Strategies to improve BCG-mediated protection from *Mycobacterium tuberculosis*

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Strategies to improve BCG-mediated protection from *Mycobacterium tuberculosis*.

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June 2004

Research Fields: Immunology & Vaccinology

Submitted in partial fulfilment of the requirements of the Open University for the degree of a Doctor of Philosophy.

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Thank-you again,

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Abstract

Background: Host resistance to pulmonary tuberculosis is associated with the induction of IFN-γ secreting T cells in the lung. Recombinant viruses used in heterologous prime-boost immunisation regimens can evoke powerful T cell immune responses and are promising candidates for novel tuberculosis vaccines. In this thesis, the immunogenicity and protection protective efficacy of viral vectors expressing the immunodominant antigen, 85A were investigated in murine and macaque models of tuberculosis disease.

Results: Recombinant modified vaccinia virus Ankara, expressing the Mycobacterium tuberculosis antigen 85A (MVA85A), strongly boosted BCG-induced antigen 85A specific- CD4+ and CD8+ T cell responses in BALB/c and C57BL/6 mice. A comparison of intranasal and parenteral immunisation of BCG showed that whilst both routes elicited comparable T cell responses in the spleen, only intranasal delivery elicited specific T cell responses in the lung lymph nodes and these responses were further boosted by intranasal delivery of MVA85A.
Following aerosol challenge of BALB/c with *Mycobacterium tuberculosis*, intranasal boosting of BCG with either BCG or MVA85A afforded unprecedented levels of protection in both the lungs (2.5 log) and spleens (1.5 log) compared to naive controls. Protection in the lung correlated with the induction of antigen 85A specific IFN-γ secreting T cells in the lung lymph nodes.

In rhesus macaques, aerosol delivery of BCG induced comparable kinetics and frequencies of T cells in the peripheral blood compared to intradermal BCG without producing abnormal pathology. MVA85A vaccination induced low level Ag85A-specific CD4+ and CD8+ T cell responses in the blood. Further vaccination with another attenuated poxvirus, Fowlpox expressing antigen 85A significantly increased 85A-specific T cell response in 5 of 6 outbred macaques. Analysis of lymphocytes in broncho-alveolar lavage showed that vaccination with either BCG or M.85A/F.85A induced high frequencies of 85A-specific T cells in the respiratory compartment.

*Conclusions:* These findings support further evaluation of mucosally targeted prime-boost vaccination approaches for tuberculosis.
List of abbreviations

PFU  plaque forming units
CFU  colony forming units
aa   amino acid
PBS  phosphate buffered saline
RT   room temperature
IFN-γ interferon gamma
SFC  spot forming cells
TB   tuberculosis

*M. tuberculosis* *Mycobacterium tuberculosis*

*P. berghei* *Plasmodium berghei*

BCG  Bacille-Calmette Guerin
MVA  Modified Vaccinia Virus - Ankara Strain
FP9  Fowlpox Virus - FP9 strain

i.n  intranasal
i.d  intradermal
i.v  intravenous
i.m  intramuscular
p    footpad
aero aerosol

ConA conconavalin A
DNA  deoxyribonucleic acid
CS   circumsporozoite
PPD  protein purified derivative

Ag85A antigen 85A
Ag85B antigen 85B

LN   lymph nodes
LLN  lung lymph nodes
FLN  facial lymph nodes
ILN  inguinal lymph nodes

pb9  peptide 9 of the circumsporozoite protein of *P. berghei*
P11  peptide 11 of the antigen 85A of *M. tuberculosis* H37Rv
P15  peptide 15 of the antigen 85A of *M. tuberculosis* H37Rv

STCL short-term cell line
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Introduction

1.1 Global Tuberculosis Epidemic

Overview

Tuberculosis in humans is predominantly caused by the bacillus, *Mycobacterium tuberculosis* (*M. tuberculosis*). *M. tuberculosis* is a respiratory pathogen passed from an infected to non-infected individual typically through coughing. The earliest reports of infection of humans with *M. tuberculosis* infection date from 5000 BC (1). Tuberculosis has spread world-wide with reports of disease in all inhabited continents. At the turn of last century, tuberculosis was a plague on the industrial world, but over the following 80 years improved public health, better diagnostics, widespread immunisation with the BCG vaccine and the introduction of chemotherapy led to a steep decline of disease incidence world-wide. Tuberculosis related morbidity and mortality again increased in the late 1980’s and in 1993, tuberculosis was declared a global epidemic by the World Health Organisation.

Eight million people are newly infected with *M. tuberculosis* annually and 2 million die each year predominately from pulmonary disease (http://www.who.int/gtb/publications/globrep01/index.html). These numbers gain even greater import in the developing world where 95% of tuberculosis cases occur (2). The re-emergence of tuberculosis as a global health threat results from a combination of factors, including limited access to chemotherapeutic drugs in developing countries, the failure of the BCG vaccine to protect from pulmonary disease in the tropics and the significantly
increased progression to tuberculosis disease in individuals who are infected with Human Immunodeficiency Virus (HIV). Today, health organisations have implemented a variety of programmes to both prevent and cure tuberculosis disease. A major goal is the development of a more efficacious vaccine to prevent pulmonary disease.

Factors decreasing the number of tuberculosis cases

Diagnostics

Early detection of tuberculosis is a central issue in minimising transmission. A definitive diagnosis of tuberculosis requires the recovery of M. tuberculosis organisms from a patient's body fluids or tissues. The key test for pulmonary tuberculosis is multiple sputum samples testing positive by microscopy. Sputum microscopy has some limitations. The sensitivity of this test is low (1x10^4 bacilli / ml), and therefore functions best in more advanced cases of disease. It also performs poorly in HIV+ individuals and is inadequate for paediatric (where it is difficult to obtain sputum) and extra-pulmonary tuberculosis. X-ray is also employed in the confirmation of pulmonary lesions and in determining the extent of disease particularly when the M. tuberculosis has undergone extra-pulmonary spread.

Skin Testing

The tuberculin skin test (TST) involves intradermal or subcutaneous injection of purified culture filtrate proteins of M. tuberculosis, commonly referred to as tuberculin. Prior M. tuberculosis exposure produces a delayed-type hypersensitivity (DTH) response to the tuberculin 48 to 72 hours after infection. The DTH response results from an influx of activated macrophages that accumulate and surround the
site of infection. The size of the DTH response is taken as a measure of the degree of exposure to *M. tuberculosis*, but does not correlate with protection from disease (3, 4). Tuberculin conversion is routinely used as an assay of recent *M. tuberculosis* exposure, but its application is limited because it can not reliably distinguish *M. tuberculosis* infection from BCG vaccination, and/or exposure to environmental mycobacteria. The US and The Netherlands rely heavily on the TST for contact tracing following *M. tuberculosis* exposure. Both countries have ended BCG vaccination programmes so as to not compromise the diagnostic value of the TST.

**DOTS programme**

The ‘directly observed therapy – short course’ approach or DOTS programme was initiated by the World Health Organisation (WHO). The programme aims to ensure that tuberculosis positive patients comply with chemotherapy regimens through regular supervision by trained health care workers. The targets for control include curing 85% of newly detected smear-positive cases of pulmonary TB and detecting 70% of existing cases by 2005 (5). The advantages originally forecast for the DOTS programme were increased cure rate, decreased incidence of multiple-drug resistant tuberculosis, increased longevity in patients suffering from Acquired Immune Deficiency Syndrome (AIDS) and increased cost-effectiveness. The programme has proved highly successful. Today, of the 22 countries reporting the highest tuberculosis burdens, 16 have cure rates greater than 70% (5). In India alone, 200 000 lives and US$ 400 million have been saved since the introduction of the DOTS programme in the late 1990s (5). A remaining area of concern is the low smear-
positive detection rates. Only 4 of the 22 high burden countries have achieved smear positive detection rates over 60%. Therefore despite high cure rates, a significant proportion of patients remain symptomatic and infectious for months before successful diagnostic testing (5).

**Therapeutic - chemotherapy**

The development of combination chemotherapy has had the greatest impact on decreasing tuberculosis incidence in the world. Anti-tuberculosis drugs work by inhibiting metabolic pathways of *M. tuberculosis*, examples being cell wall and protein synthesis.

Two factors are essential to successful chemotherapy; combination therapy (2 or more drugs) and adherence to treatment programmes which typically last 6 months (reviewed in (6)). Combination therapy was introduced after high levels of resistant strains developed following single drug treatment using streptomycin (7, 8). Adult pulmonary disease requires treatment for 6 months or more to significantly decrease the rate of relapse of disease (9). This length of treatment is necessary to achieve either sterilizing immunity or decrease the bacillary load to a level where the host immune response can contain or eradicate any residual bacilli.

**Prophylaxis - BCG Vaccine**

The only licensed vaccine against *M. tuberculosis* is the bacillus of Calmette and Guérin (BCG) (10). BCG is a live attenuated strain of *Mycobacterium bovis* and in developing countries is typically administered intradermally as a single dose to newborn infants. Each year, 80% of all infants born receive BCG (http://www.who.int/inf-fs/en/fact104.html).
Vaccine efficacy is measured in terms of the percentage reduction in disease among vaccinated individuals that is attributable to vaccination (11). The review of many studies, suggests that BCG vaccination is protective against childhood meningeal tuberculosis and systemic forms of the disease. Adult pulmonary disease is the major cause of tuberculosis mortality, and it is here, that BCG efficacy is most variable, ranging from 77% in the UK to 0% in Chingleput, India (12). The theories surrounding the variability of BCG protective efficacy are discussed is Section 1.5.

Factors increasing the number of tuberculosis cases

Host Genetics

Over 90% of immunocompetent, *M. tuberculosis* positive individuals do not develop tuberculosis (Fig. 1). The factors that control susceptibility in the remaining 10% are unknown. Studies describing an increased progression to tuberculosis in identical compared with non-identical twins demonstrated host genetics play a role in susceptibility to tuberculosis (13-15). With the exception of mutations within the IFN-γ receptor pathway (discussed in section 1.4) that appear to exert monogenic control over susceptibility to mycobacterial disease, tuberculosis susceptibility may require polymorphisms in several genes. Various genes have now been described that influence resistance and susceptibility to tuberculosis in certain ethnic populations. These include MHC genes and genes encoding proteins that may be involved in macrophage mediated killing of *M. tuberculosis* like natural resistance associated macrophage protein -1 (Nramp -1) and TNF-α (16-19).

Socio-economic factors and poor implementation of programmes
Both poverty and malnutrition have clear associations with tuberculosis incidence and the severity of clinical disease (20, 21). Overcrowding and poor ventilation can also contribute to increased transmission within a community (html://www.who.int/gtb).

Multiple-drug resistant strains of tuberculosis

Multiple drug resistant (MDR) tuberculosis is defined as resistance to at least isoniazid and rifampicin (22). The major selection factors in the development of MDR tuberculosis are inadequate drug treatment and poor adherence to recommended treatment regimens (23, 24).

Globally, the prevalence of MDR tuberculosis is low. In 2000, MDR tuberculosis accounted for 3.2% of all new cases (25). However, regions of high MDR tuberculosis incidence have emerged (26, 27).

The major problem of treating MDR tuberculosis is the cost, which has been estimated at greater than 50 times the cheapest short-course regimen for drug-susceptible *M. tuberculosis* (28).

Human Immunodeficiency Virus epidemic

Human Immunodeficiency Virus type 1 (HIV-1) infection is characterised by the progressive loss of CD4+ T cells. Human Immunodeficiency Virus positivity (HIV+) is the strongest risk factor for the progression from infection to active tuberculosis, increasing the risk of developing disease from 5-10% over one’s lifetime to 5-10% per year (29). Not surprisingly, tuberculosis is a leading cause of death amongst HIV+ -individuals (30). At the end of 2000, one third of HIV+ people were co-
infected with *M. tuberculosis* and 8.4 million (70%) lived in sub-Saharan Africa (31).

Traditional TB control measures effectively blunt the impact of HIV on tuberculosis but despite active BCG vaccination and DOTS programmes in sub-Saharan Africa, tuberculosis incidence continues to rise (32, 33). The increasing number of tuberculosis cases in HIV+ people poses an increased risk of tuberculosis transmission to the general community whether HIV+ or negative. New initiatives have been proposed to act as an adjunct to the DOTS programme (31) and include intensified case-finding, cure and preventive treatments as well as intervention against HIV. This involves the promotion of voluntary HIV testing, counselling to decrease high risk sexual behaviour, provision of condoms, treatment for sexually transmitted infections, and provision of highly active antiretroviral treatment (HAART). The development of any new tuberculosis vaccine should therefore be safe for use in immunocompromised individuals.

1.2 The Bacillus

*M. tuberculosis* – genomic structure

Epidemiology

Mycobacteria include both saprophytes and pathogens with a host range spanning plants to man. The *M. tuberculosis* complex refers to a group of mycobacteria that show greater than 99% homology at the nucleotide level and are identical at the 16S rRNA level (34, 35). Members include, *M. tuberculosis, M. bovis, M. cannetti, M. fortuitum* and *M. africanum*, which whilst genetically similar, exhibit wide species
preferences. The high level of conservation at the amino acid level within members of the *M. tuberculosis* complex produces complexity in tuberculosis vaccine development. Many of the immunodominant proteins in *M. tuberculosis* are also expressed by environmental mycobacteria that can induce cross-reactive immune responses against vaccine antigens and inhibit vaccine efficacy.

The members of the *M. tuberculosis* complex most likely descended from a common soil mycobacterium (36). The host range of the *M. tuberculosis* bacillus is limited to humans except for rare reports of infections in elephants (37). *M. tuberculosis* was formerly believed to have evolved from *M. bovis*, following the domestication of cattle by man (36). Recent genomic analysis argues that *M. tuberculosis* did not descend from *M. bovis* and that the common ancestor to both mycobacteria was already a human pathogen at the time when *M. bovis* split from the *M. tuberculosis* lineage. It is proposed that after this split, *M. bovis* acquired a broader host range, which today includes most mammals (38).

**Structure and functions**

**Growth Characteristics**

*M. tuberculosis* is a gram positive intracellular bacillus 2-4μm × 0.2-0.5μm in size. It is characterised by slow growth, a complex cell envelope and mechanisms that enable it to persist within the human host. *M. tuberculosis* can be grown in broth culture and following 3-4 weeks culture on semi-solid media produces visible off-white, roughened colonies (39).

**Analysis of the genome**
The spontaneous mutation frequency of *M. tuberculosis* is comparable to other bacteria (40). However, the allelic variation at the amino acid level is very low, with most variation associated with genes implicated in drug resistance (35). This affords a benefit to vaccine development because antigens used in vaccines will share a very high level of homology with antigens in field strains.

The H37Rv strain (H37Rv accession number AL123456) of *M. tuberculosis* is the strain most commonly used in tuberculosis research because it retains full virulence in animal models. The full sequence of H37Rv was published in 1998 (41). The *M. tuberculosis* genome contains almost 4000 genes has the ability to synthesise all essential amino acids, vitamins, enzymes and co-factors (41, 42).

The *M. tuberculosis* cell envelope has an additional layer beyond peptidoglycan, which is very rich in unusual lipids, glycolipids and polysaccarides. Genomic sequencing revealed that *M. tuberculosis* has extensive capacity for the biosynthesis and degradation of lipids with around 250 enzymes involved in fatty acid metabolism. The bacillus also has intact pathways for enzyme glycolysis, the pentose phosphate pathway, tricarboxylic acid cycle and glycoxylate cycles. Under aerobic conditions, it can generate adenosine triphosphate by oxidative phosphorylation, possesses components of several anaerobic phosphorylative electron transport chains and genes that encode haemoglobin-like proteins that may protect against oxidative stress.

Screening of novel *M. tuberculosis* genes is underway to identify candidate antigens for vaccine development or as targets for chemotherapy (43).
1.3 Clinical Aspects of Tuberculosis

Transmission

*M. tuberculosis* is transmitted by droplets of 5μm or less generated by individuals with tuberculosis by speaking or coughing. Estimates suggest 1-10 bacilli are contained in each droplet and the infectious dose can range from 5-200 bacilli (6). Transmission of *M. tuberculosis* is inefficient (Fig. 1). Only 25-50% of household contacts of patients with active pulmonary disease become infected (44). The probability of infection increases with exposure (45) and is dependant on a number of factors including the number of organisms in the contact’s sputum, the degree of exposure, ventilation, dilution effects and exposure to sunlight (6).
Figure 1: Schematic of the rates of progression to tuberculosis disease following *M. tuberculosis* exposure. Host factors encompass both acquired and genetic factors.
Primary Disease

Once inhaled, the bacilli that escape muco-cilliary clearance in the bronchioles and airways lodge in the alveoli or terminal air passages of the lung and may establish a local focus of disease. The infection of resident alveolar macrophages is followed by the recruitment of uninfected monocytes, neutrophils and the complement component, C5a. Cytokines are also released by the alveolar macrophages. Some bacilli are transported probably by dendritic cells to regional lymph nodes and an additional focus of disease develops (46).

The disease focus is called a granuloma that is a compact collection of cells comprising of many layers surrounding the bacillus. The granulomatous response to M. tuberculosis infection is perceived as protective, walling off the bacilli and preventing multiplication until such time the body can fully resolve the granuloma. Individual granulomas can exhibit different patterns of cytokine production and immunopathology within a patient suggesting that each lesion is an independent micro-environment distinct from adjacent granulomas (47). The core contains macrophages that resemble epithelial cells and are called epithelioid cells. Some macrophages fuse to form multinuclear giant cells. Surrounding this is a zone containing lymphocytes including Natural Killer (NK), αβ and γδ T cells that are responsible for macrophage activation. The majority of bacilli are killed by activated macrophages but a proportion may survive. Replication of bacilli induces apoptosis and results in central necrosis in the encasing granuloma (48). In humans, the metabolically active macrophages consume the oxygen diffusing into the granuloma
so that the necrotic interior becomes acidic and anoxic producing in caseous (cheese-like) centre which is thought to further inhibit the mycobacterial growth. The necrotic region solidifies and the lesion usually resolves by calcification and resorption without treatment and can leave a very small, calcified scar in the pulmonary parenchyma and calcification of hilar lymph node. As a result of primary infection, the individual develops delayed type hypersensitivity (DTH) to tuberculin (2-4 weeks after injection) and can also develop life-long immunity to disease.

Post Primary Disease

Post-primary disease occurs in approximately 5% of cases in immunocompetent individuals (Fig. 1). Here, the caseous core of the granuloma does not resolve but liquefies breaking down the granuloma. Liquefaction is, in part, mediated by enzymes produced by surrounding epithelioid cells. The granuloma can discharge its contents into bronchioles that can infect other parts of the lung. The large cavity that is left is CO$_2$ enriched and neutralises acid conditions and is highly permissive for extra-cellular $M.~tuberculosis$ growth. This stage is referred to as open or cavitatory tuberculosis. At this stage, patients are highly infectious because of bacilli in the bronchioles. The ability to combat the disease is severely limited because cell-mediated immune responses cannot target extracellular bacilli; which, being at very high numbers also increases the incidence of mutations within the flora and may lead to the development of antibiotic resistant mutants. $M.~tuberculosis$ can also undergo miliary dissemination, meaning spread through the bloodstream (usually gaining access via the lymphatics) and can seed in any organ of the body, including other areas of the lungs. Miliary disease can occur during primary infection or after re-
activation of disease. *M. tuberculosis* exhibits a preference for regions of high oxygen tension most commonly in the meninges but also bones, liver, spleen, brain and kidneys. Like the lung, most sites of disease can heal by granulomatous encapsulation, sometimes with calcification and necrosis.

Reactivation of quiescent primary disease and re-infection

Half of all immunocompetant individuals, who do develop tuberculosis, do not present with clinical disease until years after initial infection (Fig. 1). This phenomenon is referred to as reactivation disease and the period between primary infection and disease manifestation is described as latent infection.

The development of techniques to routinely type sub-strains of *M. tuberculosis* like spoligotyping and DNA fingerprinting have shown that a significant proportion of cases of recurrent tuberculosis arise from exogenous re-infection rather than reactivation. In non-tuberculosis endemic areas, the majority of recurrence of tuberculosis arises from reactivation of disease (49-51), whereas in endemic regions, the proportion of recurrent tuberculosis stemming from re-infection ranges from 12-75% (52-55). The risk of recurrent tuberculosis arising from re-infection is greater still in HIV-1+ individuals (56).

1.4 Host Immune Response

Overview

Resistance to tuberculosis disease is multi-factorial, invoking innate and adaptive immune responses to both contain infection and also kill bacilli. Overlapping mechanisms are involved in the control of *M. tuberculosis* infection. The first is
incoming antigen specific T cells that secrete cytokines like IFN-γ to activate locally infected macrophages and restrict bacterial growth. The second is the mounting of a DTH-like reaction in which non-infected macrophages accumulate and differentiate into a field of epithelioid cells, resulting in the containment of the infection within the granulomatous lesion and reduced dissemination (57).

The Phagocyte

Phagocytosis of *M. tuberculosis*

Phagocytosis refers to the process in which particulate material is internalised into large vacuoles called phagosomes. From the phagosome, material is transported to the late stages of the endosomal / lysosomal pathway where the phagosomal content is degraded. Phagocytes are predominately macrophages, dendritic cells and neutrophils. Following inhalation, *M. tuberculosis* bacilli are phagocytosed by a subset of alveolar macrophages (58). Phagocytosis of *M. tuberculosis* occurs via mannose receptor interactions or by complement receptors binding to complement components fixed to mycobacteria on the host cell surface (59, 60). Phagocytosis also requires the accumulation of cholesterol at the site of bacterial entry (61).

Activation of Macrophage

Activation of a macrophage results from infection and, or cytokine effects. An activated macrophage has greatly augmented microcidal activity. Infection of macrophages with *M. tuberculosis* activates Toll-like receptors 2 & 4 in a CD14 independent manner (62, 63). Toll-like receptors (TLR) are expressed on the cell surface of mammalian cells but particularly phagocytes. Binding of microbial
ligands to TLRs activates the cell by facilitating translocation the transcription factor, NF-κβ to the nucleus. This results in the transcription of genes with direct anti-microbial activity including nitric oxide (NO), genes that induce apoptosis and tissue injury like TNF-α, and cytokines that influence the adaptive immune response, like IL-12 (reviewed in (64)). Patients with defects in NF-κβ activation or phagocyte microcidal pathways present with disseminated mycobacterial diseases (65). Macrophages can also be activated directly by type I cytokines, including IFN-γ and TNF-α secreted by lymphocytes. IFN-γ and TNF-α work synergistically to augment IL-12 production in human phagocytes and increase NO production in mice (63, 66-69).

Dendritic Cells

Dendritic cells (DCs) have been detected in the lymphocyte areas of the tuberculosis granulomas (70) and are present at high density in airway epithelia and are associated with alveoli (71). Whilst DCs can phagocytose M. tuberculosis, they are poor antibacterial effector cells (72). Their main role may be the presentation of M. tuberculosis derived glycolipids and peptides to CD1- and MHC class I and II restricted T cells to induce an adaptive immune response following infection. Two papers have recently shown that the uptake of mycobacteria by DCs is mediated by the c-type lectin, DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) and identified DCs associated with M. tuberculosis derived material in the lymph nodes of patients with tuberculosis (46, 73).

BCG infected DCs induce potent antigen specific T cell responses in the mediastinal lymph nodes and afford equivalent protection to BCG vaccination following a M.
tuberculosis challenge in mice (74, 75). DCs may also produce inflammatory chemokines that attract activated lymphocytes and NK cells to the site of infection (76).

Neutrophils

In many mouse strains exhibiting increased susceptibility to *M. tuberculosis* infection, polymorphonuclear neutrophils are recruited in high numbers in place of mononuclear phagocytes (77-81). Saunders and Cooper proposed that the influx of phagocytic neutrophils have a functional role in disease as a compensatory mechanism by the host to contain infection in circumstances where normal host immunity is compromised (82).

Cytokines

The IL-12 / IFN-γ secretion pathway is important in human resistance to microbial infection

In humans, IL-12/IFN-γ signalling is important to resistance from mycobacterial disease caused by infections with environmental mycobacteria, *M. bovis* and *M. tuberculosis* (Fig. 2) (65). Casanova and colleagues observed that the level of IFN-γ signalling (from total to partial to none) within patients correlated with clinical outcome. Patients with complete IFN-γ receptor deficiency all developed disseminated disease following BCG vaccination. No mature mycobacterial granulomas were observed. In patients with partial deficiencies in IFN-γ signalling, the clinical phenotype and occurrence of mycobacterial disease was less severe and
following BCG infection, fully differentiated tuberculoid granulomas were observed (65). Most of the reported cases of mycobacterial disease in patients resulting from deficiencies in IFN-γ signalling have resulted from infections with environmental mycobacteria or BCG vaccination and not from *M. tuberculosis* infection. This probably results from the exposure of the patient to environmental mycobacteria or BCG before infection with *M. tuberculosis*. 
Figure 2: The IFN-γ signalling pathway is important in host resistance to mycobacterial disease. Molecules shaded yellow have been found mutated in certain patients with severe mycobacterial infection. Asterisks (*) indicate molecules that have been deleted in mouse models that following *M. tuberculosis* challenge show decreased survival compared to wild-type controls.
IL-12

Bioactive IL-12 is a heterodimeric molecule, comprised of constitutively expressed p35 and inducible p40 subunits. In human granulomas, IL-12p40 is predominately produced by myeloid cells and a small percentage of CD8$^+$ T cells (47, 83). IFN-γ deficiency is a consequence of IL-12 deficiency. The clinical phenotype of IL-12 deficient individuals is less severe than complete IFN-γ receptor deficiency that could be explained by IL-12 independent secretion of IFN-γ (65). Both BCG disease and environmental mycobacterial disease protected patients with IL-12 or IL-12 receptor deficiency against subsequent mycobacterial disease (84). This suggests that IL-12 is redundant for adaptive immunity to mycobacteria within most patients.

Whilst studies in humans have shown that type 1 cytokines are important in resistance to mycobacterial disease predominately caused by infection by environmental mycobacteria, studies in mouse models have shown that type 1 cytokines are necessary for resistance to \textit{M. tuberculosis} infection. IL-12 gene deletion mice cannot control bacterial growth, exhibit a significantly decreased survival times and a very severe phenotype following \textit{M. tuberculosis} infection. Compared to wild-type controls, IL-12$^-$ mice exhibited lower T cell infiltration into the lung following infection and produced minimal granuloma formation, even less than lymphocyte deficient SCID mice (85, 86). In normal mice supplemental IL-12 enhanced the clearance of mycobacteria and prevented reactivation of tuberculosis in steroid treated mice (87, 88).
IFN-γ

IFN-γ is produced by activated macrophages and lymphocytes expressing αβ γδ and NK associated markers (47, 83). Antigen specific αβ T cells are considered primary producers of IFN-γ following *M. tuberculosis* infection with CD4+ T cells providing bulk of this cytokine (89, 90).

IFN-γ can activate type 2 nitric oxide synthase in macrophages. This leads to the production of reactive nitrogen intermediates that are toxic for intracellular mycobacteria, induce enzymatic production of calcitrol (from Vitamin D3), sensitise macrophage to TNF-α and facilitate acidification of mycobacterial vacuoles in both *de novo* and established infections (91).

In mice the major role in IFN-γ is limiting mycobacterial growth but is also involved in limiting early granulocyte accumulation at inflammatory site (82, 92).

TNF-α and TNF-β

TNF-α is secreted predominately by monocytes and macrophages but also by DCs, NK and T cells (47, 83, 93). Its main role appears to be in macrophage activation. Anti-TNF-α antibody treatment (used for the treatment of rheumatoid arthritis) of patients, induced reactivation of tuberculosis, which suggested an important role for this cytokine in the control of latent infection (94, 95).

*M. tuberculosis* challenge of TNF-α−/− mice resulted in rapid death of mice, with massive bacterial load in lung and widespread dissemination to other tissues suggesting that TNF-α has a role in controlling mycobacterial growth (77). TNF−− mice have disorganised granulomas with few epithelioid macrophages, impaired
macrophage activity and lymphocytes that fail to migrate to infected tissue (77, 82). TNF\(^{-}\) mice challenged with less virulent mycobacteria produced less chemokines than wild-type mice, including factors chemotactic for macrophages and lymphocytes. Together with studies of human granulomas showing an association of TNF-\(\alpha\) with necrotic lesions this suggests TNF-\(\alpha\) may also have a role in granuloma formation and/or maintenance (83).

A possible role of TNF-\(\alpha\) in the pathogenesis of tuberculosis has emerged from human \textit{in vitro} model that showed that TNF-\(\alpha\) supports the growth of \textit{M. tuberculosis} in the first few days following infection of alveolar macrophages (58). Even so, it is clear that this cytokine plays an important role in host immunity.

TNF-\(\beta\), also called lymphotoxin-\(\alpha\), is a member of the TNF family and also binds to the TNF-\(\alpha\) receptor. TNF-\(\beta\) is primarily secreted by CD4\(^{+}\) T cells, B cells and NK cells. Deletion of this molecule increased susceptibility to \textit{M. tuberculosis} infection in mice although TNF-\(\alpha\) secretion was unimpaired (96).

Type II cytokines

Resistance to tuberculosis is most clearly associated with the secretion of TH1-type cytokines, IFN-\(\gamma\), IL-12 and TNF-\(\alpha\), but several studies have suggested that the susceptibility to disease may be associated with TH2-cytokines, particularly IL-4 and TGF-\(\beta\).

\textit{IL-4}

Rook \textit{et al} (97) argued that expression of IL-4 and IL-13 and \textit{M. tuberculosis} specific IgE production (which is IL-4 and IL-13 dependent) correlated with disease severity in tuberculosis (98-100). Conflicting studies describe depressed TH1 but not
enhanced TH2 cytokine profiles in PBMCs of patients suffering from active tuberculosis (101). Within the tuberculosis granuloma, IL-4 has been detected but never in the absence of IFN-γ. In those studies, the presence or absence of IL-4 did not correlate with clinical outcome (47).

From animal studies, there is little evidence of a functional role of IL-4 in control of bacillary load. IL-4<sup>−/−</sup> mice on a C57BL/6 background exhibit no increase in bacterial burden or histopathology different from wild-type controls even in chronic <i>M. tuberculosis</i> infection (102, 103). Conversely, following <i>M. tuberculosis</i> challenge in IFN-γ<sup>−/−</sup> or IL-12<sup>−/−</sup> mice, no shift to TH2 cytokine production was observed (86).

**TGF-β**

TGF-β is expressed by Langerhans and epithelioid cells in TB pulmonary granulomas and by blood monocytes of patients with TB (104). <i>In vitro</i> studies demonstrated that TGF-β was increased in blood monocytes following stimulation by mycobacterial antigens and could suppress T cell responses and deactivate macrophage effector functions. Antibodies that inhibit TGF-β normalised T cell proliferation and enhanced IFN-γ production (105).

Studies examining the effects of the administration of TGF-β or inhibition of TGF-β bioactivity in animal models suggest that this cytokine may impair innate and adaptive immune responses and may increase mycobacterial growth and persistence (106, 107).
IL-10

In humans, IL-10 reduced the ability of DCs to present mycolic lipid antigens to CD1-restricted T cells \textit{in vitro} (72). IL-10 induces a macrophage like phenotype in DCs that have superior antimicrobial activity than immature DCs (72).

IL-10 deficient mice are no more susceptible to \textit{M. tuberculosis} than wild-type mice in acute challenge (102). In the chronic stage of infection, mice over-expressing IL-10 showed evidence of reactivation associated with macrophage dominated lesions and decreased TNF-\(\alpha\), IL-12p40 and antigen specific IFN-\(\gamma\) production (108).

The cellular response is mainly mediated by T cells

B cells

Humoral immunity affords little contribution to resistance from tuberculosis.

Glatman-Freedman \textit{et al} (109) reviewed over 100 publications examining the clinical impact of antibody mediated immunity in tuberculosis; many presenting conflicting data on the therapeutic affects of serum therapy in humans. \textit{M. tuberculosis} infection does generate specific antibody responses but overall in humans the effectiveness of serum treatment was limited to early localised disease and relied on long periods of serum therapy (109). This suggests that the role, if any of the humoral response in human tuberculosis is minor.

B cells play a role in maintaining integrity of granulomas

B cells are abundant in murine granulomas (103). Several studies have been published showing \textit{M. tuberculosis} challenge of B cell deficient mice, reporting no
difference in bacterial load between them and wild-type mice (103, 110, 111). However, granulomas in B⁻ mice were disorganised and contained fewer lymphocytes. Mice also exhibited delayed dissemination to the spleen (103, 111). Reconstitution of B⁻ mice with naïve B cells restored the wild-type phenotype, suggesting B cells play a role in integrity of granuloma (111).

T Cells

The increased risk of TB disease within patients with impaired T cell functions (eg old age, corticosteroids or AIDS) demonstrates the pivotal role of T cells to resistance to tuberculosis (112-115).

