B, data are expressed as the absolute numbers of CD8 T cells that produce IFNg. These data are based on cell counts from the 3 right lobes of the lung.

**p<0.01 compared to BCG-vaccinated mice
* p<0.05 compared to unvaccinated mice

Error bars represent +/- SEM where n=5. All p values are based on a two-tailed Student's *t*-Test.

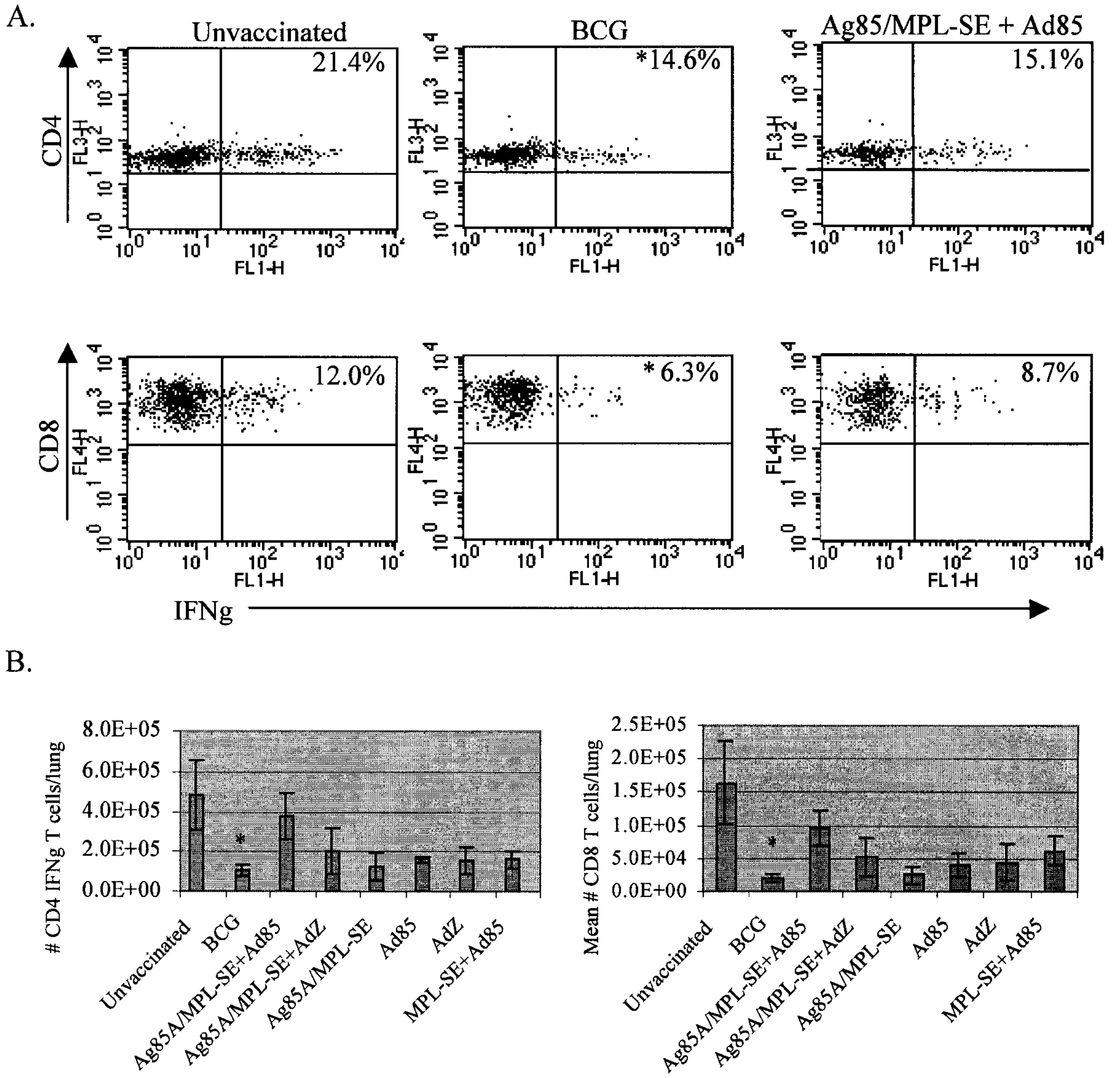


Figure 3.7 IFNg CD4 and CD8 T cell responses in the lungs of vaccinated mice 4 weeks post-challenge with *M. tuberculosis* (Experiment #3).

Mice were immunized twice subcutaneously 3 weeks apart with Ag85A protein emulsified in MPL-SE adjuvant. Two months later, mice were anesthetized and immunized twice with intranasal Ad85 or AdZ three weeks apart. Control mice were vaccinated according to the immunization schedule in Table 3.1. Each group was exposed to a low dose aerosol of *M. tuberculosis* thirty days after the last vaccination, and sacrificed thirty days post-infection. In A and B, lungs cells from each mouse were isolated, stained for CD4 or CD8, permeabilized, and then stained for intracellular IFNg. Cells were either gated on CD4 or CD8 T cells. (A) Representative dot plots depicting the mean percentages of CD4 and CD8 T cells producing IFNg. (B) Data are expressed as the absolute number of CD4 or CD8 T cells that produce IFNg. Error bars represent +/- SEM where n=5. These data are based on the right cranial and middle lobes from each mouse lung.

Group	Mean Log 10 CFU/Lungs	SEM	Log 10 Protection
Experiment #1			
Unvaccinated	5.32	0.13	0
BCG	4.06*	0.19	1.26
DNA85	4.97	0.08	0.35
DNA85+Ad85	4.76	0.28	0.55
DNA85+AdZ	5.03	0.11	0.29
Ad85	4.71	0.21	0.62
AdZ	5.05	0.19	0.27
Ag85A/DDA	4.29*	0.06	1.02
DNA85+Ag85A/DDA	4.53*	0.09	0.78
Experiment #2			
Unvaccinated	4.41	0.15	0
BCG	3.61*	0.07	0.81
DNA85+BCG	3.61*	0.16	0.81
DNA85	4.32	0.14	0.09
DNA85+Ad85	4.31	0.08	0.11
DNA85+AdZ	4.35	0.07	0.06
Ad85	4.13	0.13	0.28
AdZ	4.56	0.18	-0.15

Table 3.3 Protective efficacy of DNA85 prime-boost vaccines
In both experiments, mice were vaccinated at three-week intervals with DNA85 i. m. two times followed by one dose of Ad85 or AdZ i. n., BCG s. c., or Ag85A in DDA s. c. As controls, mice were vaccinated once with BCG s. c., Ad85 or AdZ i. n., or twice with i.m. DNA85. The mice were challenged by aerosol with *M. tuberculosis* H37Rv thirty days after the last vaccination. The CFU per lungs were measured one month later.

*p<0.05 compared to the unvaccinated mice based on a two-tailed Students' *t*-Test with n=5.

As shown in Figure 3.8, the Ag85A/MPL-SE+Ad85 prime-boost strategy in experiment #3 reduced the bacterial load in the lungs compared to the unvaccinated control, however the difference was not statistically significant. As expected, BCG conferred 0.75 $\log_{10}$ protection, which was statistically different than the unvaccinated group.

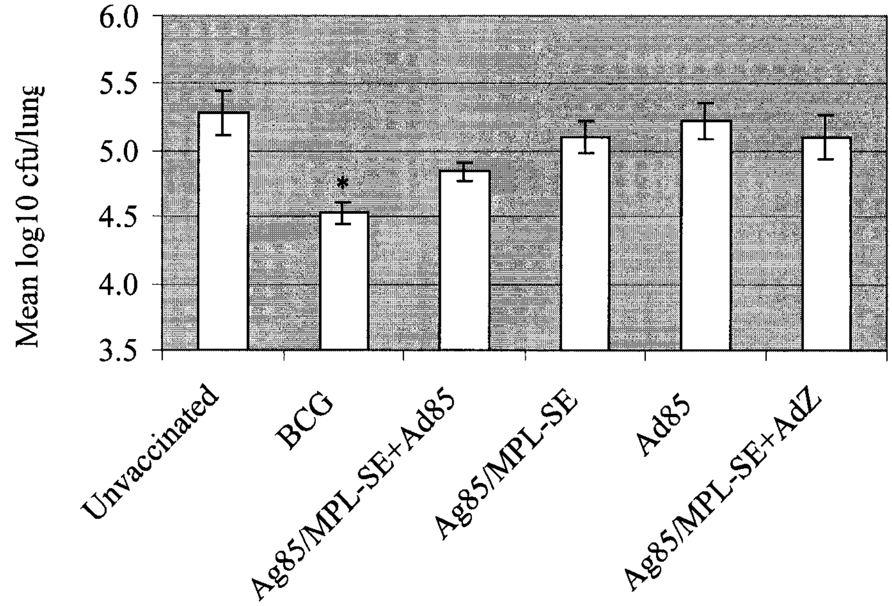


Figure 3.8 Protective efficacy of Ag85A+Ad85 prime-boost vaccine (Experiment #3)

Mice were immunized twice subcutaneously 3 weeks apart. After 2 months, the mice were anesthetized and immunized twice with intranasal Ad85 three weeks apart. Forty days after the last immunization, the mice were infected with a low-dose aerosol of *M. tuberculosis* H37Rv. The mice were sacrificed on day 30 post-infection, and the lungs were assayed for viable bacilli. Bacterial loads in the rest of the control mice were similar to the bacilli count observed in the unvaccinated group.

*$p < 0.05$ compared to the unvaccinated group using a one-tailed Student's *t*-Test. Error bars represent +/- SEM where n=5.

DISCUSSION

In this chapter, we attempted to determine if several prime-boost strategies could induce protective immunity against infection with *M. tuberculosis*. In experiment #1, we tested whether Ad85 or Ag85A (in DDA adjuvant) could boost the protective efficacy of DNA85. In experiment #2, we re-tested the ability of Ad85 to boost DNA85, as well as the ability of BCG to boost the DNA85 vaccine. In experiment #3, we used a different approach and tested whether Ad85 could boost the protective efficacy of a subunit vaccine consisting of Ag85A protein in MPL-SE adjuvant.

