The effects of environmental mycobacteria on VLP and MVA based vaccines against tuberculosis

Rahiman Sharief Faiyaz

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ABSTRACT

BCG is already established as a vaccine against a global epidemic of tuberculosis, but its efficacy remains variable. It has shown almost no protection against TB in tropical countries like Africa and India. One of the prime reasons postulated for the failure of BCG as a vaccine is associated with pre-exposure to environmental mycobacteria. Cross-sensitisation to shared mycobacterial antigens is regarded as an important factor for this variation in efficacy.

This study investigated the effects of environmental Mycobacterium avium exposure on immune responses to BCG and two novel TB vaccine candidates: Rabbit haemorrhagic disease (RHDV) virus-like particles conjugated with Antigen 85A (VLP/Ag85A) and Modified vaccinia virus Ankara expressing Antigen 85A (MVA/Ag85A). Ag85A peptide was used because it is known to be an effective immunogen. M. avium strain WAg206 was chosen for this study as it has previously been shown to interfere with BCG vaccination. RHDV VLP were generated using a recombinant baculovirus containing the VP60 capsid gene. VLP/Ag85A was prepared by chemical conjugation of mycobacterial peptide Ag85A to VLP. MVA/Ag85A is a genetically modified vaccinia virus expressing Ag85A. T cell proliferation assays, cytokine assays, total antibody and antibody isotype assays were carried out after vaccination to measure the immunogenicity.

The results suggest that among these novel vaccines, MVA/Ag85A is the best vaccine candidate following pre-exposure to WAg206 as it generated a stronger Th1 type of immune response than either BCG or VLP/Ag85A based on proliferation assays and cytokine assays specific to mycobacterial antigens. High levels of antigen-specific IFN-γ, a Th1 cytokine, were recorded when mice were vaccinated with MVA/Ag85A following pre-exposure to WAg 206. On vaccination with VLP/Ag85A, antigen-specific IFN-γ responses were low, but
the presence of higher levels of total antibody specific to mycobacterial antigens suggested induction of a predominant Th2 response which is not protective.

Future work to improve the cell-mediated response to VLP/Ag85A may include using an adjuvant which enhances Th1 responses and overcomes the inhibitory effect of environmental mycobacteria.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ag85</td>
<td>Antigen 85</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Geurin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDMEM</td>
<td>Complete Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CsCl</td>
<td>Caesium chloride</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s PBS</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidise</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>IgG</td>
<td>Immunoglobulin</td>
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</tr>
<tr>
<td>IL-5</td>
<td>Interleukin-5</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>ml</td>
<td>millilitres</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MTB</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PenStrep</td>
<td>Pencillin and Streptomycin</td>
</tr>
<tr>
<td>PPD-a</td>
<td>Purified protein derivative – avium</td>
</tr>
<tr>
<td>PPD-b</td>
<td>Purified protein derivative - bovine</td>
</tr>
<tr>
<td>RHDV</td>
<td>Rabbit haemorrhagic disease virus</td>
</tr>
<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediates</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SMCC</td>
<td>4-[N-maleimidomethyl]cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1 type cell</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2 type cell</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor – alpha</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus like particle</td>
</tr>
<tr>
<td>WAo</td>
<td>Wallaceville AgResearch</td>
</tr>
<tr>
<td>XDR</td>
<td>Extreme drug resistant</td>
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Chapter-1

INTRODUCTION
1.1 TUBERCULOSIS and MYCOBACTERIA

Tuberculosis

Tuberculosis (TB) is a contagious bacterial disease caused by *Mycobacterium tuberculosis* (MTB). It is normally a pulmonary disease mainly affecting lungs although it is capable of infecting other systems of body such as the lymphatic system, central nervous system, and the circulatory system.

TB is considered as a major cause of death worldwide, and was responsible for more than 1.6 million deaths in the year 2005 (WHO, 2007).

A major cohort of patients that die from TB are HIV-positive. A subject who is HIV-positive and has TB infection has a higher risk of morbidity when compared with a subject who is only infected with TB (WHO, 2007).

In addition to the increase in incidence of death from TB with HIV infection there are now MTB(*M. tuberculosis*) strains that are resistant to the anti-TB drugs isoniazid and rifampicin, the two most powerful anti-TB drugs. There is also extreme drug-resistant (XDR) TB, where no known antibiotic is efficacious (WHO, 2007).

Mycobacterium tuberculosis

MTB is a non-motile rod-shaped bacterium and belongs to the *Actinomycetes* family. The rods are 2-5 micrometers in length (Shinnick & Good, 1994). MTB is an obligate aerobe. For this reason, in the classic case of tuberculosis, MTB complexes are always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow doubling time, 24 hours, a physiological characteristic that
may contribute to its virulence (Cole & Brosch et al., 1998). Chains of bacterial cells in smears made from in vitro grown colonies often form distinctive serpentine cords. This observation was first made by Robert Koch who associated cord factor with virulent strains of the bacterium. MTB is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan in their cell wall.

Mycobacterium species are classified as acid-fast bacteria due to their impermeability to certain dyes and stains. However, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds. One acid-fast staining method routinely used for MTB is the Ziehl-Neelsen stain.

1.2 BCG

BCG (Bacillus-Calmette-Guerin) was first developed by Calmette and Guerin by repeated sub-culturing of virulent Mycobacterium bovis strain isolated from a bovine mastitis sample 230 times on glycerinated bile potato medium, resulting in gene deletions and duplication and attenuation. BCG was first administered as a vaccine in 1921 (Oettinger et al., 1999) and it has become since then the most widely administered vaccine globally.

Later different local sub-strains of BCG evolved (by separate closed activity) such as BCG Connaught, Danish, Glaxo, Pasteur, and Tokyo, all of which contain differences in terms of genetic and antigenic composition (Behr, 2001, Oettinger et al., 1999).

To date, more than three billion doses of BCG have been administered worldwide, making it the most widely employed vaccine in the world. It is a safe vaccine and can be given at birth (Cassanova et al., 1996). The cheap production cost makes it an ideal, cost-effective preventive measure against TB as compared to chemotherapy, which is expensive and time consuming.
Most importantly, BCG is the best vaccine tested against tuberculosis to date, as it has been shown to elicit a strong protective Type 1 immune response (Silva et al., 1999). It is currently given intra-dermally as a live, attenuated vaccine, as studies have shown that heat-killed bacteria elicit a different and ineffectual immune response (Hook et al., 1996).

1.3 Immune responses to mycobacteria

Protective immunity against MTB depends on the generation of a Type 1 cellular immune response, characterized by the secretion of interferon-γ (IFN-γ) from antigen-specific T cells (McShane et al., 2004). The human immune system relies on both non-specific innate immunity and antigen-specific acquired immunity working together in concert to protect the body against these pathogens. The innate system which includes mononuclear phagocytes, natural killer cells, interferon, and complement, has to be able to recognize pathogens and signal the presence of danger to cells of the acquired immune system (Matzinger, 2002; Janeway & Medzhitov, 2002). The acquired immune system, in turn, is responsible for further driving the effector immune response to eradicate the MTB. It also needs to form long-lived immunological memory for enhanced recall responses in order to provide life-long immunity to this pathogen.

There are two APCs involved in generating a protective immune response to MTB i.e., macrophages and dendritic cells (DC). They each have a unique role to play in this response. DC are the only cells that prime naive T cells, whereas macrophages are the cells that are primarily infected with MTB and therefore need to kill the bacteria.
In MTB infections, alveolar macrophages are the first cell line to encounter bacteria as the main route of infection is via the respiratory tract (Hingley-Wilson et al., 2003; Nicod et al., 2000). Macrophages have the microbicidal armoury to destroy most invading pathogens (reviewed by Raja, 2004). They will phagocytose mycobacteria, contain it with in a phagosome, and attempt to kill it by fusing the phagosome with a lysosome (Duclos & Desjardins, 2000; Vieira et al., 2002). Lysosomes contain a variety of toxic substances that are lethal to bacteria, for example acids, degrading enzymes such as hydrolases and peroxidases, oxygenated lipids, fatty acids, and reactive oxygen and nitrogen intermediates (Klebanoff, 2005; Nathan & Shiloh, 2000). However MTB has the ability to resist being killed in several ways, including inhibition of phago-lysosomal fusion, Reactive oxygen intermediates (ROI) and Reactive nitrogen intermediates (RNI) production, macrophage activation and antigen presentation.

Phago-lysosomal function can be constrained in macrophages infected with MTB and there are different ways to mediate this infection. Phagosomes containing live, viable mycobacteria are repressed from fusing with lysosomes as fusion of the phagosome with the lysosome has been shown to be withdrawn (Armstrong & Hart, 1971; Mwandumba et al., 2004). However, phagosomes containing mycobacteria are capable of fusing with other endosomes, which suggests they are dynamic, fusion-competent structures that are selectively prevented from fusing with lysosomal compartments (Russel et al., 1996; Mwandumba et al., 2004). A host protein found in lymphoid and myeloid cells called tryptophan aspartate-conating coat (TACO) protein has been identified, and may inhibit fusion. TACO relocalises within cells to associate with phagosomal membranes containing live MTB and is retained for longer periods (Ferrari et al., 1999).

MTB are also able to prevent damage caused by ROI and RNI produced by infected macrophages. Mycobacterial products such as LAMs and Phenoliglycolipid-1 can act as free-
radical scavengers and mitigate oxidative damage (Chan et al., 1989). MTB possesses the genes noxR1, noxR3 and ahpC that can provide resistance to nitrosative and oxidative stress (Ehrt et al., 1997; Ruan et al., 1999). Furthermore, MTB expresses a methionine sulphoxide formed from the reaction of ONOO- and methionine residues in mycobacterial proteins and can repair damage caused by RNI (St John et al., 2001).

If the macrophage is properly activated by IFN-γ, it can successfully eliminate or limit the growth of MTB (Ma et al., 2003). Alternatively, MTB persist in the macrophage due to its most evolved mechanisms to survive macrophage effector functions (Houben et al., 2006). Apoptosis, also known as programmed cell death is one more important defence mechanism against mycobacteria (Keane et al., 2000). Apoptosis prevents the spread of mycobacterial infection by sequestering the mycobacteria within membrane bound apoptotic bodies (Fratazzi et al., 1999). The ability to induce apoptosis has been shown to be dependent on the virulence of the strain involved and perhaps the multiplicity of infection (Danelishville et al., 2003) (Lee et al., 2006). So it is to be noted that macrophage’s ability to control MTB is extremely important, as this will influence the outcome of the infection (Houben et al., 2006)(Flynn and Ernst, 2000).

DC are another type of APC with a characteristic morphology of branched projections called dendrites, hence their name. Dendritic cells are primarily involved in anti-mycobacterial T cell immune response, and these are highly represented in sites of MTB infection at the onset of the inflammatory change (Giacomini et al., 2001).
Role of T-cell subsets in mycobacterial infections

The two main subsets of T cells are T helper cells (Th) cells, which characteristically express the cluster of differentiation 4 (CD4) glycoprotein; and T Cytotoxic (Tc) cells, which express the CD8 marker on its surface. CD4 and CD8 are both co-receptors for the T cell receptor. Other T cell subsets include γδ T cells, natural killer T cells, and regulatory T cells (Treg). In mycobacterial infection, these T cell subsets are known to contribute to protection (Stenger & Modlin, 1999; Mogues et al., 2001).