αβ T cells

CD4⁺ T cells

*Human Studies*

The majority of T lymphocytes in human tuberculosis granulomas express αβ TCR with about twice the number of CD4⁺ to CD8⁺ T cells (83, 116). There is a clear inverse correlation with CD4⁺ T cell number and risk of tuberculosis in HIV⁺ individuals (117). Post-mortem analysis of co-infected patients showed that increasing CD4⁺ lymphocytopenia is associated with loss of macrophage activation at the site of *M. tuberculosis* infection and loss of granuloma formation and higher bacterial loads (118). Similar observations have been made in SIV and *M. tuberculosis* co-infection experiments in macaques (119).
**Murine studies**

In mice, antibody depletion experiments, adoptive transfer and gene deletion studies have shown that CD4$^+$ T cells are required to control infection (120-123). The major effector cytokine of CD4$^+$ T cells is IFN-γ that functions to activate macrophages (124). Several reports have suggested that CD4$^+$ T cells may play other important roles in containing *M. tuberculosis* in both acute and latent infection. In CD4$^{-}$ mice, 3 weeks following *M. tuberculosis* infection, IFN-γ levels appeared similar to wild-type mice because of compensatory secretion by CD8$^+$ T and NK cells (123, 125). Even so, these mice exhibited disrupted granuloma formation characterised by the influx of macrophages rather than blood monocytes suggesting that CD4$^+$ T cells have a role in recruitment of cells to the site of infection (125). In a model of latent *M. tuberculosis* infection, CD4$^+$ T cell depletion caused reactivation of disease despite the overall levels of IFN-γ being equivalent to that observed in non CD4$^+$ T cell depleted, non-reactivated mice (126).

Flynn *et al* (127), proposed that CD4$^+$ T cells may also contribute to resistance from tuberculosis by providing CD40-dependant enhancement of DC presentation of *M. tuberculosis* antigenic ligands, provide co-stimulatory activity through CD4$^+$ T cell priming and maintenance of CD8$^+$ T cells or B cell help. Cells may also produce other cytokines such as IL-2, TNF-α, FasL, perforin and granulysin which may be involved induce apoptosis of infected macrophages.
CD8⁺ T cells

**Human Studies**

Albert *et al* solved the apparent paradox of CD8⁺ T cell recognition of intra-vacuolar pathogens by demonstrating that DCs can acquire antigen from apoptotic cells and induce CD8⁺ specific cytotoxic T cells (128). Subsequent studies have also shown that 70kDa particles can leak out of vacuoles into the cytosol (129) and mycobacterial infection of macrophages results in membrane-permeable phagosomes (130).

CD8⁺ restricted T cell lines, specific for several *M. tuberculosis* proteins have now been derived from infected patients, healthy contacts and BCG vaccinees (131-133). The presence of *M. tuberculosis* CD8⁺ T cells does not in itself demonstrate a role for these cells in human disease. *M. tuberculosis* specific CD8⁺ T cell lines secrete IFN-γ and may function like CD4⁺ cells by activating macrophages (134). Even so, the increased susceptibility of HIV⁺ patients to tuberculosis disease implies that CD8⁺ T cells induced following infection cannot fully compensate for significant decreases in the CD4⁺ T cell population.

**Murine studies**

A clear role CD8⁺ T cells has been demonstrated in mice in the control of *M. tuberculosis*. Sousa and colleagues investigated the susceptibility of a number of mice harbouring deletions in Class I pathways and demonstrated both classical and non-classical MHC I restricted T cells are involved in protection from *M. tuberculosis* infection (135). The induction of specific T cells has been shown to protect mice from challenge with *M. tuberculosis* (136). CD8⁺ T cells have been
implicated in prevention of reactivation (137). Recently Turner et al showed that early resistance to \textit{M. tuberculosis} in old mice (12-24 months) is mediated by CD8$^+$ T cells (81). This implies that vaccination regimens that can effectively boost low-level \textit{M. tuberculosis} specific CD8$^+$ T cells may be particularly effective in maintaining long-term protection from disease.

\textbf{Cytotoxic T cell killing}

In viral infections, CD8$^+$ T cells and NK cells, in addition to cytokine secretion are characterised by their cytotoxic functions, including the induction of apoptosis of infected cells through CD95/CD95L interactions, and direct killing by production of granzymes, perforin, TNF and possibly granulysin. In mice, gene deletion of perforin, granzyme or CD95 did not increase susceptibility in acute \textit{M. tuberculosis} infection, which suggested that cytotoxic effector functions may be redundant in CD8$^+$ T cell mediated control of tuberculosis (135, 138, 139).

Later studies showed that CD95/CD95L deficient mice gradually lose ability to control bacterial growth despite no impairment in the ability to generate IFN-$\gamma$. Kaufman argued (140) that cytolytic functions are necessary in the control of tuberculosis. He hypothesised that in chronic infection, cells exist that either do not express or have greatly decreased Class II expression, and are refractory to IFN-$\gamma$. These cells may activate cytotoxic mechanisms following presentation of peptides in a class I restricted manner.

\textit{In vitro} studies of human cells have demonstrated direct T cell mediated killing of \textit{M. tuberculosis} by granulysin. Granulysin is expressed in CD4$^+$ and CD8$^+$ T cells, although at a higher level in CD8$^+$ T cells. Granulysin is also produced by $\gamma$8, CD1-
restricted T cells (141, 142). Granulysin killed *M. tuberculosis* extracellularly in a
dose dependent manner by inducing osmotic lysis of the bacilli (143, 144). Another
study showed that the production of granulysin by CD8\(^+\) T cells could also kill
intracellular mycobacteria in a perforin dependent manner, although the levels
needed to induce specific lysis were at non-physiological concentrations (143).

**CD1- restricted Natural Killer T cells**

Natural Killer T (NKT) cells recognise CD1-glycolipid complexes presented by
APCs including dendritic cells. NKT cells can produce type I cytokines including
IFN-\(\gamma\) and exhibit cytotoxic effector functions. CD1-restricted T cell proliferation
and lytic responses in guinea pigs following stimulation with crude *M. tuberculosis*
extracts have been demonstrated (145). Even so, a *M. tuberculosis* specific CD1
ligand has not been reported in humans or rodent models.

CD1\(\alpha\)- mice do not differ from wild-type mice in survival following *M. tuberculosis*
infection (146). Similarly, NKT cell deficient mice successfully develop
granulomatous lesions in lungs following *M. tuberculosis* challenge (147). The only
CD1 restricted ligand so far identified that can activate NKT cells is \(\alpha\)-
galactosylceramide (\(\alpha\)-gal-cer) which is derived from the marine sponge (148).

Specific activation of NKT cells by \(\alpha\)-gal-cer significantly protected susceptible
mice from tuberculosis, increasing survival and decreasing bacterial burden and
tissue injury (146). These studies indicate that specific activation of NKT cells may
play a protective role against *M. tuberculosis* and be a good target for vaccine
development.
γδ T cells

γδ T cells recognise non-peptide organophosphate alkylamine antigens secreted by bacteria in a TCR-dependent and MHC- and CD1- unrestricted manner. In humans, γδ cells constitute 1-5% of CD3⁺ T cells in lymphoid organs but are the dominant T cell population within epithelial tissues, including skin, gut and airways (149). A recent report in macaques suggested that γδ T cells might play a role in the protective adaptive immune response to *M. tuberculosis* infection (150). The authors demonstrated that BCG-vaccinated macaques produced a γδ T cell recall response following a second BCG vaccination or *M. tuberculosis* infection that correlated with clearance of BCG bacilli and resistance to infection. Similarly in humans, Hoft et al (151) showed that following BCG vaccination, γδ T cells constitute the dominant T cell population expanding following whole BCG stimulation.

The mechanism of action of γδ T cells is unknown. No *M. tuberculosis* derived presentation molecules are known. Secretion of IFN-γ and TNF-α by γδ T cells can increase monocyte-mediated killing within two hours after exposure to live bacterial products (152). γδ T cells can also induce direct cell-to-cell cytotoxicity by the production of granulysin and perforin. γδ T cells are more efficient at producing IFN-γ cytokine on per cell basis than αβ T cells (152), but γδ T cell depletion from PBMCs did not significantly decrease the total IFN-γ secretion indicating CD4⁺ T, CD8⁺ T and NK cells are the major sources of IFN-γ (151).
Saunders et al (153) argues that in mice γδ T cells have a role in recruitment of macrophages to site of mycobacterial infection where they are then activated by αβ T cells.

In summary, whilst studies have shown that γδ T cells respond to mycobacterial infection and can secrete IFN-γ, no functional role for this T cell subset has been demonstrated in host resistance to tuberculosis.

Natural Killer (NK) Cells

So far, there is no clear role of NK cells in disease susceptibility. Nirmala et al observed no difference in the relative frequencies of NK cells in normal individuals, patients with pulmonary TB, HIV+/TB+ patients and healthy contacts (154).

Mycobacterial disease in patients that have complete deficiency in IFN-γ is more severe than in patients that lack autologous T cells (65). This suggests that other cells capable of IFN-γ signalling, like NK cells, may contribute to resistance to mycobacterial infections in immunosuppressed.

*M. tuberculosis* immune evasion mechanisms

*Inhibition of endosomal / lysosomal fusion*

*M. tuberculosis* bacilli are able to survive and even multiply within alveolar macrophages (155). *M. tuberculosis* actively arrests phago-lysosome fusion by retaining TACO (tryptophane aspartate-containing coat protein), release of which correlates with normal phagosome maturation and acidification (156-158). *M. tuberculosis* therefore actively restricts microcidal activity of macrophages particularly lysosomal hydrolase activity and avoids NO mediated killing. This
mechanism also sequesters MHC II molecules that are contained in the endosome and blocks antigen presentation in chronically infected macrophages (159). Production of the 19kDa protein expressed by \textit{M. tuberculosis} has been reported to reduce MHC II mRNA accumulation and surface expression on macrophages, but it is unclear whether this benefits the host or pathogen (160).

Down-regulation of cell surface receptors

\textit{In vitro} infection of human PBMC derived APC with live \textit{M. tuberculosis} was shown to downregulate another cell surface receptor, CD1, which subsequently decreased IFN-\(\gamma\) release by a CD1b restricted T cell line (161).

\textit{Countering reactive oxygen intermediates}

The host cell can produce highly reactive toxic molecules particularly oxygen and nitrogen radicals, which are toxic for the microbe (162). Glycolipids associated with the cell wall of \textit{M. tuberculosis} cell wall like lipoarabinmanna and phenolic glycolipid-I, are potent oxygen radical scavengers that may counter the antimicrobial mechanisms of reactive oxygen intermediates (ROI) (163, 164).

Effects on cytokines

\textit{Cytokine inhibition}

\textit{M. tuberculosis} infection, as has been observed in other bacterial infections, actively inhibits IL-12 secretion in human blood derived monocytes (165) (166, 167). There is some evidence in \textit{in vitro} systems that the mycobacterial 19kDa lipoprotein and 30kDa proteins inhibit type 1 cytokine production by macrophages, possibly decreasing T cell activation (168, 169).
Mycobacterial persistence

During latent infection, *M. tuberculosis* persists within granulatomous lesions in a non-replicating or low-replicating state capable of surviving the bacteriocidal actions of chemotherapy (170, 171).

Persistent mycobacteria are not well characterised although the trigger is thought be oxygen depletion in the granuloma causing the bacilli to shut down many metabolic pathways until more favourable conditions arise (171). Mycobacterial infection of macrophages also results in membrane-permeable phagosomes that may enable it to gain access to nutrients in chronic infection (130).

A recent study has shown that some *M. tuberculosis* genes may secure survival of the bacilli by responding specifically to host immunity. Isocitrate lyase (ICL) is a protein involved in fatty acid metabolism that is required for survival and persistence of *M. tuberculosis* in animal models (172). The expression of ICL is increased in response to activation of host macrophages and the secretion of IFN-γ but is not essential to survival of the pathogen in the absence of IFN-γ signalling (172).

1.5 The BCG Vaccine

Growth and Strains

Numerous sub-strains of BCG have been derived from the original Pasteur strain and been used in vaccination programmes over the past 70 years. The BCG sub-strains differ both genetically and in their vaccine preparations (173). Several regions of the *M. bovis* genome are missing from BCG sub-strains but only one, the RD1, is missing from every BCG sub-strain so far analysed. Conversely it is present in every
analysed *M. tuberculosis* strain (174). Loss of the RD1 region deleted 7 genes and truncated two others. None of the genes have any known function but the region itself is necessary for virulence of *M. tuberculosis* in animal models (175).

There is no clear evidence as to whether the strain evolution of BCG has impacted on vaccine efficacy against tuberculosis in man. The only trial to directly compare two BCG sub-strains reported no protective efficacy with either sub-strain (176). Some reports have suggested BCG sub-strains have attenuated with time because of decreased reactogenicity and shortened survival of bacilli at the site of vaccination (reviewed in (173)). No information is available on whether this has translated to decreased protection in man.

How does BCG protect?

Protection is reliant on replication of the bacillus

BCG must be live to mediate protection against *M. tuberculosis* (177). Replication appears necessary to produce sufficient antigen to induce an immune response by the host, particularly to secreted antigens which are highly immunogenic proteins (178-181).

BCG induces a T cell response that is CD4⁺ T cell dominated

BCG vaccination induces a strong adaptive type 1 immune response that is predominately CD4⁺ T cell dependent. CD8⁺ dependent T cell responses have also been detected although at much lower frequencies (134). Stimulation of BCG specific T cells *in vitro* induced strong IFN-γ secretion (134). Studies show that in the memory immune (BCG vaccinated or *M. tuberculosis* challenged then antibiotic treated) animals, in response to an *M. tuberculosis* challenge, IFN-γ secreting T cells
migrated to the lung, in higher numbers and much faster than in naïve animals (182, 183). Recruitment of cells to the lung correlated with decreased bacterial burden and reduced immunopathology (182).

BCG protects from tuberculosis disease not infection

Autopsy studies identified tuberculosis foci in patients vaccinated in their youth and who died from causes other than tuberculosis, suggesting that that BCG vaccination does not prevent establishment of \textit{M. tuberculosis} infection (184). Studies in animal models show that BCG vaccination does not inhibit \textit{M. tuberculosis} growth in the first 10 days following infection but affords significant protection in terms of survival following challenge and lower bacterial burdens in organs during chronic stages of infection (185).

BCG protection from other mycobacterial diseases

The mechanism of BCG protection against \textit{M. tuberculosis} is dependent on homology between proteins inducing cross-reactive immune responses. BCG protects against other mycobacterial diseases, such as leprosy. There is also evidence that BCG provides some protection against \textit{M. ulcerans} infection and glandular disease attributable to various other environmental mycobacteria in particular \textit{M. avium-intracellularare} (186-188). BCG vaccination affords 50% protection against leprosy, including in countries where BCG is not protective against \textit{M. tuberculosis} (189-191). Why BCG protects people from leprosy but not tuberculosis remains unknown.
Theories for BCG variable efficacy

Environmental mycobacteria

Wilson et al observed that geographic latitude has accounted for greater than 40% of between-study variance in BCG mediated protection, with protection decreasing closer to the equator (12, 192,193). A hypothesis has emerged that higher levels of infection by environmental mycobacteria may induce cross-reactive T cell responses to BCG, which ameliorate the protective effects of BCG (194, 195). In humans and animal models, certain species of environmental mycobacteria can provide levels of protective efficacy against tuberculosis similar to that provided by BCG (196-198). In mice, prior immunisation with M. avium inhibited subsequent BCG replication (199).

The immunological basis for this hypothesis was investigated in comparing the IFN-γ responses to PPD in UK (where BCG protects) and in Malawi (where BCG is not protective). Prior to BCG vaccination, PPD-specific IFN-γ secretion and DTH distribution to tuberculin showed that the Malawian population had a greater prior sensitisation to mycobacterial antigens, compared to the UK individuals. The average change in IFN-γ response to PPD (measured as the post-vaccination / pre-vaccination ratio) was significantly higher in the UK than Malawian cohort and correlated to the stronger protective effect of BCG in the UK than Malawi (200).

It is not only the amount of exposure to environmental mycobacteria but also the types of mycobacteria that may inhibit BCG- mediated protection. M. kansaii in
guinea pigs offers greater protection than *M. fortuitum* against *M. tuberculosis* challenge and induces much greater TST reactivity to tuberculin (198, 199). This implies that a successful tuberculosis vaccine must be able to induce higher levels of IFN-γ secreting T cells in individuals previously exposed to mycobacteria including prior BCG vaccination. Therefore, unlike BCG, future vaccines should not be growth inhibited by cross-reactive immune responses to environmental mycobacteria.

Protection wanes with time

Hart *et al* estimated the protective efficacy of BCG to last 10-15 years in the British population (196). No studies have been performed in humans correlating loss of BCG specific T cell responses and protection. Booster immunisations are commonly employed in vaccination regimens to expand the existing memory antibody and T cell pools. In one study, multiple BCG vaccinations did not increase protection against pulmonary tuberculosis, however in this region a single BCG vaccination also failed to protect (189). In contrast, repeated BCG immunisation did significantly increase protection over a single BCG vaccination against leprosy (191).

### 1.6 Strategies in Vaccines Development

**Overview**

Chemotherapy against tuberculosis is highly effective, but despite a global effort to treat tuberculosis disease, the number of new cases of disease reported in the developing world continues to rise. As such, a prophylactic vaccine is required to interrupt the cycle of *M. tuberculosis* transmission. Any such vaccine regimen must
protect where BCG has failed, namely 1) protection in the developing world, 2) protection from pulmonary disease and 3) protection beyond childhood and against adult disease. These are difficult goals but solid sterile immunity from *M. tuberculosis* infection and disease may not be necessary. Vaccines that can increase the duration of protection and decreased tuberculosis disease by 50% are predicted to afford significant savings in terms of life and cost (201, 202).

Whilst no immune correlate of protection has been identified against tuberculosis the induction of type-1 cytokines and antigen specific T cells that can activate macrophage microbial killing are strongly implicated in protection from disease. Research has focused on using vaccine vectors that effectively adjuvant the induction of type-1 cytokines and the production of antigen specific T cells. Several antigens in *M. tuberculosis* such as the antigen 85 complex have been targeted because they are highly expressed and elicit strong T cell responses in humans and animal models. Finally, induction of T cells responses in the respiratory compartment is increasingly being investigated with a view to increasing protection from pulmonary disease.

**Animal Models of Tuberculosis**

Development of a vaccine requires appropriate animal models of disease which has proved difficult because animals differ in their susceptibility to *M. tuberculosis*, in the disease pathology produced by infection and in the availability of reagents to study the immunological aspects of disease. The current approach to developing a vaccine against tuberculosis is to test vaccines in more than one mouse strain and in
other animal models, always including a BCG vaccinated group as a benchmark of protective efficacy.

Mouse Models

Over the past 5 years, most TB research groups have moved from systemic to aerosol challenge models of *M. tuberculosis*. Following low dose aerosol delivery (typically 50-500 CFU deposited in the lung), bacilli exhibit clear lag, log and stationary phases of growth (203). Aerosol challenge of *M. tuberculosis* is more virulent, induces more rapid lung pathology and significantly earlier death in mice than an intravenous challenge (204).

Protective efficacy of tuberculosis vaccines is determined either by histopathology, comparisons of survival plots or the bacillary load within organs at different time-points after challenge. BALB/c and C57BL/6 mice are most commonly used in vaccine studies. These strains exhibit similar kinetics of *M. tuberculosis* growth and very similar survival plots (surviving 225-400 and 175-325 days respectively) (205). Most vaccinology studies have focused on acute *M. tuberculosis* infection, which best parallels progressive post-primary disease in man. Increasingly models of latent infection and reactivation induced by immunosuppression have been developed but are costly and place great demands on Category III facilities.

Non-human Primates

The macaque is a highly relevant animal model for tuberculosis vaccine studies. Macaques are naturally susceptible to tuberculosis (206) and, like humans, transmit the bacillus via aerosolised droplets, produced by coughing. Lung challenge of macaques produces pathology similar to that observed in tuberculosis disease in
humans including lesions in bronchial and hilar lymph nodes, caseating lesions in the lung with eventual calcification and disseminated disease (207, 208).

Two studies have shown that following a high dose *M. tuberculosis* challenge, rhesus macaques are much more susceptible to disease than cynomolgus monkeys, and BCG vaccination produces significantly less protection from disease in rhesus than cynomolgi (207, 208).

A difficulty with the use of the cynomolgus macaque for vaccine studies in tuberculosis is that BCG vaccination, despite being highly protective, produces very low levels of specific IFN-γ following PPD stimulation of PBMCs (207). This contrasts to studies in humans and rhesus macaques where following BCG vaccination PPD and Antigen 85 (Ag85A) specific IFN-γ secretion is routinely detectable.

The benefit of using non-human primates as opposed to rodent models is that their physiology is closer to man and their larger size allows tracking of immune responses over time in the blood compartment. The availability of immune assays and reagents for the study of monkeys is increasing. Monkeys are also more relevant for safety and toxicity studies of vaccines.

**Guinea Pig**

The guinea pig is more susceptible to *M. tuberculosis* infection than mice. Guinea pigs develop a granulomatous pathology similar to that observed in human disease (203). Protection is usually measured by vaccine-enhanced survival, which has the benefit of allowing study of the effects of vaccines on chronic pathology.
Vaccine Vectors

DNA

Intramuscular or intradermal vaccination with DNA plasmids can induce both specific T cell and IgG2a antibody responses against the target antigen in mice and non-human primates (209). Huygen et al showed that DNA can express mycobacterial genes, and that vaccination with these plasmids could induce both CD4+ and CD8+ T cells specific for the recombinant antigen (210). Induction of immune responses in animals typically requires multiple vaccinations.

Clinical trials with DNA have now been performed in both the developed and developing world. Whilst plasmid DNA vaccination appears to be well tolerated in man, the strong immunogenicity and protective efficacy produced in mice and macaque models has not translated to humans (211). Methods are currently being developed to try to improve the efficiency of delivery of DNA to humans and increase its immunogenicity by the inclusion of genes encoding cytokines.

DNA still holds great potential as a vaccine vector, particularly in the developing world. DNA is lyophilisable and heat stable and enables elimination of the cold chain (209).

Poxviruses are potent inducers of T cells

Poxviruses have a large, linear double stranded DNA genome and are characterised by a complex enveloped brick-shaped virion. They replicate in the cytoplasm of cells and do not integrate to host DNA or undergo a latent intracellular stage (212). They rely on expression of viral proteins to evade host immune responses.
Modified Vaccina Virus – Ankara Strain

Modified Vaccinia Virus Ankara (MVA) is an attenuated poxvirus derived following over 500 passages in chick embryo fibroblast cells (213, 214). Generation of MVA resulted in six major deletions and the loss 30 kilobases representing 15% of the genome (215, 216), including genes that encode proteins responsible for immune evasion such as soluble receptors for IFN-γ, IFN α/β, TNF and chemokines (217). MVA can express foreign genes under early-late promoters in human cell lines but proteolytic processing of late viral polypeptides is blocked resulting in a failure to form mature virions (218).

Safety

MVA demonstrates good safety in humans and immunocompromised animal models. Non-recombinant MVA has been administered parenterally to 120000 people. Vaccination produced no side effects, despite deliberate vaccination of old, young and eczematous patients (219-221). MVA was avirulent in neonatal and irradiated mice, irradiated rabbits and irradiated macaques (220, 222, 223). MVA induces type I IFN that restricts its virulence (224).

Immunogenicity

Recombinant MVA can induce high frequencies of IFN-γ secreting CD8⁺ and CD4⁺ T cells against the target antigen in rodent and macaque models (225). MVA has been typically given intradermally and appears most effective as a boosting vaccination following a protein particle, DNA or other viral vector priming immunisation. These strategies are commonly referred to as heterologous prime-boost regimens.
Recombinant MVA is a poor inducer of antibodies. At high vaccine doses specific antibodies have been detected in mice that are predominately IgG2a sub-type (222).

Fowlpox

Fowlpox (FP) is an avipoxvirus that can also express foreign proteins in mammalian cells (226). Fowlpox is unable to replicate in mammalian cells. Conservation of genetic organisation exists between FP and vaccinia virus. Both viruses share extensive amino acid homologies despite divergent nucleotide sequence divergence. Promoter sequences function equivalently.

Fowlpox strains can strongly induce both CD4^+ and CD8^+ T cell responses in mice and macaque models (227). DNA / FP protected against SIV infection in a macaque model (228). Recombinant FP is now in Phase I trials in humans although only cancer trial results have been published (229). Like MVA, recombinant FP vaccination is a poor inducer of antibodies in mice and macaque models (230).

Recombinant protein vaccine

The efficacy of recombinant protein vaccines is dependent on the choice of adjuvant. Promising vaccine candidates are being developed that induce type 1 immune responses. Subunit protein vaccines also strongly induce specific antibodies. The main advantage of protein vaccines compared to live vectors is greater safety in immunocompromised hosts.

Live Bacterial Vaccines

Live bacterial vaccines are good candidates for tuberculosis vaccines as they are cheap to manufacture. These include recombinant BCG vaccines expressing
immunodominant antigens and or cytokines (231), attenuated strains of *M. tuberculosis* (232, 233), non-pathogenic mycobacteria including *M. vaccae* and *M. microti*, and non-mycobacterial vectors such as Salmonella and Shigella expressing immunodominant mycobacterial antigens.

**Antigen Targets**

*M. tuberculosis* secreted proteins

Over 200 proteins are released into the broth during the culture of *M. tuberculosis* (234). This group of proteins is collectively called culture filtrate proteins (CFPs). CFPs are characterised by their immunodominance. Immunisation with CFPs induces strong T cell responses and protection following *M. tuberculosis* challenge in animal models. Several CFPs have been now been purified for use in vaccines and/or diagnostic assays.

*The 85 complex*

Much attention has focused on the highly immunogenic Antigen 85 complex. This is a family of proteins comprising antigens 85A, 85B and 85C secreted by *M. tuberculosis*, BCG and many other species of mycobacteria. All three proteins are mycolyl transferases and are essential in cell wall synthesis (235). 85B and 85A that share 80% homology at the αα level, together account for over 40% of extracellular protein in broth culture (236). All three proteins are expressed in human macrophages (237) and are detectable both in the bacterial cell wall and in the phagolysosomal space (238)
Studies in this laboratory had previously focused on DNA vaccination followed by boosting with a modified vaccinia virus Ankara (MVA) expressing antigen 85A (Ag85A). DNA-MVA prime-boost strategies produce high levels of specific T cells against malarial and HIV antigens and can protect against the relevant challenge in animal models. In mice, DNA85A-MVA85A vaccination, whilst inducing high frequencies of Ag85A specific IFN-γ secreting T cells, did not achieve greater protection than BCG following *M. tuberculosis* challenge (136). Horwitz *et al* have recently developed a recombinant vaccine, which elicited greater protection than low dose BCG in a guinea pig model (239, 240). This vaccine is a recombinant BCG that over-expresses antigen 85B, suggesting that the inclusion of BCG may facilitate the design of future and more efficacious vaccines against tuberculosis.

**ESAT-6 and CFP-10**

The genes encoding ESAT-6 and CFP-10 are found in the RD1 region that is deleted from all BCG strains. Vaccination of mice and guinea pigs with ESAT-6 and CFP-10 can induce strong antigen specific T cell responses and protection equivalent to BCG.

These antigens also hold strong diagnostic value and studies showing the IFN-γ response to these antigens can show higher specificity and sensitivity than the prevailing TST in non-tuberculosis endemic regions (241, 242).

**M. tuberculosis** Cellular Proteins

Vaccination with *M. tuberculosis* heat shock proteins (HSPs) were protective in latent models of infection in mice but concerns have been raised over the safety of
microbial HSP vaccines because of potential immune cross-reactivity with human HSPs (88).

Proteins expressed during latent infection

One promising vaccine candidate is 16kDa also called α-crystallin, which is a secreted chaperone protein induced during latent infection. T cell responses to 16kDa have been detected in humans and mice models (243, 244). The development of latent models of tuberculosis will allow testing of genes expressed during latent infection.

Inducing T cell responses in the lung and regional lymph nodes

Inducing T cell responses in the lung

The lung, like the gastrointestinal and genital tissue, is part of the mucosal immune system. Mucosal membranes are in direct contact with the environment and are the site of entry for the majority of infectious pathogens. Antigens cross the mucosa via dendritic cells (DCs) resident in the epithelium or through M cells that overlay mucosal associated lymphoid tissue (MALT), rich in DCs and lymphocytes. DCs can migrate from the airways to the T cell zones of the thoracic lymph nodes but have also been shown to migrate directly to the spleen (245, 246). Once in the lymph nodes, DCs present antigen to circulating naïve T cells (247, 248).

Memory T cell homing

Once induced, antigen specific CD4+ and CD8+ T cells can migrate into lymph nodes but preferentially circulate through or reside in non-lymphoid tissues (248, 249). The
extravasation of memory T cells, from the circulation into organs is controlled by adhesion molecules and chemoattractant signals. These molecules are expressed by high endothelial venules and postcapillary venular endothelium in an organ-specific manner.

In the gut, lymphocyte trafficking is controlled by the addressin molecule MadCam-1 that is specifically expressed on the gut endothelium (250). T cells expressing α4β7 integrins bind MadCam-1 and extravasate into the gut parenchyma (251).

To date, no molecule specific to the lung endothelium has been identified but T cells recovered from the lung and bronchoalveolar-lavage express a distinctive pattern of adhesion and chemokine homing receptors that distinguish them from T cells found in other organs, particularly skin and gut (252). Comparisons of particular M. tuberculosis-susceptible and -resistant mice strains showed that whilst the splenic immune response was similar between the strains, protection in the resistant mouse strain correlated with the influx of IFN-γ secreting αβ T cells to the lung (253).

Lung vaccination studies also provide evidence of a separate lung T cell homing pathway. Rosenthal and Gallichan demonstrated that the route of vaccination was important in inducing T cell responses in the mucosal compartment in murine models (254). They and others have demonstrated that intranasal vaccination but not intraperitoneal or subcutaneous vaccination induced long-lived antigen specific T cells in the lung and lung lymphoid system (254, 255). In both macaque and guinea pigs, aerosol vaccination of BCG has been reported to offer greater protection than intradermally delivered BCG (256, 257). However, these experiments did not control
for the effect of persistent BCG in the lung inducing innate immune responses that may also have contributed to the protection observed.
1.7 Thesis Aims

The aims of this thesis were to:

* develop assays to examine the immunity in the lung,

* develop methods of delivery of vaccines into the lung,

* develop mouse and macaque models for T cell vaccinology and tuberculosis disease, and

* to use the above models to produce novel vaccine regimens against tuberculosis using recombinant subunits vaccines expressing the *M. tuberculosis* immunodominant antigen 85A.
Development of an aerosol challenge model of M. tuberculosis

2.1 Results

Description of apparatus and challenge

An initial goal of this study was to develop an aerosol challenge model of M. tuberculosis in mice, which was comparable to published models in terms of number of bacilli deposited into lungs, infection rate (100%) and kinetics of M. tuberculosis growth in lungs and spleens. A summary of published studies of murine aerosol challenge models is shown in Table 2.1. For these studies, an adapted, Henderson Apparatus was used to generate the aerosol of M. tuberculosis (258). In all challenges performed, mice were exposed to the aerosol for 20 minutes. During this time, mice were individually restrained to permit nose-only exposure that would minimise delivery of bacilli outside the respiratory compartment (259). Mice were not restrained for greater than 45 minutes. Up to 35 mice could fit in the nebulizer chamber. In challenges using greater than 35 mice, mice were randomly assigned to runs in order to control for any variation in intra-run M. tuberculosis deposition. M. tuberculosis stocks were cultured before and after challenge. Cultures typically lost 0.5 log viability over the period of the challenge (1-2 hours) (data not shown). Subsequent to the challenge and release from restraints, mice were active and
immediately groomed and fed, which are indicators that they coped with the stress of the procedure (260).
Table 2.1: Comparison of published data examining the growth rates of *M. tuberculosis* in mice organs following low dose aerosol delivery

<table>
<thead>
<tr>
<th>Study Reference&lt;sup&gt;1&lt;/sup&gt;</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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</thead>
<tbody>
<tr>
<td>Mouse Strain</td>
<td>C57BL/6</td>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>C57BL/6</td>
<td>C57BL/6</td>
<td>C57BL/6</td>
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<tr>
<td>Age at time of challenge</td>
<td>6-8 weeks</td>
<td>6-8 weeks</td>
<td>6-8 weeks</td>
<td>Middlebrook</td>
<td>Middlebrook</td>
<td>Glas-Col,</td>
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<tr>
<td>Apparatus</td>
<td>Tri Instruments</td>
<td>Middlebrook Infection Apparatus</td>
<td>Middlebrook Infection Apparatus</td>
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<tr>
<td><em>M. tuberculosis</em> strain</td>
<td>H37Rv</td>
<td>H37Rv</td>
<td>H37Rv</td>
<td>Erdman</td>
<td>Erdman</td>
<td>Erdman</td>
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<tr>
<td>Bacilli deposited in lung</td>
<td>200</td>
<td>100</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>50-100</td>
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<tr>
<td>Bacilli counts (10&lt;sup&gt;10&lt;/sup&gt; CFU/organ)</td>
<td>Day</td>
<td>Lung</td>
<td>Spleen</td>
<td>Lung</td>
<td>Spleen</td>
<td>Lung</td>
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<td></td>
<td>1-2&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>50-60</td>
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</table>

<sup>1</sup> 1 = (89); 2 = (261); 3 = (74); 4 = (138); 5 = (57); 6 = (262).
<sup>2</sup> days following aerosol challenge on which bacillary load in organs enumerated
Growth and induced histopathology following low dose aerosol challenge with H37Rv *M. tuberculosis*

Growth of *M. tuberculosis* is a function of the number of bacilli deposited in the lungs

The primary assay to determine infection levels in mice exposed to *M. tuberculosis* is the enumeration of the bacterial load in lungs and spleen at different time-points after challenge. The numbers of bacilli in the lung is a measure of the extent of lung disease and is proportional to length of survival in inbred mice strains (263). The bacterial burden in the spleen correlates with the extent of systemic dissemination of mycobacteria from the lungs (86).

