Understanding vaccine induced protective immunity to *Mycobacterium tuberculosis*

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Abstract

The current worldwide epidemic of *Mycobacterium tuberculosis* infection is a huge global health problem. Widespread BCG vaccination remains a useful tool in combating this epidemic; however, its variable efficacy requires urgent development of novel vaccines against *Mycobacterium tuberculosis*. Such a candidate vaccine is a serotype 5 adenovirus expressing antigen 85A from *M. tuberculosis* (Ad85A). In animal models Ad85A confers significant protection when administered intra-nasally. The work in this thesis demonstrates that intra-nasal immunisation with Ad85A results in inhibition of *M. tuberculosis* growth in the lung early after infection, in contrast to the late inhibition induced by parenterally administered vaccines. Early inhibition correlates with the presence in the lung of a highly activated population of antigen-specific CD8 T cells, maintained for at least 6 months post-immunisation by persistent antigen. For intra-nasal Ad85A to be effective, the vaccine must be delivered into the lower respiratory tract, as immunisation targeting only the nasal-associated lymphoid tissue (NALT) does not result in protection. Following a change of animal facility, the lung immune response to intra-dermal immunisation with Ad85A increased and this route of immunisation now induced protection, though growth of *M. tuberculosis* was inhibited only late after infection. However, this response and protection can be altered by exposure to environmental mycobacteria. Further experiments showed that simultaneous respiratory and parenteral immunisations (SIM) act additively, where local lung immunity inhibits the growth of *M. tuberculosis* early after infection and systemic immunity protects later. SIM regimes generate greatly improved protection over either immunisation alone and do not depend on priming and boosting.

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CHAPTER 1

Introduction

Chapter 1

Introduction

1.1 The tuberculosis burden

Epidemiology

Tuberculosis (TB) has been part of the human disease burden for thousands of years. Although the causative agent *Mycobacterium tuberculosis* (*Mtb*) was only identified by Robert Koch in the late 1800s, traces of mycobacterial DNA have been found in mummified remains from around the world, dating to before the start of the Common Era (1, 2). Moreover, the association of *Mtb* with humans extends considerably further (3), as evidenced by the apparent tracking of *Mtb* evolution to ancient human migrations (4).

Today the tuberculosis burden has reached pandemic proportions with approximately one third of the global population infected. The most recent available figures (2009) show as many as 9 million new cases and 1.8 million deaths due to TB per year, with the majority of cases occurring in sub-Saharan Africa and Asia (5). Once infected with *Mtb*, an individual has a 10% lifetime risk of developing active disease. However, this becomes a 10% annual risk if the individual is co-infected with HIV (6), thus making the co-pandemics of TB and HIV a huge global health crisis.

This extent of tuberculosis infection and disease is in spite of the availability of a vaccine, BCG, and effective antibiotic therapy. However, these interventions are clearly not sufficient; despite being the most widely used vaccine in history, with over 3 billion doses administered, BCG has considerable failings in preventing TB disease in adults, a problem discussed further in section 1.4. Antibiotic therapy of patients infected with *Mtb* is also problematic. Although *Mtb* is susceptible to a number of antibiotics (rifampicin, isoniazid, pyrazinamide), most treatment regimes require these to be taken together and for a number of months. Thus, the regime is expensive and patient compliance can be

low. In an attempt to improve compliance, Directly Observed Treatment and Short Course Drug Therapy (DOTS) was developed by the World Health Organisation (WHO), but despite this initiative there has been a rise in the incidence of multi-drug resistant tuberculosis (MDR-TB), resistant to at least 2 of the first line antibiotics (7). Of even greater concern is the rise of extremely drug resistant TB, or XDR-TB, resistant to even second line antibiotics. Infection with XDR-TB is almost untreatable and has mortality rates of greater than 90% in some patients (8).

Disease stages and diagnosis

Traditionally TB infection has been considered a biphasic disease; presenting as either chronic but largely asymptomatic latent tuberculosis infection (LTBI) or active disease, leading to the classic TB symptoms of a persistent cough, blood in the expectorate and cachexia. Latent infection is thought to be caused when *Mtb*-infected macrophages in the lung fail to clear the infection, thus inducing an adaptive immune response and the development of immunological memory. This immune response leads to the formation of one or more granulomata in the lung; distinct foci at the site of infection consisting of infected macrophages surrounded by other host cells including T and B cells, dendritic cells and endothelial cells (9, 10). Granuloma formation is thought to wall off the bacilli and provide an environment in which the immune system is able to prevent replication and dissemination. However, recent work has implied that granuloma formation may in fact aid mycobacterial expansion (11).

Of patients with LTBI, 90% go through life remaining in the latent stage. However, 10% progress to active disease. This is characterised by disruption of the granuloma, an increase in mycobacterial replication and up-regulation of the pro-inflammatory response. A rise in cell death in the granuloma leads to necrosis and caseation, followed by cavitation and dissemination of *Mtb* into the airway or periphery. Dissemination into the airway causes the symptoms of active disease and makes the patient

highly infectious, whereas systemic dissemination can lead to a range of pathologies throughout the body, including milliary TB or tuberculous meningitis.

However, rather than this binary, latent or active view of TB disease, the current consensus is that *Mtb* infection may exist as a continuous spectrum of bacterial replication and clinical symptoms (10, 12). Latent TB infection is often defined by the presence in an otherwise asymptomatic patient of an immune recall response to innoculation with mycobacterial antigens – a test known as the tuberculin skin test (TST). However this definition is merely sufficient to distinguish individuals who have made an adaptive immune response upon encounter of mycobacteria and gives no indication as to the extent of disease control. As such, a "latently" infected individual could have entirely cleared the infection, have controlled *Mtb* replication to a dormant, quiescent state or have actively replicating bacteria but not yet developed symptoms (12). Factors determining which of these phases of infection occurs within an individual are ill understood. Furthermore, it is becoming more evident that a range of disease stages and granuloma types can be found even within an individual patient (12).

This more complex and less defined disease structure means that accurate identification and diagnosis of TB infection becomes all the more important and challenging. As mentioned above, the conventional test for mycobacterial infection has been the use of the tuberculin skin test. However, the TST uses a range of mycobacterial proteins, known as purified protein derivative (PPD), containing peptides common to other mycobacterial species, including the BCG vaccine. Consequently, a positive TST is unable to accurately distinguish between prior BCG immunisation and *Mtb* infection, or whether that infection is active or latent. Recently, interferon gamma (IFNγ) release assays (IGRAs), such as T-SPOT.TB and QFT-G-IT, have been developed with the aim to diagnose *Mtb* infection by assaying the responses to the M*tb*-specific proteins early secreted antigenic target 6 (ESAT6) and culture filtrate protein 10 (CFP-10)(13). These proteins are encoded by the region of difference 1 (RD1) genomic segment, a region present in all virulent *Mtb* strains but absent from BCG. Therefore, these assays are able to differentiate *Mtb* infection from prior BCG immunisation. However, similarly

to the TST, IGRAs are unable to distinguish whether the patient has latent or active disease. A prospective solution to this problem may be the analysis of multiple cytokines in similar *in vitro* stimulation assays, an approach that has shown potential in some studies (14, 15). Intriguingly, recent work on transcriptional biomarkers appears to have the potential for identifying not only individuals who are latently infected with *Mtb* but also those whom are more likely to progress to active disease (16), which, if validated, could improve the way monitoring and treatments are targeted. However, all these assays require significant processing time. In order to truly improve the speed and accuracy of diagnosis, and so treatment, a test able to rapidly deliver a result at the bedside or during one clinic visit is necessary. A diagnostic tool with this potential has recently been developed: the GeneXpert MTB/RIF test is a nucleaic acid amplification test that has shown sensitivity greater than conventional smear tests at identifying active TB cases (17, 18), as well as determining their rifampicin susceptibility/resistance status, in a 15 minute assay. Such an assay could greatly improve diagnosis and time to treatment, although the major public health hurdle of distributing such technology would still need to be overcome.

1.2 Tuberculosis and the immune response

Innate immunity

Mtb is spread through the exhalation of aerosol droplets containing the mycobacteria that are subsequently inhaled by another individual. When the *Mtb* reaches the alveoli of the lung, it is phagocytosed by alveolar macrophages and dendritic cells. Phagocytosis is triggered by the binding of *Mtb* antigens to pattern recognition receptors (PRRs) on the surface of the phagocytes, such as C-type lectins. The ligands for these receptors are thought to be motifs found on the lipoproteins and glycolipids of the mycobacterial cell wall (9, 19). The binding of polysaccharide mannose motifs to the mannose receptor (MR) expressed on alveolar macrophages is known to induce phagocytosis, as is the binding of opsonised *Mtb* to complement receptor 3 (CR3) (19, 20).

Other important PRRs expressed on innate cells are the toll like recpetors (TLRs) and the C-type lectins Mincle and DC-SIGN. TLRs are a family of receptors expressed on the cell membrane or in intra-cellular vesicles of which 10 have been discovered in humans and 12 in mice (21). Each type of TLR detects specific motifs from micro-organisms, such as double stranded RNA, lipoprotein or other structural motifs. The TLRs involved in the recognition of *Mtb* are thought to be TLR2, TLR4 and TLR9, although, as TLR2 forms heterodimers with TLR1 and TLR6, these may also play a role (19). Binding of TLRs leads to the activation of myeloid differentiation primary response protein 88 (MyD88) and a signaling cascade resulting in activation and nuclear translocation of the transcription factor NF-κB (19, 21). NF-κB activation induces a pro-inflammatory immune response in the innate cells, primarily consisting of production of interleukin (IL)-12, IL-6, IL-1β, tumour necrosis factor (TNF)α and nitric oxide. The importance of this MyD88-induced pro-inflammatory response is demonstrated by the highly increased susceptibility of MyD88-knockout mice to *Mtb* infection, corresponding with a reduction in inflammatory cytokine production (21, 22). However, although this would suggest that TLR signalling is vital for host defenses against *Mtb*, knockout studies have shown conflicting results, some indicating that mice deficient in TLR 2, 4 or 9 are no more susceptible than controls, whereas others show an increase in mycobacterial load (23, 24). This could indicate some redundancy between the TLR responses to TB, although when mice deficient for all of TLR 2, 4 and 9 were challenged with *Mtb* they were able to control the infection (25). In the same study MyD88-knockout mice showed highly increased susceptibility indicating that other signalling pathways using this adaptor molecule may be able to induce macrophage effector mechanisms.

Mincle and DC-SIGN are C-type lectins expressed predominantly on macrophages and dendritic cells respectively. Mincle, or macrophage inducible C-type lectin, has recently been described as the essential receptor for trehalose-6,6'-dimycolate (TDM), otherwise known as cord factor. Binding of TDM to Mincle activates macrophages to secrete pro-inflammatory cytokines and produce nitric oxide. Notably, these anti-mycobacterial responses are lost in in Mincle-deficient mice, as is the ability to

form granulomata (26). DC-SIGN, or dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin recognises the mannose containing Man-LAM glycolipid. Unlike the pro-inflammatory response induced by TLR signalling, binding of Man-LAM to DC-SIGN is thought to induce an anti-inflammatory response, including an increase in expression of IL-10 (19). Notably, this regulatory signal may moderate the pro-inflammatory response induced by TLR signalling by altering the activation state of NF-κB (27).

Following phagocytosis, *Mtb* is able to reside in the phagosome, where it persists and replicates. However, upon engulfing the mycobacteria, an infected macrophage has a number of defences. As discussed above, binding of TLRs by mycobacterial ligands induces the production of reactive nitrogen intermediates such as nitric oxide by the host macrophage. Furthermore, the acidification of the phagosome disrupts mycobacterial growth and potentiates the actions of microbicidal effector molecules (9). Upon activation by IFNγ or TNFα signalling, the infected phagosome fuses with the lysosome, resulting in destruction of the mycobacteria and presentation of antigens to T cells through major histocompatibility complex (MHC) class II molecules. Activation by IFNγ and nitric oxide production is also thought to induce apoptosis of infected macrophages, leading to death of the mycobacteria (28). Apoptosis also allows the uptake and cross-presentation of *Mtb* antigens by dendritic cells, furthering the activation of T cells through MHC class I presentation.

Although activated macrophages have these mechanisms for destroying infecting mycobacteria, *Mtb* is an intra-cellular organism and, as such, has developed strategies to subvert this response in order to persist and replicate in resting macrophages. In particular, *Mtb* is adept at preventing the fusion of the phagosome and lysosome, thus avoiding the primary antimicrobial defence. The exact mechanism by which *Mtb* manipulates the host machinery to avoid fusion is unknown, although it appears to involve the hydrolysis of phosphatidylinositol 3-phosphate (PI3P), a host molecule necessary for maturation of the phagosome/lysosome fusion process (29). *Mtb* is then able to replicate in the phagosome until the point that it escapes into the host cell cytoplasm. This escape is mediated by a secretion system known

as the ESX-1secretion system. The ESX-1 secretion system is encoded by RD-1, similarly to ESAT6 and CFP-10 (see above), both of which are secreted by ESX-1. Whilst ESAT6 and CFP-10 are known to be essential for phagosomal escape by mycobacteria (30), the exact mechanism is unknown, although it may involve the ubiquitination or disruption of the lipid membrane leading to lysis and mycobacterial escape (31). Once in the cytosol, *Mtb* induces necrosis of the infected macrophage, causing the release of multiplying mycobacteria and allowing further infection and destruction of other macrophages (9).

As well as dendritic cells and macrophages, other cells of the innate system play important roles in the host response to *Mtb* infection. Natural killer (NK) cells are able to kill infected cells through direct cytolysis and also produce pro-inflammatory cytokines, particularly IFNγ, capable of activating infected macrophages to induce the microbicidal mechanisms outlined above. It has also been hypothesised that IL-22 production by NK cells promotes phagosome-lysosome fusion, inhibiting the growth of *Mtb* (32). However, other work has indicated that the NK response to mycobacterial infection has little effect on bacterial load (33). Neutrophils are another host cell subset important in the response to *Mtb* infection. However, whether this role is predominantly protective or pathological is unclear. Recent work has demonstrated that uptake of infected neutrophils by dendritic cells accelerates the adaptive immune response thus increasing protection (34) and others have shown that neutrophils are able to partially compensate for a lack of IFNγ production (35). Conversely, other work has shown that neutrophils do not restrict mycobacterial growth (36). Whatever the *in vivo* role of neutrophils, recent transcriptional analysis has shown that a neutrophil driven signature may be able to differentiate active and latent disease, although further validation is necessary (16).

Adaptive immunity

Although the above innate immune responses play a significant role in controlling *Mtb* infection, and may in some case be able to clear the infection entirely, the adaptive immune system remains a vital

component of the host defenses. The adaptive immune response is dependent on infected antigen presenting cells (APCs), such as macrophages and dendritic cells, migrating to the draining mediastinal lymph node and presenting to circulating naïve T cells. However, as discussed above, *Mtb* has evolved a number of immune-modulatory mechanisms with which to disrupt this process. In the course of most respiratory infections, activation of CD4 and CD8 T cells occurs approximately 3 days following infection. However, in the case of *Mtb* infection, the response is delayed in mice until approximately 9 days post-infection and activated T cells are not detected in the lungs until 14 days (37-39). Whether the delay in activation is due to the low infectious dose or active inhibition of the innate system by *Mtb* is unclear. This delay in activation becomes significant when the importance of adaptive immunity in protection from *Mtb* is understood.

CD4 T cells are a subset of T cells that respond to antigen peptides presented by MHC class II molecules on the surface of APCs, recognised by the binding of a specific αβ T cell receptor and CD4 co-receptor. The importance of CD4 cells in mediating protection during *Mtb* infection is highlighted by the greatly increased susceptibility of HIV+ patients, an increase in risk that inversely correlates with the patient's peripheral blood CD4 count (40). Furthermore, depletion and adoptive transfer of the CD4 subset in mice infected with *Mtb* highlights their importance in protection (41, 42). Upon recognition of their cognate peptide, CD4 cells become activated and can differentiate into a number of subclasses, as defined by cytokine expression and transcription factor activation. In *Mtb* infection the major protective CD4 phenotype is the T helper 1 (Th1) CD4 T cell. Th1 cells are characterised by IFNγ production and the translocation of the transcription factor signal transducer and activator of transcription 4 (STAT4) (43). It is thought that IFNγ production by CD4 Th1 cells upon recognition of infected macrophages activates the phagocytes and induces the protective antimicrobial responses outlined above. The importance of IFNγ in mediating protection is highlighted by the increased susceptibility to mycobacterial disease of patients deficient in components of the IFNγ receptor (44), as well as those deficient in IL-12 mediated signaling pathways, a critical factor in promoting Th1

differentiation and IFNγ production by CD4 cells (45). However, other work has demonstrated IFNγ mediated protection independent of CD4 cells (41), as well as CD4 mediated protection in the absence of IFNγ (46), indicating that other protective mechanisms are capable of inhibiting mycobacterial growth.

Another cytokine thought to play a significant role in the immune response to *Mtb* infection is tumour necrosis factor alpha (TNFα). As well as being produced as part of the innate response, this cytokine is a significant product of the adaptive immune response to TB. Similarly to IFNγ, TNFα signalling activates macrophages, inducing the protective maturation of the phagosome and also appears to be necessary for formation of organised granulomata and prevention of subsequent caseation (47, 48). Mice deficient in the TNF receptor or depleted of TNFα show an enhanced susceptibility to *Mtb* infection, further highlighting its importance (49). The use of TNFα-blockade in the treatment of rheumatoid arthritis allows investigation of what is *de facto* depletion in humans. These investigations have shown an increase in reactivation of latent TB in patients receiving anti- TNFα treatment (50), indicating the importance of TNFα in host defence against *Mtb* infection across species.

As well as which effector cytokines are produced, recent investigation has highlighted the significance of so-called multifunctional T cells. These are individual cells producing multiple different cytokines. Work on another intra-cellular pathogen, *Leishmania sp*, showed that vaccine induced CD4 cells producing IFNγ, IL-2 and TNFα simultaneously correlated with protection (51). Further work has gone on to describe the importance of multifunctional T cells in other infections, including TB (15, 52). However, the data are contradictory, with only some work indicating a correlation with vaccine induced protection (53, 54). Importantly, recent investigation into BCG induced responses indicates that induction of multifunctional T cells gives no indication of future susceptibility to TB (55). It should be noted that this paper also indicates that measurement of T cell cytokine responses does not predict the ultimate risk of TB disease.

As well as the Th1 subset, other sets of CD4 T cells play important roles in TB immunity. These include the Th17 and T-reg cells. Th17 cells are a recently described subset of cells characterised by the production of IL-17, IL-23 and activation of the RAR-related orphan receptor gamma T (RORγt) transcription factor. Th17 cells were initially defined as potent pro-inflammatory cells causing autoimmune diseases such as experimental autoimmune encephalomyelitis and human demyelinating diseases (56). However, as such potent pro-inflammatory cells, their role in infection was soon investigated, particularly in TB where the balance between a protective response and immuno-pathology is so important. Although IL-17 was shown to be required for protection against extracellular bacterial pathogens, the effect was less pronounced for intracellular pathogens, although the small effects observed were associated with reduced neutrophil recruitment (57). In a mouse model of *Mtb* infection, IL-17 is produced early after challenge, although in this case production is dominated by γδ T cells (58). Th17 cells have been shown to play an important part in the recall response in the lungs of vaccinated mice following *Mtb* challenge, aiding the recruitment of protective Th1 cells (59). However, mice deficient in IL-23 signalling do not produce IL-17 but show no increase in susceptibility (60), indicating the exact role of Th17 cells in *Mtb* infection requires futher investigation.

T-reg cells are another subset of CD4 cells, defined by expression of CD25 and the transcription factor forkhead box p3 (FoxP3), although other markers are sometimes used. Expression of IL-10 by T-regs has the effect of moderating the Th1 (and possibly Th17) response, reducing the production of IFNγ (61). In *Mtb* infection, inhibition of IL-10 increases the Th1 response and leads to a reduction in mycobacterial load (62). Furthermore, when T-reg cells are depleted prior to TB challenge, mice also show a reduction in colony forming units (CFU) of *Mtb* in the lung (63). However, although T-regs appear to be detrimental to the host in allowing mycobacteria to persist at higher levels, it must be remembered that they play an important part in avoiding immuno-pathology. The importance of regulation of the immune response is evident in cases when that regulation has been removed.

Programmed death-1 (PD-1) is a molecule known to down regulate the CD4 immune response and blockade of PD-1 has been shown to benefit the host in some viral infections (64). However, if PD-1 deficient mice are infected with *Mtb* they demonstrate a significant increase in pro-inflammatory cytokines, lung bacterial load, immuno-pathology, and die considerably earlier than controls (65). Furthermore, this increase in pathology and susceptibility is driven by the CD4 response (66). At this juncture it is worth noting that, although a pro-inflammatory response is required for destruction of *Mtb*, the mycobacterium itself needs the immuno-pathology and granuloma caseation driven by the same response before it can spread to another host. Therefore both host and mycobacteria are fighting to shift the balance of inflammatory and regulatory immune responses in their favour.

Although classically it has been thought that a CD4 Th1 response is the mediator of protection, recent work has shown the importance of *Mtb*-specific CD8 T cell responses during infection. CD8 T cells recognise antigenic peptides derived from the cytosol and presented on the MHC class I molecules of host cells. Upon recognition of their cognate peptide, activated CD8 cells have a range of potential effector functions, including cytokine production similar to CD4 cells or direct killing of cells through cytotoxic mechanisms. Initial work into the role of CD8 T cells in protection from TB showed mice depleted of CD8 cells exhibited no change in mycobacterial growth (67). Equally, adoptive transfer of memory CD8 T cells conferred no increase in protection (68). In both cases, the equivalent procedure with CD4 cells did affect the course of infection. However, recent work has demonstrated that CD8 cells are an important component of a protective response, particularly following vaccination. In mice, CFP-10-specific vaccine-induced CD8 T cells are sufficient to confer protection (69) and in non-human primates depletion of CD8 cells following BCG vaccination led to increase pathology and mycobacterial load (70). However, it should also be noted that, similarly to CD4 T cells, CD8 cells have been reported to contribute to immuno-pathology in cows through increased IFNγ production (71). CD8 cells are also present in significant numbers in granulomata (12), involved in the activation of macrophages through cytokine secretion alongside their cytotoxic role. Increasingly, therefore,

CD8 T cells are being recognised as an important subset in immune responses to *Mtb* infection, although their precise role and mechanism of action has yet to be fully elucidated.

As well as classical T cells, non-conventional T cells are now coming to be recognised as important subsets in *Mtb* infection. As mentioned above, γδ T cells have been identified as a source of IL-17 production early after infection, possibly a requirement for mature granuloma formation (72). Direct cytolysis of mycobacterially infected phagocytes by γδ T cells has been reported (73) and mice deficient in γδ T cells exhibit increased susceptibility to *Mtb* infection (74). Another recently described subset of non-conventional T cells is the mucosally associated invariant T cells (MAIT cells). Restricted to MR-1, these cells are enriched in the lungs of active TB patients, where they have been detected within the lesions (75, 76). In particular, MAIT cells have been shown to produce TNFα in response to *Mtb*-infected lung epithelial cells (75). However, the precise role of these newly described cells in *Mtb* infection remains to be elucidated.

1.3 Animal models of tuberculosis infection

In order to further understand the immune response to *Mtb* infection as well as determine the importance and function of different subsets within that response, accurate animal models are needed. As well as aiding the understanding of the primary host immune response, these models are just as important for the analysis of potential vaccine candidates and therapeutic interventions. However, as described above, TB is a complex disease with a range of disease stages characterised by different types of granulomatous lesions involving various levels of necrosis, fibrosis and cell infiltration. Therefore, developing an animal model that is able to reproduce these conditions whilst remaining manageable for laboratory work, both in terms of cost and available techniques, is a major challenge for TB research.

Mice

The laboratory mouse is a well used animal model across a range of immunological and genetic fields. As a result a wide range of tools are available for the investigation and manipulation of mice as a model, thus further potentiating their common usage. Much of the work discussed above investigating the roles of various immune cell subsets was performed in the mouse model, whether by generating specific gene knockout strains or by otherwise depleting or enhancing the presence or effect of each subset. This has allowed high resolution analysis of various aspects of the host immune response to *Mtb* infection. However, the mouse model does present problems. Unlike humans, mice exposed to pulmonary *Mtb* infection all become infected and if left untreated will control the mycobacterial burden after approximately 2-3 weeks, at which point *Mtb* load will remain at a high but maintained set point for approximately 1 year until mice succumb to infection and die (77). This is contrary to human disease, where the majority of individuals infected contain *Mtb* in latent disease, often to the point that few or no mycobacteria can be detected, and only a small proportion progress to active disease. The type of lesions developed in mice during infection is also different. The classical human lesion, found in active and latent disease, consists of a central hypoxic caseous/necrotic region surrounded by macrophages and neutrophils with a cuff of lymphocytes (12). In mice, however, the granuloma structure is less organised, more cellular and non-necrotic. It is therefore argued that the mouse model is not comparable to human infection, particularly latent disease, but rather that infection in mice is a chronic disease. One attempt to circumvent this problem is the Cornell model of infection. In this model, infected mice are treated with antibiotics 12 weeks post-infection. As a result, mycobacterial load can be reduced to levels at which they can no longer be cultured or isolated from the infected mice. However, if the mice are then left without further treatment, approximately one third of these mice spontaneously develop TB disease. Furthermore, if the other two thirds of the antibiotic treated mice are immuno-suppressed almost all go on to develop TB disease (78). It has thus been

argued that antibiotic treatment induces a form of latent infection in the mice that is comparable to human latency, although the true validity of this model is still debated.

Guinea pigs

The other major small animal model for modelling *Mtb* infection is the guinea pig. This model differs from the mouse model in the type of lesion that is formed following infection with *Mtb*. Rather than the disorganised, non-necrotic lesion seen in the mice, guinea pig granulomata more closely resemble those observed in humans, developing into caseous, cavitating lesions in late stage disease. As such, the guinea pig model is often seen as a more rigorous and relevant small animal model for the testing of human vaccine candidates and therapeutics. This is particularly the case for agents designed to treat latent infection, once granulomata have formed (78), although true latent TB does not occur in guinea pigs. However, the guinea pig model lacks the ease of manipulation available in the mouse model, with far fewer immunological reagents and no genetic knockouts or transgenics available. Thus, although guinea pig lesions may be more comparable to human disease, the processes behind their formation are more difficult to elucidate. However, the validity of the guinea pig model has been questioned. Guinea pigs are highly susceptible to *Mtb*, with only one or two mycobacteria sufficient to establish lethal infection. This increased susceptibility has been associated with increased eosinophil recruitment and degranulation in guinea pig granulomata leading to early necrosis (79). Hence, the high susceptibility of guinea pigs to *Mtb* may be due to an immunological deficit of sorts, thus questioning the legitimacy of the model (79).

Cows

Rather than being a bespoke model of human *Mtb* infection, bovine tuberculosis represents a disease burden in its own right, with a huge impact on agricultural economies. Caused by *Mycobacterium bovis* rather than *Mtb*, bovine TB is a natural host-pathogen infection, rather than an experimental model *per se*. The immunological responses in cows following *M. bovis* infection show many

similarities to the responses seen in human infection, both in terms of innate pathogen recognition and cell mediated immunity (80). Granuloma formation and structure follow similar patterns to that seen in humans, with a range of heterogeneous caseous granulomata detectable in the lungs and lymph nodes of individual cows. Furthermore, as the granulomata age some progress to liquefaction, as seen in humans, although this appears only to occur in advanced disease (81). In terms of latent infection, exposed but skin test negative cows have developed mycobacterial reactivity many years after exposure, despite no further contact with infectious individuals, implying that latent infection can occur naturally if not experimentally (81). As well as being a useful model for examining primary responses to infection, the experimental bovine TB system can be used to assess human vaccine candidates. As discussed below, BCG vaccination of children shows variable efficacy across individuals and populations, a problem that, as yet, has no definitive explanation. BCG vaccination of neonatal calves has similar variability in efficacy, although the cause of this variation is equally unknown (82). A further parallel between cow and human responses to BCG vaccination is that skin test responses following BCG do not correlate with protective immunity (83, 84). These similarities in response to BCG indicate that the bovine model may present a realistic model for other candidate human vaccines, as well as highlighting the potential value of vaccine research in either species to those working on the other (84, 85). A small caveat with assessing human vaccine efficacy in cows is that a different challenge organism is being used in the bovine system. Although *M. bovis* has considerable similarity to *Mtb*, the two organisms are known to have phenotypic differences. For example, although *M. bovis* and *Mtb* cause similar pathologies in their own host species, cows and humans respectively, each is significantly less virulent when infecting the opposing species (80, 86). This may particularly affect the assessment in cattle of human vaccines based on attenuated strains of *Mtb*, where the immunogenicity may be somewhat diminished in cattle compared to humans (80). However, the other similarities of the cattle-*M. bovis* model to human disease, alongside the availability of a wide range of immunological reagents with which to manipulate and study the

response, means that it is a valuable experimental model for human TB, as well as for bovine TB disease itself.

Non-human primates (NHPs)

The closest experimental model to human TB disease is *Mtb* infection in primates. Upon low dose pulmonary infection, outbred cynomolgus macaques develop a range of disease similar to that seen for a human population, with some developing active disease and other TST positive animals remaining free of clinical symptoms (87). The pattern of lesions and granulomata also resemble the range observed in human TB patients (88), with granuloma formation showing similar cytokine dependencies as observed in humans (89). Primate models also have the advantage of being able to assess concurrent infection with *Mtb* and simian immunodeficiency virus (SIV), the simian analogue of HIV, allowing the elucidation of one infection's effect on the other (90). As these models are deemed so similar to human disease, analysis in primates is often the final stage of assessment of any therapeutic or vaccine against human TB before they progress into clinical trials, particularly as more immunological reagents for macaques become available. However, although a rigorous model, significant problems remain with the NHP model, including the possibility of transmission between individuals in a colony, ethical considerations and the almost prohibitive cost (87).

Of the models discussed above, as well as others, the one chosen by any particular investigator is dependent on a number of variables, including cost, the hypothesis being investigated and the readout desired. Although primates allow the most accurate representation of TB disease on the scale of an individual or group, if cellular dynamics are being investigated then the imaging advantages of the zebrafish-*M. marinum* model would probably be preferred. Equally, the genetic and immunological manipulability of the mouse model may make that the model of choice. As said elsewhere, a good model should restrict the variables of a system such that the overall context remains but hypothesis testing becomes clearer (91).

1.4 Vaccines against TB

BCG

As mentioned above, the current global TB burden is despite the availability of a vaccine: the attenuated *M. bovis* Bacille Calmette-Guérin, or BCG. BCG is the only licensed TB vaccine and has been given to over 3 billion people. It is given at or soon after birth in countries with endemic TB disease. However, although BCG vaccination protects infants from severe disseminated disease as well as lung disease, this protection falls over time until by late adolescence much of the effect has waned (92). The efficacy of BCG vaccination across populations is also variable, ranging from 80-90% in Northern America and Europe, to 0% efficacy in parts of Asia and sub-Saharan Africa (93). The cause of this variation is as yet unknown, although there are a number of hypotheses, of which any or all could be playing a part. One hypothesis is that co-infections with other microorganisms may be affecting the response following BCG immunisation, specifically co-infection with helminths. Helminth infection is thought to bias the immune system to a T helper 2 (Th2) type response, away from the Th1 response thought to protective against TB. As helminth infection is common in regions in which BCG efficacy is poor (94) and chemotherapy for helminth infection has been shown to lead to improved responses to subsequent BCG (95) it is possible that co-infections with helminths may be playing a part in the variable efficacy of BCG. However, this requires further investigation, particularly as no work has been done on the effect of helminth infection on prior BCG vaccination.

Another explanation or contributing factor to the variation in the efficacy of BCG vaccination is the degree of exposure of the individual to other species of *Mycobacteria*. Largely non-pathogenic in immuno-competent individuals, non-tuberculous mycobacteria (NTM) exist in soil or water and are more common at the lower latitudes and around the tropics where BCG efficacy is most variable. Furthermore, children from these regions show greater immune responses to NTM compared to those in Britain, where BCG is highly effective (96). Exposure to NTM could increase variation in two

ways: by conferring a greater degree of natural immunity to *Mtb* infection, thus making it more difficult to observe an improvement in protection conferred by BCG – an effect termed "masking" – or by inducing an immune response that kills or inhibits the growth of BCG, thus reducing its efficacy as a vaccine – termed "blocking". Studies have shown both that blocking can occur, with NTM-specific responses inversely correlating with BCG response (97), but equally that pre-existing NTM immunity has no effect on the magnitude of response to BCG (96). However, both these studies measured the IFNγ response following BCG, a response that has been shown not to correlate with future risk of acquiring *Mtb* infection (55). Equally, as BCG is often given at or soon after birth, prior exposure to NTM would be limited. Further investigation into the effect of maternal exposure to NTM or other infections is needed (98), as well as the effect of NTM exposure on pre-existing BCG induced responses.

It is widely recognised that improvement is needed over the current BCG vaccine. As BCG provides protection against more severe forms of TB disease in infants and the infrastructure and logistics for its distribution are well established, it is likely that any improvement will involve BCG or a modified version. Boosting BCG with a further homologous BCG immunisation shows no improvement in risk of acquiring infection (99, 100) and induces only a modest increase in response (101), possibly due to inhibitory effects of the primary vaccination. As such, other strategies for improving over BCG are needed. These fall generally into one of two categories: boosting prior BCG vaccination with a heterologous subunit immunisation or developing new strains of attenuated mycobacteria which could replace BCG.

Subunit vaccines

Subunit vaccines are designed to boost the immunity induced by a prior BCG priming immunisation by delivering antigens common to BCG and *Mtb* in a heterologous prime-boost regime. By potentiating the response to a particular antigen or antigens, as well as by modulating the type of response induced, these vaccines hope to improve the degree of protection conferred against TB disease. Subunit vaccines can be administered either as protein plus adjuvant or delivered within a viral vector.

Protein subunit vaccines can consist of whole individual mycobacterial proteins, individual peptides or fusions of multiple antigens. These are then formulated with an adjuvant designed to act either as a vehicle, as a depot for the antigen or as an immuno-modulatory agent, or a combination of all three (102). Currently alum (and its derivatives) and oil and emulsion are the only adjuvants licensed for human use but a range of others are in development, from cytokines to bacterial components to particle delivery systems (102). At the time of writing, the TB vaccine using protein plus adjuvant furthest through the clinical trial process is M72 plus AS02A. This fusion protein consists of two *Mtb* antigens, Mtb32 and Mtb39, formulated with a proprietary adjuvant containing a detergent QS-21 and monophosphoryl lipid A (MPL), a bacterial derivative known to induce TLR4 signalling (94, 103). M72 conferred an increase in protection over BCG when administered as a boost in animal models (104, 105) and has completed Phase IIa trials in South Africa and the Philippines (94).

