Prime-boost vaccination strategies using the *Mycobacterium tuberculosis* Apa (alanine-proline rich antigen)

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This thesis is dedicated to the memory of Dr. M. Joseph (Jo) Colston (1948-2003)

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Arminda made me a better man. To her, all my love, forever.

ABBREVIATIONS

Most used abbreviations

Ala	Alanine
AIDS	Acquired immunodeficiency syndrome
Apa	Alanine-proline rich protein
APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDC	Centres of Disease Control
cDNA	Complementary DNA
CMV	Cytomegalovirus
CTL	Cytolytic T lymphocyte
DCs	Dendritic cells
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DOTS	Directly-observed therapy short course
DTH	Delayed type hypersensitivity
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunoabsorbant assay
FACS	Fluorescence cell-activated sorter
Fc	Fragment crystallisable
FCS	Fetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GATA3	GATA binding protein 3
GFP	Green fluorescent protein
Gly	Glycine
HCl	Hydrochloric acid
His	Histidine
HLA	Human leukocyte antigen

HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HSP	Heat shock protein
Ig	Immunoglobulin
IFN-γ	Interferon-gamma
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IPTG	Isopropyl thiogalactoside
kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
KO	Knockout
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
MDR-TB	Multidrug-resistant tuberculosis
MHC	Major histocompatibility complex
MPB	*Mycobacterium bovis*-derived protein
MPT	*Mycobacterium tuberculosis*-derived protein
MPTR	Major polymorphic tandem repeat
MVA	Modified *Vaccinia* virus Ankara
NaCl	Sodium chloride
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate reduced oxidase
NaOH	Sodium hydroxide
NBT-BCIP	Nitroblue-tetrazolium and 5-bromo-4-chloro-3
NKT	Natural killer T cell
NO	Nitric oxide
NOS2	Nitric oxide synthase 2
NRP	Non-replicating persistence
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Proline-glutamic acid motif
PGRS	Polymorphic guanine+cytosine rich sequence

PMA	Phorbol 12-myristate 13-acetate
PPD	Purified protein derivative
PPE	Proline-proline-glutamic acid motif
PVDF	Polyvinylfluoride
RANTES	Regulated upon activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room temperature
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SFC	Spot-forming cells
STAT	Signal transducer and activator of transcription
TAP1	Transporter for antigen presentation 1
TB	Tuberculosis
TBE	Tris-borate EDTA
TBS	Tris-buffered saline
TCR	T cell receptor
TEMED	N,N,N',N' - tetramethylethylene diamine
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TU	Tuberculin units
WHO	World Health Organisation

CHAPTER ONE

Introduction

1.1 Historical aspects

"This is arterial blood: I cannot be deceived by its colour. It is my death warrant."

John Keats (Cook, 1995)

On a winter evening in London in 1818, the Romantic poet John Keats coughed up blood on his pillow. As a qualified doctor, he knew what it meant. In February 23, 1821, after hopeless treatments, Keats was pronounced dead.

Tuberculosis (TB) is one of the most historically important diseases. A number of characteristic and pathognomonic lesions in prehistoric skeletons and in Egyptian mummies have been attributed to it (Zink, 2003). A slowly but inexorably progressive and debilitating disease called *pthisis* (a Greek word; the lay equivalent would be "consumption") was certainly known to Hippocrates and his circle. Roman doctors described terminal tuberculosis with accuracy. The Black Death of the fifteenth century is said to have wiped out a third of Europe's population and Cholera epidemics and other diseases still killed more than tuberculosis. But TB was unlike any of these. It was a killer but it also transformed the lives of its victims; it became a formative influence in art, music and literature (Dormandy, 1999), it challenges doctors, governments and society from antiquity well into our days.