Mice were challenged with increasing concentrations of *M. tuberculosis* (10^5-10^7 CFU/ml *M. tuberculosis*) to determine the optimal dose for future challenges (Fig. 3). Bacillary load was measured at 24 hours and 8 weeks post challenge (Fig. 3A&B). The results demonstrate that the mean CFU in both the lungs and spleens, 8 weeks post challenge was proportional to the concentration of the original inoculum in mice. These data concur with observations made in previous studies (204).
Figure 3: Reproducible aerosol delivery of *M. tuberculosis* to mice lungs is dose dependent. BALB/c mice were challenged by aerosol for twenty minutes by nebulising $10^5$, $10^6$ and $10^7$ CFU/ml of *M. tuberculosis*. Lungs were harvested at 24 hours post challenge (A) and lungs, spleen and liver at 8 weeks post challenge (B). Total CFU in the lung are shown for individual mice in (A). In (B), the log$_{10}$(mean CFU) +/- SEM is shown for each organ ($n=10$)
The challenge dose of $10^5$ CFU/ml was too variable, producing an infection of only 1 of 3 mice at 8 weeks post challenge. Both the $10^6$ and $10^7$ CFU challenge doses produced 100% infection of mice. The bacterial burdens in the lungs and spleens at 8 weeks post challenge in the $10^6$ CFU/ml group showed low intra-group variability and mean loads that were comparable to published studies described in Table 2.1. The titre of $10^7$ CFU/ml was more virulent and produced bacterial burdens in the organs 2-3 logs higher than similar time-points in published studies (Table 2.1). Challenge titres around $10^6$ CFU/ml best agreed with published studies and were used for further experiments to examine the kinetics of *M. tuberculosis* growth in this model.

**Kinetics of *M. tuberculosis* growth in BALB/c mice lungs and spleen**

**Lung**

Mice were challenged with $10^6$ CFU/ml of *M. tuberculosis* by aerosol. Forty-eight hours after infection an average of 100 CFU of *M. tuberculosis* was recovered from the lungs of an individual mouse (n=5) (Fig. 4). *M. tuberculosis* bacilli entered logarithmic growth between day 8 and 15 following challenge. Beyond day 15, growth slowed and was stationary from day 26 to day 60.
**Figure 4:** *M. tuberculosis* delivered as an aerosol undergoes lag, log and stationary phases of growth in mice lungs. BALB/c mice were challenged for 20 minutes with nebulised *M. tuberculosis* (2×10⁶ CFU/ml). The number of *M. tuberculosis* colonies in lungs and spleens were enumerated at different time-points after challenge and are expressed as log₁₀(mean CFU) +/- SEM (n=5)
Spleen

Colonies were first detected in the spleen at day 15, and grew logarithmically over the next 10 days (Fig. 4). Growth stabilised between day 26 and 33 but small though significant increases in bacterial burden were observed at day 46 ($P < 0.01$) and 60 ($P < 0.01$). Similar observations (gradual increase in splenic the mean [CFU]) have been made in C57BL/6 mice (reviewed in Table 2.1, studies 1 & 6).

Histopathology following *M. tuberculosis* infection in BALB/c mice

Gross Pathology

Lungs were visibly normal at day 15. By day 26, all mice had numerous abscesses (2-4 mm long) on their lungs. At this time-point and thereafter, mediastinal lymph nodes (MLN) (that drain the lung) and spleens were enlarged in all mice.

Histopathology

Granulomata (tubercular lesions with central epithelioid cells and peripheral lymphocytes) with ZN positive organisms were first detected in the MLN at day 15 (Table 2.2). At subsequent time points, all mice analysed contained ZN positive granulomata in their MLN.

Lungs remained histologically normal until 8 days following infection (Table 2.2). By day 15, inflammatory cells were observed in the lung parenchyma and AFB bacilli detected. By day 26, mice lungs contained peribronchial / perivascular lymphocytic infiltrates and pyogranulomata (granulomata that also contain some polymorphs). Similar pathology was observed in all mice at later time points.

Disease pathology in the spleens was first detected 26 days post challenge, when pyogranulomata but no ZN positive organisms were detected in one animal (Table
2.2). At day 33, one of two mice had pyogranulomata with ZN positive organisms. At days 49 and 60, all mice spleens contained pyogranulomata with ZN positive organisms.
<table>
<thead>
<tr>
<th>Days post challenge</th>
<th>Lung</th>
<th>ZN Mediastinal lymph node</th>
<th>ZN Spleen</th>
<th>ZN +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>bronchioles normal range - no inflammatory cells seen.</td>
<td>N/A</td>
<td>normal range.</td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>bronchioles normal range.</td>
<td>N/A</td>
<td>normal range.</td>
<td></td>
</tr>
<tr>
<td>Day 15</td>
<td>bronchioles normal range. very occasional herophil and aveolar macrophages seen.</td>
<td>N/A</td>
<td>normal range.</td>
<td></td>
</tr>
<tr>
<td>Day 26</td>
<td>Focal, mild acute inflammation with ZN+ve bacteria Focal, subacute pneumonitis with ZN+ve bacteria. foci of necrosis and lymphocyte and heterophil infiltrates with ZN+ve bacilli</td>
<td>Y</td>
<td>mycobacterial granulomatous lymphadenitis</td>
<td>Y</td>
</tr>
<tr>
<td>Day 33</td>
<td>perivascular cuffing with lymphocytes. Pyogranulomatous pneumonitis with ZN+ve bacilli peribronchial/perivascular cuffing with lymphocytes. Pyogranulomatous pneumonitis with ZN+ve mycobacteria.</td>
<td>Y</td>
<td>mycobacterial granulomatous lymphadenitis</td>
<td>Y</td>
</tr>
<tr>
<td>Day 49</td>
<td>Peribronchial/perivascular cuffing with lymphocytes. Pyogranulomatous pneumonitis with ZN+ve mycobacteria.</td>
<td>Y</td>
<td>mycobacterial granulomatous lymphadenitis</td>
<td>Y</td>
</tr>
<tr>
<td>Day 60</td>
<td>Peribronchial/perivascular cuffing with lymphocytes. Pyogranulomatous pneumonitis with ZN+ve mycobacteria.</td>
<td>Y</td>
<td>mycobacterial granulomatous lymphadenitis</td>
<td>Y</td>
</tr>
</tbody>
</table>

1 Blinded examination H&E and ZN stained wax embedded sections performed RCVS Specialist in Veterinary Pathology
2 BALB/c mice infected by aerosol deposition of 60 CFU M. tuberculosis.
3 N/A = sample not available
4 Y = ZN positive bacteria detected by ZN staining
BCG mediated protection in lungs and spleen following aerosol *M. tuberculosis* infection

Parenteral BCG vaccination of inbred mice affords significantly greater protection in lungs and spleens compared with non-vaccinated animals (264). BALB/c mice were vaccinated with BCG then challenged by aerosol using a titre of $2 \times 10^6$ CFU/ml *M. tuberculosis*. The parameters of the experiment, BCG vaccination in the footpad giving a dose $5 \times 10^5$ CFU of Pasteur BCG and challenge 4 weeks later had been used in a number of published studies (265). Bacterial loads were determined in lungs and spleens 4, 6 and 8 weeks after challenge (Fig. 5 A&B). Again, in accordance with published studies, significant protection was observed in both the lungs and spleens in BCG vaccinated mice compared to naïve controls at all time-points examined ($P < 0.001$).
Figure 5: BCG induces significant protection from an aerosol *M. tuberculosis* challenge in the lungs and spleen. Total CFU burden is shown in lungs (A) and spleen (B) of naïve or BCG (5×10² CFU in footpad) vaccinated mice at different time-points following aerosol challenge with *M. tuberculosis* (1×10⁶ CFU/ml). CFU burden is expressed as log₁₀ mean CFU +/- SEM (n=10).
2.2 Discussion

These studies established that a titre of $1 \times 10^6$ CFU/ml of *M. tuberculosis* delivered as an aerosol for 20 minutes would infect 100% of mice. In this model, the standard deviation within groups with respect to organ bacillary load was small and enabled significant differences to be calculated between naïve and BCG-immunised mice at all time-points tested after challenge. The kinetics of growth of *M. tuberculosis* following challenge with $10^6$ CFU / ml *M. tuberculosis* is in accordance with published studies (204). Following infection, the *M. tuberculosis* showed clear lag, log and stationary phases of growth in lungs and spleens with dissemination of bacilli to the spleen occurring around day 15 post challenge (reviewed in Table 2.1). The disease pathology induced in this model, was also characteristic of *M. tuberculosis* infection in other murine models, involving lung peribronchial and perivascular lymphocytic infiltrates and pyogranulomata in the lung, pyogranulomatous splenitis and granulomatous lymphadenitis of the mediastinal lymph nodes (266).

The analysis of BCG vaccinated mice in the lungs and spleen at different time-points following *M. tuberculosis* challenge demonstrated the strong protective efficacy of BCG in acute infection. BCG remains the gold standard against which all vaccination regimens are compared in tuberculosis challenge models. Chapters 4 and 5 of this thesis deal with the development of new vaccination regimens against tuberculosis in mice. In the challenge model developed in this study the protection in the spleen produced by BCG Pasteur strain at 4 weeks, which is a time-point often
used in other published studies, was below the limit of detection of the assay. This implied that a comparison of bacterial burdens in the spleen between alternate vaccine regimens and BCG might have been uninformative at the 4 week time point. As such, in the challenge studies performed in Chapters 4 and 5, organs were harvested 6 weeks post challenge to ensure all BCG-vaccinated mice had \textit{M. tuberculosis} burdens in both the lungs and spleens significantly above the limit of detection of the assay.
3 Inducing antigen specific T cells in the respiratory compartment

3.1 Background to Chapter

Immunogenicity assays, dissection and vaccination techniques for this project were developed in mice using malaria vaccines. These techniques were then applied to the development of tuberculosis vaccines strategies described in Chapters 4 and 5.

In BALB/c mice, the induction of specific CD8+ T cells, are crucial to protection from pre-erythrocytic liver stage malaria. Intravenous vaccination with DNA then MVA, both expressing the circumsporozoite (CS) gene from *Plasmodium berghei* (*P. berghei*) afforded complete protection from a subsequent sporozoite challenge (267). This protection was mediated by a single immunodominant CD8+ T cell restricted epitope called pb9, which is recognised by H2d BALB/c mice following vaccination (268). The magnitude of the splenic T cell response to pb9 following vaccination is a correlate of protection from *P. berghei* challenge that enables the comparison of different vaccination regimens (including different routes of vaccination) within this model. Other studies by this group had shown that intradermal (ear) or intravenous vaccination with DNA / Adenovirus, Adenovirus/MVA, DNA/Fowlpox (FP9), MVA /FP9 or FP9/MVA expressing CS afforded full or partial protection following a *P. berghei* parasite challenge (269, 270).
The specific aims of this chapter were to investigate 1) whether lung delivery of recombinant viral or DNA vaccine constructs is immunogenic in the spleen and the respiratory compartment and 2) if immunogenic, whether lung immunisation of these malaria vaccines could afford protection against a *P. berghei* challenge.

### 3.2 Results

**Description of the regional lymph nodes in mice**

Regional lymph nodes were identified in BALB/c mice (Fig. 6) (271) (272). The same lymph node sets were identified in C57BL/6 mice with the exception of MLN, which were not visible, even after intranasal influenza A (A/PR/8/34) infection. The patterns of afferent and efferent lymphatic drainage to and from lymph node sets in rodents were first described by Tilney *et al* (271). The lymphatic drainage of those LNs that are relevant to these studies is described in Table 3.1.

<table>
<thead>
<tr>
<th>Lymph Nodes</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Facial Lymph Nodes</td>
<td>drain the skin of head and ventral aspect of sides of neck</td>
</tr>
<tr>
<td>Superficial Cervical Lymph Node</td>
<td>drain tongue and nasolabial lymphatic plexus</td>
</tr>
<tr>
<td><em>Internal Jugular, Paratracheal and Mediastinal Lymph Node</em></td>
<td>pooled LNs drain the lung and pharynx, larynx and proximal part of oesophagus through pharyngeal lymphatics</td>
</tr>
<tr>
<td>Inguinal Lymph Node</td>
<td>draining the thigh and genital mucosa,</td>
</tr>
</tbody>
</table>

*a* these regional lymph nodes are pooled and collectively called lung lymph nodes (LLN) in the text
Figure 6: Anatomical positioning of lymph node sets found in BALB/c mice.
Intranasal MVA.CS vaccination induces pb9-specific responses in the draining lung lymph nodes

8-10 week old female BALB/c mice were immunised once with $10^6$ PFU MVA.CS intranasally (i.n-), intradermally in the ear (i.d-) or intravenously (i.v-) in the lateral tail vein (Table 3.2). Intranasal immunisations were given in 10μl and 50μl volumes to identify the minimum volume necessary to achieve optimal induction of specific T cells in the lower lung. Previous studies had reported that volumes of 30μl or greater were necessary to deposit virus in the lung and also induce T cell responses in the lung and draining lymph nodes (272, 273).

Five and ten days after vaccination, facial lymph nodes (FLN), lung lymph nodes (pooled paratracheal and mediastinal lymph nodes) (LLN), inguinal lymph nodes (ILN) were taken from mice from each group, pooled and assayed by IFN-γ ELISPOT. Spleens were also removed and assayed individually.

Intranasal vaccination in a 10μl bolus did not induce any pb9-specific T cells in the lymph nodes and produced only a few spots at day 5 in the spleen. The 50μl bolus induced strong, specific T cell responses in the LLN (185 SFC/10^6 cells) and low but detectable responses in the spleen at day 5.
Table 3.2 The induction of pb9-specific IFN-γ responses in lymph nodes is dependent on route of immunisation of 10^6 PFU MVA.CS

<table>
<thead>
<tr>
<th>Day 5</th>
<th>i.n-10^6</th>
<th>i.n-50^d</th>
<th>i.d-Ear</th>
<th>i.v</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLN</td>
<td>0</td>
<td>7</td>
<td>280</td>
<td>32</td>
</tr>
<tr>
<td>LLN</td>
<td>0</td>
<td>185</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>ILN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>3 (2)</td>
<td>25 (8)</td>
<td>380 (121)</td>
<td>875 (160)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 10</th>
<th>i.n-10^6</th>
<th>i.n-50</th>
<th>i.d-Ear</th>
<th>i.v</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLN</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>LLN</td>
<td>0</td>
<td>23</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>ILN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>0</td>
<td>98 (4)</td>
<td>52 (19)</td>
</tr>
</tbody>
</table>

a SFC / 10^6 cells in pooled lymph node cells of 3 mice taken 5 or 10 days post vaccination
b day organs removed and assayed by ex-vivo ELISPOT
c i.n-10 = MVA.CS given intranasally in 10μl
d i.n-50 = MVA.CS given intranasally in 50μl
e average of SFC / 10^6 cells in 3 spleens +/- SEM shown in brackets

Both i.d- and i.v- MVA.CS immunisation elicited strong pb9 specific responses in the spleen 5 days after vaccination (267). Antigen specific responses in the draining lymph nodes differed between these routes of vaccination. Intravenous vaccination produced low level pb9-specific responses in all LNs assayed 5 days after vaccination. In contrast, i.d-MVA.CS vaccination in the ear produced strong responses in the FLNs, which drain the head, including the ears. Low level responses were also detected in the LLN and are probably due to antigen specific T cells in the paratracheal LN which also drain cells from the FLN (271). No responses were detected in the ILN that drain the flank and genital mucosa (Table 3.2).

The induction of pb9-specific T cells and draining LNs typically decreased by 10-fold between day 5 and 10 days post vaccination.
Intranasal FP9.CS vaccination induces pb9 specific responses in the draining lung lymph nodes

Having shown that a 50μl bolus of MVA.CS was a sufficient volume to induce T cell responses in the LLN, similar experiments were conducted with FP9.CS. Cervical lymph nodes (CLN) that drain the nasal mucosa were also assayed to determine whether i.n- vaccination also induced antigen specific responses in the nasal mucosa. Mice were i.v- or i.n- (50μl) vaccinated and the CLN, LLN and ILN were removed 5 and 10 days after vaccination (Table 3.3). Intravenous immunisation of FP9.CS induced low level pb9-specific T cell responses in the ILN and CLN and strong responses in the spleen. Intranasal vaccination of FP9.CS, as was observed with MVA.CS induced responses primarily in the LLN and to a lesser extent in the CLN and showed that vaccination at this volume induces T cell responses primarily in the respiratory compartment. As was observed following i.n-MVA.CS, i.n- FP9.CS vaccination also induced low level responses in the spleen.
Table 3.3. The induction of pb9 specific IFN-γ responses in lymph nodes is dependent on route of immunisation of 10⁶ PFU FP9.CS

<table>
<thead>
<tr>
<th>Day 5</th>
<th>i.n-50ᵇ</th>
<th>i.v</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLN</td>
<td>24.5</td>
<td>15</td>
</tr>
<tr>
<td>LLN</td>
<td>149</td>
<td>0</td>
</tr>
<tr>
<td>ILN</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>45 (7)</td>
<td>327 (94)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 10</th>
<th>i.n-50</th>
<th>i.v</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLN</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>LLN</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>ILN</td>
<td>0</td>
<td>5.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>33(14)</td>
<td>103 (15)</td>
</tr>
</tbody>
</table>

ᵇ i.n-50 – FP9.CS given intranasally in 50μl
ᶜ average of SFC /10⁶ cells in 6 spleens
ᵈ average of SFC /10⁶ cells in 3 spleens

a Day 5 - average of 2 independent experiments. In each experiment lymph node cells from 3 mice were pooled. Day 10 - SFC /10⁶ cells in pooled lymph node cells of 3 mice
+/- SEM shown in brackets
CLN and LLN cells were also cultured separately with pb9 peptide for 7 days. A 
\(^{51}\)Cr release assay was performed using P815 cells incubated with pb9 as targets and 
compared to non-specific lysis of target cells pulsed with an irrelevant K\(^d\) restricted 
nucleoprotein peptide (Fig. 7). Intravenous FP9.CS vaccination induced detectable 
lysis above background in both the LLN and CLN cell cultures (Fig. 7A). Intranasal 
vaccination in a 50μl bolus induced specific lysis in the CLN to similar levels as 
intravenous vaccination but lysis in the cultured lung lymph nodes was 2-fold greater 
than that produced by i.v-FP9.CS vaccination (Fig. 7B). These results support the 
ELISPOT data that i.n- vaccination with recombinant poxviruses targets the 
respiratory compartment.
Figure 7: Intranasal delivery of FP9.CS induces pb9-specific CTLs in the lung lymph nodes. Lymph nodes from the lung and upper thoracic region were removed from mice (n=3), 5 days after receiving FP9.CS (10^6 PFU) given either intravenously (i.v) (A) or intranasally (i.n) (B). Individual ^51Cr release assays were performed on pb9 peptide stimulated lung lymph node (LLN) and cervical lymph node (CLN) 7 day old cultures. The average % specific lysis of P815 target cells pulsed with pb9 or an irrelevant 9 aa flu peptide, NP are shown.
Intranasal delivery of naked DNA.CS does not induce detectable T cell responses in the lung lymph nodes

Mice were immunised with 50μg of DNA expressing CS (DNA.CS) either i.n- (50μl), or intramuscularly (i.m). The DNA was suspended in sterile PBS without adjuvants. No pb9-specific T cell responses were detected in ELISPOT in the LLN following 1, 2 or 3 vaccinations with i.n-DNA.CS, where each vaccination was given 2 weeks apart (data not shown). Splenocytes from mice vaccinated with i.n- or i.m- with DNA.CS were taken 10 days after the third vaccination, and cultured with pb9 peptide. Cells were then tested for their ability to specifically lyse pb9-pulsed target cells (Fig. 8). Cultured cells taken from i.m-DNA.CS immunised animals produced about 60% lysis at the highest effector: target (100:1) ratio. Specific lysis induced by i.n-DNA.CS immunisation was lower at 20% but demonstrated that i.n- DNA immunisation can, albeit inefficiently induce T cell responses in the spleen.
Figure 8: Repeated intranasal administration of naked plasmid DNA induces low level pb9-specific CTL in the spleen. Mice were immunised 3 times with 50μg DNA.CS given 2 weeks apart either intranasally (i.n) or intramuscularly (i.m). 10 days following the last vaccination, spleens were harvested, cultured for 7 days with pb9 peptide and 51Cr release assays were performed. Results are expressed as the average % specific lysis of P815 target cells pulsed with pb9 minus the average % specific lysis of P815 target cells cells pulsed with an irrelevant 9 aa flu peptide, NP.
Heterologous vaccination of recombinant viral vectors delivered intranasally induces higher frequencies of T cells than homologous intranasal vaccination.

Parallel studies within the laboratory had shown that heterologous i.v-MVA.CS/ i.v-FP9.CS or i.v-FP9.CS / i.v-MVA.CS vaccination produced significantly higher pb9-specific T cell responses in the spleen that homologous vaccination of either vaccine (270). Heterologous MVA.CS / FP9.CS prime-boost regimens were tested in combination with i.n- delivery of vaccines to investigate whether, CS-specific CD8+ T cells could be expanded at the level of the LLN. Mice were vaccinated with i.n- FP9.CS and immunised two weeks later either with FP9.CS given i.n- or i.v- or with MVA.CS given i.n- or i.v-. Ten days later, LLN and spleens were assayed by IFN-γ ELISPOT (Fig. 9).
Figure 9: Heterologous vaccination with FP9.CS and MVA.CS induces higher responses in the lung lymph nodes and spleen than homologous viral vaccination. Mice were immunised intranasally with FP9.CS and 14 days later immunised either intranasally (i.n) or intravenously (i.v) with FP9.CS or MVA.CS. 10 days after the second vaccination, spleens and lung lymph nodes were removed and assayed for p99-specific responses in IFN-γ ELISPOT. Responses for pooled lung lymph nodes (LLN) (A) and individual spleens (B) are shown (n=3). Results are representative of two experiments and are expressed as spot forming cells / 10⁶ cells (SFC/10⁶ +/- SEM).
Intranasal prime and i.n- boosting vaccinations produced the strongest pb9-specific T cell responses in the LLN (Fig. 9A). Heterologous vaccination with i.n-MVA.CS / i.n-FP9.CS produced a higher response (~3-fold greater) in the LLN than homologous vaccination 2× i.n-FP9.CS.

Intranasal vaccination with FP9.CS was sufficiently immunogenic in the spleen to prime a subsequent boosting vaccination with i.v-MVA.CS that was significantly higher than vaccination with i.v-MVA.CS alone ($P < 0.01$), or 2× i.n-FP9.CS ($P < 0.01$) (Fig. 9B).

Heterologous vaccination produced significantly higher pb9-specific T cell responses than homologous regimens in the spleen. This applied to mice given vaccination by the purely i.n- route (i.n-FP9.CS / i.n-MVA.CS > 2× i.n-FP9.CS) ($P < 0.01$), or mice give a priming vaccination i.n- and then boosted i.v- (i.n-FP9.CS / i.v-MVA.CS > i.n-FP9.CS / i.v-FP9.CS) ($P < 0.01$).
<table>
<thead>
<tr>
<th>Vaccination</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>0</td>
</tr>
<tr>
<td>i.v-FP9.CS / i.v-MVA.CS</td>
<td>50</td>
</tr>
<tr>
<td>i.v-MVA.CS / i.v-FP9.CS</td>
<td>40</td>
</tr>
<tr>
<td>i.n-FP9.CS / i.v-MVA.CS</td>
<td>10</td>
</tr>
<tr>
<td>i.n-MVA.CS / i.v-FP9.CS</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>0</td>
</tr>
<tr>
<td>i.v-FP9.CS / i.v-MVA.CS</td>
<td>80</td>
</tr>
<tr>
<td>i.v-MVA.CS / i.v-FP9.CS</td>
<td>60</td>
</tr>
<tr>
<td>i.n-FP9.CS / i.n-MVA.CS</td>
<td>0</td>
</tr>
<tr>
<td>i.n-MVA.CS / i.n-FP9.CS</td>
<td>0</td>
</tr>
<tr>
<td>i.n-FP9.CS / i.v-MVA.CS</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 3.4** Intravenous *P. berghei* sporozoite challenge of BALB/c mice 14 days following vaccination with heterologous viral vectors expressing the circumsporozoite gene

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Vaccination</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naive</td>
<td>0</td>
</tr>
<tr>
<td>2000 sporozoites</td>
<td>i.v-FP9.CS / i.v-MVA.CS</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>i.v-MVA.CS / i.v-FP9.CS</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>i.n-FP9.CS / i.v-MVA.CS</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>i.n-MVA.CS / i.v-FP9.CS</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Naive</td>
<td>0</td>
</tr>
<tr>
<td>1000 sporozoites</td>
<td>i.v-FP9.CS / i.v-MVA.CS</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>i.v-MVA.CS / i.v-FP9.CS</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>i.n-FP9.CS / i.n-MVA.CS</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>i.n-MVA.CS / i.n-FP9.CS</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>i.n-FP9.CS / i.v-MVA.CS</td>
<td>0</td>
</tr>
</tbody>
</table>

\( ^{a} \) Mice (n=10) were vaccinated 2 weeks apart and challenge 14 days post the second vaccination; i.v = intravenous immunisation of \( 10^6 \) PFU in 100\( \mu \)l; i.n = intranasal immunisation of \( 10^6 \) PFU in 100\( \mu \)l

\( ^{b} \) Survival scored as number of mice that were negative for blood stage parasitaemia 8 days post challenge

\( ^{c} \) Mice challenged with sporozoites by intravenous injection in 100\( \mu \)l

Intranasal administration of MVA.CS / FP9.CS does not afford protection from *Plasmodium berghei* challenge in BALB/c mice

The protective efficacy of i.n- MVA.CS / i.n-FP9.CS and i.n-MVA.CS / i.v-FP9.CS vaccination regimens was tested in an i.v- challenge with *P. berghei* sporozoites. The dissections, parasite challenge and subsequent scoring of Geimsa stains slides for detection of blood stage parasitaemia were performed by Richard Anderson and Carolyn Hannan (NDM, Oxford University). The results are summarised in Table 3.4. In both experiments, all naïve animals were blood stage positive for malaria at
day 8 post challenge. The strongest protection observed was afforded by FP9.CS / MVA.CS vaccination that produced 50% and 80% protection in experiment 1 and 2 respectively. The level of protection reflected the strength of the parasite challenge being 2000 and 1000 sporozoites in experiment 1 and 2 respectively. In experiment 1, 1 animal vaccinated with i.n-FP9.CS / i.v-MVA.CS was protected but this was not reproduced in the subsequent lower dose challenge. Purely i.n- vaccination using heterologous viral vectors afforded no protection from disease.

3.3 Discussion

The results in this chapter illustrate that in BALB/c mice, the induction of antigen specific T cells in secondary lymphoid organs following vaccination with replication deficient viruses is highly compartmentalised. The induction of specific T cells in the draining LN but not non-draining lymph nodes most likely results from the specific migration of antigen-loaded dendritic cells (DCs) from the site of immunisation. A previous study has shown that following subcutaneous footpad immunisation, labelled antigen loaded murine DCs migrated to the T cell areas of the draining popliteal LNs. Very little migration to non-draining LNs, the thymus and spleen was detected (274).

In this study, i.n- vaccination of either MVA.CS or FP9.CS produced strong pb9-specific T cell responses in the LLNs and concords with the study of Vermaelen et al showing that airway DCs selectively transport antigen to the LLN (245). Intranasal vaccination also produced low level responses in the spleen, which may have resulted from transport of virus to the circulation from the lung or possibly through the gut from reflux during immunisation or low level migration of lung antigen
loaded DCs to the spleen. Both i.d- and i.v- immunisation induced high frequencies of pb9-specific T cells in the spleen. These routes of immunisation differed in the T cell responses in the lymph node compartments. Intradermal vaccination in the ear induced strong T cell responses in the draining FLN, low level responses in the LLN but no detectable responses in the distal LNs. Intravenous vaccination induced low level responses in all lymph node sets, which most likely reflect the deposition of virus in organs throughout the body following vaccination.

The frequency and kinetics of pb9-specific T cells induced by a single vaccination with FP9.CS or MVA.CS were comparable. Prime-boost strategies using these heterologous viral vectors given i.n- or i.v- were examined. Heterologous viral vaccination (FP9/MVA) induced higher numbers of specific T cells in the LLN than homologous vaccination (FP9/MVA>FP9/FP9), when both vaccinations were given intranasally. An intranasal vaccination of FP9.CS induced pb9-specific T cells in the spleen and when boosted by i.v-MVA.CS produced responses significantly greater than i.v- vaccination of MVA.CS alone. These results are in concordance with other studies that demonstrate in mice, optimal induction of T cell responses in the respiratory compartment is achieved following i.n- vaccination with viral or DNA vectors (275). Moreover, the induction of T cell responses in both the lung and systemic compartments can be achieved by a combination of systemic and mucosal vaccination that is optimised when heterologous vaccine vectors are used (255).

An i.n- / i.n- or i.n- /i.v- vaccination regimen with FP9.CS followed by MVA.CS failed to protect mice from an intravenous P. berghei sporozoite challenge. These vaccination regimens produced significantly lower pb9-specific T cell responses (3-4
fold lower) in the spleen than systemic vaccination of FP9.CS / MVA.CS that were protective following challenge (270). These results support earlier observations that the induction of splenic IFN-γ secreting T cells in the spleen is the best known correlate of protection against liver stage malaria in mice models (267).

The induction of specific T cells in the LLN did not correlate with protection against malaria that is essentially a disease of the liver and blood. However, the induction of T cells in the LLN has been shown to correlate with protection from viral respiratory pathogens, like influenza (272). Further studies were undertaken to investigate whether the induction of M. tuberculosis-specific T cells in the LLN could correlate with protection from the tuberculosis.
4  

*M. tuberculosis* prime-boost vaccination regimens – targeting the respiratory compartment

4.1 Background

The only licensed vaccine against *M. tuberculosis*, Bacille-Calmette Guerin (BCG) (10), is an attenuated strain of *Mycobacterium bovis*. Despite the poor protective efficacy afforded by the BCG vaccine against pulmonary tuberculosis, 80% of infants throughout the world receive BCG each year (http://www.who.int/inf-fs/en/fact104.html). This implies that trials of future tuberculosis vaccine candidates will likely be performed in BCG vaccinated communities.

In mice, DNA.85A - MVA.85A vaccination, whilst inducing high frequencies of Ag85A specific splenic IFN-γ secreting T cells, did not achieve greater protection than BCG following *M. tuberculosis* challenge (136). The aim of this part of the study was to determine whether in a mouse *M. tuberculosis* aerosol challenge model, BCG-induced protection can be improved by mucosal lung delivery and/or boosting with a second dose of BCG or recombinant MVA expressing Ag85A (MVA.85A).
4.2 Immunogenicity Studies

BCG vaccination

Choice of BCG-sub-strain

A variety of BCG sub-strains are used in immunogenicity and protection studies in animal models (264). In studies performed in this laboratory, both the Pasteur 1173 P2 and Evan-Medeva BCG protected BALB/c mice equally following infection with *M. tuberculosis* using the aerosol challenge model described in Chapter 2 (compare Fig. 5 and (136)). The Pasteur 1173 P2 vaccine strain was chosen for immunogenicity studies because Pasteur BCG-vaccinated mice induced very low levels of non-specific IFN-γ secretion in splenocytes in ELISPOT assays (0-10 SFC/10^6 cells). A similar observation has been reported elsewhere (276). In contrast, mice vaccinated with Evan-Medeva BCG, produced higher levels of non-specific IFN-γ secretion in immune assays (100-200 SFC/10^6 cells) (data not shown).

Antigen 85A is immunodominant following BCG vaccination

The PPD- and 85A culture filtrate protein- T cell responses induced by parenteral (p-) BCG vaccination were compared over the course of a year using IFN-γ ELISPOT (Fig. 10). PPD is the purified protein derivative from *M. tuberculosis* broth culture. The level of response to 85A protein ranged between 20-60% of the overall PPD response. PPD and 85A protein T cell responses were abrogated by CD4^+ T cell depletion (PPD – 93-98% depletion, 85A – 85-94% depletion of total
response of non-depleted cells in IFN-γ ELISPOT). No more that 5% of the total response in ELISPOT was lost following CD8⁺ T cell depletion showing very low levels of CD8⁺ dependent PPD or 85A protein responsive cells induced following BCG immunisation. Responses to Ag85A class I- and II- restricted epitopes (P11 and P15 respectively) (277), were also assayed to determine the strength of the Ag85A peptide specific response produced by BCG vaccination. T cell responses specific for the major CD4⁺ epitope in Ag85A, the P15 peptide, accounted for over 80% of the T cell response to the whole protein at all time-points and BCG doses tested (Fig. 10). T cells specific for the CD8⁺ epitope, P11 were not detected in either ELISPOT or chromium release assays (data not shown).
Figure 10. Antigen 85A is immunodominant following BCG immunisation. BALB/c mice (n=3-6) were immunised with 5x10^5 CFU BCG in the footpad. At the time-points indicated, spleens were assayed by ex-vivo ELISPOT for responses to PPD, 85A CFP and 85A - CD4+ (P15) and CD8+ (P11) peptides. Responses are expressed as IFN-γ secreting spot forming cells (SFC) / 10^6 cells +/- SEM.