The challenge study results from experiment #1 showed that the DNA85 vaccine on its own did not elicit a significant level of protection against tuberculosis, thereby making it impossible to conclude whether Ad85 or Ag85A in DDA adjuvant were capable of boosting the protective efficacy of the DNA vaccine. This was a surprising finding because this plasmid DNA construct has been shown to be nearly as effective as BCG in past studies [12]. To verify that the DNA85 encoded our gene of interest (i.e. Ag85A), we ran a diagnostic restriction enzyme digest on the plasmid and found that it indeed contained the Ag85A gene (Figure 3.1). The other interesting finding in this experiment was that Ag85A emulsified in DDA adjuvant conferred a statistically significant level of protection (1.02 Log_{10} better than in the unvaccinated mice) against aerosol challenge with *M. tuberculosis*. These results are not consistent with a recent study published by Huygen's group which showed that Ag85A as a subunit vaccine was not protective against intravenous TB challenge [25]. In their study, they immunized mice with the same amount of protein as we did here in experiment #1, however, they used MPL as the adjuvant rather than DDA. The discrepancy in the protection conferred by Ag85A protein between these two studies emphasizes the importance of including effective adjuvants in vaccine design.

While our intent in experiment #2 was to test the ability of Ad85 or BCG to boost the protective efficacy of the DNA85 vaccine, we ran into the same problem that we did in experiment #1; DNA85 was not protective on its own, despite the fact that we used a different stock of the plasmid. The fact that the DNA85 vaccine failed to protect mice in two separate studies brings into question its ability and reliability as a protective vaccine against TB in humans.

That said, however, DNA85 vaccination was immunogenic in experiment #2. For instance, while BCG vaccination resulted in significantly more CD4 IFNγ T cells in the lungs, priming the BCG vaccine with DNA85 recruited an even higher percentage of CD4 T cells capable of producing IFNγ to the lungs (Figure 3.3). The fact that the lungs of mice immunized with DNA85+BCG had more CD4 IFNγ T cells than BCG alone suggests that DNA85 was successful in priming CD4 T cells that were capable of making IFNγ. A possible explanation for the increase of CD4 IFNγ T cells in the DNA85+BCG vaccinated mice compared to BCG alone could be that the BCG boost may have focused a larger pool of protective CD4 IFNγ T cells that were primed by the DNA85 vaccine into the lungs. Accordingly, DNA85, when given alone, did not recruit CD4 IFNγ T cells to the lung. This recruitment of CD4 IFNγ T cells into the lungs by BCG vaccination appeared to be essential for protection since the only mice that were protected in experiment #2 were either BCG- or DNA85+BCG-vaccinated mice.

Interestingly, intranasal Ad85 was also able to focus CD4 IFNγ T cells that were primed by DNA85 vaccination to the lungs. However, since DNA85+Ad85 vaccinated mice were not protected against TB challenge, the number of CD4 IFNγ T cells that the Ad85 boost recruited to the lungs may not have been sufficient to confer protection, especially considering that this prime-boost vaccination could only stimulate T cells specific for Ag85A protein. The BCG boost may have been more successful because it presumably stimulated a more diverse population of mycobacterial-specific CD4 IFNγ T cells, and not just Ag85A-specific T cells. Another explanation for the lack of protection by DNA85+Ad85 could be that the CD4 IFNγ cells were transient in the lungs because they were Th1 effector cells that died shortly after vaccination rather than protective memory cells that persisted until the time of TB infection. It may be, therefore, worthwhile to sample the lungs for CD4 IFNγ T cells at different time points after vaccination to determine the kinetics of this cell population that was induced by the prime-boost vaccine. In addition, we could not detect an increase in CD8 IFNγ T cells in the lungs of mice after immunization with DNA85+Ad85, however, we cannot rule out the possibility that the vaccine generated CTL responses. It may have been more useful to determine if the DNA-virus prime-boost vaccination strategy induced Ag85A-specific CTL responses since it is not clear by which mechanism, IFNγ production or cytotoxicity, CD8 T cells help protect against TB.

While the DNA85+BCG and DNA85+Ad85 prime-boost vaccines in experiment #2 were successful in recruiting CD4 IFNγ T cells to the lungs, we were unable to detect a significant number of CD4 IFNγ T or CD8 IFNγ T cells in the lungs of mice vaccinated with the Ag85A/MPL-SE+Ad85 prime-boost vaccine in experiment #3. However, we did observe a significant increase in IFNγ production from spleen cells isolated from Ad85-vaccinated mice, which suggests that the Ad85 vaccine was immunogenic on its own, despite the fact that it did not confer significant protection against TB as it has in past studies. In addition, more IgG2a antibody (anti-Ag85A) was detected in the sera of mice that were immunized with Ag85/MPL-SE+Ad85 than in the sera of mice that were vaccinated with Ag85/MPL-SE alone (Figure 3.4B). This suggests that the Ad85 booster was able to enhance Th1-type immunity in the Ag85A/MPL-SE primed mice. Since the Ag85A/MPL-SE+Ad85 vaccine was unable to significantly reduce the bacterial load in the lungs, and since Ag85A/MPL-SE alone did not confer significant protection, it seems reasonable to conclude that the lack of protection by this prime-boost strategy was due to the fact that Ag85A/MPL-SE prime was ineffective in stimulating memory CD4 IFNγ T cells, and therefore, only a small pool of CD4 IFNγ T cells may have been available for Ad85 to recruit to the lungs. In fact, the antibody isotype data in Figure 3.4B shows that the subunit vaccine generated high levels of IgG1 antibody against Ag85A, suggesting that a stronger Th2-type immune response was generated by the protein immunization. In addition, since it is unlikely that the Ag85A/MPL-SE subunit vaccine would enter the class I MHC presentation pathway, and since the Ad85 vaccine seemed to be ineffectual in the lungs in this experiment, it follows that we could not detect any differences in the number of CD8 IFNγ T cells among the groups.

The failure of the Ag85A/MPL-SE subunit vaccine to induce Th1-type immunity could have been due to poor immunostimulation by the adjuvant. It would be worthwhile to test this protein-virus prime-boost strategy again using Ag85A subunit vaccine emulsified in DDA rather than MPL-SE, since the Ag85A/DDA vaccine protected mice almost as well as BCG in experiment #1, and the Ag85A/MPL-SE on its own did not confer any protection in the lungs. The reason we chose to use MPL-SE was because it has been shown to induce Th1-type responses such as macrophage activation in terms of increased cytokine release, antigen uptake, processing, and presentation [20]. The fact that Ag85A-specific IgG1 antibody, which is associated with Th2-type responses, predominated the sera of Ag85A/MPL-SE vaccinated mice suggests that MPL-SE was not effective in promoting Th1-type responses. It may be worthwhile therefore, to use a combination of adjuvants, as it has been shown that DDA and MPL were most immunogenic and protective when used together in an ESAT-6 subunit vaccine, possibly because the MPL and ESAT-6 became incorporated into the DDA micelles allowing for optimal delivery to macrophages [4]. It should also be noted that ten times more Ag85A protein was used in the Ag85A/DDA formulation in Table 3.3 compared to the Ag85A/MPL-SE formulation. The increased amount of protein in the Ag85A/DDA vaccine may have accounted for the increase in protection against TB, as it has been demonstrated that large amounts of peptides that achieve high density on the surface of antigen-presenting cells tend to stimulate Th1-type cell responses whereas low-density antigen presentation usually elicits Th2-type cell responses [6]. These combined data suggest that the amount of antigen and the type of adjuvant are important factors to consider when designing effective subunit vaccines.

The most interesting finding in this study was that more CD4 IFNγ T cells were observed in the lungs of BCG-vaccinated (s.c.) mice before challenge, and that an increase in this cell population correlated with a significant reduction in the bacterial load in the lungs after TB challenge. Interestingly, following challenge with *M. tuberculosis,* the ratio and number of CD4 IFNγ T cells were lower in BCG-vaccinated mice compared to the unvaccinated group. This suggests that the cells generated in the lungs of the BCG-vaccinated mice were efficient memory T cells that were able to clear the tubercle bacilli soon after infection. However, we could not detect a memory phenotype (i.e. $CD44^{hi}CD45RB^{lo}$) in the BCG-vaccinated mice before or after challenge, perhaps because any memory cells that were generated by the vaccine may have migrated out of the lungs once they had controlled the growth of the tubercle bacilli. Other unpublished data within our laboratory have shown that IFNγ-producing CD4 T cells are sequestered to the lungs after subcutaneous vaccination with BCG, and that a cell population with this phenotype is still present in the lungs at the time of TB infection (i.e. 3 months after vaccination), presumably poised and ready to activate bacilli-infected macrophages.

How and why these BCG-activated cells were able to migrate to the lungs after subcutaneous vaccination is unknown. A possible explanation is that the activated CD4 IFNγ T cells homed to the lung, which may be constitutively activated at low levels [11]. If the vascular endothelium were activated, even at low levels, upregulation of adhesion molecules on the endothelium may have helped to promote extravasation of the activated T cells into the lung. Another possibility is that BCG itself drained into either the thoracic lymph nodes or the bronchus-associated lymphoid tissue (BALT) where the bacilli may have infected macrophages thereby activating them. These cells may have then migrated to the lungs. Once in the lungs, the BCG infection could have attracted protective T cells that were generated in the lymph nodes. To address whether the BCG organism makes its way to the lungs or regional lymph nodes, each of these organs could be assayed for the presence of viable bacilli at different time points after subcutaneous vaccination.