Two other protein based vaccine candidates that have entered clinical trials are Hybrid 1 and HyVac4. Hybrid 1 is a fusion protein consisting of antigen 85B and ESAT6, and HyVac4 is a fusion of 85B and TB10.4 (106, 107). HyVac4 was developed from Hybrid 1 in an attempt to avoid conflict with TB diagnostic assays based on immune responses to ESAT6 (see section 1.1). In animal models both vaccines have shown efficacy when adjuvanted with MPL along with cationic lipid vesicles (106, 108, 109). Both vaccines have also been used with the adjuvant IC31, a proprietary adjuvant containing a

TLR9 nucleotide agonist and a cationic peptide, and have shown protection in animal models (107, 110). Currently, these formulations of Hybrid 1 and HyVac4 with IC31 have either recently finished or are undergoing early clinical trials (94). A further iteration of these hybrid fusion vaccines has also recently begun development. H56 is similar to Hybrid 1, containing 85B and ESAT6 but it also includes Rv2660c, a latency-associated antigen (111). H56, formulated with a cationic liposome adjuvant CAF01, has shown protection as a prophylactic vaccine when given alone and or as a boost to BCG, with increased efficacy over Hybrid 1 observed late after challenge (111). Interestingly, when administered post-antibiotic treatment in an adapted Cornell model of infection, H56 also showed efficacy as a therapeutic vaccine, indicating its potential as a post-exposure intervention (111).

Other subunit vaccines in development use recombinant viral vectors to deliver antigens. Viral vectors have become an increasingly popular method for vaccine delivery for a number of diseases as they are capable of inducing potent cell mediated responses to vectored antigens as well as generating high antibody titres (112). In terms of TB vaccine development, the most common vectors used are recombinant adenoviruses and modified vaccinia virus Ankara (MVA). These vectors allow delivery of a particular antigen or antigens whilst utilising the natural tropism and adjvanticity of the viral vector.

Of the adenovirus based viral vector vaccines currently in development, the furthest advanced is the Aeras-402 vaccine. This is a serotype 35 adenovirus containing 3 *Mtb* antigens as a fusion protein, 85A, 85B and TB10.4. This vaccine was protective when given intra-nasally or intra-muscularly to mice (113) and is currently being evaluated in Phase II clinical trials in Kenya and South Africa, where it has induced significant CD4 and CD8 T cell responses detectable in the blood up to 6 months post-immunisation (94, 114). Interestingly, by basing this vaccine on serotype 35 the investigators hope to avoid potential problems with anti-vector immunity common in the human population (see below).

Another adenovirus based TB subunit vaccine is Ad85A. This is based on a human serotype 5 replication deficient adenovirus containing antigen 85A. Ad85A will be further discussed below but it should be noted here that is currently undergoing a Phase I first-in-man study in Canada to evaluate its safety and immunogenicity in humans.

Of the current viral vector based vaccines in clinical trials, the most advanced is MVA85A, consisting of the replication deficient vaccinia virus containing antigen 85A. MVA85A effectively boosts antigen-specific T cell responses when given as a parenteral boost to BCG in animals and humans (115-118). In mice, MVA85A improved protection over BCG alone when both immunisations were administered to the respiratory tract (119). However, when administered parenterally as a boost to parenteral BCG to mice no improvement in protection was detected (120). In cattle and non-human primates any improvement in protection has been weak or absent (116-118). Extensive trials have been performed using MVA85A administered parenterally in both the UK and Africa and a Phase IIb efficacy study is currently underway in South Africa (94).

Mycobacterial vaccines

Rather than boosting the response to BCG antigens using subunit vaccines, another strategy for improving vaccine efficacy has been to use recombinant BCG or attenuated strains of *Mtb*. The initial attenuation of *M. bovis* to BCG was not targeted to particular virulence factors, so re-introduction of antigens lost during this attenuation may enhance the immunogenicity of BCG without increasing virulence. Likewise, BCG was not designed to induce a specific profile of response, opening the possibility that recombinant strains could improve the balance of vaccine induced immunity. For similar reasons, targeted attenuation of *Mtb* also has the potential to generate vaccine strains that induce responses to a broader range of infectious strains, thus conferring greater protection.

rBCGΔureC:Hly, one of the recombinant BCG strains, has not had any mycobacterial antigens re-introduced but rather has been designed to improve the type of response induced by BCG. Hly, or

listeriolysin from *Listeria monocytogenes*, enables *Listeria* to escape the host phagosome. When included in recombinant BCG, Hly promotes the translocation of BCG antigens to the cytoplasm, thus increasing the presentation of these antigens to CD8 T cells (121). Furthermore, by upregulating the rate of apoptosis of infected macrophages, Hly is thought to increase the cross-priming of CD8 cells by dendritic cells (121). By improving the CD8 response to BCG, it was expected that this recombinant BCG would confer greater protection than the parental BCG strain, a hypothesis borne out in mouse models (120, 121). This vaccine is currently progressing through Phase I safety trials in South Africa (94).

Similar phagosome-disrupting recombinant BCG strains have also been developed. The AFRO-1 recombinant BCG expresses perfringolysin rather than Hly to potentiate endosomal escape (122). As well as perfringolysin, this strain over expresses three mycobacterial antigens common to BCG and *Mtb*, 85A, 85B and TB10.4 (122). By over expressing these antigens and improving cross-priming this vaccine has demonstrated improved immunogenicity in mice and guinea pigs; however this did not result in improved protection over the parental BCG strain in mice (122). A Phase I clinical trial of this vaccine will soon be starting in healthy subjects in the USA (94).

Rather than re-introducing antigens to BCG, another strategy has been to attenuate *Mtb* so that it can replace BCG. This has advantages in possibly conferring greater protection due to greater similarity between vaccine and pathogen, and attenuated pathogens have been used successfully as vaccines for other diseases. However, attenuated strains must present no risk of spontaneous reversion to virulence and therefore should contain at least two attenuating mutations (92). The SO2 strain of attenuated *Mtb* is PhoP deficient, a transcription factor thought to be required for virulence factor expression (123). This strain is more attenuated than BCG in immuno-deficient mice and is highly immunogenic (116, 123). However, although a further attenuated mutation is in development (92), SO2 is no more protective than the parental BCG strain in mice, guinea pigs or monkeys (116, 123).

A further strategy has been to use inactivated mycobacteria as a therapeutic vaccine and adjunct to chemotherapy. Immunisation of newly diagnosed TB patients with inactivated *M. vaccae* aimed to boost the host mycobacteria-specific response during chemotherapy, thus accelerating the time to effective treatment (124). Initial trials demonstrated that a single *M. vaccae* immunisation had no effect on the time to negative culture (124). However, in a recently completed Phase III trial multiple immunisations of HIV-positive patients led to improvement in definite TB disease diagnosis rates, although no effect was seen on disseminated or probable TB disease rates (125).

Although using recombinant BCG or attenuated *Mtb* strains in place of the current BCG vaccine presents some interesting possibilities, some caveats are needed. Any new live mycobacterial vaccine would need to be safe in HIV positive and immuno-compromised individuals, a quality not displayed by BCG. It is possible that strains more attenuated than BCG but equivalent in terms of protection, such as SO2 or rBCGΔureC:Hly, may be useful in allowing a greater proportion of people to receive vaccination. However, the costs of developing a new vaccine that did not improve an individual's protection would need to be justified. The use of attenuated *Mtb* strains as potential vaccines has further problems, as ensuring spontaneous reversion of attenuating mutations cannot occur would need to be conclusively proven. Furthermore, until more accurate diagnostics for *Mtb* infection are available, inclusion of *Mtb* variants as part of the vaccine program will most likely hinder the differentiation of vaccinated and infected individuals. Despite this however, recombinant mycobacteria technology presents exciting possibilities for development of improved vaccines, particularly if these are used alongside the candidate subunit vaccines described above.

1.5 Adenoviruses and Ad85A

Adenoviruses as viral vectors

Adenoviruses are double stranded DNA viruses that can cause mild upper respiratory or gastro-intestinal infections, although more serious infections can occur in immuno-compromised individuals.

In humans there are 6 subclasses (A-F) into which the various serotypes are divided based on sequence homology (126). The viral genome is split into a number of early and late units expressed over the course of infection. Of these, the early 1 (E1) unit is necessary for replication as it initiates viral DNA synthesis, as well as inhibiting the immune response to adenoviral infection. Adenovirus entry is most commonly mediated by binding of the viral fiber knob to the coxsackie-adenovirus receptor (CAR) expressed on host cells, followed by interaction of the penton base with host integrins leading to internalisation (127). Upon host cell entry, adenoviruses escape the endosome and locate to the nucleus, where replication begins (127).

Adenoviruses were initially developed as potential gene therapy vectors, particularly for diseases of the lung due to the natural tropism of adenoviruses to the respiratory tract. Deletion of the E1 unit ensured replication deficiency and further deletion of E3 in subsequent generations of vectors allowed large transgenes to be inserted. Treatment of cystic fibrosis using adenoviral transfer of the functional cystic fibrosis transmembrane regulator (CFTR) gene initially showed promise in restoring function, although ultimately problems arose due to increased inflammation and lack of persistent gene expression (128). However, these qualities were seen as potentially beneficial for effective vaccine delivery and consequently adenoviruses have become more popular for this purpose.

Adenoviruses are highly immunogenic, able to induce maturation of dendritic cells as well as induce pro-inflammatory cytokine secretion through a MyD88 dependent signalling pathway (129). Replication deficient adenoviral vectors have had E1 and often E3 deleted, both of which are important for virus immune evasion. As such, these vectors retain the highly immunogenic characteristics of wild type viruses. The E3 unit actively interferes with the MHC class I presentation pathway as well as preventing apoptosis of infected cells (126), hence deletion of this element in viral vectors should even enhance immunogenicity. Adenoviral vectors induce both antibody and cellular responses to the transgene more effectively than other viral vectors, with the cellular response dominated by CD8 T cells (130). Although wild type adenovirus DNA is known to persist in mucosal lymphocytes long

after infection, this persistence is thought to be dependent on E3 mediated inhibition of apoptosis (131). Hence, adenoviral vectors lack the persistent gene expression required for gene therapy. However, in terms of vaccination, lifelong transgene expression is not necessarily required, although the persistence of antigen may be important in inducing the most effective phenotype of response. The rate of viral clearance appears to vary between tissues. Antigen can be detected following up to 40 days following intra-muscular immunisation, with transgene sequences detectable up to 1 year (132, 133). However, if administered intra-nasally, adenoviral clearance has been reported to occur within 10-14 days (134, 135).

As a result of these qualities, adenoviral vectors have become a popular and useful base for the development of a number of vaccine candidates against various pathogens. These include vaccines against Ebola (136), rabies (137), HIV (138), malaria (139) and tuberculosis (54, 114, 140) amongst others (126). Of these vaccines, the farthest developed is a human serotype 5 adenovirus expressing the Gag, Pol and Nef proteins from HIV. This vaccine underwent a Phase III trial in which the effect of vaccination on rate of HIV acquisition and subsequent HIV viral load was measured (141). Unfortunately, no reduction in either measurement was conferred by the vaccine. Furthermore, the rate of infection was raised amongst men with high titres of adenovirus serotype 5 specific antibodies prior to vaccination (138). Whilst the cause behind this increase is unclear, it raises concerns about the use of serotype 5 adenoviruses as viral vectors; more so given anti-adenovirus serotype 5 antibodies are common in the human population and many of the current vaccine candidates are based upon it. Whether or not it has an effect on disease acquisition, pre-existing anti-vector immunity has been shown to limit the efficacy of a vaccine (130). To avoid these potential problems, other serotypes of adenovirus have been investigated as potential vectors. As discussed above, the Aeras-402 vaccine uses human serotype 35, although antibodies to this serotype are also present in the human population. Others have investigated the use of adenoviruses from chimpanzees as these do not circulate in the human population but have similar immunological properties (126).

Ad85A

In this thesis, much of the work reported was performed using Ad85A – a human serotype 5 E1/E3 deleted adenovirus containing the 85A antigen from *Mtb*. 85A is a 31kDa protein and part of the antigen 85 complex, a group of mycolyl transferases required for synthesis of the mycobacterial cell wall (142). 85A is one of the major products secreted by *Mtb* and BCG as well as other species of *Mycobacteria* (143), with epitopes recognised in both animals and humans (144, 145). As the antigen 85 complex is essential for mycobacterial growth and highly immunogenic in *Mtb* infected individuals (145), it represents a promising vaccine target.

In BALB/c mice, Ad85A immunisation has demonstrated potent immunogenicity, inducing significant CD4 and CD8 cell cytokine responses in the lung when administered either intra-muscularly (i.m.) or intra-nasally (i.n.) (140). Furthermore, upon challenge with virulent *Mtb*, i.n. immunised mice show a reduction in lung mycobacterial load. However, although i.m. immunised mice are partially protected, this protection is only transient (140). Similarly, previous work in our lab demonstrated that Ad85A administered intra-dermally (i.d.), conferred no protection to mice despite strong systemic immune responses (52). However, i.n. immunisation with Ad85A consistently protects from *Mtb* challenge in animals, whether administered alone or as a boost to BCG (52, 140, 146-148). Ad85A has also been assessed in the cattle-*M. bovis* challenge model (118), where it was administered i.d. as a boost to BCG and demonstrated significant immunogenicity. Upon *M. bovis* challenge these animals showed decreased lung pathology compared to naïve controls. However, no statistical difference was observed between Ad85A boosted animals and BCG immunised only controls, although there was a trend towards reduced total lung and lymph node pathology (118). These data indicate that Ad85A shows promise as a TB vaccine candidate and, as mentioned above, is currently undergoing a Phase I clinical trial assessing safety.

1.6 Respiratory immunisation

Although many effective vaccines are currently administered systemically, the concept of mucosally targeted vaccination against mucosal pathogens is gaining momentum. As well as the practical advantages of needle free, non-invasive immunisation, respiratory immunisation has immunological benefits. It is becoming clearer that a common mucosal immune system (CMIS) connects mucosal tissues around the body, such as the gastro-intestinal tract, the skin, the genito-urinary tract and the respiratory tract. The degree to which one tissue is connected to another remains unclear, as does the extent of separation between the CMIS and systemic immune system. However, mucosal immunisation has been shown to induce mucosal antibody and cellular immune responses both at the site of immunisation and in distal mucosa (149, 150). By inducing immune responses at the site of infection, mucosal vaccines hope to improve protection compared to systemic vaccines, which are reliant on recruitment from the periphery. Respiratory mucosal immunisation is particularly effective against a number of respiratory pathogens. Early studies demonstrated that respiratory immunisation of humans was as effective as systemic vaccination against plague, tularaemia and anthrax (150) and i.n. immunisation with live attenuated influenza virus (LAIV) induces more effective homologous and cross-reactive immunity than i.m. immunisation with inactivated influenza virus (151). Furthermore, a respiratory immunisation regime against measles has been widely administered to children in Mexico. Here, aerosol immunisation with attenuated virus was more effective than systemic immunisation with the same vaccine (152, 153), demonstrating the possibility of widespread respiratory immunisation.

Similarly to measles and influenza, *Mtb* is primarily a respiratory pathogen, indicating that respiratory immunisation may be an effective strategy against tuberculosis. Intra-nasal immunisation of BALB/c mice with BCG induced systemic cellular responses equivalent to parenteral immunisation and is highly immunogenic in the lungs. This led to considerable protection after challenge, particularly if 2 i.n. doses of BCG were given (119). Respiratory immunisation regimes using subunit vaccines against TB are also being developed. The Ag85B-ESAT6 fusion protein administered i.n. with adjuvant

effectively boosted systemic responses following BCG and conferred an increase in protection (154). As mentioned previously, adenoviral vectors have a natural tropism for the lung, lending them to use as in respiratory vaccines. Ad85A and Aeras-402 (serotype 35 adenovirus) both confer significant protection when administered i.n. in mice (113, 140) as well as guinea pigs (Ad85A) (148). Aeras-402 is currently being formulated into a dry powder for further aerosol delivery to NHPs and humans (155). Respiratory immunisation is particularly advantageous for adenoviruses as, unlike other immunisation routes, it appears to bypass any host pre-existing anti-vector immunity (156), an important consideration for any vaccine based on serotype 5 adenovirus (Section 1.5).

A number of mechanisms exist for delivery of pharmaceutical agents to the human respiratory tract, whether intra-nasally, by aerosol or by spray. Choice of delivery mechanism is vital in terms of delivering the agent to the desired region of the respiratory tract, an important consideration when attempting to induce the correct immune response whilst avoiding unnecessary side effects. A number of variables, including particle size, density and inspiratory flow rate are known to affect whether the majority of a respiratory agent is deposited in the nasopharynx, the bronchial airways or the alveoli (157). Whilst a number of proprietary nebulisers and inhalers are available, the choice for any particular drug or vaccine will depend on the targeted region of delivery, as well as the agent's stability under formulation (150).

In terms of vaccines, respiratory immunisation with LAIV is performed using the AccuSpray^{TM} device, primarily targeting the nasopharynx and upper respiratory tract, whereas the Mexican measles vaccine was administered by nebuliser, thought to target more into the lower respiratory tract. The lower respiratory tract is obviously a tissue highly sensitive to damage and perturbation. Although no side effects were reported following the respiratory measles vaccine, there may be some concern about delivery of a vaccine to this site. In the case protein vaccines, the development of safe mucosal adjuvants is necessary before they can become effective in humans. There is also concern over the administration of viruses, as translocation of virus to the central nervous system has been reported for

adenoviruses in mice (158) and for influenza in humans (159). However, other respiratory immunisations have proved to be safe, including in the case of TB, where BCG has been given by the respiratory route to humans without harmful effect (160). Thus the development of the respiratory route as an effective administration strategy against *Mtb* infection remains a potential improvement over current approaches.

1.7 Aims of thesis

When starting this project, previous work on the Ad85A vaccine had established that it was protective when administered by one route (i.n.) but not when by another (i.d.) (52). The aims of this thesis were therefore: 1) to establish the durability of the immune response following Ad85A i.n. immunisation and whether protection was maintained to time points long after immunisation; 2) to ascertain the mechanism by which intra-nasal immunisation confers protection; 3) to learn whether immunisation restricted to the upper respiratory tract was sufficient to induce protection or if delivery to the lung was required; 3) to investigate why the immune response induced by i.d. immunisation was altered following a change in animal facility and what effect this had on protection; 4) if i.n. immunisation protected by a distinct mechanism to systemically administered vaccines, to determine if the two could be combined to generate a more efficacious immunisation regime.

CHAPTER 2

Materials and Methods

2. Materials and Methods

A list of standard solutions and their composition is given in Section 2.7.

2.1 Mice

These studies used mice 6-8 week old female BALB/c (H-2^d) or C57BL/6 (H-2^b) mice, as indicated (Harlan, Blackthorn, UK). All the work was approved by the animal use ethical committee of Oxford University and complied with Home Office guidelines.

2.2 *Mtb* challenges

2.2.1 *Mtb* stock

Mycobacterium tuberculosis, Erdman strain was kindly provided by Dr. Amy Yang, (FDA Centre for Biologics Evaluation and Research, Bethesda, USA) and was stored frozen at -80^{o}C.

2.2.2 Challenge methodology

Aerosol challenge

Aerosol challenges were performed using a modified Henderson apparatus, described in Phillpotts *et al* (1997). Briefly, mice were restrained and placed in a sealed chamber. The chamber was then filled with an aerosol of a diluted *Mtb* mixture for 20 minutes, before the chamber was flushed with air for a further 10 minutes. *Mtb* deposition in the lungs was measured at 24 hours post-challenge.

Intra-nasal challenge

Intra-nasal (i.n.) challenges were performed by diluting the *Mtb* stock in PBS to the empirically determined concentrations for the desired challenge dose. Mice were anaesthetised with isoflurane and

allowed to inhale 40-50µl of the *Mtb* suspension placed drop-wise on alternate nostrils. *Mtb* deposition in the lungs was measured 24 hours post-challenge.

Intra-tracheal challenge

Intra-tracheal (i.t.) challenges were performed by diluting the *Mtb* stock in PBS to the empirically determined concentrations for the desired challenge dose. Mice were anaesthetised with Ketaset/Domitor and positioned vertically with ventral side facing the operator and the mouth open. A gel loading pipette tip was inserted into the trachea until the bronchial bifurcation was reached (in order to confirm that the tip was inserted in the trachea rather than the oesophagous), the tip withdrawn partially and 40µl of the *Mtb* challenge dose ejected into the trachea.

Comparison of challenge route

All routes of challenge were found to give comparable results in terms of mycobacterial load in naïve and immunised mice (Table 1) (54).

	Aerosol ± SD	i.n. ± SD	i.t. ± SD
Naïve	6.36 ±0.22	6.45 ±0.33	6.58 ±0.41
BCG	5.75 ±0.32	5.53 ±0.39	5.36 ±0.60
B-Ad i.d.	5.35 ±0.63	5.41 ±0.36	5.07 ±0.23
B-Ad i.n.	4.95 ±0.48	4.62 ±0.14	3.20 ±0.57

Table 1: Comparison of protection after different methods of *Mtb* infection. BALB/c mice were immunised with BCG, or primed with BCG and 8 weeks later boosted with Ad85A given intra-dermally (B-Ad i.d.) or intra-nasally (B-Ad i.n.). Naïve mice were used as controls. Mice were challenged with *Mtb* 4 weeks after the last immunisation either by aerosol, intra-nasally (i.n.) or intra-tracheally (i.t.). Deposition in the lungs was measured 24 h after challenge and was 880 CFU after aerosol, 1000 CFU per lung after i.n. and 620 CFU after i.t. *Mtb* challenge. Mice were sacrificed 4 weeks later and lung CFU enumerated. Data represent the mean +/- SD from 6–8 mice per group. Adapted from Ronan *et al* (54).

2.2.3 *Mtb* colony forming unit (CFU) quantification

Tissue homogenisation

At the desired time point post-challenge, mice were culled and the lungs and spleen removed. The tissues were then disrupted in order to release any *Mtb*. Each individual tissue was placed in a conical vial containing borosilicate beads (Sigma Aldrich, UK) and 1ml PBS and beaten for 1 minute using a Mini Beadbeater (Stratech Scientific Ltd, UK).

Plating of homogenate

Ten-fold serial dilutions of the individual tissue homogenates were made and a known volume plated on 7H11 Middlebrook agar plates (E&O laboratories, UK). The plates were incubated for approximately 3 weeks at 37^{o}C and 5% CO_2, after which the number of colonies was counted.

2.3 Immunisation

2.3.1 Anaesthesia

Mice were anaesthetised in one of two ways depending on the period of anaesthesia required:

Intra-peritoneal ketamine

Intra-peritoneal (i.p.) injection with 100µl of a mixture of 100mg/ml Ketaset (ketamine hydrochloride) and 1mg/ml Domitor (medetomidine hydrochloride) each diluted 1:10 in endotoxin free water. Following anaesthesia by this method, mice were roused with an intra-peritoneal injection of 100µl of Antisedan (atipamezole hydrochloride), diluted 1:10.

Inhalation of isofluorane

Inhalation of a mixture of isofluorane (3-5%) and oxygen (0.8-1L/min) in air. Mice were placed in an anaesthesia box until cessation of body movement and a noticeable increase in respiratory rate occurred.

Although there was a concern that inhalation of anaesthetic would affect the response after delivery of vaccines to the lungs, direct comparison of inhaled isofluorane with intra-peritoneal Ketaset/Domitor anaesthetised mice immunised i.n. showed no effect due to method of anaesthesia.

2.3.2 Routes of Immunisation

Intra-nasal

Intra-nasal immunisation was performed by holding anaesthetised mice on their back and allowing them to inhale 40 or 50µl of the vaccine (unless otherwise indicated) pipetted drop-wise onto alternate nostrils. Initially i.n. immunisations were given in 50µl but following unexpected deaths of mice immunised with this volume, it was reduced to 40µl under veterinary advice. For the small volume immunisations (5-6µl) half the total volume was pipetted directly into each nostril using a P2 pipette tip.

Intra-dermal

Intra-dermal (i.d.) immunisation was performed by injecting 25µl of the vaccine into the dermis of the upper side of each ear of an anaesthetised mouse, such that a distinct bolus could be seen. Injection was done using a narrow gauge (29G) insulin syringe.

Intra-muscular

Intra-muscular (i.m.) immunisation was performed by injecting 25µl of the vaccine into the quadriceps of each leg of an anaesthetised mouse.

Subcutaneous

Subcutaneous (s.c.) immunisations of BCG were performed either by injecting 100µl of the vaccine into the flank or scruff of the neck, or by injecting 30µl into the hind foot pad of anaesthetised mice.

2.3.3 Oral administration of mycobacteria

In experiments where mycobacteria were introduced orally to the mice, this was done in a manner that would closely resemble environmental exposure. Mycobacterial species *M. abscessus* and *M. smegmatis* were cultured for 48 hours in 25ml LB broth at 37^{o}C with shaking. The resulting culture was spun at 1100xg for 10 mins and the pellets resuspended in 500ml of autoclaved DI water in the cage water bottles. For BCG administration, 10 vials of BCG (SSI) were dissolved in 500ml of autoclaved DI water in the cage water bottles. The final concentration of all species was between 10^3 and 10^4 CFU/ml. After 1 week, *M. abscessus* and *M. smegmatis* could be recovered from the water at the same concentration; however no BCG growth could be detected.

2.4 Vaccines

2.4.1 BCG

BCG (SSI strain) was kindly provided by Dr. Amy Yang, (FDA). For each mouse, one vial of BCG was resuspended in 50µl of sterile, endotoxin-free PBS (Sigma Aldrich) and administered subcutaneously, unless otherwise indicated, resulting in an immunisation of approximately $2x10^5$ CFU per mouse.

2.4.2 Ad85A

The human tissue plasminogen activator leader sequence was fused to the coding region of *Mtb* 85A (nt 99797 to nt 98910, lacking the leader sequence) (Geneart) (Appendix I), subcloned into pDONR221 vector, then recombined into E1/E3 deleted AdHu5 (pAd/CMV/V5-Dest) (Invitrogen) following the manufacturer's instructions. A replication-deficient AdHu5-85A recombinant virus clone was amplified in E.coli, digested with PacI and transfected into 293A cells to produce an adenoviral stock. Bulk virus was produced by infecting 293A cells with the stock, and purified with the AdenoPure kit (Puresyn, USA) according to the manufacturer's instructions (54). Concentration of purified virus was determined by Nanodrop spectrophotometry (Thermo Scientific, USA) and validated by serial dilution of cytopathic effect on 293A cells.

Each vaccination was given at $2x10^9$ v.p. per mouse, diluted in endotoxin-free PBS (Sigma Aldrich). If the virus was given simultaneously by more than one route, the total dose remained the same. When Ad85A was administered along with cholera toxin (CT) (Sigma Aldrich), CT was dissolved in the vaccination mixture such that the desired immunisation volume contained 2µg CT and $2x10^9$ v.p. of Ad85A.

2.4.3 r85A

The recombinant antigen 85A protein was kindly provided by Professor Tom Ottenhoff (Leiden University Medical Centre, Leiden, The Netherlands) and was produced as described in (161). For s.c. immunisations, 4µg of protein was dissolved in 200µl monophosphoryl lipid A Sigma adjuvant system (MPL) (Sigma Aldrich) according to the manufacturer's instructions. 100µl was then injected into the flank s.c. and 100µl intra-muscularly. For i.n. immunisations, 4µg of protein and 2µg of cholera toxin (Sigma Aldrich) was dissolved in 40µl PBS. The protein was administered 3 times at 2 weekly intervals.

2.4.4 ESAT6

The ESAT6 antigen was a synthetic peptide consisting of the first 20 amino acids of the ESAT6 protein ($ESAT6_{1-20}$ MTEQQWNFAGIEAAASAIQG) (Peptide Protein Sythetics, UK). Sub-cutaneous and intra-nasal immunisations were performed as for rec85A, but using 20µg of the peptide per immunisation. The peptide was administered 3 times at 2 weekly intervals.

2.5 Assay of immune responses

2.5.1 Cell isolation

All cell isolation was done under sterile conditions except where indicated.

Lung cells

Mice were killed by cervical dislocation at the desired time point post-immunisation and the thorax opened. The lungs were perfused by injecting 2-5ml of PBS into the right ventricle until the lungs inflated. The lungs were then removed, cut to ~1mm^3 pieces, and incubated in 5ml Dulbecco's Modified Eagle Medium α (DMEM) containing 0.7mg/ml collagenase type 1 (Sigma Aldrich) and 30 µg/ml DNase (Sigma Aldrich) for 40 min at 37°C and 5% CO_2. The tissue suspension was then mashed through a 70µm cell strainer using the plunger from a 5ml syringe. The resulting cell suspension was centrifuged at 400xg for 7 minutes. The supernatant was discarded and the cell pellet resuspended in 10ml complete DMEM. In order to remove erythrocytes, this suspension was layered onto 5ml of Lympholyte (VH Bio Ltd, UK) and centrifuged at 1100xg for 30 minutes with the brake off. After density separation, the cell-containing interface was removed with a Pasteur pipette, diluted to 10ml in complete DMEM and centrifuged at 400xg for 7 minutes. The supernatant was discarded and the cells resuspended in the residual medium. If cells were counted, a sample of the suspension was diluted 1:10 in complete DMEM and then 1:1 in trypan blue (Invitrogen) and counted on the Countess Automated Cell Counter (Invitrogen).

Splenocytes

Mice were killed by cervical dislocation at the desired time point post-immunisation, the abdomen and peritoneum opened and the spleen removed. The spleen was then mashed in PBS using the plunger of a 5ml syringe and passed through a 70µm cell strainer. The cell suspension was centrifuged at 400xg for 7 minutes and the supernatant discarded. The cell pellet was then resuspended in 5ml Red Blood Cell Lysis Solution (Qiagen/Gibco, UK) and incubated at room temperature for 5 minutes to lyse the erythrocytes. This suspension was diluted to 50ml with PBS and centrifuged at 400xg for 7 minutes. The supernatant was discarded and the cell pellet was resuspended in 2ml complete DMEM. If cells were counted, a sample of the suspension was diluted 1:10 in complete DMEM and then 1:1 in trypan blue (Invitrogen,) and counted on the Countess Automated Cell Counter (Invitrogen).

NALT

At the desired time point post-immunisation, the mice were sacrificed by cervical dislocation and the head removed from the body. The lower jaw, tongue and connective tissue were then removed to expose the soft palette of the upper jaw. The front incisors were then removed to expose the anterior end of the soft palette. The palette was peeled back from the nose towards the rear of the head, removing the palette as well as the paired NALT structures at the posterior end. Using a needle, the NALT structures were mashed into complete DMEM in order to release the cells. The resulting suspension was then rested upright for 10 minutes in order for any pieces of large debris to settle out. After this debris was removed, the cell suspension was centrifuged at 400xg for 10 minutes. The supernatant was then discarded and the cell pellet resuspended in the residual medium. For all experiments, the NALT cells from all mice in a group were pooled. If cells were counted, a sample of the suspension was diluted 1:10 and counted on the Countess Automated Cell Counter (Invitrogen) and averaged over the number of mice per group.

2.5.2 Flow Cytometry

Surface staining

If staining cells immediately after isolation, 150µl of the required cell suspensions, approximately $1x10^6$ cells, were transferred to a V-bottomed plate and centrifuged for 5 minutes at 400xg. Cells were then resuspended in 100µl of PBS 1% BSA (FACS buffer) containing Fcγ block (anti-mouse anti-CD16/CD32 antibody, eBioscience, UK) diluted 1:50 according to manufacturer's instructions and incubated at 4°C for 15 minutes. 100µl of FACS buffer was then added and the plate centrifuged at 400xg for 5 minutes and the supernatant discarded. 50µl of a cocktail of the desired antibodies, diluted to the correct concentration in FACS buffer, was added and the plate incubated for 20 minutes at 4°C in the dark. 150µl of FACS buffer was then added and the plate centrifuged at 400xg for 5 minutes, the supernatant discarded and the cells resuspended in 200µl of FACS buffer for analysis. The antibodies used were as follows: CD4 (clone RM4–5); CD27 (LG.7F9); CD127 (A7R34); CD62L (MEL-14) (all eBioscience, UK); CD8 (53-6.7) (BD Biosciences, UK). All the preceding antibodies were used at 1:100 dilution of the stock solution.

Tetramer staining

Some BALB/c derived samples were stained with a MHC class I tetramer specific for the dominant CD8 epitope of 85A, MPVGGQSSF (NIH tetramer facility, Bethesda, USA). If cells were stained with the tetramer, this was done after the Fcγ block step but prior to staining for other surface markers described above. 50µl of tetramer, diluted 1:50 in FACS buffer, was added to each sample and the plate incubated in the dark for 20 minutes at room temperature. It should be noted that the dilution and incubation temperature was optimized for each batch of tetramer by staining cells with different

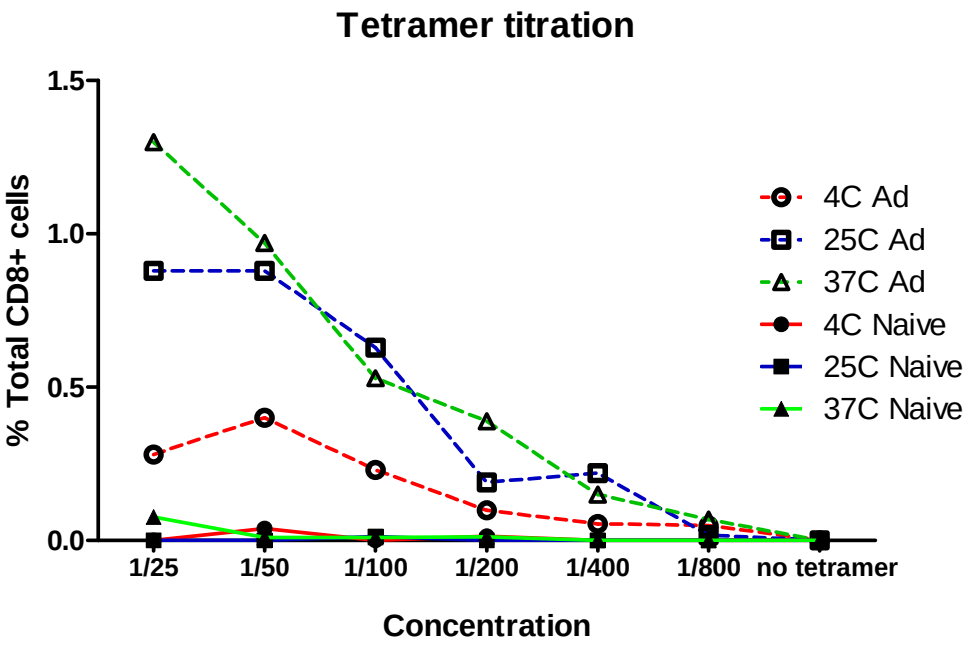


Figure 1: Titration of tetramer to determine optimal staining concentration and temperature. Splenocytes from BALB/c mice immunised i.d. with Ad85A 4 weeks previously were stained for CD4, CD8 and with a MHC class I tetramer specific for the dominant CD8 epitope of 85A, with the tetramer staining performed at the range of concentrations and temperatures indicated. Splenocytes from unimmunised mice were also stained to determine the level of non-specific binding.

concentrations of tetramer at different temperatures, as shown in Figure 1. The cells were then washed with 150µl of FACS buffer and centrifuged at 400xg for 5 minutes. Surface staining for other markers was then performed as outlined above.