Figure 11. Mice were vaccinated with 10^5 or 10^7 CFU of BCG in a 100μl bolus. Within 5 minutes of vaccination, lungs were removed, homogenised and culture for 3 weeks on middlebrook plates. Results are expressed as the log_{10}[CFU] +/- SEM (n=5)
Intranasal BCG immunisation induces strong T cell responses in the spleen, lung and lung lymph nodes

Deposition studies with intranasal delivery of BCG

BALB/c mice were immunised intranasally (i.n-) (as described in Chapter 3) with $10^5$ or $10^7$ CFU of BCG (Pasteur). BCG was cultured from lungs immediately after vaccination (Fig. 11). Approximately, 1% of the bacilli delivered are recovered from the lungs using this technique.

Immunogenicity

The frequency and localisation of IFN-γ secreting T cells induced by either parenterally or i.n- delivered BCG was compared. The responses to i.n-BCG and p-BCG produced comparable levels of PPD- and Ag85A peptide specific CD4⁺ T cell responses in the spleen (Fig. 12A). In contrast, the frequency of antigen specific T cells induced in the lungs 12 weeks after vaccination was significantly higher following i.n- vaccination than p- vaccination (Fig. 12B). The cell yields between naïve mice and mice receiving BCG either intranasally or in footpad did not significantly differ (data not shown).
Figure 12. Intranasal BCG (i.n-BCG) immunisation induces higher frequencies of specific T cells in the lung compartment than parenteral BCG (p-BCG) immunisation. BALB/c mice (n=9) were immunised with $10^7$ CFU of BCG either i.n- or p- in the ear. 12 weeks later spleens (A), lungs (B), facial lymph nodes (FLN) (C), and draining lung lymph nodes (LLN) (D) were harvested and assayed by ELISPOT, for responses to PPD, and the 85A - CD4+ (P15) and CD8+ (P11) epitopes. Spleens and lungs were assayed individually. Results for these organs are the average of two independent experiments, 4 mice per group. For the lymph node assays, 9 mice were divided into 3 sub-groups from which nodes were pooled. Results are expressed as the mean of the 3 sub-groups +/- SEM. * indicates significant differences ($P$ value < 0.05) by Student's $t$ tests in the induction of T cell responses by the different routes of vaccination.
Parenteral BCG immunisation in the ear produced specific IFN-γ secretion in the draining FLN whereas intranasal delivery produced responses exclusively in the LLN reflecting the pattern of response in the lung (Fig. 12C & D respectively). No responses were detected in the distal ILN (data not shown). This suggests that similar to the observations made with poxviruses, i.n-BCG vaccination induces T cell responses that are compartmentalised and dependent on route of vaccination.

In summary, whilst both i.n-BCG and p-BCG produce strong splenic T cell responses, i.n-BCG elicited stronger T cell immunity in the lung and its draining lymph nodes.

**MVA.85A boosts both CD4+ and CD8+ T cell responses induced by BCG**

MVA can boost both DNA- and FP9- induced T cells, specific to a common recombinant antigen (267, 270). Studies were conducted to determine whether MVA.85A could also boost BCG-induced Ag85A specific T cell responses. Fourteen weeks after $10^7$ CFU BCG was given parenterally, mice were boosted with $10^6$ PFU MVA.85A. This time-point was chosen because the frequency of Ag85A specific T cell responses had plateaued and small standard deviations were observed within groups of mice. Parenteral boosting with MVA.85A doubled the PPD response and increased the Ag85A specific CD4+ and CD8+ T cell responses 4 and 5 fold respectively in the spleen (Fig. 13A). Immune responses in the FLN showed a similar
pattern of boosting in PPD and Ag85A CD4$^+$ and CD8$^+$ peptide assays (data not shown).
Figure 13: MVA.85A specifically boosts both the CD4+ and CD8+ 85A T cell responses induced by BCG. BALB/c mice were parenterally immunised with 10^7 CFU of BCG. 14 weeks later, mice were boosted (25μl/ear) with 10^6 PFU MVA.85A (A) or 10^6 PFU MVA.CS (B). MVA or BCG only controls were included. Spleens were harvested 10 days post boost and assayed by ELISPOT for responses to PPD and 85A peptides. Results are the mean +/- SEM of two experiments, 3-4 animals per group. * indicates significant differences (P value < 0.05) by Student’s t tests in the induction of T cell responses by the different vaccine regimens.
Parallel experiments were conducted in which mice were immunised with BCG and boosted with an irrelevant MVA expressing the malarial CS protein (MVA.CS), to determine whether BCG was specifically priming the Ag85A CD8⁺ T cell response (Fig. 13B). The frequency of CS-specific CD8⁺ T cells was not significantly different between BCG/MVA.CS and MVA.CS-only groups, suggesting that the Ag85A CD8⁺ restricted T cell response (whilst undetectable following the BCG immunisation) is specifically boosted by MVA.85A. In separate experiments, MVA.CS immunisation did not affect the frequency of IFN-γ secreting T cells specific for PPD, P15 and P11 induced by BCG vaccination (Fig. 14A). Conversely, prior BCG vaccination did not significantly affect the magnitude of pb9-specific lysis produced by splenocytes from mice vaccinated with MVA.CS (Fig. 14B).
Figure 14: PPD, 85A peptide and CS peptide responses in ELISPOT are shown in BCG immunised animals boosted with MVA.CS (A). 51Cr release assays were also performed on splenocytes from BCG immunised animals boosted with MVA.CS (B). Results for ELISPOT are expressed as the average (SFC) / 10^6 cells +/- SEM of individual spleens from 3-4 mice, assayed in duplicate and in half-fold dilutions. The results for 51Cr release assays are expressed as average % CS peptide specific lysis minus non-specific lysis of P815 target cells.
Histology studies of mice immunised intradermally with BCG-MVA.85A

Ear immunisation of BCG produced a hard lump at the site of immunisation, palpable 3 months after vaccination. ZN staining of the ear showed that ZN-positive bacilli were retained at the site of immunisation, 1 month after vaccination (Appendix 1). At this time-point, bacilli were also detected in the FLN (Appendix 2). MVA.85A vaccination in the ear of BCG-immunised mice did not exacerbate pathology at the site of immunisation, FLN or the lung (data not shown).

MVA.85A can significantly boost T cell responses following an intranasal vaccination of BCG

Similar to the wholly parenteral BCG / MVA.85A prime-boost regimen described above, parenteral MVA.85A boosting of i.n-BCG immunised mice also significantly increased the level of PPD and Ag85A CD4⁺ and CD8⁺ T cell responses in the spleen (Fig. 15A). Intranasal MVA.85A boosting of i.n-BCG immunised mice did not significantly increase Ag85A-specific T cell responses in the spleen (Fig. 15A) or the FLN (data not shown) but produced a 3-5 fold increase in the level Ag85A-specific CD4⁺ T cells in the LLN (Fig. 15B).
Figure 15: MVA.85A specifically boosts i.n-BCG induced T cell responses in the spleen and lung lymph nodes. Mice were immunised i.n- (100μl) with 10^7 CFU of BCG and after 14 weeks boosted i.n- or i.d- with either 10⁶ PFU MVA.85A or 10⁶ PFU MVA.CS. 10 days later spleens (A) and lung lymph nodes (LLN) (B) were harvested and assayed by ELISPOT for responses to PPD, 85A. Results for ELISPOT are expressed as the average (SFC/10⁶ cells +/- SEM of individual spleens or pooled lymph nodes from 3-4 mice, assayed in duplicate and in half-fold dilutions. Data shown are representative of 2 experiments. * Indicates a P value < 0.05, as determined by LRA comparison of all groups.
MVA.85A can significantly boost T cell responses following an parenteral vaccination of BCG.

Parenteral BCG immunisation, although a poor inducer of specific T cell responses in the LLN, did elicit specific T cell responses in the lung parenchyma. Studies were conducted to examine whether the T cells induced in the lung compartment by p-BCG immunisation could be boosted by i.n-MYA.85A vaccination. Intranasal MVA.85A boosting did not significantly increase T cell responses in the spleen (Fig. 16A). However, i.n-MVA.85A immunisation did increase p-BCG induced 85A CD4⁺ T cell responses in both the FLN and the LLN (Fig. 16B & C).
Figure 16: i.n-MVA.85A specifically boosts p-BCG induced T cell responses in the lung lymph nodes. Mice were immunised p- with 10^7 CFU of BCG and after 14 weeks boosted i.n- or p- with either 10^6 PFU MVA.85A or 10^6 PFU MVA.CS. 10 days later spleens (A), facial lymph nodes (B) were harvested and assayed by ELISPOT for responses to PPD, 85A. P15 CD4+ dependent responses in the LLN of p-BCG immunised mice is shown in (C). Results for ELISPOT are expressed as the average (SFC)/10^6 cells +/- SEM of individual spleens or pooled lymph nodes from 3-4 mice, assayed in duplicate and in half-fold dilutions. Data shown are representative of 2 experiments. * Indicates a P value < 0.05, as determined by LRA comparison of all groups.
MVA.85A boosting following a low dose i.n-BCG vaccination

Mice were immunised i.n- with BCG at doses ranging from $10^{2.6}$ CFU. Twelve weeks later, mice were boosted either i.n- or p- with MVA.85A (Fig. 17). Lungs were homogenised and BCG bacilli recovered (Fig. 17A). Analysis of splenic T cell responses showed that $10^4$ CFU BCG was the lowest dose capable of inducing a detectable PPD-specific T cell response in the spleen (Fig. 17B-D). Whilst the 85A CD4$^+$ T cell response was significantly boosted by MVA.85A following vaccination of $10^5$ and $10^6$ CFU of BCG, no boosting of the PI1-specific CD8$^+$ T cell response was observed (Fig. 17E-F). These data suggest that lower doses of BCG do not induce or inadequately induce 85A specific CD8$^+$ T cells that can be expanded by subsequent MVA.85A vaccination.
Figure 17A: MVA.85A vaccination of BCG immunised mice does not affect the recovery of bacilli from mouse lungs 12 weeks after vaccination. BALB/c mice were immunised i.n- with increasing doses of BCG (1x10^2 - 1x10^6 CFU). 12 weeks later, mice were boosted i.n- or p- with 10^9 PFU MVA.85A. 10 days later, lungs were removed, homogenised and cultured to recover residual BCG bacilli. The average CFU +/- SEM are shown for each group (n=4).

Figure 17B-F: MVA.85A vaccination boosts 85A specific- CD4+ but not CD8+ T cell responses induced by low dose BCG vaccination. BALB/c mice were immunised i.n- with increasing doses of BCG (1x10^2 - 1x10^6 CFU) (B)-(E). 12 weeks later, mice were boosted i.n- or p- with 10^6 PFU MVA.85A. 10 days later spleens were harvested and 85A specific T cell responses were examined in ELISPOT. Results are the mean SFC/10^6 cells +/- SEM (n=4). * indicates significant differences (P value < 0.05) by Student’s t tests in the induction of T cell responses by the different vaccine regimens. Bacteriology from lungs is shown in Figure15(4).
Table 4.1. MVA.85A\textsuperscript{a} vaccination 8 weeks after BCG immunisation of BALB/c mice does not exacerbate gross lung pathology

<table>
<thead>
<tr>
<th>Immunisation Regimen\textsuperscript{b}</th>
<th>Lung Pathology</th>
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<tbody>
<tr>
<td>i.n-BCG $10^{4.5}$</td>
<td>NAD\textsuperscript{d}</td>
</tr>
<tr>
<td>i.n-BCG $10^{5.5}$/i.n-MVA.85A</td>
<td>NAD</td>
</tr>
<tr>
<td>i.n-BCG $10^{5.5}$/p-MVA.85A</td>
<td>NAD</td>
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<tr>
<td>i.n-BCG $10^{6}$</td>
<td>Abscess\textsuperscript{c}</td>
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<tr>
<td>i.n-BCG $10^6$/i.n-MVA.85A</td>
<td>Abscess</td>
</tr>
<tr>
<td>i.n-BCG $10^7$/p-MVA.85A</td>
<td>Abscess</td>
</tr>
<tr>
<td>i.n-BCG $10^7$/i.n-MVA.85A</td>
<td>Flecked\textsuperscript{l}</td>
</tr>
<tr>
<td>i.n-BCG $10^7$/p-MVA.85A</td>
<td>Flecked</td>
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\textsuperscript{a}MVA.85A dose = $10^6$ PFU
\textsuperscript{b}i.n = intranasal; p= parenteral (50\textmu l in footpad)
\textsuperscript{c}description of lung pathology observed in 4/4 mice
\textsuperscript{d}NAD = no abnormality detected
\textsuperscript{e}Abscess = 2-3 x 3-5 mm white and raised / 5-10 over lungs
\textsuperscript{f}Flecked describes 2-4 mm flat and off-white and circular lesions/ > 20 over lungs

One mouse in the group that received $10^4$ CFU i.n-BCG then p-MVA.85A produced strong T cell responses to P11, P15 and PPD that increased the mean and the SEM within the group (Fig. 17D). In this mouse, the BCG recovery from the lung 8 weeks after vaccination was higher than other mice within the group (Fig. 17A). This implies that this animal received a much higher dose of BCG at the time of vaccination that induced stronger T cell responses that were effectively boosted by p-MVA.85A vaccination.

Records were made of the gross pathology induced by vaccination (Table 4.1). Intranasal BCG vaccination alone induced pathology at higher doses, but MVA.85A
vaccination did not exacerbate gross pathology. No further pathology studies were performed because in humans, lung immunisation would be more likely given by aerosol. Pathology studies following aerosol delivery of BCG in rhesus macaques that were given a human dose BCG are described in Chapter 6.

Intranasal DNA.85A vaccination requires a lipid adjuvant to induce T cell responses in the spleen

In Chapter 3, repeated i.n- immunisation of naked DNA, failed to induce detectable T cell responses in the LLN and spleen. In this chapter, intranasal delivery of DNA was examined in conjunction with lipid adjuvant and MVA.85A boosting vaccinations. BALB/c mice were immunised with DNA.85A (DNA.85A) with or without a cationic lipid, GL-67 either i.n- or i.m-. GL-67 (67) is a cationic lipid that had been previously shown to significantly increase gene expression of plasmid DNA in the murine lung (278). These experiments were performed with Steve Hyde (Nuffield Department of Clinical Biochemistry. Oxford University) Two weeks after the first vaccination, mice were immunised with a second dose of DNA.85A +/- 67 or with MVA.85A given i.n- or i.v-. 10 days later spleens and LLN were removed and analysed by IFN-γ ELISPOT (Fig. 18).
Figure 18: Lipid GL-67 mixed with naked DNA.85A adjuvants the induction of 85A specific CD4+ and CD8+ T cell responses in the spleen. BALB/c mice were immunised with DNA.85A pCIK85A (60µg) either i.m- or i.n- with or without GL-67. Two weeks later mice were revaccinated with either a second dose of DNA.85A with or without GL-67 or i.n- or i.v- with 10^6 PFU MVA.85A. 10 days later, spleens (A) and lung lymph nodes (LLN) (B) were removed and assayed for 85A specific CD4+ and CD8+ peptide specific responses in ELISPOT. Results are expressed as the average SFC 10^6 cells for individual spleens (+- SEM) or pooled LLN (n=4).
Vaccination of 2x i.n-DNA.85A failed to elicit responses in the spleen but low level responses were observed following inclusion of lipid 67 (Fig.18A). The adjuvant effect of 67 on DNA.85A was clearer in the spleen when mice were boosted with MVA.85A. Interestingly, naked DNA.85A / MVA.85A produced responses in the LLN equivalent to DNA.85A+67 / MVA.85A (Fig.18B). This suggests that naked DNA given intranasally can induce low levels of T cells in the LLN but that the induction of splenic T cells requires an adjuvant. Intranasal DNA.85A+67 prime followed by systemic boost with MVA.85A induced the strongest response in the spleen for both CD4+ and CD8+ 85A specific T cell responses (Fig. 18A). This vaccination regimen gave higher levels of response in the spleen than the classical i.m-DNA.85A / i.v-MVA.85A vaccination regimen. Lipid 67 did not adjuvant DNA.85A induced T cell responses when the vaccination was given intramuscularly. A mucosal prime and boost using heterologous vaccine vectors DNA.85A/MVA.85A induced the strongest responses in the LLN.

4.3 Aerosol *M. tuberculosis* Challenge

Acute Aerosol Challenge of Vaccinated BALB/c mice

Immunogenicity studies using BCG / MVA.85A vaccination regimens had shown that the frequency of specific IFN-γ secreting T cells induced by BCG could be significantly increased. Further, i.n- vaccination with either BCG or MVA.85A was able to induce responses in the LLN. These regimens were chosen as the most promising for *M. tuberculosis* challenge studies. Animals were vaccinated with i.n-
BCG. Twenty-two weeks later, mice were given MVA.85A either i.n- or p-. After 1 month, mice were challenged with *M. tuberculosis* given as an aerosol. Lungs and spleens were harvested and cultured for the presence of *M. tuberculosis* colonies 6 weeks after challenge (Fig. 19A & B). There was no significant difference in the number of mycobacterial colonies on plates with or without TCH indicating no detectable BCG in the lungs or spleens, six weeks after challenge (data not shown).
Figure 19: Protection of BALB/c mice against aerosol M. tuberculosis challenge by BCG boosting regimens. Mice were immunised with BCG given either i.n- (10^5 CFU) or p- in the footpad (5x10^5CFU). 22 weeks later groups were boosted either i.n- or p- (footpad) with BCG or MVA.85A (10^6 PFU). Four weeks following the second immunisation mice (n = 9-12) were challenged and approximately 250 CFU M. tuberculosis H37rv was deposited into the lung. After six weeks, lungs and spleens were harvested and plated out in serial dilutions on Middlebrook agar plates containing TCH (5µg/ml). Results are shown for lung and spleens in (A) and (B) respectively. Results are expressed as the mean (log_{10}[CFU]) of either the lung or spleen +/- SEM. * indicates significant differences (P value < 0.05) by Student’s t tests in bacterial load between the various groups clustered by arrows. The origin of the arrow (circle) designates the reference group to which statistical comparisons were made.
Two doses of BCG afforded greater protection than one in BALB/c mice compared to the naïve controls, significant protection was conferred in the lung but not the spleen by a single parenteral dose of BCG administered 22 weeks before challenge in BALB/c mice (lung: $P<0.01$, spleen: $P=0.84$). In previous studies, BCG immunisation 4 weeks prior to aerosol challenge yielded highly significant protection in both the spleen and lungs (Fig. 5, Chapter 2), indicating that the protective effect of BCG in the spleen diminishes with time (265). When mice were immunised with a second dose of BCG, protection in both the lungs and spleens was highly significant when compared to naïve controls (lung: $P<0.001$, spleen: $P=0.001$) and to the $1\times$ p-BCG group (lung: $P=0.022$, spleen: $P=0.001$).

Intranasal BCG offers greater protection than p-BCG in BALB/c mice

A single i.n-BCG immunisation (0.5 log CFU lower than the parenteral dose) resulted in significant protection in the lung compared to the naïve control group ($P<0.001$), but this was not significantly different from the p-BCG group ($P=0.065$) (Fig. 19A). Numbers of bacilli in the spleen were similar between the i.n-BCG, p-BCG and naïve control group (all comparisons: $P>0.5$) (Fig. 19B). When a second i.n-BCG immunisation was given, protection in both the lung and spleen was significantly improved when compared to the naïve, $1\times$ i.n-BCG and $1\times$ p-BCG groups (all comparisons - lung: $P<0.001$, spleen: $P<0.001$). Two doses of i.n-BCG also showed significantly greater protection in the lung than immunisation with 2× p-BCG ($P<0.001$).
MVA.85A boosting of BCG provides comparable protection to two BCG immunisations & 2.5 log protection over naives

Boosting i.n-BCG, either i.n- or p- with MVA.85A produced significantly higher levels of protection compared with the 1× i.n-BCG group against challenge (Fig. 19). Protection in the i.n-MVA.85A boosted group was striking, showing the same levels of protection as the 2×i.n-BCG group (lung: 2.5 logs and spleen: 1.5 logs lower than the naïve group). When compared to the i.n-BCG group, the mice boosted parenterally with MVA.85A did not show increased protection in the lung ($P=0.494$) (Fig. 19A). However, the level of protection in the spleen was significantly more than the i.n-BCG group ($P<0.05$) and comparable to the 2×i.n-BCG and i.n-BCG / i.n-MVA.85A groups (Fig. 19B).

Protection in the lung correlates with T cell responses in the LLN

On the day of challenge (4 weeks post 2nd immunisation), four mice from each group were sacrificed for immunogenicity studies. Only mice in the 2×i.n-BCG and i.n-BCG / i.n-MVA.85A groups produced detectable T cell responses to the P15 Ag85A CD4+ T cell epitope in their LLN at the time of challenge (Fig. 20A). These groups showed the highest levels of protection in both the lungs and spleen following challenge, which indicates that Ag85A specific T cell responses in the LLN correlate better with protection in the lung than splenic responses. No T cell response was detected in the LLN to the 85A CD8+ P11 peptide in any group, most likely because the BCG dose used in the challenge experiment was too low to induce a specific CD8+ T cell response.
**Figure 20:** Protection in the lung against aerosol challenge with *M. tuberculosis* correlates with the presence of 85A specific-IFN-γ secreting CD4+ T cells in the lung lymph nodes (LLN). LLN from mice were pooled and examined for responses to PPD and the 85A peptides P15 and P11 by ELISPOT(A). Splenocytes were examined using ELISPOT for responses to PPD (B), 85A protein (C), P15 (D), P11(E). Specific lysis of P11 pulsed P815 target cells are examined in (F). Results are expressed as the mean SFU/10^6 cells. * indicates significant differences (*P* value < 0.05) by Student’s *t* tests in the induction of T cell responses from BCG or MVA.85A only vaccine regimens.
Some differences were observed between groups in the splenic assays. Whilst all groups produced comparable responses in IFN-γ ELISPOT to PPD and 85A proteins (Fig. 20B & C respectively), the Ag85A CD4<sup>+</sup> T cell response to P15 in the i.n-BCG / i.n-MVA.85A and i.n-BCG / p-MVA.85A groups were increased two- and three-fold respectively over the i.xi.n-BCG group (Fig. 20D). Responses to the P11 CD8<sup>+</sup> T cell epitope were detectable at low levels in the i.n-BCG / p-MVA.85A group in ELISPOT (Fig. 20E). In chromium release assays performed on cultured splenocytes, only this group produced significantly higher levels of specific lysis (30%) of P11-pulsed targets compared with control cells (Fig. 20F). Again, because of the lower BCG dose used in the challenge experiment, the detection of 85A-specific CD8<sup>+</sup> T cells in the spleen was most likely induced by MVA.85A vaccination alone.

4.4 Discussion

Recombinant MVA effectively induces both CD4<sup>+</sup> and CD8<sup>+</sup> IFN-γ secreting T cells to foreign antigens, including Ag85A and is particularly effective as a boosting vector (136, 267, 279). MVA.85A immunisation boosted Ag85A specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell pools induced by BCG, demonstrating that a recombinant virus can also boost T cell responses induced by a bacterial prime.

The protective efficacy of the BCG - MVA.85A prime-boost regimen was tested in aerosol *M. tuberculosis* challenge. This regimen was compared to single and double
vaccinations with BCG given either parenterally or intranasally. In BALB/c mice, irrespective of route of delivery, two BCG immunisations were more protective than one in the lungs and spleens. In humans, repeated intradermal BCG immunisation did not provide any greater protection against pulmonary tuberculosis irrespective of age at time of boost or time between BCG immunisations in Malawi (189). However, in the region examined, a single BCG immunisation conferred no protection against pulmonary tuberculosis. Multiple BCG doses or indeed boosting with MVA.85A may still provide some benefit in a population where a single BCG immunisation is partially protective. Repeated BCG immunisation has been found to increase protection from leprosy by about 50% (191). The antigen 85A protein derived from *Mycobacterium leprae*-Ag85A shares 90% amino acid identity with *M. tuberculosis*-Ag85A, is protective in mice models, and can induce specific T cell responses in infected humans (280, 281). In addition, *Mycobacterium leprae* (*M. leprae*) specific cytolytic CD8+ T cells have been identified in leprosy patients (282, 283). Future clinical trials could also assess whether a BCG-MVA.85A regimen could boost *M. leprae* specific- CD4+ and CD8+ T cell populations and what impact this could have on protection from leprosy.

The protective effect of eliciting lung-localised T cell populations through mucosal delivery of tuberculosis vaccines was also examined. Intranasal BCG was as effective as p-BCG in inducing peripheral T cell responses but induction of responses in lymph nodes was highly dependent on the route of immunisation. Intranasal delivery of BCG elicited robust responses in the LLN that could be further
boosted by i.n- immunisation with MVA.85A (284). A single dose of i.n-BCG gave the same level of protection as p-BCG following a *M. tuberculosis* challenge. In mice that were immunised intranasally with BCG and again boosted intranasally with either a second BCG dose or MVA.85A, the levels of protection in the lungs and spleens were both significantly higher than a single BCG immunisation. This level of protection (2.5 logs) has not previously been reported in murine vaccination studies.

At the time of challenge (4 weeks after the last vaccination), only the i.n-prime / i.n.-boost vaccination regimens produced detectable responses in LLN and the highest levels of protection. The frequency of specific T responses in the spleen did not correlate with the strong protection observed in these groups. These data suggest that the frequency of antigen specific IFN-γ secreting CD4⁺ T cells in the LLN may constitute a better indicator of protection in the lung than systemic responses against pulmonary tuberculosis. Two explanations exist for this observation. Firstly, in mice that have been infected aerogenically with *M. tuberculosis*, bacilli disseminate first to the LLN, possibly via dendritic cells, and then to periphery (285, 286). The induction of *M. tuberculosis* specific T cells follows dissemination of bacilli. The responses are induced first in the LLN (but not other LN compartments) and later in the spleen (285). The strong protection observed in both the lungs and spleens in the i.n-prime / i.n-boosted groups may have resulted from the microcidal actions of resident *M. tuberculosis* specific T cells in the LLN limiting dissemination of bacilli back to the lungs and periphery. Whether *M. tuberculosis* dissemination from the
lung parenchyma to the lung lymph nodes also occurs in humans is unknown but in both symptomatic and asymptomatic individuals pathology is often found in the hilar lymph nodes (6). Alternatively, antigen specific T cells in LLN may reflect lymphatic drainage of protective T cell populations resident in the lung (271, 284, 287). Several murine studies have shown a correlation between an influx of IFN-γ secreting CD4+ and CD8+ T cells into the lung during acute infection and protection from tuberculosis (253, 288-290). In humans, Condos et al show that the bronchoalveolar lavage (BAL) of patients with less clinically advanced pulmonary tuberculosis contained higher levels of IFN-γ secreting lymphocytes than patients with advanced tuberculosis who conversely had increased numbers of neutrophils in their BAL (291).

The acute *M. tuberculosis* challenge model used here did not in itself distinguish between the utility of BCG and MVA.85A as boosting agents after BCG priming. Previous studies have shown that BCG is still recoverable from the lung, albeit in very low numbers one month after lung immunisation (86, 292). Histological analysis showed an ongoing inflammatory response in the lungs and numbers of macrophages, neutrophils, NK cells and anti-microbial mediators such as IL-12 and TNF-α in lung lavage were still elevated (292, 293). The level of protection observed following challenge of 2x i.n-BCG immunised mice, could in part be due to ongoing innate immune reactions induced by the residual BCG bacilli in the lungs. In contrast, it is unlikely that innate immune mechanisms contributed to the protection observed in the i.n-BCG / i.n-MVA.85A group. Unlike BCG, the MVA
virus does not persist in rodent lungs (294). In cotton rats that are highly susceptible to vaccinia virus infection, MVA could not be detected in lungs five days after intranasal immunisation and no pathology was observed. Similarly, intranasal immunisation of BALB/c mice with $5 \times 10^7$ PFU (1.5 logs greater than the vaccination dose in this study) of non-recombinant MVA did not provide any protection from challenge with the lung pathogens, respiratory syncytial virus (RSV) or parainfluenza virus (PIV) (295, 296). In those studies, groups of mice vaccinated with recombinant MVA expressing RSV or PIV proteins were protected following intranasal challenge a month after vaccination.

Whilst the i.n-prime / i.n-boost vaccination regimen was highly protective in this study, was intranasal delivery of the vaccines necessary for both the prime and the boost? In this study, a single BCG vaccination, whether parenteral or intranasal, afforded equivalent protection in lungs and spleens. A similar observation was recently reported by Palendira et al (182). These results conflict with earlier studies in other animal models in which showed aerosolised BCG afforded better protection than parenterally delivered BCG (256, 297). In these early studies, the time between vaccination and challenge was relatively short, and as described above any ongoing inflammatory reaction in the lung may have contributed to increased levels of protection. In this study and that of Palendira et al, BCG was eliminated from the lung by either resting the animals after BCG vaccination (22 weeks) or by antibiotic treatment. Palendira et al also showed that irrespective of the route of BCG vaccination, the recruitment of IFN-γ secreting CD4+ T cells to the lung following
aerogenic *M. tuberculosis* challenge was similar. This suggests, in this model at least, that following a single immunisation of BCG, protection is independent of route of vaccination. Previous studies in viral systems have similarly suggested that following a single intranasal immunisation, T cell responses first develop in the LLN, then extravasate stochastically from the blood to organs like spleens and lungs (255, 273, 287). When the animals were challenged intranasally, the presence of antigen drew T cells back to the lung (182, 255, 287, 298). Applying these observations to the prime-boost regimen in this study, an intranasal MVA.85A boost of BCG-immunised mice, will express Ag85A in the lung and not only expand Ag85A specific T cells resident in the respiratory compartment but also pull in memory T cells from the periphery. This is supported by the observation that intranasal boosting with MVA.85A of parenterally BCG-immunised mice also significantly increased PPD and Ag85A CD4+ T cell responses in the LLN (see also (255)). This view is not in conflict with the wealth of vaccinology publications showing that mucosal vaccination affords longer lasting and greater protection than parenteral vaccination from mucosal pathogens (reviewed in (299)). In the majority of these studies, vaccination regimens have involved repeated mucosal vaccination, each time not only boosting but also targeting memory T cells to the mucosal tissue.

These data suggest that in mice the induction of T cell responses in the respiratory compartment is important in protection from pulmonary disease. Even so, strong peripheral T cell responses remain central to protection in the spleen. Following *M. tuberculosis* challenge, i.n-MVA.85A boosting gave greater protection in both the
lung and spleen than i.n-BCG immunisation alone. Parenteral MVA.85A boosting of mice immunised with BCG intranasally improved the level of protection in the spleen but not the lung. No Ag85A specific immune responses were detected in the i.n-BCG / p-MVA.85A group in the LLN. However, splenic immune responses in this group were significantly higher than other groups. Parenteral immunisation of MVA has been previously reported to elicit strong lytic T cells responses in the periphery but not the mucosa (255). Together this suggests that parenteral boosting of BCG-immunised mice with MVA.85A expanded peripheral T cell populations that, following challenge controlled better the levels of *M. tuberculosis* bacilli that had disseminated from the LLN. In humans, parental BCG vaccination is protective against systemic forms of disease and parenteral BCG vaccination in rhesus macaques appears to protect from haematogenous spread of bacilli (12, 207). In summary, whilst lung lymph node T cell responses may correlate with protection in the lung, specific T cell responses in the spleen may constitute a good indicator of protection from systemic forms of disease.

In this chapter, BCG-mediated protection against an aerosol *M. tuberculosis* challenge was significantly increased by MVA.85A boosting of BCG immunised mice and/or intranasal vaccine delivery. The induction of specific IFN-γ secreting cells in the LLN appears to be predictive of protection in the lung, although in the present study the contribution of innate immune responses to the protection observed in the 2×i.n-BCG group cannot be excluded. The potential of lung delivery of
vaccines to increased protection from pulmonary tuberculosis is further investigated in the rhesus macaque model in the following chapters.