As mentioned, there were fewer CD4 IFNγ-producing cells in the lungs of BCG-vaccinated mice after challenge with TB compared to the unvaccinated control. A possible explanation for this may be that the memory CD4 IFNγ-producing cells in the BCG-vaccinated mice exerted their protective function early after TB infection, and then re-entered the circulation. In the unvaccinated control mice, the CD4 IFNγ were still present in large numbers in the lungs 30 days post-infection most likely in efforts to control the bacilli. Since neither the DNA85+Ad85 vaccine in experiment #2 nor the Ag85/MPL-SE+Ad85 vaccine in experiment #3 protected mice against challenge with TB, we expected the number of CD4 IFNγ T and CD8 IFNγ T cells in the lungs 30 days after infection to be similar to the unvaccinated controls. This was the case in the lungs of mice vaccinated with Ag85A/MPL-SE+Ad85 in experiment #3, however, the data from the lungs of mice vaccinated with DNA85+Ad85 in experiment #2 were not consistent with these findings. While the DNA85+Ad85 vaccinated mice recruited significantly more CD4 and CD8 IFNγ T cells to the lungs after vaccination compared to unvaccinated mice, the vaccine did not protect the mice during infection with *M. tuberculosis*. Although the DNA85+Ad85 vaccinated mice had the same number of CD4 and CD8 IFNγ T cells as the unvaccinated mice during TB infection, the DNA-virus prime-boost recruited significantly more CD4 and CD8 T cells to the lungs after infection than any of the other groups. This suggests that DNA85+Ad85 was highly immunogenic, and that it recruited subsets of CD4 and CD8 T lymphocytes that were not capable of making IFNγ in the lungs. It seems reasonable to speculate that a proportion of the CD4 T cells may have been Th2-type cells that were capable of secreting Th1-suppressive cytokines such as IL-10, which can down-regulate macrophage function thereby limiting the ability of macrophages to destroy intracellular tubercle bacilli [10]. It is also possible that a proportion of the CD8 T cells that did not make IFNγ were CTLs, as it has been shown that Ag85A is recognized by CTLs in BCG-vaccinated individuals [23]. Additionally, the Ag85A-specific antibody profiles in the serum after vaccination supports the notion that the DNA85+Ad85 prime-boost vaccine was more immunogenic than the other vaccines in experiment #2, and that it induced a mixed Th1/Th2 phenotype (Figure 3.4A).

In summary, based on the results from the BCG-vaccinated mice, it appears that in order for a vaccine strategy to successfully protect against tuberculosis infection, the vaccine must focus or sequester protective CD4 IFNγ T cells to the lungs prior to infection. Ad85, although it was not as effective as BCG in recruiting CD4 IFNγ T cells to the lungs after DNA85 vaccination in experiment #2, it was able to sequester a significant number of these T cells to the lungs after prime-boost vaccination. However, the mice were not protected against TB. In fact, this DNA-virus prime-boost strategy may have been too immunogenic in the sense that it may have stimulated not only a large pool of Ag85-specific CD4 IFNγ T cells, but also a subset of Th1-suppressive T cells, which may have interfered with protective responses during TB infection. The inability of the protein-virus prime-boost vaccine in experiment #3 to generate CD4 IFNγ T cell responses in the lung and to protect against challenge with *M. tuberculosis* may have been due to the fact that MPL-SE was not effective in stimulating macrophage presentation of Ag85A peptides to CD4 TH1-type cells, and therefore, a protective CD4 IFNγ T cell subset was not available for recruitment to the lungs by the Ad85 boost. Future prime-boost studies should employ a combination of adjuvants with the subunit vaccine, such as DDA and MPL-SE, since the Ag85A/DDA subunit vaccine on its own was significantly protective, and as mentioned above, these two adjuvants may work synergistically to increase the protective efficacy of protein vaccines [4]. Our study not only emphasizes the need for effective adjuvants when designing a vaccine to target Th1-type immunity, but also the necessity of focusing protective CD4 IFNγ T cells to the lungs prior to aerosol infection with *M. tuberculosis*.

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CHAPTER 4:

PULMONARY NECROSIS RESULTING FROM DNA VACCINATION AGAINST TUBERCULOSIS

INTRODUCTION

Disease caused by *Mycobacterium tuberculosis* is the leading cause of death from an infectious agent. The World Health Organization estimates that over 2 million people die from tuberculosis every year, and that 1.7 billion people are infected with the disease [5]. The pathogenesis of tuberculosis is different from most bacterial infections in that the bacilli can be harbored within the host as a latent infection for several weeks to several decades before active disease arises [8]. Current estimates are that approximately 10% of latent infections reactivate, resulting in active tuberculosis that can be spread from contact to contact [29]. Furthermore, in people co-infected with human immunodeficiency virus, the risk of reactivation disease increases to 5-15% annually [22].

There has been a gradual realization that the protective efficacy of the existing vaccine against tuberculosis, *Mycobacterium bovis* BCG, wanes in people as they enter adulthood [3], [30], and in certain clinical trials, its overall effectiveness was zero [19]. As a result, there is now a concerted effort to develop more effective vaccines against tuberculosis, most of which are prophylactic vaccines against *M. tuberculosis*, that is, vaccines that are administered before exposure to *M. tuberculosis*. However, since one third of the world's population is latently infected with the bacilli, it has been proposed that development of a therapeutic or post-exposure vaccine that would confer protection against reactivation in latently infected people is a worthwhile pursuit [7].

Until recently, no effective prophylactic vaccine had been shown to reduce the bacterial load in the lungs after infection had occurred. Turner *et al.* showed that BCG, Ag85A DNA, and CFP subunit vaccines, all of which have been shown to confer significant protection in the mouse model of tuberculosis infection, failed to protect mice when they were given after aerosol infection [33]. Recently, however, Lowrie *et al.* published results showing that a DNA vaccine expressing the 65 kDa heat shock protein from *Mycobacterium leprae* (hsp60 DNA), which has also been shown to significantly protect mice against tuberculosis as a prophylactic vaccine [32], was remarkably successful as a therapeutic vaccine against intravenous challenge with *M. tuberculosis.* The vaccine was also shown to protect mice against reactivation in a Cornell type model in which bacteria were eliminated by chemotherapy, leaving only bacilli that were in a latent or dormant form [15].

In the study presented here, the hsp60 DNA vaccine was tested using the more realistic pulmonary infection model in the mouse, in which the animal was exposed to a small challenge dose by aerosol exposure. In this model, the hsp60 DNA vaccine was ineffective in both the prophylactic and Cornell modes. Moreover, this vaccine, as well as the previous described highly protective Ag85A DNA vaccine [12], both induced progressively severe pulmonary necrosis in an immunotherapeutic vaccination model, especially when given to a susceptible mouse strain. While this class of DNA vaccines appears to be completely safe in terms of inoculation into naïve individuals, the data presented here suggest that if given unknowingly to individuals already exposed to tuberculosis, there may be a risk of exacerbating disease or triggering reactivation of latent tuberculosis. This previously unanticipated problem thus raises a significant safety issue.

MATERIALS AND METHODS

Mice

Specific-pathogen-free female BALB/c, C57BL/6, CD8 knockout, perforin knockout, and B cell knockout mice, 6 to 8 weeks old, were purchased from the Jackson Laboratories, Bar Harbor, Maine. CD8 knockout (KO) mice lacked the α-chain of the CD8 molecule and therefore lacked in functional CTL. The perforin KO mice were unable to produce perforin due to a mutation in the gene encoding this protein, which in turn caused a functional deficiency in granzyme as well. BKO mice lacked mature B cells. The background strain of the knockouts was C57BL/6. Mice were maintained under barrier conditions in the biosafety level 3 biohazard facility at Colorado State University. They were given sterile water, mouse chow, bedding, and enrichment for the duration of the experiment.

Plasmids

A 3.1 kb *Xmn*I genomic DNA fragment containing the *M. leprae* hsp60 gene was cloned into the *Eco*RV site of the pHMG plasmid [10], [16]. The hsp60 DNA plasmid was made by cloning a 3.3 kb *Bam*HI/*Not*I fragment which contained the hsp60 gene from the pHMG65 into the polylinker site of plasmid pCDNA3 (5.4 kbp from Invitrogen).

Vaccinations

The DNA vaccine encoding the hsp60 protein antigen of *M. leprae* was constructed using the plasmid vector pCDNA3 [32], [15]. Ag85 DNA (TB) was prepared and used as previously described [1], [12]. Mice were injected intramuscularly (i.m.) three to four times at three-week intervals with 50 μg hsp60 DNA per quadriceps muscle using a 30-gauged needle and syringe. As negative controls, mice received injections with control plasmid DNA (pCDNA3) or with saline. As a positive control, mice received subcutaneous injections with BCG at 10^6 CFU in 200 μl of sterile 1X phosphate buffered saline (single inoculation).

Animal challenge studies

In all experiments, mice were challenged by low-dose aerosol exposure with *M. tuberculosis* strain H37Rv using a Glas-Col (Terre Haute, Ind.) aerosol generator calibrated to deliver 50-100 bacteria into the lungs. Bacterial counts in the lungs were determined by plating serial dilutions of individual whole-organ homogenates on nutrient 7H11 agar and counting colony-forming units following 3 weeks incubation at 37°C. In the prophylactic model, mice were challenged 4 weeks after the last DNA injection and lungs were harvested for bacterial counts and histological analysis 4 and 10 weeks following aerosol infection. In the Cornell model, mice were aerogenically infected with *M. tuberculosis* 4 weeks prior to receiving isoniazid (Sigma) in their drinking water at 100 mg/ml for 60 days. Immediately following drug treatment, the mice received i.m. injections with hsp60-DNA 3 times as described above. Five weeks after the last DNA injection, mice received intraperitoneal (i.p.) injections with 200ul of dexamethasone (Sigma) at 6 mg/kg every 2 days for 8 weeks. Lungs were harvested 2.5 weeks later for bacterial counts and histological analysis. In the therapeutic model, mice received the first hsp60 DNA injection 8.5 weeks after the aerosol infection. Lungs were harvested for bacterial counts and histological analysis 4 and 7 weeks after the last DNA injection. In the knockout study, mice received the first of four hsp60 DNA injections 8.5 weeks after the aerosol infection. Lungs were harvested for histological analysis 68 days after the last vaccination.

Histological analysis

The middle right lung lobe was obtained from each mouse and immediately inflated with and stored in 10% formalin. Tissues were processed routinely and sectioned for light microscopy such that the maximum surface area of each lobe could be viewed. Sections were then stained with hematoxylin and eosin. Slides were examined by a veterinary pathologist with no prior knowledge of the experimental groups and were subjectively graded for both quality and quantity of cellular accumulation.

RESULTS

Vector confirmation

To verify that the hsp60 DNA plasmid contained the gene encoding for the hsp60 protein, the plasmid was digested with restriction enzymes *Bam*HI and *Not*I. The agarose gel in Figure 4.1 shows that the restriction digest yielded two bands of the expected sizes with one at 3.3 kbp (DNA fragment containing the hsp60 gene) and one at 5.4 kbp (pCDNA3 vector control).