Tuberculosis was one great engine pushing the Romantic Movement in the eighteenth and nineteenth century. It seemed to single out the young, talented and famous – writers, poets and playwrighters; artists; musicians; courtesans; society beauties and scientists. The list of famous people who died from TB is long: from Renee Laënnec to Edward Trudeau; from Moliére to Chekov; Dostoyevsky; the Brontë family; Modigliani; writers like Franz Kafka, D.H. Lawrence and George Orwell (Reichman & Tanne, 2001). Although TB picked out and killed a few princes, it slaughtered the poor by the million. Overcrowding, poverty and malnutrition were all found together during the Industrial Revolution. It is not surprising that especially during this period, death toll by tuberculosis had a massive increase, particularly between the young and poor in England (Dormandy, 1999). The phenomenon is transposable, with the appropriate adjustments, to other countries. In America, TB became epidemic closely after the arrival of industrialisation. Tuberculosis spreads more easily when people are in close contact with an infected person. Men, women and children working packed against each other into poorly ventilated factories ("the dark satanic mills", as the poet William Blake used to call them), were the perfect breeding site for TB (Reichman & Tanne, 2001).

Escape from illness by moving to another place is probably as ancient as hope. In Europe, the pilgrimage of consumptives initially started to leave the northern fog for more gentle skies in Italy or the south of France. Following this, the sanatorium movement arrived, which combined the features of a hospital with those of a luxury hotel, in towns situated at high altitude. The beginning of this movement (between other reasons) could have its origins from a mistaken belief that tuberculosis was caused by a weakened heart and that reduced atmospheric pressure could help (Dormandy, 1999). As expected, sanatoriums, with its regimen of an intended healthy lifestyle, good nutrition and gradually increasing exercise (sometimes too

forceful on TB sufferers) became very fashionable on the 19th and first half of the 20th century. *The Magic Mountain* (1927), a novel written by the 1929s Nobel Prize winner Thomas Mann, described this lifestyle superbly. By 1923, there were 656 sanatoriums with 66.000 beds in the United States alone. However, the sanatoriums, with its draconian demands, had absolutely no effect in curing TB (Reichman & Tanne, 2001), although the effects of direct sunlight, more specifically ultraviolet rays, are fatal for TB bacteria (some forms of skin tuberculosis were even cured by a special lamp in the 19th century), the death rate in and out of the sanatoriums were about the same (Reichman & Tanne, 2001). In reality, perspectives of cure and prevention of this terrible disease looked distant and were yet to come.

1.2 The causative agent: *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (MTB) is a bacterium and causes tuberculosis. However this concept has only been known for 122 years. Before, tuberculosis was commonly attributed to several causes, from heredity to "dampness of the soil", but few believed it would be contagious and caused by a microbe. However the past is punctuated with clues. The Italian physician Girolamo Fracastoro postulated in 1546 that diseases such as *phtisis* were transmitted by invisible particles that could survive outside the body and still infect. Jean Antoine Villemin (1827-1892), a French army doctor performed a series of convincing and controlled experiments on the infectious nature of tuberculosis in animals. However, the medical establishment at the time could not accept such "heretic ideas" (Dormandy, 1999). It came to Robert Koch, a brilliant 38-year-old German physician, to present to a stunned audience on March 24, 1882, that through an elegant four-step procedure (the "Koch-Henle postulates"),

tuberculosis was indeed an infectious disease caused by a bacterium. He won the Nobel Prize for medicine and physiology in 1905 (Kaufmann, 2001).

The genus *Mycobacterium*, belonging to the order Actimicetales and to the Mycobacteriaceae family, consists of members of the *Mycobacterium tuberculosis* complex and more than 80 species of non-tuberculous mycobacteria, including pathogenic, opportunistic, and non-pathogenic species (Soini & Musser, 2001). *M. tuberculosis* and *M. leprae* (causing leprosy) are the human pathogens of most notable significance in this genus. In the tissues, *M. tuberculosis* are non-motile straight rods, measuring 0.4 X 3 micrometers, which do not sporulate. They are characterised by a resistance to destaining by 95% ethanol, 3% hydrochloric acid (acid-fast stain), a feature that depends on the thick and complex cell envelope, an additional layer after the peptidoglycan, rich in lipids and glycolipids. *M. tuberculosis* is a slow-growing organism (average generation time of 24h) and is considered a Gram-positive bacteria since they possess a single cytoplasmic membrane (Black, 2001). The *M. tuberculosis* complex comprises *M. tuberculosis* together with *M. africanum*, *M. bovis*, *M. canettii* and *M. microti*. The subspecies are very alike (99.9% similarity at the nucleotide level) and can only be distinguished by a limited number of phenotypic or genotypic characteristics, but differ remarkably with respect to host range and pathogenicity; whereas *M. microti* is an almost exclusive rodent pathogen, *M. bovis* infects a variety of species including humans (Brosch, 2001).