**CD4 T cells**

The host-pathogen interaction in MTB is usually based on communication between T cells and infected macrophages. The result of this interplay may lead to control of the infection or activation of the disease. CD4+ T cells have crucial role in limiting the MTB bacilli growth with the help of macrophages.

MTB specific CD4 T-cells are primed in the lymph node by DC expressing MTB antigens such as Ag85A,B,C, ESAT-6 and CFP-10. These immunodominant antigens are secreted and hence only induce protective immune responses against live bacteria. When activated by MTB, CD4+ T cells secrete the macrophage-activating cytokines like IFN-γ and TNF-α which help macrophages in limiting the growth of intracellular mycobacteria, as those cytokines are cytotoxic for those infected macrophages.

At present, the most widely accepted theory regarding the immune functioning of Th cells is the model proposed by Mosmann et al in 1996. According to this model, CD4 T cells can be subdivided into two independent subsets, Th1 or Th2 cells, on the basis of cytokine secretion.
and bioactivities (Mosmann et al, 1986, Coffman, 2006). Each subset has very distinct effector functions. Th1 cells secrete large amounts of pro-inflammatory cytokines, such as IL-2, IL-12, IFN-γ, TNF-β and TNF-α upon activation. These cytokines are essential for the smooth functioning of cell-mediated immune response against intracellular pathogens, such as viruses and mycobacteria (Liew, 2002). By contrast, Th2 cytokines regulate humoral or antibody responses by B cells. A Th2 immune response is associated with allergic reactions, parasitic and extracellular bacterial infections (Murphy et al., 1999; Abbas et al., 1996).

Both Th1 and Th2 cells have a cross-regulatory role, where only one pathway pre-dominates at any one time (Coffman, 2006). Cytokines of a certain subtype will promote the expansion of that particular subtype, while inhibiting the development of the other subset. This in turn induces the development of distinctive effector function specific to the immunogen (Murphy & Reiner, 2002) e.g. the sequential action of IFN-γ and IL-12 leads to the development of Th1 cells, while down-regulating Th2 responses. Depending on the balance of Th1 versus Th2 cytokines produced during immunity, the immune system can be skewed to either a cell-mediated pathway or humoral pathway (Coffman, 2006).

It has been well established that CD4 T cells provide protection against MTB. Following reinfection by MTB, contact between recirculating T cells and the mycobacteria should ideally lead to rapid cytokine secretion by memory T cells, at a lower threshold of antigen stimulation. This will result in the rapid accumulation of monocytes and other immune cells to the infection site to eliminate pathogen (Reinhardt et al., 2001).

CD8+ T cells (Tc) are another subset of T cells which can be activated in response to MTB and BCG (Szereday et al, 2002). These cells secrete IFN-γ as well, but in lower quantities than CD4+ T cells. It is established that CD8+ T cells offer protective immunity by helping
macrophages in controlling intracellular mycobacteria in later stages of infection (Szereday et al., 2002). Once activated, Tc cells become effector cells that can directly lyse target cells via cytotoxic granules such as perforin and granzymes (Serbina et al., 2000), or induce apoptosis in cells infected intracellularly with mycobacteria (Bonato et al., 1998; Silva et al., 2000). It was demonstrated by Lazrevic et al (2005) that CD8+ cells in the granuloma periphery possessed high lytic ability, but produced minute amounts of IFN-γ during acute TB infection. However in chronic TB infection, this switched to high IFN-γ production, with low lytic ability (Lazrevic et al., 2005). This shows the dynamic activity of CD8+ cells within granuloma.

γδ TCR+ T cells are characterised by unique T cell antigen receptor comprised of γ and δ chains and mainly express Vδ9 and Vδ2 elements. These cells also have the function of killing infected macrophages by IFN-γ secretion. Unlike the previously mentioned subsets, γδ TCR+ T cells (Vδ2+ T cells) recognise different types of mycobacterial molecules based on phospho antigens which are processed and presented to these cells with co-stimulation by macrophages (Boom et al., 2003). The prominent role of γδ T cells in protective immunity is also established by the fact that number and reactivity to phosphate antigens (TUBAg3-4, isopentyl-ATP, Pyrophosphate molecules such as TUBag1-2, isopentyl pyrophosphate(IPP), mono-ethyl pyrophosphate(MEPP), and others) increases during primary infection and challenging after BCG vaccination (Boom et al., 2003).

**NKT cells**

NKT cells tend to secrete large amounts of both Th1 and Th2 cytokines on stimulus and establish the ability to regulate immune function. Remarkably, in some disease models, pro-inflammatory immune responses are modulated by NKT cells (Stock & Akbari, 2008). NKT
cells are also known to induce functionally distinct immune responses depending on the conditions of activation (Wang et al., 2008). The previous studies shows that CD4− NKT cells from human peripheral blood are Th1-biased, whereas the CD4+ subset produced both Th1 and Th2 cytokines. Thus, the CD4+ and CD4− NKT-cell subsets may be accountable for different after effects. It has been established that T and NKT cells play a vital role in immunity against TB (Apostolou et al., 1999). Increased NKT cell counts indicate the severity of pulmonary tuberculosis whereas decreased counts imply wrong diagnosis of the disease (Zahran et al., 2006).

<table>
<thead>
<tr>
<th>Function</th>
<th>Th1</th>
<th>Th2</th>
</tr>
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<tbody>
<tr>
<td>Type of immune response</td>
<td>Cell-Mediated</td>
<td>Humoral</td>
</tr>
<tr>
<td>Type of cytokines</td>
<td>Pro-inflammatory</td>
<td>Anti-inflammatary</td>
</tr>
<tr>
<td>Action</td>
<td>Protection against intracellular pathogens (MTB, virus, etc)</td>
<td>Extracellular pathogens (Bacteria, Protozoa etc)</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IFN-γ, TNF-α, IL-2, IL-12</td>
<td>IL-4, IL-5, IL-6, IL-10</td>
</tr>
</tbody>
</table>

Table 1. Table featuring the major attributes of the immune profiles of Th1 and Th2 cells
Immune mediators in Tuberculosis

Interleukin 2

Interleukin 2 (IL-2) and its receptor (IL-2R) were the first cytokine and cytokine receptor to be cloned. The first function attributed to IL-2 was a potent capacity to enhance in vitro T-cell proliferation and differentiation. IL-2 was therefore originally named T-cell growth factor (TCGF). Bachmann & Oxenius (2007) showed that IL-2 also allows for the differentiation of naive T cells into effector and memory cells.

IL-2 seems to have strong effect on bacterial cell counts after BCG infection in mice. The decrease in viable bacteria counts indicates that IL-2 affects the replication of organisms not only the degradation of dead organisms. There might be several pathways for IL-2 to perform its action. It is reported that IL-2 ceases the replication of MTB in human macrophages (Jeevan & Asherson, 1988).

IL-2 may also act indirectly through a T cell on macrophages via by inducing the release of IFN-γ from activated lymphocytes, as it is known that IFN-γ accelerates elimination of mycobacteria by macrophages. IL-2 may also enhance an effective immune response by inactivating suppressor cells (Jeevan & Asherson, 1988).

IL-2 can constrain pro-inflammatory IL-17 production and exert its immunosuppressive function by stimulating the generation and homeostasis of regulatory T cells (T-reg). Indeed, IL-2 is a non-redundant factor for the in vivo homeostasis of T-reg, which constitute a fundamental part of immunological self-tolerance and immune regulation. (Bachmann & Oxenius, 2007).
Interferon-\(\gamma\)

IFN-\(\gamma\) can be secreted by Th1 cells, Tc cells and NK cells (Emoto et al., 1999, Demangel et al., 1999). Also known as immune interferon, IFN-\(\gamma\) is the only Type II interferon. It is serologically distinct from Type I interferons as it is acid-labile, while the Type I variants are acid-stable. (Schroder et al., 2004)

IFN-\(\gamma\) has shown anti-viral, anti-tumour and immunoregulatory properties (Schroder et al., 2004). IFN-\(\gamma\) promotes Th1 differentiation by up-regulating the transcription factor T-bet (Andre A. Lighvani et al, 2001). IFN-\(\gamma\) is the prominent cytokine of Th1 cells.

IFN-\(\gamma\) is a crucial cytokine for the control of mycobacterial infections because IFN-\(\gamma\) plays a critical role in the stimulation of macrophages to produce nitric oxide (NO), and it is demonstrated that NO plays a pivotal protective role in MTB infection (Cooper et al, 2002) because granulomas from NOS2-deficient mice had reduced acid phosphatase activities suggesting that NO is required for macrophage activation. It is also established that absence of NOS2 affects the cytokine production of the Th1 type of immune response, ultimately leading to death (Garcia et al., 2000). It was established to be important for a vaccine to induce an early response characterized by the production of IFN-\(\gamma\) (Walravens et al., 2002).

IL-12

The importance of interleukin 12 (IL-12) for the generation of protective immune response during primary infection with mycobacteria has been well established from various murine and human epidemiological studies (reviewed by Trincheiri, 2003).

IL-12 is the primary cytokine that drives a Type 1 T helper cell immune response in MTB infections (Dong and Flavell, 2001). It promotes the differentiation of naive T cells into Th1 cells; stimulates the proliferation of T cells; and sustains the Th1 response (Park et al., 2000,
O’Shea and Paul, 2002). This allows the formation of a stronger effector T cell response and long term protection against TB (Stobie et al., 2000). IL-12 also stimulates the growth of activated CD8 T cells and NK cells, both of which contribute towards protection in TB infection (Serbina et al., 2001, Xing et al., 2000).

In addition, IL-12 triggers the production of important pro-inflammatory cytokines such as IFN-γ and TNF-α by activated T cells, APCs and NK cells. IL-12 conveniently suppresses IL-4 induced IgE production, thus helping to skew the immune response away from a Th2 pathway (Yoshimoto et al., 1997) which is really helpful when the host immune system is fighting intracellular pathogens such as MTB, where a strong, inflammatory Th1 response is necessary for protection (Gately et al., 1998).

**TNF-α**

Tumor Necrosis Factor-alpha (TNF-α) is an important pro-inflammatory cytokine predominantly released by macrophages, monocytes, dendritic cells, neutrophils, T cells, NK cells and B cells. TNF-α plays multiple roles in the immune and pathological responses during mycobacterial infections (Henderson et al., 1997). It works accordingly with IFN-γ to activate macrophages to inhibit growth of MTB via the induction of NOS2 expression (Saunders et al., 2005). TNF-α also greatly increases the phagocytic activity and production of pro-inflammatory cytokines such as interleukin 1 (IL-1) by macrophages (Flynn & Chan, 2001). Sustained autocrine activation of macrophages by this cytokine is not only essential to enhance the clearance of MTB in acute TB infections, but also to prevent reactivation of latent disease (Mohan et al., 2001; Scanga et al., 1999).
TNF-α also influences T cell responses in a number of ways (Watts, 2005). It plays an important role in initiating and sustaining T cell responses, and therefore promotes long lived immunity against MTB (Croft, 2003). Other roles of TNF-α in MTB infections include inducing T cell colony formation, enhancing cytotoxic T cell and NK cell activity, and inducing apoptosis in mature T cells.