Prophylactic vaccination studies

In a first series of experiments, BALB/c mice were immunized three times with hsp60 DNA prior to challenge by aerosol with *M. tuberculosis* strain H37Rv. In these

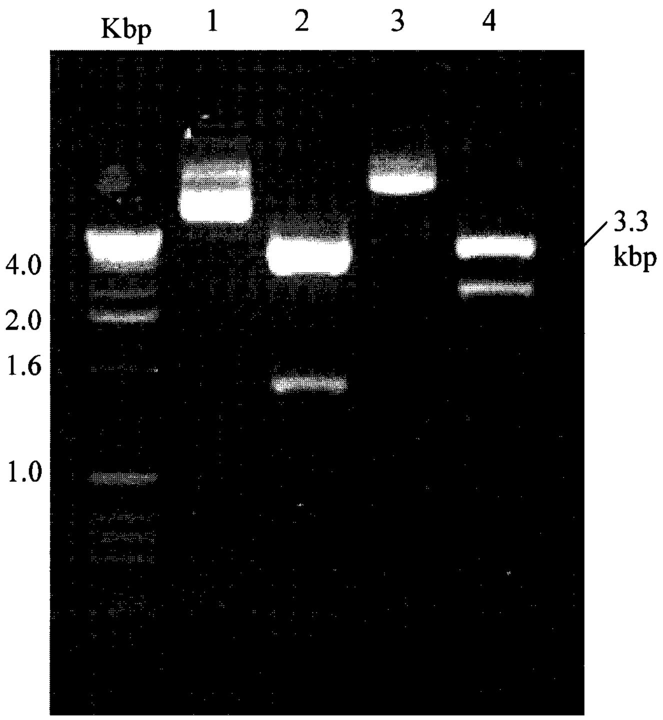

Figure 4.1. *Bam*HI/*Not*I DNA fragment was present in the hsp60 DNA construct. The 3.3 kb DNA fragment was removed from the hsp60 DNA plasmid by digestion with restriction enzymes *Bam*HI and *Not*I. The fragments were run on a 0.8% agarose gel containing ethidium bromide. The restriction digest yielded bands of the expected sizes in Lane 4 with the linear pCDNA3 vector DNA running at 5.4 kbp and the fragment containing the hsp60 gene running at 3.3 kbp. The uncut hsp60 DNA plasmid ran at approximately 8.7 kbp in Lane 4.

studies, no evidence of protection was seen, and so the experiments were repeated using an intrinsically more resistant strain, C57BL/6, in which good results have been previously obtained with other DNA vaccines [12], [31]. However, here again, no reduction in the bacterial load in the lungs was observed (Figure 4.2). Because of previously observed [34] detrimental pathology using a similar vaccine (hsp60 TB DNA) the lungs of these mice were carefully examined. In sections of lungs examined 30 days after challenge with *M. tuberculosis*, the overall pathology observed was similar in the vaccinated mice and in saline controls, with both showing widespread granulomatous inflammation (Figures 4.3A and B). In sections examined 80 days later, the aggregates of lymphocytes and macrophages were large and appeared to be much more extensive in the mice receiving the DNA vaccine, suggesting an increased cellular influx (Figure 4.3C and D).

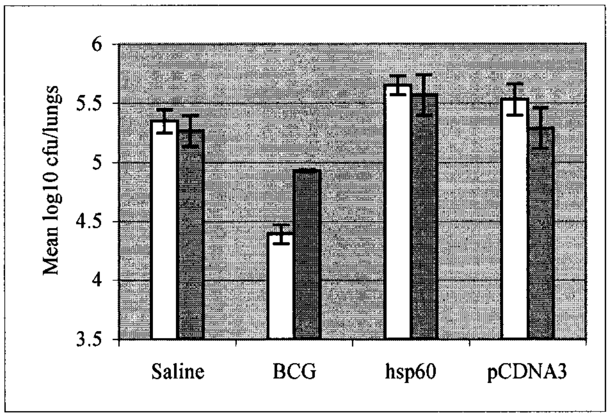

Figure 4.2. Prophylactic vaccination in C57BL/6 mice. Mice were given hsp60 DNA or control vector DNA three times at three-week intervals. The mice were exposed to a low dose aerosol infection with *M. tuberculosis* thirty days after the last vaccination and mice were sacrificed thirty (white bars) and eighty days (gray bars) after the infection. Serial dilutions of organ homogenates were plated on 7H11 agar plates. The number of viable bacteria per organ was assessed by counting the number of colonies that had grown after 21 days at 37°C.

*p<0.01 compared to unvaccinated mice using a two-tailed Student's *t*-Test. Error bars represent +/- SEM where n=4.

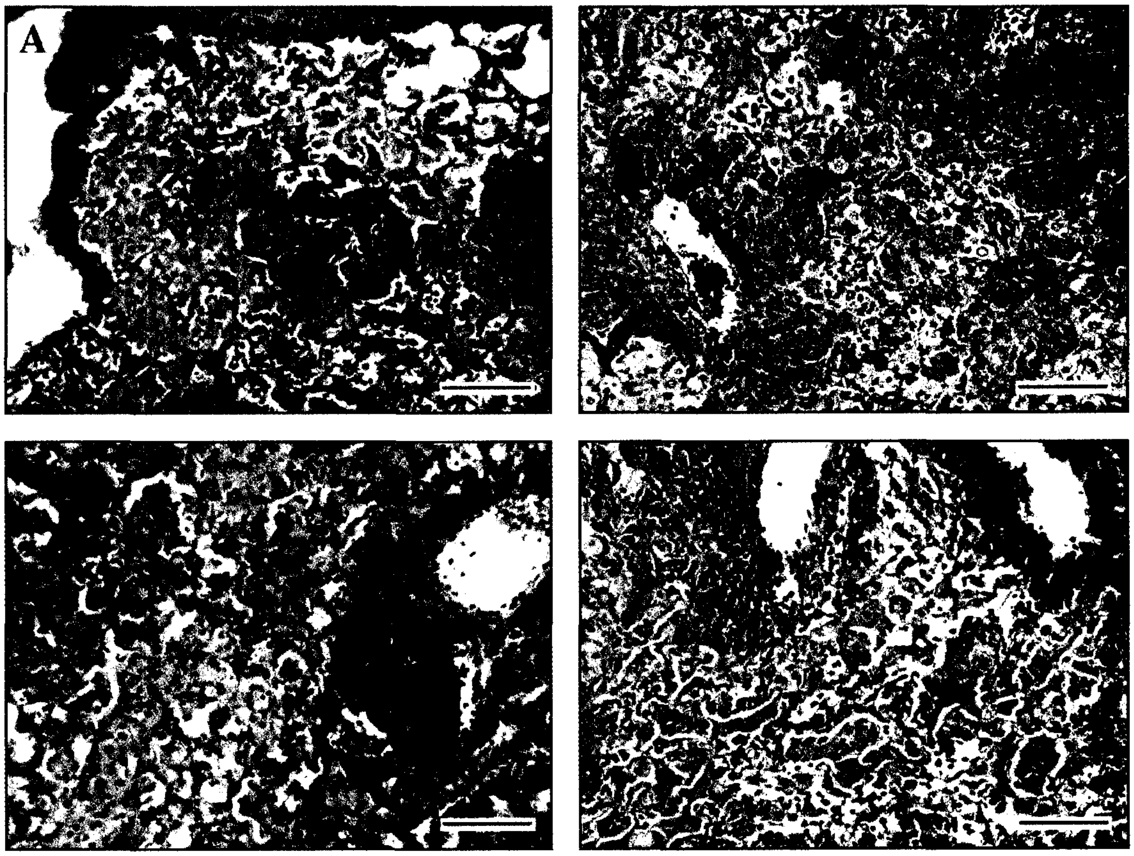

Figure 4.3. Photomicrographs of lungs of C57BL/6 mice harvested 30 days (A and B) or 80 days (C and D) after aerosol exposure to *M. tuberculosis*. Mice received prophylactic vaccination with hsp60 DNA (A and C) or saline (B and D). Alveoli contain large numbers of macrophages and lower numbers of lymphocytes, but there is no appreciable neutrophil infiltration or necrosis. At day 80 perivascular accumulations of lymphocytes are seen; these were particularly prominent in the hsp60 DNA vaccinated animals. Photos representative of all four mice per group.

Post-chemotherapeutic vaccination studies

A second series of studies were performed in both BALB/c and C57BL/6 mice using the Cornell protocol [26], in which infected mice were given chemotherapy to reduce the bacterial load to below detectable levels, after which steroid immunosuppression was given to try to reactivate residual surviving bacteria. Following the chemotherapeutic protocol previously described for the hsp60 DNA vaccine [15], inoculation with this vaccine was given prior to steroid reactivation. Despite the large number of mice used, only a small percentage of mice showed evidence of reactivation (range of one to three mice having a detectable bacterial colony in their lungs per group of 15 animals), which did not permit analysis of any potential differences between groups. In view of this, we attempted to address this problem by repeating the experiment using a deliberately less effective chemotherapeutic regimen, isoniazid alone for 60 days, that we knew from previous experience would reduce the bacterial load considerably but would not completely eliminate the bacterial load in the infected mice, at least not to the extent that this could be determined bacteriologically. In this study (Figure 4.4), isoniazid treatment reduced the bacterial load by 3 logs in the lungs of the infected mice. Groups of mice were then given hsp60 DNA, vector alone, or saline, and the bacterial load was assessed 30 days after the third vaccination. As shown, none of these materials had any effect on the re-growth seen in the bacterial load in the lungs. Moreover, since these values were still relatively low, a second group of treated mice were given 8 weeks of treatment with daily doses of dexamethasone to see if this altered any of the groups. As shown, the steroid therapy exacerbated the bacterial load to a similar degree in all three groups, measured 18 days after cessation of 8 weeks of dexamethasone treatment. These data thus indicate that the DNA vaccine had no protective effect, even when the bacterial load was very low.