Intra-cellular cytokine staining

For intra-cellular cytokine staining (ICS), cells from the desired tissues were first prepared as outlined above. In order to assess the specific response of the cells within a tissue to a particular antigen, the cells were stimulated with that antigen for 1 hour at 37^{o}C and 5% CO_2, unless otherwise indicated. Brefeldin A (Golgiplug, BD Bioscience, UK) was then added according to the manufacturer's instructions (stock solution diluted 1:250 and 50µl added to 200µl of cell suspension) and the whole suspension incubated for a further 5 hours at 37^{o}C and 5% CO_2, unless otherwise indicated. For the

makeup of specific antigens and the concentrations used, see below. After the 5 hour incubation, the cells were removed from the incubator and stored in the fridge overnight. The following day the cells were surface stained with relevant antibodies as described above. Following surface staining and washing, cells were resuspended in 100µl Cytofix/Cytoperm (BD Biosciences) in order to fix and permeabilise them. The cells were then incubated in the dark at 4^{o}C for 15 minutes. Following incubation, 100µl of Perm/Wash Buffer (BD Bioscience) was added and the plate centrifuged at 400xg for 5 minutes. 50µl of a cocktail of the desired antibodies, diluted to the correct concentration in FACS buffer, was then added and the plate incubated for 20 minutes at 4^{o}C in the dark. 150µl of FACS buffer was added and the plate centrifuged at 400xg for 5 minutes, the supernatant discarded and the cells resuspended in 200µl of FACS buffer for analysis. The antibodies used were as follows: IFNγ (clone XMG1.2), IL-2 (clone JES6-5H4), TNFα (MP6-XT22) and IL-17A (eBio17B7) (all from eBioscience). All the above antibodies were used at a concentration of 1:100. For IL-17 ICS, cells were incubated with the stimulating antigen for 2 hours prior to the addition of Brefeldin A, followed by 5 hours further incubation.

Antigen 85A: For 85A stimulation, cells were either stimulated with a pool of peptides or with a mix of 3 epitopes. The pool of peptides consisted of 66 15-mers, over-lapping by 10 amino acids, spanning the whole 85A protein (Peptide Protein Synthetics) (individual peptide sequences are given in Appendix II). All peptides were dissolved to make a stock solution at a concentration of 50µg/ml. The epitope mix consisted of the BALB/c CD4 epitope and the dominant and sub-dominant CD8 epitopes ($Ag85A_{99–118aa}$ TFLTSELPGWLQANRHVKPT; $Ag85A_{70–78aa}$ MPVGGQSSF and $Ag85A_{145–152aa}$ YAGAMSGL respectively) (Peptide Protein Synthetics) (144, 162) at a stock concentration of 50µg/ml. All stimulations were done at a final concentration of 2µg/ml.

ESAT6: For ESAT6 stimulation, cells were stimulated with the same synthetic peptide used for immunisation. Specifically, $ESAT6_{1-20}$ MTEQQWNFAGIEAAASAIQG at a final concentration of 2µg/ml.

PPD: PPD was supplied by the Statens Serum Institute. For stimulation it was used at a final concentration of 10µg/ml and was incubated for 6 hours at 37°C in 5% CO_2 before adding Brefeldin A and then further incubated overnight.

Analysis

Stained samples were run on a LSRII Flow Cytometer (BD Biosciences). Flow cytometry data were analysed using Flow Jo software (Tree Star Inc., USA) and multi-functional cytokine analysis was done using the Pestle and Spice software (163).

2.6 Cell depletion

2.6.1 CD8 depletion

For CD8 depletion, 100µg of azide-free anti-CD8 antibody (clone 2.43) (Santa Cruz Biotechnology, USA) was given i.p.. One dose of depleting antibody was given 2 days before the time of challenge (-2 days) and another dose was given 2 days after the day of challenge (+2 days).

2.6.2 CD4 depletion

For CD4 depletion, 40µg azide-free anti-CD4 antibody GK1.5 (Santa Cruz Biotechnology) was given i.p.. One dose of depleting antibody was given 2 days before the time of challenge (-2 days) and another dose was given 2 days after the day of challenge (+2 days).

2.7 Working solutions

PBS

1 tablet of Phosphate Buffered Saline (Dulbecco 'A') (Oxoid, UK) dissolved in 100ml DI water, giving a solution consisting of: 0.16 mol Sodium chloride; 0.003 mol Potassium chloride; 0.008 mol Disodium hydrogen phosphate; 0.001 mol Potassium dihydrogen phosphate.

FACS buffer

PBS containing 1% w/v bovine serum albumin (BSA)(Sigma Aldrich).

Complete medium

Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, UK), supplemented with 1% L-glutamine (200mM, Invitrogen) or already containing GlutaMAX (Trademark, Invitrogen), supplemented with 10% heat inactivated foetal calf serum (FCS)(Gibco/Qiagen) and containing 1% Penicillin-Streptomycin (Gibco/Qiagen).

2.8 Statistics

Data analysis and presentation was done using Prism (GraphPad Software, USA). Where data conformed to parametric assumptions the ANOVA test was used with Tukey's or Dunnet's post-test. If only two groups were compared Student's t-test was employed. When data did not fit the assumptions required for parametric tests the alternative non-parametric Kruskall-Wallis and Mann-Whitney tests were employed.

CHAPTER 3

Immune response and protection following Ad85A intra-nasal immunisation

Chapter 3

Immune response and protection following Ad85A intra-nasal immunisation

Sections of this chapter have already been published in Ronan *et al.* PLoS One (2009) (54) and have been approved for use by the co-authors in accordance with University of Oxford regulations.

3.1 Introduction

As described in the Introduction, there are currently many different vaccine candidates at various stages of development that aim to improve upon the protection afforded by BCG. This may be by introducing a more effective strain of BCG or by following the BCG immunisation with a second, heterologous immunisation with an antigen conserved between BCG and *Mtb* – termed a prime-boost regimen. A popular antigen for prime-boost immunisations is antigen 85A, part of the *Mtb* antigen 85 complex of mycolyl transferase molecules. Various vehicles can be employed for delivery of vaccine antigens but the work in this chapter will concern the delivery of antigen 85A using an adenoviral vector.

3.1.1 Ad85A

Adenovirus vectors were initially developed for the delivery of gene therapy agents but have since been shown to be effective vaccine vectors, with a number currently in development as TB vaccine candidates (113, 148). One of these candidates is Ad85A; a replication-deficient, E1/E3 deleted, human serotype 5 adenovirus containing antigen 85A from *Mtb*. This vaccine has been shown to induce a potent CD8 T cell response in BALB/c mice (52, 140). Although it is thought that control of *Mtb* infection is mediated principally by CD4 T cell helper type 1 (Th1) cells, a view supported by the increased susceptibility of HIV+ patients to active TB disease in inverse correlation to their CD4 count, there is increasing evidence that CD8 cells also play a significant role in contributing to protection (70, 164).

3.1.2 Route of administration

It has been shown that the route of administration of Ad85A affects the localisation of the induced CD8 T cells and that this has a direct effect on the degree of protection afforded. Intra-dermal (i.d.) immunisation has been shown to generate a strong systemic antigen-specific CD8 T cell response detectable in the spleen but with little response detectable in the lungs (52) (this is further discussed in Chapter 5, where some changes in the response following i.d. immunisation are investigated). Conversely, intra-nasal (i.n.) immunisation induces a strong CD8 T cell response in the lungs but only a weak response is detectable in the spleen (52). This is the case whether Ad85A is administered as a boost following BCG (52) or as a single immunisation (140). The impact on protection after *Mtb* challenge is marked: i.n. immunised animals show a reduction in mycobacterial load in the lung of ~1 Log compared to control animals, whereas i.d. immunised animals show no reduction in mycobacterial load despite a strong systemic response (52).

3.1.3 *Mtb* growth kinetics

Following pulmonary infection, the initiation of the immune response to *Mtb* is significantly delayed. T cell activation is not detected in the mediastinal lymph nodes until approximately 9 days post-infection and mycobacteria-specific T cells are not seen in the lungs of naïve mice until day 14 post-infection (37-39). This delay in activation of a primary acquired immune response could be due to a combination of physiological factors, such as the low infectious dose and naturally low inflammation in the lung, as well as modulation of the innate immune compartment by *Mtb* itself (37). However, mice only begin to control *Mtb* growth in the lungs after 21 days, when the mycobacterial load comes under the control of the adaptive immune response (37, 165). Although BCG immunised mice show a 1 Log reduction in *Mtb* numbers in the lung 28 days after infection, the kinetics of *Mtb* growth after challenge are not altered until after day 14 (166).

In these models of *Mtb* growth the acquired immune response responsible for the control is either a primary response induced after antigen presentation in the mediastinal nodes or, in the case of immunised mice, is a secondary response generated from circulating memory cells encountering antigen in the lymph nodes. Either way, both are vulnerable to any delay in antigen arrival to the lymph nodes. In contrast, i.n. immunisation with Ad85A induces a population of *Mtb*-specific T cells in the lungs (52, 167). Rather than being dependent on recruitment from a circulating pool, these cells already present at the point of infection may be able to act earlier to control *Mtb* growth.

3.1.4 Aims

It is clear that generating a strong antigen-specific response in the periphery is not sufficient to confer protection. Rather, antigen-specific cells must be present in the lung and an effective method of generating this response in the lung is i.n. administration of Ad85A. The aim of this chapter is first to confirm the above data as well as to investigate the kinetics and characteristics of the antigen-specific response following Ad85A i.n. immunisation.

The presence of a population of *Mtb*-specific T cells in the lungs at the time of challenge, rather than in the periphery, could mean that these cells are able to bypass the delay in antigen arrival in the lymph node. By investigating the growth kinetics of *Mtb* in mice immunised with Ad85A, either as a single immunisation or in a prime-boost regime, it will be possible to establish whether the presence of a lung resident population of immune cells impacts on *Mtb* growth. This would also shed light on the mechanism by which i.n. immunisation with Ad85A confers protection.

3.2 Results

3.2.1 Time course of the antigen-specific response after Ad85A i.n. immunisation

It has been previously shown that intra-nasal immunisation with Ad85A induces a potent population of antigen-specific CD8 T cells in the lung and that these correlate with protection following *Mtb*

challenge 4 weeks post-immunisation (52). However the kinetics of the response following Ad85A i.n. immunisation have not been described and neither the peak of the antigen-specific response nor its durability are known. To determine these factors, BALB/c mice were immunised i.n. with Ad85A, either as a single immunisation or as a boost 8 weeks after BCG immunisation, and culled at day 4 and weeks 1, 2, 3, 4, 6, 9, 15 and 23 post-Ad85A. Lung and spleen cells were isolated, stimulated with the pool of antigen 85A peptides and IFNγ, IL-2 and TNFα production measured by flow cytometry (Figures 1 and 2).

In the lung, the peak of the CD8 T cell response was 4 weeks post-immunisation. At this time point the mice that received BCG before Ad85A immunisation showed a greater proportion of 85A-specific cytokine expression compared to mice which received only Ad85A (Figure 1A). This indicates that prior BCG immunisation can prime a response that can be boosted by subsequent Ad85A immunisation. However, it should be noted that in another experiment no boosting effect was evident. Equally, only at the peak of the response was any boosting effect evident, with other time points showing comparable levels of cytokine production between mice in the prime-boost regimen and those receiving a single immunisation (Figure 1A). The CD8 T cell response peaked at 8% and 3% of CD8+ cells expressing IFNγ for prime-boost and single immunisation groups respectively. Following the peak, the responses declined in both groups such that by 9 weeks post-immunisation the percentage of IFNγ+ T cells for both groups had fallen to approximately 1.5%. This level of response was maintained at 15 and 23 weeks post-immunisation. CD8 T cell TNFα responses were also measured and followed a similar kinetic to IFNγ, although with a slightly lower percentage of cells producing TNFα at both the peak and late time points. As shown previously, many fewer CD8 T cells produce IL-2 at the peak (52) and there was no IL-2 production by CD8 cells at the late time points from either immunisation group (Figure 1A).

A CD4 T cell response to antigen 85A was also detected in the lungs of i.n. immunised mice (Figure 1B). This was not as strong as the CD8 response, with a maximum of 0.4% of CD4+ cells producing

IFNγ, and no evident prime-boost effect. The peak of the CD4 response was also broader than that seen for the CD8 response. IFNγ production peaked at 2 weeks post-immunisation and this level of response was maintained, with a marginal reduction, until 4 weeks. At 6 weeks post-immunisation the IFNγ response had fallen to approximately 0.13% and this level of response was maintained out to the late time points (Figure 1B). As in the CD8 response, CD4 TNFα production followed a similar kinetic to that seen for IFNγ, although at a lower frequency. Although the CD4 IL-2 response was at the limit of detection, thus increasing the noise in the results, there was very little IL-2 production at the late time points, echoing the results seen for the CD8 response (Figure 1B).

Intra-nasal immunisation, although designed to elicit a response in the lung, does induce a systemic response detectable in the spleen (Figure 2). The splenic response followed a similar kinetic to that seen in the lung, although at an approximately 10-fold lower level. The CD8 IFNγ response peaked at 4 weeks, when a small prime-boost effect was evident. However, this was not statistically significant and other time points showed no effect of prior BCG immunisation on the magnitude of response (Figure 2A). At the later time points the response appeared to fluctuate more than the steady level seen in the lung, although this may be inherent variation due to the smaller number of responding cells. As in the lungs, the CD8 TNFα response followed a similar kinetic to that seen for IFNγ (Figure 2A). A very small, highly variable CD4 response was detectable in the spleen but only during the first 4 weeks after immunisation. Any IL-2 production by CD8 or CD4 cells in the spleen following Ad85A i.n. was too low to be detected.

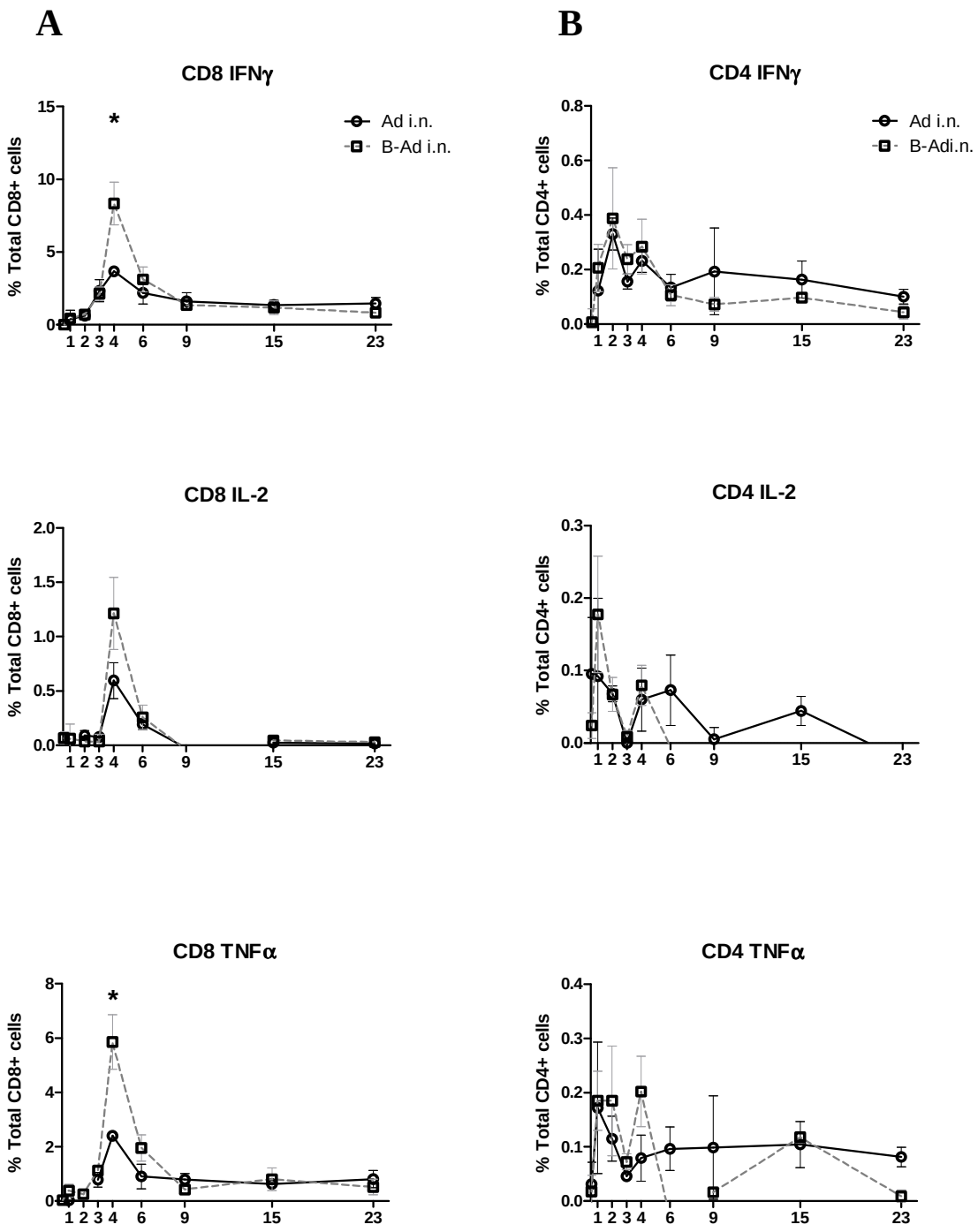


Figure 1: Lung cytokine response kinetics following Ad85A i.n. immunisation. Mice were immunised with Ad85A i.n. (Ad i.n.) or primed with BCG and boosted 8 weeks later with Ad85A intra-nasally (B-Ad i.n.). Lung cells were isolated at the indicated times after the last immunisation and stimulated with pooled 85A peptides for 6 hours. The frequencies of IFNγ, IL-2 and TNFα producing cells were determined by flow cytometry on CD8 (A) and CD4 (B) gated cells. * $p < 0.05$ Ad i.n. vs B-Ad i.n. at 4 weeks. Adapted from Ronan *et al* (54).

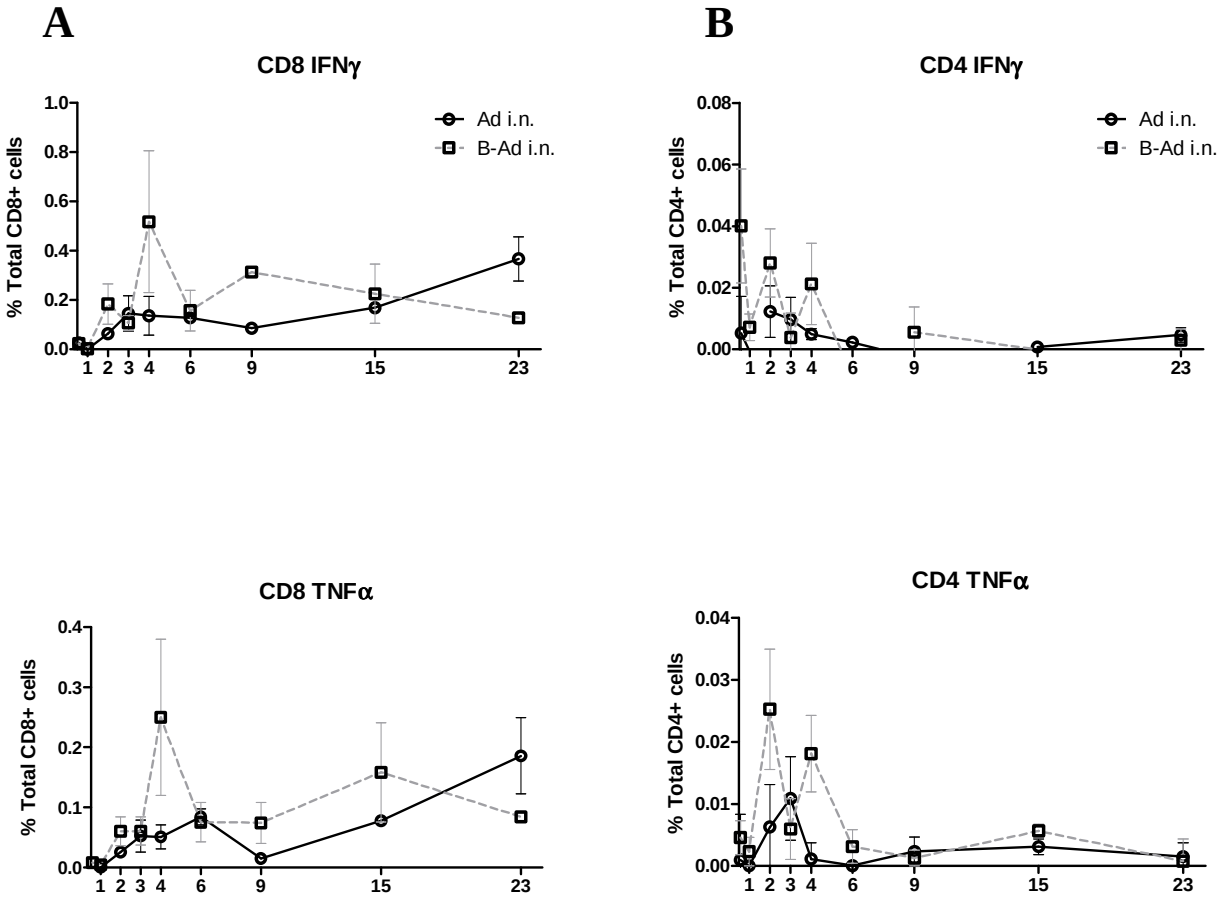


Figure 2: Spleen cytokine response kinetics following Ad85A i.n. immunisation. Mice were immunised with Ad85A i.n. (Ad i.n.) or primed with BCG and boosted 8 weeks later with Ad85A intra-nasally (B-Ad i.n.). Spleen cells were isolated at the indicated times after the last immunisation and stimulated with pooled 85A peptides for 6 hours. The frequencies of IFNγ and TNFα producing cells were determined by flow cytometry on CD8 (A) and CD4 (B) gated cells.

3.2.2 Duration of protection following Ad85A i.n. immunisation

It has previously been demonstrated that Ad85A i.n. immunisation confers protection when mice were challenged with *Mtb* 4 weeks post-immunisation (52). However, protection was only assayed at the peak of the response to 85A. Yet, it is evident that an Ad85A i.n. response is also detectable in the lung 6 months post-immunisation (Figure 1). If a population of 85A-specific cells in the lung is a reliable correlate of protection after Ad85A immunisation, then mice challenged with *Mtb* 6 months post-immunisation should also show a reduced mycobacterial load. This is also important in regard to the value of Ad85A as a potential vaccine, for vaccine induced protection is only useful if that response is durable. To this end, the protection after *Mtb* challenge at 4 and 24 weeks post-Ad85A immunisation was investigated (Figure 3).

When mice were challenged with *Mtb* by aerosol 4 weeks after a single immunisation with Ad85A i.n. there was a ~ 1 Log reduction in *Mtb* CFU in the lungs compared to naïve animals. Mice that had received Ad85A i.n. as a boost following BCG immunisation showed a reduction in mycobacterial load in the lungs of ~ 1 Log compared to BCG only immunised controls (Figure 3A). In the spleen, Ad85A alone was able to protect animals to a level equivalent to that seen after BCG only, although there was no increase in protection when Ad85A i..n. was given as a boost following BCG immunisation (Figure 3B). This confirms the previous data on the effect of Ad85A i.n. on *Mtb* challenge early after immunisation. Interestingly, when mice were challenged 24 weeks after Ad85A i.n. immunisation the level of protection in both lung and spleen was maintained (Figure 3 C + D). Immunisation with Ad85A i.n. alone was able to protect mice to a level equivalent to that seen after BCG only immunisation, an approximately 1 Log reduction in mycobacterial load in the lung and a 1.5 Log reduction in the spleen compared to naïve animals (Figure 3 C + D). Unfortunately the protection in mice that received the prime-boost regime could not be tested at this late time point, but the data show that not only is Ad85A i.n. immunisation able to induce an immune response that persists for at

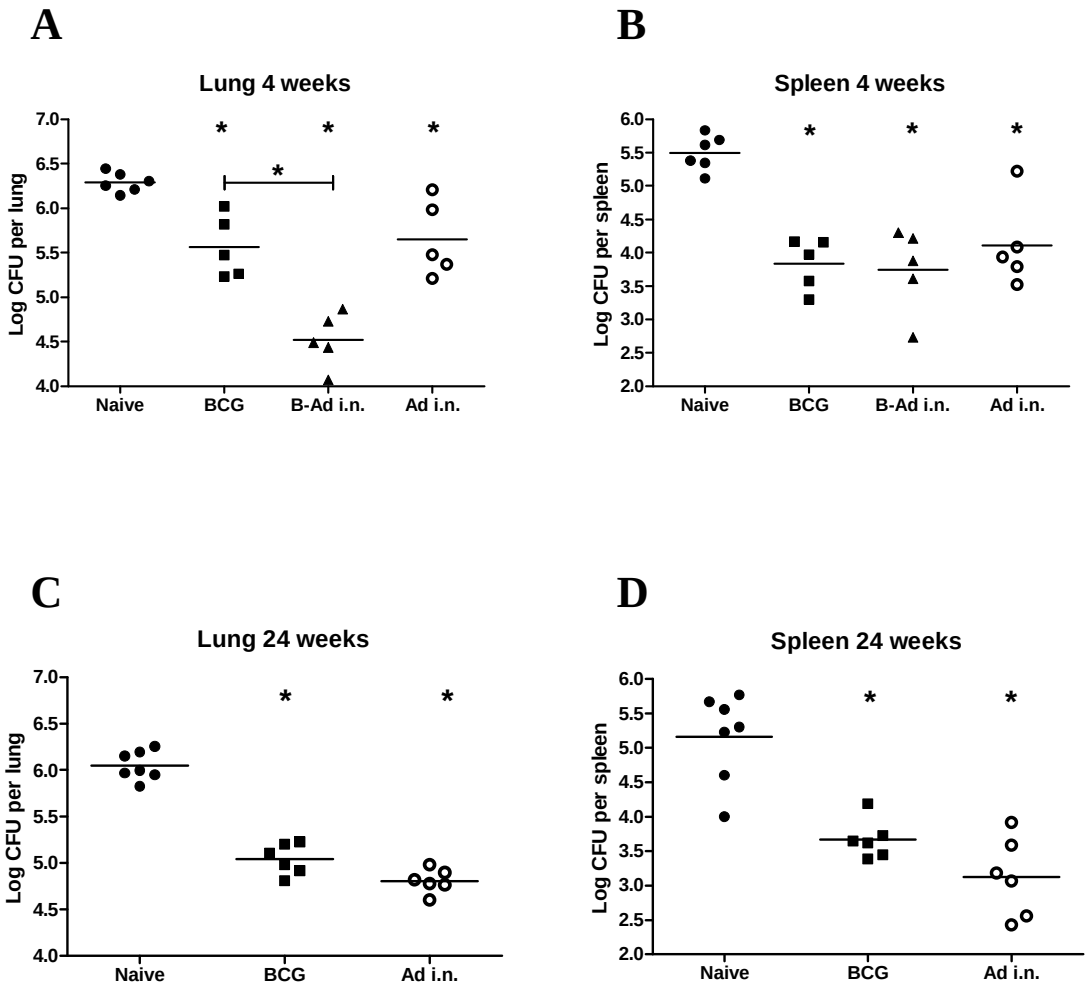


Figure 3: Effect of Ad85A i.n. immunisation after *Mtb* challenge 4 or 24 weeks post-immunisation. BALB/c mice were immunised with BCG, or primed with BCG and 10 weeks later boosted intra-nasally with Ad85A (B-Ad i.n.), or immunised only with Ad85A i.n. (Ad i.n.). Naïve mice were used as challenge controls. Mice were challenged with *Mtb* by aerosol 4 (A + B) or 24 (C + D) weeks post-immunisation.. Deposition in the lungs was measured 24 h after challenge and was ~200 CFU/lung. Mice were sacrificed 6 weeks later and lung (A + C) and spleen (B + D) CFU enumerated. Symbols show CFU counts of individual mice and the horizontal line indicates the mean. * $p<0.0001$ versus Naïve unless indicated. Adapted from Ronan *et al.* PLoS One (2009) (54).

least 6 months but that this response correlates with a level of protection equivalent to that observed after BCG immunisation.

3.2.3 Phenotyping of Ad85A i.n. induced CD8 T cells

As intra-nasal immunisation with Ad85A induces durable protection against *Mtb* challenge and this correlates with the presence of a population of CD8 T cells in the lung as late as 6 months post-immunisation, it was necessary to characterise these antigen-specific cells. CD8 T cells can be divided into 3 activation states: effector, effector memory and central memory cells, based on the expression levels of CD62L and CD127. Effector cells were defined as CD62L- CD127-, effector memory cells as CD62L- CD127+ and central memory cells as CD62L+ CD127+ (Figure 4A) (168). In the lung at 4 weeks post-immunisation, ~70% of the IFNγ+ cells were of effector phenotype and ~30% of effector memory phenotype (Figure 4B). It was possible that the stimulation with 85A prior to staining could alter the expression of these surface markers leading to over-estimation of the effector population, but *ex vivo* staining of 85A-specific MHC class I tetramer positive cells gave the same result. In contrast to the lung, antigen-specific cells in the spleen following i.n. immunisation showed a predominantly effector memory phenotype, with > 90% of IFNγ cells exhibiting the less activated state (Figure 4B). Only a small number of antigen-specific cells were detectable in the spleen following i.n. immunisation so further subdivision of this population could be misleading, though this finding was replicated in 3 experiements.

A high degree of activation amongst 85A-specific cells would be expected at the peak of the response but, interestingly, similar activation was detected at 23 weeks post-immunisation (Figure 4C). Although at this time point the proportions of effector and effector memory antigen-specific cells had equalised, this still meant that 50% of antigen-specific cells were highly activated 6 months after immunisation. To validate this unexpected finding, cells were examined for expression of another

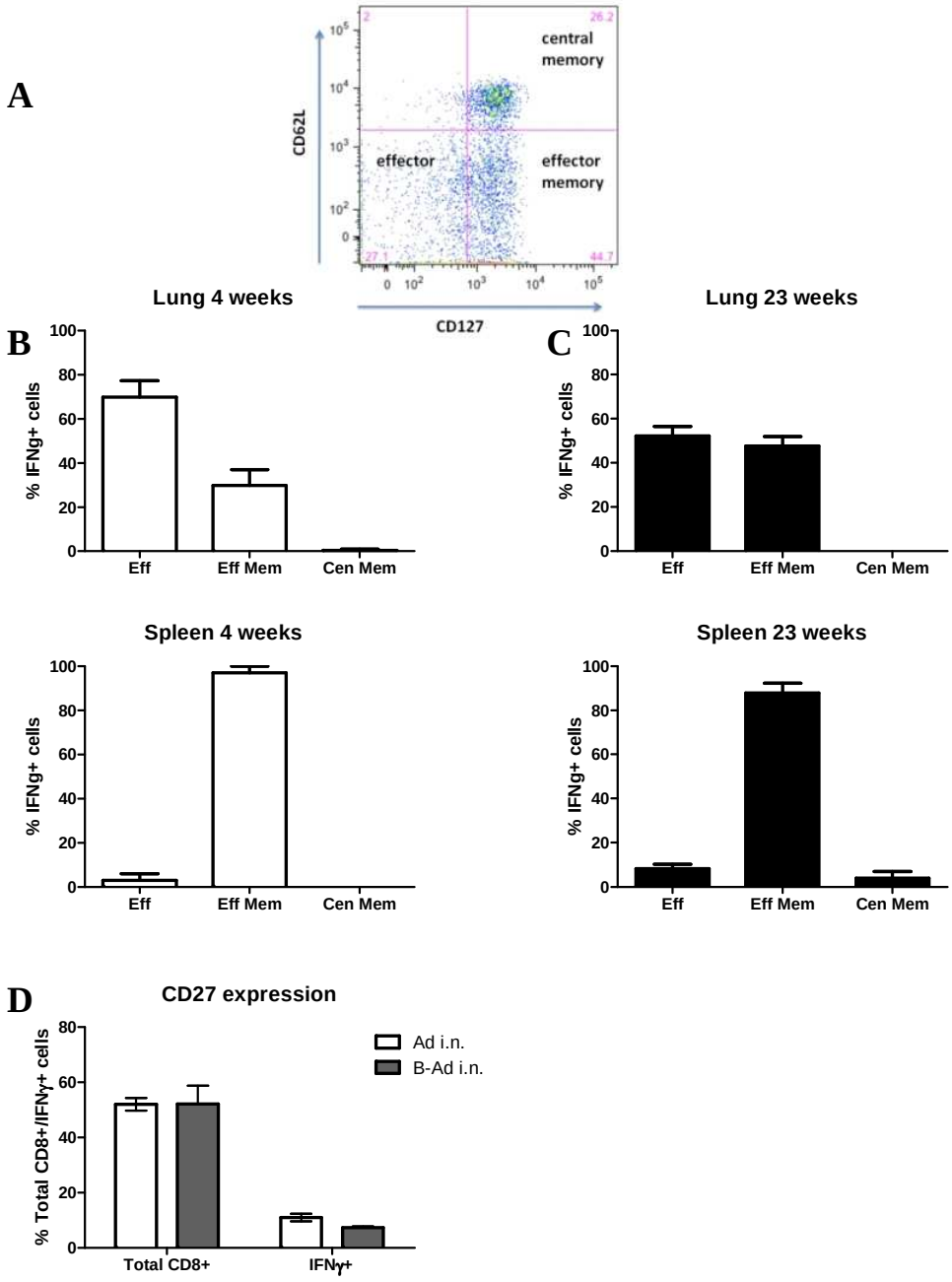


Figure 4: Phenotype of antigen-specific cells following i.n. immunisation with Ad85A. (A) FACS plot indicating the characterisation of activation status of CD8 T cells by CD62L and CD127 expression (showing total lung CD8 T cells from a mouse immunised i.n. with Ad85A). (B + C) Activation status of antigen-specific cells from the lung or spleen of mice immunised with Ad85A i.n. 4 (B) or 23 (C) weeks previously. (D) CD27 expression of total CD8 T cells and antigen-specific CD8 cells in the lung of mice immunised 23 weeks previously. Results are expressed as the mean and SEM from 3 or 4 mice per group. Representative of 3 experiments. Adapted from Ronan *et al* (54).