In the clinic, irrespective of route of delivery, MVA.85A boosting may be of more benefit than multiple BCG vaccination for several reasons. Increasing evidence suggests that exposure to environmental mycobacteria have a negative effect on BCG-induced protection (193). Unlike BCG and as outlined above, the strong immunogenicity and protective capacity of MVA.85A occurs without any viral replication and therefore is unlikely to be compromised by exposure to environmental mycobacteria (222). In addition, MVA has been given without adverse effects to severely immunocompromised primates suggesting it would carry less risk in HIV positive individuals than subsequent vaccinations with live BCG (300). Increasing evidence suggests that CD8\(^+\) T cells are also necessary for protection from disease, particularly against reactivation of latent tuberculosis infection (137). MVA.85A expanded low-level BCG induced Ag85A specific CD8\(^+\) T cell response as well as CD4\(^+\) T cell responses. However, CD8 T cell boosting was only observed following high dose BCG vaccination and would not have contributed strongly to the protection observed in BCG / MVA.85A vaccinated groups following challenge. The ability to boost CD8\(^+\) T cell responses with recombinant viruses should provide an advantage, particularly for long-term protection, over multiple BCG immunisations and possibly over other subunit vaccination regimens that induce T cell responses that are predominately CD4\(^+\) dependent. In this Chapter and
Chapter 2, DNA plasmids and poxvirus vectors (FP and MVA) were shown to effectively combine to induce high levels of specific CD8+ T cell responses. As such, studies in monkeys sought to further augment 85A specific CD8+ T cell responses by including fowlpox expressing Ag85A vaccination to BCG MVA.85A vaccination regimens (Chapters 6 & 7).
5 Intranasal vaccination with, and/or MVA.85A boosting of BCG does not improve BCG-mediated protection of C57BL/6 mice

5.1 Background to Chapter:

Host genetic heterogeneity in humans and animal models directly impacts on the type and strength of the immune response to intracellular pathogens (301). The promising levels of protection achieved with BCG / MVA.85A vaccination of BALB/c mice warranted repeat of these experiments in a second mouse strain, C57BL/6 mice. Several 85A specific- CD4\(^+\) but no CD8\(^+\) restricted T cell epitopes have been identified following vaccination with BCG, MVA.85A or D.85A in C57Bl/6 mice (302, 303).

5.2 Results

Intranasal vaccination with, and/or MVA.85A boosting of BCG does not improve BCG-mediated protection of C57BL/6 mice

C57BL/6 mice were vaccinated parenterally in the footpad (p-) or i.n- with 10\(^5\) CFU of BCG. 36 weeks later, mice were given a second vaccination of BCG or MVA.85A (10\(^6\) PFU) given i.n- or p-. Four weeks after the last vaccination, mice were
challenged with *M. tuberculosis* given as an aerosol. Bacterial burdens were determined from lungs and spleens harvested 6 weeks after challenge.

All vaccinated groups were significantly protected in both the lung (average -0.7 log) and spleen (average 1.1 log) compared to naïve controls (lung - $P < 0.001$, spleen - $P < 0.001$) (Table 5.1). Boosting of BCG immunised mice with either a second dose of BCG or MVA.85A did not significantly increase the level of protection in either organ.

**Table 5.1: Bacillary load (log_{10} (CFU)) in organs 6 weeks after aerosol *M. tuberculosis* challenge of C57BL/6 mice**

<table>
<thead>
<tr>
<th>Group$^a$</th>
<th>Lung</th>
<th>SEM</th>
<th>Spleen</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAIVES</td>
<td>5.86$^{b,c}$</td>
<td>0.07$^d$</td>
<td>4.44$^{b,c}$</td>
<td>0.15</td>
</tr>
<tr>
<td>p$^e$-BCG</td>
<td>5.03</td>
<td>0.06</td>
<td>3.20</td>
<td>0.16</td>
</tr>
<tr>
<td>p-BCG/ p-BCG</td>
<td>5.22</td>
<td>0.11</td>
<td>3.46</td>
<td>0.17</td>
</tr>
<tr>
<td>p-BCG/ p-MVA.85A</td>
<td>5.10</td>
<td>0.10</td>
<td>3.21</td>
<td>0.17</td>
</tr>
<tr>
<td>i.n-BCG$^f$</td>
<td>5.14</td>
<td>0.12</td>
<td>3.51</td>
<td>0.16</td>
</tr>
<tr>
<td>i.n-BCG/i.n-BCG</td>
<td>5.15</td>
<td>0.09</td>
<td>3.38</td>
<td>0.16</td>
</tr>
<tr>
<td>i.n-BCG/ i.n-MVA.85A</td>
<td>5.22</td>
<td>0.04</td>
<td>3.39</td>
<td>0.16</td>
</tr>
<tr>
<td>i.n-BCG/ MVA.85A</td>
<td>5.14</td>
<td>0.05</td>
<td>3.37</td>
<td>0.14</td>
</tr>
</tbody>
</table>

$^a$ in each group n=10

$^b$ value is geometric mean of the log_{10}(CFU) in the lung

$^c$ comparison of naives to all other groups by Student's $t$ test produced $P < 0.01$

$^d$ +/- SEM

$^e$ p.= footpad immunisation of $5 \times 10^7$ CFU BCG or $10^6$ PFU MVA.85A

$^f$ i.n = intranasal immunisation of $10^7$ CFU BCG or $10^6$ PFU MVA.85A
Figure 21: p-MVA.85A vaccination of p-BCG immunised C57BL/6 mice induces significantly higher 85A specific- IFN-γ secreting splenic T cells. At the time of challenge (4 weeks post second immunisation), spleens (n = 4) from vaccinated mice were assayed for PPD (A) and 85A culture filtrate protein (B) and 85A peptide specific (C) responses in IFN-γ ELISPOT (SFC/10⁶ cells +/- SEM) (A) * indicates significant differences (P value < 0.05) by Student’s t tests in the induction of T cell responses to BCG and MVA.85A only vaccine regimens.
FIGURE 22: p-M.85A vaccination of p-BCG immunised C57BL/6 mice induces significantly higher 85A specific proliferation. At the time of challenge (4 weeks post second immunisation), spleens (n = 4) from vaccinated mice were assayed for PPD and 85A protein specific proliferation.
Immunogenicity Results in C57BL/6 mice

Four mice from each group were sacrificed at time of challenge (4 weeks following the second immunisation) for immunogenicity studies. Additional groups, PBS/p-MVA.85A, PBS/p-BCG, PBS/i.n-MVA.85A and PBS/i.n-BCG were included as controls. Splenic T cell responses to PPD, Ag85A protein, Ag85A peptide pools or individual epitopes were tested in IFN-γ ELISPOT and proliferation assays (Fig. 21 & 22 respectively).

There was no significant difference in the frequency of the PPD specific IFN-γ secreting T cells in ELISPOT between BCG vaccinated mice whether given BCG parenterally or intranasally or vaccinated once or twice (Fig. 21A). In contrast, the p-BCG/p-MVA.85A vaccinated mice showed significantly higher 85A protein and peptide specific responses than other groups (Fig. 21B & C). This group also produced significantly higher proliferation to the 85 protein than mice vaccinated with BCG and MVA.85A alone (Fig. 22). Intranasal delivery of BCG induced specific IFN-γ and proliferative responses comparable to p-BCG (Fig. 21 & 22) but unlike BALB/c mice, i.n-BCG/p-MVA.85A vaccination did not induce PPD- and 85A- specific T cell responses greater than in-BCG alone (Fig. 21A&B).

5.3 Discussion

In this chapter, vaccination regimens that produced protection greater than parenterally delivered BCG in BALB/c mice failed to increase protection in C57BL/6 mice. Failure to improve on BCG-mediated protection occurred even in a group (p-BCG/p-MVA.85A) that at the time of challenge, exhibited significantly
higher specific IFN-γ secretion and proliferative T cell responses than that induced by BCG vaccination alone. These data contrast with the results from BALB/c mice studies where increases in splenic 85A specific IFN-γ secreting CD4⁺ T cells correlated with increased in protection in the spleen.

Both C57BL/6 and BALB/c mice strains are described as resistant to *M. tuberculosis* infection (204). C57BL/6 mice live slightly longer following i.v. challenge (204). In this study, bacterial loads in the lung and spleen did not differ significantly between strains 6 weeks after challenge. Whilst C57BL/6 and BALB/c mice are similarly resistant to *M. tuberculosis* infection, differences have been reported in the immune responses of these strains following BCG vaccination and resistance to infection with other bacteria. Following intratracheal BCG vaccination, C57BL/6 mice secrete higher levels IL-12 and IFN-γ into lung lavage than BALB/c mice (292) and also clear a second BCG infection more rapidly than BALB/c mice (304). BALB/c mice are also strikingly more susceptible than C57BL/6 mice to other infectious pathogens like *Leishmania major*, *Yersinia enterocolitica* and *Chlamydia trachomatis* infections (305-307). In each case, the increased susceptibility of BALB/c compared to C57BL/6 mice has been associated with lower IL-12 and IFN-γ but higher levels of TH2-type cytokine secretion following infection (305-307). Shibuya *et al* suggested that the mechanism for strain differences may related responsiveness to IL-12 and demonstrated that optimal induction of IL-12 in BALB/c mice needed IL-1α and TNF-α as cofactors which were not required for C57BL/6 mice (308).

Therefore, despite significant increases in specific IFN-γ secreting CD4⁺ T cells by MVA.85A boosting BCG vaccinated C57BL/6 mice this may have been insufficient
to significantly improve the strong protective effect afforded by BCG from *M. tuberculosis* challenge. So far, no other vaccination regimen has afforded greater protection than BCG in this mouse strain, which may be because the αβ CD4+ T cell responses induced by BCG vaccination affords maximal protection from a *M. tuberculosis* challenge in C57BL/6 mice. Alternatively, the induction of other arms of immune response like CD8+ T cells (induced in BALB/c but not C57BL/6 mice following MVA.85A vaccination) may be necessary to increase BCG-mediated protection in this strain.

This study demonstrates that MVA.85A vaccination can significantly increase 85A specific T cell responses induced by BCG in C57BL/6 mice but also highlights the strong protective effect of BCG in this strain and the genetic differences between BALB/c and C57BL/6 mice. Such divergent protection results in inbred mouse strains both classified as resistant to *M. tuberculosis* strains, prompted investigation of BCG / MVA.85A vaccination regimens in outbred non-human primates, which are described in the subsequent chapters.
6 Prime-boost vaccination regimens in rhesus macaques

6.1 Outline

A 2 year study using the rhesus macaque (Macaca mulatta) model was conducted to directly compare aerosol and intradermal BCG delivery. Animals were also boosted with MVA.85A and different recombinant virus, Fowlpox (F) expressing 85A (FP9.85A) to determine whether the significant boosting of T cell responses observed in the murine model could be replicated in non-human primates. This study involved the development of several immune assays that are described in detail in the Materials and Methods section.
Figure 23: Vaccination schedule. Each arrow indicates the week of and type of immunisation given. The animals (1 to 6) immunised at each time point are shown in boxes above the time line. 1=Glyn; 2=Hector; 3=Golf; 4=Gulp; 5=Hal; 6=Harry. The asterisk indicates the week of autopsy of animals 1 to 4. The following abbreviations are used: i.d-BCG, intradermal BCG \((4 \times 10^5 \text{ CFU})\) given into the upper right arm; aero-BCG, aerosolised BCG \((4 \times 10^5 \text{ CFU})\) delivered by a Halolite nebulizer; MVA.85A \((5 \times 10^8 \text{ PFU})\); FP9.85A \((5 \times 10^8 \text{ PFU})\). Both poxvirus vaccinations were given intradermally into the upper left and right arms.
6.2 Results

Induction of T cell responses in peripheral blood mononuclear cells

BCG delivered intradermally or by aerosol elicits similar kinetics and T cell responses in the blood compartment.

Antigen specific T cell responses to PPD, 85 culture filtrate protein and 85A peptides were compared fortnightly in the first 12 weeks following either intradermal or aerosol vaccination of $4 \times 10^5$ CFU BCG in 4 macaques. The schedule of vaccination is shown in Fig. 23. Prior to vaccination, animals produced no T cell response to either PPD or 85 protein in ELISPOT but a high frequency of IFN-γ secreting cells were observed in all animals following non-specific mitogenic stimulation with concanavalin A (conA) ($>1000$ SFC/10$^6$ cells) (Sigma-Aldrich, Dorset, England) (data not shown). Responses to PPD and Ag85A were first detected 3 weeks after vaccination in both BCG intradermally (Fig. 24A&B) and aerosol immunised animals (Fig. 24C&D). The frequency of response increased until weeks 8-12 reaching comparable levels in all 4 animals regardless of route of vaccination. No PPD- or 85A protein- specific T cell responses were detected over the same period in the non-vaccinated macaques, Hal and Harry (data not shown). In summary, BCG vaccination either intradermally or via aerosol induced a high frequency of PPD- and 85 protein- specific T cell responses. Similar to the observations made in BALB/c
and C57Bl/6 mice, Ag85A was immunodominant in rhesus macaques following BCG vaccination.

**ELISPOT performed on short-term cell lines**

Cultured ELISPOT performed on short-term cell lines (STCLs) were optimised by week 10 of the study. Cells were stimulated with BCG-infected autologous macrophages and cultured for 14 days to expand any low frequency 85A-specific T cell pools. Subsequent ELISPOTS on these cell lines detected strong PPD- and 85A protein-specific responses in Glyn, Golf, Gulp and Hector at week 12. Golf also produced a response to the P25-33 (Table 6.1). STCLs specific for the Ag85A CFP were detected in Harry. No antigen specific STCLs were derived from Hal, although strong IFN-γ secretion to mitogenic stimulation with Con A was observed.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Glyn</th>
<th>Hector</th>
<th>Golf</th>
<th>Gulp</th>
<th>Hal</th>
<th>Harry</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>85 protein 1-20</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31 con A</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

*STCL generated by stimulation with BCG-infected autologous macrophages. Cells were fed every 3 days with 10 units / well of lymphocult-T for 2 weeks. STCLs were stimulated for 18-20 hours with the antigen indicated in the presence of equal numbers of γ-irradiated autologous fresh PBMCs. + designates a positive ELISPOT response produced by stimulation of STCLs. A response was designated positive for the response was > 50 SFC / 10⁵ cells greater that control wells which were 1) non-stimulated STCLs 2) γ-irradiated autologous fresh PBMCs plus antigen.
MVA.85A vaccination of rhesus macaques with or without previous BCG vaccination

Between week 12-16, all macaques were immunised with $5 \times 10^8$ PFU of MVA.85A (Fig. 24A-D). Bloods were taken 2 weeks after vaccination and fortnightly thereafter. The dose of vaccine given was deduced from previous studies and was predicted to both induce T cells specific to the recombinant antigen and be well tolerated (225, 309).

*Ex-vivo* ELISPOT

The frequency of BCG induced T cells responses between weeks 12-16 were strong but fluctuating from bleed to bleed in all 4 animals (Fig. 24A-D). There is no clear data that MVA.85A boosted the frequency of PPD or Ag85 specific IFN-γ secreting T cells in BCG immunised animals (Fig. 24A-D). No IFN-γ secretion to either PPD or the 85 protein was detected two weeks after vaccination in the two animals that received MVA.85A alone (data not shown). A second MVA.85A immunisation was given 5-9 weeks later (Fig. 24A-D). Again, there was no evidence that T cell responses were boosted following the second vaccination.
Figure 24: Induction of T cell responses following i.d- or aero-BCG vaccination and subsequent MVA.85A boosting. Animals were immunised intradermally (A & B) or by aerosol administration (C & D) with $4 \times 10^6$ CFU of BCG (Danish strain 1331). All 4 macaques were given two doses of MVA.85A i.d- at the time-points indicated. The frequency of IFN-γ secreting T cells specific for PPD and 85A culture filtrate protein in the blood, are shown. The x-axis shows the week of the study where the week of BCG vaccination is week 0. Responses are the average of duplicate wells minus background and are expressed as spot forming cells/10⁶ cells (SFC/10⁶ cells).

Figure 25: FP9.85A vaccination significantly boosts pre-existing 85A specific T cell responses. Each macaque was immunised with two doses of FP9.85A given intradermally at the weeks 42 and 47, as indicated by the arrows below the x-axis. The x-axis itself shows the week of the study The left panel (A, C, E, G, I & K) shows the frequency of IFN-γ secreting T cells specific for PPD and 85A culture filtrate protein and r85B protein in the blood. The right panel (B, D, F, H & J) shows the frequency of IFN-γ secreting T cells specific for 85A peptides split into pools of peptides 1-8, 9-16, 17-24 and 25-33. Responses are the average of duplicate wells minus background and are expressed as spot forming cells/10⁶ cells (SFC/10⁶ cells).
Animals produce responses to 85A peptides

Pools of 20mer peptides overlapping by 10 amino acids spanning Ag85A and recombinant 85B (r85B) were included in assays from week 33 (Fig. 25).

Responses in BCG-MVA.85A vaccinated animals:

Whilst all BCG vaccinated animals produced responses to 85A protein, strong r85B responses were also detected in Glyn (80 SFC/10⁶ PBMCs) and Golf (145 SFC/10⁶ PBMCs) (Fig. 25A&E). Low level 85A peptide specific responses were also detected in Golf to P1-8 (12.5 SFC/10⁶ PBMCs), P9-15 (15 SFC/10⁶ PBMCs) and P25-33 (25 SFC/10⁶ PBMCs), all of which produced responses over 100 SFC/10⁶ PBMCs in subsequent bleeds (Fig. 25F).

For the first time, low frequency responses were detected to 85A culture-filtrate protein (30 SFC/10⁶ PBMCs) in Harry’s blood (Fig. 25J). Stronger responses to r85B protein (65 SFC/10⁶ PBMCs) and 85A P9-15 (85 SFC/10⁶ PBMCs) were also detected showing that MVA.85A vaccination alone did induce T cell responses (Fig. 25J). It is unclear why responses to MVA.85A were not detected earlier in this animal but the response identified at week 33 was maintained at week 42 showing that these responses were real and specific and induced by vaccination. The 85A peptide pool responses in Harry were CD8⁺ T cell restricted which may explain the prior failure to detect a response following stimulation of cells with 85A protein.
alone (see Table 6.2). No responses to PPD, 85A protein, r85B (Fig. 25A) or the 85A peptides (data not shown) were detected in Hal at week 42.

**ELISPOT performed on short-term cell lines after MVA.85A boosting**

Cultured ELISPOTs were performed between weeks 14 and 27. All BCG vaccinated animals produced PPD- and 85A protein- specific STCLs (Table 6.1). A STCL specific for 85A protein was also derived from Harry, which reinforced the observations in ex-vivo assays showing that MVA.85A vaccination of Harry had induced a de novo T cell response.

In addition, Glyn, Hector and Golf produced peptide specific responses at multiple time-points to P31. Hector also produced a response to P23 (Table 6.1).

**FP9.85A vaccination of MVA.85A immunised macaques with or without BCG vaccination induce both CD8⁺ and CD4⁺ T cell responses**

All macaques were immunised with $5 \times 10^8$ PFU of FP9.85A at weeks 42 and 47. Bloods were taken on the day of vaccination, 7 days post vaccination and 21 days post vaccination. The FP9.85A had been previously tested in BALB/c mice and like MVA.85A induced specific CD8⁺ and CD4⁺ T cell responses to the Ag85A peptides P11 and P15 respectively (data not shown).

Glyn (i.d-BCG / MVA.85A / FP9.85A)

Glyn who had a strong BCG-induced immune response prior to FP9.85A vaccination, did not exhibit any appreciable increase in protein response clearly attributable to poxvirus vaccination (Fig. 25A). The response to P25-33 increased slightly as did the response peptide 31 (P31) found within this pool (Fig. 25B).
Hector (i.d-BCG / MVA.85A / FP9.85A)

Hector did not exhibit any increase in protein-specific response to FP9.85A vaccination (Fig. 25C). At week 42, a low-level response to P25-33 (22.5 SFC/10^6 PBMCs) was detected. At week 43, 7 days after the first FP9.85A vaccination, the P25-33 responses doubled and a response to P16-24 (37.5 SFC/10^6 PBMCs) was also detected. The peptide pool responses dropped to background levels by 21 days then rose again (~5-fold increase), 7 days after the second vaccination. These two peptide pools contained P31 and P23 that induced a positive response in cultured ELISpot after MVA.85A vaccination. Responses to individual peptides were also examined. No response to P31 was detectable at week 42 but FP9.85A vaccination induced a response of 150 SFC/10^6 PBMCs at week 43, dropping to less than 50 SFC/10^6 PBMCs at week 45 and then doubling in response to the second FP9.85A vaccination (data not shown) (Fig. 25D). Low level responses to P23 (25 SFC/10^6 cells) were also detected 7 days after each FP9.85A vaccination that then dropped back to background levels.

Golf (aero-BCG / MVA.85A / FP9.85A)

The effects of FP9.85A vaccination were much stronger in the remaining animals. Golf showed a significantly increased in response to PPD, 85A protein following the first vaccination (Fig. 25E). The second vaccination did not appear to boost protein responses. Responses to P1-8 and P25-33 were boosted significantly at week 43. Pool 1-8 responses increased over ten-fold and responses to P25-33 from 160 SFC/10^6 PBMCs to greater than 400 SFC/10^6 PBMCs (Fig. 25E). The response to P31 doubled but did not account for level of boosting observed in P25-33, which was
later attributed to P29 (Table 6.2). The second FP9.85A vaccination did not boost but rather maintained the level of protein or peptide responses at week 48.

Gulp (aero-BCG / MVA.85A / FP9.85A)

Gulp’s protein specific T cell responses did not appreciably increase after FP9.85A vaccination (Fig. 25G). The T cell response to P1-8 increased ten-fold to ~500 SFC/10^6 PBMCs and to P16-24 increased from undetectable levels to over 800 SFC/10^6 PBMCs (Fig. 25H). Here the second FP9.85A vaccination had an effect, boosting the peptide pool response to P16-24 (from 100 to ~400 SFC/10^6 PBMCs), though not to the same levels as the first vaccination.

Harry (MVA.85A / FP9.85A)

After the first FP9.85A vaccination, responses to both PPD and 85A protein increased from less than 50 SFC/10^6 PBMCs to several hundred in Harry (Fig. 25I). These responses were maintained for 5 weeks. Responses to peptide pools 9-15 and 16-24 were impressive. Peptide pool 9-15 T cell responses were less than 100 SFC/10^6 PBMCs at week 42 (Fig. 25J). Responses were boosted to 1200 SFC/10^6 PBMCs after the first FP9.85A vaccination. Responses to P16-24 were boosted from undetectable levels to over 500 SFC/10^6 PBMCs at week 43. These responses decreased by week 47 (although were higher than pre-FP9.85A vaccination levels) and were again boosted by the second vaccination. As observed in Gulp, the level of boost to the second homologous FP9.85A vaccination was not as great as the first.

Hal (MVA.85A / FP9.85A)

MVA.85A vaccination did not induce detectable T cell responses in Hal in ex-vivo or cultured ELISPOT assays despite production of IFN-γ to non-specific mitogenic
stimulation at all time points. Following FP9.85A vaccination responses to PPD, 85A protein and r85B were clearly induced (Fig. 25K). Interestingly, in contrast to the other animals for which the peak of response to FP9.85A vaccination was day 7, for Hal the response peaked later, at 3 weeks. These responses dropped back down to background levels at week 47 and were not boosted by the second FP9.85A vaccination. No responses were produced to the 85A-peptide pools following FP9.85A vaccination (data not shown).

Characterisation of T cell responses

PPD and 85A protein T cell responses were CD4+ dependent

Depletions were performed using human dynabeads. Flow cytometry showed that the dynabeads were less efficient in depleting CD4+ T cells than CD8+ T cells in rhesus macaque PBMCs. This was reflected in the quality of the results in the ELISPOT assays performed on the depleted cells (Table 6.2 and 6.3). Even so, the ELISPOT results following CD4+ and CD8+ T cell depletions showed responses to PPD and 85A protein were predominately CD4+ dependent (Table 6.2).
85A peptide specific responses

Responses to peptide pools for each individual animal were dependent with the exception of P2 (NH2-SGTHSWE Y W G A Q L N A M KPDL-COOH) detected by cultured ELISPOT and then confirmed in ex vivo assays. A positive response was clearly detectable in one animal in ex vivo ELISPOT assays.

Hector - 342, Goh - 21A S607 (10^7 cells). The HLA sequences of these animals have been used and described in detail in previous studies. The HLA specific T cell responses in BCG-MVA 85A and MVA 85A vaccinated macaques are summarized. P2 85A vaccination with P2 85A elicited a strong CDP and CD8+ 85A specific T cell response in BCG-MVA 85A and MVA 85A vaccinated macaques. Overall, the second MVA85A vaccination which was capable of boosting T cell responses was not as effective as the first (311).

Figure 26: Peptide 31 from the 85A aa sequence contains a putative MAMU-DR supermotif to which 3 of the macaques responded following BCG vaccination 41. The boxed residues fit the predicted anchor residues of MAMU-DR supermotif.

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Further anchor residues that could modulate allelic specificity
85A peptide specific responses

Responses to peptide pools (data not shown) and individual peptides were CD8' dependent with the exception of P31 (Table 6.3). Peptide 31 was recognised by CD4' T cells in 3 of the 4 BCG immunised animals (Glyn, Hector and Golf), first detected by cultured ELISPOT and then confirmed in ex-vivo assays. At the time of necropsy (14 weeks after the last FP9.85A vaccination) a P31-specific T cell response was clearly detectable in these animals in ex-vivo ELISPOT (Glyn - 57, Hector - 342, Golf -214 SFC/ 10^6 cells). The p31 sequence fits a predicted MAMU DR supermotif for macaques (Fig. 26) (310). It is unclear why the Ag85A peptides did not detect more CD4' T cell responses, despite strong CD4' T cells response to 85A protein in all macaques. In other studies performed in this group these same peptides have been used and detected CD4' T cell responses in BALB/c (Chapter 3) and C57Bl/6 mice (Chapter 4), cattle (E. Taracha, personal communication) and humans (311). Together these observations suggest that there may be some impairment in the presentation or recognition of CD4' peptide epitopes by macaque antigen presenting cells or specific CD4' T cells respectively. Further studies are needed to investigate these observations.

In summary, FP9.85A vaccination significantly boosted CD4' and CD8' 85A specific T cell responses in BCG-MVA.85A and MVA.85A immunised macaques. Overall, the second FP9.85A vaccination whilst capable of boosting T cell responses was not as effective as the first (312).
Vaccination with MVA.85A / FP9.85A induces responses to r85B protein

Ag85A shares significant homology at the amino acid level with 85B (80%) also expressed in the 85 complex. Not surprisingly, vaccination with recombinant virus expressing 85A induced strong responses to stimulation with r85B, which generally followed the kinetics of responses induced following 85A protein stimulation and were predominately CD4+ dependent (Fig. 25J & 25J, Table 6.2).
Table 6.2: PPD, 85A and r85B protein responses are predominately CD4⁺ dependent

<table>
<thead>
<tr>
<th>Animals</th>
<th>Week</th>
<th>CD4 depletion %</th>
<th>CD8 depletion</th>
<th>CD4 depletion %</th>
<th>CD8 depletion</th>
<th>CD4 depletion %</th>
<th>CD8 depletion</th>
</tr>
</thead>
<tbody>
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<td>Glyn</td>
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<td>72</td>
<td>0</td>
<td>74</td>
<td>25</td>
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<td>50</td>
<td>85</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hector</td>
<td>27</td>
<td>93</td>
<td>0</td>
<td>71</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>84</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Golf</td>
<td>27</td>
<td>100</td>
<td>61</td>
<td>94</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>96</td>
<td>8</td>
<td>72</td>
<td>6</td>
<td>82</td>
<td>9</td>
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<tr>
<td>Gulp</td>
<td>27</td>
<td>82</td>
<td>40</td>
<td></td>
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<td>100</td>
<td>0</td>
<td>70</td>
<td>0</td>
<td>74</td>
<td>14</td>
</tr>
<tr>
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<td>88</td>
<td>0</td>
<td>75</td>
<td>15</td>
<td>88</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>95</td>
<td>6</td>
<td>88</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hal</td>
<td>82</td>
<td>78</td>
<td>2</td>
<td>84</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Week bead depletions were performed

b % depletion = 100-((number of IFN-γ specific responses in ELISPOT in depleted cells / number of IFN-γ specific responses in ELISPOT in non-depleted cells) × 100)
Table 6.3: 85A peptide specific responses are predominately CD8⁺ dependent

<table>
<thead>
<tr>
<th>Animals</th>
<th>P 1-8</th>
<th>P 9-15</th>
<th>P 16-24</th>
<th>P 25-33</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>peptide</td>
<td>% CD4 depletion</td>
<td>% CD8 depletion</td>
<td>peptide</td>
</tr>
<tr>
<td>Glyn</td>
<td>P31*</td>
<td>90</td>
<td>0</td>
<td>P31*</td>
</tr>
<tr>
<td>Hector</td>
<td>P23*</td>
<td>40</td>
<td>100</td>
<td>P23*</td>
</tr>
<tr>
<td>Golf</td>
<td>P2</td>
<td>2</td>
<td>92</td>
<td>P29</td>
</tr>
<tr>
<td></td>
<td>P28</td>
<td>6</td>
<td>88</td>
<td>P29</td>
</tr>
<tr>
<td></td>
<td>P31*</td>
<td>78</td>
<td>5</td>
<td>P31*</td>
</tr>
<tr>
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<td>100</td>
<td>P19</td>
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<td></td>
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</tr>
<tr>
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<td>100</td>
<td>P22</td>
</tr>
<tr>
<td></td>
<td>P14</td>
<td>4</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*Week bead depletions were performed

b% depletion = 100 - ((number of IFN-γ specific responses in ELISPOT in depleted cells / number of IFN-γ specific responses in ELISPOT in non-depleted cells) × 100)
Tuberculin Skin Testing

Intradermal BCG vaccination produces a stronger tuberculin skin test (TST) than aerosol BCG vaccination

Intradermal or subcutaneous BCG vaccination or infection with *M. tuberculosis* induces a strong DTH reaction to tuberculin PPD in humans. Rhesus macaques also produce a positive tuberculin skin test (TST) following *M. tuberculosis* infection or intradermal BCG vaccination. The routine TST for macaques is intradermal injection of 1250 units of *M. bovis* tuberculin into the eyelid (S Wolfensohn, Veterinary Services Oxford University, personal communication). In order to align the macaque TST with studies in humans, the animals were injected with *M. tuberculosis* tuberculin in increasing doses (10, 100 and 1000 units). The rhesus macaques were tested for TST responses at week 53 (Table 6.4). Tuberculin doses at 10 and 100 units lacked the sensitivity to detect differences within the group. In response to injection of 1000 tuberculin units, the animals intradermally vaccinated with BCG produced a positive TST of 3-4 whereas the animals immunised by aerosol produced negative response 0-1.

MVA.85A / FP9.85A vaccination alone can induce a strongly positive TST.

Harry and Hal were also given a TST after MVA.85A-FP9.85A vaccination (Table 6.4). Hal was negative but Harry produced a very positive TST of 4-5 showing
recombinant 85A viral vaccinations alone can induce a strong delayed type hypersensitivity to tuberculin.
Table 6.4: Tuberculin Skin Testing (TST) Results

<table>
<thead>
<tr>
<th>Animal</th>
<th>Vaccination Regimen</th>
<th>Tuberculin Dose (units)</th>
<th>Weeks post last vaccination</th>
<th>TST Grading</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyn</td>
<td>i.d-BCG / 2xMVA.85A / FP9.85A</td>
<td>10^1</td>
<td>6 (53)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^3</td>
<td>3-4</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hector</td>
<td>i.d-BCG / 2xMVA.85A / FP9.85A</td>
<td>10^1</td>
<td>6 (53)</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^3</td>
<td>3-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golf</td>
<td>Aero-BCG / 2xMVA.85A / FP9.85A</td>
<td>10^1</td>
<td>6 (53)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gulp</td>
<td>Aero-BCG / 2xMVA.85A / FP9.85A</td>
<td>10^1</td>
<td>6 (53)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^3</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hal</td>
<td>2xMVA.85A / 2xFP9.85A</td>
<td>10^1</td>
<td>6 (53)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^4</td>
<td>23 (70)</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2xMVA.85A / 2xFP9.85A / i.d-BCG</td>
<td>10^3</td>
<td>12 (82)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Harry</td>
<td>2xMVA.85A / 2xFP9.85A</td>
<td>10^1</td>
<td>6 (53)</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^3</td>
<td>4.5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2xMVA.85A / 2xFP9.85A / i.d-BCG</td>
<td>10^3</td>
<td>12 (82)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

---

- **TST** - 1000 units tuberculin given intradermally into right eyelid. Reactions were scored 48 hours later.
- **Vaccination regimen** detailed further in Fig. 23.
- **Number in brackets** is reference week number described in timeline in Fig. 23.
- **TST eyelid grading**:
  - 0 = no reaction (-);
  - 1 = bruise (-);
  - 2 = erythema of palpebrum, no swelling (-);
  - 3 = Erythema of palpebrum and slight swelling (-/+);
  - 4 = Swelling of palpebrum, drooped eyelid and erythema (+);
  - 5 = Swelling and/or necrosis, eyelid closed (+).
Induction of T cell responses in secondary lymphoid organs and respiratory compartment

The CD4^+/CD8^- subset of T cell responses induced by vaccination is the same in blood, spleen, lymph nodes and broncho-lavage compartments. The BCG-MVA.85A-FP9.85A vaccinated macaques were necropsied at week 67. Sections of spleen and various lymph nodes were removed for immune analysis. Cell preparations were arbitrarily considered viable if conA stimulation produced a response in ELISPOT > 1000 SFC/10^6 cells. Two observations were made from the successful assays. The character of T cell responses was maintained between the blood and secondary lymphoid tissue (Fig. 27A & B; Table 6.5). Secondly, the immunisation with BCG whether given intradermally or by aerosol followed by intradermal poxvirus boosting, elicited 85A specific T cell responses in the majority of lymph node compartments tested (Table 6.4).
Figure 27: The restriction of 85A peptide specific T cell responses observed in the blood are conserved in the spleen and lymph node sets of rhesus macaques. No specific homing of T cell responses was observed. The BCG immunised macaques were necropsied at week 67. Blood, spleens and lymph nodes (LN) sets were removed and 85A specific T cell responses were assayed by ELISpot. Comparisons of T cell responses are shown between blood and other compartments. Gulp: blood vs. spleen (A); Hector: blood vs. LNs (B); Harry: Blood vs. BAL (C). Results shown are the average of duplicate wells minus background and are expressed as spot forming cells/10^6 cells (SFC/10^6 cells). Assay results for spleens and lymph nodes were only accepted if the Con A responses was > 1000 SFC/10^6 cells.
Table 6.5: BCG vaccination delivered intradermally or as an aerosol induces specific T cell responses in lymph nodes both distal and proximal to the site of injection.