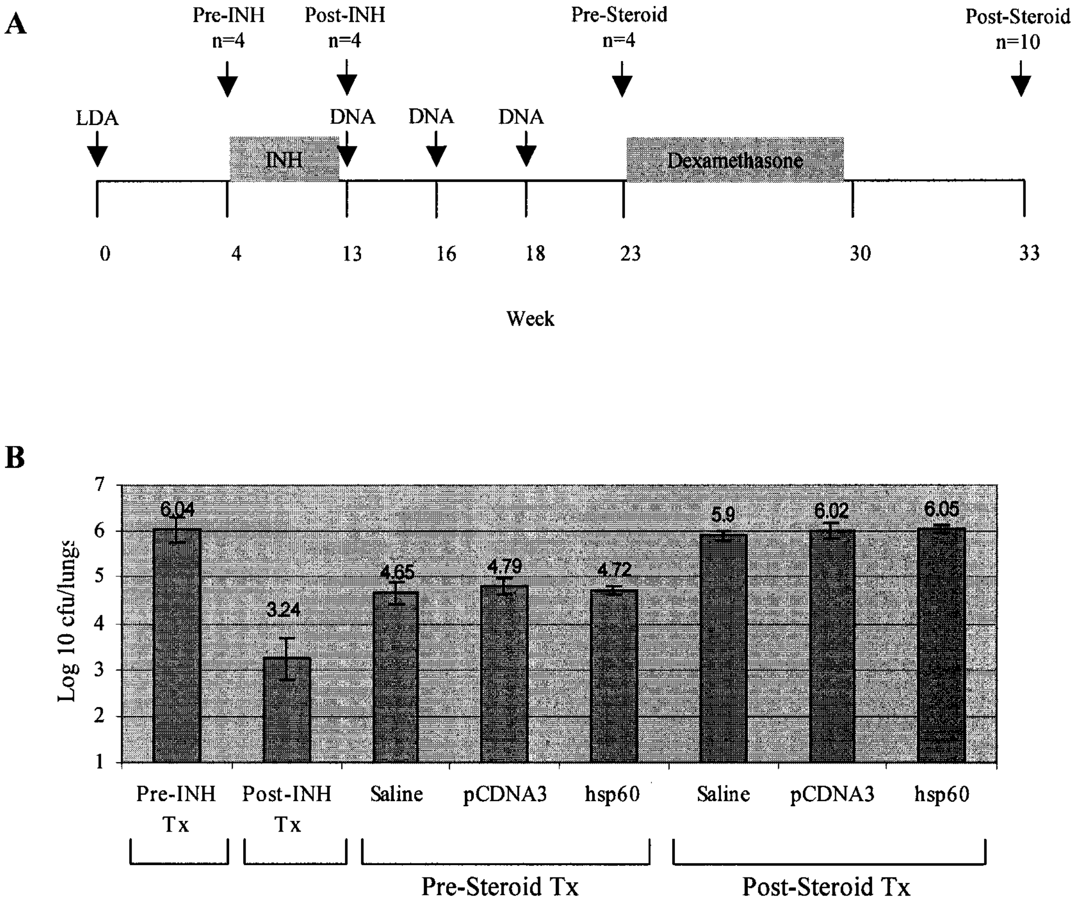

Figure 4.4. Effect of hsp60 DNA vaccine in the modified Cornell model. (A) The schedule of treatments and sampling. (B) Four weeks after exposure to low-dose aerosol challenge with *M. tuberculosis* H37Rv, the bacterial load was assessed after 30 days post-infection (Pre-INH Tx), and then the remaining mice were treated with isoniazid for 60 days, after which the reduction in bacterial load was assessed (Post-INH Tx). Mice were then given three vaccinations with the hsp60 DNA or control injections. Thirty days after the final vaccination the bacterial load was determined, and it was then determined again after 8 weeks of treatment with dexamethasone. Error bars represent +/- SEM where n=5.

Immunotherapeutic vaccination studies

In a third series of experiments, we examined the ability of the hsp60 DNA and the Ag85A DNA vaccines to reduce the bacterial load in the lungs if given after the mouse had already been infected with *M. tuberculosis*. The first set of studies was performed in BALB/c mice, which are relatively susceptible to aerosol infection. The protocol was designed to follow the bacterial load starting 1 month after the final DNA vaccine administration, but a few days after this vaccination was completed, many of the mice began to die, and others appeared to be in severe distress, and were therefore euthanized in accordance with our animal usage approval protocol.

The lungs from these mice were then examined and animals receiving saline or control DNA had expected discrete granulomas consistently seen 4-5 months after aerosol exposure. These lesions contained mixtures of lymphocytes and macrophages, with some of the latter cells appearing foamy and being gradually surrounded by increasing fibrosis (Figure 4.5A). In contrast, granulomas in mice receiving hsp60 DNA were very big (as much as 10-fold larger than those in controls) and contained multiple areas of destructive necrosis throughout these structures (Figure 4.5B). Higher magnification of these areas revealed foci consisting of groups of dead macrophages, often containing large clumps of acid-fact staining bacteria (Figure 4.5C and D). Similar areas of necrotic foci were seen in BALB/c mice that were given the Ag85A DNA vaccine (data not shown).

Again, given these unexpected results, the studies were repeated using C57BL/6 mice, with the protocol modified to reduce the vaccine frequency given from four to three. In these subsequent experiments, no deaths were observed in any of the animals,

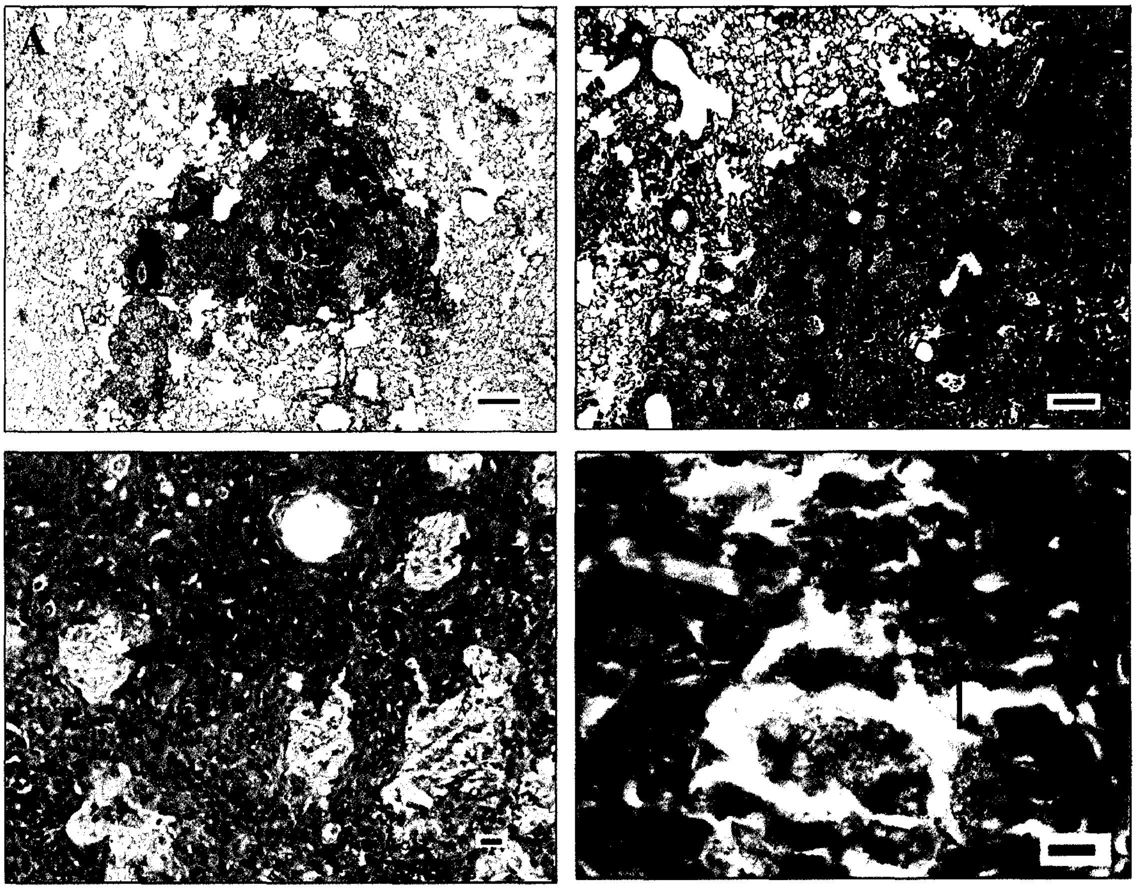

Figure 4.5. Representative histologic appearance of lungs from BALB/c mice given immunotherapeutic vaccination with hsp60 DNA. (A) In mice given saline, only small granulomatous foci were seen in the lungs of these animals 130 days after low-dose aerosol challenge with *M. tuberculosis* H37Rv. Perivascular aggregates of lymphocytes can be seen extending into the adjacent parenchyma, which is variably surrounded by macrophages. Size bar = 100 μm. (B) In mice given hsp60 DNA the lesions are very large (size bar = 100 μm) and show evidence of extensive tissue destruction. There is a sharp line of demarcation with normal lung parenchyma, and alveoli are filled with infiltrates of mononuclear cells and some neutrophils. There is loss of alveolar wall detail, with multifocal necrosis. (C) At a higher power of magnification (size bar = 10 μm), these multiple necrotic foci are characterized by fibrillar acidophilic cellular debris with clear clefts that represent extracellular cytoplasmic lipid deposition (arrow). These foci contain a mixture of normal to completely degenerate macrophages. (D) Acid-fast staining revealed multiple bacilli (example indicated with an arrow) visible within the cytoplasm of foamy macrophages as well as in the extracellular space of necrotic foci. Such bacilli were only rarely seen in lesions of control mice at this stage of the infection.

This experiment was conducted by Erik Rush, a research assistant in the Orme laboratory.

but no reductions in the bacterial load were seen 30 days after the third inoculation with the DNA vaccine (Figure 4.6). Examination of the lungs of these mice at this time point again revealed large areas of degenerative pathology in the DNA vaccinated mice (Figure 4.7). Interestingly, a similar pattern was seen in the lungs of mice 100 days after the final vaccination, but in many sections the lesions seemed less pronounced, suggesting that some degree of tissue repair had taken place (Figure 4.8).

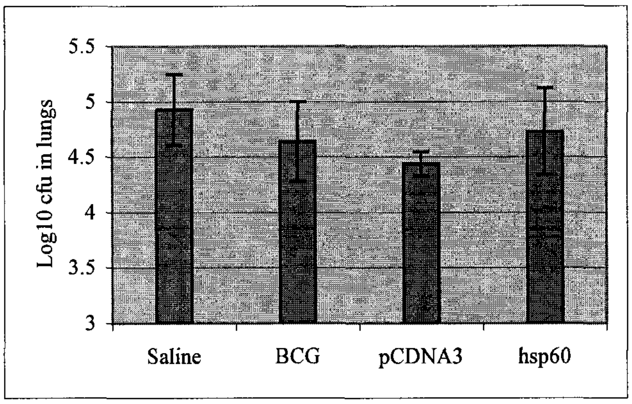

Figure 4.6. Immunotherapeutic vaccination with C57BL/6 mice. Mice were given three injections of hsp60 DNA, vector DNA control, or BCG on days 60, 81, and 102 after exposure to low-dose aerosol challenge with *M. tuberculosis* H37Rv. The data shown are the mean numbers of bacteria recovered from the lungs after a further 30 days. No protection was seen in any groups at this time, or after a further seventy days (data not shown). Error bars represent +/- SEM where n=5.