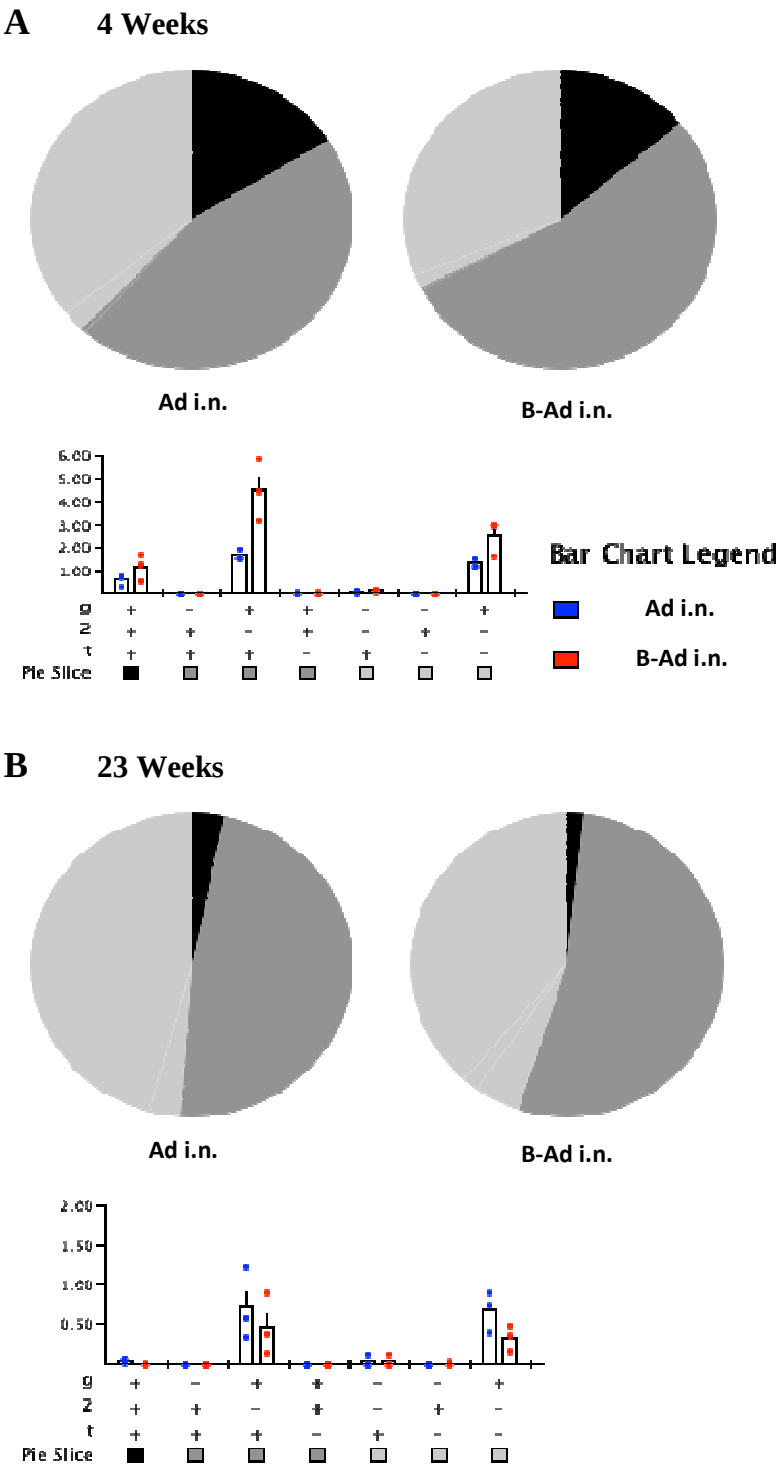


Figure 5: Polyfunctionality of antigen-specific lung CD8 T cells after Ad85A i.n. Pie charts indicate the proportion of CD8 antigen-specific cells producing 1 (light grey), 2 (dark grey) or all 3 (black) of the cytokines IFNγ, IL-2 and TNFα in mice immunised 4 (A) or 23 weeks previously (B). Bar charts indicate the percentage of total CD8 cells producing the various possible combinations of the 3 cytokines. Representative of 2 experiments. Adapted from Ronan *et al* (54).

activation status marker, CD27 (Figure 4D). A lack of CD27 expression is thought to indicate recent antigen exposure and terminal effector differentiation (169). At 23 weeks post-immunisation, antigen-specific CD8 cells showed markedly reduced CD27 expression compared to the rest of the lung CD8 T cell population (Figure 4D), confirming their highly activated state. The reduced expression of CD27 suggests persistent antigen exposure as a possible explanation for the maintained level of high activation. Further work in our lab confirmed that antigen persisted as late as 23 weeks post-immunisation in a form sufficient for mononuclear cells isolated from the lung to drive proliferation of antigen-specific splenocytes (data not shown) (54).

It has been suggested that cells producing multiple cytokines play an important role in protection from *Mtb* infection, with increased triple cytokine producing cells correlating with increased protection (170). Although there is a proportion of 3+ CD8 cells at 4 weeks (Figure 5A), there are very few detectable at 23 weeks as there is little IL-2 production. Despite this, there is no decrease in the level of protection; this implies 3+ cells do not correlate with protection seen in this model.

3.2.4 Kinetics of *Mtb* growth in mice immunised with Ad85A i.n.

Having identified this highly activated population of *Mtb*-specific cells in the lung, it was interesting to investigate what effect they would have on mycobacterial growth immediately following infection. Mice were immunised with Ad85A i.n., either as a single immunisation or as a boost following BCG immunisation 10 weeks previously. Four weeks after the Ad85A immunisation the mice were challenged with *Mtb* and culled on days 1, 3, 8, 14, 21 and 28 post-infection. The *Mtb* load in the lung and spleen was then quantified. Naïve and BCG only immunised mice were used as controls.

The growth of *Mtb* in the lungs of naïve mice followed the same pattern as previously described (165), with control of growth only occurring around 21 days. The results for BCG immunised mice also repeated previously reported data, with no reduction in CFU compared to naïve mice occurring until

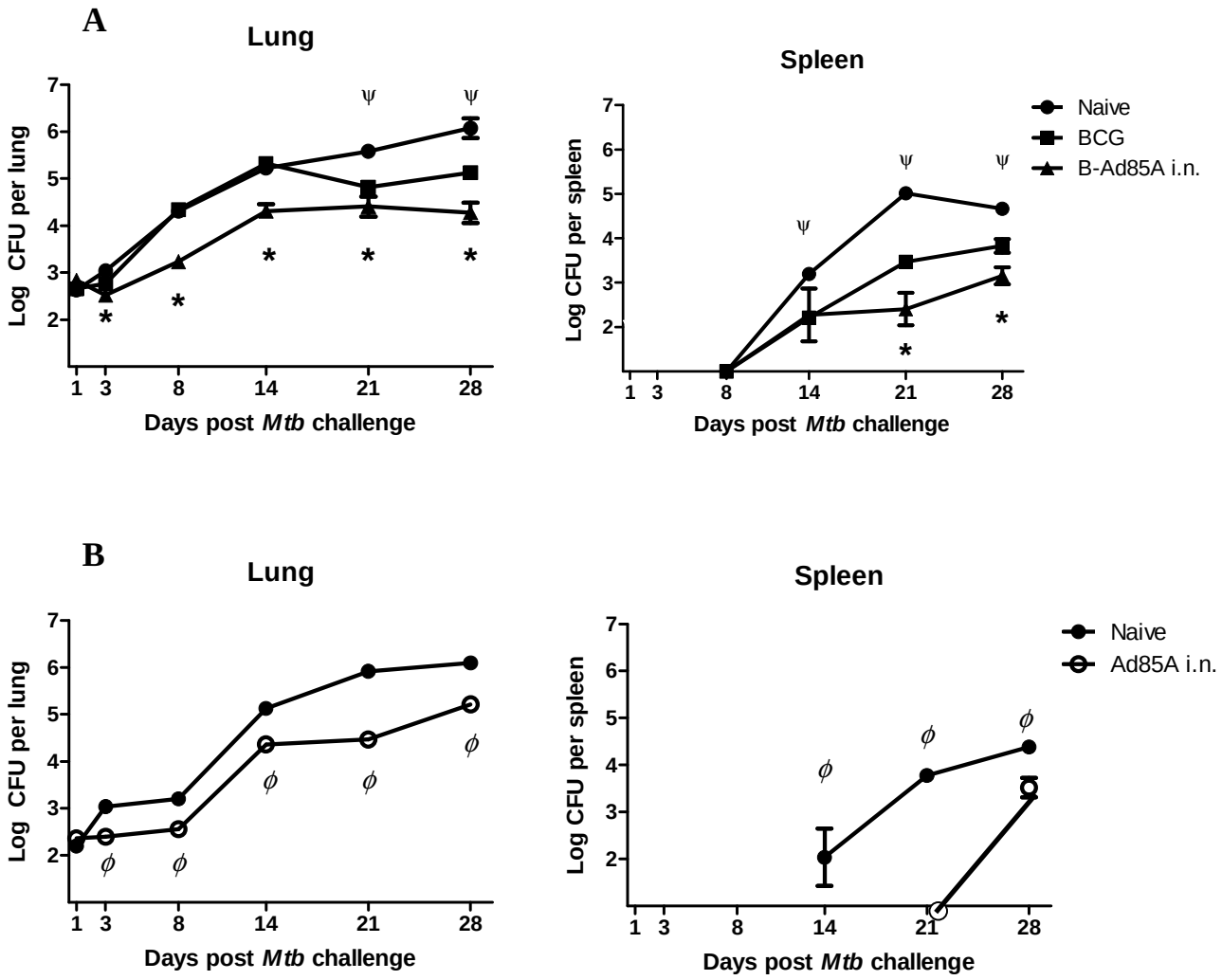


Figure 6: Early inhibition of *Mtb* growth in mice immunised with Ad85A i.n. BALB/c mice were immunised with BCG or primed with BCG and 10 weeks later boosted with Ad85A i.n. (A) or immunised only with Ad85A i.n. (B) Naïve mice were used as controls. Mice were challenged with *Mtb* by aerosol 4 weeks after the Ad85A immunisation and sacrificed at days 1, 3, 8, 14, 21 and 28. Lung and spleen CFU were enumerated. Results are expressed as the mean counts and SEM of 5–7 mice per group. * $p < 0.05$ B-Ad85A i.n. versus BCG. Ψ $p < 0.05$ BCG versus Naïve. *Φ* $p > 0.05$ Ad85A i.n. versus Naïve. Adapted from Ronan *et al* (54).

after day 14 (Figure 6A) (166). However, in mice immunised with BCG and boosted with Ad85A i.n., a significant reduction in *Mtb* CFU compared to naïve and BCG immunised mice is detectable from day 3 post-infection (Figure 6A). This indicates that the protection induced by Ad85A i.n. controls *Mtb* growth by a mechanism different from that observed following parenteral BCG immunisation, occurring significantly earlier after infection. To exclude any prime-boost effect, the growth curve following a single Ad85A immunisation was also examined (Figure 6B). It is again evident that Ad85A i.n. immunisation is able to significantly reduce the mycobacterial load compared to naïve mice from day 3 post-infection. This difference of approximately 1 Log is maintained at least as far as 28 days post-infection. Combined, these data suggest that Ad85A induced cells in the lung are able to inhibit growth of *Mtb* in the first 3-8 days after challenge. Such early inhibition could work additively with the parenteral BCG protection by acting on the early and late phases of growth respectively; an effect notably different from an archetypal prime-boost.

3.2.5 Early inhibition is CD8 T cell dependent

In order to determine if the early inhibition of mycobacterial growth was mediated by antigen-specific CD8 T cells, the effect of T cell depletion on *Mtb* burden was investigated. As the hypothesis was that the CD8+ cells inhibited *Mtb* growth immediately following infection, mice needed to be depleted before the day of challenge and remain depleted for the 7 days following challenge, at which point they were to be culled. To this end, Ad85A i.n. immunised mice were given 100µg of anti-CD8 or anti-CD4 antibody i.p. 2 days prior (Day -2) and 2 days following challenge (Day 2). This regime was sufficient to deplete CD8 or CD4 cells from the lung for the required time period (Figure 7A + B). Mice were challenged with *Mtb* i.n. 2 days following the first depletion dose (Day 0) and culled on Day 7, when lung *Mtb* CFU were enumerated (Figure 7C). As expected, Ad85A i.n. in undepleted mice conferred significant protection compared to naïve mice at day 7. However, if CD8 cells had been depleted then this protection was lost (Figure 7C). No loss of protection was seen when mice

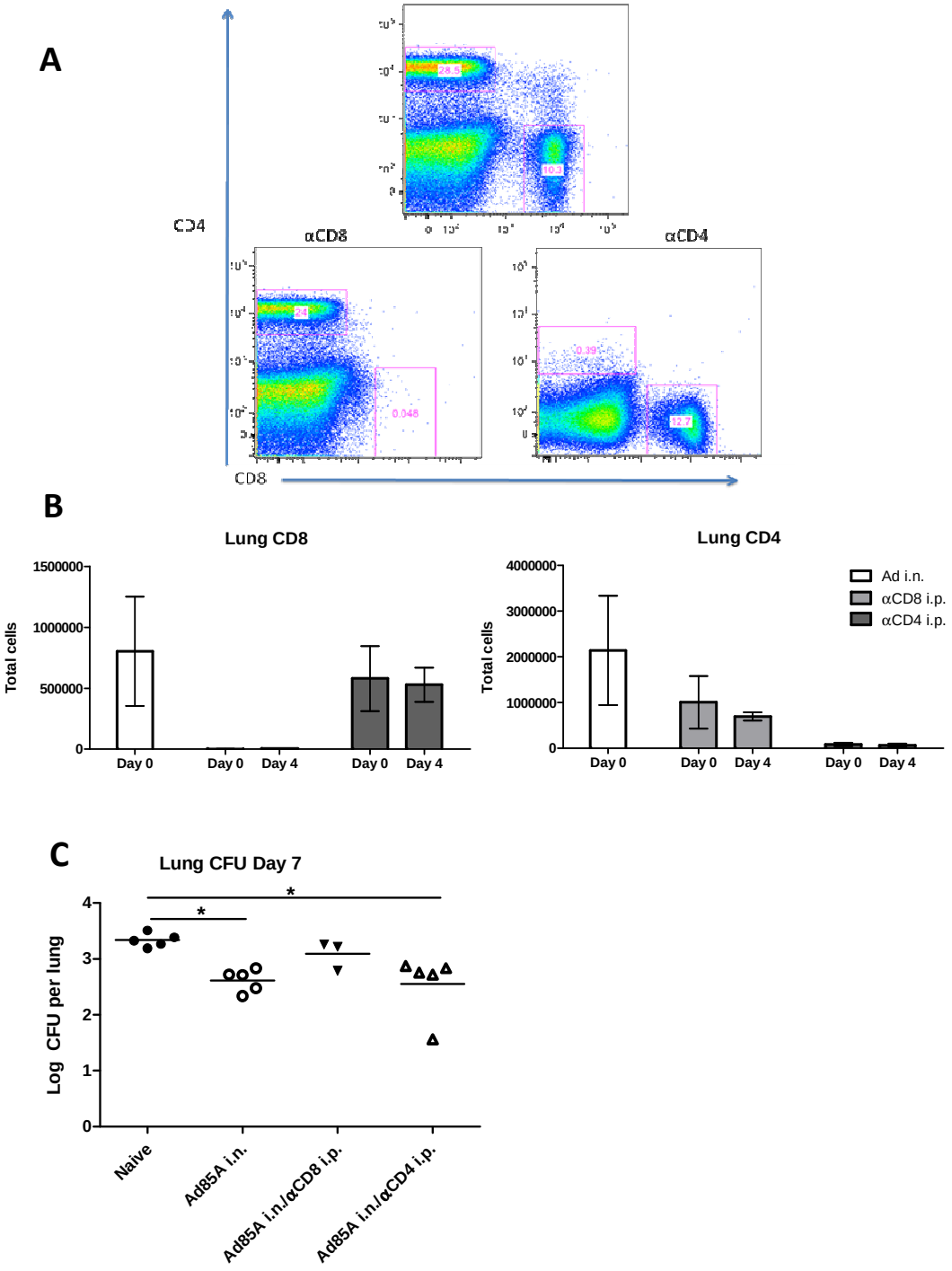


Figure 7: Effect of CD8 or CD4 depletion on Ad85A i.n. induced protection. Anti-CD8 (αCD8) or anti-CD4 (αCD4) depleting antibody was administered i.p. two days prior to challenge (Day -2) and a further dose administered 4 days later (Day 2). Mice were challenged on Day 0 and culled on Day 7. (A) Representative FACS plots showing levels of CD8 and CD4 cells in undepleted mice and levels on day 4 following both doses of αCD8 or αCD4 antibody. Data are from unchallenged mice. (B) Total number of CD8 and CD4 cells in depleted, unchallenged mice on days 0 and 4. (C) Mice were immunised with Ad85A i.n. and 4 weeks later challenged i.n. with *Mtb*. Graph shows lung *Mtb* CFU on Day 7 post-challenge. * $p < 0.01$.

were depleted of CD4 cells. Although this experiment has only been performed once, these data add further support to the hypothesis that Ad85A i.n. immunisation protects through the early inhibition of *Mtb* growth after infection, and this inhibition is mediated by vaccine induced CD8 T cells.

3.3 Discussion

Adenoviral vectors were originally developed for gene therapy, particularly for cystic fibrosis. Although they began to fall out of favour for this purpose as replacement gene expression was not sufficiently reliable, their use as vaccine vectors has become more common (126). This is possibly due to the ease with which they can be rendered replication-deficient through deletion of the E1 unit and their ability to accommodate large transgenes (126) (reviewed in Introduction). As such, human serotype adenoviruses and, increasingly, chimpanzee strains are becoming popular subunit vectors for vaccines against a number of pathogens including HIV and TB (113, 171).

Adenoviruses containing antigens from *Mtb* have shown to be protective in various animal models (52, 113, 140, 148). It has been demonstrated that immunisation with an adenovirus containing antigen 85A confers protection to mice when administered intra-nasally but that protection is only transient or totally absent when the vaccine is administered parenterally (52, 140).

The work in this chapter confirms that i.n. immunisation with Ad85A alone confers protection equivalent to BCG and Ad85A i.n. given as a boost following BCG immunisation provides protection better than BCG alone. One of the obvious advantages of giving a TB vaccine i.n. is that the antigen is delivered directly into the lung, thus inducing an immune response at the point of pathogen entry. It appears to be these lung-resident cells, specifically 85A-specific CD8 T cells induced following i.n. but not parenteral immunisation, that are responsible for conferring protection in this model (52, 167). However, the mechanism by which protection is mediated was unknown.

Mtb infection is different from other pulmonary infections in that the induction of the immune response is much delayed (38, 166). Activation of *Mtb*-specific T cells is dependent on the dissemination of the mycobacteria to the mediastinal lymph node and this can take between 9 and 11 days (172), probably delayed by the actions of *Mtb* on infected pulmonary dendritic cells (39). Even once dissemination has occurred, the arrival of activated T cells in the lung is further delayed until 14 days post-infection (37) and only after this point does the immune response begin to control *Mtb*. This is the case for both naïve and BCG immunised mice (166). This presented an indication as to the mechanism by which Ad85A i.n. immunisation might confer protection. If antigen-specific CD8 cells were already present in the lung at the time of infection it is possible this delay in dissemination to the lymph node and subsequent activation of systemic cells could be bypassed, thus allowing earlier control of infection. Mapping the growth kinetics of *Mtb* in the lungs of Ad85A i.n. immunised mice showed control of mycobacterial growth in the first 7 days following infection (Figure 6). Depletion of T cell subsets indicated that early inhibition is dependent on CD8 but not CD4 T cells. This is in line with other work suggesting that Ad85A i.n. not only protects if CD4 cells are depleted at the time of challenge, but also that the induction of the CD8 response can occur, and importantly still confer protection, without CD4 help at the time of immunisation (173). Interestingly, it appears that early inhibition is independent of any prime-boost effect with BCG; rather that it is a separate event able to act in an additive manner with the late protection seen after parenteral BCG. This may point to a reason for the ineffectuality of some parenteral subunit boosts to BCG, including parenteral Ad85A. This is discussed further in Chapter 5 and 6.

The mechanism by which these CD8 cells protect is as yet unclear. It is unknown whether the lower mycobacterial burden seen earlier after infection is due to active killing of *Mtb* by CD8 cells or whether they are just inhibiting growth. The CD8 cells are able to produce large amounts of IFNγ and TNFα upon antigen stimulation (Figure 1), indicating the ability for cytokine-mediated activation of infected macrophages. However the 85A-specific cells in the lung also show a highly activated effector

status with reduced CD27 expression, a phenotype characteristic of high granzyme B and perforin expression (169), indicating that a cytolytic mechanism may at least be playing a part (164). Further work involving blockade of perforin or other cytolytic molecules *in vivo* may shed more light on the exact mechanisms involved.

Conferring protection is obviously a vital characteristic of any vaccine, but equally important is that the protection be durable. A vaccine which protects against infection years later is far more valuable than one protective only at the peak of the vaccine response. Ad85A i.n. immunisation induces a durable response that is able to protect at 6 months post-immunisation to the same level as that seen at 4 weeks (Figure 3). Unusually, the 85A-specific CD8 cells in the lung at 6 months display an activation status similar to that seen at the peak of the response (Figure 4). This highly activated phenotype appears to correlate with early protection (174), particularly as the antigen-specific cells in the lung after non-protective parenteral immunisation show a mainly effector memory activation status. Such a high level of activation and differentiation, particularly the low levels of CD27 expression on 85A-specific cells, suggests that this phenotype is being maintained by the persistence of antigen (169). Antigen vectored by adenoviruses has been shown to persist for up to 40 days following intra-muscular immunisation, leading to a protracted memory contraction phase and a high degree of activation long after immunisation (132). Transgene sequences have been detected in muscle tissue 1 year following intra-muscular adenoviral injection (133), although others have shown that mice clear the transgene from the lung within 14 days of i.n. inoculation (134). In the case of Ad85A, 85A-specific cells were shown to proliferate in the lung in an antigen-dependent manner (174) and lung tissue from mice immunised 6 months previously was able to drive proliferation of antigen-specific splenocytes *in vitro*, suggesting that antigen is persisting and driving the activated phenotype (54).

Not only is it important to have a population of activated antigen-specific cells in the lung, but the location of these cells within the lung is also important. Xing and colleagues have shown that, in order to protect, the cells must be present in the airways of the lungs rather than in the lung parenchyma

(146, 167). Data from our lab support this and indicate that a substantial proportion of cells localised in the airway express the chemokine receptor CXCR6 (175). The airway population appears to be independent of the periphery, able to proliferate and protect with no recruitment from secondary lymphoid tissues (174). By residing in the airway, activated antigen-specific CD8 cells are likely to be able to act on the cells initially infected by *Mtb* and thus inhibit the early growth stages. In contrast if cells are in the lung but only in the parenchyma then they are subject to the delay in activation induced by *Mtb* and are unable to act at the early growth stage (176)(Chapter 5).

3.4 Summary

This work has shown that the antigen-specific response following Ad85A i.n. immunisation consists predominantly of a highly activated CD8 T cell population that persists for at least 6 months. Importantly, protection following *Mtb* challenge is equivalent at this late time point post-immunisation to that seen at 4 weeks. It would be interesting to perform further investigation into the durability of this protection and the correlating activated phenotype of antigen-specific cells. The protection seen following Ad85A i.n. immunisation occurs during the early phase of *Mtb* infection, inhibiting growth in the first 7 days following challenge. This protection mechanism is distinct from that seen following parenteral BCG immunisation, where growth is only controlled after day 14 post-infection. This implies that, rather than acting as a traditional prime-boost effect, the protection seen following immunisation with the BCG-Ad85A i.n. regime is down to two separate inhibitory effects acting additively at different points of the *Mtb* growth curve. Further demonstration of this effect using non-interacting antigens is described in Chapter 6.

CHAPTER 4

The role of the nasal-associated lymphoid tissue (NALT) in Ad85A induced protection

Chapter 4

The role of the nasal-associated lymphoid tissue (NALT) in Ad85A induced protection after *Mtb* challenge

Sections of this chapter have already been published in Ronan *et al.* Vaccine (2010) (177) and have been approved for use by the co-authors in accordance with University of Oxford regulations.

4.1 Introduction

As shown in Chapter 3, Ad85A given by the intra-nasal route affords protection equivalent to that seen following parenteral BCG vaccination. This protection correlates with the induction of a large population of antigen 85A-specific CD8+ T cells in the lung and we and others have shown that the location of these cells within the lung airways is important (146, 175). However there is little information on the role of the upper respiratory tract (URT) associated lymphoid tissue in protection against *Mtb* challenge.

4.1.1 NALT

The importance of mucosal immunity in response to pathogens is becoming more and more evident. However, the extent to which mucosal and systemic immunity are separate has only recently become more clear. It is thought that a common mucosal immune system (CMIS) connects a series of organised inductive sites, termed mucosal-associated lymphoid tissue (MALT), with mucosal effector sites such as the genito-urinary tract, the lamina propria of the intestine and sections of the respiratory tract. Among the important MALT structures are Peyer's patches in the intestine and the bronchus-associated lymphoid tissue (BALT), located mainly at the bronchial bifurcations in the lung. It is not clear if all MALTs are connected equally to all others or if there is further sub-compartmentalisation of mucosal responses within the CMIS. Here, however, I will be focusing on the response in the MALT

tissue of the upper respiratory tract, the nasal associated lymphoid tissue, or NALT and its relationship to responses in the lower respiratory tract.

In rodents, the NALT is one of the major lymphoid tissues in the upper respiratory tract and consists of two separate compartments: the first is the diffuse, or D-NALT, consisting of small populations of immune cells dispersed throughout the nasal cavity; the second is the organised, or O-NALT. The O-NALT is made up of two paired lymphoid structures situated above the soft palate and composed of secondary lymphoid B cell follicles and T cell parafollicular areas (reviewed in (178)). The O-NALT, as a discrete, organised structure, was chosen for analysis due to greater consistency in isolating the whole tissue and will be what is referred to as NALT in the rest of this chapter.

4.1.2 NALT immunology

As mice are obligate nose breathers, the NALT is the first lymphoid structure to encounter inhaled antigen, primarily sampled through the M cells located within the NALT epithelium (179). The NALT is thought to be the primary site of induction of an airway immune response, followed by draining of cells to the cervical lymph nodes (180), although this may not always be the case (181). A number of studies have investigated the importance of the NALT in response to primary infections with a range of bacteria and viruses, as well as its role in vaccine induced protection against these pathogens. In a pneumococcal challenge model, cauterisation of the NALT did not affect protection induced by intra-nasal vaccination (182). However, the cauterization was performed on infant mice and at this stage NALT development may not be complete. In a model of influenza infection, virus specific CD8+ T-cells were shown not to accumulate in the NALT until after clearance of the infection, indicating that the NALT is not the inductive site for an immune response (181). A similar model of influenza challenge showed that ablation of the NALT had no effect on T-cell recruitment, serum or nasal cavity IgG and IgA levels or the speed at which the virus was cleared (183). However, in contrast, an intra-nasal model of reovirus infection showed the NALT to be the inductive site of both humoral and

cellular immune responses (184) and in another influenza virus model, depletion of T-cells prior to virus challenge increased viral load in both the lungs and nose, implying that T-cells restrict viral replication in both sites (185). In terms of vaccine-induced immune responses, NALT targeted vaccines have been shown to induce potent mucosal responses, both at local and distal sites (186, 187) and Fernandez *et al* have recently shown that the NALT is essential for recombinant staphylococcal enterotoxin B vaccine (rSEBv)-mediated protection in an intra-nasal SEB-induced toxic shock challenge model (188).

4.1.3 Aims

As the NALT clearly plays a significant role in the immune response to respiratory infections, it was important to investigate if it contributed to the protection from *Mtb* after Ad85A was given intra-nasally. Therefore immunisation regimes that might generate an equivalent response in the NALT but not in the lungs were investigated. This would allow investigation of the NALT response and its effect on subsequent *Mtb* challenge.

4.2 Results

4.2.1 Kinetics of the antigen-specific response in the NALT

Since the kinetics of the NALT response to adenoviruses are not known, it was necessary to determine at what point post-immunisation the maximal antigen-specific response occurred, in order that immunisation regimes could best be quantified and compared. To this end, a time course was performed where the NALT was extracted from mice immunised with Ad85A i.n. 7, 14, and 21 days previously. Figure 1 shows that the maximal NALT response was at 3 weeks post-immunisation and so this was the time point chosen for subsequent experiments.

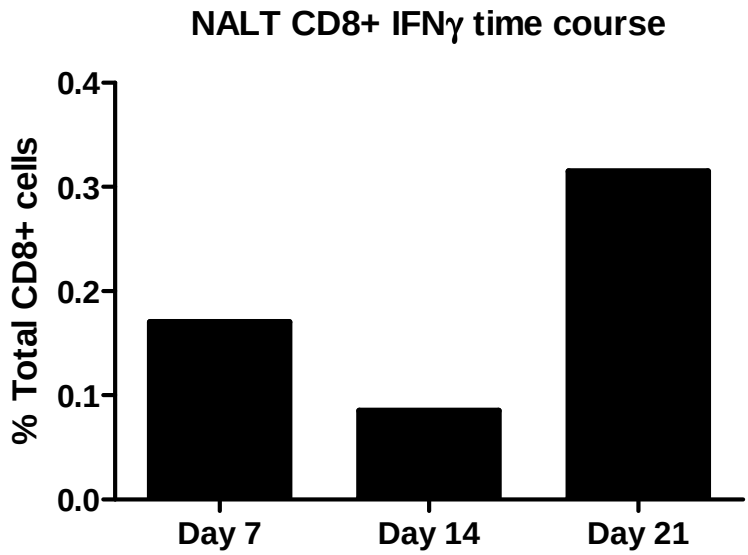


Figure 1: Time course of NALT CD8+ antigen specific IFNγ production. BALB/c mice were immunised i.n. with Ad85A in 50µl and the NALT extracted at the indicated number of days post-immunisation. NALT cells from 4 mice were pooled for analysis. ICS was performed following 6 hour stimulation with the dominant T cell epitopes of antigen 85A.

4.2.2 Optimisation of inoculum volume to specifically target NALT alone

It has been shown that the location within the respiratory tract of a response to an i.n. administered antigen is dependent on the volume of the inoculum (189). Therefore, by reducing the volume in which the Ad85A was delivered it should be possible to restrict it to the URT, and so to the NALT. As the same number of viral particles (v.p.) was delivered in each inoculum, there was a concern that reducing the volume too much would lead to an unacceptable increase in the variation because small changes in the delivered volume of a highly concentrated mixture would lead to large variation in the number of viral particles delivered. The standard, protective i.n. immunisation of Ad85A is given in 50µl and induces a strong antigen-specific response in the lung as well as a response in the NALT (Table 1). Therefore, in an attempt to restrict the response to the NALT only, inocula of 20µl, 10µl, 6µl and 5µl, all containing $2x10^9$ v.p., were administered and the antigen-specific responses in the

	NALT CD8+ IFNγ+	Lung CD8+ IFNγ+
5µl i.n.	0%-0.3%	0%
6µl i.n.	0.2% - 0.6%	0%-0.3%
10µl i.n.	0.4%	5.7%
20µl i.n.	1.2%	13.3%
50µl i.n.	0.05-0.5%	2-11%

Table 1: NALT and lung antigen-specific response following immunisation with inocula of different volumes. Percentage of antigen-specific IFNγ producing CD8+ cells in the NALT and lung after i.n. immunisation with Ad85A in different volumes. Data presented as the range of responses seen in at least two experiments with 4 mice per group or as single values if only one experiment was performed. Adapted from Ronan *et al* Vaccine 2010 (177).

NALT and lung were investigated (Table 1). Delivery of Ad85A in any volume greater than 6µl generated an antigen-specific response in the lungs, and although 6µl did induce a small lung response on occasion, administration in 5µl did not consistently induce a NALT response. Therefore, subsequent experiments were carried out using Ad85A in 5-6µl to target the NALT and 50µl to target the whole respiratory tract.

4.2.3 Immune response following NALT-specific or whole respiratory tract immunisation

The same number of Ad85A v.p. were given in either 6µl or 50µl and the number of antigen-specific cells in the lung and the NALT determined. Figure 2 shows the number of IFNγ+ T cells, as determined by ICS, in both the lung and the NALT after stimulation with the 3 dominant T cell epitopes of 85A. In the NALT, the 6µl and 50µl regimes induced comparable numbers of antigen-specific CD8+ T cells (Figure 2A). Although the numbers are very low this is a yield of cells from mouse NALT comparable to that reported by others (181). In contrast, 50µl induced a strong CD8+ response in the lung, with a higher frequency and large number of antigen-specific CD8+ T-cells ($\sim 3\times10^4$), while the 6µl inoculum induced fewer than 2000 antigen specific CD8+ cells in the lung ($p < 0.05$) (Figure 2B). The number of CD4+ antigen-specific cells induced in the lung and NALT by a 6 or 50µl inoculum ofAd85A was also compared and although there appeared to be a trend toward a higher response in the lung after administration of 50µl, the difference was not statistically significant

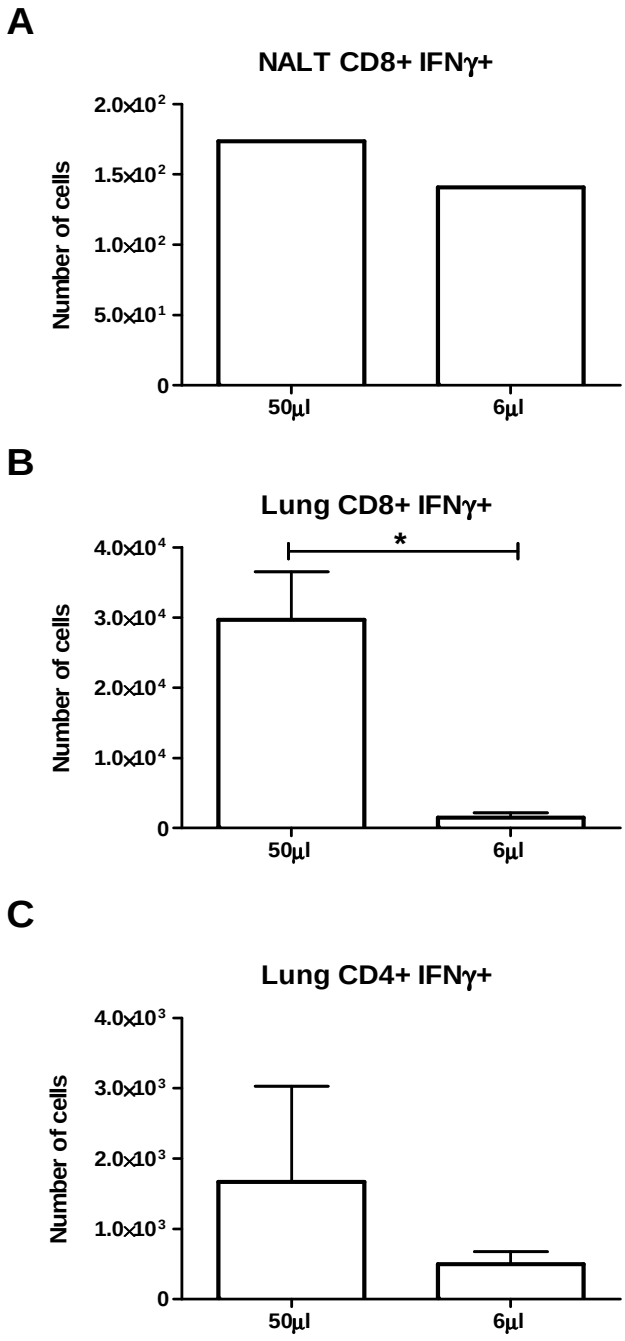


Figure 2: Number of IFNγ+ cells in lung and NALT. BALB/c mice were immunised i.n. with 50 or 5–6µl of Ad85A. Cells were extracted from the tissues 3 weeks after Ad85A immunisation. IFNγ production was quantified by ICS. Bars represent (A) the total number of CD8+ IFNγ+ cells per NALT based on the pooled cells from 4 mice (% IFNγ+ CD8 cells from pooled mice for 50µl = 0.36% and for 5–6µl = 0.57%), (B) mean total number of CD8+ IFNγ+ cells per lung for individual mice (mean % IFNγ+ CD8 cells for 50µl = 1.87% and for 5–6µl = 0.31%). (C) Mean total number of CD4+ IFNγ+ cells per lung for individual mice (mean % IFNγ+ CD4 cells for 50µl = 0.08% and for 5–6µl = 0.05%) *$p < 0.05$ as determined by Mann–Whitney test. Representative of at least three experiments. Adapted from Ronan *et al* Vaccine 2010 (177).

(Figure 2C). No CD4+ response was detectable in the NALT. Thus, immunisation with 6 or 50µl induce comparable CD8+ responses in the NALT but, although a 6µl inoculum induces a very small CD4+ and CD8+ response in the lung, a 50µl inoculum generates a much stronger lung CD8+ response.