<table>
<thead>
<tr>
<th>Lymph Nodes</th>
<th>Glyn</th>
<th>Hector</th>
<th>Golf</th>
<th>Gulp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hilar</td>
<td></td>
<td></td>
<td>×</td>
<td></td>
</tr>
<tr>
<td>Axillar</td>
<td>×</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inguinal</td>
<td></td>
<td>×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iliac</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submandibular</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tonsil</td>
<td></td>
<td></td>
<td></td>
<td>×</td>
</tr>
</tbody>
</table>

\[a\] IFN-γ ELISPOT assays performed on lymph nodes harvested from macaques at week 67 of study
\[b\] The '×' indicates a positive antigen specific response to either 85A protein or peptide pools. A positive response is taken as greater than 20 SFC/10^6 cells over background. Responses in media only wells were < 5 SFC/10^6 cells for all lymph node assays.
\[c\] The shaded cell indicates assays where the frequency of cells responding to concA stimulation was > 1000 SFC/10^6 cells.

The BCG vaccinated animals were also repeatedly bronchoscoped and lung lavage containing lung lymphocytes were collected. No erythrocyte contamination was observed in the bronchoalveolar lavage fluid (BALF). Responses of BALF T cells to PPD and 85A protein and peptide antigens exhibited the same T cell restriction as observed in the blood but were 1-2 logs higher than the blood compartment (Fig. 27C; Table 6.6). BALF was also taken from Hal and Harry following MVA.85A-FP9.85A vaccination. Similarly, strong 85A-specific T cell responses were detected showing that intradermal vaccination with poxviruses alone is sufficient to induce specific and strong T cell responses in the macaque lung (Fig. 27C; Table 6.6).
Table 6.6: Comparison of IFN-γ responses in Broncho-alveolar lavage fluid (BALF) and blood

<table>
<thead>
<tr>
<th>Animal</th>
<th>Vaccination *</th>
<th>Time</th>
<th>PPD Blood</th>
<th>BALF</th>
<th>Blood</th>
<th>BALF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyn</td>
<td>i.d-BCG/2×MVA.85A/2×FP9.85A</td>
<td>6 (53)*</td>
<td>180d</td>
<td>2186</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hector</td>
<td>i.d-BCG/2×MVA.85A/2×FP9.85A</td>
<td>6 (53)</td>
<td>50</td>
<td>1697</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golf</td>
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<td>6 (53)</td>
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<td>11455</td>
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</tr>
<tr>
<td>Gulp</td>
<td>aero-BCG/2×MVA.85A/2×FP9.85A</td>
<td>6 (53)</td>
<td>67.5</td>
<td>4100</td>
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<td></td>
</tr>
<tr>
<td>Hal</td>
<td>2×MVA.85A/2×FP9.85A</td>
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<td>0</td>
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<td>20 (67)</td>
<td>175</td>
<td>2300</td>
<td>187</td>
<td>1100</td>
</tr>
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<td>6 (53)</td>
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<td>800</td>
<td>140</td>
<td>1300</td>
</tr>
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</table>

* Vaccinations received at time of lavage
* Weeks since last immunisation. Number in brackets refers to week of study outlined in Fig. 23.
* PPD or 85A protein specific responses expressed as SFC / 10^6 cells in *ex vivo* ELISPOT
BALF samples produced a similar cytokine profile regardless of vaccination regimen.

The luminex system enables the simultaneous detection of cytokines within a single sample. This assay was used to analyse BALF samples taken from the macaques to determine whether aerosol BCG vaccination had induced a different cytokine milieu in the lung. The concentration of cytokines differed between animals. This was probably a reflection of the quality of the sample. All animals produced very similar cytokine profiles even from different time-points (Fig. 28). Both type 1 and 2 cytokines, IFN-γ, IL-4 and IL-13 were detected in all animals (Fig. 28A-F) and the pro-inflammatory cytokine, IL-8 was detected in three animals, Hal, Harry and Golf (Fig. 28A,B & E).
Figure 28: Aerosol BCG vaccination does not produce a different cytokine profile in broncho-alveolar lavage supernatant from intradermally vaccinated animals, 30 weeks after vaccination. Concentrated BALF fluid was obtained at the time-points indicated and assayed for the presence of cytokines using the luminex system. Results are shown for each animal: Glyn (A), Hector (B), Golf (C), Gulp (D), Hal (E), Harry (F). Results are expressed as the average pg/ml for 10× BALF concentrate for duplicates.
pg/ml in BALF [10x]

E.

pg/ml in BALF [10x]

F.

pg/ml in BALF [10x]

D.

pg/ml in BALF [10x]

E.

pg/ml in BALF [10x]

D.

pg/ml in BALF [10x]

E.

pg/ml in BALF [10x]

D.

pg/ml in BALF [10x]
Necropsies of BCG vaccinated animals

Pathology

Glyn, Hector, Golf and Gulp were necropsied at week 67. In the mice studied in Chapter 4, high dose intranasal BCG immunisation produced severe pathology in the lung. A major question in this section of the study was whether aerosol delivery of human doses of BCG to non-human primates also induced pathology in the lung.

Thorough gross and histological examination was performed on all organs including the lungs for any evidence of vaccine induced pathology (G. Hall, CAMR; Rest Associates, Cambridge UK). No abnormalities or ZN positive bacilli were detected (detailed pathology reports comprise Appendices 3-7).

Culture of ZN -positive mycobacteria.

Lungs, spleen and secondary lymphoid tissue including the hilar lymph nodes were cultured for viable mycobacteria. One acid-fast colony was grown from the homogenised lung of Hector (i.d-BCG). Following biochemical testing it was identified as *Mycobacterium fortuitum* (*M. fortuitum*), which is a fast, growing bacterium that is found in water, soil and dust that can transiently colonise the respiratory tract in humans (313, 314).
BCG vaccination of MVA.85A/FP9.85A vaccinated macaques induces a recall response to PPD- and 85A- specific CD4⁺ T cells

Background

At week 70, Hal and Harry were both producing strong T cell responses, in response to either PPD or 85A-protein stimulation. Both animals were vaccinated intradermally with 4x10⁵ CFU BCG.

BCG vaccination induced lesions at the site of previous MVA.85A and FP9.85A vaccination.

BCG vaccination induced lesions at the site of prior poxvirus vaccination in both macaques (Table 6.7). The lesions did not occur at the site of BCG vaccination but appeared to correspond with the site of prior poxvirus vaccination. Notably, no skin reaction had been observed following vaccination with MVA.85A and FP9.85A in these animals which suggests that the lesions were induced by BCG vaccination. The lesions may have been caused by 85A-specific T cells induced by BCG vaccination responding to residual live virus or virally encoded antigen persisting at the site of poxvirus immunisation. Whilst each poxvirus immunisation was given over 5 sites, only two lesions were produced following BCG vaccination and may be explained by viral antigen persisting at only some injection sites.
Table 6.7: BCG vaccination of animals previously immunised with MVA.85A / FP9.85A induces lesions at the site of prior poxvirus immunisation

<table>
<thead>
<tr>
<th>Animal</th>
<th>weeks post BCG</th>
<th>Description of pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hal</td>
<td>2xMVA.85A/2xFP9.85A/BCG</td>
<td>1 small lesion on right upper arm</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1 small red lump (no scab) on right upper arm.</td>
</tr>
<tr>
<td>Harry</td>
<td>2xMVA.85A/2xFP9.85A/BCG</td>
<td>2 small lesions on right upper arm</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2 small lesions - both red, one with scab at the top of wound.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2 raised plaques of 5-8mm diameter. Well demarcated, crusty lesions. Axillary lymph nodes slightly enlarged.</td>
</tr>
</tbody>
</table>

* MVA.85A and FP9.85A vaccinations given in 5 sites between upper left and right arms
* BCG vaccination given in 1 site in upper right arm
* Pathology descriptions for week 1 and 3 made by an animal technician and week 4 by an Oxford University Veterinary Officer.
* at all time-points animals were described as being in good health

Ex-vivo responses post vaccination

Animals exhibited a classical memory T cell response following BCG vaccination to PPD and 85A protein stimulation.

BCG immunisation induced increased in the frequency of T cells specific for 85A and PPD protein in both Hal and Harry (Fig. 29A&B). T cell responses were detected a 7 days post BCG vaccination and appeared to peak at week 3. This was earlier than in the other BCG vaccinated animals for which responses peaked between 8-12 weeks post vaccination (inserts Fig. 29A&B). Responses in Hal and Harry declined over the next 4 weeks and then plateaued at a steady state level. The peak of response was not any greater than the peak observed in the other BCG immunised
animals. This data shows boosting of specific T cell responses by BCG but also illustrates a memory recall response in which antigen specific T cells induced by poxvirus vaccination expand faster in response to BCG immunisation than naïve cells. CD4+ and CD8+ bead depletions again showed T cell responses to the PPD and 85A protein to be CD4+ dependent (Table 6.2).
Figure 29: BCG vaccination of MVA.85A-FP9.85A vaccinated macaques induces a PPD and 85A specific CD4+ T cell recall response to but does not boost pre-existing 85A specific CD8+ T cells. The MVA.85A-FP9.85A vaccinated macaques were intradermally BCG vaccinated at week 70 as indicated by the arrows. PPD (A) and 85A (B) specific IFN-γ secretion was assayed over the following 12 weeks and are shown in bold. Responses of Glyn and Hector after intradermal BCG vaccination from the earlier part of the study are shown in dashed lines to highlight the differences in the kinetics of T cell responses between the animals. Weeks are shown on the x-axis where the week of BCG vaccination is week 0.
Figure 30: The arrows designate the week and type of vaccination. The inset shows the individual 85A peptide response assayed over the same period. Responses are the average of duplicate wells minus background and are expressed as spot forming cells/10⁶ cells (SFC / 10⁶ cells).
BCG vaccination does not boost 85A specific CD8⁺ responses

The CD8⁺ restricted peptide responses to P22 and P14 in Harry were unchanged by BCG vaccination showing clearly that BCG vaccination did not boost 85A specific-CD8⁺ T cell responses (Fig. 30).

MVA.85A / FP985A vaccination induces CD8⁺ T cell responses lasting for over a year in rhesus macaques.

At week 112 of the study, 42 weeks after BCG vaccination PPD and 85A T cell responses were still strong. CD8⁺ T cell responses to P14 (43 SFC/10⁶ PBMCs) and P22 (43 SFC/10⁶ PBMCs) induced by MVA.85A / FP9.85A vaccination were still detectable in Harry 65 weeks after the last FP9.85A vaccination (Fig. 30).

Increases in T cell responses with time in the absence of vaccination

The T cell response to FP9.85A vaccination induced low level PPD and 85A protein specific T cell responses at week 43 and 45 in Hal. These responses dropped down to undetectable levels by week 48. At week 67, Hal produced responses to PPD and 85A in the order of hundreds of SFC/10⁶ cells that were maintained between weeks 67, 70 and 71 (compare Fig. 25K and Fig. 30).
Figure 31: Analysis of frozen PBMCs shows that the MVA.85A/FP9.85A- induced T cell responses specific for 85A culture filtrate protein were detectable at weeks 50 and 71 in Hal. Antigen specific T cell responses in frozen PBMCs from different time points were compared in ELISPOT (A) and 5 day proliferation assays (B).
The mechanism of the apparent increase in Hal's 85A-specific T cell response was unclear. To investigate this observation, ELISPOTs were performed on frozen cells from bleeds taken at week 39 through to week 76 (Fig. 31). The magnitude of the response of frozen cells were similar to that observed in ex-vivo assays and like the assays on the fresh cells, showed the clear induction of a memory recall response to both 85A and PPD at weeks 74 and 76. However, in contrast to ex vivo assays where no specific T cell responses were detected at week 50 (despite strong IFN-γ production to ConA) (Fig. 25K), frozen cells from this time point produced specific 85A- and PPD- T cell responses (140 SFC and 80 SFC/10⁶ cells respectively) (Fig. 31A). This suggests that low level T cell responses were not detected in the original ex-vivo assays, rather than an increase in the frequency of 85A-specific T cells between weeks 50 and 67. This observation was supported by proliferation assays performed in parallel on the frozen cells in which a positive stimulation index was recorded in Ag85A stimulated cells (SI = 7.6) (Fig. 31B). Assay parameters such as the quality of antigen used at weeks 50 and 67 (4 months apart) may explain the differences in the frequency of T cell responses observed in the ex vivo assays.

MVA.85A and FP9.85A vaccination induces 85A-specific antibodies.

Plasma samples were screened for Ag85A specific immunoglobulin, induced following the various vaccination regimens (Fig. 32). All 6 macaques had pre-
existing 85A specific antibodies (endpoint titres were between 4-16) (Fig. 32 and data not shown). Non-vaccinated rhesus macaques have been reported to have antibodies to *M. tuberculosis* culture filtrate proteins (315). The 85A specific antibodies may have been induced by exposure of the macaques to environmental mycobacteria, most of which express the 85 complex of proteins; like the *M. fortuitum* cultured from Hector’s lung (316). No 85A specific antibodies were induced following intradermal or aerosol BCG vaccination. In 3 of the 6 macaques, MVA.85A vaccination boosted antibody titres (Fig. 32). Responses were strongest following the second vaccination in 2 animals (Fig. 32B&C). A similar observation following 2 recombinant MVA vaccinations has been made previously in monkeys (317). 85A-specific antibody levels also increased after FP9.85A vaccination, but were modest despite significant increases in specific CD4’ and CD8’ T cells observed in the peripheral blood at these time points (Fig. 32 B&C).
Figure 32: MVA.85A and FP9.85A but not BCG vaccination induce 85A specific antibody responses detectable in plasma. 85A specific antibody titres in plasma samples were assayed over the course of the study. The week of the study is shown on the x-axis and the arrows denote the time and type of vaccination received by the animal. A response was deemed positive if the average Abs405nm from duplicate wells was $> 2 \times$ mean (control wells).
6.3 Discussion

This study was conducted to examine the immune responses induced in a non-human primate model by aerosol administration of BCG and also by subsequent boosting with recombinant MVA and FP9 viruses expressing the BCG immunodominant antigen, 85A.

Deposition of BCG into the lungs as an aerosol induced comparable kinetics and frequencies of T cell responses in the blood as intradermal BCG vaccination. PPD and 85A specific IFN-γ secreting T cells were detected 3-4 weeks after vaccination. The frequency of response rose and peaked between 8-12 weeks post vaccination. T cell responses to proteins were CD4+ dependent. Similar responses (slow induction of response, gradual rise and CD4+ T cell dominated) had been observed in splenocytes when comparing intranasal (given as a 100µl bolus) and parenteral BCG vaccination in BALB/c (Chapter 3).

At the time of necropsy, Hector had a *M. fortuitum* infection providing some evidence of prior exposure to environmental mycobacteria in these animals and most likely explains the pre-BCG vaccination 85A specific antibody responses detected. Black and colleagues have hypothesized that exposure to environmental mycobacteria interferes with the induction of IFN-γ secreting BCG induced T cells (200). Many mycobacteria express the 85A protein including *M. fortuitum* (although the degree of amino acid homology is not known) (316). Brandt and colleagues
showed in mice, that certain isolates of environmental mycobacteria (but not *M. fortuitum*) could inhibit BCG replication and diminish BCG-induced IFN-γ secretion (199). In this study in macaques, prior exposure to environmental mycobacteria whilst sufficient to induce antibody responses did not induce PPD or 85A specific T cell responses detectable in IFN-γ ELISPOT. BCG vaccination of these animals induced sustained numbers IFN-γ secreting T cells. However, it is not possible to exclude that the frequency of IFN-γ secreting T cells induced by BCG vaccination may have been higher in naïve macaques.

In humans, T cell responses against *M. tuberculosis* in the respiratory compartment are important in protection from pulmonary disease (291). In this study no evidence was found of specific mucosal homing to either the regional lymph nodes or BALF produced by aerosol administration of BCG. Rather, the results from analysis of BALF and necropsy lymphoid samples suggests that intradermal vaccination in non-human primates induces high frequency T cell responses in the respiratory compartment and in secondary lymphoid compartments distal and proximal to the site of injection. The strong T cell responses induced in the BALF of Hal and Harry after intradermal MVA.85A-FP9.85A vaccination contrasts with studies in mouse models, which show high levels of compartmentalisation of immune responses following different routes of viral infection (Chapter 3 see also (275). The homing patterns of T cells are not fully characterised in man but it is possible that the T cell responses induced in macaques following vaccination are more comparable to man than the observations made in inbred mice. As such, the implication of these data is that in humans, intradermal vaccination of live bacterial vaccines or attenuated
recombinant viruses are sufficient to induce high frequencies of antigen specific cells in the lung. In addition, the characterisation T cell responses in the peripheral blood (the compartment most available for human studies), appears to be a good surrogate for profile of T cell responses in the lung compartment and secondary lymphoid tissue, including the spleen.

To my knowledge, this is the first study, albeit preliminary, to perform multi-cytokine profiling of the macaque BALF. As such the cytokine profile of a healthy lung is not known. All 6 macaques irrespective of vaccination regimens harboured similar cytokine profiles in their BALF including both TH1 (IFN-γ), TH2 cytokines (IL-4 and IL-13) and the chemokine, IL-8. To date no studies have been published describing cytokine profiles using the luminex system in humans. However, cytokine ELISAS in man and animal models have detected each of these cytokines in lung lavages or secreted by lung cells. A wide literature exists showing increased or depressed levels of these cytokines associated with susceptibility or resistance to pulmonary diseases. For example, IL-13 has anti-inflammatory effects by acting on TNF-α and IFN-γ in sarcoidosis (318). IL-8 is elevated in HIV-1 patients with (319). IFN-γ is depressed and IL-4 elevated in lungs of patients with pulmonary TB (99, 291). In the present study, cytokines were detected in the BALF in the absence of abnormal lung pathology, which suggests that the profiles observed reflect cytokines present in healthy lungs including the two macaques receiving BCG as an aerosol. Further confirmation of this observation could be provided by cytokine profiling of lung lavages of non-vaccinated healthy macaques.
One difference between aerosol and intradermal BCG administration was observed in the TST response of the macaques. Here, aerosol delivery of BCG was as immunogenic as intradermal BCG but produced a lower response to tuberculin (320). These observations may reflect different homing patterns of immune cells to the skin following the different routes of immunisation, although since TST is not predictive of immunity the relevance this may have to protection from disease is unclear (196, 321). This observation may have benefits to tuberculosis diagnostics. The TST is an important tool in the determination of recent *M. tuberculosis* exposure but displays poor specificity in people previously intradermally vaccinated with BCG. Further studies are needed, but delivery of BCG as an aerosol may help avoid issues of specificity in the use of the TST for *M. tuberculosis* contact tracing.

The hand-held aerosol device engineered for humans enabled delivery of a defined BCG dose without adverse effect to infant macaques through a paediatric mask and suggests that widespread human aerosol BCG vaccination is tenable. Aerosol delivery of vaccines provides several advantages over intradermal delivery. Aerosol vaccination is non-invasive and therefore avoids risks associated with parenteral injections like issues of safe disposal of syringes, particularly relevant in HIV endemic areas. There is some experience of aerosol delivery of vaccines in man. The measles vaccine, which is an attenuated strain of the airborne measles virus has been delivered successfully as an aerosol to over 1.5 million people including children for over a decade in Mexico (322, 323). A theoretical risk associated with any nasal
route of immunisation is vaccine-induced encephalitis, produced by infection of the CNS via the olfactory lobe that is also accessible via the respiratory compartment. Necropsies of macaques in this study detected no AFB or histological abnormality in the nasal turbinates or olfactory bulb nor any inflammation of the meninges. No clinical features of tuberculosis-like disease such as coughing, lethargy or weight loss in the macaques were recorded during in the year following vaccination. BCG has been given previously as an aerosol to adults and children and repeatedly and very high doses to metastatic cancer patients (324, 325). Respiratory function tests and post-mortem examination of the cancer patients who received aerosolised BCG showed no pulmonary dysfunction or disseminated BCG infection. Household contacts and relatives remained PPD skin test negative throughout the study period, suggesting vaccinees were not infectious after aerosol BCG administration (325). Prima facie, BCG vaccination by aerosol is safe and well tolerated however further testing is required, particularly toxicology studies at earlier time points following vaccination. In summary, we show that delivery with BCG is possible and safe and that it induces comparable levels of responses as intradermal immunisation.

The six macaques were twice immunised intradermally with MVA.85A (5-9 weeks apart) then 21 weeks later received two FP9.85A vaccinations (5 weeks apart). MVA.85A vaccination induced no 85A specific CD4+ T cell responses in ELISPOT in the naïve macaques. No CD4+ restricted T cell responses by MVA.85A vaccination was recorded in the 4 BCG immunised animals but a small but bona fide increase in 85A antibody titre was recorded in 3 animals. It is unclear why
MVA.85A did not boost 85A specific CD4⁺ T cell responses induced by BCG as was observed in BALB/c and C57BL/6 mice. It is possible T cell boosting in response to MVA.85A vaccination may have been missed because the BCG induced T cell response was still strong and fluctuating between weeks 12-16 or because of technical issues.

The inclusion of peptides spanning 85A-protein sequence in ELISPOST assays identified T cell responses produced by MVA.85A vaccination in Harry and Golf. In BALB/c mice, MVA.85A vaccination was able to boost low level CD8⁺ T cell responses induced by BCG. In this study we observed no evidence MVA.85A boosting of BCG induced CD8⁺ T cell responses. The differences between these studies may be a function of dose. Boosting of BCG induced CD8⁺ T cells in mice was only observed following high dose BCG vaccination (10⁷ CFU) and lost at lower doses. Here, the macaques received the human BCG dose, which can induce 85 specific CD8⁺ T cell responses in man, but at low frequencies (133).

The macaques were further vaccinated with FP9.85A, which has been shown to significantly boost CD8⁺ T cell responses following a DNA or poxvirus prime (326). FP9.85A vaccination either induced or significantly boosted existing CD4⁺ and CD8⁺ T cell responses in all 4/6 macaques. Responses peaked at day 7 with the exception of Hal, in which responses peaked 3 weeks after vaccination. A second FP9.85A vaccination further boosted responses in several animals but not as strongly
as the first vaccination. Peptide pool responses were CD8\(^+\) whilst PPD and Ag85A protein responses were almost wholly CD4\(^+\) dependent.

Hal and Harry that had both received two vaccinations each of MVA.85A and FP9.85A were intradermally vaccinated with BCG at week 70. At this time both animals were producing strong CD4\(^+\) restricted T cell responses to both PPD and 85A protein and CD8\(^+\) restricted T cell response to p22 and p14 were clearly detectable in Harry. Following BCG vaccination both animals exhibited a recall response to CD4\(^+\) T cell PPD and 85A protein. Responses peaked at 3-4 weeks post vaccination rather than 8-12 weeks as had been observed in the study. The peak of response was no greater than earlier observed. BCG failed to boost the CD8\(^+\) dependent T cell responses in Harry.

Animals were given TST to determine whether MVA.85A-FP9.85A vaccination had any effect of BCG induced TST. Prior to BCG vaccination but after MVA.85A-FP9.85A vaccination, Harry was strongly positive demonstrating that vaccination with recombinant viruses expressing just 85A is sufficient to produce positive skin test to tuberculin. In people, DTH to TST has been shown to peak 2-3 months after BCG vaccination (327). Animals were skin tested 12 weeks and again 40 weeks after intradermal BCG vaccination. At both time-points, the animals failed to produce a positive skin test. Losses of TST reactivity have been reported in both primates and humans. Prior BCG vaccination can render *M. tuberculosis* infected macaques unresponsive to tuberculin (256, 320). In humans, Hoft and co-workers showed that
a TST response was no longer detectable in intradermally vaccinated humans when they have been previously primed with oral BCG vaccination (321). Together this suggests that vaccination with MVA.85A / FP9.85A may render macaques anergic to subsequent DTH reaction to tuberculin. The mechanism of this inhibition is unclear but could result from differences in the phenotype of the T cells induced by MVA.85A/FP9.85A or BCG vaccination. Whilst both vaccination regimens were given intradermally and induced IFN-γ secreting T cells, differences in cytokine production or expression of integrins that mediate trafficking of the T cells to and from the skin may exist. Further characterisation using flow cytometry and multi-cytokine secretion assays of the T cells induced by MVA.85A/FP9.85A vaccination compared with BCG vaccination will help to elucidate the differences in TST responses observed.
BCG-MVA.85A-FP9.85A

vaccination of cynomolgus macaques

7.1 Results

Challenge

Cynomolgus macaques (*Macaca fascicularis*) were intradermally vaccinated with $4 \times 10^5$ CFU of BCG (BCG Copenhagen, SSI). 12 weeks later, the animals were immunised with MVA.85A ($5 \times 10^8$ PFU) and after 5 weeks vaccinated with FP9.85A ($5 \times 10^8$ PFU) (Fig. 33). Both poxvirus vaccines were given intradermally to both upper left and right arms. Three control animals were included that had received an adjuvant preparation that had been shown previously to afford no protection from tuberculosis disease (J. Langermans, personal communication). The third group in the study received $4 \times 10^5$ CFU of BCG alone. Animals were challenged intratracheally with 1000 CFU *M. tuberculosis* (Erdman strain) 5 weeks after their last vaccination and monitored for 6 months. The challenge was supervised by Jan Langermans (BPRC, Rotterdam, Holland). The challenge parameters were designed following consultation between Jan Langermans, Adrian Hill and myself. A summary of challenge results presented in Table 7.1 but is not part of this thesis. All animals in the control (no BCG) group had caseating granulomas in their bronchial lymph nodes. In the BCG only group, one animal showed no abnormalities following
gross examination whereas the remaining two animals had involvement in the bronchial lymph nodes though to a lesser extent than the control animals. In the BCG-MVA.85A-FP9.85A group, two of the three animals were normal on gross examination but the third had open cavitory tuberculosis with lesions in the spleen and lung. The results from the challenge were promising and suggested that the BCG/MVA.85A/FP9.85A vaccination regimen may increase BCG-mediated protection in cynomolgus macaques. Further, studies are underway to understand to explain the severe disease pathology (worse than control animals) observed in one of the BCG/MVA.85A/FP9.85A vaccinated animals. All macaques in this study were outbred and this animal may have been particularly susceptible to mycobacterial disease. The immunogenicity data presented was performed in Oxford in an effort of optimise immunogenicity assays in this species of macaque.
Figure 33: Vaccination schedule for cynomolgous macaques. Each arrow indicates the week of and type of immunisation given. All animals were challenged with *M. tuberculosis* at week 27. The following abbreviations are used: i.d-BCG, intradermal BCG ($4 \times 10^6$ CFU) given into the upper right arm; MVA85A ($5 \times 10^8$ PFU); FP9.85A ($5 \times 10^8$ PFU). i.t - intratracheal. Both poxvirus vaccinations were given intradermally into the upper left and right arms.
Table 7.1 Gross pathology results following *M. tuberculosis* challenge of BCG-MVA.85A-FP9.85A vaccinated cynomolgus macaques

<table>
<thead>
<tr>
<th>Reference</th>
<th>Vaccination</th>
<th>Description of Gross Pathology at necropsy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>J-455</td>
<td>MPL/DDA adjuvant</td>
<td>Involvement of BLN granuloma &amp; caseation / some lesions</td>
</tr>
<tr>
<td>J-462</td>
<td>MPL/DDA adjuvant</td>
<td>Involvement of BLN granuloma &amp; caseation / some lesions</td>
</tr>
<tr>
<td>J-466</td>
<td>MPL/DDA adjuvant</td>
<td>Involvement of BLN granuloma &amp; caseation / some lesions</td>
</tr>
<tr>
<td>J-450</td>
<td>BCG</td>
<td>Normal</td>
</tr>
<tr>
<td>J-460</td>
<td>BCG</td>
<td>Limited involvement BLN</td>
</tr>
<tr>
<td>J-463</td>
<td>BCG</td>
<td>Limited involvement BLN</td>
</tr>
<tr>
<td>J-457</td>
<td>BCG-MVA.85A-FP9.85A</td>
<td>Normal</td>
</tr>
<tr>
<td>J-458</td>
<td>BCG-MVA.85A-FP9.85A</td>
<td>Normal</td>
</tr>
<tr>
<td>J-459</td>
<td>BCG-MVA.85A-FP9.85A</td>
<td>Open TB large lesion lower left lobe of lung lesions in liver &amp; spleen</td>
</tr>
</tbody>
</table>

*macaques were challenged intratracheally with 1000 CFU *M. tuberculosis* (Erdman strain) 5 weeks after the last vaccination. Necropsies were conducted 6 months after challenge.

*4 × 10^5 CFU* BCG (Danish 1331) given intradermally

*5 × 10^7 PFU MVA.85A or FP9.85A given intradermally

BLN=bronchial lymph nodes

Optimisation of immunogenicity assays in cynomolgus macaques

ELISPOT assays

Frozen PBMCs from a non-vaccinated cynomolgus macaque were assayed by IFN-γ ELISPOT using the same protocol described in rhesus macaques (Fig. 34). Cells were stimulated for 20 hours with PPD, 85A protein, 5μg/ml streptokinase: streptodornase (SKSD) and the non-specific mitogen, ConA. The response from non-
stimulated cells was about to 10 SFC/10^5 at a concentration of 2×10^5 cells/well. PPD stimulation did not produce a response above control wells. Low level responses were observed at the 2×10^5 cell/well concentration to 85 protein (<10 SFC / 10^6 cells) and SKSD (~10 SFC / 10^6 cells) (Fig. 34A). ConA stimulation demonstrated that cynomolgus PBMCs could produce strong IFN-γ secretion (>1100 SFC / 10^6 cells) in this assay stimulation (Fig. 34B). The frequency of ConA-specific responses decreased with cell concentration.

Immunogenicity results from vaccinated cynomolgus macaques

PBMCs frozen at different time-points following vaccination were then tested in ELISPOT (Fig. 35). Cells from control animals (adjuvant only) were also included in the assays. Responses to conA were consistent and strong (>600SFC/10^6 cells) in all samples tested. Non-stimulated cells produced low background (<20 SFC / 10^6 cells) in two of the four animals (Fig. 35A&C) and higher non-specific responses (~50 SFC / 10^6 cells) in the others (Fig. 35B&D). The level of background was consistent between all the time-points tested. No 85A peptide-specific T cell responses were detected at any time-point. Following stimulation with PPD, a response of ~ 200 SFC / 10^6 cells was detected on a naive macaque (adjuvant only) but not to any of the BCG-MVA.85A-FP9.85A vaccinated animals. 85A protein responses were consistently above background (100-500 SFC / 10^6 cells), including pre-vaccination time-points and in the control animal.
Figure 34: PBMCs of non-vaccinated cynomologous macaques produce IFN-γ in response to stimulation with Concanavalin A (ConA). PBMCs from a naïve macaque were stimulated with several antigens as well as ConA in different cell concentrations in an ELISPOT well. Different scales for the y-axis are shown in (A) & (B). Responses are shown as the average spot-forming cells from duplicate wells.

Figure 35: BCG vaccination of cynomologous macaques does not induce PPD, 85A culture filtrate protein or 85A peptide specific- IFN-γ secreting T cells in ELISPOT. Assays were performed on PBMCs from an animal from the control group (A) and on macaques from the BCG-MVA.85A-FP9.85A vaccinated groups (B)-(D). Responses are shown as the average spot-forming cells per million PBMCs (SFC/10⁶ cells) from duplicate wells from different time-points. The time of the bleed is expressed as the last vaccination plus a numeral, where the numeral represents the number of weeks post-vaccination.
Proliferation Assays

Previous studies in rhesus macaques had shown that the level of proliferation following incubation of $^3$H in either 3 or 5 day old cultures was similar (data not shown). Cells from a non-vaccinated cynomolgus macaque were tested to confirm whether the assay also worked in this species. A stimulation index (SI) of $> 10$ to con A was observed (Fig. 364). In the single sample provided from a non-vaccinated cynomolgus macaque, strong proliferation to 85A protein but not to PPD was observed.
**Figure 36:** BCG vaccination does not induce proliferation to PPD or 85A culture filtrate protein in cynomolgous macaques. Proliferation assays were performed following stimulated with PPD, 85A culture filtrate protein or Concanavalin A (ConA) a naïve macaque (A), on macaques from the BCG-MVA.85A-FP9.85A vaccinated groups (B), (C), (D) and an animal from the control group (E). The SI index is expressed as the fold increase in proliferation of cells stimulated with antigen over non-stimulated cells.
Proliferation assays performed on cells from the animals in the study (control and BCG-MVA.85A-FP9.85A vaccinated) did not proliferate in response to PPD but proliferated strongly to the 85A protein (Fig. 36 B-E). The pattern of 85A-specific proliferation over the various time-points in the 3, BCG-MVA.85A-FP9.85A vaccinated animals reflected the conA responses. This, together with the positive proliferation to 85A protein in non-vaccinated animals suggests cells were responding to non-specific mitogenic component in 85A culture-filtrate and not 85A antigen itself.