1.4 Current Vaccination strategies

The only commercially available vaccine for TB is Bacille Calmette Guérin, a live attenuated strain of *M. bovis*. Unfortunately, the effectiveness of BCG is inconsistent. BCG vaccination can effectively prevent forms of childhood TB involving dissemination (Rodrigues et al., 1993), probably due to its ability to control blood-borne bacilli (Weigeshaus et al., 1989). However, it is estimated that effectiveness of the BCG vaccine in adults is approximately 50% (Colditz et al., 1994). The effectiveness varies substantially between zero to 80% protection. Additionally, in some studies (Sterne et al., 1998; Comstock, 1994) the effectiveness of BCG wanes over time, whereas in others similar levels of protection have been observed up to 60 years of vaccination (Aronson et al., 2004).

There are several possible explanations for the variation in effectiveness between BCG vaccinated populations. There are multiple substrains of BCG in use, due to passaging in different laboratories, which have diverged genetically and immunologically from one another (Oettinger et al., 1999). Different BCG substrains can confer different levels of protection against TB (Gheorgiu & Labrange, 1983). Variation may also be a result of host genetics, with genetic elements prevalent in different populations contributing to the variable protection afforded by BCG. Moreover, the virulence of different strains of MTB can vary
and the virulence of the strain of MTB endemic to a population could affect the effectiveness of the BCG vaccine.

Finally, there may be environmental factors that affect the effectiveness of the BCG vaccine. Populations that exhibit low BCG effectiveness are commonly in developing countries, which have greater exposure to helminth infections and environmental mycobacteria than populations in developed countries (Elias et al, 2006). Helminth infection at the time of BCG vaccination is hypothesized to result in either immunosuppression of the BCG immune response or skew the response to an unprotective Th2 profile. Similar hypotheses have been suggested for the interaction between environmental mycobacteria and BCG vaccination (Rook et al., 2005).

1.5 Environmental mycobacteria and their influence on BCG vaccine

Environmental mycobacteria are omnipresent inhabitants of the environment (Primm et al., 2004). They can be found in a wide variety of environmental sources such as water, soil, air, and food sources, and lead life as saprophytes, commensals or symbionts (Yoder et al., 1999). Environmental mycobacteria are not like other members of MTB complex, as they are not regarded as obligate pathogens (Herdman & Steele, 2004).

Humans generally tend to expose to these organisms from environmental sources. Water is considered as the primary source of mycobacterial infection in humans (Vaerewijck et al., 2005), and this infection happens mainly by aerosols produced during the daily activities such as drinking, bathing and swimming. Poor hygiene practises are also a common way of getting infection via food and soil contaminated with environmental mycobacteria (Reed et al, 2006).
The *Mycobacterium avium* complex (MAC) comprises a heterogeneous group of closely related acid fast organisms that includes *M. avium*, *Mycobacterium intracellulare*, *M. avium* subspecies paratuberculosis (MAP), *M. avium* subspecies silvaticum (the 'wood pigeon' bacillus) and *Mycobacterium lepraemurium*. Subspecies of *M. avium* are known to harbour multiple copies of a number of different genomic insertion elements. Those characterised to date include IS900 IS901/902, IS1110, IS1245, IS1311 (GenBank accession number U16276), IS1626 and IS1612. Most of these insertion elements are common to strains of *M. avium* that exist ubiquitously in the environment. They rarely cause disease in immunocompetent hosts (Lauchumroonvorapong et al, 1996). However, unique possession of IS900 or IS901 by *M. avium paratuberculosis* and certain strains of *M. avium* respectively, divides the species into at least two clearly distinct biological subtypes which differ in terms of their host range and pathogenicity (Kunze et al, 1992). In the absence of any reports confirming the replication of either *M. avium paratuberculosis* or IS901+ *M. avium* in the environment, it is generally assumed that these organisms exist as obligate pathogens. Exposure, usually by ingestion, can result in infection of the intestine leading to chronic inflammatory enteritis in ruminants and various monogastric species including primates. Given the very close genetic relationship between the different subspecies of *M. avium* (up to 98% genomic homology), the genetic basis for these biological differences is unclear. However, the observation that IS901 in *M. avium* concurs with increased pathogenicity for experimentally infected mice is supported by several reports of insertion element-mediated phenotypic change in other bacterial species (Pseudomonas, Streptococcus, Mycobacterium, Legionella, Escherichia and Yersinia) (Inglis et al., 2006).

There are currently two main hypotheses regarding the effects of cross-sensitisation on the variable efficacy of BCG Vaccination. A famous large scale study involving guinea pigs...
(Palmer & Long., 1966) suggested that prior exposure to environmental mycobacteria may lead to natural immunity to other strains of mycobacteria. This may be beneficial to the host but it also limits the protection that BCG can offer, which may explain its variable efficacy in different places of the world. However, this degree of natural immunity is obviously insufficient to protect against virulent MTB infection, as evidenced by high disease burdens in places where there is also widespread sensitisation to environmental mycobacteria, such as South Africa and India (Black et al., 2002 & 2001).

Another widely acknowledged hypothesis is that prior exposure to environmental mycobacteria can prime the host’s immune system against shared mycobacterial antigens (Brandt et al. 2002). This subsequently interferes with the immune response elicited towards BCG after vaccination, ultimately leading to its failure as a vaccine. This was demonstrated experimentally in mouse studies by Brandt et al., (2002) which revealed that certain \textit{M. avium} strains from Malawi, a region where BCG does not confer protection, affected \textit{in vivo} BCG multiplication in mice. Their study revealed that prior exposure to live environmental mycobacteria can result in a wide range of immune response that is recalled immediately after BCG vaccination and controls the replication of the vaccine. In their experiments involving sensitized mice, BCG elicited only a transient immune response with a marginal frequency of mycobacterium-specific cells and almost no protective immunity against TB. In contrast, the efficacy of TB subunit vaccines was unaffected by prior exposure to environmental mycobacteria (Brandt et al., 2002).

Human epidemiological evidence supporting this hypothesis is also available in the form of several large scale vaccination trials, in which BCG failed to provide any protection to populations in areas with widespread environmental sensitization (such as Egypt, India and
Africa) as compared to places with minimal exposure to environmental mycobacteria (such as Denmark) (Anderson & Doherthy, 2005).

From these observations, the potential of environmental mycobacteria (in particular the *M. avium* species) to interfere with the efficacy of BCG is clearly evident from many human and livestock vaccination programs, as well as laboratory experimental trials. However, the immunological mechanisms underlying these findings have yet to be completely elucidated. A much better understanding of how certain environmental mycobacterial strains affects the human immune system is thus needed in order to uncover the reasons behind the widespread failure of BCG in humans. This information will also be extremely valuable for the development of new or improved vaccines against TB that will be effective in areas of the world where BCG has failed.

**M. avium WAg 206**

The study described in this thesis utilises a unique strain of environmental mycobacteria *M. avium* strain WAg 206, a IS901 positive strain, isolated from tuberculosis lesions in farmed cattle by researchers at AgResearch, Wallaceville, New Zealand (de Lisle et al., 2005). WAg 206 affected the performance of BCG to induce a protective immune response in vivo in different animal models, including cattle, guinea pigs, and mice (Buddle et al., 2002, de Lisle et al., 2005). Recent studies have shown that following oral infection, *M. avium* WAg 206 strain had the capacity to invade and persist much longer within the gastrointestinal tract lymphatic tissues of guinea pigs (de Leslie et al., 2005) and mice (Young et al., 2007) and induce a strong Th2 immune response.
1.6 New Vaccination strategies for MTB

Since the BCG fails to consistently protect against TB, for the reasons described above significant effort has been directed towards developing new vaccine strategies against TB testing them in situations where BCG does not work.

Such novel vaccines may include:

Virus like particles coupled with the mycobacterial antigen 85 (VLP-Ag85A) and recombinant modified vaccinia virus Ankara expressing antigen 85A (MVA85A).

**Rabbit haemorrhagic disease virus as vaccine carriers**

Virus-like particles represent a specific class of subunit vaccine that mimic the structure of authentic virus particles. They can be helpful in presenting antigenic, chemically-conjugated proteins such as Ag85A to the immune system, as VLPs are recognised readily by the immune system and are very effective as candidate vaccines (Noad & Roy, 2003).

Rabbit haemorrhagic disease virus (RHDV), also known as rabbit calicivirus (RCV), is the type species of the genus Lagovirus belonging to Caliciviridae family. The RHD virion is 30–40 nm in diameter, shows a characteristic morphology, and the capsid is composed of a major protein of 60 kDa (VP60). The 7.5 kb positive-sense ssRNA genome encodes a large precursor polyprotein which undergoes proteolytic cleavage to yield the mature protein. The C-terminal region of the polyprotein gives rise to the p60 polypeptide, which is detected by antibodies against the capsid protein. This polypeptide produced from the cloned gene in vitro, spontaneously forms VLP. The production of VLPs start with infecting insect cells using recombinant baculovirus, and culturing the cells. Post culture, the supernatants and lysates are separated and purified usually by sucrose gradient centrifugation, and examined for recombinant VLPs by SDS-PAGE and immunoblotting techniques (Gromadzka et al., 2006).
**Antigen 85 complex**

The MTB cell wall is made up of mycolic acid, arabinogalactan and peptidoglycan linked covalently. Some of the enzymes playing a vital role in biosynthesis of the mycobacterial cell wall are three dominant exported fibronectin-binding proteins, consisting of Antigen 85 complex. The Antigen 85 complex (Ag85A) from MTB consists of three well enough secreted proteins (FbpA, FbpB and FbpC2) which play a key role in the pathogenesis of tuberculosis (Kremer et al., 2002).

To determine whether Ag 85A could induce antigen-specific cellular immune response IFN-γ production was examined in immunised mice. At one month after immunization, the spleen cells from mice immunized with Ag 85A or BCG could produce IFN-γ in response to Ag 85A. IFN-γ level in response to Ag 85A was four times higher in Ag 85A-immunized than in BCG-vaccinated mice (Naito et al., 1999).

**MV85A (Modified vaccinia virus Ankara expressing antigen 85A)**

Vaccinia virus can be genetically modified to become carriers that produce recombinant proteins. Vaccinia viruses are reconstructed to express foreign genes, and are used as vectors (vaccine candidates) to deliver antigens.

MV85A is a recombinant modified vaccinia virus Ankara, that expresses the antigen 85A derived from mycobacteria. It was found to induce high levels of antigen-specific IFN-γ-secreting T cells when used alone in bacille Calmette-Guérin (BCG)-naive healthy volunteers. In these volunteers who had been vaccinated 0.5–38 years previously with BCG, substantially higher levels of antigen-specific IFN-γ-secreting T cells were induced. At 24 weeks after vaccination these levels were 5–30 times greater than in vaccines administered simply a single BCG vaccination. Therefore MVA85A could be used clinically to boost the capacity of the standard BCG vaccine. (McShane et al., 2004)
1.7 Objectives of this study

- To establish base-line responses in BCG-vaccinated mice using lymphocyte transformation and cytokine assays.

- To produce RHDV VLPs and chemically couple Ag85A peptide from *Mycobacterium bovis* to them.