4.2.4 Protection against *Mtb* challenge afforded by NALT-specific or whole respiratory tract immunisation

As shown in Chapter 3, administration of Ad85A i.n. in a 50µl volume affords protection equivalent to that seen by a single BCG s.c. immunisation. By developing an immunisation regime that gave an antigen-specific response in the NALT equivalent to that seen after 50µl immunisation but with a vastly reduced response in the lung, it was possible to isolate the role of the NALT in providing this protection. Mice were immunised with Ad85A in either 5-6µl or 50µl, either alone or in a prime boost regime, where BCG was given s.c. and Ad85A given 10 weeks later. All groups of mice, plus a naïve control, were challenged with *Mtb* by aerosol 4 weeks after the Ad85A immunisation. Immunisation with Ad85A i.n. in 50µl decreased mycobacterial load in the lung by ~1 log compared to unimmunised animals when given alone (5.48 Log vs. 6.23 Log; $p = < 0.01$) and when given as a boost after BCG by ~1 log more than BCG (4.49 log vs. 5.47 log; $p = < 0.01$) (Figure 3A). Immunisation with Ad85A i.n. in 50µl also decreased mycobacterial load in the spleen by ~1 log compared to unimmunised controls, though no additional protection was seen when Ad85A was given as a boost after BCG (Figure 3B). However when Ad85A was administered in 5-6µl, either alone or as a boost after BCG, no effect on mycobacterial load was detected in lung or spleen (Figure 3A and B).

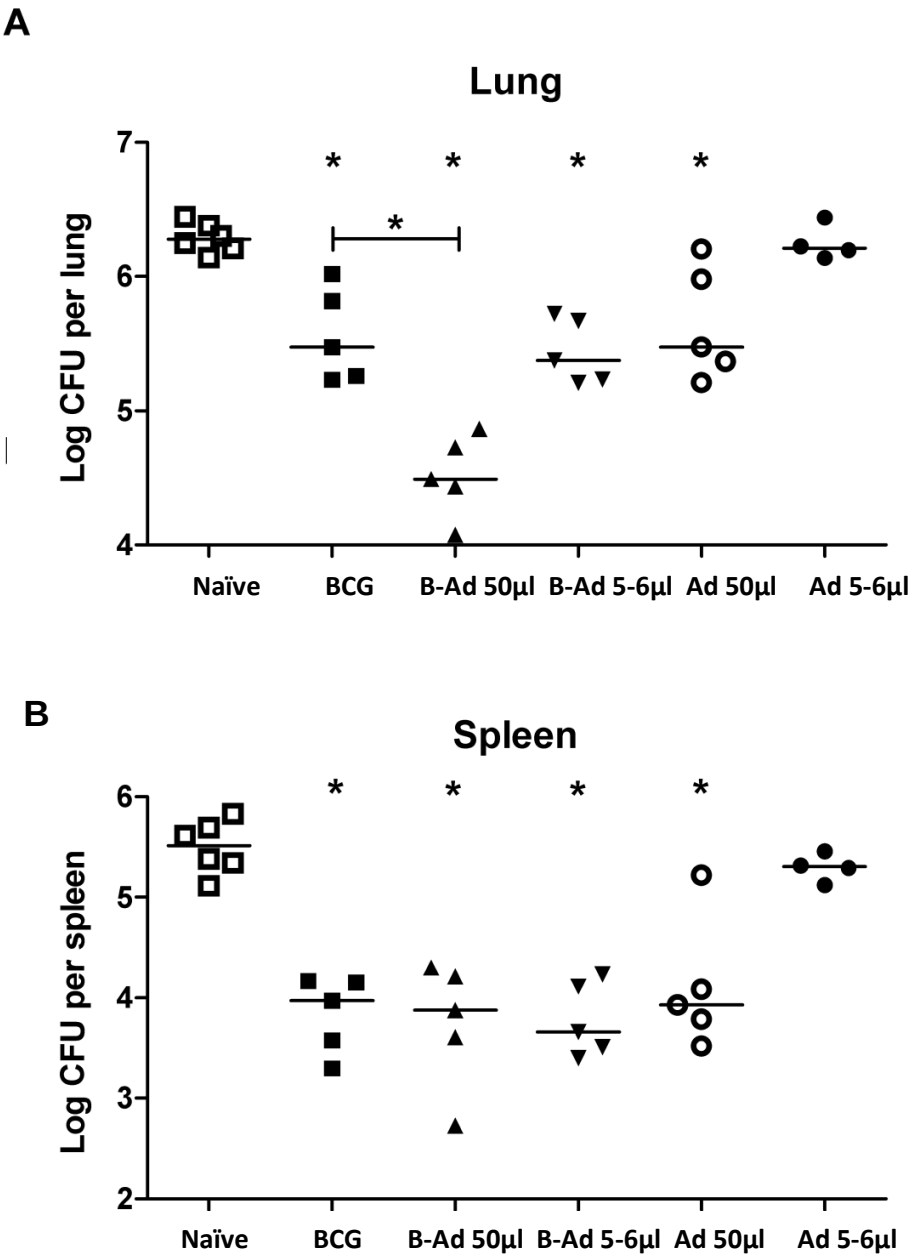


Figure 3: Mycobacterial CFU in lung and spleen after *Mtb* aerosol challenge. BALB/c mice were immunised with BCG and 10 weeks later immunised i.n. with Ad85A either in 50 or 5-6µl (B-Ad 50µl and B-Ad 5-6µl). Other mice were given Ad85A only in 50 or 5-6µl (Ad 50µl and Ad 5-6µl). Mice were challenged with 200CFU of *Mtb* 4 weeks post final immunisation, culled 6 weeks post-*Mtb* challenge and CFU enumerated for the lungs (A) and spleen (B). The horizontal line represents the median log CFU per group with each symbol representing one mouse. *$p < 0.05$. Statistical significance is compared to Naive group unless indicated and determined by Kruskal–Wallis test. Representative of at least three experiments. Adapted from Ronan *et al* Vaccine 2010 (177).

4.2.5 Quality of the lung immune response

As shown in Chapter 3, the presence of 85A-specific cells in the lung correlates with the protection seen after *Mtb* challenge. Although immunisation with Ad85A in 5-6µl i.n. did not provide any protection from challenge, there were still 85A-specific cells detectable in the lung, even if at a much reduced number and frequency (Figure 2 and Table 1). Investigations were therefore performed to determine if there were qualitative differences between the cells induced in the lung after immunisation in 5-6µl or 50µl, or whether the difference in protection was due to the number and frequency alone.

Cytokine response

Lung cells of mice from both regimes underwent ICS after stimulation with the mix of the 3 dominant 85A T cell epitopes and the total number of CD8+ T cells producing IFNγ, IL-2 or TNFα was compared. The mice immunised with 50µl had a greater number of cytokine producing CD8+ cells compared to those immunised with 5-6µl for all 3 of the cytokines measured (Figure 4A). As polyfunctional antigen-specific cells have been reported to be important in a number of diseases including *Mtb* infection (51, 170), the proportion of CD8+ T cells that were producing all three (3+), any two (2+) or only one (1+) cytokine was assessed (Figure 4B). Immunisation with 50µl induced a greater proportion of single cytokine producing CD8+ cells than immunisation with 5-6µl and this difference was made up of cells producing IFNγ (Figure 4A).

Activation phenotype of antigen-specific cells

Although the number of cytokine producing cells was higher in the 50µl group for all three cytokines, the most marked difference was in the number of IFNγ+ cells (Figure 4A). As shown in Chapter 3, it is not just the number or frequency of IFNγ+ cells that is important but also their activation state as defined by CD62L and CD127 expression (133). It is a high proportion of highly activated effector

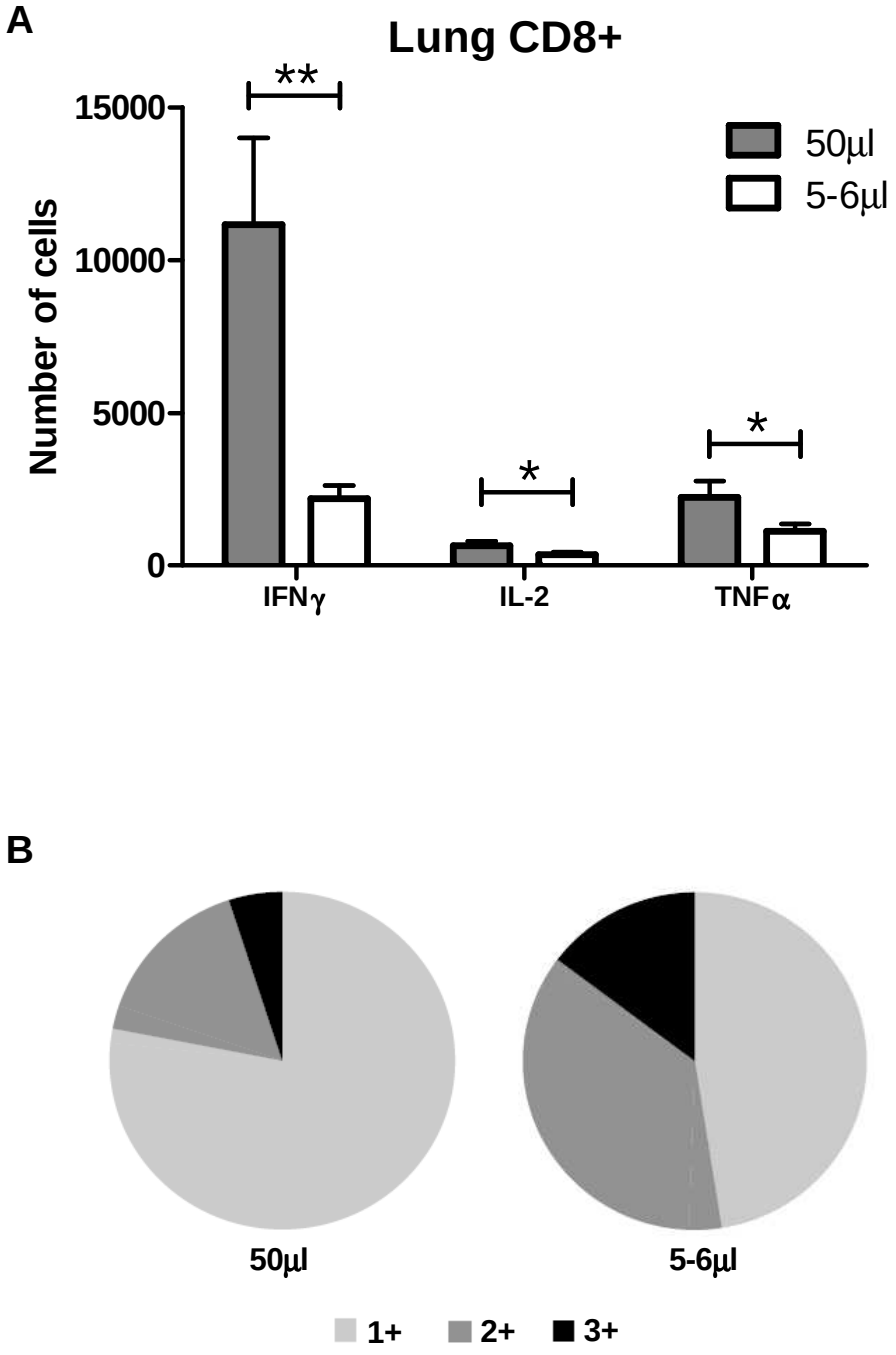


Figure 4: Cytokine production 3 weeks post-Ad85A immunization with 6 or 50µl. (A) The total number of CD8+ cells producing either IFNγ, IL-2 or TNFα per lung ± SEM (12 mice/group). **$p < 0.005$; *$p < 0.05$ as determined by Student's t-test. (B) The quality of the lung CD8+ response, showing the proportions of cells producing 1, 2 or all 3 of IFNγ, IL-2 and TNFα. Representative of two experiments. Adapted from Ronan *et al* Vaccine 2010 (177).

	Effector		Effector Memory	
	Total cells	**% antigen specific cells**	**Total cells**	**% antigen specific cells**
5-6µl	682 (±315)*	47.0 (±3.84)*	675 (±333)*	37.5 (±8.16)
50µl	17430 (± 4063)	60.9 (±0.98)	11732 (±2776)	37.8 (±1.14)

Table 2: Total number and proportion of antigen specific cells of effector and effector memory phenotype in the lung after immunisation with small or large volumes. Both the mean number of CD8+ IFNγ+ cells present per lung within the effector subsets and the percentage of all CD8+ IFNγ+ cells in each subset. Effector cells are defined as CD62L− CD127− and effector Memory as CD62L− CD127+. The gating strategy and a FACS plot showing the indicated populations is shown in Chapter 3. Data are represented as the mean of 4 mice ±SEM. Statistical significance was determined by Mann–Whitney test. *$p < 0.05$. Adapeted from Ronan *et al* Vaccine 2010 (177).

cells (CD62L-, CD127-) compared to effector memory cells (CD62L-, CD127+) that correlates with protection. The IFNγ+ CD8+ cells from both the 50µl and 5-6µl regimes were therefore examined for CD62L and CD127 expression. Immunisation with 50µl induced significantly higher numbers of both effector and effector memory cells, as well as a greater frequency of effector cells (Table 2).

4.2.6 Augmenting the NALT response

Following Ad85A i.n. immunisation, the NALT response does not contribute to protection. However, it was unclear whether this was due solely to the low magnitude of the response. To determine if a stronger NALT response may have an effect, Ad85A was administered in 5-6µl in combination with Cholera Toxin (CT), a potent mucosal adjuvant in the NALT, and compared with administration in 50µl and 5-6µl alone (Figure 5). Administration of Ad85A with CT as an adjuvant greatly increased the level of response in the NALT compared to Ad85A alone given in 5-6µl (Figure 5A). However, when these mice were challenged with *Mtb* there was no change in the level of protection afforded (Figure 5B).

4.3 Discussion

It is now becoming clear that targeting a vaccine to the mucosa is not only possible but an effective means by which to immunise against mucosal pathogens. Vaccines against measles and influenza are licensed in some countries for delivery by nebulizer or nasal spray, showing that the respiratory route is a safe and efficacious route of delivery. As Ad85A has been shown in pre-clinical models to protect against *Mtb* challenge when given into the airway (Chapter 3, (54, 148)), the importance of different respiratory tract lymphoid areas in contributing to protection needs to be investigated. This is particularly important if such a mucosal vaccine is going to be developed to combat *Mtb* infection in humans as it could have a huge impact on the method by which the vaccine is delivered.

In rodents, the major upper respiratory lymphoid tissue is the nasal associated lymphoid tissue, or NALT. It is located at the rear of the soft palate in a position to sample any antigen carried through the airways that settles in the upper respiratory tract. It seems that there is no NALT structure in adult humans, although diffuse NALT can be found in children under 2 years old (190). However, the structure in rodents is thought to be analogous to Waldeyer's ring in humans, consisting of the adenoids, tonsils and other lymphoid structures. By investigating the NALT and its role in mice, the significance of these upper respiratory lymphoid structures in protecting the human lung may be established.

As shown in Chapter 3, intra-nasal immunisation with Ad85A confers protection by inhibiting *Mtb* growth early after infection and this inhibition correlated with the presence of antigen specific CD8+ T cells. As Ad85A induces an antigen-specific response in the NALT as well as the lung, it is possible that some of the early inhibition is due to an immune response in the URT as well as in the lung. Here an immunisation regime is established that maintains an antigen-specific response in the NALT equivalent to that following 50µl immunisation but that has a greatly reduced lung response.

There is little information on the kinetics of the immune response in the NALT, particularly the T cell

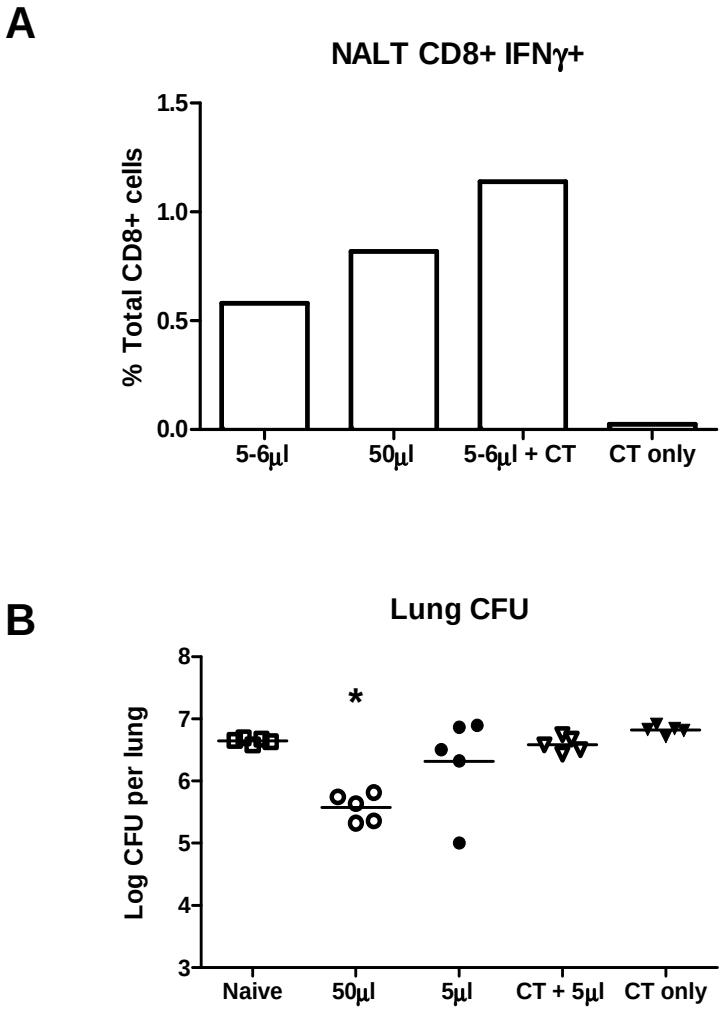


Figure 5: Immune response and protection against *Mtb* challenge following CT immunisation. (A) The percentage of CD8+ NALT cells producing IFNγ 3 weeks post-immunisation. BALB/c mice were immunised as before, with Ad85A with CT in 5-6µl or with CT alone. ICS was performed after 6 hour stimulation with the T cell epitopes from 85A. (B) Mice were intra-nasally challenged with ~300CFU *Mtb* 3 weeks post-immunisation and culled 4 weeks post-challenge. * $p < 0.001$ versus naïve as measured by ANOVA.

response. Wiley *et al* have shown that, after influenza infection, accumulation of T cells in the NALT does not begin until 8 days post infection (183), peaks at day 10 and then declines (181). After reovirus infection, the peak IgA response in the NALT is found at day 7 before tailing off, although a significant level is maintained at least as far as day 15 (184). After vaccination with pneumococcal polysaccharide the serum antibody response also peaks 7 days post-immunisation. In comparison to these studies the peak response to Ad85A occurred much later, at 21 days post-immunisation, although a response can be detected at 7 days. This mirrors the response kinetics seen in the lung after Ad85A and, although delayed in comparison to other infections, is in line with the kinetics of the NALT after other adenovirus infections (191).

The importance of the NALT in protection against various respiratory tract pathogens is still under debate. After primary influenza infection the response in the NALT is only detectable after pathogen-specific responses have been seen in the nasal mucosa and cervical lymph nodes (CLNs), implying that the NALT is not necessary for induction of a protective response (181). Further, when the NALT is surgically removed the rate of clearance of influenza from the lungs goes unchanged (183). Similarly, surgical removal of the NALT has no effect on the induction of IgA antibodies after i.n. immunisation with a pneumococcal vaccine (182), although it should be noted that this was in infant mice where the NALT may not be fully developed. However, other work has shown that the NALT is the inductive site after reovirus infection (184) and that if T cells are depleted from the nose during influenza infection the viral titres significantly increase (185). Recently, Fernandez *et al* have shown that a functional NALT is necessary for protective immunity against staphylococcal enterotoxin B (SEB) after i.n. immunisation with a recombinant vaccine (188). Others have shown that the innate immune compartment in the NALT can significantly affect the systemic response following intra-nasal immunisation with a candidate tuberculosis vaccine (192). However, in this model, an 85A-specific response that is restricted to the NALT alone, without a concurrent response in the lung, is unable to confer any protection after *Mtb* challenge.

To conclusively show that it plays no role in protection, it is necessary to bypass the NALT and induce a lung response only. To this end, intra-tracheal immunisations were performed to bypass the URT. This technique proved difficult and variable in effect and the data are therefore not included here; however, when mice were challenged with BCG after intra-tracheal Ad85A immunisation, mice with a response in the lung, but no response in the NALT, showed protection equivalent to that conferred by 50µl i.n. immunisation.

Another consideration must be that the lack of protection is due to the low magnitude of response seen in the NALT. Although it has been shown that adenovirus delivered to the NALT does infect and persist in the NALT tissue (191), it may be that this does not deliver a sufficient PAMP signal to induce a protective response. Cholera toxin (CT), produced by *Vibrio cholera,* is a potent mucosal adjuvant and, as shown in Chapter 6, can be used to elicit a protective lung response. Although unsafe for use in humans in its native form, safer mutant versions are currently in development (193). To determine if a more potent antigen-specific response would afford protection, Ad85A was administered i.n. in 5-6µl along with CT. This regime successfully increased the level of response but provided no protection after subsequent *Mtb* challenge (Figure 5). This indicates that it is the location of the response within the airway, rather than its magnitude that is significant in affording protection from *Mtb* challenge.

As it is the location of the antigen-specific cells within the airway that is important for protection, it is worth considering the small population of cells induced in the lung by 5-6µl immunisation and why these cells do not provide any detectable protection after challenge, at least in experiments of this size. When the memory phenotype of the antigen specific CD8+ cells induced in the lung by each regime was examined, it was clear that 50µl immunisation induced far more effector cells than 5-6µl immunisation, both in terms of relative frequency and total cell numbers (Table 2). In Chapter 3 it was demonstrated that highly activated effector cells in the lung correlated with protection and the low frequency of these cells after NALT specific immunisation may contribute to the lack of protection.

After 50µl immunisation, it is thought that the activated effector phenotype is driven by the persistence of antigen. However, adenovirus administered in 6µl has been shown to locate only to the NALT (191) and the cells in the lower respiratory tract after this immunisation will not be driven by local antigen to maintain the effector phenotype. Another possibility is that the cells detected in the lung after the two regimes have different locations within the tissue. Migration between different mucosal compartments through common homing mechanisms has been widely described (178, 194) and it may be that the 85A-specific cells detected after 5-6µl immunisation are located in the bronchial associated lymphoid tissue (BALT). This may also explain the difference in memory phenotype as highly activated effector cells are likely to be resident in the tissue rather than migrating to other lymphoid tissue.

The most substantial difference between the regimes was that NALT-specific immunisation induced far fewer antigen-specific cells, whether this was measured by IFNγ, IL-2 or TNFα production (Figure 4) and it may be that this is the most important factor in determining the extent of protection. Immunisation with Ad85A in 50µl induces an immune response in the draining mediastinal nodes as well as inducing inflammation in the lung. This means that antigen-specific cells leaving the mediastinal lymph nodes will be recruited back to the lungs, including the airways, because of local inflammation (195). Any activated, non-antigen-specific cells in the blood will most likely also be recruited into the lungs. In contrast, immunisation with a small volume induces a weak immune response in the NALT and perhaps the cervical nodes, but because the lungs are not inflamed, cells leaving these inductive sites will not be preferentially recruited to the lungs. It would be interesting to investigate the administration of a NALT immunisation concurrently with an empty adenovirus delivered to the lower respiratory tract to see if this had any effect on protection.

These data have important implications for the design of TB vaccines that are to be administered to the respiratory tract. A range of instruments and techniques exists for the respiratory delivery of pharmaceuticals, all with various particle size, speed and delivery method (150, 157). These data show that, for protection against *Mtb*, a method must be chosen that delivers viable vaccine to the lower

respiratory tract and alveoli. This may be beneficial for clinical trials, for if the NALT is not necessary then the vaccine could be inhaled through the mouth from a nebulizer or similar device, rather than through the nose. This may reduce the risk of the vaccine translocating to the cranial nerves, a risk associated with intra-nasal administration (159, 196). However, it should be noted that although the greater lung inflammation caused by lower respiratory tract administration of Ad85A may contribute to protection, vaccine-induced inflammation leading to lung pathology has been reported for other vaccines (197).

4.4 Summary

This work has shown that the peak of the immune response in the NALT after Ad85A i.n. vaccination is 21 days post-immunisation and that this NALT response can be isolated from the response in the lower airways by the delivery of the virus in 5-6µl. By challenging mice with a NALT-specific response only it was possible to show that the NALT response alone was not sufficient to confer protection after *Mtb* challenge and that a response in the lower respiratory tract was necessary, and possibly sufficient to provide any reduction in mycobacterial load. Of further interest, the lack of protection after NALT-specific immunisation was in spite of a small population of 85A-specific cells present in the lung. Whether these cells were unable to provide protection purely due to paucity of numbers or whether it was due to their possible location in the BALT rather than the airway could be an interesting avenue of future work. Similarly, it is unclear whether non-specific lung inflammation and cell infiltration may be necessary for NALT-targeted immunisation to protect. Investigation into these factors may indicate what type of administration techniques would be optimal for future TB vaccine development.

CHAPTER 5

Immune responses and protection in the lung following intra-dermal immunisation with Ad85A in different animal facilities

Chapter 5

Immune response and protection in the lung following intra-dermal immunisation with Ad85A in different animal facilities

5.1 Introduction

As shown in Chapter 3, Ad85A administered intra-nasally (i.n.) is able to confer protection against *Mtb* challenge equivalent to that seen following BCG immunisation. This protection correlates with an antigen-specific, highly activated population of CD8 T cells in the lung and with inhibition of mycobacterial growth in the first week following infection. It has previously been shown that, although protective when administered i.n., Ad85A does not protect if given intra-dermally (i.d.) (52). This lack of protection is not due to poor immunogenicity following immunisation, as a significant systemic response is detected in the spleen. Rather, the lack of protection is due to the location of the response; intra-dermal (i.d.) immunisation induces only a minimal response in the lung and it is this that is thought to be required for protection (52). Forbes *et al* show that Ad85A immunised i.n. generates a lung antigen-specific response consisting predominantly of CD8 cells. However, when Wang *et al* use the same vaccine the i.n. response is split between CD4 and CD8 cells, with a greater number of CD4 cells responding. When they give Ad85A parenterally (intra-muscularly; i.m.) they see a CD8 response in the lung equivalent to that seen following i.n. immunisation (140) and in a paper by Santosuosso *et al* i.m. immunisation even generates a stronger CD8 lung response than i.n. immunisation when tested at 2 weeks (167).

Due to closure of the animal house at the Centre for Ecology and Hydrology (CEH), a change in animal facility was necessary during the course of this work. Anecdotally, it is often said that changes between facilities can lead to unexpected changes and variation in the outcomes of previously consistent experiments. Despite the anecdotal evidence, little experimental work appears to have been published on the subject, though it has been shown that mice raised in cleaner, specific pathogen free

(SPF) facilities can have different susceptibilities to autoimmune diseases compared to those raised in less stringently clean animal houses (198-200). In the case of the work described here, the change in animal house was from a non-SPF facility at CEH to a tightly regulated SPF facility, Biomedical Sciences level 2 (BMSL2). As a switch in facilities had the potential to affect the outcome of the Ad85A immunisation regimes, confirming that our model remained robust in the new facility was necessary.

As it happened, changing animal facility did have an effect on the response; specifically, the response following Ad85A i.d. immunisation (see Results below). This change in response allowed exploration into the underlying causes as well as the effect of the change on protection. Fortunately, no alteration was observed in the response following intra-nasal administration, giving a baseline constant against which the i.d. responses could be compared.

5.1.1 Aims

The change in the response to Ad85A under SPF conditions raised three questions: whether increased environmental antigen exposure was behind the response seen in CEH; whether a specific microorganism or group of microorganisms might be behind the change and whether the altered Ad85A i.d. lung response, which exhibited some of the characteristics of the protective i.n. profile, also reflected a change in the protective efficacy of i.d. immunisation.

5.2 Results

5.2.1 Ad85A i.d. response in the CEH versus BMSL2 animal facilities

As shown in Chapter 3, Ad85A i.n. generates a lung response able to protect following *Mtb* challenge. It has also been shown that the same vaccine administered parenterally does not protect, the proposed reason being that there was very little response induced in the lung (52). Data generated in the same animal facility (CEH) used for the work in (52) replicated these findings: Ad85A i.n. generated a

strong lung response but little in the spleen and Ad85A i.d. induced a strong splenic response but little in the lung (Figure 1). When Ad85A immunised mice from CEH were challenged with *Mtb* only the mice immunised i.n. showed a reduction in mycobacterial load compared to naïve mice, confirming the data in (52)(Figure 1C). This suggested that protection correlated with a strong lung response and that the minimal lung response following i.d. immunisation was the reason for this regime not conferring protection, despite a strong systemic response.

Upon switching animal facilities from CEH to BMSL2, it was necessary to confirm that the results found in the previous facility were reproduced. Mice were immunised with Ad85A either i.d. or i.n. and culled after 4 weeks and the lung and spleen response determined. Ad85A i.n. immunised mice showed the same pattern of response as that seen in the CEH facility, with a strong lung response but a negligible splenic response (Figure 2). Surprisingly, however, the mice immunised i.d. showed a markedly different response compared to that seen in CEH. The response in the spleen was again strong, made up predominantly of CD8 cells, although responding CD4 cells could also be detected. The differences occurred in the lung. The CD4 response following i.d. immunisation was now closer in magnitude to that seen after i.n. immunisation. However, the most striking difference was that the CD8 IFNγ response was notably stronger after i.d. than after i.n. immunisation (Figure 2). The responses showed a similar profile for TNFα production, and if CD8 cells were stained with an 85A-specific MHC class I tetramer i.d. and i.n. immunised mice showed approximately equal levels of tetramer-positive cells (data not shown).

As Ad85A i.d. immunisation in BMSL2 was giving a strong lung response, it was necessary to characterise the phenotype of these cells and determine how they compared to the antigen-specific cells in the lung induced by i.n. immunisation. Chapter 3 showed that 85A-specific cells in the lung following i.n. immunisation exhibited a highly activated phenotype, with the majority displaying effector activation status. In order to determine if the i.d. induced cells in the lung displayed a similar phenotype, these cells were stained for the surface markers CD62L and CD127. As in Chapter 3,

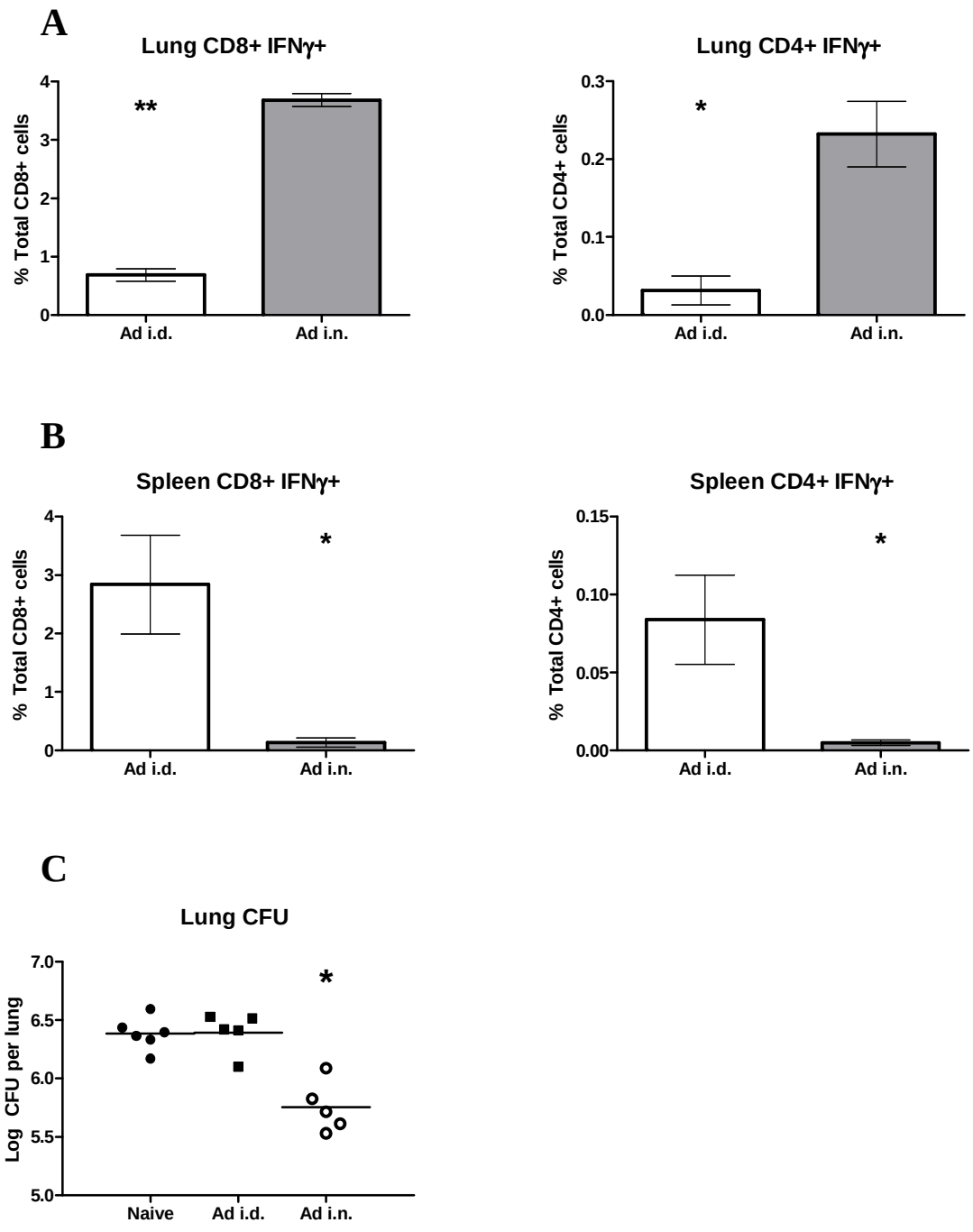


Figure 1: IFNγ response of mice immunised with Ad85A i.n. or i.d. in the CEH animal facility. BALB/c mice were immunised i.n. or i.d. with Ad85A and 4 weeks post-immunisation mice were culled, lung and spleen cells isolated and stimulated with the pool of 85A peptides (similar results were obtained with the mix of 3 dominant T cell epitopes). The CD8 and CD4 IFNγ response in the lung (A) and spleen is shown (B), as measured by intra-cytoplasmic staining (ICS). (C) Immunised mice were challenged with *Mtb* by aerosol 4 weeks post-Ad85A, culled 6 weeks post-challenge and lung CFU enumerated. ** $p < 0.0001$; * $p < 0.05$ by Student's t-test. Representative of 3 experiments.