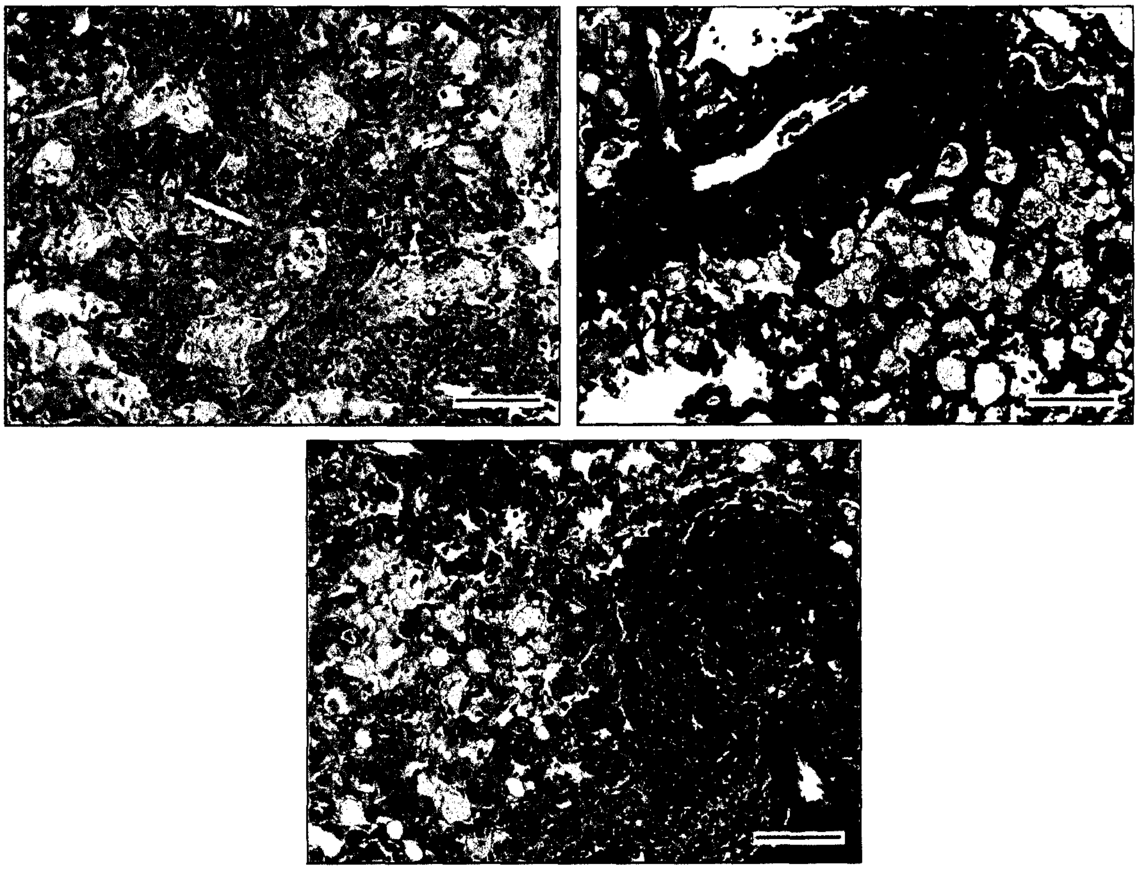

Figure 4.7. Histologic appearance of lungs of C57BL/6 mice 30 days after the third immunotherapeutic vaccination with hsp60 DNA. In mice given hsp60 DNA (A) alveoli and interalveolar sepate are effaced by infiltrates of macrophages, some of which have abundant foamy cytoplasm. There are random foci of necrosis containing karyohectic and cellular debris with intralesional aggregates of neutrophils (indicated by an arrow). Mice given saline (B) or DNA vector control (C) have prominent perivascular accumulation of lymphocytes and intra-alveolar macrophages but no necrosis or neutrophilic infiltration. Hematoxylin and eosin. 100X total magnification. Photos are representative of all four mice per group.

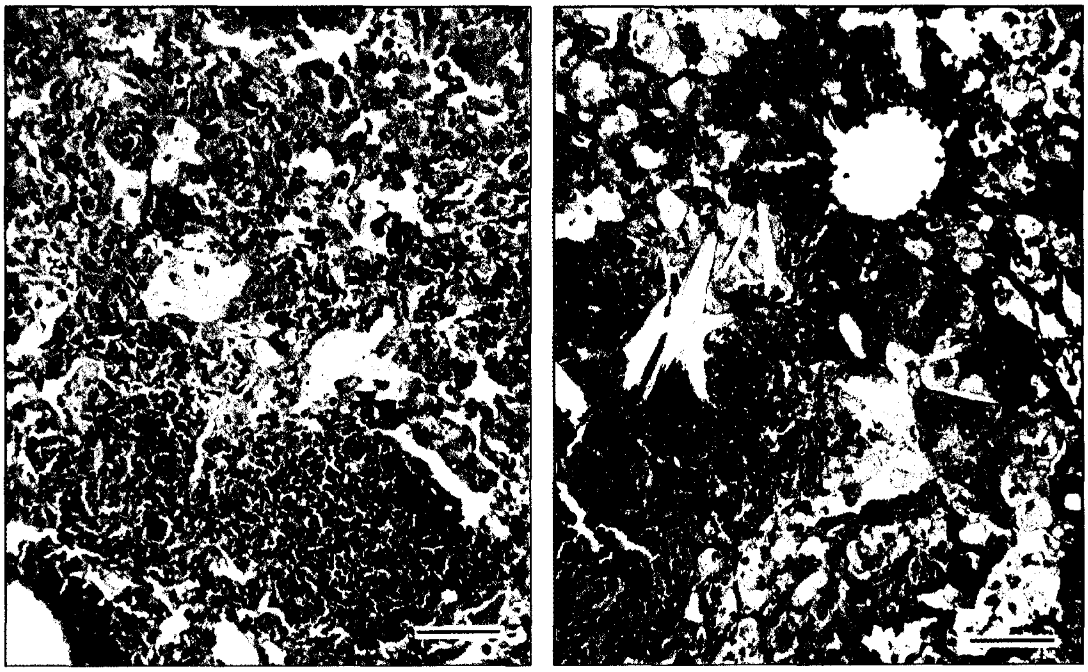

Figure 4.8. Histologic appearance of lungs of C57BL/6 mice 100 days after immunotherapeutic vaccination with hsp60 DNA. Foci of necrosis contain debris (small arrows) and clefts of accumulated cholesterol (large arrows). Alveoli contain macrophages with abundant foamy cytoplasm, and aggregates of lymphocytes are prominent. Overall, the appearances of lungs from vaccinated (A) and saline controls (B) were similar. Hematoxylin and eosin. 100X total magnification. Photos are representative of all four mice per group.

Knockout mice studies

In order to determine which immune cell population or effector molecule might be responsible for the degenerative pathology observed in the lungs of mice that were treated therapeutically with hsp60 DNA, we examined the lungs of B cell deficient, perforin deficient, and CD8 deficient mice, all of which were therapeutically treated with four intramuscular injections of hsp60 DNA at two-week intervals beginning 50 days after aerosol infection with *M. tuberculosis*. At day 160 after the aerosol challenge, histological analysis of the lung tissue revealed that wildtype C57BL/6 mice, CD8 KO, and perforin KO mice exhibited variable sized multifocal granulomatous structures that were characterized by epithelioid and foamy macrophages and aggregates of lymphocytes (Figure 4.9). In the B cell KO mice that received the hsp60 DNA vaccine (Figure 4.10), however, severe necrotizing granulomatous pneumonia was evident in the lungs, with multiple foci of neutrophils and eosinophils associated with the areas of necrosis. In several sections, the lesions were diffuse and fibrinous, and in some cases the lumen of large airways were plugged with fibrin and necrotic cellular debris. These combined histopathologic observations strongly suggest that B cells play a role in perhaps curtailing the pathological immune responses in hsp60 vaccinated mice that were initially infected with *M. tuberculosis*.

DISCUSSION

The results of this study show that a plasmid DNA expressing the hsp60 protein of *M. leprae*, which has been shown previously to confer highly significant protection against intravenous and intraperitoneal infections with *M. tuberculosis* strain H37Rv, failed to protect mice when the more realistic aerosol model of infection was used.

Figure 4.9. Representative photomicrographs of lungs from mice 160 days post aerosol infection with *Mycobacterium tuberculosis.* C57BL/6 mice (A), CD8 KO mice (B) and perforin KO mice (C) received 4 intramuscular injections with hsp60 DNA at 2-week intervals beginning 50 days after aerosol exposure to *M. tuberculosis.* Histologic samples were harvested 68 days after the last vaccination. In each panel, a focus of granulomatous pneumonia is characterized by epithelioid and foamy macrophages filling parenchyma, which in turn is punctuated by variable sized aggregates of lymphocytes (arrows). The architecture of these lesions is similar in each group, with the suggestion however, that those in the perforin KO mice tend to be smaller and with fewer lymphocytes than those in the other two groups. Hematoxylin and eosin.100X total magnification. Photos are representative of all five mice per group.