- To evaluate the effects of exposure to environmental mycobacteria on BCG vaccination as well as on MVA/Ag85A and VLP/Ag85A vaccines by orally pre-sensitising mice with WAg206 prior to vaccination.
Chapter-2

MATERIALS & METHODS
2.1 Animals

Specific pathogen free (SPF) BALB/c mice were obtained from the Department of Animal Laboratory sciences, University of Otago, Dunedin. All the experiments using mice were covered by a permit from the Otago University Animal Ethics Committee (AEC No: 51/08).

2.2 Media

Culture medium comprised Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen corporation) + 5% (vol.), Foetal Calf Serum (FCS) + 0.1% (vol.), 2-Mercaptoethanol (Invitrogen corporation) (net conc. at 55mM in D-PBS) + 5% (vol.), PenStrep (Penicillin at 10000 units/ml, Streptomycin at 10,000 µg/mg) (Invitrogen corporation).

2.3 Novel Vaccines used

2.3.1 VLP-Ag85A preparation

RHDV VLP preparation was initiated by infecting Sf-21 cells (Spodoptera frugiperda IPLB-sf21) (400 ml; 10^6 cells/ml) with recombinant baculovirus containing the VP60 gene in medium supplemented with PenStrep (20 U (12µg) Pencillin / 20 µg Streptomycin). An MOI of 1 was used. The cell count was calculated using haemocytometer and plaque-assayed viral inoculum. The culture was incubated for 4 days at 28 °C with shaking followed by treatment with 2 ml (0.05%) TritonX-100 detergent (SIGMA, MO, USA) for 1 hour at RT. The culture was then centrifuged at 10,000g for 20min in a JA14 rotor (Beckman Coulter, CA, USA) with the supernatant being transferred to Ti70 rotor (Beckman Coulter, CA, USA). After centrifuging for 90 min at 100,000 g, the pelleted VLP was re-suspended overnight (at 4 °C in 500µl coupling PBS) (0.26% NaH₂PO₄.2H₂O + 1.18% Na₂PO₄ + 0.87% NaCl in distilled water, pH at 7.2) after discarding the supernatant.
Residual debris was removed from the re-suspended pellets by centrifuging them at 10,000g for 20 minutes, and then the supernatant were collected and loaded onto 3 ml of caesium chloride (CsCl at 1.2g/cm³) and then further overlayed with 3ml of CsCl at 1.4 g/cm³ in SW32.1 tubes. The tubes were then topped up with insect PBS and then subjected to centrifugation at 100,000g at 4.0°C for 18 hours using a SW 32 Ti rotor (Beckman Coulter).

The translucent band produced on the gradient was harvested using a Pasteur pipette over a light box. SDS-PAGE was used to confirm the presence of RHDV VLP, and the sample was dialysed for 2 hours twice using Slide-A-Lyzer dialysis cassette (Pierce technologies, Aalst, Belgium), and then finally overnight. The VLP production for this work was carried out by Stephanie Win with help from Ms. Vivian Young, Dept of Microbiology, University of Otago.

Sulfo-SMCC (No.22322, Pierce, Illinois) are often used to prepare antibody-enzyme and hapten-carrier protein conjugates in a two step reaction scheme. First, the amine-containing protein is reacted with a several fold molar excess of cross linker followed by removal of excess (non reacted) reagent by desalting or dialysis and, finally, the sulfhydryl-containing molecule is added to react with the melamide groups already attached to the first protein.

The procedure carried out for this project was as follows.

Vaccine of volume 1.7 mg was prepared (1.5mg for inoculation (in vivo)) by chemical conjugation technique.

SMCC (Sulfo succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate) was taken at 20 times molar excess of 1ml of RHDV VLP at 5.5mg/ml (9.17 x 10⁻⁸ moles). So the molarity of Sulfo-SMCC (436.37 g/mol) was approximately 1.8 X 10⁻⁶ moles. When converted to weight, it was about 0.8mg of SMCC. 10µl of Dimethyl formamide (DMF) was added to 0.8mg of sulfo-SMCC.
0.8mg of Sulfo-SMCC was added to 1ml of VLP (conc. at 5.5mg/ml) and incubated at room temperature with shaking (rotation) for 30 minutes (total volume 1.5 ml).

Two hundred times the sample volume (300ml) of PBS (Phosphate buffered solution with NaCl) (0.26% NaH$_2$PO$_4$.2H$_2$O + 1.18% Na$_2$HPO$_4$ + 0.87% NaCl in Distilled water, PH 7.2) was prepared and used to dialyse the sample. The sample was dialysed twice for 2 hours, and then a third time overnight.

Sulfo-SMCC-VP60 was harvested from Slide-A-Lyzer dialysis cassette (Pierce technologies, Aalst, Belgium) using a syringe, and 5mg of Sulfo-SMCC-VP60 was retained after dialysis.

Ag85A CD4 (Auspep) peptide molecule was used as the antigen to couple to the VLP. To couple Ag85A (2648 g/mol) with Sulfo-SMCC-VP60 Ag85A peptide was prepared at 10 times molar excess of Sulfo-SMCC-VP60, therefore 8.3 X 10$^{-7}$ moles of Ag85A (2.2mg peptide) was added to Sulfo-SMCC-VP60. This was incubated at room temperature for 1 hour. The sample was again dialysed twice for 2 hours, and then third time overnight.

VP60-Sulfo-SMCC-Ag85A was again harvested from Slide-A-Lyzer dialysis cassette (Pierce technologies, Aalst, Belgium) and SDS-PAGE was performed to confirm the coupling.

**SDS-PAGE (Polyacrylamide gel electrophoresis)**

All samples (VP60, VLP/Ag85A, BSA) were diluted in equal volume of 2 X SDS-PAGE sample buffer and boiled for 5 minutes prior to loading on 10% acrylamide gels. For a Coomassie stained gel Broad Range ladder (New England Biolabs, Ipswich, UK) was used. The gel was run at 170 V until the dye front had reached the end of gel in a gel tank (Biorad, CA, USA). The gels were then removed and stained with Coomassie blue for 30 minutes and then placed in destain (10% Methanol + 10% Acetic acid in distilled water) with tissue paper to remove all unwanted stain. Protein bands were captured using a ChemiDoc system (Biorad).
2.3.2 MVA/AG85A

The MVA/AG85A vaccine used in this study was obtained as a gift from Oxford University (Dr Sarah Gilbert).

2.4 Vaccination schedule

The mice were divided into 6 groups based on the vaccination and are tabulated as follows:

TABLE 2. Vaccination strategy in different mice groups used in the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Treatment</th>
<th>Quantity (per mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stage-1 (Preliminary experiments)</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>PBS</td>
<td>100 µl subcutaneous</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>BCG</td>
<td>$10^6$ CFU subcutaneous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stage-2 (Pre-sensitisation experiments)</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>PBS</td>
<td>100 µl subcutaneous</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>BCG</td>
<td>$10^6$ CFU subcutaneous</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>BCG + WAg206</td>
<td>$10^9$ CFU orally (WAg206)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ BCG ($10^6$ CFU subcutaneous)</td>
</tr>
<tr>
<td>Group</td>
<td>Number</td>
<td>Treatment</td>
<td>Quantity (per mouse)</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>----------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>PBS</td>
<td>100 µl subcutaneous</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>BCG</td>
<td>$10^6$ CFU subcutaneous</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>BCG + WAg206</td>
<td>$10^6$ CFU orally (WAg206)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ BCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(10^6 CFU subcutaneous)</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>VLP + WAg206</td>
<td>$10^6$ CFU orally (WAg206)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ VP60</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(100 µl @ 1 mg/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(subcutaneous)</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>VLP/Ag85A + WAg206</td>
<td>$10^6$ CFU orally (WAg206)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+ VLP/Ag85</td>
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<td></td>
<td></td>
<td>(100 µl @ 1 mg/ml)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(subcutaneous)</td>
</tr>
<tr>
<td>Group</td>
<td>Number</td>
<td>Treatment</td>
<td>Quantity (per mouse)</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>----------------------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>MVA/Ag85A + WAg206</td>
<td>$10^6$ CFU orally (WAg206) +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MVA/Ag85A (5 x $10^7$ pfu into ear)</td>
</tr>
</tbody>
</table>

All the *M. Avium* WAg206 pre-sensitisation mice groups were orally given $10^6$ CFU WAg206 (diluted in 7H9 media). The mice were administered with respective vaccines eight weeks after pre-sensitisation (Week 8).

Four weeks after immunisation (Week 12) in stage-3 (Final experiment), Groups 4, 5 and 6 were boosted (same quantity as before) with either VP60 or VLP/Ag85 or MVA/Ag85A accordingly.

Four weeks later (Week 16), the mice were assessed for immunological responses to mycobacterial antigens.

**2.5 Splenocyte suspension preparation**

The whole spleen was isolated from the euthanized mice (cervical dislocation). The spleen was then transferred on to a 10ml Petri dish containing 5ml media and macerated using 5ml syringe rubber. Clumps were removed using cell strainer under a sterile environment. The cell suspension was increased to a volume of 10ml by topping up with media (DMEM + 5% FCS). The cell suspension was centrifuged at 300x g and supernatant was removed. The cells were re-suspended with media in 20ml FALCON tubes (BD, USA). The cells were counted.
using Coulter-Counter, and then the cell suspension was diluted to a concentration of $2 \times 10^6$ cells/ml using media.

2.6 Splenocyte culture for cytokine data

Twenty four well flat bottom plates (Nunc A/S, Roskilde, Denmark) were used to culture splenocytes for measuring the concentrations of cytokine produced by the cells with different antigens used in our study. Within the same plates, cells were distributed evenly across wells using pipettes for different antigens and controls: media only (negative control), ConA (positive control)(2.5 µg/ml), PPD-b (33 µg/ml), PPD-a (33 µg/ml) and Ag85A (20 µg/ml). One millilitre of cell suspension was added to each well, and 500µl of antigen was added to respective wells, making the total volume to 1.5ml

Two different plates were prepared for each group of mice to examine two different cytokines: IL-2 at 24 hour time point, and IL-5 and IFN-γ at 72 hours.

The plates were then cultured in an incubator at 37°C, 10% CO₂. Supernatants (1.2ml approximately) were removed at appropriate time-points and carefully transferred in to sterile eppendorf tubes. These were spun at 800 x g for 5 minutes in order to remove debris, then transferred to new eppendorf tubes, and frozen (at -80°C) until the time of analysis.

2.7 Splenocyte proliferation assay

Ninety six well round bottom plates (Nunc A/S, Roskilde, Denmark) were used to carry out the proliferation assay of splenocytes. The antigens (50µl volume) were added to the wells. Cell suspension was added at concentration of $2 \times 10^6$ cells/ml and the plates were incubated at 37°C, 10% CO₂ (Sanyo CO₂ incubator Model: 17AIC) for 72 hours. The plates were taken out to add 1 µCi of [methyl-3H]thymidine to each well diluted in 50 µl media. The cells
were then incubated overnight at 37 °C, 10% CO₂ before harvesting the cells with a *Tomtec Harvester™ 96* (Model: Mach III M). The cells were harvested onto a filter mat printed with grids, and the filter mat was dried using a microwave oven for 150 seconds.