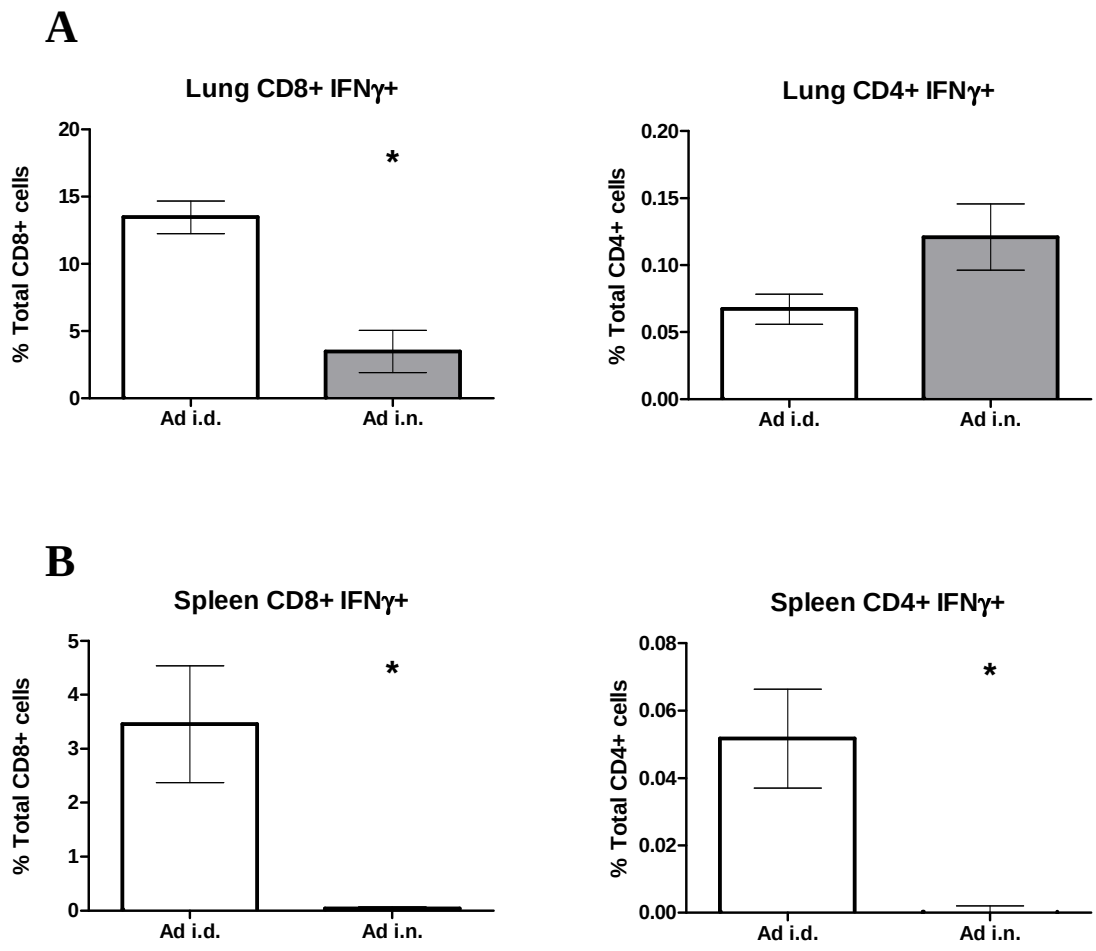


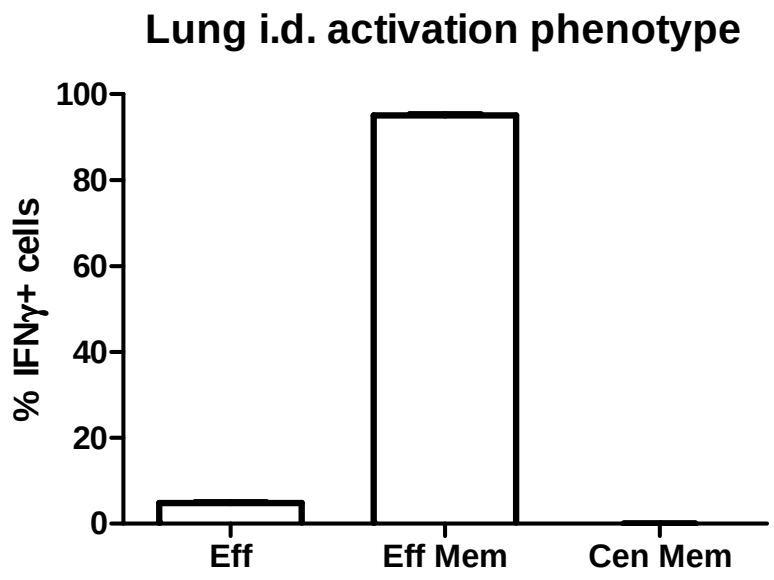
Figure 2: IFNγ response of mice immunised with Ad85A i.n. or i.d. in the BMSL2 animal facility. BALB/c mice were immunised i.n. or i.d. with Ad85A as a single immunisation. 4 weeks post-immunisation mice were culled, lung and spleen cells isolated and stimulated with the mix of the 3 dominant T cell epitopes. The CD8 and CD4 IFNγ response in the lung (A) and spleen is shown (B), as measured by ICS. * $p < 0.05$ by Student's t-test. Representative of 3 experiments.

effector cells were defined as CD62L- CD127-, effector memory cells defined as CD62L- CD127+ and central memory defined as CD62L+ CD127+ (168). Characterisation by these markers identified that, contrary to that seen following i.n. immunisation, the majority of the antigen-specific cells induced in the lung by i.d. immunisation were of effector memory phenotype (Figure 3). A less differentiated, less activated phenotype following i.d. immunisation may be due to the lack of antigen in the lung following i.d. immunisation, as we have shown the persistence of antigen in the lung following i.n. immunisation may be driving the increase in activation (Chapter 3)(54). One of the signs of recent antigen exposure is down regulation of CD27 (169). Lung antigen-specific cells induced following i.n. immunisation showed very low levels of CD27 expression (Chapter 3, Figure 3) but, following i.d. immunisation, a greater proportion of the 85A-specific cells remained CD27+ (Figure 3). However this was only a small increase, with the vast majority of i.d. induced antigen-specific cells remaining CD27-. This may indicate that reduced antigen exposure is not the reason for the lower activation state of i.d. induced CD8 cells.

5.2.2 Protective effect of Ad85A i.d. immunisation

In CEH, an antigen-specific CD8 T cell population in the lung had correlated with protection. Therefore it was interesting to investigate whether the mice from the BMSL2 animal house were protected following Ad85A i.d., and if this protection was of the same magnitude and occurred by the same mechanism as that seen after i.n. immunisation. In Ad85A i.n. immunised mice mycobacterial growth was inhibited in the first 7 days following infection and dependent on the vaccine induced CD8 response (Chapter 3). It was hypothesised that this early control was due to the highly activated cells present in the lung at the time of challenge acting immediately upon infected cells, thus avoiding the delay in mycobacterial dissemination to the lymph nodes (Chapter 3). After Ad85A i.d. immunisation in BMSL2 the cells, although present in the lungs, were less activated. It was therefore possible to tell if the presence of cells in the lung was sufficient for protection or if those cells needed to be highly activated in order to inhibit *Mtb* growth. Mice from the BMSL2 facility were immunised either i.d. or

A



B

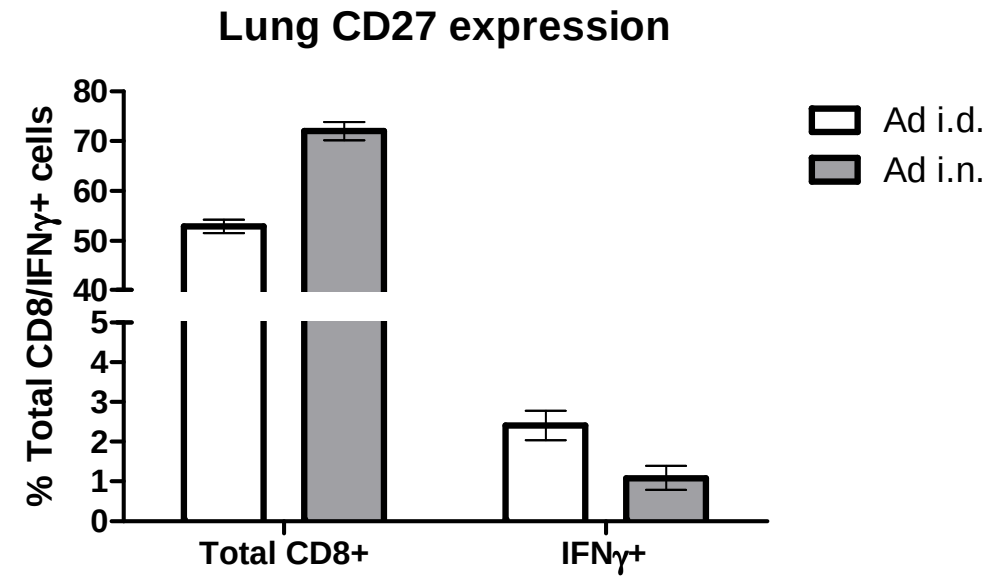


Figure 3: Activation status of Ad85A i.d. induced cells in the lung in BMSL2. (A) Activation status of antigen-specific cells from the lung of mice immunised with Ad85A i.d. 4 weeks previously. Cell subsets were defined as in Chapter 3 Fig. 4. (B) CD27 expression of total CD8 T cells and antigen-specific CD8 cells in the lung of mice immunised i.d. or i.n. 4 weeks previously. Results are expressed as the mean and SEM from 3 or 4 mice per group. Representative of 3 experiments.

i.n. with a single dose of Ad85A and 4 weeks later were challenged i.n. with *Mtb*. On days 7, 14, 21 and 28 following infection mice were culled and the *Mtb* CFU in their lungs enumerated (Figure 4). As seen in Chapter 3, mice immunised with Ad85A i.n. were able to inhibit *Mtb* growth in the first 7 days, showing a significantly reduced mycobacterial load compared to naïve mice. This reduction was then maintained for all time points measured (Figure 4). However, i.d. immunised mice showed no reduction in CFU compared to naïve mice for days 7, 14 or 21. Only at day 28 was there a significant reduction in mycobacterial load compared to naïve mice, although the *Mtb* burden remained significantly higher than that seen in the i.n. immunised mice (Figure 4).

These data indicate that, despite inducing a stronger lung cytokine response, Ad85A i.d mice were less able to reduce the *Mtb* CFU in the lungs than i.n. immunised mice. This also indicates that the mechanism by which the Ad85A i.d. induced cells protect is distinct from that seen following i.n. immunisation, with i.d. mice only limiting growth in the late phase of growth, as opposed to the early phase inhibition exhibited in i.n. immunised mice.

5.2.3 Investigations into the change in Ad85A i.d. lung response

The switch of animal facilities from CEH to BMSL2 had significantly altered the response seen following i.d. immunisation and so further investigation into the possible causes was warranted. The most obvious difference between the two facilities was the level of cleanliness. BMSL2 is a specific pathogen free (SPF) facility, with mice kept in sealed, isolated cages receiving only filtered air. All food, bedding and water is autoclaved and mice are only handled within a laminar flow hood. All these measures and others are present to ensure the mice are exposed to as few pathogens or antigens as possible. In contrast, the CEH facility did not have SPF status; mice were kept in open top cages exposed to the air and drank water drawn from the tap. As such, these mice were likely to have been exposed to far more environmental microorganisms and their immune systems to have experienced more antigenic challenge. As the most notable difference between the facilities, it was this contrast that

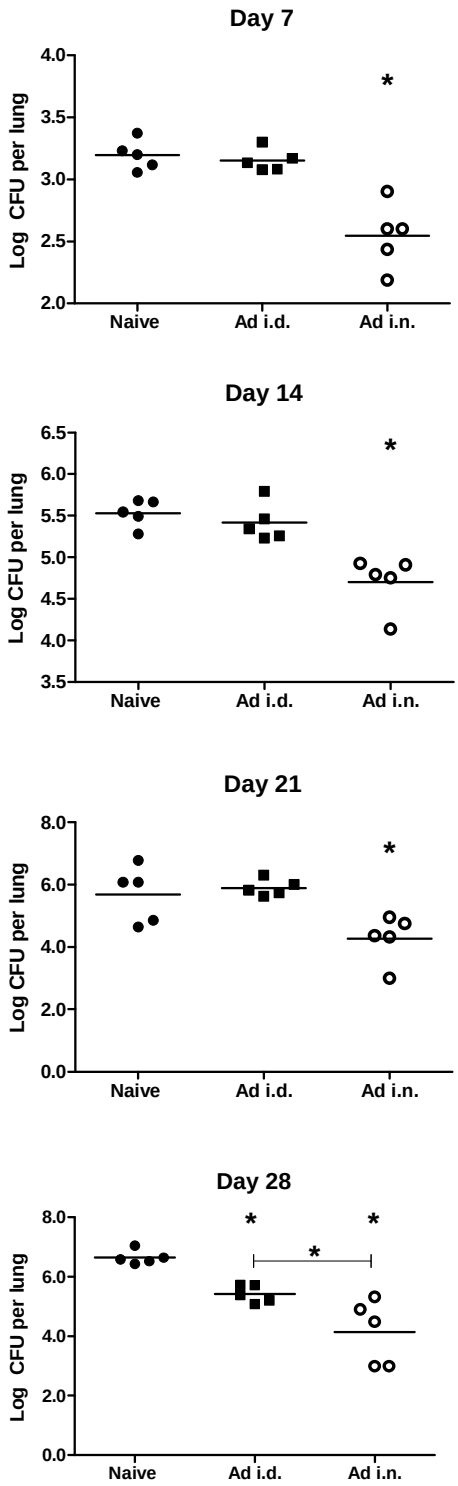


Figure 4: Time course of *Mtb* load in BMSL2 mice following low dose i.n. challenge. BALB/c mice were immunised with i.d. or i.n. with Ad85A and 4 weeks later challenged i.n. with a low dose (~200 CFU) of *Mtb*. Naïve mice were used as a control. On days 7, 14, 21 and 28 post-challenge mice were culled and *Mtb* CFU in the lung enumerated. * $p < 0.005$ versus naïve, unless indicated.

was explored in an attempt to explain the change in responses.

A direct comparison between the two facilities, along with a comprehensive screen of any environmental microorganisms present, would have been the ideal way to investigate this problem. Unfortunately, at no point was concurrent access to both the BMSL2 and CEH facilities available, thus precluding any direct comparisons between the two. However, in order to determine if the difference was down to mice being housed in “clean” (BMSL2) or “dirty” (CEH) environments, other “dirty” animal facilities were sought. Of the suitable locations in Oxford, the BMS level 1 and Anatomy animal facilities were chosen as neither are SPF and both house mice in open top cages. If mice kept in one or both of these facilities gave the same response following Ad85A i.d. immunisation as those kept in CEH, then it might have been possible to elucidate the reason for the change in response seen in BMSL2. Mice were housed in the two facilities for 1 week following arrival from the supplier (Harlan supplied all the mice used in all facilities mentioned), then immunised i.d. or i.n. with Ad85A. After 4 weeks further housing in the facilities, the mice were culled and the lung response measured (Figure 5). In both animal houses, the response was comparable to that seen in BMSL2, with the CD8 response following Ad85A i.d. immunisation stronger than that seen following i.n. immunisation. The CD4 response following i.d. or i.n. immunisation in either animal house was not different from that seen in the CEH facility. These results indicate that, rather than the BMSL2 mice having unusual responses, it is the response in mice from CEH that is exceptional. They also show that this exceptional response was not being driven purely by the fact that it was a “dirty” facility.

5.2.4 Environmental exposure to mycobacteria

Housing mice in other “dirty” facilities did not lead to the same response as that seen in CEH, indicating that general exposure to environmental microorganisms and antigens is not sufficient to reduce the lung response following i.d. immunisation. This implies that it was a factor specific to CEH that was driving the change in response, most likely exposure to a specific microorganism found in the

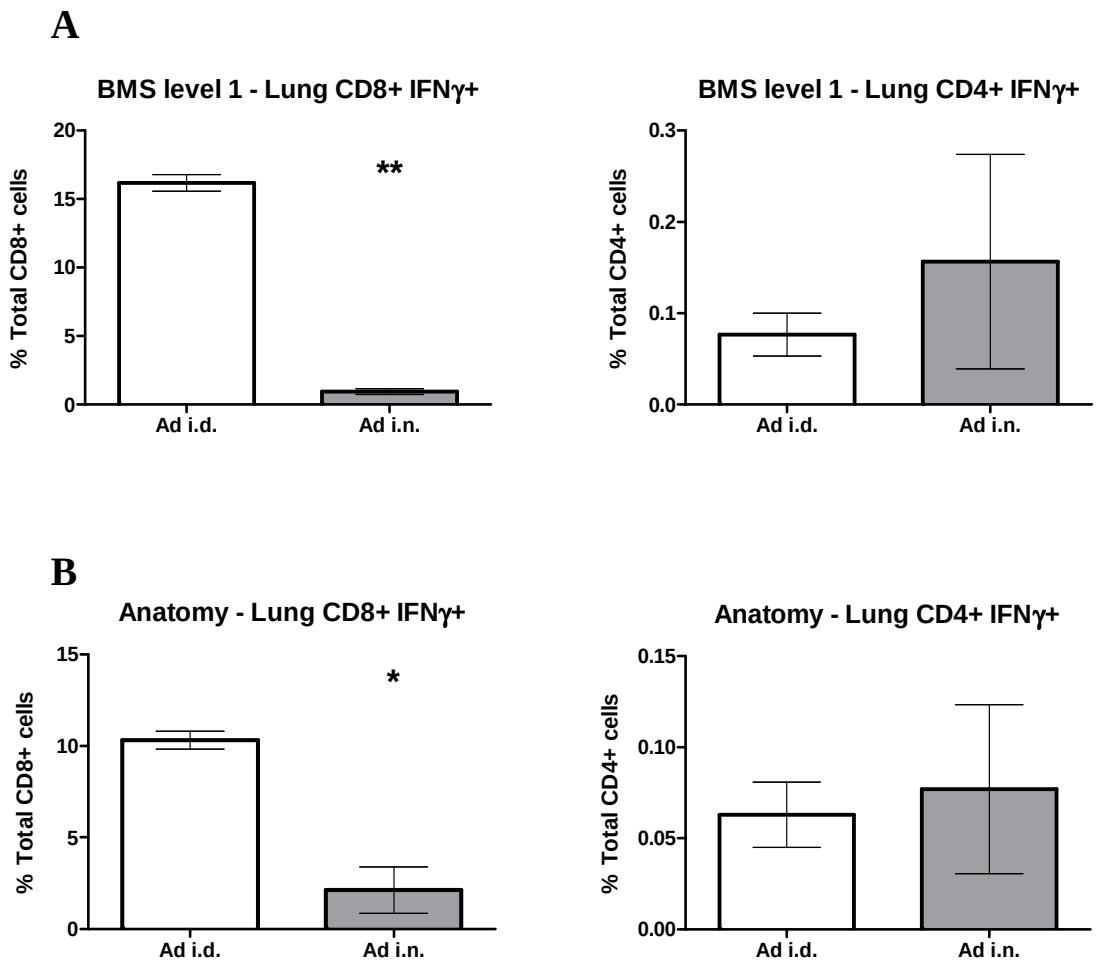


Figure 5: Lung IFNγ response in mice housed in other "dirty" facilities. BALB/c mice were housed in one of 2 different "dirty" animal facilities: BMS level 1 (A) or Anatomy (B). Mice were housed in the dirty facility for 1 week before being immunised i.d. or i.n. with Ad85A. At 4 weeks post-immunisation mice were culled, lung and spleen cells isolated and stimulated with the mix of the 3 dominant T cell epitopes. Lung IFNγ response is shown, as measured by ICS. ** $p < 0.0001$, * $p < 0.005$. Comparable results were also seen in the Institute of Molecular Medicine animal house.

facility. Whilst this specific microorganism could be anything, it has been shown that TB vaccines can be affected by prior exposure to environmental mycobacteria (201). It could be that exposure to environmental mycobacteria in CEH altered the response to antigen 85A following i.d. immunisation. In order to test this, rather speculative, hypothesis, mice kept in BMSL2 were immunised i.d. with Ad85A as normal. However, rather than having sterile drinking water, BCG was added to the cage water bottle and the animals allowed to drink from it for 3 weeks following immunisation. At 4 weeks post-immunisation the mice were culled and the lung response measured (Figure 6A). Interestingly, in the first experiment, despite a low overall response, the mice that had received BCG water showed a statistically significant reduction in the lung CD8 response compared to mice that drank only sterile water (Figure 6A).

Potentially this might be a very exciting result, but it of course needed to be validated and repeated, especially as it was so unexpected. It was also interesting to investigate whether other species of mycobacterium would have the same effect, particularly as it is unlikely that the mice were exposed to the BCG vaccine strain. Therefore, as well as repeating the BCG water, other cages of Ad85A i.d. immunised mice were given water containing *M. smegmatis*. Other Ad85A i.d. mice were given water containing heat killed (HK) BCG or BCG Pasteur, in order to determine if the effect was common to all BCG strains and also whether ingestion of viable mycobacteria was necessary (Figure 6B). The overall response in this experiment was much higher, but the HK BCG water and the *M. smegmatis* water both appeared to reduce the response in the lung by 2-3%, and the mice that received the BCG water showed a reduction of ~5% compared to i.d. immunised mice given clean water (Figure 6B). However, none of these differences were statistically significant. To further clarify whether mycobacteria in the water was having an effect on the response, the experiment was repeated again with the mice exposed to BCG, *M. smegmatis* or *M. abscessus*. *M. abscessus* inhabits water and, as the CEH facility was supplied by an independent roof-top water tank, could represent one of the potential environmental mycobacteria that the mice in CEH could have been exposed to. When the response 4

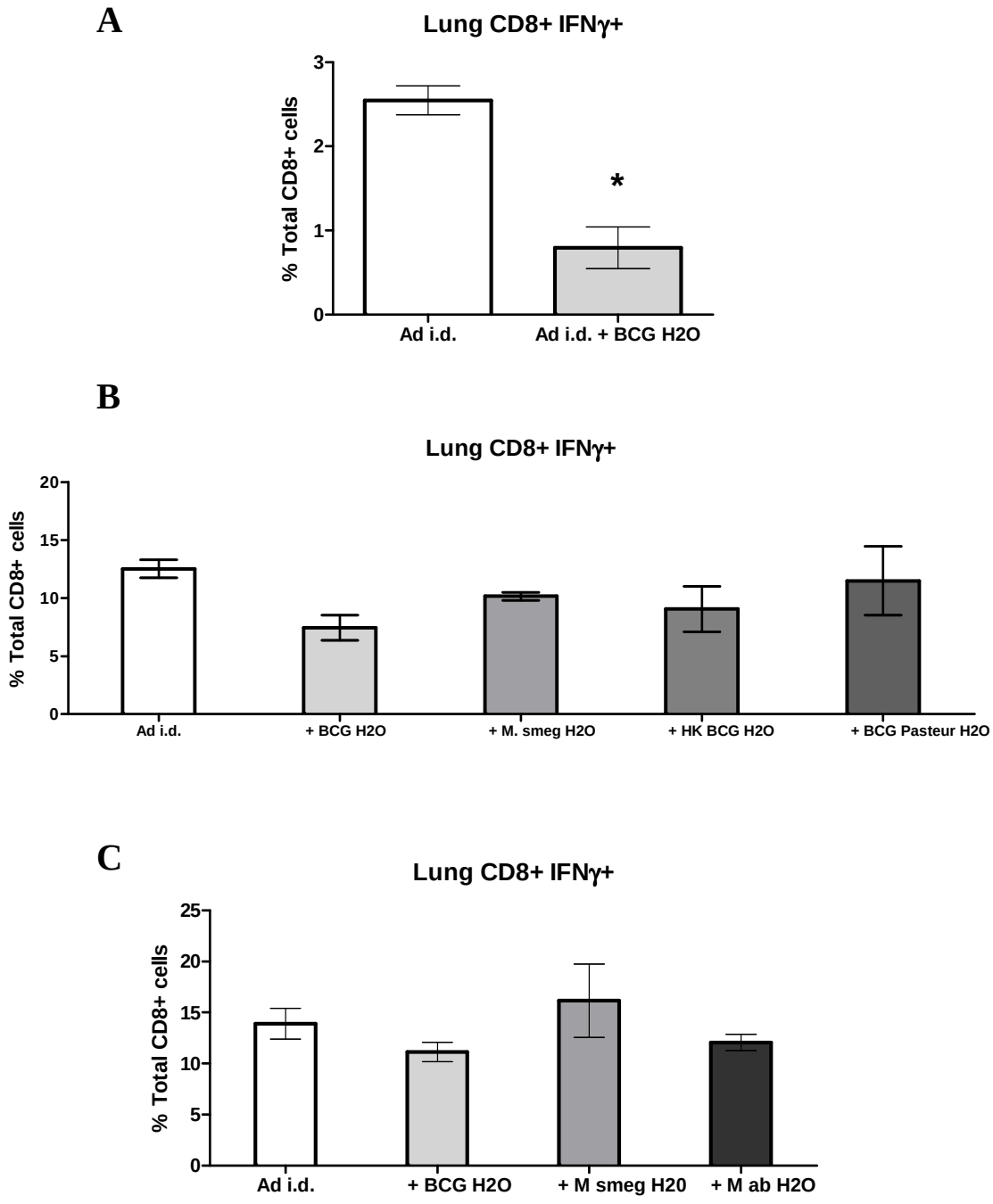


Figure 6: Effect of mycobacterial exposure on lung response following Ad85A i.d. immunisation. BALB/c mice were immunised with Ad85A i.d. and immediately following immunisation a particular variety of mycobacterium was added to the cage water bottle. One group of immunised water was left with sterile water. Mice were able to drink from the bottle *ad libitum* for 2 weeks, when it was replaced with sterile water. At 4 weeks post-immunisation mice were culled, lung cells isolated and stimulated with the mix of the 3 dominant T cell epitopes. Lung IFNγ response is shown, as measured by ICS. HK BCG – Heat killed BCG; M. smeg – *Mycobacterium smegmatis*; M. ab – *M. abscessus*. * $p < 0.005$ by Student's t-test.

weeks post-immunisation was examined in these groups, a similar effect was seen. The BCG and *M. abscessus* water groups both showed a reduction in the CD8 IFNγ response of 2-3%, although in this experiment *M. smegmatis* water had no effect (Figure 6C). However, as in the experiment shown in Figure 6B, the differences seen here were not statistically significant. This, along with another experiment where none of the mycobacteria-inoculated waters had any effect (data not shown), indicate that if there is a true effect then it is weak and variable.

A further hint that the response in the lung following i.d. immunisation could be affected by mycobacterial exposure came when the effect on *Mtb* challenge was investigated. As BCG water had shown a trend towards reducing the level of antigen-specific cells in the lung, it was hypothesised that this should have an effect following infection, as the presence of a lung response correlates with protection. Mice were immunised and given BCG water as described above and subsequently challenged at 4 weeks post-immunisation. When the mycobacterial CFU in the lung 4 weeks post-challenge were enumerated, Ad85A i.d. gave a significant reduction in *Mtb* load (Figure 7). However, if Ad85A i.d. immunised mice had been exposed to BCG water prior to challenge, this protective effect was lost. There appeared to be a small reduction in CFU in this group, although it was not significantly different from the level seen in naïve mice (Figure 7). A second independent experiment has recently confirmed these findings.

5.3 Discussion

Parenterally administered subunit vaccines against TB have been shown to be highly immunogenic, eliciting strong responses in the systemic compartment, whether in the blood or spleen (114, 115, 140, 202). However, despite these strong responses, some of these vaccines have shown variable protection upon mycobacterial challenge (118, 120) and, when administered as a boost to BCG, add little to the protection seen after BCG vaccination alone (52, 147). For one of these vaccines, Ad85A, Forbes *et al*

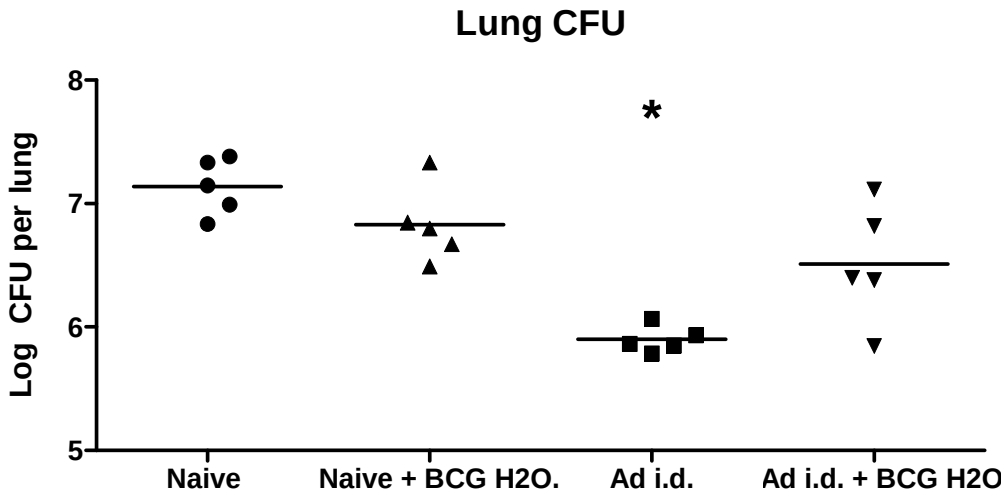


Figure 7: Effect of mycobacterial exposure on protection following Ad85A i.d. immunisation. BALB/c mice were immunised with Ad85A i.d. and either the water left sterile (Ad i.d.) or immediately following immunisation BCG was added to the cage water bottle (Ad i.d. + BCG H2O). Mice were able to drink from the bottle *ad libitum* for 2 weeks, when it was replaced with sterile water. At 4 weeks post-immunisation mice were challenged i.n. with ~200 CFU of *Mtb*. At 4 weeks post-challenge mice were culled and the lung *Mtb* CFU burden enumerated. It should be noted that these data are from mice from the same experiment as those shown in Figure 6B. * $p < 0.01$ versus naïve.

have shown that this lack of protection following i.d. immunisation may be due to a very low antigen-specific response in the lung, despite a strong systemic response (52). However, if the same vaccine is given i.n. the lung mycobacterial load is reduced, corresponding with a strong antigen-specific response in the lung (52). The work in Chapter 3 and (54), demonstrates that this protection following i.n. Ad85A immunisation is due to the lung resident cells inhibiting *Mtb* growth in the first week following infection. Intra-dermal immunisation is not able to protect as the cells are not in the lung and so cannot act in this early phase of growth.

However, in this chapter it has been shown that, following a change in animal facilities, i.d. immunisation resulted in a strong response in the lung, contrary to the results of Forbes *et al*. Whilst unexpected, it was not wholly surprising; Ad85A given intra-muscularly (i.m.) has been shown to induce a lung CD8 T cell response at 2, 6 and 8 weeks post-immunisation that is at least comparable in magnitude to that seen following i.n. immunisation (140, 167). As previous work had shown that a

population of antigen-specific cells in the lung correlated with protection, the mice immunised with Ad85A i.d. in the BMSL2 should have displayed a reduction in CFU at least equivalent to i.n. immunised mice. However the protection afforded by the stronger lung CD8 i.d. response is significantly weaker than that seen in i.n. immunised mice, with CFU reduced by only ~0.5 Log in i.d. mice versus a reduction of approximately 1.5 Log in i.n. mice. Parenteral immunisation has been shown by others to offer a similar level of protection when mice are challenged 4 weeks after i.m. immunisation (140). However, this protection appears to be transient, with no protection if mice are challenged at 12 weeks.

A notable difference between the lung responses induced by i.d. and i.n. immunisation is the activation status of the antigen-specific cells. Following i.n. immunisation, the majority of cells display an effector phenotype (Chapter 3), whereas i.d. induced cells almost all display an effector memory phenotype (Figure 3). This marked difference may provide an indication as to why Ad85A i.d. immunisation is less effective at conferring protection than i.n. immunisation, despite a stronger antigen-specific response. Chapter 3 demonstrated that i.n. immunisation confers protection by inhibiting mycobacterial growth early after infection, that this control is dependent on CD8 T cells and that the majority of these cells are highly activated effectors. However, following challenge of Ad85A i.d. immunised mice, there is no control of *Mtb* in the first 7 days. Rather, reduction of *Mtb* CFU only occurs after 21 days post-challenge (Figure 4). This delay in action may be down to the difference in activation status. If highly activated effector cells are required for killing or inhibition of *Mtb* then i.d. induced protection becomes limited by any delay in further activation and differentiation of the antigen-specific cells. As described in Chapter 3, *Mtb* is adept at delaying the adaptive immune response in the lung until at least 14 days post-infection (37, 165).

Location of vaccine-induced cells within the lung is likely also to play a significant role in the protection conferred by the different regimes. Others have shown that antigen-specific cells need to be present in the airway lumen, as detected by bronchio-alveolar lavage (BAL), in order to confer

protection (146, 167). Antigen-specific cells following i.m. immunisation are entirely within the lung parenchyma and interstitium, whereas a significant proportion of i.n. induced lung cells are present in the airways (167). Similarly, we have shown that there are no cells in the BAL following Ad85A i.d. immunisation (175). What causes i.n. induced cells to reside in the airway is unknown; however work by others in our lab has shown that the chemokine receptor CXCR6 is present on many of the cells in the airway (175). Approximately half of antigen-specific CD8 cells in the airway lumen display this receptor and, notably, adoptively transferred CXCR6+ CD8 cells from the lungs of i.n. immunised mice are able to protect recipient naïve mice, whereas CXCR6- cells are not (175).

The significance of CXCR6 expression may be an indicator of the importance of general inflammation in the lungs in influencing protection. Santosuosso *et al* have shown that 85A-specific cells can be detected in the BAL of i.m. immunised mice if they have received another adenovirus containing an irrelevant antigen intra-nasally (146). However, this effect was transient and only a few cells were detectable in the BAL by 2 months. However, if repeated doses of antigen 85 protein were given into the lung of Ad85A i.m. immunised animals then antigen-specific cells remained in the airway (146). Our own data confirm the importance of both inflammation and antigen in the lung to recruit cells to the airway. Cells were detectable in the BAL of i.d. immunised mice only if they had received a combination of recombinant antigen with CXCL16, the ligand for CXCR6, but not when either antigen or chemokine were given alone (175). Further evidence for the importance of inflammation within the lung is found if the *Mtb* challenge dose is raised. If ~2000 CFU are administered rather than a couple of hundred then i.d. mice are as protected as i.n. mice (data not shown). Whether this is due to a relative loss of protective ability in i.n. immunised mice or the greater inflammation activating and recruiting i.d. induced cells earlier in infection is difficult to determine, particularly as this challenge method leads to a more rapid deterioration of all groups of mice. However, it should be noted that in the work performed by the Xing group, an intra-nasal challenge dose of 10,000 CFU of H37Rv *Mtb* is used (140, 167).

The significant change in the response seen following i.d. immunisation upon switching animal house led to some interesting questions. Differences in susceptibility to autoimmune diseases between mice housed in different animal facilities have been reported (198-200). However, these were transgenic mice that had been kept in these different animal houses since birth. Indeed, it has been suggested that it is the difference in commensal bacteria acquired in the neonatal weeks that drives these differences, with changes later in life having a weaker effect (200). However, in the case outlined in this chapter, mice for all experiments came from the same SPF source (Harlan) and arrived at the various facilities at 6-8 weeks old. Mice were then immunised after approximately a week in each of the facilities. This suggests that, whatever was causing the change, it was capable of altering the response in either the week following arrival in CEH or in the weeks following immunisation. The initial hypothesis was that mice kept in open topped cages in "dirty" facilities were exposed to more environmental antigens, possibly leading to a more tolerising environment, particularly in the lungs. However, the data from the mice housed in the Anatomy and BMSL1 facilities showed that this was not the case. Therefore the cause must have been specific to CEH. It has been shown that aspects of the immune response in the gut can be dependent upon the presence of specific classes of commensal microorganisms (203), so it is possible that the mice at CEH had been exposed to a particular microorganism not present in the other "dirty" facilities. The initial investigations into the possibility that this microorganism may be a mycobacterium were largely speculative; however the results presented the interesting prospect that exposure to environmental mycobacteria could lead to a change in the immune response in the lung following Ad85A i.d. immunisation, leading to a loss of efficacy. This is a problem that has been extensively investigated with regard to the BCG vaccine and its variable effects (201, 204), but the possibility that it could affect subunit vaccines could be concerning for future vaccine initiatives. However, the effect seen here was weak and highly variable and warrants further investigation before any significant conclusions can be drawn. It would also be interesting to determine if the same effect was observed with vaccines containing antigens not present in the environmental mycobacteria, whether *Mtb*-specific antigens such as ESAT6, or antigens from other pathogens entirely.