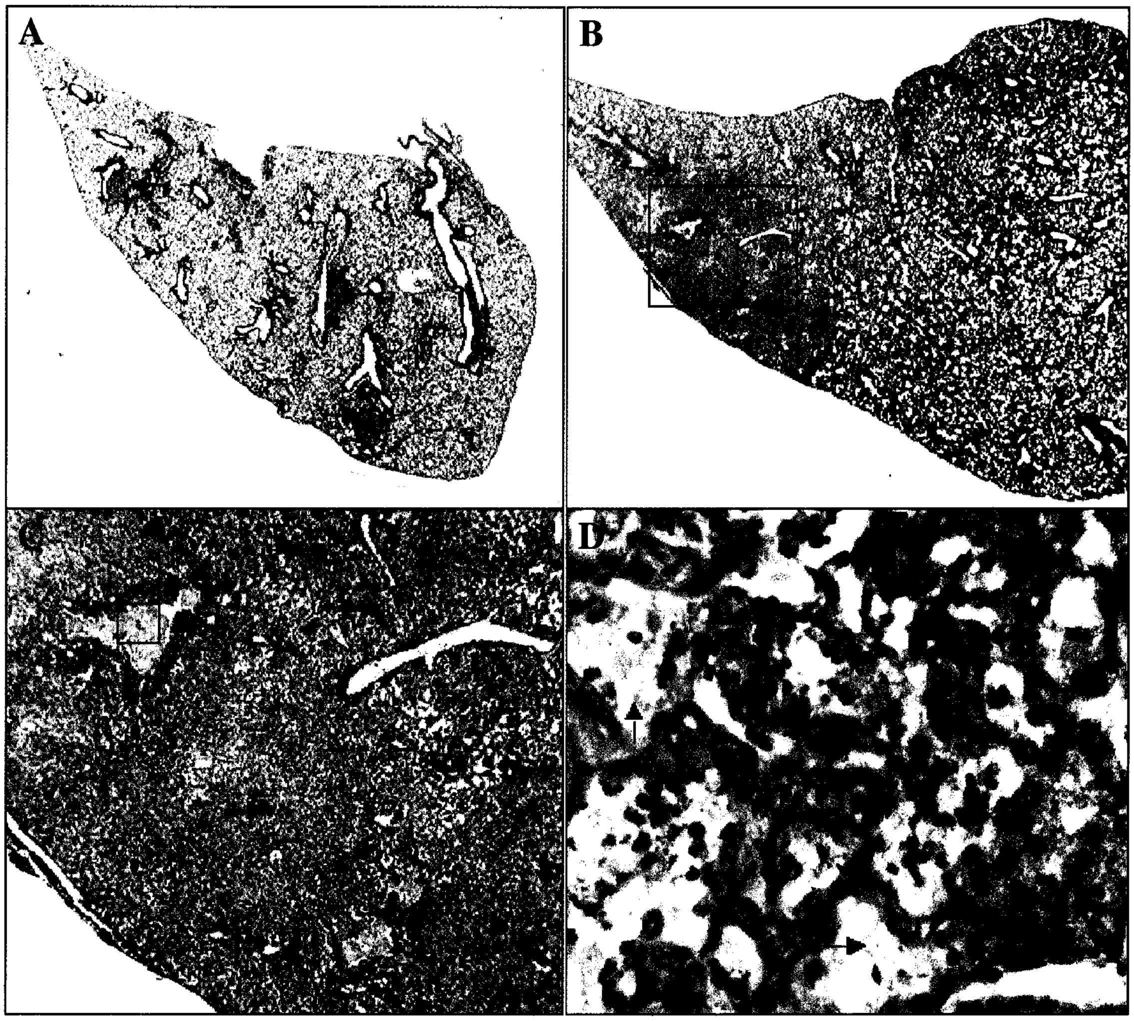

Figure 4.10. Representative photomicrographs of lungs from B cell KO mice 160 days post aerosol infection with *Mycobacterium tuberculosis.* Panel A mouse did not receive a vaccine, Panels B-D mice received 4 intramuscular vaccinations with hsp60 DNA. Panel A shows a moderate, multifocal granulomatous pneumonia (arrows). Panel B shows a severe, diffuse, fibrinous, necrotizing granulomatous pneumonia. In some cases, the lumen of large airways are plugged with fibrin and necrotic cellular debris (*). Panel C shows the area inside black box of B at higher magnification. Multiple foci of neutrophils and eosinophils (arrows) are now visibly associated with areas of necrosis. Panel D shows a focus of neutrophils and eosinophils, and an abundance of fibrin (arrows). Hematoxylin and eosin. A and B: 10X total magnification. C: 40X total magnification. D: 400X total magnification. Photos are representative of four out of five mice.

The previous observation [32] that the hsp60 DNA vaccine was highly protective when mice were given this construct as a prophylactic vaccine is very interesting because it implies that the bacilli produce large amounts of this protein antigen early during the course of tuberculosis infection. In contrast, however, the fact that culture filtrates from *M. tuberculosis* grown in culture broth contain only trace amounts of hsp60 protein, and that such cultures only begin to produce substantial amounts of the antigen when the bacilli are deprived of nutrients or are exposed to an environment of low oxygen tension [18], [27] allows one to speculate that an hsp60 antigen-based vaccine would not be protective against *M. tuberculosis* challenge. Whatever the explanation, the data in the present study suggest that the hsp60 DNA vaccine did not protect prophylactically for three possible reasons. First, the alveolar and/or local resident macrophages in the lungs may not present the hsp60 antigen, and therefore, protective CD4 TH1-type cells against hsp60 antigen were not induced. Second, the bacilli may not produce hsp60 protein early during the infection due to the very high local oxygen tension. Third, the hsp60 antigen may be presented on the surface of infected macrophages in the lungs during infection, but immune T cells may fail to reach the infected macrophages due to the influx of other uninfected macrophages which eventually give rise to the characteristic epithelioid macrophage granuloma. Such a scenario may occur because, unlike secreted proteins which are recognized very early during the tubercle infection [20], the kinetics of the presentation of hsp60 may be slower since the bacteria probably only start producing this protein when they are exposed to host defenses such as reactive oxygen and nitrogen intermediates produced by activated macrophage [4], [9]. If any of these explanations is correct, then it implies that this vaccine would most certainly be ineffective under clinical conditions. That said, however, it is still important that the DNA class of vaccines be tested under such conditions, especially considering that nearly a century has passed since BCG was first developed as a vaccine against tuberculosis, and the field has failed to provide a better vaccine since then.

Our failure to observe no more than minimal reactivation in our initial study using the Cornell model was almost certainly due to two reasons. First, the combination chemotherapy protocol is extremely potent in mice, and four months of treatment will reduce the number of potentially reactivating mice to a low percentage (in this study, the highest reactivation observed was 3 out of 15 mice), as well as increasing the number of mice needed per group to preserve statistical power. Second, although most, if not all, mice can be induced to reactivate if sufficient steroid treatment is subsequently given, this treatment generally has to be given for an extended period of time to be fully effective [26]. Whereas only three injections of dexamethasone resulted in full reactivation of all control animals, and more than 10^4 bacterial colonies were detected in the lungs of each of these mice in the study described by Lowrie *et al.* [15], in our own experience [2], at least seven injections of the more potent hydrocortisone only gave rise to partial reactivation of mice and bacterial counts of less than 100 colony forming units. In a more recent, very comprehensive analysis of the type of model, a study by Scanga *et al.* took even longer to induce reactivation in mice given a similar chemotherapeutic regimen, and again, the recovery of bacterial colonies was very low (a mean of 70).

To try to address this, we used a modified protocol that we knew would not completely reduce the bacterial load to undetectable levels. We reasoned that although it was probable that this protocol would not drive all bacteria into a truly latent state, it would still be a fair assessment of whether the hsp60 DNA vaccine could reduce or eliminate the presence of the remaining bacilli. However, as shown above, we were unable to find any evidence that the hsp60 DNA vaccine could reduce the bacterial load in the lungs.

Administration of both the hsp60 DNA and the Ag85A DNA as immunotherapeutic vaccines to BALB/c mice already in a state of chronic disease resulted in death of the animals within a few days of receiving the final intramuscular inoculation. Necropsy of the few remaining mice in both cases revealed multiple foci of small, circular areas of necrotic tissue damage throughout the granulomatous structures, with similar damage seen in mice receiving either vaccine. Moreover, in both cases, lesions were noticeably much larger than in controls, suggesting a florid lymphocytic influx induced by the vaccines. A repeat of this study in resistant C57BL/6 mice in which the DNA immunization frequency was reduced did not result in fatalities, but the vaccination had no effect on the bacterial load in the lungs. Some lung damage was observed in the DNA-vaccinated mice, but interestingly this did not appear to have further progressed in animals examined 100 days later, possibly indicating that some degree of tissue repair had taken place.

The results shown here, as well as very similar recent results by others [23] attempting DNA vaccination in an immunotherapeutic or Cornell style model, clearly show that the generation of protection against an active infection using vaccines in an immunotherapeutic manner creates a major challenge. Since the vaccine would be given at a distant site, T cells generated as a result would have to be able to rapidly home to the site of infection and have the necessary molecules to effectively invade the granulomatous tissue, which by that time could be in an advanced state of formation. Moreover, by that time the increasing local fibrosis and micronecrosis that are primary characteristics [24] of chronic tuberculosis in the mouse lung would almost certainly be a further hindrance. Having said that, however, the data presented here seem to suggest that the hsp60 DNA vaccine induced a considerable lymphoctyic influx, which was seen in both the prophylactic and immuntherapeutic models.

Collectively, these data indicate that the previously described hsp60 DNA vaccine, so effective in intravenous infection models [15], lacks the capacity to protect mice against pulmonary tuberculosis when delivered by the more realistic aerogenic route. In addition, a previously unanticipated observation concerned the apparently accelerated development of widespread necrotic lesions in the lungs of such animals, especially if the vaccine was given in an immunotherapeutic mode.

The multiple pockets of necrosis seen in the lungs of the DNA-vaccinated mice in the immunotherapeutic studies are reminiscent of the famous Koch reaction in which Koch found that immunization of mice with a suspension of mycobacterial antigens triggered necrosis in preexisting lesions in the lungs of these animals [25]. A potential explanation for these observations is the generation of potent cytotoxic T lymphocyte CD8 responses induced by the hsp60 DNA vaccine, as Silva *et al.* have demonstrated [28]. While the basis of the Koch reaction is not precisely known [17], we used various mouse models with gene disruptions to try to identify if it is indeed CD8 T cells, or perhaps others, that are responsible for the adverse histopathologic and potentially fatal responses that we observed in the therapeutic vaccination models in this study. We were surprised to find that there were no appreciable differences in the lung pathology of CD8 and perforin knockout mice that received the hsp60 DNA vaccine compared to the vaccinated wildtype mice. Surprisingly, the lungs of the hsp60 DNA vaccinated B cell knockout mice were severely damaged with diffuse lymphocytic recruitment, necrosis and fibrin-filled airways. Taken together, these unexpected results suggest that immune B cells play a role in somehow dampening the cytotoxic effects of the cell-mediated immunity evoked by the hsp60 DNA vaccine.