These were placed in plastic bags, with 5ml liquid scintillant (BetaPlate Scint™, Wallac) added on to filter mat just before sealing. These were transferred onto cassettes of the liquid scintillation counter (1450 Microbeta Plus Scintillation Counter™, Wallac), and counted. The results were obtained on the computer using the software Microbeta windows workstation.

### 2.8 IFN-γ/IL-2/IL-5 ELISA of splenocyte culture supernatants

Nunc-Immuno™ 96 Microwell™ plates (Nunc A/S, Roskilde, Denmark) were incubated overnight at 4 °C with 100 µl of anti-mouse cytokine capture antibody (BD Biosciences Pharmingen) diluted 1:250 in ELISA Coating buffer (0.1M NaHCO₃). Wells were washed 4-6 times with ELISA wash buffer (PBS + 0.05% Tween-20) and incubated with 200µl of blocking buffer (PBS + 1% Bovine serum albumin) for 2 hours at room temperature to block non-specific protein binding. After blocking, the plates were washed 6 times with ELISA Wash buffer. Then 100 µl of appropriate samples (thawed supernatants) and doubling dilutions of the appropriate recombinant cytokine standard (BD Biosciences, Pharmingen) was added. Plates were sealed and incubated at 4 °C overnight, then washed 6 times with wash buffer. Plates were incubated for 1hour at room temperature with 100µl of biotinylated anti-mouse cytokine detection antibody (BD Biosciences, Pharmingen) diluted 1:250 in blocking buffer. Plates were washed 6 times again in wash buffer. Streptavidin-horseradish peroxidase(HRP) conjugate enzyme(BD Biosciences Pharmingen) diluted 1:3000 in blocking buffer was added to each well and incubated for 30 minutes at RT. Plates were washed 6 times with ELISA wash buffer and 100µl of TMB substrate reagent (BD Biosciences Pharmingen) was added to develop the colour reaction.
Plates were left until a clear gradient of colour was observed across the recombinant cytokine standard dilutions, at which point 100 µl of 1N H$_2$SO$_4$ was added to stop the reaction. After stopping the reactions, the absorbance of each well was read on Microplate reader (BIORAD Model:550) at 450nm which converted absorbance values to the quantity of cytokine in pg/ml using the standard curve plotted from the recombinant cytokine standard dilutions using the software Microplate manager(ver.4.2.1, BIORAD). The duplicated samples were averaged to give a mean measurement of cytokine production for each sample.

2.9 Total serum antibody ELISA (PPD-b / Ag85A)

Blood extracted from the mice by cardiac puncture (and allowed to clot), was used to provide the serum.

The plates were coated with 50ul of antigen [PPD-b (Biocor) at 50 µg/ml and Ag85A (H – Cys - Leu - Thr - Ser - Glu - Leu - Pro - Gly - Trp - Leu - Gln - Ala - Asn - Arg - His - Val - Lys - Pro - Thr - Gly - Ser – NH$_2$) (Auspep) at 20 µg/ml] diluted in coating buffer (0.1M NaHCO3) and incubated at 4 °C overnight. Plates were washed 6 times in wash buffer and then blocked with 200ul of blocking buffer, incubated for 2 hours at room temperature. Plates were washed again with wash buffer (0.05% Tween-20 in PBS), and 100 µl aliquots of serum samples were added to wells in doubling dilutions starting at 1:20 diluted in blocking buffer, and incubated for 1 hour at room temperature.

Plates were washed again with wash buffer 6 times, and 100 µl of goat anti-mouse IgG HRP conjugate diluted 1:5000 in blocking buffer was added to each well, and the plates were incubated for 45 minutes at room temperature.

Plates were washed 6 times, and 100 µl per well of TMB substrate was added to develop the reaction. When colour developed, 100 µl of 1N H$_2$SO$_4$ was added to each well to stop the reaction.
reaction, and the plates were read on Microplate reader (BIORAD Model: 550) at 450nm and converted to Optical density values (O.D) values for the relevant amount of antibody present in the serum.

2.10 Isotyping Serum antibody ELISA (PPD-b / Ag85A)

Plates were coated with 50ul per well of antigens (PPD-b at 50ug/ml and Ag85A at 20ug/ml) diluted in coating buffer(0.1M NaHCO3) on separate plates, and doubling dilutions of standards (100ul per well), with doubling dilutions starting at 500ng/ml for IgG1, and 250ng/ml for IgG2a) were added to appropriate wells. There were four different sets of plates (IgG1-PPD-b, IgG1-Ag85A, IgG2a-PPD-b, IgG2a-Ag85A).

The plates were incubated at 4 °C overnight and washed 6 times with wash buffer. The plates were blocked with 200 µl per well of blocking buffer for 2 hours at room temperature. One hundred µl per well of serum samples diluted in blocking buffer was added to sample-containing wells, and 100ul per well of blocking buffer was added to wells containing standards in doubling dilutions starting at 1:20 dilution. The plates were incubated for 2 hours at room temperature.

Plates were washed 6 times with wash buffer, and 100ul of biotinylated anti-mouse antibodies to IgG1 or IgG2a (IgG1 antibody diluted 1:8000, and IgG2a at 1:4000 in blocking buffer) was added to wells, and the plates were incubated for 45 minutes at room temperature. The plates were washed 6 times with wash buffer, and 100ul of Streptavidin-HRP (Zymed) diluted 1:3000 in blocking buffer was added to wells, and incubated for 30 minutes at room temperature.

Plates were washed for 6 times, and developed with TMB as described previously.
2.11 Statistical Analysis

For statistical analysis paired t tests were used for comparison between two groups and One-way ANOVA was used to compare whole data sets, generated by PRISM (version 5.0) software. P Values less than 0.05 were considered significant.
Chapter 3

RESULTS
3.1 Preliminary experiments demonstrating that BCG vaccination induces immune responses to bovine-PPD and Ag85A in mice:

**Proliferation responses:**

The proliferation of splenocytes was greatest when challenged with PPD-b at 33 µg/ml and Ag85A at 20µg/ml (Figure 3.1). There was a large difference between naive mice and BCG vaccinated mice with regard to responses at both PPD-b concentrations showing that BCG vaccination elicited immune response specific to PPD-b, and T-cells had become activated. A smaller but distinct response was elicited to Ag85A. Since antigen-specific proliferation was detected in vaccinated mice it was of interest to investigate IL-2 production.

**Cytokine responses:**

**Splenocytes from BCG Vaccinated mice produce low levels of antigen-specific IL-2 compared with naive mice where IL-2 was undetectable.**

Splenocytes from naive mice produced almost no IL-2 to re-stimulation with antigen therefore low IL-2 levels produced by splenocytes from BCG vaccinated mice were considered a positive result (Figure 3.2). Based on the repeated results (results from one of three experiments are shown) IL-2 production was highest at PPD-b concentration of 33 µg/ml. The dose/response assay failed to detect differences in response to three different concentrations.

Since it was established that the BCG sensitised splenocytes could produce IL-2 in response to either PPD-b or Ag85A after 24 hours, their ability to express IFN-γ after 72 hours was investigated.
BCG Vaccinated mice produce antigen-specific IFN-γ but this is undetectable in naive mice.

Repeating the trend, naive mice did not produce IFN-γ in response to any of the antigens, whereas BCG vaccinated mice produced increasing levels of IFN-γ with increasing dose of the challenge antigens (Figure 3.3). Again, IFN-γ production was highest at PPD-b concentration of 33µg/ml. No difference was detectable at all Ag85A concentrations where very low levels of IFN-γ were produced.

Splenocytes from BCG vaccinated mice were shown to produce Th-1 type cytokines IL-2 and IFN-γ in response to PPD-b and Ag85A. It was of interest to compare this with Th2 cytokines. So, the IL-5 response, representative of a Th2 response, was investigated.

No difference exists between naive mice and BCG vaccinated with regard to IL-5 production in response to mycobacterial antigen

Splenocytes from both naive mice and BCG vaccinated mice produced very low, almost undetectable, amounts of IL-5 to all concentrations of antigen. There was no significant difference between these groups in response to any of the antigen after 72 hours (Figure 3.4).

To determine whether this weak Th2 response correlated with antibody antigen-specific IgG in serum of vaccinated mice was investigated.

Antibody responses:

No difference in serum antibody levels in response to PPD-b and Ag85 between naive mice and BCG vaccinated mice.

There was no detectable difference in the serum antibody levels in response to the antigens PPD-b and Ag85A (Figure 3.5). This correlated with the IL-5 production suggesting that there was almost no Th2 cytokine response in BCG vaccinated.
FIGURE 3.1 Comparison of proliferation of splenocytes from BCG vaccinated mice and naïve (control) mice with different antigens (PPD-b, Ag85A). Data shown is mean ± standard error, results from two mice in each group.
FIGURE 3.2  IL-2 expression (Cytokine ELISA) produced by cultured splenocytes from BCG vaccinated mice and naïve (control) mice in response to in vitro challenge with with antigens PPD-b and Ag85A at different concentrations. No cytokine was detected in the naive mice to any of the antigens. Data shown is mean ± standard error, results from two mice in each group.
FIGURE 3.3 Comparison of IFN-γ expression (Cytokine ELISA) produced by cultured splenocytes from BCG vaccinated mice in response to challenge with different antigens PPD-b, Ag85A at different concentrations. No cytokine was detected in the naive mice to any of the antigens. Data shown is mean ± standard error, results from two mice in each group.
FIGURE 3.4 Comparison of IL-5 expression produced by cultured splenocytes from BCG vaccinated mice versus naïve mice in response to challenge with different antigens PPD-b, Ag85A at different concentrations. Data shown is mean ± standard error, results from two mice in each group.
FIGURE 3.5 Comparison of Serum antibody levels to PPD-b obtained from BCG vaccinated mice group and naïve (control) mice.
3.2 Pre-sensitising mice by oral administration of *M. Avium* WAg206 affects the immune response to BCG in mice

**Proliferation responses:**

Splenocytes from BCG vaccinated mice re-challenged with PPD-b mycobacterial antigen *in vitro* recorded higher proliferation than BCG vaccinated mice exposed to *M. avium* WAg206 (Fig 3.6). This trend was reversed when splenocytes were challenged with PPD-a *in vitro*. No responses to Ag85A were detected either from BCG vaccinated mice or BCG vaccinated mice exposed to *M. avium* WAg206. It was of interest to determine whether these results were reflected in IL-2 production.

**Cytokine responses**

**Modulation of IL-2 production to PPD-b by oral pre-sensitisation with *M. Avium* WAg 206**

Prior exposure to *M. avium* strain WAg 206 resulted in a trend towards decreased levels of IL-2 in BCG immunised mice in response to PPD-b, but increased levels of IL-2 in response to PPD-a (Figure 3.7). In response to Ag85A, *M. avium* WAg 206 presensitised group produced (statistically significant) more IL-2, though the difference was small.

Since differences in IL-2 production were detectable in pre-sensitised mice, it was of interest to investigate whether these were reflected in IFN-γ production.
Modulation of IFN-\(\gamma\) production:

Exposure to \textit{M. avium} strain WAg 206 prior to immunisation with BCG resulted in decreased levels of IFN-\(\gamma\) following challenge with PPD-b, PPD-a and Ag85A, and increased levels of IFN-\(\gamma\) in response to PPD-a (\(P < 0.05\)) (Figure 3.8). This was in contrast to IL-2 production where pre-sensitisation with WAg206 resulted in an increase of this cytokine when the splenocytes were challenged with Ag85A. Since this result suggested that the predominant Th1 response to PPD-b and Ag85A was reduced by WAg206 pre-sensitisation, the Th2 response exemplified by IL-5 production was investigated.