5.4 Summary

The immune response following Ad85A i.d. immunisation can vary depending upon the animal facility in which the mice are housed. Change to a SPF facility led to i.d. immunised mice developing a response in the lung that was absent when they were housed in a non-SPF facility. This new response was associated with protection upon *Mtb* challenge, although not to the same degree or at the same time point post-infection as the protection seen after i.n. immunisation. This lower efficacy is likely due to a lower level of activation of Ad85A i.d. induced cells, along with their location in the lung interstitium rather than in the airway. The change in response between animal houses was not a general difference between SPF and non-SPF facilities, but a specific characteristic of the CEH animal facility. Tantalisingly, there appears to be a hint that the change in response seen in CEH may have been due to environmental exposure to mycobacterial species, although this possibility requires extensive further investigation.

CHAPTER 6

Simultaneous immunisation with TB vaccines

Chapter 6

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At the time of writing sections of this chapter have been submitted for publication as Tchilian *et al* 2011, and their reproduction here has been approved by the co-authors in accordance with University of Oxford regulations.

6.1 Introduction

The data presented in Chapters 3 and 5 have shown that Ad85A immunisation is able to confer protection following *Mtb* challenge when administered by respiratory (i.n.) immunisation as well as by systemic (i.d.) immunisation when an antigen-specific lung response is induced (Chapter 5). However, respiratory immunisation with Ad85A is more protective than i.d. immunisation, despite the latter inducing a stronger immune response in both the lung and the spleen. Furthermore, i.n. immunised mice inhibit *Mtb* growth in the first week post-infection but i.d. immunised mice not until 3 weeks post-challenge. This is possibly because i.n. immunisation induces a highly activated antigen-specific CD8 response in the lung airways that is able to inhibit *Mtb* growth early, whereas the interstitial lung response induced by i.d. immunisation acts only after day 7, following *Mtb* dissemination to the draining lymph nodes. Similarly, parenteral BCG immunisation also shows inhibition of mycobacterial growth in this late phase of growth (Chapters 3 & 5).

Ad85A i.n. administered as a boost to prior BCG s.c. immunisation confers protection significantly better than either immunisation given alone, due to the additive effect of Ad85A-induced early protection and BCG-induced late protection (Chapter 3)(54). As the increase in protection was due to the combination of two stages of protection, it was reasoned that protection would be equally as effective if parenteral and respiratory vaccines were administered simultaneously. This idea was investigated using the immunisation of BALB/c and C57BL/6 mice with BCG and subunit vaccines.

As Ad85A i.d. immunisation protects in the late phase, it was interesting to investigate whether the same additive effect of combined early and late phase inhibition would be apparent in mice immunised with Ad85A i.n. and i.d. simultaneously. However, it was also necessary to establish the concept in other model systems. To this end the effect of BCG and subunit immunisation in C57Bl/6 mice was investigated. Parenteral immunisation with recombinant protein or peptide plus an adjuvant has been shown to be effective in mice and other animals (106, 108, 201). Although these regimes often afford protection equivalent to that seen in BCG immunised controls, when they are given as boosts after prior BCG immunisation any further increase in protection is variable (111, 205). Protein immunisations have been shown to be effective when administered into the respiratory tract, indicating that this route is effective across vaccine vehicles, and have been shown to boost protection in BCG immunised mice (154). BCG has also been shown to be highly protective when administered into the respiratory tract of guinea pigs and mice (119, 206), further emphasising the efficacy of this route of administration.

It is possible that the variable effect of parenteral boosting of BCG is due to any additional protection being masked as both vaccines act at similar times post-infection. However, as respiratory immunisation generates a response that acts early after challenge, respiratory and systemic immunisation with two protective vaccines should act additively on the two different phases of growth. As this would be two distinct effects acting additively, it should be independent of prime-boosting and the vaccines could be administered simultaneously. This approach of **s**imultaneous **im**munisation by the respiratory and systemic route was termed SIM.

6.1.1 Aims

In this chapter, investigation into simultaneous immunisation with candidate TB vaccines, including Ad85A, recombinant proteins and peptides, and BCG aimed to demonstrate that combining respiratory and systemic immunisation is an effective way of increasing protection against *Mtb*. Furthermore,

experiments were designed to test whether increased protection is due to a prime-boost effect or, alternatively, because the two immunisations inhibit *Mtb* growth at distinct times following infection.

6.2 Results

In the light of the data presented in Chapter 5, it should be noted that the immunisations described in this chapter were performed in the BMSL2 animal facility.

6.2.1 Simultaneous immunisation (SIM) with Ad85A in BALB/c mice.

Ad85A has been shown to be protective when administered either i.n. or i.d. in BMSL2 but with each type of administration inhibiting growth at different time points following infection (Chapters 3 and 5). Further, Ad85A i.n. has been shown to act additively with late inhibition induced by s.c. BCG immunisation. Therefore, it was hypothesised that parenteral (i.d.) and respiratory (i.n.) immunisation with Ad85A may also be able to act additively and confer protection greater than either alone.

BALB/c mice were immunised with Ad85A either i.d. or i.n. alone or simultaneously with both i.d. and i.n. Ad85A (i.d./i.n.). To avoid any effect of an increase in total antigen dose, the dose for a single immunisation was split between the two routes. Therefore, all mice received a total of 2×10^9 v.p. of Ad85A. Two weeks post-immunisation mice were culled and the antigen-specific response in the lung and the spleen measured (Figure 1). As shown in Chapter 5, single administration of Ad85A i.d. or i.n. induced a strong lung CD8 response, with the response in i.d. mice the stronger. Interestingly, the mice that had received the i.d./i.n. regime showed a reduction in CD8 response compared to those that had received i.d. alone (Figure 1A). The most striking difference between the regimes, however, was that mice in the i.d./i.n. group had a significant population of antigen-specific CD4 cells in the lung, compared to the minimal response detected in the single administration groups (Figure 1A). The origin of this new population of CD4 cells was unclear. It is possible that cells from the i.d. induced CD4 population circulating in the periphery had been recruited to the lung by the i.n. immunisation, a theory

supported by the apparent drop in CD4 response seen in the spleen in the i.d./i.n. group (Figure 1B). A similar pattern is observed when total cell numbers are analysed (data not shown). As well as a reduction in the CD8 lung response, i.d./i.n. immunisation appeared to alter the activation profile of antigen-specific lung CD8 cells. As shown in Chapter 5, single Ad85A i.d. induced cells were mainly effector memory phenotype as defined by CD62L and CD127 expression. However, in the i.d./i.n. group the activation phenotype was more similar to that seen following i.n. immunisation, with an increase in the proportion of effector cells (Figure 1C). These patterns of response were detectable at least as long as 3 months post-immunisation (data not shown).

A strong CD8 lung response had previously correlated with protection following Ad85A immunisation (Chapter 3), so it was possible the reduced CD8 response in the i.d./i.n. group would have an effect on mycobacterial load. However, antigen-specific CD4 cells are also known to be important for protection. Therefore, the effect of the i.d./i.n. immunisation regime on protection following *Mtb* challenge was determined. Mice were immunised with single i.d. or i.n. doses of Ad85A or with the i.d./i.n. regime and challenged i.n. with *Mtb* 4 weeks post-immunisation. Four weeks post-challenge the mice were culled and the lung mycobacterial load enumerated. As expected, Ad85A i.n. conferred a reduction in CFU of approximately 1 Log (Figure 2). In this experiment, i.d. immunisation protected to a similar extent as i.n. immunisation, although it should be noted that 3 of the measurements (highlighted in red) may not be as reliable as the others as they were calculated using a different batch of agar plates, a factor known to increase variability. The i.d./i.n. group, despite the change in the antigen-specific response profile compared to the single immunisation controls, showed no difference in the level of protection (Figure 2). This indicates that the protection seen following individual systemic or respiratory immunisation with Ad85A is not able to act additively when the two are given simultaneously.

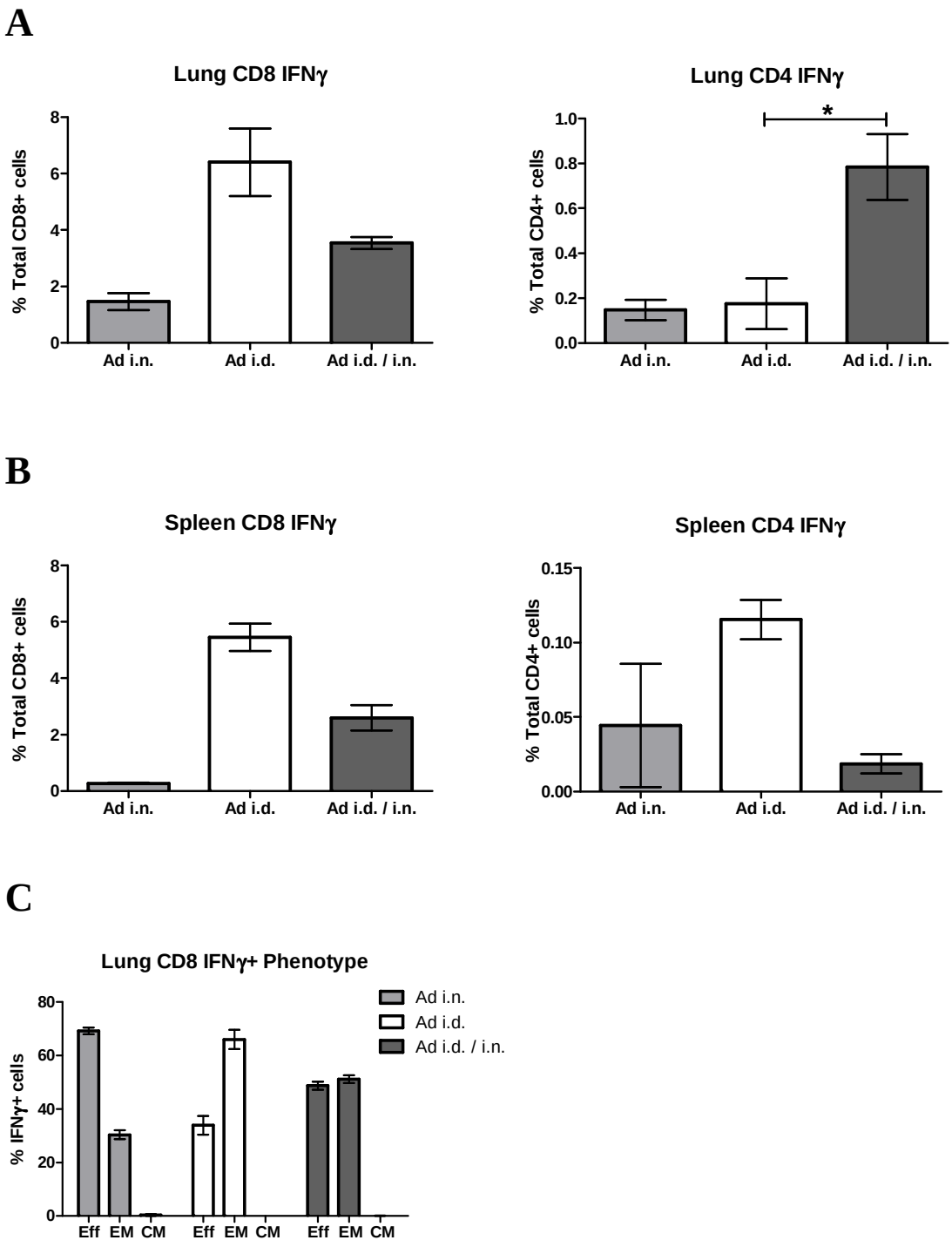


Figure 1: Immune response following single or simultaneous i.d. or i.n immunisation with Ad85A. BALB/c mice were immunised with a total of $2x10^9$ v.p. of Ad85A in either a single i.n. or i.d. immunisation or simultaneously with concurrent i.d. and i.n. immunisations (i.d./i.n.) and culled 4 weeks later. Lung and spleen cells were stimulated with the mix of 3 85A T cell epitopes. IFNγ expression was measured by ICS for the lung (A) and spleen (B). (C) Activation status of lung CD8+ IFNγ+ cells as defined by CD62L and CD127 expression (Chapter 3). Representative of 4 experiments. * $p < 0.05$ by one way ANOVA.

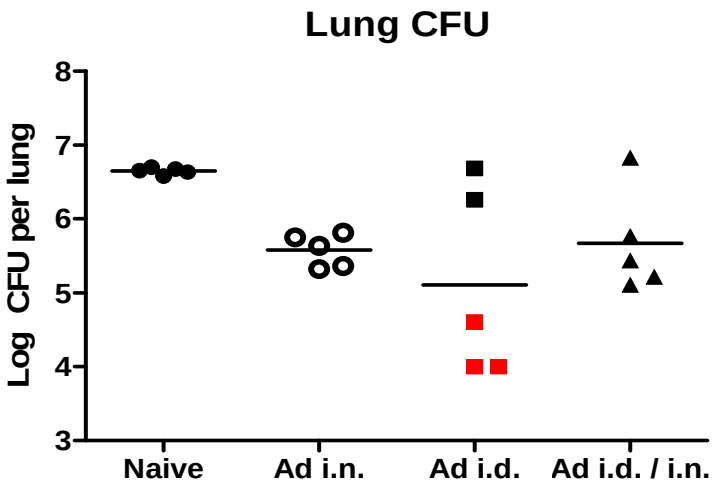


Figure 2: Protection following single or simultaneous immunisation with Ad85A i.d. or i.n. BALB/c mice were immunised with a total of $2x10^9$ v.p. of Ad85A either i.n. or i.d. or simultaneously (i.d./i.n.) and challenged i.n. with *Mtb* 4 weeks post-immunisation. Four weeks post-challenge mice were culled and lung CFU enumerated. Data points indicated in red were enumerated from possibly defective plates and should be interpreted with caution. Representative of a single experiment.

6.2.2 Lack of protection following Ad85A immunisation in C57Bl/6 mice.

Initially, the phenomenon of additive protection had been seen with parenteral BCG and i.n. Ad85A in BALB/c mice. However, this effect needed to be demonstrated in other experimental systems. To this end, the model was tested in C57Bl/6 mice. Mice were immunised i.n. with Ad85A as previously but in this model the lung response was considerably weaker and more equally divided between CD4 and CD8 cells than the response seen in BALB/c mice (Figure 3A). Moreover, neither Ad85A i.d. or i.n. protected C57Bl/6 mice (Figure 3B). Although adenoviral vector immunisation has been shown by others to be protective when administered twice intra-muscularly (53), the inefficacy demonstrated here precluded further use of Ad85A in C57Bl/6 mice in our hands.

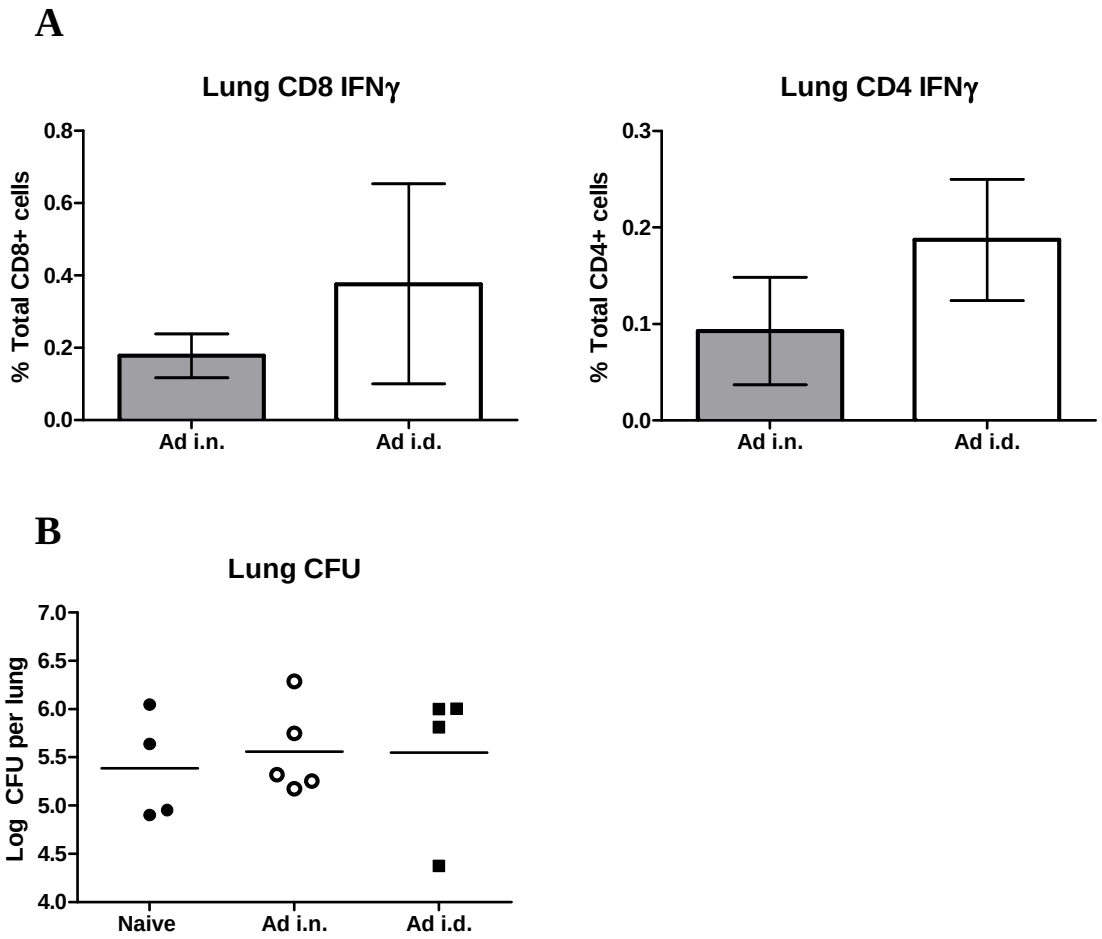


Figure 3: Ad85A lung response and lack of protection in C57Bl/6 mice. C57Bl/6 mice were immunised either i.d. or i.n. with 2x 10^9 Ad85A. (A) 4 weeks post-immunisation mice were culled and lung cells stimulated with the pool of 85A peptides. Similar results were obtained for TNFα expression. (B) 4 weeks post-immunisation, mice were challenged i.n. with *Mtb*, culled 4 weeks post-challenge and lung CFU enumerated. Representative of 2 experiments.

6.2.3 Simultaneous immunisation (SIM) with subunit protein vaccines in C57Bl/6 mice.

As Ad85A did not protect C57Bl/6 mice, other protective vaccines were required before the idea of additive, distinct phase protection could be tested. Protein with adjuvant has been shown to be protective against *Mtb* in C57Bl/6 mice (111, 205) and so was chosen to further investigate the efficacy of SIM regimes. The antigens chosen were recombinant 85A protein (r85A) and a recombinant peptide consisting of the first 20 amino acids of the 6 kDa early secreted antigenic target (ESAT6). Intra-nasal immunisations were adjuvanted with Cholera toxin (CT), known to be a potent mucosal adjuvant, and MPL was used as the adjuvant for parenteral immunisations. Intra-nasal immunisation with these vaccines generated a strong antigen-specific lung response, although this differed from the response to Ad85A in BALB/c mice as it consisted of CD4 cells, with no CD8 response detectable (Figure 4A & B). Parenteral immunisation with either antigen elicited a splenic response, also consisting of CD4 cells, although the response following r85A s.c. was ~5-fold higher than the response following ESAT6 s.c. immunisation (Figure 4A & B). No responding CD8 cells were detectable in the spleen. Upon i.n. *Mtb* challenge mice immunised with r85A or ESAT6 by either route, whether s.c. or i.n., were protected to a level equivalent to that seen following parenteral BCG immunisation (Figure 4C).

As both antigens had shown protection in C57Bl/6 mice, they were combined in a SIM regime; ESAT6 administered s.c. and r85A administered i.n. (ESAT6 s.c./r85A i.n.). In the Ad85A - BALB/c model the efficacy of respiratory vaccines correlated with a strong antigen-specific response in the lung. When the immune responses in this SIM protein - C57Bl/6 system were investigated, a strong lung CD4 response was always observed specific to the to the i.n. antigen, with little response to the systemic antigen detected in the lung (Figure 5A & B). In the spleen, there was always a strong response to the systemically administered antigen, with only weak responses detected to the antigen administered by the respiratory route (Figure 5C & D). Antigen-specific responses by CD8 cells were too weak to be detected.

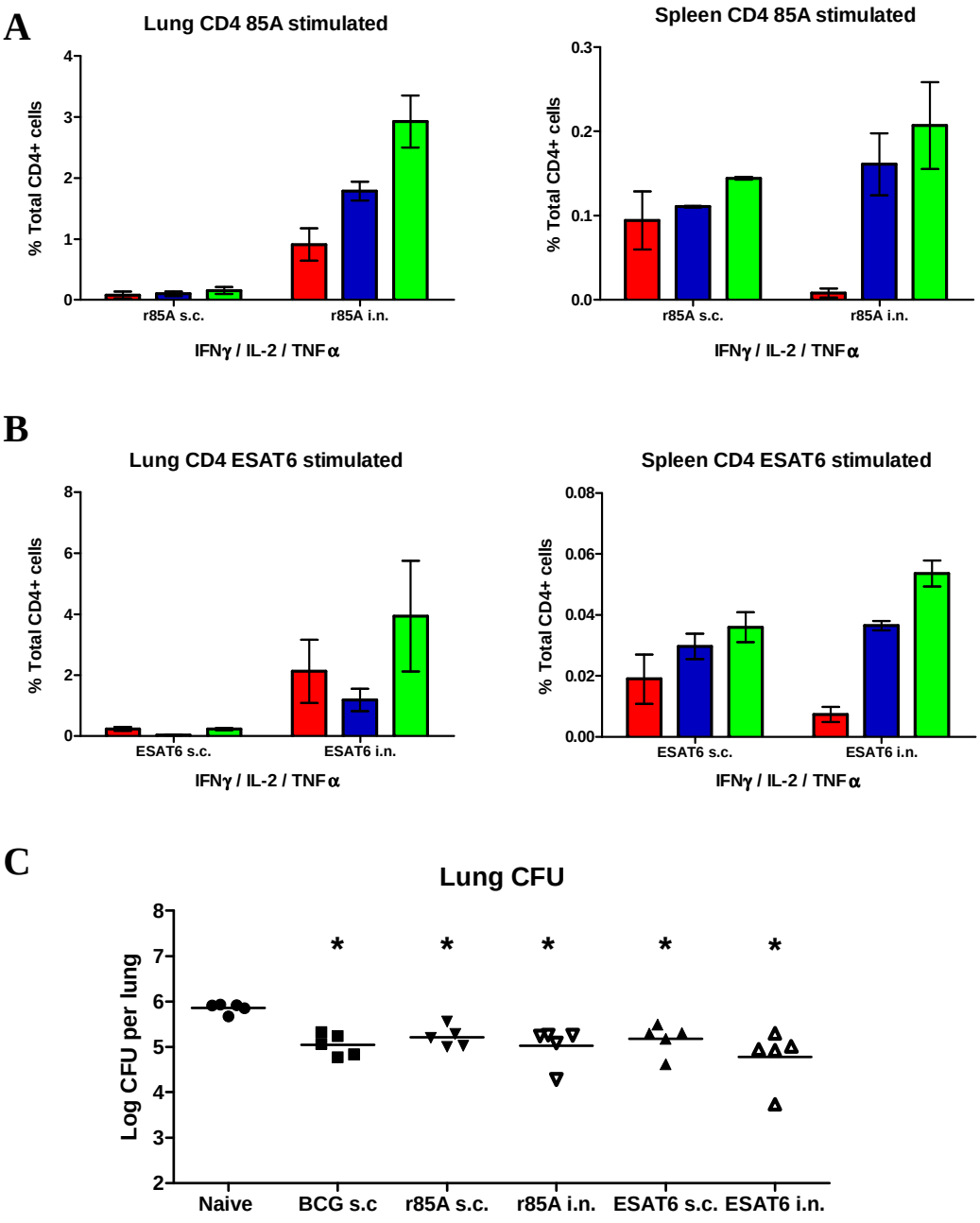


Figure 4: Immune response and protection of r85A or ESAT6 protein immunisation s.c. or i.n. in C57Bl/6 mice. C57Bl/6 mice were immunised with r85A or ESAT6 either i.n. or s.c. as described in Materials and Methods. Immunisations were given 3 times at 2 week intervals. (A&B) 3 weeks following the last immunisation, mice were culled, lung and spleen cells isolated, stimulated with the pool of 66 overlapping 85A peptides or the ESAT6 peptide used for immunisation and cytokine expression measured by ICS. Red – IFNγ; Blue – IL-2; Green – TNFα. (C) 4 weeks post-immunisation mice were challenged i.n. with *Mtb*, culled 4 weeks later and lung CFU enumerated. Naïve and BCG s.c. immunised mice were used as controls. * $p < 0.001$ versus Naïve.

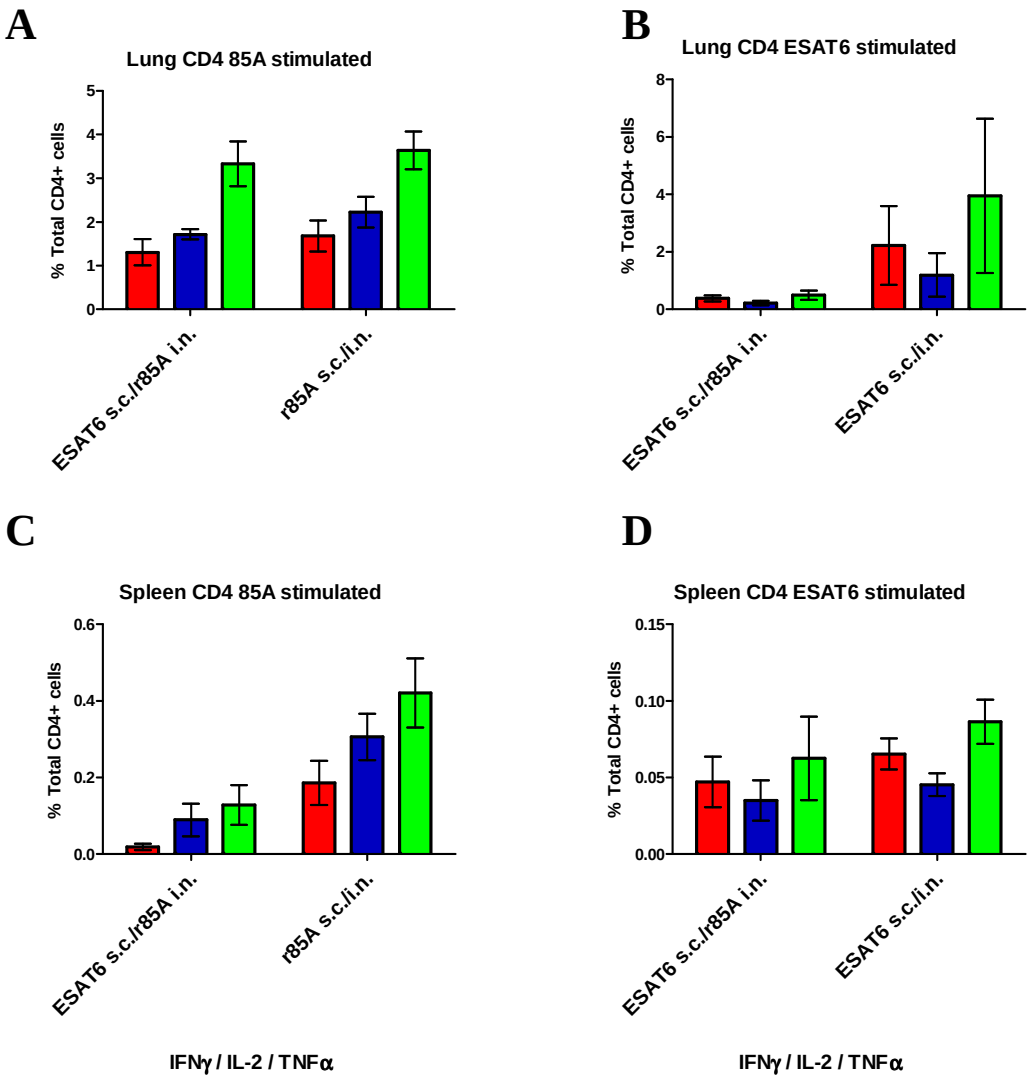


Figure 5: Immune responses to component antigens in mice immunised with a SIM regime using r85A and ESAT6. C57Bl/6 mice were immunised 3 times at 2 week intervals with r85A or ESAT6 s.c. and i.n. alone or simultaneously in the combinations shown. 3 weeks following the final immunisation lung (A+B) and spleen (C+D) cells were stimulated with either the pool of 85A peptides (A+C) or the same ESAT6 20 amino acid peptide used for immunisation (B+D) and the cytokine expression by CD4 cells measured by ICS. Red – IFNγ; Blue – IL-2; Green – TNFα. Bars and whiskers represent the mean and SEM of 3-4 mice per group. Representative of 3 experiments.

Upon mycobacterial challenge, the SIM regime of ESAT6 s.c./r85A i.n. conferred a significant improvement in protection over either antigen given alone by their respective routes (Figure 6A). Interestingly, if both antigens were simultaneously given s.c. or i.n. (in separate immunisations rather than mixed), no such improvement over the individual vaccines was seen (Figure 6B & C respectively). This implied that the effect was due to two separate protective effects, one resulting from the respiratory immunisation and one from the systemic immunisation. It should be noted, however, that if the routes of the SIM regime were reversed and ESAT6 given i.n. and r85A given s.c., then no increase in protection was observed (data not shown). These data support the hypothesis that the i.n. and s.c. immunisations act separately by inducing distinct immune responses in the lung and systemic compartments respectively; responses that are able to act additively when the antigens are given in a SIM regime. Interestingly, however, if r85A or ESAT6 was given by both respiratory and systemic immunisation, a homologous SIM regime, then no increase in protection was observed compared to an individual immunisation with that antigen (data not shown). This was despite a strong response to the antigen in both the lung and the periphery (Figure 5).

6.2.4 Simultaneous immunisation (SIM) with protein subunit vaccines and BCG in C57Bl/6 mice.

As BCG is the only licensed vaccine for use in humans it was necessary to show the efficacy of SIM regimes using BCG as one of the components. As shown in the Ad85A-BALB/c system, BCG s.c. immunisation inhibits *Mtb* growth in the late phase of growth. Therefore, if BCG s.c. was combined with simultaneous i.n. protein immunisations then an additive increase in protection should be observed. However, if the protein immunisation was administered s.c. then no such increase should be detected. In order to investigate this hypothesis, mice were immunised parenterally with BCG and simultaneously given r85A either parenterally (BCG s.c./r85A s.c.) or by respiratory immunisation (BCG s.c./r85A i.n.). Ten weeks following immunisation the mice were challenged with *Mtb* and the lung mycobacterial load enumerated 5 weeks post-challenge. As hypothesised, despite receiving two

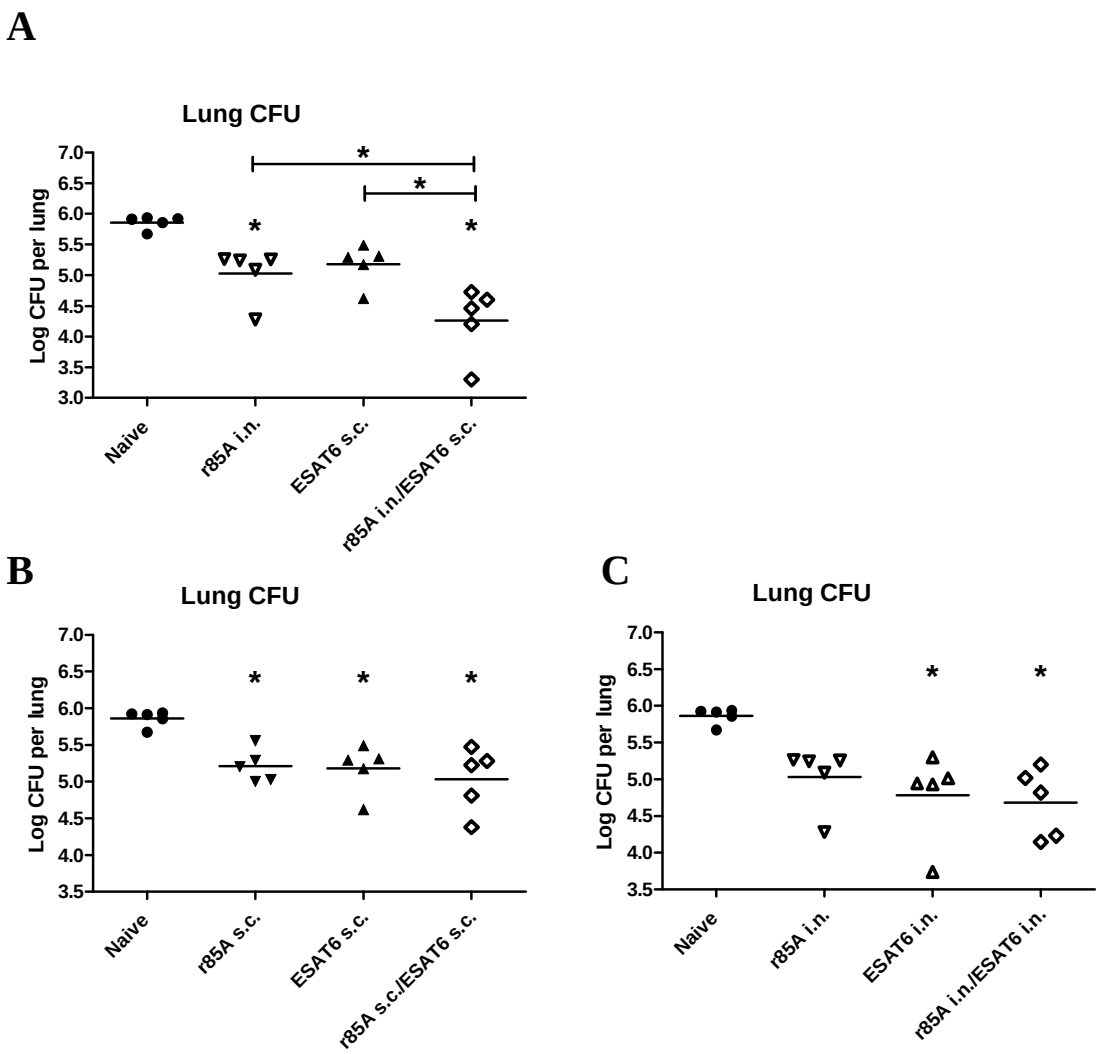


Figure 6: Simultaneous systemic and respiratory immunisation (SIM) with r85A and ESAT6 confers protection following *Mtb* challenge. C57Bl/6 mice were immunised 3 times at 2 week intervals with (A) r85A i.n. or ESAT6 s.c. alone or in combination; (B) r85A s.c. or ESAT6 s.c. alone or in combination; (C) r85A i.n. or ESAT6 i.n. alone or in combination. 6 weeks after the final immunisation mice were challenged with *Mtb* i.n., culled 5 weeks post-challenge and the lung CFU enumerated. * $p > 0.005$ versus Naïve unless indicated, as measured by ANOVA. Representative of 2 experiments. Adapted from Tchilian *et al* (submitted).

protective immunisations, BCG s.c./r85A s.c. immunised mice were no better than the BCG s.c. only controls, with only one mouse showing an increase in protection (Figure 7A). However, mice that received SIM BCG s.c./r85A i.n. showed a reduction in mycobacterial load of approximately 1 Log compared to groups that had received only parenteral immunisations (Figure 7A).