It has been said that *M. tuberculosis* has evolved from *M. bovis* (which causes bovine TB) by adaptation of the animal pathogen to the human host (Stead, 1995), approximately 10,000 years ago, when people first settled down to tend their cattle. In a comparative genomic analysis between the *M. tuberculosis* H37Rv genome (see below) with the partially sequenced *M. bovis* genome, Brosch and colleagues (2002) have proposed that *M. bovis* has undergone several deletions relative to MTB; in fact,

it is the final member of a separate lineage inside of the MTB complex that branched from a progenitor of MTB isolates (Brosch, 2002). Whether this progenitor, at the time of branching of MTB strains and *M. africanum* → *M bovis* lineage, was already a human pathogen is subject to speculation. Complete sequencing of the *M. bovis* genome has confirmed these predictions (Garnier, 2003).

The completion of the whole-genome sequence for *M. tuberculosis* H37Rv strain (Cole, 1998) is a hallmark in tuberculosis research. The annotated sequence has revealed a 4.41 megabase genome with regions of higher than average G (guanine) + C (cytosine) content. Few organisms display the extensive array of genes for lipophilic molecules identified in MTB (Cole, 1998). The genome of MTB is rather stable, single-nucleotide polymorphisms are rare; insertion and deletion events (InDels) are the principal source of genome plasticity (Brosch, 2001). However, a recent study (Fleischmann, 2002) comparing whole genomes *in silico* of laboratory strain H37Rv and clinical isolate CDC1551 has identified frequent polymorphisms and suggests that these may be not so rare between MTB strains.

The MTB genome sequencing also brought to light two large families (roughly 10% of the genome) of novel glycine-rich proteins; they were characterised by a conserved amino-terminal segment with either a proline-glutamic acid (PE) or a proline-proline-glutamic acid (PPE) motif; some were combined with a carboxy-terminal domain comprising varying numbers of short repetitive motifs (PE-PGRS or PPE-MPTR). These proteins are highly polymorphic and were suggested to be a source of antigenic diversity in the bacillus (Cole, 1998; Banu, 2002). In addition, members of this family have been implicated in the pathogenesis of *M. tuberculosis* (Camacho, 1999), *M. marinum* (Ramakrishnan, 2000) and *M. bovis* BCG (Brennan, 2001). The Gly-Ala-rich domain (PGRS) present in some PE proteins may influence

proper antigen presentation to the host and may prevent the development of an effective cellular immune response (Delogu, 2001).

1.3 The disease: notes on diagnostic, treatment and epidemiology

In the nineteenth century, health officials prohibited the act of spitting in public; they thought erroneously that it was a major cause of tuberculosis infection (Reichman & Tanne, 2001). However, virtually all *M. tuberculosis* is transmitted by airborne particles that are 1 to 5 μm in diameter (fig.1.1). Usually, only the pulmonary form of tuberculosis is transmissible. Transmission is greatly influenced by characteristics of the source case (number of bacteria excreted, etc.) and the nature of the encounter (such as the duration and closeness of exposure). However, regardless of these factors, infection can result when as few as one to five bacteria are deposited in a terminal alveolus (Small & Fujiwara, 2001).

After infection, a precarious balance is struck between the host and the pathogen. In about 5 percent of persons, the infection progresses from a latent form to active disease within two years after infection, and an additional 5 percent have active disease at some later point in their lives (Small and Fujiwara, 2001). Although less common, exogenous reinfection instead of reactivation can occur, especially in individuals who are highly exposed or HIV-infected (van Rie, 1999).