Modulation of IL-5 production:

Though the pre-sensitisation with WAg206 made no difference to IL-5 production, it did result in increased responses to PPD-a and Ag85A (\(P < 0.05\)) (Figure 3.9). However the actual amounts of IL-5 produced in all the groups were very low, suggesting that there was a limited Th2 type response.

Antibody responses:

There was no difference observed between the groups with regard to total serum antibody levels specific to PPD-b (Figure 3.10(a)), all the levels being very low.

Levels of IgG1 antibody specific to PPD-b were lower in \textit{M. avium} pre-sensitised BCG vaccinated mice than in BCG vaccinated mice but this was not statistically significant. It was opposite in the case of IgG1 antibody specific to PPD-a (Figure 3.10(b)), though again the results were not statistically significant. Low levels (but statistically significant) of IgG2a antibody specific to antigens PPD-b were produced in all the groups, and showing that more antibody produced by \textit{M. avium} pre-sensitised BCG vaccinated mice when compared to BCG vaccinated mice.
FIGURE 3.6 Comparison of proliferation of splenocytes from BCG vaccinated mice, BCG vaccinated mice presensitised orally with \textit{M. avium} strain WA9206 and naïve (control) mice in response to PPD-b, PPD-a and Ag85A. ** \(p < 0.005\), compared to cells stimulated with BCG alone. Data shown is mean ± standard error, results from three mice in each group.
FIGURE 3.7 Comparison of IL-2 expression produced by cultured splenocytes from BCG vaccinated mice, BCG vaccinated mice pre-sensitised orally with *M. avium* strain WAg206 and naïve (control) mice in response to different antigens (PPD-b, PPD-a, Ag85A). *p < 0.05*, BCG vaccinated mice pre-sensitised orally with *M. avium* strain WAg206 compared to cells stimulated with BCG alone. Data shown is mean ± standard error, results from three mice in each group. This result is representative of two repeat experiments.
FIGURE 3.8 Comparison of IFN-γ expression (cytokine ELISA) produced by cultured splenocytes from BCG vaccinated mice, BCG vaccinated mice pre-sensitised orally with *M. avium* strain WAg206 and naïve (control) mice in response to different antigens (PPD-b, PPD-a, Ag85A). * p < 0.05, ** p<0.005, *** p < 0.0005, Data shown are mean ± standard error; results from three mice in each group. This result is representative of two repeat experiments.
FIGURE 3.9 Comparison of IL-5 production by cultured splenocytes from BCG vaccinated mice, BCG vaccinated mice pre-sensitised orally with *M. avium* strain WAg206 and naïve (control) mice in response to different antigens (PPD-b, PPD-a, Ag85A)(* p < 0.05). Data shown is mean ± standard error, results from three mice in each group. This result is representative of two repeat experiments.
FIGURE 3.10 (a). Comparison of Serum total-antibody levels specific to PPD-b obtained from BCG vaccinated mice, BCG vaccinated mice pre-sensitised orally with *M. avium* strain WAg206 and naïve (control) mice in response to PPD-b and PPD-a. Data shown is mean ± standard error, results from three mice in each group.

(b). Comparison between levels of PPD-b specific IgG1, IgG2a antibody response and PPD-a specific IgG1, IgG2a antibodies in sera obtained from BCG vaccinated mice, BCG vaccinated mice pre-sensitised orally with *M. avium* strain WAg206 and naïve (control) mice in response to PPD-b and PPD-a (*p < 0.05).
3.3 VLP can be coupled with Ag85A using a chemical conjugation technique

VLP (VP60) peptide was chemically conjugated with Ag85AA peptide using Sulfo-SMCC, a hetero bi-functional linker, as described in Materials and Methods. VLP conjugated with Ag85A (VLP/Ag85A) was analysed on a standard 10% SDS-PAGE gel before being stained with Coomassie blue and pictured using a Chemidoc. Figure 3.11 shows the well defined bands of VLP/Ag85A (at around 65 kDa) compared to VP60 (VLP only) peptide (around 60 kDa). This confirmation of coupling was essential to establish that this novel vaccination candidate VLP/Ag85A was ready for the test to check its efficacy as a novel vaccine candidate against TB.
Figure 3.11 Chemically conjugated VLP/Ag85AA run on a 10% SDS-PAGE gel along with VP60 (VLP only) and titrations of BSA used as positive control. A Broad range marker (New England Biolabs) was used to assign VLP/Ag85A size.
3.4 Vaccination with VLP/Ag85A generated low antigen-specific IFN-\(\gamma\) response only in mice.

**Proliferative responses:**

Surprisingly, the splenocytes from VP60 vaccinated mice had higher proliferation than VLP/Ag85A vaccinated mice under all conditions (sensitised *in vitro* with antigens PPD-b, PPD-a and Ag85A) and the responses were statistically significant (Figure 3.12).

**Cytokine responses:**

No IL-2 was detected in response to any of the antigens PPD-b, PPD-a and Ag85A with any of the vaccinated groups (naive, VP60 and VLP/Ag85A) (data not shown).

Very low levels of IFN-\(\gamma\) that were almost negligible were detected in response to the antigens PPD-b, PPD-a and Ag85A in both the vaccinated groups (VP60 and VLP/Ag85A) (Figure 3.13). Significantly more IFN-\(\gamma\) was detected to PPD-b and PPD-a in mice vaccinated with VLP/Ag85A.

As the splenocytes were shown to produce Th-1 type cytokines IL-2 and Interferon-\(\gamma\) in response to PPD-b and Ag85A, IL-5 response (Th2 response) was investigated. No IL-5 levels were detected in any of the groups.
FIGURE 3.12 Comparison of proliferation of splenocytes from VLP alone (VP60) administered mice and VLP/Ag85A vaccinated mice in response to PPD-b, PPD-a and Ag85A (** p < 0.005, VLP/Ag85A vaccinated mice compared to VLP alone (VP60) administered mice. Data shown is mean ± standard error; results from six mice in each group).
Figure 3.13 Comparison of IFN-γ produced by cultured splenocytes from VP60 (VLP only)-vaccinated mice and VLP/Ag85A-vaccinated mice in response to antigens PPD-b, PPD-a and Ag85A. ** p < 0.005, VLP/Ag85A vaccinated mice compared to VLP alone (VP60) administered mice. Data shown is mean ± standard error; results from six mice in each group.
3.5 Pre-sensitising mice with *M. avium* WAg206 has less effect on antigen-specific immune response to MVA85A than on those to VLP/Ag85A

**Proliferation responses:**

When all groups were exposed to *M. avium* strain WAg206 prior to vaccination, the splenocytes from MVA85A vaccinated mice had significantly higher proliferative response to all mycobacterial antigens PPD-b, PPD-a and Ag85A) when compared to rest of the groups (Figure 3.14).

Interestingly, splenocytes from VLP only vaccinated mice had a higher proliferative response to PPD-b than VLP/Ag85A vaccinated mice. The reverse was observed in response to Ag85A although the degree of proliferation was low.

The splenocytes from VLP/Ag85A vaccinated mice had equal or slightly higher proliferation than BCG vaccinated mice in response to PPD-a and Ag85A but a trend towards a lower response to PPD-b.

**Cytokine responses:**

**Modulation of IL-2 production:**

When all mice groups were pre-sensitised with *M. avium* WAg206, the mice vaccinated with novel TB vaccine candidates VLP/Ag85A and MVA85A unexpectedly produced less IL-2 in response to PPD-b when compared to BCG (Figure 3.15). The levels of IL-2 in all groups were, however, low.
By contrast, the levels of IL-2 to PPD-a in the novel vaccination groups were higher than in the BCG vaccinated, the levels being higher than in response to PPD-b. In addition the levels of IL-2 to Ag85A in the novel vaccination groups were higher than in BCG vaccinated mice. The MVA85A group had higher levels of IL-2 production when compared to VLP/Ag85A except in response to PPD-a.

**Modulation of IFN-γ production**

When all vaccination groups of mice were pre-sensitised with *M. avium* WAg206, the levels of IFN-γ were higher in MVA85A group than in VLP/Ag85A response to PPD-b, PPD-a and Ag-85. The VLP/Ag85AA vaccinated group produced lower levels of IFN-γ than BCG vaccinated mice except in response to PPD-a repeating the trend of IL-2.

Surprisingly the VP60 (VLP only) group (negative control for VLP/Ag85A) produced similar amounts of IFN-γ when compared to VLP/Ag85A vaccinated mice except when challenged with Ag85A (Figure 3.16).

**Modulation of IL-5 production:**

When all vaccination groups of mice were pre-sensitised with *M. avium* WAg206, only the mice immunised with VLP/Ag85AA produced IL-5 in response to Ag85A and even this resulted in low levels of this cytokine (Figure 3.17).

**Antibody responses:**

There was no significant difference in serum antibody levels to PPD-b between vaccinated groups pre-sensitised with *M. avium* WAg 206 (Fig. 3.18 (a)). The levels were low in all
groups. However VLP/Ag85AA vaccinated mice produced relatively higher amounts of antibody specific to Ag85A.

IgG1 antibody isotype production was higher when compared to IgG2a, specific to PPD-b and Ag85A.

The BCG vaccinated group of mice produced higher levels of IgG1 specific to PPD-b than any other group, and VLP/Ag85A vaccinated mice had significantly higher levels of IgG1 and IgG2a specific to Ag85A (Figure 3.18(b)).

No other group of vaccinated mice produced significant levels of IgG2a specific to PPD-b and Ag85A.
Figure 3.14  Comparison of proliferation of splenocytes (CPM) of different vaccination groups (BCG, VLP only, VLP/Ag85AA, MVA85A) pre-sensitised with *M. avium* strain WAg206 and PBS (control mice) in response to PPD-b, PPD-a and Ag85A (* p <0.05, ** p < 0.005, ***p<0.0005). Data shown is mean ± standard error, results from six mice in each group.
FIGURE 3.15 Comparison of IL-2 expression in different vaccination groups (BCG, VLP only, VLP/Ag85AA, MVA85A) pre-sensitised with *M. avium* strain WAg206 and PBS (control mice) in response to PPD-b, PPD-a and Ag85A (*p <0.05, **p < 0.005, ***p<0.0005). Data shown are mean ± standard error, results from six mice in each group.
FIGURE 3.16 Comparison of IFN-γ expression in different vaccination groups (BCG, VLP only, VLP/Ag85AA, MVA85A) pre-sensitised with *M. avium* strain WAg206 and PBS (control mice) in response to PPD-b, PPD-a and Ag85A (*p < 0.05, **p < 0.005, ***p < 0.0005). Data shown are mean ± standard error; results from six mice in each group.
Figure 3.17 Comparison of IL-5 expression in different vaccination groups (BCG, VLP only, VLP/Ag85AA, MVA85A) pre-sensitised with *M. avium* strain WAg206 and PBS (control mice) in response to PPD-b, PPD-a and Ag85A. *p < 0.05 data shown are mean ± standard error; results from six mice in each group.
a. PPD-bovine

Reciprocal of Serum dilution

* Serum dilution @ 1/20

b. IgG1 - PPD-b

Conc. in ng/ml

***

IgG1 - Ag85

Conc. in ng/ml

*

IgG2a - PPD-b

Conc. in ng/ml

*

IgG2a - Ag85

Conc. in ng/ml

*
FIGURE 3.18 (a). Comparison of total serum antibody levels specific to PPD-b and Ag85A through (1 in 20 dilution) of sera obtained from in different vaccination groups (BCG, VLP only, VLP/Ag85AA, MVA85A) pre-sensitised with *M. avium* strain WAg206 and PBS (control mice). * p < 0.05, ** p < 0.005, ***p<0.0005 data shown is mean standard error; results from six mice in each group.