In order to show that this improvement over BCG s.c. alone was not restricted to 85A-specific immunity, SIM was carried out with other *Mtb* antigens: ESAT6 peptide and recombinant protein TB10.4. When these antigens were given i.n. simultaneously with BCG s.c. (BCG s.c./ESAT6 i.n. and BCG s.c./10.4 i.n. respectively) they induced a significant increase in protection over single BCG s.c. immunisation and, in the case of ESAT6, over simultaneous parenteral immunisation (BCG s.c./ESAT6 s.c.) (Figure 7B & C). Although with both immunisations given simultaneously there was little chance of a prime-boost effect in the r85A or TB10.4 experiments, the use of ESAT6 confirmed that the increase in protection was not due to a prime-boost effect as ESAT6 is not present in BCG.

6.2.5 Simultaneous immunisation (SIM) with BCG s.c. and BCG i.n. in C57Bl/6 mice.

These data indicate that SIM immunisation can be highly effective; therefore, the effect of administering BCG only in a SIM regime was investigated. As seen in the experiments outlined above, BCG is protective when administered s.c.; however it has also been shown to be protective when administered i.n. (119). C57Bl/6 mice were therefore given the same dose of BCG either in a SIM regime (BCG s.c./i.n.), s.c. only or i.n. only and subsequently challenged i.n. with *Mtb*. The BCG SIM regime was able to reduce lung mycobacterial CFU by over 2 Log compared to those receiving BCG by only one route, an improvement of up to 3 Log vs naïve mice (Figure 8A). In a repeat experiment, a similar improvement in protection was observed, on this occasion the SIM regime conferred a 1 Log improvement over single immunisation and a 2 Log reduction in CFU compared to naïve mice (Figure 8B).

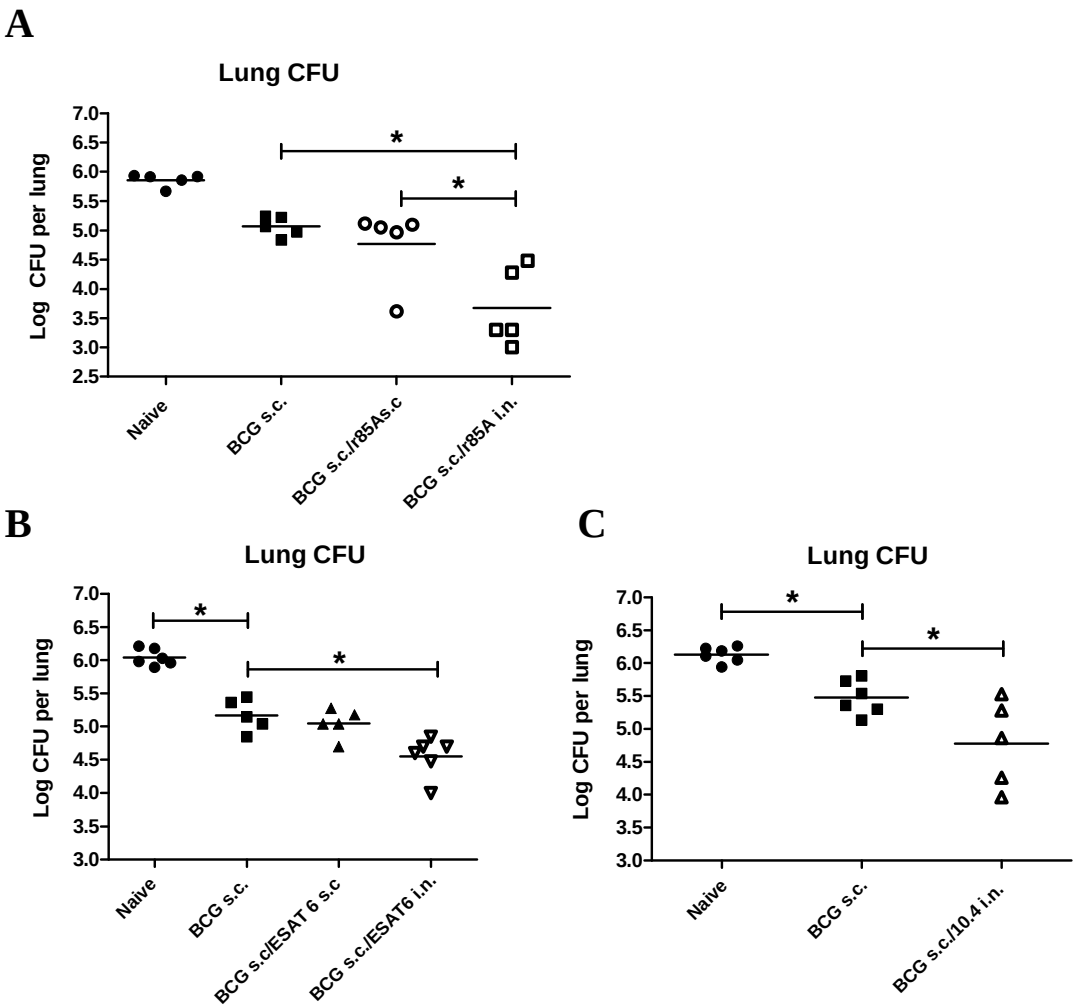


Figure 7: Additive protection of parenteral BCG and respiratory subunit immunisation. C57Bl/6 mice were immunised with either BCG s.c. only, or simultaneously with BCG s.c. and either (A) r85A i.n. or s.c., (B) ESAT6 i.n. or TB10.4 i.n. (C). 10 weeks post-immunisation mice were challenged i.n. with *Mtb*. 5 weeks post-challenge mice were culled and lung CFU enumerated. * $p < 0.05$ between groups as indicated. Representative of 2-3 experiments. Adapted from Tchilian *et al* (submitted).

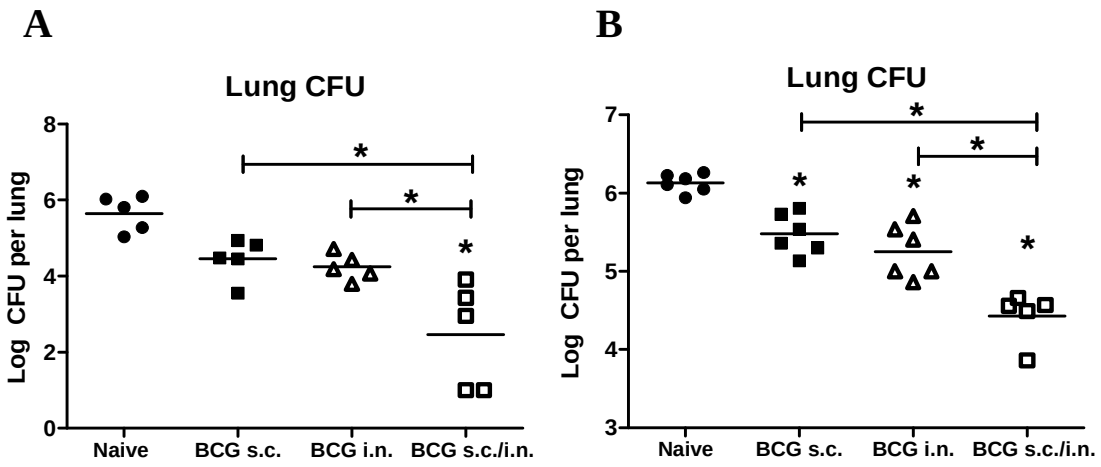


Figure 8: Simultaneous systemic and respiratory immunisation (SIM) with BCG improves protection following *Mtb* challenge. In two identical experiments (A+B) C57Bl/6 mice were immunised with BCG s.c. or i.n. or with the same dose split between simultaneous s.c. and i.n. immunisations (BCG s.c./i.n.). 10 weeks later the mice were challenged i.n. with *Mtb* and 5 weeks post-challenge were culled and the lung CFU enumerated. * $p < 0.0005$ versus naïve unless indicated, measured by ANOVA. Adapted from Tchilian *et al* (submitted).

6.2.6 Kinetics of *Mtb* growth following s.c., i.n. or SIM immunisation.

The above data suggest that respiratory and systemic immunisations act by different mechanisms that can work additively with each other. The work in Chapters 3 and 5 indicates that this is because respiratory immunisation inhibits mycobacterial growth early after infection and systemic immunisation inhibits growth late after infection. As the immune response following protein immunisation echoed that seen in the Ad85A – BALB/c model, with i.n. generating a strong lung response and systemic immunisation a strong splenic response, it was hypothesised that a similar pattern of early and late inhibition was occurring in this model. C57Bl/6 mice were therefore immunised either s.c. or i.n. with r85A, ESAT6 or BCG. Mice were also immunised with BCG in a SIM regime (BCG s.c./i.n.). Following subsequent *Mtb* challenge, mice were culled at 7 day intervals to track at what point after infection each vaccination inhibited growth. In all mice that had received one of the vaccines intra-nasally, *Mtb* growth was inhibited compared to naïve mice by day 7 post-

challenge. However, mice that had received a systemic immunisation with any of the vaccines did not inhibit mycobacterial growth until day 14 post-challenge (Figure 9). In the case of ESAT6 a significant reduction in *Mtb* load was not reached until day 28 (Figure 9). Importantly, in the mice that had received BCG in a SIM regime, two phases of protection were observed. At day 7, mice that had received i.n. BCG immunisation, either alone or in the SIM regime, showed a reduction in lung CFU compared to naïve mice. By day 14, the mice that had received BCG s.c. alone showed a reduction equivalent to those that had received i.n. BCG only. However, at this time point, the mice that had received the SIM regime showed a significant improvement in protection compared to mice that had received either immunisation alone (Figure 9). These data, along with the data presented in Chapters 3 and 5, confirm the hypothesis that respiratory immunisation induces a response in the lung that is able to protect early after *Mtb* challenge, within 7 days. Systemic immunisation induces a strong peripheral response that is able to protect but this acts later: between 14 and 21 days post-infection. It would be of further interest to map the kinetics of mice that have received the other SIM regimes to determine if the two phases of protection can also be observed in these mice.

6.3 Discussion

As discussed in Chapter 3, *Mtb* growth in the lungs of naïve mice is not controlled until 21 days post-infection and although parenteral BCG immunisation confers protection, this does not occur until at least 14 days after challenge. The work in Chapter 3 demonstrated that mice immunised i.n. with Ad85A are able to control *Mtb* growth in the first 7 days following infection, an effect able to act additively with the late protection conferred by parenteral BCG. In those experiments, the two vaccines were given 10 weeks apart. However, as the increase in protection was due to the cumulative effects of a lung and systemic response, simultaneous administration of a protective respiratory immunisation alongside a protective parenteral vaccine (SIM) should confer protection in the same manner.

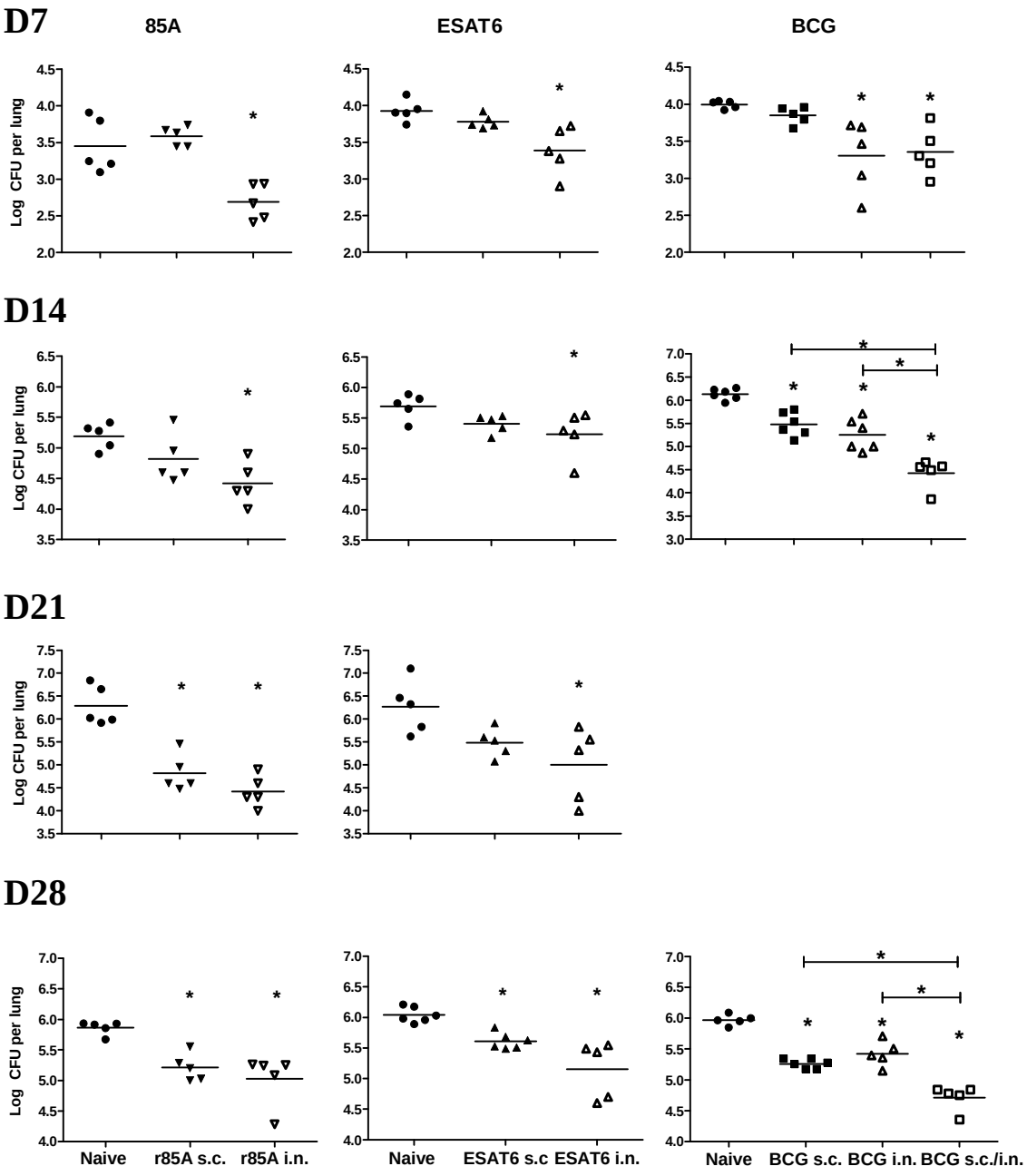


Figure 9: Kinetics of *Mtb* growth following systemic or respiratory immunisation. C57BL/6 mice were immunised s.c. or i.n. either 3 times at 2 weekly intervals with one of r85A or ESAT6 or once with BCG s.c., i.n. or both (s.c./i.n.). 4 weeks following the final protein immunisation or 10 weeks post-BCG mice were challenged i.n. with *Mtb*. Groups of mice were then culled on days 7, 14, 21 and 28 post-challenge and the lung CFU enumerated. Only 3 mice were available for the day 21 BCG s.c./i.n. group and so the data have been omitted. * $p < 0.001$ versus naïve, as measured my ANOVA. Adapted from Tchilian *et al* (submitted).

The data in this chapter demonstrate that it is the combination of vaccines in a SIM regime, targeting both early and late phase growth, that is protective. Two separate vaccines, protective when administered alone, administered together by the same route were no more protective than either given alone. This may be because, when given by the same route, the protective effect of each vaccine would potentially be acting at the same point in the growth curve, whether that is early after infection if both were given i.n., or late if both were given systemically. Only if the two protective vaccines target separate phases do they combine most reliably to improve efficacy. Dietrich and colleagues have shown that simultaneous parenteral immunisation with BCG and a recombinant fusion protein is able to increase protection over either immunisation alone, but only when administered along with liposomal vehicles, possibly due to establishment of an antigen depot (205). Interestingly, using the same fusion protein, Olsen *et al* demonstrated that the antigens were protective only when administered as a fusion protein but not when given as separate proteins in the same immunisation cocktail (207). The reason behind this effect is unclear, although it may be due to differential antigenic processing or increased immunogenicity of the fusion protein (207). As potent protection is seen following SIM using non-cross-reactive vaccines, whether that is BCG with ESAT6 or two different proteins, the improvement in protection following SIM is not due to a prime-boost effect and may in fact offer an alternative paradigm for immunisation schedules.

Interestingly, although SIM showed significant improvement in protection when using heterologous vaccines, if the same antigen is administered by both the respiratory and systemic routes then no improvement in protection is seen. Ad85A has been shown to protect BALB/c mice early after infection in i.n. immunised mice and late in i.d. immunised mice (Chapter 3 & 5). The same can be said of r85A and ESAT6 in C57Bl/6 mice (Figure 9). However, if any of these immunisations are given together in a homologous SIM regime no improvement in protection over individual immunisations is detected (Figure 2 and data not shown). This is despite strong immunogenicity in both the lung and periphery (Figure 1 & 5). The exception to the inefficacy of homologous

immunisation is SIM with BCG. When BCG is given simultaneously by respiratory and systemic routes the improvement in protection is striking (Figure 8). It is clearly evident that the two immunisations within the SIM regime act at different time points after challenge, combining to confer a significantly greater degree of protection (Figure 9). Why BCG is effective in a homologous SIM regime when other antigens are not is unclear. It is possible that responses to two different antigens within BCG are responsible for the inhibition at each phase. However, BCG is complex, with many immuno-modulatory mechanisms and a direct comparison of a replicating organism with recombinant proteins may be difficult.

The marked increase in efficacy using BCG in a SIM regime is an important finding, particularly as BCG is currently the only licensed TB vaccine. Previous efforts to boost prior BCG with further BCG immunisations have found no increase in efficacy (208), likely due to host immunity to BCG following the primary immunisation. However, by simultaneously administering the vaccine by each route this problem can be circumvented. SIM offers another advantage over the prime-boost paradigm in that it requires only one clinic visit by the patient. In areas where patient compliance and tracking can be problematic, removing the need for a follow up visit could greatly aid coverage. However, the long term protection conferred by BCG has been widely reported as variable, particularly in sub-Saharan Africa. This, combined with safety concerns surrounding BCG immunisation of HIV+ individuals (209), highlights the importance of developing novel vaccines against *Mtb*, whether they be new recombinant mycobacteria or subunit vaccines. By demonstrating the efficacy of SIM using protein plus adjuvant, the work here shows that many new vaccine candidates could potentially be combined in a SIM regime to greater protective effect.

There are two significant caveats to the development of SIM as an immunisation regime. The first is that, perhaps obviously, the vaccines used for each route must be protective on their own. The vaccines used here were highly mouse strain specific: Ad85A was not protective in C57Bl/6 mice (Figure 3) and therefore was ineffectual when combined in a SIM regime (data not shown) and immunisation

with any of the protein subunits was not protective in BALB/c mice, whether alone or combined into SIM (data not shown). Thus, as the SIM regime depends on two distinct protective effects acting separately, it is necessarily reliant on an effective systemic response and an effective respiratory response.

This highlights the second caveat. No TB vaccine is currently approved for administration by the respiratory route, a fact that evidently precludes SIM immunisation in humans. There are obviously safety concerns surrounding vaccine delivery to such a sensitive site as the respiratory tract, particularly as previous efforts have resulted in significant side effects (159, 196, 197). However, influenza and measles vaccines have been approved in some countries for respiratory immunisation (150) and BCG has been shown to be safe when delivered by nebuliser to adolescents (160). Nonetheless, if the aforementioned safety concerns around BCG are to be avoided using subunit immunisation, then a suitable mucosal adjuvant will be required. The work here used Cholera toxin (CT) as an adjuvant for the respiratory protein immunisations, resulting in potent lung responses (Figure 7). However, CT is too toxic to be used in humans and the related mucosal adjuvant heat labile toxin (LT) from *Escherichia coli* has been shown to cause Bell's palsy (196). Modified, non-toxic versions of these adjuvants are being developed (210, 211), hopefully furthering the potential for efficacious subunit respiratory immunisation.

6.4 Summary

The work in this chapter has shown the efficacy of a new immunisation paradigm: simultaneous respiratory and systemic vaccination (SIM). SIM can confer a significant increase in protection over either of the constituent immunisations given alone. However, this is not due to a prime-boost effect, but rather due to the combined effect of the respiratory immunisation inhibiting the early phase of *Mtb* growth following infection and the systemic immunisation inhibiting late phase growth. Importantly, the work shows that the protection seen following BCG – the so-called "gold standard" for TB vaccine

efficacy – can be markedly increased if BCG is administered in a SIM regime. Such a significant increase in vaccine efficacy following a simple change in immunisation strategy highlights the potential in SIM based strategies for future TB vaccines.

CHAPTER 7

Concluding remarks

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7.1 Summary

The work in this thesis set out to discern why a candidate TB vaccine, Ad85A, administered by the respiratory route was able to protect BALB/c mice following *Mtb* infection but not when administered parenterally. Investigations determined that i.n. immunisation induced antigen-specific cells in the lung that were able to inhibit the growth of *Mtb* early after infection, before systemic immunity began to act. Examination of the role played by upper respiratory tract immune responses indicated that for respiratory immunisation with Ad85A to be protective, a lung response must be generated. A move from one animal house to another altered the immune responses seen in the lungs following i.d. immunisation, resulting in a degree of protection. However, this protection occurs only in the late phase following infection, contrary to that seen after i.n. immunisation. This is most likely because i.n. immunisation elicits a response in the airway, whereas i.d. immunisation does not. There are tantalising hints that these changes in response and protection following i.d. immunisation may be caused by exposure to environmental mycobacteria.

These results indicate that different immunisation routes (respiratory and parenteral) target different phases of *Mtb* growth. Simultaneous immunisation by the two routes (SIM) has the potential to target both phases of growth and thus additively improve protection, whereas 2 vaccines targeting the same phase may interfere with each other. By using non-cross-reactive protein vaccines to rule out any possible prime-boost effect, this work demonstrated that using SIM was more effective than 2 respiratory or 2 parenteral immunisations, most likely due to targeting the two distinct phases. Furthermore, SIM using the only currently licensed TB vaccine, BCG, significantly improved protection, thus indicating a potential augmentation of current TB vaccination programs and setting a new gold-standard by which candidate vaccines can be assessed.

7.2 Further questions

The work described above has outlined a regime representing a departure from conventional prime-boost methodology for vaccination against *Mtb*, or that at the very least could act as a potential adjunct to it. However, a number of questions remain.

The primary unknown in this work is what is occurring in the lung immediately after infection. Although depletion of CD8+ cells from the lung of Ad85A i.n. immunised mice resulted in the loss of early inhibition, a positive transfer of antigen-specific CD8 T cells would be needed before it could conclusively be said to be the mediating population. Furthermore, the mechanism by which mycobacterial growth is restricted is unknown. Is *Mtb* replication just inhibited, thus preventing the exponential growth seen in naïve mice, or are infected cells and mycobacteria actively killed by the CD8 T cells, or is it a combination of the two? Moreover, whatever effect is occurring, the question of whether it is driven by direct cytolytic action of the CD8 cells or by cytokine-mediated activation of infected cells is unknown. Further investigation of these mechanisms, whether in knock-out mice or using antibody blockade, would go some way to understanding how early phase inhibition occurs.

One argument in favour of vaccine-induced early inhibition being cytokine-mediated is that it is observed following protein immunisation in C57Bl/6. In these mice, no CD8 T cell responses can be measured, but potent CD4 antigen-specific cytokine production can be detected. Although far from conclusive evidence, the fact that these mice are able to inhibit early growth may be an indication that cytokine production is at least playing a role in early *Mtb* control. However, this line of reasoning may be confounded by the fact that the only antigen specificity readout for these experiments was cytokine production, thus risking a circular argument.

A number of other unknowns also remain about early inhibition. Although it is clear that respiratory immunisation is able to restrict mycobacterial growth in the first week following infection, it does not eliminate *Mtb* entirely. Eventually mycobacterial growth escapes and enters an exponential phase until

brought under control, after which the *Mtb* burden reaches a plateau. As outlined in Chapter 3, an adaptive immune response cannot be detected in the lymph nodes of naïve mice until 9 days post-infection, followed by arrival in the lungs at 14 days – an arrival that correlates with control of *Mtb* growth. This raises that possibility that the early inhibition of *Mtb* following respiratory immunisation is acting only as a delay in this process. Once *Mtb* growth escapes the early control and enters log phase, will the same kinetic be observed but merely a week later, resulting in no long term protection? Or is there a sufficient burden of *Mtb* in the lungs of vaccinated mice for dissemination to the lymph node to occur, thus generating a primary response at the same rate as in naïve mice? The data presented here suggest that the latter is the case, as *Mtb* burden in i.n. immunised mice reaches a plateau at a similar time to naïve mice, but at approximately one order of magnitude lower. This implies a primary response is generated at the same speed in immunised mice and hence can exert control earlier during the log phase growth, resulting in a lower mycobacterial load. However, experiments into the long term protection post-challenge of i.n. immunised mice need to be performed, as well as careful mapping of any primary immune response to non-vaccine antigens in the lung draining lymph nodes.

Similarly, the question as to why *Mtb* growth is controlled only for the first few days following infection remains. One possibility is that *Mtb* is escaping under immune pressure. Although not conventionally assumed to be as capable of escape as viral infections, recent work has demonstrated that mutation rates of *Mtb* may be higher than previously expected (212). Thus, if under sufficient immune pressure, it is possible that escape mutations could occur, particularly if vaccination only targeted one antigen. Such escape mutations could be behind the lack of increased efficacy in homologous protein SIM regimes. These regimes induce strong systemic and respiratory responses and the individual immunisations, when given alone, are protective. However, no additive effect is observed. It is possible that the early inhibition in the lung exerts sufficient immune pressure to drive escape within that antigen, leading to a loss of efficacy of the late response, as both the systemic and

respiratory responses are specific to the same antigen. However, although the *Mtb* mutation rate is higher than expected, it is still considerably slower than for pathogens such as HIV. This, combined with low mycobacterial numbers early after infection and a long generation time may make early phase escape implausible. However, sequencing of *Mtb* isolates from the late phase following early inhibition may provide some interesting insights.

If escape is occurring, or even if not, the protective efficacy of SIM regimes could be enhanced by the use of fusion proteins. Olsen and colleagues have demonstrated that when mice are immunised with two antigens administered as a fusion protein, the protective effect of both antigens combines to improve efficacy. However, no such improvement is observed if the two antigens are just mixed (106). One of the advantages of SIM as a concept is that it imposes no restriction on what vaccines are used; only that one is protective when given by the respiratory route and the other protects when given systemically. Thus, incorporation of such fusion proteins into a heterologous SIM regime could enhance the protection observed following SIM vaccination with single antigens.

The work here also raises some causes for concern. One of these is the apparent strain specificity and efficacy variation of the different vaccines. Ad85A was highly protective in BALB/c mice when administered i.n., as well as conferring a degree of protection when given systemically. However, the same vaccine given by either route to C57Bl/6 mice provided no protection at all, despite being highly immunogenic. Similarly, the protein vaccines protective in C57Bl/6 mice were not protective when given to BALB/c mice (data not shown). Further variation, possibly equally due to strain differences, appears when the systemic immunisations of Ad85A and protein vaccines are compared. Both confer protection in the late phase of growth when analysed in their respective strain; however Ad85A i.d. gained efficacy only after switching animal houses, once a lung response could be detected. This contrasts with systemic protein immunisation, which induces very little response in the lung but yet is still protective. The reason behind this difference unfortunately remains unclear. Inter-strain variation of susceptibility is widely acknowledged in mice; however, the disparity of efficacy across strains

highlights the importance of choosing the correct experimental model for vaccine testing. Equally, Ad85A i.d. induced protection in BALB/c mice correlated with the presence of CD8 T cells in the lung, whereas protein induced protection in C57Bl/6 mice correlated with an antigen-specific CD4 response in the periphery. It may be the case that CD8 cells need to be resident in lung tissue at the time of infection before protection can occur, whereas different migration mechanisms for CD4 cells allow more effective recruitment from the periphery.

There are also causes for concern when considering the wider application of the work presented here. Ad85A and SIM regimes both require delivery of vaccines to the respiratory tract and subsequent activation of pulmonary immune cells. Widespread administration of an attenuated measles vaccine to the lower respiratory tract has appeared to demonstrate such a route is safe. However, the generation of a large population of activated T cells could have unforeseen consequences or side-effects, particularly in terms of immuno-pathology. Furthermore, the importance of the common mucosal immune system is becoming more evident. Although the work here suggests that responses in the NALT mucosa are not necessary for protection following respiratory immunisation, i.n. infection has been demonstrated to induce a significant population of activated T cells in the vaginal tract (213). Generating such populations of activated T cells in distal mucosa may have a significant impact, positive or negative, on the outcome of other mucosal infections. Following the unexpected outcome of the STEP trial (138), such bystander immunological effects of vaccination need to be carefully considered in HIV endemic areas.

As mentioned, the concept of SIM vaccination has an advantage as almost any effective vaccine can be included in such a regime. However, a recent finding has raised a potential problem with the current crop of vaccine candidates. Many of these candidates are based on antigens such as the antigen 85 complex, ESAT6 and similar proteins as they are highly immunogenic and protective in animal models. Upon analysis of the evolution of the *Mtb* genome, it appears that many of these antigens, particularly the epitope regions within them, are under purifying selection (214). If these antigens are

hyper-conserved despite being well recognised by the host immune system, it may imply that such recognition is actually beneficial to the pathogen. This presents the risk that, by raising such responses with vaccination, one could inadvertently worsen the outcome of infection.

Despite these caveats, I believe the work here presents a notable addition to the field of TB vaccinology. The early protection observed following respiratory immunisation highlights the importance of inducing effective immune responses at the site of pathogen entry. Furthermore, by combining respiratory immunisation with a simultaneous parenteral vaccination, one can target distinct phases of infection. Using such a regime, significant improvements in protection can be achieved by a mechanism notably different from conventional prime-boost methodology.

Chapter 8

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Appendix I Antigen 85A insert sequence plus tPA leader sequence

atggatgcaatgaagagagggctctgctgtgtgctgctgctgtggagcagtcttcgtttcgcccagccaggaaatccatgcccgattcag
aagattttcccggccgggcttgccggtggagtacctgcaggtgccgtcgccgtcgatgggccgtgacatcaaggtccaattccaaagtggtggtgc
caactcgcccgccctgtacctgctcgacggcctgcgcgcgcaggacgacttcagcggctgggacatcaacaccccggcgttcgagtggtacgac
cagtcgggcctgtcggtggtcatgccggtgggtggccagtcaagcttctactccgactggtaccagcccgcctgcggcaaggccggttgccagact
tacaagtgggagaccttcctgaccagcgagctgccggggtggctgcaggccaacaggcacgtcaagcccaccggaagcgccgtcgtcggtcttt
cgatggctgcttcttcggcgctgacgctggcgatctatcacccccagcagttcgtctacgcgggagcgatgtcgggcctgttggacccctcccaggc
gatgggtcccaccctgatcggcctggcgatgggtgacgctggcggctacaaggcctccgacatgtggggcccgaaggaggacccggcgtggca
gcgcaacgacccgctgttgaacgtcgggaagctgatcgccaacaacacccgcgtctgggtgtactgcggcaacggcaagccgtcggatctgggt
ggcaacaacctgccggccaagttcctcgagggcttcgtgcggaccagcaacatcaagttccaagacgcctacaacgccggtggcggccacaacg
gcgtgttcgacttcccggacagcggtacgcacagctgggagtactggggcgcgcagctcaacgctatgaagcccgacctgcaacgggcactggg
tgccacgcccaacaccgggcccgcgccccagggcgcctag

Bold characters indicate tPA leader sequence.

Appendix II Pool of peptides

Peptide number	Sequence	Molecular weight
1	MQLVDRVRGAVTGMS	1619.907
2	RVRGAVTGMSRRLVV	1656.998
3	VTGMSRRLVVGAVGA	1472.754
4	RRLVVGAVGAALVSG	1424.691
5	GAVGAALVSGLVGAV	1240.447
6	ALVSGLVGAVGGTAT	1272.446
7	LVGAVGGTATAGAFS	1278.409
8	GGTATAGAFSRPGLP	1359.486
9	AGAFSRPGLPVEYLQ	1604.806
10	RPGLPVEYLQVPSPS	1638.864
11	VEYLQVPSPSMGRDI	1690.916
12	VPSPSMGRDIKVQFQ	1688.947
13	MGRDIKVQFQSGGAN	1607.789
14	KVQFQSGGANSPALY	1566.714
15	SGGANSPALYLLDGL	1447.589
16	SPALYLLDGLRAQDD	1646.798

17	LLDGLRAQDDFSGWD	1707.797
18	RAQDDFSGWDINTPA	1692.742
19	FSGWDINTPAFEWYD	1847.936
20	INTPAFEWYDQSGLS	1727.827
21	FEWYDQSGLSVVMPV	1756.974
22	QSGLSVVMPVGGQSS	1432.597
23	VVMPVGGQSSFYSDW	1658.829
24	GGQSSFYSDWYQPAC	1695.763
25	FYSDWYQPACGKAGC	1695.872
26	YQPACGKAGCQTYKW	1703.939
27	GKAGCQTYKWETFLT	1732.955
28	QTYKWETFLTSELPG	1799.977
29	ETFLTSELPGWLQAN	1705.865
30	SELPGWLQANRHVKP	1731.955
31	WLQANRHVKPTGSAV	1663.88
32	RHVKPTGSAVVGLSM	1538.813
33	TGSAVVGLSMAASSA	1308.455
34	VGLSMAASSALTLAI	1404.671
35	AASSALTLAIYHPQQ	1570.747

36	LTLAIYHPQQFVYAG	1720.968
37	YHPQQFVYAGAMSGL	1668.871
38	FVYAGAMSGLLDPSQ	1555.75
39	AMSGLLDPSQAMGPT	1475.684
40	LDPSQAMGPTLIGLA	1483.729
41	AMGPTLIGLAMGDAG	1374.623
42	LIGLAMGDAGGYKAS	1423.632
43	MGDAGGYKASDMWGP	1542.689
44	GYKASDMWGPKEDPA	1651.795
45	DMWGPKEDPAWQRND	1844.959
46	KEDPAWQRNDPLLNV	1794.965
47	WQRNDPLLNVGKLIA	1737.015
48	PLLNVGKLIANNTRV	1621.925
49	GKLIANNTRVWVYCG	1693.968
50	NNTRVWVYCGNGKPS	1694.869
51	WVYCGNGKPSDLGGN	1566.692
52	NGKPSDLGGNNLPAK	1481.61
53	DLGGNNLPAKFLEGF	1591.764
54	NLPAKFLEGFVRTSN	1692.915

55	FLEGFVRTSNIKFQD	1801.011
56	VRTSNIKFQDAYNAG	1683.821
57	IKFQDAYNAGGGHNG	1548.615
58	AYNAGGGHNGVFDFP	1522.576
59	GGHNGVFDFPDSGTH	1543.552
60	VFDFPDSGTHSWEYW	1872.945
61	DSGTHSWEYWGAQLN	1750.78
62	SWEYWGAQLNAMKPD	1795.97
63	GAQLNAMKPDLQRAL	1625.892
64	AMKPDLQRALGATPN	1582.823
65	LQRALGATPNAVPAP	1463.638
66	GATPNAVPAPQGA	1150.238