A subgroup of infected people may develop primary tuberculosis, which in most cases is a self-limited, mild pneumonic illness that generally goes undiagnosed. Briefly, at the site of the bacilli, there is an alveolitis with arrival of monocytes and polymorphonuclear cells. The tubercle is then organised with histiocytes, surrounded by epithelioid cells. These are differentiated macrophages with large endoplasmic reticulum. They fuse to form giant multinucleated Langerhans cells (Roitt, 2001). At

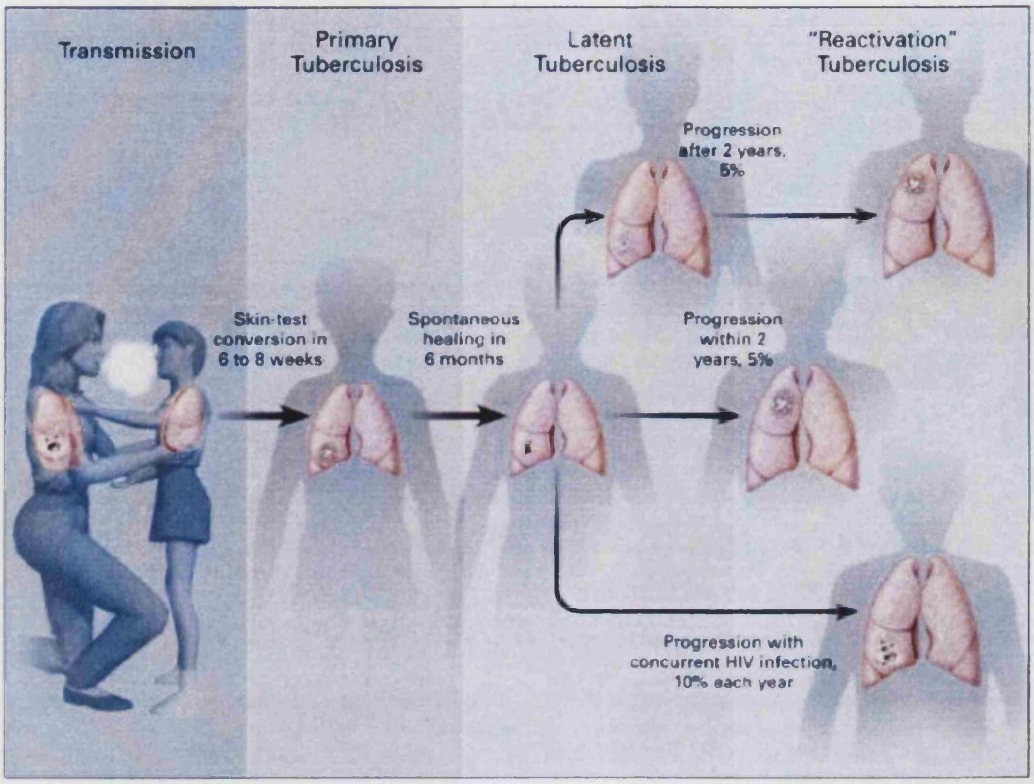


Figure 1.1 Transmission of tuberculosis and progression from latent infection to reactivated disease. Approximately 10% of infected, HIV-seronegative individuals will develop tuberculosis at some point in their lives. In five percent of people with latent infection, active disease will develop within two years, and in an additional 5%, progression to active disease will occur later. The rate of progression to active disease is dramatically increased among people co-infected with HIV. Modified from Small and Fujiwara (2001).

the periphery, there are lymphocytes and fibroblasts. The centre of the tubercle may suffer caseous necrosis (caseification). The described structure is called a tuberculous granuloma and is a hallmark of tuberculosis (Robbins, 1986). From this structure, the bacilli may be drained to the regional lymph nodes where other granulomas may also form. The complete structure is called the primary complex (Ghon's complex). Often, the infection is aborted at this stage without further significance; a calcified nodule is eventually observed in a x-ray of the area. Nonetheless, viable bacilli may persist for years (Tarantino, 2002).

Again, in a small proportion of the infected, usually children, there is progression of the primary disease. Exudative infiltration with variable degree of consolidation can be seen in the pulmonary parenchyma. If caseation is present, the progression of the disease is faster and the prognosis is worse. Miliary dissemination can occur; the term "miliary TB" refers to the small, discrete granulomatous lesions in lungs and other organs that result when blood-borne tubercle bacilli seed many tissues (Fanning, 1999). Pathology of pulmonary lymph nodes during the primary disease is a frequent finding in children. An increase in volume of the hilar-paratracheal nodes can seriously compress the bronchi and restrict airflow (Tarantino, 2002).