7.2 Discussion

In humans, a hallmark of BCG vaccination is the induction of IFN-γ following PPD stimulation of PBMCs (200). Failure to produce IFN-γ in response to BCG vaccination can lead to uncontrolled growth of BCG within patients (65). In this study, the ELISPOT and proliferation assays developed in cynomolgus macaques demonstrated that BCG vaccination despite affording significant protection against *M. tuberculosis* challenge, did not induce PPD-specific IFN-γ secreting T cells. These data are supported by a previous study showing PPD stimulation of PBMCs from BCG vaccinated cynomolgus macaques produce 3 fold less IFN-γ in ELISA compared with BCG-vaccinated rhesus macaques (207). In either the absence of T cell derived IFN-γ or very low levels of IFN-γ (below the limit of detection of the ELISPOT assay), it is unclear by what mechanism BCG induced protection in these animals.
An alternative explanation for these results is that BCG vaccination did induce IFN-γ secreting T cells but that the in vitro immune assays failed to detect the specific cells. It is possible that either the assay lacked sensitivity generally or the frequency of IFN-γ secreting T cells was below the limit of detection of the assay. The former is less likely as ex vivo cells did produce strong IFN-γ and proliferative responses to non-specific mitogen stimulation and these animals. The latter point could be examined by the culture of cells to expand the mycobacterial specific T cell pools. Cynomolgus PBMCs can produce IFN-γ to PPD and 85A protein in ELISA and ELISPOT after M. tuberculosis challenge when lungs contain very high numbers of replicating bacilli (J. Langermans, personal communication). This suggests that that BCG vaccination is not sufficiently virulent to induce IFN-γ secretion in this species and that the protection from disease observed in this study was afforded by immune responses other than IFN-γ production.

Identification of the immune mechanisms induced by BCG in cynomolgus macaques will be important to understanding the broader host immune response that may be involved in protection from tuberculosis disease in humans. Until such time, the contribution of this macaque model to testing T cell vaccines is limited because the lack of informative immunogenicity data prevents determination of correlates of protection from disease. Development of a wider array of immune assays examining the production of other cytokines such as TNF-α and characterising other
lymphocyte subsets induced by BCG vaccination in cynomolgus macaques is warranted.
8 Conclusion Remarks

8.1 Summary of results

In humans, the protection afforded by the BCG vaccine is primarily mediated by the induction of IFN-γ secreting T cells, predominately of a CD4$^+$ T cell phenotype, that cross-react with *M. tuberculosis* proteins (200). Despite the induction of specific IFN-γ secreting T cells in most BCG vaccinees, protection from BCG is variable and wanes with time (11).

In this thesis, strategies to increase the protective efficacy of BCG have been investigated in animal models of tuberculosis disease. These include, 1) boosting BCG-induced, IFN-γ secreting CD4$^+$ T cells 2) the *de novo* induction or the boosting of Ag85A specific CD8$^+$ T cells and the 4) induction of higher frequencies of CD4$^+$ and CD8$^+$ T cells in the lung mucosa by intranasal or aerosol delivery of BCG or recombinant poxvirus vaccines.

*BCG vaccination*

BCG vaccination induced long-lived T cells in both BALB/c and C57BL/6 mice. In BALB/c mice, PPD- and 85A- specific CD4$^+$ T cell responses were detectable 1 year after vaccination, although there is some evidence of waning of the BCG-mediated protective response following *M. tuberculosis* challenge. PPD- specific T cells were also detectable in C57BL/6 mice 9 months after vaccination at which time BCG conferred significant protection from challenge.
In BALB/c, C57BL/6 and rhesus macaques 85A specific T cells were strongly induced in the lungs and spleen following BCG vaccination. At all time-points tested in BALB/c, C57BL/6 and rhesus macaques, 85A-specific protein or peptide responses constituted 20-80% of total PPD response, suggesting that Ag85A is an immunodominant following BCG vaccination.

In cynomolgus macaques, although BCG vaccination afforded significant protection from M. tuberculosis challenge, no PPD- or 85A-specific T cell responses were detected following BCG vaccination.

**Poxvirus vaccination**

The kinetics of the frequency of T cells induced by recombinant MVA or FP vaccination differed greatly from BCG vaccination. In mice, responses in the spleen or LN peaked at 5 days post vaccination and decreased by a log, 10 days following MVA.CS vaccination. Similar kinetics were observed in FP9.85A vaccinated macaques in which 85A peptide specific T cell responses peaked at day 7 post vaccination dropping down ~ 3-fold by 3 weeks post vaccination. 85A-specific CD4+ T cell restricted responses were detectable 6 months after vaccination (last time-point available before BCG vaccination) in Hal and Harry. CD8+ T cell responses were detectable 15 months after FP9.85A vaccination.

**Heterologous Poxvirus boosting**

Some work was performed on heterologous poxvirus boosting (without BCG) using malaria antigens in BALB/c mice and using 85A antigen in rhesus macaques. The results show in mice that heterologous prime-boost induces higher frequencies of T cells in the LLN than homologous vaccination. Further testing is needed to compare
MVA-FP9 or FP9-MVA directly against BCG-MVA-FP9 and other prime-boost strategies like DNA-MVA in mouse and macaque models.

**Boosting 85A specific CD4⁺ T cells**

In BALB/c, C57BL/6 mice and rhesus macaques significant increases in either the frequency of BCG-induced 85A specific IFN-γ secreting CD4⁺ T cells or 85A specific proliferation was observed following poxvirus boosting with either MVA.85A or FP9.85A. Recombinant poxviruses have been shown previously to boost DNA and protein induced T cells but this is the first demonstration of boosting of bacterial prime with a virus in mice and non-human primates. Conversely, BCG immunisation of rhesus macaques previously immunised with MVA.85A and FP9.85A induced a recall T cell response to 85A.

**The induction of 85A specific CD8⁺ T cells**

Poxvirus boosting of BCG-induced CD8⁺ T cells was dependent on the BCG dose. The human dose of BCG given to the rhesus macaques did not induce CD8⁺ T cells in *ex vivo* assays and did not appear sufficient to prime a CD8⁺ T cell response after MVA.85A vaccination. The induction of 85A specific T cells was demonstrated using DNA.85A / MVA.85A vaccination in mice or MVA.85A / FP9.85A vaccination in macaques. Together this suggests that in humans a single vaccination with either MVA.85A or FP9.85A may be insufficient to significantly expand specific CD8⁺ T cell responses. Heterologous vaccination regimens such as DNA/MVA or MVA/FP9 may be required to induce CD8⁺ T cells, independent of BCG vaccination.
The induction of \( \text{CD}4^+ \) and \( \text{CD}8^+ \) T cells in the lung mucosa by intranasal or aerosol delivery of vaccines

There was a clear difference between murine and non-human primate models in the induction of T cells in secondary lymphoid tissue following vaccination. The studies in BALB/c mice demonstrated that the induction of specific T cell responses in regional lymph nodes is highly compartmentalised and dependent on route of vaccination. Vaccination with poxviruses or BCG induced responses in the LN draining the site of injection but not in distal LN. As such, in BALB/c mice, intranasal immunisation was the best route of immunisation for the induction of specific T cells in the respiratory compartment. In rhesus macaques, parenteral vaccination induced very high numbers of specific T cells in the lung compartment and T cell responses in most lymph node sets. In terms of induction of T cells in the lung, there was not clear benefit of aerosol administration compared with parenteral vaccination of BCG.

Intranasal boosting increases specific T cell responses in the respiratory compartment

The impact of intranasal boosting by BCG or MVA.85A significantly increased protection from aerosol \( M. \text{tuberculosis} \) challenge in BALB/c mice in both the lungs and spleen. This correlated with the detection of 85A-specific CD4\(^+\)T cells in the LLN at the time of challenge and provided a correlate of protection from lung disease in this mouse strain. These same vaccination strategies failed to increase protection in C57BL/6 mice highlighting the impact of genetic heterogeneity in protection from tuberculosis disease.
8.2 Future Directions

Overall, these data provide promise that BCG-induced specific T cell responses can be boosted with recombinant poxviruses expressing Ag85A in humans. The question remains whether the boosting of a BCG immunodominant antigen that is also expressed by *M. tuberculosis* is sufficient to increase protective efficacy against human tuberculosis. This question is further complicated by the many clinical manifestations of tuberculosis disease (primary / reactivation / re-infection disease and/or pulmonary / systemic disease) against which poxviral boosting or mucosal vaccination may have variable success. In addition, factors like HIV-1 infection, the variability of BCG protective efficacy, exposure to environmental mycobacteria and anti-poxvirus vector immunity may all impact on the success of these vaccine regimens in man. These questions can only be answered by Phase III field trials that in the past have run over decades and involved thousands of patients (196). As such, the development of animal models mimicking factors such as latent infection, environmental exposure and HIV-1 will be beneficial to further test the efficacy of poxvirus boosting and/or mucosal vaccination regimens and expedite the whole process.

Protection against reactivation disease

The aerosol mouse challenge developed early in this project is a model of primary or acute disseminated disease. Extrapolating to humans, the protection results and significant increases in specific CD4+ T cells produced by intranasal vaccination with BCG / MVA.85A in BALB/c mice are promising for protection against childhood and systemic disease. Collaborative studies in guinea pigs have shown that
BCG / MVA.85A / FP9.85A afforded significantly greater protection than BCG after *M. tuberculosis* challenge. The guinea pig model is also an example of post-primary disease where the read-out of protection is survival following lung infection with *M. tuberculosis*.

These studies are not informative as to whether these vaccine regimens will afford any protection from reactivation of disease that often manifests in adulthood following years of latent *M. tuberculosis* infection. Models of latent *M. tuberculosis* infection have been developed in mice models where tuberculosis is reactivated spontaneously or by immunosuppression (328, 329). Experiments are planned to test the efficacy of BCG boosting and/or mucosal vaccination regimens in this model to determine whether vaccination can prevent reactivation of disease.

Antigens other than Ag85A may be more effective to prevent reactivation of disease. During latent infection, *M. tuberculosis* bacilli are believed to persist in a non-replicating state that no longer expresses high levels of the 85 complex (170). The 16kDA antigen, like the 85 complex is a secreted protein by *M. tuberculosis* and BCG but is also expressed *in vivo* and *in vitro* in persistent non-replicating mycobacteria; and is also being investigated as a target antigen for the prevention of reactivation (170).

**Protection from re-infection**

The rate of tuberculosis, arising from re-infection increases in tuberculosis endemic regions and increases against in HIV-1 positive individuals. No animal models of re-infection have been reported, most likely because guinea pigs, mice and rhesus macaques are highly susceptible to *M. tuberculosis* infection and cannot contain the
primary infection without antibiotic treatment. Cynomolgus macaques are more resistant to *M. tuberculosis* infection and can contain infection at a sub-clinical state (208). However, the lack of specific IFN-γ secretion following mycobacterial infection and poor TST responses produced in this animal suggests it would be difficult to identify latent infection for which the test is specific IFN-γ secretion to ESAT-6 antigen or a positive TST. Future clinical trials will have to include extensive characterisation of *M. tuberculosis* sub-strains to distinguish between re-infection and reactivation of disease.

*Protection in HIV-1 infected individuals*

The progressive loss of CD4⁺ T cells caused by HIV-1 infection significantly increases progression to tuberculosis disease (117). It is unknown whether a tuberculosis T cell vaccine will be successful in preventing tuberculosis in HIV-1 / *M. tuberculosis* co-infected in the absence of either effective anti-retroviral therapy to maintain CD4⁺ T cell levels and/or an effective HIV-1 vaccine. Several studies have been published in rhesus macaques modelling co-infection with *M. tuberculosis* and SIV or SHIV (SIV / HIV chimeric virus) (119). To date, none have tested vaccines beyond BCG. These models would be highly informative to determine the contribution of CD8⁺ T cell mediated protection against tuberculosis disease in CD4⁺ T cell depleted animals. This model would also enable the examination of combinations of *M. tuberculosis* vaccines with anti-retroviral therapies or HIV-1 vaccines. The demonstration that BCG can be delivered as an aerosol will be of particular use in HIV-1 endemic areas to minimise usage of syringes and decrease reliance on
expertise in vaccine delivery. However, studies examining the aerosol delivery of BCG to SIV⁺ macaques are necessary to exclude questions arising of increased toxicity of the vaccine delivered by this route.

*Protection against pre-existing environmental mycobacterial infection*

Studies in both mice and guinea pigs have shown that exposure to certain types of environmental mycobacteria inhibit BCG replication and protective efficacy. Recently, Brandt and colleagues have published that immune responses induced by a subunit vaccine expressing 85B- and ESAT-6- fusion protein were not inhibited by prior exposure to environmental mycobacteria (199). Similar studies need to be performed to test the protective efficacy of poxviruses expressing 85A. It is unclear whether aerosol delivery of BCG or poxviruses will be more or less inhibited by prior exposure to environmental mycobacteria than parenteral vaccination. Most environmental mycobacteria are found in soil and drinking water and would infect via the gut.

No studies have been published in macaques on the effects of environmental mycobacterial exposure to BCG-mediated protection. The strong induction of PPD-specific IFN-γ in BCG vaccinated rhesus macaques like that observed in humans suggests that parallel studies such as by Black *et al* could be conducted in macaques looking at effects of environmental mycobacteria on BCG IFN-γ production (200). These studies could be taken further and examine the effects of exposure to environmental mycobacteria on the immunogenicity and protective efficacy of MVA.85A and FP9.85A without BCG priming.
**MVA.85A / FP9.85A as a post-exposure vaccine**

BCG is ineffective as a post *M. tuberculosis* exposure vaccine (262). Indeed pathology worsened following BCG infection of animals chronically infected with *M. tuberculosis*. This is referred to as the Koch phenomena and is believed to result from exacerbation of the immune response produced by BCG infection.

It is possible that MVA.85A or FP9.85A may work as a post-exposure vaccine because neither vaccine is dependent on replication for expression of its recombinant antigen. Immunisation with recombinant poxviruses, in *M. tuberculosis* infected though healthy individuals, has two implications. The issue is the timing of poxviral boosting of BCG immunised children. In the developing world, the high levels of *M. tuberculosis* exposure mean that whilst children are BCG vaccinated at birth, it is likely that many will become *M. tuberculosis* infected during childhood. Prior *M. tuberculosis* exposure will have an impact on when children should be boosted ie early in infancy before *M. tuberculosis* infection or perhaps 10-15 years later when BCG protective efficacy is thought to wane. The second issue is whether MVA.85A or FP9.85A vaccination of *M. tuberculosis* infected individuals will exacerbate pathology and can be investigated using histological analysis.

*Using poxviruses as vaccine vectors for other diseases*

The reintroduction of the vaccinia virus vaccine and the use of MVA as vaccines in the study of other infectious diseases (most notably, HIV-1, hepatitis and malaria) may induce cell-mediated responses against the viral vector. It is unknown whether anti-vector immunity will inhibit the induction of 85A specific T cells produced by MVA.85A vaccination, although studies are ongoing. Other groups are investigating
the use of different viral vectors (examples include - Semliki Forest virus and Sindbis virus) that do not cross-react with vaccinia virus to circumvent this problem.

**Identifying correlates of protection**

There is clear evidence in humans that IFN-γ signalling and CD4+ T cells are essential to host immunity and protection from mycobacterial disease. Accordingly, in BALB/c mice, the induction of 85A specific IFN-γ secreting CD4+ T cells in the LLN correlated with protection. However, unlike disease such as measles where antibody titres clearly correlate with protection, T cell vaccines may require the analysis of susceptible and resistant phenotypes that is using more than 1 assay and 1 cytokine. Both TNF-α and Th2 cytokines have been implicated in resistance and susceptibility to disease. In this study multi-cytokine analysis was used successfully and this and similar techniques hold promise for broader characterisation of disease resistant and susceptible phenotypes.

**How have these studies contributed to vaccine development against tuberculosis disease?**

*M. tuberculosis* is a patient and insidious pathogen. However, in many countries the incidence of tuberculosis disease has decreased from epidemic proportions to low levels though the use of effective contact tracing, chemotherapy and improved living conditions. The degree of contribution of BCG vaccination to this decrease is controversial.

In the developing world, despite the efforts of the DOTS programme, tuberculosis disease continues to rise. This is due to sub-optimal levels of case detection, non-compliance with chemotherapy, HIV-1 infection and poorer standards of living like
overcrowding and malnutrition and the low protective efficacy of BCG from pulmonary disease. It is in this population that an improved BCG vaccine will have the greatest impact.

These studies have advanced the use of poxviral vectors as tuberculosis vaccines and have also strengthened the case for lung delivery of vaccines for the induction of mucosal immunity and as an alternate and safe route of vaccination. Poxviral vaccines and mucosal delivery of vaccines are approaches to tuberculosis vaccine development that are immunogenic, inexpensive and prima facie are safe. As such, further studies are justified to develop these regimens as realistic tuberculosis vaccines for the developing world.
9 Materials & Methods

9.1 Vaccines

DNA Plasmids

The vectors, pSG2.CS were constructed by Sarah Gilbert (NDM, Oxford University). pSG2.CS contains entire coding region of *P. berghei* circumsporozoite protein. pCIK85A was constructed by Deborah Gill (NDCS Oxford University) by sub-cloning the 85A coding region from PSG285A (136). pCIK85A contains αα 1-323 of Ag85A from the H37Rv *M. tuberculosis* strain. Eighteen αα from the C-terminus end of Ag85A were deleted (5'-ALGATPNTGPAQGA-3') during the cloning process of PSG2.85A and replaced with 5'-GSIPNPLLGLD-3'. The highlighted sequence is an epitope called a Pk tag that is recognised by the monoclonal antibody MCA136 (Serotec, Oxford UK) and was used to confirm expression of the recombinant protein

Recombinant Viral Vectors

The construction of MVA.85A and FP9.85A has been described previously (136, 330). Both vectors express αα 1-323 of the 85A coding sequence under the control of the vaccinia virus P7.5 early/late promoter.

Briefly, chicken embryo fibroblasts (CEFs) were infected with wild-type MVA and transfected with the vaccinia virus shuttle vector containing the 85A or CS coding sequence. Plaques containing recombinants expressed β-galactosidase that produces
a blue precipitate in the presence of its substrate, X-galactosidase. This property enabled the recombinants to be picked and purified.

Construction of FP9.85A involved infection of primary CEFs with wild-type fowlpox and transfection with a shuttle vector containing the 85A sequence (αα 1-323) and green-fluorescence protein (GFP) as the selection marker. GFP expressing recombinants were sorted separately from non-recombinant CEFs by use of a fluorescence-activated cell sorter. Recombinants were further purified by plaque picking and virus stocks were grown in primary CEFs and purified through a sucrose cushion by ultracentrifugation and diluted in endotoxin free PBS for injection.

Preparation for Vaccination

DNA plasmid purification

DNA plasmids were stored at -20°C until use. Vials were thawed and diluted in endotoxin free PBS within 2 hours of use.

Viruses

Virus stocks were stored at -80°C until use. Vials were thawed on ice, sonicated (3×15 seconds bursts) then diluted in endotoxin free PBS (Sigma, Dorset.UK). The virus was kept on ice until vaccination.

BCG

The Pasteur vaccine strain (P1172D2) (Micheline Lagranderie. Pasteur Institute. Paris) used in mouse studies was stored at -80°C until use. Stock vials (1-2×10⁹ CFU/ml) were thawed on ice, sonicated (2×15 seconds bursts) then diluted in endotoxin free PBS (Sigma, Dorset.UK). The BCG was kept on ice until vaccination.
Mice were immunised within 1.5 hours of thawing of the BCG. The stock solution of BCG was vortexed (~15 seconds) just prior to vaccination.

The Copenhagen strain (Batch # 10012: Statens Serum Institut. Copenhagen S Denmark) used to vaccinate the rhesus and cynomolgus macaques, was stored as a lyophilised powder -80°C until use. The BCG was diluted in the recommended diluent, briefly vortexed and maintained on ice until vaccination. Macaques were vaccinated within 1 hour of reconstitution of BCG.

*Mycobacterium tuberculosis* H37Rv

The *M. tuberculosis* strain H37Rv (Trudeau Mycobacterial Culture Collection #102) was grown in Dubos medium at 37°C for 21-28 days. The broth culture was centrifuged, suspended in a tryptic soy broth-glycerol and stored at -70°C after titration. Stock solutions were sonicated before use.

Ziehl-Neelson Stain

The bacterial culture was smeared on a glass slide and dried. The culture was fixed in absolute methanol for 1 minute, then covered with 1% carbol fuchsin (concentration), heated to steaming and then allowed to stand for 5 minutes. The slides were rinsed gently with water and the excess drained from the slide. The slide was decolourised with 3% acid – 70% alcohol solution until the colour no longer ran from the slide then counter-stained with 0.1% methylene blue for 30 seconds. The slide was rinsed gently and excess fluid drained. The slide was air dried and examined with a 1000× light microscope.
9.2 Animal Welfare

All procedures were performed by myself (personal license PPL 30/5011), veterinary officers or licensed technicians in accordance with the Home Office Guidance and Codes of Practice (on the Housing and Care of Animals used in Scientific Procedures, the Housing and Care of Animals in Designated Breeding and Supplying Establishments) and the Humane Killing of Animals under Schedule I to the Animals (Scientific Procedures) Act 1986).

9.3 Murine Studies

Mouse Strains

8-10 week old female BALB/c (H-2\textsuperscript{d}) and C57BL/6 (H-2\textsuperscript{b}) mice (Harlan Orlac, Blackthorn, UK) were used for the studies described. Mice were housed in an animal breeding facility in conventional conditions.

Immunisations

Intranasal Immunisation

Intranasal vaccinations were performed in a Class I hood. Animals were anaesthetised with halothane (vaporising within a glass container) (Merial Animal Health, Harlow UK). Mice were deemed fully anaesthetised when body movement was no longer observed and breathing patterns changed to a visible, rapid movement of the rib cage. 7-10 seconds later, mice were removed from the container and the vaccine given drop-wise using a pipette to the nares over 3-5 seconds.

Intravenous Immunisation
Mice were warmed for ~5 minutes at 37°C or until vasodilation of the lateral tail vein was visible. Mice were restrained and immunised with 50μl or 100μl in the lateral tail vein using a U-100 29G needle (BD Consumer Healthcare, Le Pont de Claix, France).

Intradermal Ear Immunisation

Mice were anaesthetised intraperitoneally with 50–100μl Midazolan (Antigen Pharmaceuticals Tipperary, Ireland): Hypnovel (Merial Animal Health Harlow, Essex, England): water (1:1:2), then vaccinated with 25μl/ear using U-100 29G needle (BD Consumer Healthcare, Le Pont de Claix, France)

Footpad immunisation

Mice were restrained and 50μl bolus was injected using a U-100 29G needle (BD Consumer Healthcare, Le Pont de Claix France) beneath the skin of the left- hind footpad.

Intramuscular Injection

Mice were anaesthetised intraperitoneally with 50 – 100μl Midazolan: Hypnovel: water (1:1:2). Fur was removed using dilipatatory cream and 50μl was injected into the lower tibialis of each leg using a 26 G needle (Becton Dickinson, Oxford). The needle had been sheathed to ensure it did not penetrate beyond the muscle.
Immune Assays

Preparation of single cell suspensions

*Splenocytes*

Splenocytes were removed aseptically and kept at room temperature (RT) in PBS until use. A single cell suspension was produced by crushing the spleen with the butt of a 10ml syringe and straining cells through a 70µm cell strainer (Becton Dickinson Le Pont De Claix France). Cells were pelleted (7minutes, 300g with brake) and residual erythrocytes lysed by incubation in ACK lysis buffer (0.15M NH₄Cl, 1mM KCO₃, 0.1mM Na₂EDTA) for 5 minutes, during which cells were gently mixed. Lysis was stopped after diluting the cell suspension 1 in 5 with PBS. Cells were spun, suspended and counted either by crystal violet staining or using a Casy 1 TT (settings 5.47 – 15µm) (Schärfe System, Reutlingen, Germany). This method yielded over 98% cell viability and negligible erythrocyte contamination.

*Lymph Node Cells*

Lymphocytes were removed aseptically and kept at RT in PBS until use. Residual fat was removed prior to crushing of LN through a wet cell strainer. Cells were washed twice in PBS, suspended in CTL media and counted. Cells showed over 98% viability.

*Lung cells*

Isolation of lymphocytes from murine lungs was adapted from a protocol by Gonzales-Juarrerro *et al* (331). Lungs were perfused with 10 ml of PBS / heparin following the insertion of an 18G needle 2-3mm into the apex of the heart (left...
ventricle). Lungs were diced into 1 mm cubes with razor blades in 2 ml of RPMI. An equal volume of 1.4 mg/ml collagenase XI (Sigma C7657) and 60μg/ml Dnase IV (Sigma D5025) in RPMI was added. The tissue was incubated for 30 minutes at 37°C with occasional mixing. The reaction was stopped with 5% FCS in RPMI. The resulting slurry was mixed thoroughly and pushed through a 70μm cell strainer. Cells were spun for 5 minutes at 300g. Residual erythrocytes were lysed with ACK lysis buffer. Cells were suspended in R10 (RPMI, 10% foetal bovine serum) and counted using trypan blue exclusion. Cells typically showed 80-90% viability.

Cell Culture and Peptides

All cells were cultured in CTL media comprising α-MEM (GibcoBRL, Paisley UK) supplemented with 10% foetal bovine serum (FBS) (Sigma, Watford UK), 0.05mM β-mercaptoethanol (GibcoBRL, Rockville USA), 10μM HEPES buffer (GibcoBRL), 20mM L-glutamine (GibcoBRL) and 100μg/ml penicillin/streptomycin (GibcoBRL).

The panel of 20’mer 85A peptides from Ag85A protein from M. tuberculosis H37Rv (Genbank Accession Number: P17944) used in these studies are shown in Table 9.1.
### Table 9.1. Antigen 85A peptides from H37Rv *M. tuberculosis* strain

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Other peptides used in assays include pb9 -- SYIPSAEKI (amino acids: 372-80) from circumsporozoite protein from *P. berghei* (Genbank Accession Number: AAA29541) (Research Genetics, Huntsville USA); NP peptide- TYQRTRALV (amino acids: 147-155) from nucleoprotein expressed by influenza A virus (Genbank Accession Number: CAA36234).

*Ex vivo* IFN-γ Elispot

Specific IFN-γ secretion by spleen or lymph node cell suspensions was assayed by ELISPOT. MAHA plates (Millipore, Bedford, MA USA) were coated overnight at 4°C in 50μl of 10μg/ml of IFN-γ coating antibody (MABTECH Nacka Sweden) in PBS. Plates were blocked in 10% FBS in RPMI overnight at 4°C. Cells were incubated for 18-20 hours with antigen cultured in CTL media (267). Following incubation, cells were discarded and the plate washed 2× with PBS, followed by a 5 minute incubation with distilled water to lyse any residual cells. The plates were washed a further 3× with PBS and then incubated overnight at 4°C with 1μg/ml of biotinylated anti-IFN-γ antibody (MABTECH Nacka Sweden). Plates were then washed 5 times in PBS and incubated with 50μl of 1μg/ml of streptavidin alkaline phosphatase (MABTECH Nacka Sweden) either overnight at 4°C or at room temperature for 2-4 hours. Plates were developed by eye using a BioRad ALP Kit according to the manufacturer’s instructions. The colour reaction was stopped using tap water. Well images were captured with an AID ELISPOT Reader System and spots enumerated using the companion software (Autoimmun Dignostika imBH, Straßberg, Germany). The final concentration of antigens used in the assays was PPD at 10μg/ml, individual peptides at 2μg/ml and Ag85A protein at 10μg/ml.
Results are expressed as the mean spot-forming cells/10^6 cells (SFC/million) +/- SEM.

51Chromium Release Assays

Cells were stimulated with 2 μg/ml of peptide P11 and cultured for 7 days. Cells were fed with 10 units/ml of Lymphocult-T (Biotest, Dreieich Germany) on day 3. Cells were harvested then incubated for 4 hours with 51Chromium-loaded P815 cells (ATCC: TIB-64). Specific lysis was calculated as follows: 100 × [(experimental radioactivity - spontaneous radioactivity) / (total radioactivity - spontaneous radioactivity)]. Results are expressed as the mean percentage (%) specific lysis +/- SEM.

Proliferation Assays

Cells (1-2×10^5 cells/well) were stimulated with 1 μg/ml of peptides, 10 μg/ml PPD or Ag85A protein or ConA in a final volume of 200 μl for 3 days. Wells were pulsed with 1 μCi / 20 μl RO (RPMI) of 3H-Thymidine. Plates were frozen 12-18 hours later. The proliferation response or stimulation index (SI), was calculated as the fold increase in incorporation of [3H] thymidine by cells stimulated with antigen over cells cultured in media alone. A SI equal to or greater than 2 was reckoned as a positive proliferative response to antigen. All cell samples produced a SI > 10 to ConA.

M. tuberculosis Challenge

Aerosol Challenge
Four weeks after the second immunisation, mice were challenged by aerosol with \textit{M. tuberculosis} using a modified Henderson Apparatus, which was enclosed within a Category III isolator (258). Mice were individually restrained to allow exposure of only the nose during challenge (ADG Developments, Hertfordshire, UK). Deposition in the lungs was estimated 24-48 hours after challenge.

Enumeration of bacterial load

Bacterial load in organs was calculated by plating 10-fold serial dilutions of organ homogenates on both Middlebrook 7H11 agar and thiophene-2-carboxylic acid Hydrazide (TCH) (5\(\mu\)g/ml) supplemented Middlebrook 7H11 plates. TCH (Sigma-Aldrich, Dorset, UK) at this concentration prevents BCG growth (39). Organs were fully homogenised in 1ml PBS in screw cap tubes containing glass beads (0.8mm) using a Mini-Bead Beater (Stratech Scientific Soham UK) for 60-100 seconds at 50 revolutions per minute (rpm) and then left to settle for 10 minutes. Plates were examined at 1 week to ensure no contamination with rapidly growing mycobacteria. Protection results are expressed as \(\log_{10}\) (mean (CFU)) +/- SEM (n=9-12).

\section*{9.4 Macaque Studies}

Housing and behaviour

Six infant rhesus male macaques (12-22 months) weighing between 2.0-2.7 kg were used in this study. The schedule for vaccination is described in Fig. 23.

Animals were housed in troupes of 20-30 juvenile males and were monitored daily for signs of distress or any changes in behaviour in their interactions with other
macaques and animal technicians. All observations were recorded and dated. No adverse behaviour was reported during the study. Body weight was recorded monthly. All animals showed a 45-60% gain in body weight in the year following BCG vaccination (Fig. 37).
Figure 37: All macaques gained weight at similar rates throughout the study.
BCG immunisations

Intradermal Vaccination

Animals were intradermally immunised with BCG (Danish strain 1331, SSI, Copenhagen, Denmark) as a single injection of $4 \times 10^5$ CFU in a 100μl bolus to the upper left arm.

Aerosol Delivery of BCG

Two animals were given $4 \times 10^5$ CFU BCG (Danish strain 1331, SSI, Copenhagen, Denmark) by aerosol. This vaccine dose has been reported to be well tolerated in macaques (256). Prior to vaccination, macaques were screened with an emulator to mimic the breathing pattern of each animal and to calculate the time required for the specified dose of aerosolised BCG to be administered to the lungs. A HaloLite™ nebulizer (Profile Therapeutics, Bognor Regis, UK) was used to generate aerosols of between 1-5 μm based on previous estimates to achieve optimal deposition in the lower lung. Aerosols expelled by the HaloLite™ were delivered to an anaesthetised macaque via a paediatric mask with an outlet valve connected to a filter (0.22μm, Pall). Light pressure on the mask created a seal that also blocked the nasal passage ensuring the macaque breathed only through the oral cavity. The mask was held in position for 12 minutes during which the macaques breathed with no apparent discomfort. Macaques were monitored for several hours following the procedure. No visible signs of respiratory discomfort or coughing were observed immediately after vaccination or during the course of the study (67 weeks).
MVA.85A and FP9.85A vaccinations

MVA.85A and FP9.85A vaccinations were given intradermally. Immediately before vaccination, viruses were vortexed for 1 minute. The maximum volume injected into any one site was 100μl. Animals typically received 5 injections divided between the left and right upper arms.

Tuberculin Skin Testing

Animals were anaesthetised and 10, 100 or 1000 tuberculin units (TU) of Tuberculin Purified Protein Derivative (Evans VACCINES, Liverpool, UK) diluted in 100μl of water was injected in the upper left or right eyelid, between the medial and lateral canthi. 48 hours later, any local reaction was read and scored on a scale of 0-5 by a qualified veterinarian. 0 = no reaction (-); 1 = bruise (-); 2 = erythema of palpebrum, no swelling (-); 3 = erythema of palpebrum and slight swelling (+/-); 4 = swelling of palpebrum, drooped eyelid and erythema (+); 5 = swelling and/or necrosis (+); 5+ eyelid closed (+). The + or - symbols in parenthesis indicate whether the tuberculin skin test (TST) is deemed positive or negative respectively. This scale was developed by S. Wolfensohn (Oxford University, Senior Veterinary Officer).