(b). Comparison between levels of PPD-b specific IgG1, IgG2a antibodies and PPD-a specific IgG1, IgG2a antibodies (in ng/mL) in sera obtained from in different vaccination groups (BCG, VLP only, VLP/Ag85AA, MVA85A) pre-sensitised with *M. avium* strain WAg206 and PBS (control mice).
3.6 Summary of results

- In the preliminary experiments, splenocytes from BCG vaccinated mice had maximum proliferation in response to PPD-b at 33ug/ml, and the cytokine assay (high IFN-γ) supported this result, suggesting that BCG elicits good Th1 type immune response generally.

- When BCG vaccinated mice, pre-exposed to *M. avium* WAg206, were compared with BCG-only vaccinated mice, the antigen-specific proliferation of splenocytes was higher in the latter group to PPD-b, and the trend was opposite in response to PPD-a. The same trend was seen in cytokine assays (levels of IL-2, IFN-γ). This trend was reflected in the antigen-specific IgG1 antibody response, where levels were higher than IgG2a.

- VLP was coupled with Ag85 (by me with the help of Stephanie Win and Vivian Young) using chemical cross-linker Sulfo-SMCC. This was confirmed by SDS-PAGE as the VLP/Ag85 was at 65 KDa on the broad-range ladder and VP60 (VLP only) was seen at 60 KDa.

- Vaccination with VLP/Ag85A generated poor antigen-specific proliferative response, and the same trend was seen in cytokine assays (IFN-γ) as well. No IL-2 was recorded at all conditions. VLP-only vaccinated mice generated higher responses than VLP/Ag85A.

- In the final experiment where novel vaccination groups VLP/Ag85A and MVA85A were pre-exposed to *M. avium* WAg206, MVA85A had higher proliferative responses to all antigens than other vaccination groups. The same trend was seen in levels of IL-2 and IFN-γ (cytokine assay) except IL-2 being higher in VLP/Ag85 group in response to PPD-a. High levels of IL-5 were observed in VLP/Ag85A vaccinated mice in response to Ag85A.
• There was no significant difference in total antibody levels in response to PPD-b in any of the vaccinated groups, but VLP/Ag85 vaccinated mice had higher levels in response to Ag85.

• High levels of IgG1 antibody to were recorded in mice group vaccinated with VLP/Ag85A when compared with MVA85A vaccinated group. This suggests that VLP/Ag85A vaccinated mice group elicited a predominant Th2 type response, which is not protective. MVA85A vaccinated mice strong Th1 type of response (Protective) which seems to be a better vaccine candidate.
Chapter-4

DISCUSSION
4.1 Protective immune response conferred by BCG

BCG is an attenuated live bacterial vaccine that is capable of inducing a protective immune response against TB under normal conditions. BCG has been widely distributed worldwide for use against TB with over three billion doses having been administered. As such it is regarded as a safe vaccine and can be given at birth (Cassanova et al., 1996). The low production cost makes it an ideal, cost-effective preventative measure against TB, as compared to using antibiotics to treat disease, particularly in developing countries. BCG is the best vaccine tested to date against TB (Silva et al., 1998). It is currently given intradermally as a live, attenuated vaccine, as studies have shown that heat-killed bacteria elicit a different and ineffectual immune response (Hook et al., 1996, Duagelat et al., 1995). The immune response associated with protection that follows BCG vaccination, is a Th1 type T-cell mediated immune response (Arnaud Marchant et al, 1999).

To confirm the generation of an immune response to BCG vaccination under normal conditions existing in our laboratory, preliminary experiments were carried out. Splenocytes from BCG-vaccinated mice proliferated on re-stimulation with immunodominant antigens PPD-b and Ag85A in vitro. In addition we wished to confirm the type of immune response that was induced. As expected, BCG vaccinated mice generated good levels of IL-2 when compared to naive mice, which produced no detectable IL-2. PPD-b-specific IL-2 production was higher than Ag85A-specific IL-2 levels in BCG vaccinated mice. The same trend was repeated with antigen-specific IFN-γ levels. The high levels of IFN-γ suggest that BCG elicits strong Th1 type of immune response, as IFN-γ and IL-2 are associated with this response. Very low levels of antigen-specific IL-5, a Th2 type of cytokine, were detected. The low levels of PPD-specific antibody correlates with the results of Young et al following the administration of BCG.
4.2 Modulation of immune response by BCG following exposure to environmental mycobacteria

When BCG is efficacious it induces a Th1 immune response in the host. However the efficacy of BCG varies greatly. Previous studies show that prior exposure of animals to environmental mycobacteria *M. avium* interferes with the immune response elicited towards BCG after vaccination (Young et al, 2007), and ultimately suppresses the protective immune response leading to failure of BCG vaccine (Brandt et al., 2002).

As BCG is a live vaccine, it is particularly sensitive to pre-existing immune responses to antigens that it has in common with certain strains of environmental mycobacteria. At the antigen level, many mycobacterial species are also cross-reactive, with certain immunodominant antigens being highly conserved between strains (Harboe et al., 1979, Chapras et al., 1970).

Previous studies by Brandt et al (2001) show that animals exposed to several environmental mycobacteria raise an immune response that controls the multiplication of BCG, thereby limiting the vaccine-induced immune response before it is completely generated. These studies hypothesised that prior exposure to environmental mycobacteria can prime host’s immune system against shared mycobacterial antigens, and subsequently interferes with the immune response elicited towards BCG after vaccination. In this situation the animals made a strong Th1 immune response to the *M. avium* strains and thus when animals were given BCG post *M. avium* infection, a strong cross-reactive Th1 immune response ensued, which removed BCG before it could secrete any immunodominant antigens associated with priming a protective immune response.
Previous experiments by Young et al (2007) investigated one strain of *M. avium* WAg206 and showed that animals exposed to these environmental mycobacteria were capable of suppressing antigen-stimulated IFN-γ production in BCG-immunized mice and of inducing a de novo IgG antibody response to *M. bovis* antigens. This is indicative of a Th2 immune response, which is known to be non-protective. They used the same strain of *M. avium* that has been used in this current study.

Therefore the next sets of experiments described in this thesis were carried out to confirm that the inhibitory effect of environmental mycobacteria on the immune response elicited by BCG was reproducible. This was necessary before experiments could be performed to investigate whether a different vaccine strategy could overcome this.

Although tested at a much earlier time-point than Young et al, our results suggested that the protective Th1 immune response was lowered in BCG vaccinated mice pre-sensitised with *M. avium* prior to vaccination. This is supported by the fact that *M. avium* in particular shares a large number of similar antigens with BCG, such as Antigen 85 (Ag85) and certain heat shock proteins (Demangel et al, 2005). In this study, *M. avium* strain WAg206, a New Zealand isolate was used. The choice of this strain was based on the experiments carried out by Young et al. Their findings show that WAg206 has significantly higher inhibitory effect. WAg206 is an IS901 (Insertion element) positive strain, and this insertion element is shown to be associated with the virulence (Young et al, 2007). Studies in guinea pigs showed it affected the protection that BCG offers to live challenge.

The T-cell proliferation assay indicates that the PPD-b specific T-cell response to BCG was suppressed when the mice were pre-exposed to environmental mycobacteria. Splenocyte proliferation in response to mycobacterial antigen PPD-b was significantly higher in mice vaccinated with BCG only when compared to mice pre-sensitised to WAg 206. This trend was
also observed in T-cell responses to PPD-a. This might be because of the previously established hypothesis that prior exposure to environmental mycobacteria can prime host’s immune system against shared mycobacterial antigens which subsequently interferes with the immune response elicited towards BCG after vaccination, ultimately leading to lowered protective immune response.

The proliferative responses seen were mimicked in cytokine levels detected. The cytokine responses were typically Th1 type, and supported the results of the proliferation assay.

PPD-b specific IL-2 was lowered in mice group pre-exposed to WAg206. Interestingly in response to PPD-avium, the opposite result was seen, as IL-2 production was higher in BCG vaccinated mice pre-exposed to WAg206 than in BCG only vaccinated mice. This may be because of the ‘Original antigenic sin’ concept described in 1960 by Thomas Francis, Jr. The inclination of the body's immune system to preferentially utilize immunological memory based on a previous infection when a second slightly different version, of that foreign entity (e.g. a virus or bacterium) is encountered. This leaves the immune system "trapped" by the first response it has made to each antigen, and unable to elicit potentially more effective responses during subsequent infections. The phenomenon of original antigenic sin has been described in relation to influenza virus (Krause & Richard, 2006), dengue fever, human immunodeficiency virus (HIV), and to several other viruses.

IFN-γ production in response to mycobacterial antigens showed similar response to IL-2. The IFN-γ production was low in mice pre-exposed to WAg206 and vaccinated with BCG when re-stimulated with mycobacterial antigens PPD-b and Ag85. This contrasts with the response to PPD-avium where IFN-γ production was higher in mice pre-exposed to WAg206 than BCG-only vaccinated mice. These results are similar to those from previous work which shows that environmental mycobacteria can cross-sensitize the mice to the subsequent IFN-γ
response to BCG immunization (Demangel et al., 2005, Brandt et al., 2002). This is because environmental mycobacteria can prime the host’s immune system against shared mycobacterial antigens (Brandt et al. 2002). This subsequently interferes with the immune response elicited towards BCG after vaccination. As IFN-γ is considered as immune mediator for a Th1 immune response, these lowered IFN-γ levels to PPD-b and Ag85A can be interpreted as ability of WAg206 to inhibit the vaccine-mediated protection (Young et al., 2007).

As both IL-2 and IFN-γ were indicative of Th-1 type of immune response, to determine whether there was evidence of a Th2 response IL-5 production was investigated.

Antigen-specific IL-5 production was very weak (around 20-30 pg/ml) in response to all antigens suggesting that there was a poor to non-existent Th2 type immune response.

The total antibody levels in serum were no different in both the vaccinated mice groups when compared to naive (control) mice in response to PPD-b. So, we measured the levels of IgG subtypes specific to PPD-b and PPD-avium to determine whether there was any difference in the isotypes which in turn could be indicative of a predominant Th1 or a Th2 response. Overall, antigen-specific IgG1 isotype levels were higher than IgG2a antibody levels, confirming previous work (Young et al, 2007). Levels of IgG1 specific to PPD-b were lower in BCG vaccinated mice pre-exposed to WAg206 than in BCG-only vaccinated mice, and this trend was reversed with regard to PPD-avium specific IgG1 levels. These results suggest that antibody levels are directly related to the specific antigen (PPD-b for M. bovis and PPD-avium for M. avium) derived from particular mycobacterial species, but the cross-reactivity to these antigens might induce high level of responses when mice are sensitised to both M. avium and BCG. This is because both MTB and M. bovis (BCG) are within the genus Mycobacterium, they share a close relationship at antigen level with environmental
mycobacteria as well. This similarity in the nature of antigens shared by them induces a response with the other antigen which leads to similar but non-protective response.