There are clinical and experimental evidence that *M. tuberculosis* can persist in tissues for months to decades without replicating, yet with the ability to resume growth and activate disease, a characteristic named "dormancy" or "nonreplicating persistence (NRP)" (Wayne & Sohaskey, 2001). *Post-mortem* examination of tissues from asymptomatic individuals living in high-incidence areas and that died from causes unrelated to tuberculosis frequently reveals the bacillus in culture (Young, 2002). With time to adapt, the tubercle bacilli can survive on microaerophilic or anaerobic environments, restrictive conditions found inside granulomas and necrotic

tissues *in vivo*; in these conditions of hypoxia, genes coding for regulatory functions are activated (Wayne & Sohaskey, 2001). In response to alternative energy sources, the NRP bacilli increase expression of isocitrate lyase, an enzyme from the glyoxylate cycle that allows the bacteria to use acetate or fatty acids as the sole carbon source (McKinney, 2000). Changing the cell wall architecture seems also important for the persistence of MTB *in vivo* (Seiler, 2003). In such cases of latent tuberculosis, the host mounts a strong immune response that frequently contains but does not eliminate the infection; disruption of immune mechanisms can lead to reactivation of the disease in both humans and animals (North & Jung, 2004).

Secondary tuberculosis could start with an early exudative-inflammatory process with pneumonic characteristics. The lesion, as seen in x-rays, shows 1-2 cm of diameter. With progression, classical symptoms may appear: cough, fever, and expectoration, sometimes with traces of blood; anorexia, night sweats and weight loss. Caseation and cavitation can be present in the lungs, frequently in the upper lobes. The dissemination of caseous material can spread the infection to other regions of the lung. If this process develops in an acute form it can result in caseous pneumonia with extensive consolidation areas or caseous bronchopneumonia, both being very serious forms of TB. When the early infiltrate is driven to chronicity, the process can stabilise and fibrosis develops (Tarantino, 2002). The person with the disease can live for many years, however periodic episodes of haemoptisis are not uncommon and can be life-threatening.

Besides its preference for the lung, the tubercle bacillus can ultimately infect and cause disease in any human organ (Tarantino, 2002). The extrapulmonary forms of tuberculosis are again raising in frequency nowadays, an event particularly linked to the AIDS epidemic. Extrapulmonary TB accounts for 62% of cases in HIV-infected people compared to 20% in non-HIV-infected (Fanning, 1999). The most

commonly affected extrapulmonary site is the lymph node. TB meningitis has the highest rates of mortality (Fanning, 1999).

Definitive diagnosis of mycobacterial disease usually involves the isolation and identification of the infecting organism in the laboratory. The recommended procedure for clinical specimens involves decontamination and solubilisation of the material, microscopic examination for the presence of acid-fast bacilli, isolation by culture, identification and drug susceptibility testing. However, because of the slow growth rate of the organism, these procedures can take several weeks (Hale, 2001). Recently, several molecular methods have been developed to reduce diagnostic time. Nucleic-acid amplification (NAA) with genus-specific primers based on the 16S ribosomal RNA gene (Amplicor; Roche Diagnostic Systems, Branchburg, NJ) can detect specifically *M. tuberculosis* complex bacteria directly from smear-positive clinical specimens in a few hours (Watterson & Droboniewski, 2000). Another NAA kit, the Enhanced Mycobacterium Tuberculosis Direct Test (E-MTD, Genprobe, San Diego, CA), based on isothermal amplification of reverse transcribed ribosomal RNA from mycobacteria, has been approved by the FDA to aid the diagnosis of smear-negative specimens (Soini & Musser, 2001). Culture of mycobacteria in liquid and solid media is still required because of its higher sensitivity, growth of the organism for precise species identification and drug susceptibility testing. In this regard, the use of nucleic-acid probes (AccuProbe®, Gen-Probe Inc) allows differentiation between tuberculous and non-tuberculous bacteria such as *M. avium* complex (Hale, 2001). Identification of species within the *M. tuberculosis* complex is a difficult task. High-performance liquid chromatography (HPLC), for the analysis of mycolic acid esters, allows differentiation of BCG from *M. tuberculosis* and *M. bovis* (Floyd, 1992). Although imperfect (cross-reactivity with BCG and environmental bacteria), the tuberculin skin test, an intradermal injection of purified protein derivative in the