There are two possible mechanisms by which B cells may have dampened the pathologic response that we observed in hsp60 DNA vaccinated mice. First, B cells produce IL-10 which can down-regulate cell-mediated responses, such as IFN-g production by Th1 cells [6] by interfering with the ability of macrophages to activate Th1 cells. Although IL-10 production does not directly affect Th1 cells and CTLs, the down-regulation of IFNγ results in decreased expression of class I and II MHC molecules [14]. Down-regulating Th1 responses also results in less IL-2 release, which also may contribute to weaker T-helper and CTL responses. When IL-10-producing B cells were removed, it is possible that hsp60-specific CD4 and CD8 T cells that were primed and expanded after vaccination may have responded too potently and unchecked to the bacterial infection. The second possible mechanism is that antibodies specific for hsp60 protein may have blocked the presentation of hsp60 to antigen-specific CD4 T cells. Blocking the activation of effector or memory CD4 T cells in turn blocks the secondary expansion of cytotoxic CD8 T cells [13]. At any rate, it appears that B cells play an important role in preventing substantial damage in the lungs put forth by potent hsp60-specific CD4 and CD8 T cells. Studies in progress aim to determine whether the Ag85A DNA vaccine will evoke the same damage in the lungs of B cell knockout mice.

Two findings should be strongly stressed here. The first is the common finding that both DNA vaccines tested here (hsp60 and Ag85A DNA), as well as our previous experience with a DNA vaccine encoding hsp60 protein from *M. tuberculosis* [34], seems to imply that the pathological effects observed appear to be a general phenomenon of DNA vaccines encoding immunogenic mycobacterial antigens and are clearly not limited to vaccines encoding the heat shock chaperonin proteins. The frequency of immunization is also an important factor, since no adverse effects were seen until the mice had received at least three injections. In a previous study in which Ag85A DNA was given just twice, we saw no protection, but we also saw no evidence of untoward pathological effects [33]. Additionally, the relative resistance of the host seems to be important, given the lack of mortality seen in the C57BL/6 mice. It is interesting that it has previously been demonstrated [11] that BALB/c mice infected with *M. tuberculosis* react much more strongly to hsp60 protein than mice on the B6 background.

The second finding to be stressed is that all of the DNA vaccines tested to date in this laboratory appear to be completely safe in the context of the inoculation procedure itself. It is only after the animal has been exposed to *M. tuberculosis* infection that these pathological effects become evident, and only then if they are given repetitively. Moreover, since these pulmonary lesions take time to develop, this undoubtedly explains why they were not noted in the intravenous challenge model described by Lowrie *et al.* [15], in which no histological data appear to have been collected. Nevertheless, these findings raise a safety issue and may imply that DNA vaccine given to individuals who unknowingly already have an undetected lesion in their lungs containing chronic or latent bacilli run the risk of inducing necrotic reactivation. In this regard, there are several DNA vaccines under test that have given good results to date [21] including ESAT-6, MPT64, and others, and it is unknown if such potential vaccines would also induce these problems.

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CONCLUDING REMARKS

The fact that BCG does not provide consistent protection to all individuals, and that its protective efficacy wanes in the adult population has spurred on vigorous research efforts to develop an improved vaccine to combat tuberculosis. Unfortunately, no published vaccine candidate to date has been shown to confer protection in animal models that surpasses the level of protection provided by BCG.

One of the most interesting findings in the studies presented here was that BCG was able to focus CD4 T cells capable of producing IFNγ to the lungs after subcutaneous vaccination. Recruitment of this protective cell population to the lungs prior to infectious challenge with *M. tuberculosis* seemed to be essential in controlling the growth of the bacilli. Ad85, therefore, is an appropriate candidate vaccine against pulmonary tuberculosis due to its ability to infect epithelial cells in the respiratory tract, which thereby enables the virus-based vaccine to focus immunity to the lungs. It is the opinion of this author that, based on its ability to focus previously primed CD4 T cells capable of producing IFNγ to the lungs, the Ad85 vaccine may be most useful in prime-boost strategies, especially since the Ad85 vaccine was only partially protective on its own. In the results presented here, the number of CD4 IFNγ T cells recruited to the lungs by DNA85+Ad85 was lower than that which was recruited by BCG vaccination alone. This decrease in recruitment of CD4 IFNγ T cells into the lungs was most likely due to the fact that the DNA85 vaccine was ineffective.

Several reasons probably account for the superior protective response that was induced by BCG compared to Ad85. One possible reason could be that BCG produces a much broader range of mycobacterial antigens than does Ad85. Another potential explanation is that BCG, because it is a replicating, intracellular organism, is able to induce strong Th1-type immune responses, unlike the replication-defective Ad85 vaccine that, according to these studies, seemed to induce stronger antibody responses to Ag85A protein. Perhaps an improved adenoviral system could be developed to remedy these two weaknesses of the Ad85 vaccine. For instance, a gutless adenoviral vector, which contains a larger cloning capacity than the first-generation adenovirus used in these studies, could be cloned which expresses several more mycobacterial antigens than just Ag85A alone. Another approach could be to include a gene that encodes not only a secreted form of Ag85A with a tissue plasminogen activator signal sequence, but also a second copy of the gene that would target Ag85A to the cytoplasm. This could be accomplished by tagging the second Ag85A gene with ubiquitin, which would target the protein to proteasomes in the cell. This same vector could be engineered to also encode Th1-stimulating components such as IL-12 or IL-2, which would help to direct the immune response toward a Th1-type response.

While directing the immune response toward Th1-type immunity is essential, the presentation mode of the vaccine is also extremely important. For instance, although we are not certain that the Ad85 vaccine used in these studies expressed a secreted form of Ag85A protein, even if it did, producing extracellular, soluble protein may not be the best approach since the protein would then be vulnerable to clearance by specific antibodies before they could be phagocytosed by APC. It was interesting that each of the recombinant vaccines used in this project (i.e. Ag85A subunit, DNA85 and Ad85) induced relatively strong Ag85A specific antibody responses, and that they were less protective than BCG, while BCG did not induce any detectable antibodies against Ag85A and was highly protective. It seems possible, therefore, that inducing strong antibody responses against the target mycobacterial antigen may inhibit effective CD4 T cells responses. For example, specific antibodies may bind to important epitopes on the antigen, thereby altering presentation on class II MHC to CD4 T cells.

To insure that CD4 T cells capable of producing IFNγ are efficiently stimulated, therefore, the best approach may be to prime this cell population with a subunit vaccine emulsified in micelle-type adjuvant(s) that would allow for the most efficient uptake by antigen presenting cells while somewhat avoiding clearance by antibodies. This priming vaccination could then be followed by a boost with the gutless adenoviral vector mentioned above in order to boost not only CD4 T cells responses, but also CD8 T cell responses which may be important for protection during the chronic stages of tuberculosis infection. The best Ag85A subunit vaccine for use in this prime-boost approach would be Ag85A in DDA/MPL-SE, based on the fact that Ag85A emulsified in DDA conferred over 1 Log_{10} protection in the lungs in this study, and the fact that other researchers have found that adjuvants such as MPL-SE and DDA are superior when used in combination. DNA85 could also be used in this system as the priming vaccine since, in theory, it should be capable of priming both CD4 and CD8 T cells against Ag85A. However, in this author's hands, the DNA85 vaccine repeatedly failed to protect mice against aerosol challenge with *M. tuberculosis*.

The fact that the DNA85 vaccine did not confer consistent protection in these studies brings into question whether this vaccine would be realistically valuable in humans. This is an unfortunate finding since DNA vaccines are easier to store and cheaper to produce than either the Ad85 or Ag85A subunit vaccines. Moreover, if the inconsistency in the protective efficacy of this vaccine were due to technical difficulties in administering the vaccine intramuscularly to mice, this would be a major problem because it is unlikely that the health workers who would administer the vaccine in the villages of developing countries would be well-trained in administering intramuscular injections.

The other major finding in the work presented here was that hsp60 DNA (*M. leprae)*, which has been shown to protect prophylactically and therapeutically against intravenous *M. tuberculosis*, was completely ineffective in this laboratory on both accounts, and that it in fact caused severe pulmonary necrosis remindful of the classical Koch reaction when the vaccine was delivered in a therapeutic mode. Again, B cells seemed to have played an important role in these studies. However, instead of possibly interfering with the generation of protective immunity as proposed above in the Ag85A vaccine studies, hsp60-specific antibody may have actually protected the mice against potent hsp60-specific CD4 T cell responses, which may have been directly or indirectly involved in evoking necrosis in the lungs later during the tuberculosis infection.

The danger in using a highly conserved protein like hsp60 in a vaccine is that hsp60-reactive T cells that were primed and boosted during multiple immunizations may cross-react with host hsp60 proteins that are probably expressed later during the tuberculosis infection. For instance, it is possible that infected macrophages within the granuloma upregulate the production of hsp60 protein as they become more and more stressed due to the infection. As macrophages begin to die, extracellular hsp60 (self or mycobacterial) could be phagocytosed by incoming macrophages and presented to CD4 T cells, which in turn could activate CTLs to kill cells presenting hsp60 as well as produce IFNγ, thereby further activating macrophages. In these studies, hsp60-specific antibodies may have been important in down regulating this response by binding to the extracellular hsp60 (self or mycobacterial).

This theory of autoimmunity to explain the lung damage in hsp60-vaccinated mice must be used with caution, however, since DNA85 had a similar effect in a susceptible mouse strain when delivered in a post-exposure mode. It may be that the infection itself generates strong antigen-specific T cell responses, which we then boosted with each vaccination resulting in perhaps too much cellular immunity. If allowed to run unchecked, we would expect to see substantial pathological damage in the lungs, which we did in the B cell knockout mice. In this regard, it is possible that the production of IL-10 by B cells may have played a part in down-regulating destructive cell-mediated immune responses that presumably became more potent after repeated injections with hsp60 DNA. Studies in progress will determine whether DNA85-vaccinated B cell knockout mice will present with similar lesions in the lungs.

Although DNA vaccines, particularly DNA85 and hsp60 DNA, appear to be perfectly safe when they are delivered in a prophylactic mode, the results reported in this study may have important implications for the use of DNA vaccines in general because if given unknowingly to individuals who have already been exposed to tuberculosis, there is the possibility that susceptible individuals may present with exacerbated disease or reactivated tuberculosis.