Based on the results of proliferative and cytokine responses, it was concluded that exposure to environmental mycobacteria leads to inhibitory effect, and makes BCG vaccine less immunogenic. This outcome may be influenced by other factors such as dose, route of immunisation, and interval between pre-sensitisation with *M. avium* and BCG vaccination. Though a dose of $10^6$ CFU WAg206 used in this study was generally used in previous studies by Young et al and others, a more prominent response might be generated by higher doses, or the use of a more virulent strain (Howard et al., 2002). Brandt et al (2002) used a dose of $2 \times 10^6$ CFU of environmental mycobacteria to pre-sensitise. They showed no statistically significant protection against TB when BCG was given. Studies in cattle suggest that animals that had naturally acquired a higher level of response to *M. avium* elicited a comparatively poor response to BCG vaccination and a lower level of immunity compared to animals with a lower level of naturally acquired response to *M. avium* tested on previous occasions (Howard et al., 2002). In addition, the route of exposure might influence the response: natural oral or respiratory infection may elicit a varied immune response compared to subcutaneous exposure and have altered outcomes for vaccination (Howard et al., 2002).

### 4.3 When pre-exposed to environmental mycobacteria, MVA85A proves to be a better vaccine candidate than VLP/Ag85A

The main challenge now is to create vaccines that overcome the inhibitory effect created by environmental mycobacteria, and offers a protective immune response even when the host is pre-exposed to environmental mycobacteria.

The two main categories of TB candidate vaccines that are being trialled are live mycobacterial vaccines and subunit vaccines (Reviewed by Anderson & Doherty, 2005).
Live vaccines are developed by attenuating virulent organisms, or by genetically modifying microbes in various ways. This includes adding back deleted genes or increasing the expression of immunodominant genes. These recombinant vaccines may prime the immune system more efficiently than BCG, thereby generating a better and more sustained immunity against TB. One such vaccine is Modified Vaccinia virus Ankara expressing Ag85A (MVA85A). This vaccine is showing promise as a candidate for BCG because it produces higher levels of long-lasting cellular immunity when used together with the conventional BCG (McShane et al., 2004).

Previous work done by Ibanga et al (2006) demonstrated that MVA85A was safe and this vaccine was shown to induce protective and strong cellular immune responses in humans. Therefore it was of interest to determine whether the immune response to this live vaccine was adversely affected after pre-exposure to *M. avium* WAg206. Responses to this vaccine were compared with those to VLP/Ag85A, again following pre-exposure to Wag206, because VLP/Ag85A is an inert vaccine. The use of an effective inert vaccine may avoid public resistance to the use of genetically modified virus for immunisation.

VLP based vaccines normally induce antibodies and neutralize disease-related proteins, but they can also induce cell-mediated responses necessary to offer an effective means to treat mycobacterial diseases (Jennings & Bachmann, 2009).

On the basis of this information the efficacy of two novel vaccines VLP/Ag85A and MVA85A was compared with BCG following pre-exposure to environmental mycobacteria *M. avium* WAg 206.

WAg206 were administered to all groups prior to immunisation and then animals were vaccinated with different vaccines. MVA85A vaccine proved to be more efficacious than novel vaccine VLP/Ag85A vaccine with respect to generation of a Th1 immune response.
Splenocytes from MVA85A vaccinated mice had higher proliferative response than any other vaccinated mice in response to all the antigens (PPD-b, PPD-avium, Ag85). VLP/Ag85 did not induce such high levels of proliferation as seen with MVA85, but was slightly better than BCG in terms of proliferative response. The response of MVA and VLP vaccinated mice to PPD a and b may be as a result of *M. avium* infection (cross reactivity).

Antigen-specific IL-2 responses were unexpectedly different from one antigen to another. In response to PPD-b, BCG produced slightly higher levels of IL-2 than novel vaccines, although the levels of IL-2 were low in all groups but, again, in response to PPD-avium and Ag85, IL-2 production was higher in novel vaccination groups (VLP/Ag85 and MVA85A) than BCG vaccinated mice. This might be due to specificity of PPD-b to BCG (*M. bovis*) as PPD-b antigen is purified protein derivative of *M. bovis* (BCG). In contrast, the novel vaccine groups contain the antigen Ag85A so it is likely that they may elicit a better immune response to Ag85A. These are the reasons most likely to effect to cause variation in cytokine response among vaccine groups.

Antigen-specific IFN-γ levels were higher in MVA85A mice than any other vaccinated mice, suggesting a strong Th1 type of immune response on MVA85A vaccination. The point to note here is IFN-γ levels in response to PPD-b in VLP/Ag85 vaccinated mice was lower than BCG vaccinated mice, giving a hint that VLP/Ag85 vaccination might not overcome the Th2 type response produced to *M. avium*.

IL-5 levels specific to Ag85 were much higher in VLP/Ag85 group suggesting the Th2 type of immune response to VLP/Ag85 vaccination. There were no significant levels of IL-5 produced that were specific to any of the antigens (PPD-b, PPD-avium, Ag85) in either BCG vaccinated mice or MVA85A vaccinated mice, suggesting that these two groups elicit protective Th1 immune response.
Though there was no significant difference in the levels of serum total antibody levels specific to PPD-b between different vaccinated mice groups pre-exposed to WAg206 before vaccination, VLP/Ag85 vaccinated mice produced significantly much higher levels of serum total antibody specific to Ag85 than any other group. This further supports that VLP/Ag85 vaccinated mice might have shifted to Th2 type of immune response, which is antibody mediated.

Overall, IgG1 isotype levels were much higher than IgG2a isotype levels, and VLP/Ag85 vaccinated mice pre-exposed to WAg206 produced significantly higher levels of IgG1 specific to Ag85 than other groups pre-exposed to WAg206. As IgG1 isotype of antibodies are indicative of Th2 type of immune response, this result further gives the strength to the concept that VLP/Ag85 vaccination might have elicited Th2 type of immune response, instead of protective Th1 immune response. The predominantly Th2 immune response (high levels of Ag-85 specific IL-5; high level of serum total antibody specific to Ag85; high levels of IgG1 specific to Ag85) might be because VLP generates higher B-cell mediated immune response (Bachman et al., 1993). A contributing factor may be that no adjuvant is used in construction of VLP/Ag85 vaccine. Some adjuvants are known to boost Th1 immune response and increase the protective immunity (Peacey et al., 2007; Storni et al, 2004; Young et al., 2006). It may be possible to influence the response to VLP/Ag85 by the selection of one of these.

Based on results MVA85A vaccine was shown to be a better vaccine candidate than VLP/Ag85 as it elicited strong Th1 response on vaccination (high proliferative response and higher IFN-γ production), and this type of cell-mediated immune response is necessary to develop immunity against TB. This confirms previous work that has established that MVA85A is an effective vaccine candidate, (McShane et al, 2004).
The definitive test of protection against TB is actually challenging the vaccinated animals with optimal doses of virulent MTB. Trials in which WAg206-treated, MVA/Ag85 or VLP/Ag85-immunised mice were challenged with live MTB would give further insight into the value of these vaccines in overcoming the problem of inhibitory environmental mycobacteria. These trials are underway.

It should be mentioned that the mouse model used in the experiments described in this thesis has its own disadvantages. Results obtained using this model may not be extrapolated directly to humans, as the process of granuloma formation in mice after TB infection is different to that seen in humans and other naturally susceptible hosts (e.g., guinea pigs). Mice are innately resistant to tuberculosis and the pathogenic effects of MTB do not occur in these animals unless they are immunodeficient. They generate strong protective cellular responses against TB (Reviewed by Gupta & Katoch, 2009). Despite the differences the mouse model is the most widely used model of Tb infection. Mice are easily housed and their genome is well characterised. There are also a wide variety of immunological tools, such as murine-directed antibodies and gene-deficient strains of mice, which can be utilised when working with murine models. Subcutaneous BCG vaccination of mice results in Th1 immune response against mycobacterial antigens. After an aerosol MTB challenge, a $\approx 1 \log_{10}$ decrease in lung bacterial numbers is observed in BCG-vaccinated mice compared to naive controls. This $\approx 1 \log_{10}$ decrease is regarded as the gold standard level of protection in pre-clinical vaccine trials, against which novel vaccines are easily compared. Other animal models such as guinea pigs that show pathology typical of MTB in humans may provide good vaccine candidates once the proof of principle is established in mice (Reviewed by Gupta & Katoch, 2009). The limitations to the use of guinea pigs early in investigations of potential vaccine candidates are the poor availability of reagents to assess immunologic factors involved in protection in
vaccine studies coupled with a limited range of inbred animals enabling repetition of experiments against a constant genetic background.

### 4.4 Future directions

Through this research we have been able to conclude that MVA85A is a successful vaccine candidate that may overcome the inhibitory effect of environmental mycobacteria, by inducing a strong Th1 type of immune response. However, a VLP based vaccine may also be successful if an appropriate adjuvant is used in conjunction with this (Jennings and Bachmann, 2009). Various successful adjuvants being currently used in TB vaccine preparations listed by Doherty and Andersen (2005) are as follows:

- LTK63 (a modified and detoxified heat-labile toxin from E. coli tested in human volunteers as an influenza vaccine)

- Lipovac (a stable emulsion containing the cationic surfactant dimethyl dioctadecyl ammonium bromide and the synthetic mycobacterial cord factor trehalose dibehenate)

- AS2 (an oil-in-water emulsion containing 3-deacylated monophosphoryl lipid A (a detoxified form of lipid A from Salmonella enterica serovar Minnesota), and a purified fraction of Quillaria saponaria, known as Quil A)

- IC31 (A mixture of oligodeoxynucleotides and polycationic amino acids)

- Montanide (A water-oil emulsion, two variants exist, based on mineral and non-mineral oil)
• ISCOM (A formulation of *Quillaja saponins*, cholesterol, phospholipids, and protein)

• OM-174 (A modified and detoxified lipid A from *E. Coli* (diphosphate triacyl))

Some of the above mentioned adjuvants might be linked with VLP/Ag85 vaccine in future studies introducing an immunodrug that offers protective immune response against TB.

Prime-boost immunisation strategies have also been shown to bring about high levels of cellular immunity against different pathogens. Various studies have demonstrated the improved effectiveness of these strategies in animal models and the increased immunogenicity of a BCG prime-MVA85A boost strategy has been established in humans. Supplementary work on the mode of action of this effective strategy will allow optimisation of such vaccination strategies, and finally lead to development of an improved vaccine against TB (McShane and Hill, 2005). So, a combination of priming with MVA85A and boosting with VLP/Ag85A might lead to better efficacy than a single vaccination as this proved successful with other novel vaccines. Future work in this area might include strategies of large scale production of safe and cost-effective VLP based vaccines coupled with specific mycobacterial antigens that also provide protective immunity against XDR strains of MTB, that are posing a serious threat.
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