surface of the forearm, remains the gold standard for the diagnosis of latent tuberculosis (Small & Fujiwara, 2001). A significant induration at the site, measured in millimeters, 48-72 hours after injection, when correlated with the medical history of the patient, can frequently indicate a long-standing infection.

Tuberculosis cases must be reported to public health authorities; besides treating the patient, protection of the community is essential. Five first-line antimicrobial agents (isoniazid, rifampin, pyrazinamide, ethambutol and streptomycin), form the basis of the currently recommended anti-tuberculosis therapy. The recommended protocol include initial treatment with three or four drugs for two months, followed by a four-month continuation phase in which two drugs are administered (Small & Fujiwara, 2001).

Second-line medications (capreomycin, ciprofloxacin and others) could be less effective and more toxic than first-line drugs; only patients who cannot tolerate first-line drugs or are infected with organisms that are resistant to them should use second-line drugs. HIV-infected persons with full compliance to standard treatment for tuberculosis do not have increased risk of treatment failure (Small & Fujiwara, 2001). In addition, anti-tuberculous chemoprophylaxis has been shown to reduce the incidence of tuberculosis in HIV-infected patients (Maartens, 2002). Preventive therapy of immunocompetent adults with latent tuberculosis infection has a protective efficacy in the range of 60-80% (Borgdorff, 2002).

If untreated, TB is frequently lethal. Modern chemotherapy is highly successful provided a well-designed drug regimen is taken as planned. Programs of directly observed therapy (DOT) where an individual is assigned to assure compliance of TB patients to chemotherapy have been devised. A review of ten years of trials has concluded that, if well implemented, such programs are indeed superior to unsupervised pill taking (Hill, 2002). DOTS, an acronym used since 1995,

originally stood for directly observed therapy short course, but actually, as developed by the International Union against Tuberculosis and Lung Disease (IUATLD), consists of a menu of five specific elements: a national TB control program, laboratory monitoring, directly observed therapy, a constant supply of first-line anti-mycobacterial drugs, and good recording and reporting. It represents the current World Health Organisation (WHO) TB control strategy (Elzinga, 2004).

Multidrug-resistant tuberculosis (MDR-TB) is defined as infection with strains resistant to at least isoniazide and rifampicin. Rifampicin is bactericidal to mycobacteria and the most important anti-tuberculous drug. It inhibits the synthesis and function of mycobacterial DNA-dependent RNA polymerase (Sefton, 2002). A mutation in a region of the mycobacterial *rpoB* gene that encodes the β-subunit of RNA polymerase leads to an alteration of the rifampicin-binding site and resistance (Hirano, 1999). Isoniazide resistance is most commonly associated with missense or null mutations in the *katG* gene, which codes for a catalase-peroxidase enzyme that converts isoniazide into its bioactive form (Pym, 2002). MDR-TB strains with resistance up to twelve anti-mycobacterial drugs at the same time have been found in patients from a study in Peru (Mitnick, 2003). Nonetheless, MDR-TB is curable, but therapy is complex. A community-based method to treat MDR-TB named DOTS-plus has been reported to attain high rates of treatment success (Mitnick, 2003). Second-line drugs are used and the regimen includes two or more drugs to which the isolate is susceptible, including one drug given parenterally for six months. The duration of this observed treatment is 18-24 months (Sterling, 2003).