Necropsies

The aerosol and intradermally BCG-immunised animals were necropsied at week 67. A full autopsy was performed recording gross appearance of external condition and all major internal organs of each animal. Organs either whole or in part were fixed, sectioned and haematoxylin / eosin stained, then examined and scored for evidence of pathology (Rest Associates, Cambridge, UK). Other sections were stained by the
ZN method to detect the presence of acid-fast mycobacteria (AFB) (Rest Associates, Cambridge, UK). No AFB were detected by this method.

**Bacteriology**

Sections of the right apical and left caudal lung lobes, spleen and lymph nodes not used for histological examination, were homogenised and cultured for 4 weeks at 37°C on middlebrook plates to detect viable mycobacteria. One ZN positive colony grew from a right caudal lung homogenate from Hector. The colony was identified as *M. fortuitum* (Public Health Laboratory Service, Dulwich Hospital, UK).

**Broncheo-alveolar Lavage**

Animals were lavaged several times during the study to sample cells in the lung compartment. Repeated lung lavage is well tolerated by macaques (332). None of the animals in this study displayed any adverse effects following lavage. The procedures were performed by animal technicians (Harlan, Loughborough UK).

Animals were anaesthetised and tubing (5mm ID / 8.0 mm OD) (Portex, Hythe, Kent, England) was inserted past the level of the third bifurcation of the bronchus. Warm sterile Hank's solution (~ body temperature) (Sigma-Aldrich, Dorset, England) was introduced into the lung through the tube. The fluid was left in the lung for 1 minute and then withdrawn under suction (not exceeding 40mm/hg) over several minutes. Typically a 20ml volume was introduced into the lung and 10-15ml was recovered. Broncheo-alveolar lavage fluid (BALF) was stored on ice until processing.
Preparation of single cell suspensions

Peripheral Blood Mononuclear Cells

Blood was diluted 1 in 2 with RPMI (GibcoBRL, Strathclyde, UK) and then isolated using a Lymphoprep gradient (Axis- Shield, Oslo, Norway). Cells were spun for 45 minutes at 950g with no brake. Macaque peripheral blood mononuclear cells (PBMCs) did not form a clean interface between the Lymphoprep and plasma layer. As such, the majority (around 15mls) of the interface and ficoll gradient was removed with a Pasteur pipette and diluted into 50mls RPMI. Cells were then washed twice (no brake during centrifugation). Cells formed a loose pellet with significant erythrocyte contamination, which produced high levels of background in IFN-γ ELISPOT. Residual contaminating erythrocytes were hypotonically lysed with 5-7 ml ACK buffer for 5 minutes. Cells were then washed and pelleted. If erythrocyte contamination was still observed, cells were again lysed. The degree of erythrocyte contamination appeared to be a function of the animal not protocol. Golf’s blood was typically lysed 2-3 times to achieve a pure lymphocyte pellet. The plasma (50%) layer was frozen for antibody studies. Cells were washed and suspended in RPMI, 10% foetal bovine serum (R10). Cells were either immediately used for proliferation and cultured ELISPOT, frozen in 10% DMSO/FBS for later analysis or cultured overnight without antigen at 37°C degrees in R10 medium (RPMI, 10% FBS, 20mM L-glutamine, 100µg/ml penicillin/streptomycin) at a concentration of 2-4 $10^6$ cells/ml for use indirect ELISPOT assays.

Following PBMC isolation, cells were diluted 1/1000 and counted using a Casy 1 TT (Schärfe System, Reutlingen Germany) on settings 5.7 - 12 μm. Cells showed 98%
viability and at every time point produced a strongly positive response to ConA stimulation.

Lymph Nodes

Lymph nodes were maintained in PBS at RT until preparation for immune assays. All manipulations were then carried out under aseptic conditions. Fat was removed and LN were then crushed with the butt of a 10ml syringe and put through a 70μm cell strainer. Cells were washed and left overnight at RT without antigen for analysis in indirect ELISPOT assays.

Splenocytes

Spleens were maintained in PBS until cell preparation. Spleens were roughly cut with sterile scissors then crushed with the butt of a 10ml syringe into a metal strainer. 25% of cell suspension was removed for bacteriology. The remainder was then strained over 5, 70μm cell strainers and washed twice with PBS. Three rounds of lysis with ACK buffer were required, to achieve complete lysis of erythrocytes. The cells were suspended in 100ml of R10 and left overnight. Both the spleen and LN cell suspensions congealed overnight yielding far lower cells than predicted for a lymphoid organ of that size. Further optimisation of these cell-isolation techniques is needed.

Broncho-alveolar lavage cells

All manipulations of BAL cells were conducted under aseptic conditions. All reagents and instruments were firstly cooled to 4°C. BALF was strained though a 70 μm filter to remove mucus then spun (10 minutes, 950g) with no brake. Cells were counted by trypan exclusion and cell morphology recorded. Lung lavage yielded 2-4
× 10⁶ cells. 55-60% of BALF cells had a lymphocyte morphology being small, round and highly refractive. Cell viability ranged from 50-90%. The majority of dead cells did not appear to be lymphocytes being larger with a fibroblast or endothelial cell morphology.

**Lymph Nodes and Splenocytes**

Cell concentration and percentage viability were determined by trypan blue exclusion. Cell viability for lymph nodes and splenocytes prior to use in immune assays ranged between 90-98%. Cells were small, round and highly refractive and in culture responded strongly to the mitogen, conA by forming budding colonies and secreting IFN-γ.

**Thawing of Frozen Cells**

Frozen cells that had been stored in 90% FBS /10% dimethyl Sulfoxide in liquid N₂ were thawed in a 37°C water-bath. A solution of 60μg DNase I (type IV) / RPMI (Sigma-Aldrich, Dorset UK) (RT) was added drop-wise to the thawed vial and the frozen cells gently mixed with the diluent. The cells were then added to a tube containing 10ml DNase I / RPMI, centrifuged for 5 minutes at 300g. The cell pellet was suspended and another 3 ml of DNase I / RPMI added for 2-3 mins. 10 mls of R10 was added, cells were spun then suspended in 5mls R10. Cells were left at 37 C for 45-60 mins, then spun, suspended and counted using trypan blue exclusion. 70-85% of cells frozen were recovered with this technique. Thawing of cells without adding DNase I, recovered ~20% fewer cells.
Indirect Elispot

The Monkey / Human IFN-γ kit (MABTECH, Nacka, Sweden) was used in all ELISPOT assays. MAIP plates (Millipore, Bedford, MA USA) were coated with 10μg/ml of IFN-γ antibody (clone 7-B6-1) diluted in 100μM Carbonate Buffer, pH 9.6 (Sigma-Aldrich, Dorset UK) overnight at 4°C. Plates were blocked with 2% BSA/H2O and incubated overnight at 4°C. Cells were aliquoted into wells at a final concentration of 2-4 × 10⁵ cells/well for PBMCs. Splenocytes and lymphocytes were used at 2-8 × 10⁵ cells/well and BALF cells at 1.25×10⁴ - 10⁵ cells/well. All samples were assayed in duplicate and/or with titrations of cells. Antigens were diluted in R10 and added to wells to achieve a final volume of 100μl. Cells were stimulated with antigens at the following final concentrations - PPD - 10μg/ml (SSI, Copenhagen Denmark), 85A protein -5 μg/ml (K. Huygen, Institut Pasteur), 85B 5 μg/ml (Lionex GmbH, Braunshweig Germany), 20’mer peptides spanning Ag85A-25 μg/ml (Research Genetics, Huntsville US), concanavalin A-10 μg/ml (Sigma-Aldrich Dorset UK). Cells were left at 37°C for 18-22 hours then washed 6× with PBS/ 0.05% Tween. 100μl of biotinylated anti-IFN-γ antibody (clone GZ-4) at 1μg/ml was added to each well and incubated overnight at 4°C. Plates were washed and streptavidin-ALP (1/1000 dilution) was added and incubated either overnight at 4°C or at RT 2-4 hours. Plates were washed 6 times then developed using an AP Conjugate Substrate Kit (BioRad Laboratories, Hercules, CA USA) according to manufacturer's instructions. Well images were captured with an AID ELISPOT Reader System and spots enumerated using the companion software (Autoimmun
Dignostika GmBH, Straßberg, Germany). Background responses in non-antigen stimulated wells were typically <25 SFC / 10^6 cells and responses to the non-specific mitogen, ConA >1000 SFC / 10^6 cells. Table 9.2 compares the percentage of lymphocytes before and after ACK lysis and after overnight culture without antigen. The results show that the percentage of B, T and NK cells did not differ between the ACK lysis stage and overnight culture. Overnight culture of cells did significantly deplete large, granular cells in the monocyte gate most likely reflecting the adherence of cultured monocytes to the tissue culture dishes (46-50% to < 22-33% in 3 independent samples) (Fig. 38).

Flow cytometry

PBMCs were stained with a series of human antibodies. Cross-reactivity with rhesus macaque PBMCs was confirmed for the antibodies listed (data not shown). The following volumes/stain were used: ^aCD3-PE (SP34) - 20μl; ^aCD4-FITC (M-T477) 20μl; CD8-FITC, ^bCD8-TC 5μl (RPA-T8) 5μl; ^bCD14-TC (M5E2) 5μl; ^aCD16-FITC (3G8) 20μl; ^bCD20-FITC (2H7) 5μl; ^aCD45RA-PE (SH9) 20μl; IgG1-TC; ^aIgG2a-FITC, ^aIgG3-PE, ^aIgG3-APC. ^a= Becton-Dickinson. Oxford UK; ^b= Caltag-MedSystems, Silverstone UK.

10^6 PBMCs were washed with PBS then 2× with FACS buffer (2% FBS/ 0.05mM EDTA / PBS). Cells were incubated with antibodies for 20 minutes at 4 °C then washed twice with 0.5ml FACs buffer. Cells were pelleted then fixed with 2% FBS / 1% PFA / 0.05mM EDTA / PBS for analysis the following day.
<table>
<thead>
<tr>
<th>within lymphocyte / NK gate</th>
<th>LP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Next Day&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+/CD4+/CD8-</td>
<td>67.24</td>
<td>72.65</td>
<td>74.5</td>
</tr>
<tr>
<td>CD3+/CD4-/CD8+</td>
<td>20.4</td>
<td>15.6</td>
<td>15.95</td>
</tr>
<tr>
<td>CD3+/CD4+/CD8+</td>
<td>2.23</td>
<td>1.64</td>
<td>2.43</td>
</tr>
<tr>
<td>CD3+/CD4-/CD8-</td>
<td>10.12</td>
<td>10.12</td>
<td>7.13</td>
</tr>
<tr>
<td>CD3-/CD8+/CD16+</td>
<td>16.8</td>
<td>30.7</td>
<td>27.8</td>
</tr>
<tr>
<td>CD20+ /CD3-/CD14-</td>
<td>24.8</td>
<td>13.74</td>
<td>17.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>LP = PBMCs purified from lymphoprep gradient  
<sup>b</sup>LP + ACK = PBMCs after ACK hypotonic lysis of residual erythrocytes  
<sup>c</sup>Next Day = non-adherent PBMCs after overnight culture in R10, no antigen  
<sup>d</sup>cells first gated on the population in bold
Figure 38: Overnight incubation of macaque PBMCs decreases cells in the monocyte gate by about 50%. Forward vs Side Scatter density plots were generated of PMBCs after isolation from whole blood subsequent ACK buffer lysis (A) and after overnight incubation in R10 (B). The monocyte gate (boxed) shows that the density of monocytes, decrease following overnight incubation.
CD4⁺ / CD8⁺ T cell depletions

The restriction of peptide specific T cell responses was determined by using ELISPOT following depletion of cells CD4⁺ (M-450) and CD8⁺ (M-450) human Dynabeads (DYNAL, Wirral, Merseyside, UK) used in accordance with the manufacturer's instructions. The efficacy of depletions (ranging between CD4 beads: 80-94%; CD8 beads: 95.4- 99.8%) was confirmed using flow cytometry.

Cultured Elispot-

PBMCs were left O/N in round bottom 96 well plates at 4 × 10⁶ cells / ml without antigen. The following day, non-adherent cells were harvested. The remaining adherent cells were infected with 2× 10⁴ CFU BCG/well (SSI, Denmark Copenhagen) for 2-4 hours in RPMI (no additives). Cells were washed and non-adherent cells added back at a concentration of 2 × 10⁵ cells/ well. Cells were fed with 10 units Lymphocult-T every 3 days. After 14 days culture, non-adherent cells harvested and washed. 10⁵ cultured cells were added with equal numbers of γ-irradiated (12 minutes, 3000 rads in 1ml 5% FBS/RPMI) autologous cells (freshly separated PBMC's from subsequent bleed). Cells were stimulated with 10μg/ml PPD, 5μg/ml 85A protein or 25μg/ml 85A peptides for 18-20 hours. Control wells contained γ-irradiated autologous cells plus antigen and did not produce IFN-γ (< 20 SFC / 10⁶ cells). The development of the cultured ELISPOT was the same as described for the indirect ELISPOT.
Proliferation Assays

Quadruplicate wells each containing $1 \times 10^5$ cells were seeded in 96-well RB plates were stimulated for 3 or 5 days with antigen at the following concentrations (PPD - 10 µg/ml, 85A protein -µg/ml, peptide pools- 10 µg/ml, Conconavalin A-5 µg/ml).

The proliferation response or stimulation index (SI), was calculated as the fold increase in incorporation of $[^3]$H thymidine by cells stimulated with antigen over cells cultured in media alone. A SI equal to or greater than 4 was reckoned as a positive proliferative response to antigen. All cell samples produced a SI > 10 to ConA.

Antibody ELISA

Preliminary experiments were performed to determine saturating concentrations of coating antigen and peroxidase conjugated antisera.

Nunc-Immuno Polysorp plates (Fisher Scientific U.K. Ltd. Loughborough, Leicestershire UK) were coated overnight at 4°C with 50 µl/well of 5 µg/ml of 85A-culture filtrate protein diluted in carbonate buffer. Plates were washed 6 times with 0.05% Tween 20 / PBS and then blocked with 2%BSA/water for 1 hour at 37°C. This and all subsequent incubations at 37°C were performed in a humidified chamber. The 50% plasmas/RPMI were thawed, centrifuged for 3 minutes at 670g to remove any precipitate and then added to plates in a final volume of 50µl/well. Serial half fold dilutions were made (1 - 1/1024) with 1%BSA/0.05% Tween 20/PBS (diluent buffer).

Each plate contained duplicate blank wells (no plasma) and half fold dilutions of a reference rhesus macaque serum (1/100 - 1/3200) (NMonS from Nordic Immunologicals, Tilberg Netherlands) that was positive for 85A antibodies at titres of 400 -800. Plates were incubated for 2 hours at 37°C, washed and 50µl of peroxidase
conjugated rhesus macaque immunoglobulin antisera (containing IgG, IgA, IgM, Fc, Fab) was added to each well at a dilution of 1/250 with diluent buffer (GAMON-Ig from Nordic Immunological Laboratories Tilberg, Netherlands). Plates were incubated for a further hour at 37°C and then developed using the ABTS Microwell Peroxidase Substrate System (KPL Gaithersburg, Maryland US) according to manufacturer's instructions. The reaction was stopped after 10 minutes incubation with an equal volume of 1% SDS. A single absorbance reading was made at 405 nm using an endpoint or multi-plate titration protocol on a Model 550 Microplate Reader (BIO-RAD Hemel-Hempstead, Hert UK)

Multi-cytokine analysis of BALF

The BALF supernatants were concentrated 5 - 25 times by centrifugation using a 3kDa Centriprep device (Millipore, Bedford USA). The BALF was then analysed for the presence of 13 cytokines using a Lincoplex Multiplex Immunoassay Kits Human Cytokine Panel as per manufacturer's instructions (Lincoplex, St Charles, USA). Assays were performed in duplicate. Briefly, 25 µl of concentrated BALF was mixed with Luminex beads and incubated on a blocked MABV plate for 1 hour at RT with shaking. Secondary and tertiary antibodies were then added and incubated for 30 minutes with shaking. The samples were read using a Luminex-100 machine (1000 events in total / minimum of 50 events per cytokine). Positive controls (provided with the kit) fell within the forecast range and standard curves were acceptable for all cytokines.
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Appendix

Animal models are important in the development of novel vaccines not only for testing the immunogenicity and protective efficacy of vaccine regimens but also for toxicology studies. At various stages in the immunology studies described in this thesis, organs were examined for evidence of vaccine-induced pathology. Whilst further pathology studies are warranted, from the studies conducted there was no evidence of adverse pathology produced in the animal models from aerosol delivered BCG, MVA.85A or FP9.85A. The reports from the pathology studies are tabled below and have been referenced in the text.
### Appendix I: Summary of H/E staining and ZN pathology in Ear

<table>
<thead>
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<th>Vaccination Regimen</th>
<th>Sex</th>
<th>Features</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 / 7</td>
<td>accompanying mild acanthosis</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>2 / 7</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>1 / 7</td>
<td>dilatation of infundibulum of follicle within inflammation with keratin. both have mild lymphatic dilatation</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td></td>
<td>5</td>
</tr>
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<td>6</td>
<td>1 / 7</td>
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<tr>
<td>7</td>
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<td>mild dilatation of both</td>
<td>6</td>
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<tr>
<td>8</td>
<td>2 / 7</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>1 - lymphatic dilatation of both</td>
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<td>11</td>
<td>1 / 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>both have mild diffuse expansion of dermis by mixed, degenerating inflammatory cells and lymphatic dilatation.</td>
<td>6</td>
</tr>
<tr>
<td>13</td>
<td>7</td>
<td>both have focal diffuse expansion of dermis by mixed, degenerating inflammatory cells and lymphatic dilatation.</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>1 / 7</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>7</td>
<td>both have diffuse expansion of dermis by mixed, degenerating inflammatory cells and lymphatic dilatation.</td>
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</tr>
<tr>
<td>16</td>
<td>7</td>
<td>one has focal fibrosis and the other focal inflammation of the dermis with lymphatic dilatation of dermis by mixed inflammatory cells. the second main piece has lymphatic dilatation</td>
<td>6</td>
</tr>
<tr>
<td>17</td>
<td>1 / 3 / 7</td>
<td>the second main piece has lymphatic dilatation</td>
<td>6</td>
</tr>
<tr>
<td>18</td>
<td>1 / 3 / 7</td>
<td></td>
<td>6</td>
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<td>6</td>
</tr>
<tr>
<td>20</td>
<td>2 / 7</td>
<td>both have lymphatic dilatation</td>
<td>6</td>
</tr>
</tbody>
</table>

*1. one piece has focal expansion of dermis by mixed, degenerating inflammatory cells / 2 both have focal expansion of dermis by mixed, degenerating inflammatory cells / 3 the second has lymphatic dilatation / 4 normal range apart from mild lymphatic dilatation / 5 focal subacute dermatitis associated with mycobacteria / 6. oedema / 7. ZN +ve long beaded organisms seen*
### Appendix 2: Summary of H/E staining and ZN pathology in Facial Lymph nodes

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Sex</th>
<th>Features&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Interpretation&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>1 / 2 - foci of large, pale macrophages in central area</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1 / 2 - foci of large, pale macrophages in medullary cord areas</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2 - foci of large, pale macrophages in various areas</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1 / 2 / 3 - foci of large, pale macrophages and plasma cells in medullary cords</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>2 / 3 / 4 - Scattered foci of large, pale macrophages and plasma cells mainly in medullary cords</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>2 / 3 / 4 - large, pale macrophages and plasma cells mainly in medullary cords</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>3 / 5</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>2 / 3 / 4 - scattered foci of large, pale macrophages and plasma cells mainly in medullary cords</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>3 / 5</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>3 / 5</td>
<td>6</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>1 / 2 / 5</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>1 / 2 / 5</td>
<td>6</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>1 / 2 / 5</td>
<td>6</td>
</tr>
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<td>14</td>
<td></td>
<td>1 / 2 / 5</td>
<td>6</td>
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<td>15</td>
<td></td>
<td>1 / 2 / 5</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>2 / 4 / 5</td>
<td>6</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>1 / 2 / 5</td>
<td>6</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>2 / 5</td>
<td>6</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>2 / 4 / 5</td>
<td>6</td>
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<tr>
<td>20</td>
<td></td>
<td>1 / 2 / 5</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1 some pale centred active follicles / 2 ZN +ve long beaded organisms seen / 3 LN structure clearer / 4 foci small and inactive / 5 foci of large, pale macrophages in various areas

<sup>b</sup> 6 microgranulmata associated with mycobacteria
### Appendix 3. Report on gross pathology of macaques at time of necropsy

<table>
<thead>
<tr>
<th>Condition</th>
<th>Glyn</th>
<th>Hector</th>
<th>Golf</th>
<th>Gulp</th>
</tr>
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<tbody>
<tr>
<td><strong>External Condition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>NAD - Good condition</td>
<td>NAD - Good condition</td>
<td>NAD</td>
<td>NAD - hair loss from back</td>
</tr>
<tr>
<td>Eyes</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Orifices</td>
<td>No discharges</td>
<td>No discharges - slight faecal staining at base of tail</td>
<td>No discharges</td>
<td>No discharges</td>
</tr>
<tr>
<td><strong>Body Condition</strong></td>
<td>Lean</td>
<td>NAD - Lean</td>
<td>Lean</td>
<td>Lean</td>
</tr>
<tr>
<td><strong>Digestive system</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>NAD</td>
<td>NAD - hepatic LN undetectable</td>
<td>NAD - hepatic LN undetectable</td>
<td>Small area of mild diffuse capsular fibrosis</td>
</tr>
<tr>
<td>Gall bladder bile duct pancreas</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Mouth tongue</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Pharynx tonsils</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Stomach</td>
<td>NAD</td>
<td>Slight reddening of the gastric mucosa in the fundus</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Small intestine</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Caecum</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Caecal LN</td>
<td>-</td>
<td>NAD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Colon rectum</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>MLN</td>
<td>NAD</td>
<td>Anterior MLN enlarged</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td><strong>Respiratory System</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nares</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Larynx trachea</td>
<td>Blood stained froth at tracheal bifurcation</td>
<td>Blood stained froth at tracheal bifurcation</td>
<td>Blood stained froth at tracheal bifurcation</td>
<td>Blood stained froth at tracheal bifurcation</td>
</tr>
<tr>
<td>Thyroid parathyroids thymus</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>lungs</td>
<td>X</td>
<td>X</td>
<td>NAD</td>
<td>X</td>
</tr>
<tr>
<td>pleura</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Tracheo-bronchial LN</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td><strong>Urinary System</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidneys adrenals</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Ureters</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Bladder</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Urethra</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td><strong>Reproductive System</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testes</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
</tr>
<tr>
<td>Prostate</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
<td>NAD - immature</td>
</tr>
<tr>
<td>Penis</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
<td>NAD</td>
</tr>
</tbody>
</table>

* NAD = no abnormality detected

* Bright red mottling - attributed to hypostatic congestion
**Appendix 4 - Summary of H/E staining following rhesus macaque necropsies at week 67**

<table>
<thead>
<tr>
<th>Glyn - Organs</th>
<th>Features</th>
<th>Interpretation</th>
<th>ZN</th>
</tr>
</thead>
<tbody>
<tr>
<td>L Inguinal LN</td>
<td>Normal range</td>
<td>-ve</td>
<td></td>
</tr>
<tr>
<td>R Axillary LN</td>
<td>Active follicles</td>
<td>Normal range</td>
<td>-ve</td>
</tr>
<tr>
<td>R Cervical LN</td>
<td>Few small but active follicles only</td>
<td>Normal range</td>
<td>-ve</td>
</tr>
<tr>
<td>R Submandibular LN</td>
<td>Large and secondary active follicles. Paracortical expansion.</td>
<td>Hyperplasia, B and T</td>
<td>-ve</td>
</tr>
<tr>
<td>L Tonsil</td>
<td>Large and secondary active follicles. Paracortical expansion.</td>
<td>Hyperplasia, B and T</td>
<td>-ve</td>
</tr>
<tr>
<td>L Turbinates</td>
<td>Foci of fibrosis with a few neutrophils</td>
<td>Subacute rhinitis, focal and minimal</td>
<td></td>
</tr>
<tr>
<td>R Turbinates</td>
<td>Foci of eosinophils</td>
<td>Eosinophilic rhinitis, focal and minimal</td>
<td></td>
</tr>
<tr>
<td>Nasal Septum</td>
<td>Nodules of lymphocytes and a few histiocytes, eosinophils and neutrophils in the submucosa</td>
<td>Subacute rhinitis with mixed inflammation, mild</td>
<td></td>
</tr>
<tr>
<td>Hilar LN</td>
<td>Few active follicles, oedema of sinuses</td>
<td>Normal range</td>
<td>-ve</td>
</tr>
<tr>
<td>Spleen</td>
<td>Follicles active. T cell areas less active. Red pulp contains significant number of eosinophils and neutrophils.</td>
<td>Hyperplasia B white pulp with neutrophilic / eosinophilic infiltrates of red pulp</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Staining of periacinar hepatocytes less dense (glycogen)</td>
<td>Normal range</td>
<td></td>
</tr>
<tr>
<td>L Lung x 2</td>
<td>Terminal congestion and oedema, peribronchial / perivascular lymphocyte nodules</td>
<td>Previous antigenic stimulation, agonal oedema</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Normal Range</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Appendix 5 - Summary of H/E staining following rhesus macaque necropsies

<table>
<thead>
<tr>
<th>Hector - Organs</th>
<th>Features</th>
<th>Interpretation</th>
<th>ZN</th>
</tr>
</thead>
<tbody>
<tr>
<td>R Internal Iliac LN</td>
<td>Normal range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L Inguinal LN</td>
<td>Sinus histiocytosis. Few eosinophils in sinuses. Artefact one are</td>
<td>Sinus histiocytosis</td>
<td>-ve</td>
</tr>
<tr>
<td>L Axillary LN</td>
<td>Secondary active follicles. Paracortical expansion. Sinus histiocytosis</td>
<td>Sinus histiocytosis, haemosiderosis</td>
<td>-ve</td>
</tr>
<tr>
<td>R Retropharangeal LN</td>
<td>Secondary active follicles. Paracortical expansion.</td>
<td>Hyperplasia, B and T</td>
<td>-ve</td>
</tr>
<tr>
<td>R Submandibular LN</td>
<td>Secondary active follicles. Paracortical expansion.</td>
<td>Hyperplasia, B and T</td>
<td>-ve</td>
</tr>
<tr>
<td>R Tonsil</td>
<td>Active follicles, neutrophil and eosinophil exocytosis</td>
<td>Hyperplasia with mild acute tonsillitis</td>
<td>-ve</td>
</tr>
<tr>
<td>L Turbinates / nasal septum</td>
<td>Lymphocytes and a few histiocytes, eosinophils and neutrophils in the submucosa. Focal cartilaginous proliferation</td>
<td>Subacute rhinitis with mixed inflammation, mild</td>
<td></td>
</tr>
<tr>
<td>R Turbinates / nasal septum</td>
<td>Lymphocytes and a few histiocytes, eosinophils and neutrophils in the submucosa and focally with exocytosis.</td>
<td>Subacute rhinitis with mixed inflammation, mild</td>
<td></td>
</tr>
<tr>
<td>Hilar LN</td>
<td>Follicles active, T cell areas less active. Red pulp contains significant number of neutrophils.</td>
<td>Hyperplastic white pulp with neutrophil infiltrates of red pulp</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>Normal range</td>
<td></td>
<td>-ve</td>
</tr>
<tr>
<td>Liver</td>
<td>Normal range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung x 2</td>
<td>Terminal congestion and oedema, peribronchial / perivascular lymphocyte nodules</td>
<td>Previous antigenic stimulation, agonal oedema</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Normal range</td>
<td></td>
<td></td>
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</table>
### Appendix 6 - Summary of H/E staining following rhesus macaque necropsies

<table>
<thead>
<tr>
<th>Golf - Organs</th>
<th>Features</th>
<th>Interpretation</th>
<th>ZN</th>
</tr>
</thead>
<tbody>
<tr>
<td>R Internal Iliac LN</td>
<td>Some secondary follicles</td>
<td>Mild hyperplasia, B</td>
<td></td>
</tr>
<tr>
<td>L Inguinal LN</td>
<td>Sinus histiocytosis</td>
<td>Sinus histiocytosis</td>
<td>-ve</td>
</tr>
<tr>
<td>L Axillary LN</td>
<td>Expanded paracortex, focal histiocytosis</td>
<td>T-cell hyperplasia</td>
<td>-ve</td>
</tr>
<tr>
<td>L Retropharangeal LN</td>
<td>Expanded paracortex</td>
<td>T-cell hyperplasia</td>
<td>-ve</td>
</tr>
<tr>
<td>R Submandibular LN</td>
<td>Few secondary follicles</td>
<td>Mild hyperplasia, B</td>
<td>-ve</td>
</tr>
<tr>
<td>R Tonsil</td>
<td>Active follicles, neutrophil exocytosis</td>
<td>Hyperplasia with mild acute tonsillitis</td>
<td>-ve</td>
</tr>
<tr>
<td>L Turbinates / Nasal septum</td>
<td>Lymphocyte follicles, few plasma cells, histiocytes, eosinophils and neutrophils in the submucosa with eosinophil exocytosis.</td>
<td>Subacute rhinitis with mixed inflammation, mild</td>
<td></td>
</tr>
<tr>
<td>R Turbinates / Nasal septum</td>
<td>Lymphocyte follicles and a few histiocytes, eosinophils and neutrophils in the submucosa.</td>
<td>Subacute rhinitis with mixed inflammation, mild</td>
<td></td>
</tr>
<tr>
<td>Hilar LN</td>
<td>Expanded paracortex</td>
<td>T-cell hyperplasia</td>
<td>-ve</td>
</tr>
<tr>
<td>Spleen</td>
<td>Follicles and T cell areas active, Red pulp contains significant number of neutrophils and eosinophils. Macrosopic nodule structure identical.</td>
<td>Hyperplastic white pulp with neutrophil infiltrates of red pulp</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>Normal range</td>
<td></td>
</tr>
<tr>
<td>Lung x 2</td>
<td>Terminal congestion and oedema, peribronchial / perivascular lymphocyte nodules</td>
<td>Previous antigenic stimulation, agonal oedema</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td>Normal range</td>
<td></td>
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</tbody>
</table>
### Appendix 7 - Summary of H/E staining following rhesus macaque necropsies

<table>
<thead>
<tr>
<th>Gulp - Organs</th>
<th>Features</th>
<th>Interpretation</th>
<th>ZN</th>
</tr>
</thead>
<tbody>
<tr>
<td>L inguinal LN</td>
<td>Expanded paracortex</td>
<td>T-cell hyperplasia</td>
<td>-ve</td>
</tr>
<tr>
<td>R Axillary LN</td>
<td>Expanded paracortex, one with brown pigment (probably melanin)</td>
<td>T-cell hyperplasia</td>
<td>-ve</td>
</tr>
<tr>
<td>L Retropharangeal LN</td>
<td>Secondary active follicles. Paracortical expansion.</td>
<td>Mild hyperplasia, B and T</td>
<td>-ve</td>
</tr>
<tr>
<td>R Submandibular LN</td>
<td>Secondary active follicles. Paracortical expansion.</td>
<td>Mild hyperplasia, B and T</td>
<td>-ve</td>
</tr>
<tr>
<td>L Tonsil</td>
<td>Active follicles</td>
<td>Mild hyperplasia</td>
<td>-ve</td>
</tr>
<tr>
<td>L Turbinates</td>
<td>Few histiocytes, eosinophils and neutrophils in the submucosa.</td>
<td>Subacute rhinitis with mixed inflammation, mild</td>
<td></td>
</tr>
<tr>
<td>L Nasal Septum</td>
<td>Lymphocytes, histiocytes, eosinophils and neutrophils in the submucosa.</td>
<td>Subacute rhinitis with mixed inflammation, locally moderate</td>
<td></td>
</tr>
<tr>
<td>R Turbinates / Nasal Septum</td>
<td>Nodules of lymphocytes, histiocytes, eosinophils and neutrophils in the submucosa with exocytosis</td>
<td>Subacute rhinitis with mixed inflammation, locally moderate</td>
<td></td>
</tr>
<tr>
<td>Hilar LN</td>
<td>Paracortical expansion. Sinus histiocytosis</td>
<td>Mild T-cell hyperplasia and sinus histiocytosis</td>
<td>-ve</td>
</tr>
<tr>
<td>Spleen</td>
<td>Follicles and T cell areas active. Red pulp contains significant number of neutrophils.</td>
<td>Hyperplastic white pulp with neutrophil infiltrates of red pulp</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Capsule normal</td>
<td>Normal range</td>
<td></td>
</tr>
<tr>
<td>Lung x 2</td>
<td>Terminal congestion and oedema, peribronchial / perivascular lymphocyte nodules</td>
<td>Previous antigenic stimulation, agonal oedema</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td>Normal range</td>
<td></td>
</tr>
</tbody>
</table>