Pathogens from the *M. tuberculosis* complex cause epidemics that run over centuries (Dye, 2002). A consensus statement by a panel of experts from the WHO estimated that in the year of 1997, there were 7.96 million new cases of TB in the world with around 2 million deaths and 1.86 billion people infected (Dye, 1999). TB

is the most frequent cause of death due to a single pathogen. It is a leading killer between people with HIV/AIDS. Comments such as "TB is arguably the most successful pathogen on the planet" (Jacobs, W. R., in Enserink, 2001) are not out of place. In the later 1980s, after decades of steady declining, TB was considered a disease of the past in first-world countries. However, the synergistic combination of a deteriorating public health infrastructure, inadequate institutional control, urban crowding, the epidemic of HIV infection, and immigration resulted in the resurgence of tuberculosis in the United States between 1985 and 1992 (Small & Fujiwara, 2001). A highly infectious MDR strain (strain W), invariably untreatable with five anti-mycobacterial drugs, was identified and disseminated rapidly in New York City, an epidemic epicenter (Bifani, 2002). In this scenario, molecular techniques, like DNA-fingerprinting, have been important to study pathways of TB transmission. The transposable element IS*6110* is the most used marker; it varies both in copy number and location inside the genome (Bifani, 2002).

The perceived threat of pandemic drug-resistant TB is enormous, with the potential to destabilise society. However, MDR-TB is at critical levels only in specific regions of the world. Highest rates of MDR strains have been observed mainly in Eastern Europe and in some provinces of China (Espinal, 2003). Most of the cases are generated by poor treatment protocols with first-line drugs.

1.4 Th1-Th2 immune responses

The immune response has primarily evolved to defend the host against pathogens and to avoid reactivity to self. Thymus-derived lymphocytes (T cells) are the prime conductors of this response and they vary in type and function. By the early 1970s, it was commonly accepted that T cells could be divided into two distinct

subsets on the basis of their cell-surface markers: CD4+ (Lyt 1) T cells that could "help antibody synthesis" (therefore, helper T cells) and CD8+ T cells (Lyt 2), which lysed pathogen-infected target cells on direct contact (Liew, 2002). Evidences suggesting that helper CD4+ T cells were heterogeneous were confirmed in 1986 when Mosmann, Coffman and colleagues characterised two distinct types of CD4+ T cells from a panel of murine T helper (Th) cell clones, according to patterns of cytokine production (Mosmann, 1986). Adopting an earlier nomenclature (Tada, 1978), they named **Th1** the T cell clone producing interleukin-2 (IL-2), interferon-gamma (IFN-γ), granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-3 in response to antigen presented by antigen-presenting cells (APCs). Type 2 T helper cells (**Th2**) on the other hand, produced a B-cell stimulating factor (identified later as IL-4), mast-cell growth factor (IL-5) and IL-3 (Mosmann, 1986). In addition, the Th2 clone had the ability to enhance the production of immunoglobulin (Ig) E and IgG1 by lypopolysaccharide (LPS)-induced B cells. Th1 cells are responsible for the induction of IgG2a switching by B cells (Stevens, 1988).

These findings were confirmed later in humans with the characterisation of specific clones producing either IFN-γ or IL-4/IL-5 in response to *M. tuberculosis* or *Toxocara canis*, respectively (Romagnani, 1991). After encountering antigen, i.e., engagement of the T cell receptor (TCR) by the appropriate peptide-major histocompatibility complex (MHC) molecule, which triggers clonal expansion, naive Th cells rapidly undergo programmed differentiation to become effector Th1 or Th2 cells. The products of these lymphocytes can act as autocrine growth factors to promote further expansion of these cells, as well as reciprocal inhibitory agents for the opposite cell type (fig.1.2). In general terms, IL-4 can help the clonal expansion of Th2 population and limit the proliferation of Th1 cells; conversely, IFN-γ enhanced Th1-cell growth and inhibits Th2-cell development (Liew, 2002).

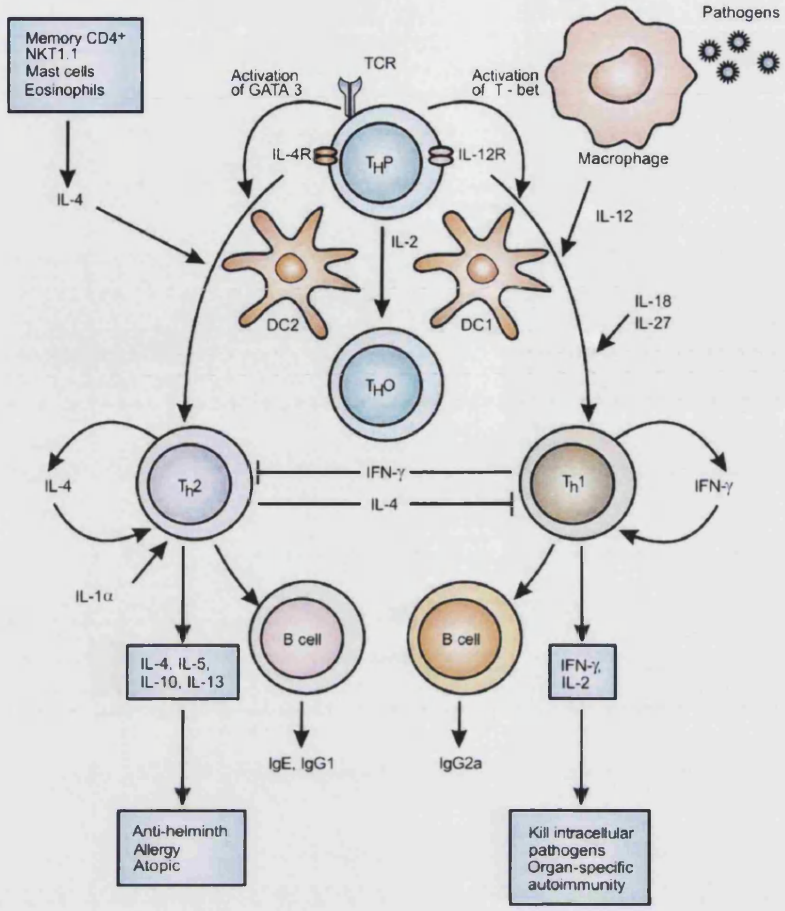


Figure 1.2 Schematic representation of induction and regulation of Th1 and Th2 cells. The Th-precursor (ThP) cell can differentiate into T-helper (Th)1, Th2 or Th0 cells depending on factors such as the cytokine microenvironment. Interleukin (IL)-12 drives Th1 cells, and IL-4 can promote Th2 cells. Th0 cells differentiate in neutral conditions with optional IL-2. Interferon-γ (IFN-γ) and IL-4, produced by Th1 and Th2, respectively, can also act as autocrine growth factors as well as inhibitory factors for the opposite subset. Functionally, Th1 mediate the destruction of intracellular pathogens and organ-specific autoimmunity. Th2 cells are anti-helminthic and increase allergic reaction through IL-4, IL-5 and IgE. IL-4R, IL-4 receptor; NKT, natural killer cells; TCR, T-cell receptor. Modified from Liew (2002).

In addition to TCR engagement, IL-12 produced by macrophages and dendritic cells, has been shown to be a major factor in the production of IFN-γ and the development of the Th1 phenotype (Hsieh, 1993; Robinson & O'Garra, 2002). On the other hand, IL-4, which besides T cells, is produced by different cell types such as natural killer T cells and mast cells, has been shown to be essential for Th2 cell development (Seder, 1992), although inducible Th2 responses *in vivo*, have been reported in absence of IL-4 (Fallon, 2002). Therefore, Th1/Th2 T cell populations may share a common precursor and at the onset of the response, the cytokine milieu is primordial in lineage decision. Nonetheless, antigen dose and affinity, MHC haplotypes and co-stimulators may have crucial roles in determining the dominance of a Th response (Liew, 2002; Murphy & Reiner, 2002; Szabo, 2003). This is actually an area of intense investigation and lively controversy.

The Th2 cells are typically involved in optimal antibody production, non-phagocytic immunity (protection against extracellular invaders such as helminthes) and in inappropriate immune responses generated in atopic diseases and asthma. Cross-linking of IgE bound to its receptor results in degranulation of mast cells, basophils and eosinophils (Roitt, 2001). Besides IL-4 and IL-5, Th2 cells often produce IL-6, IL-9, IL-10 and IL-13 cytokines, and this cytokine network can operate redundantly to sustain a Th2 response even in the lack of one or more key cytokines (Fallon, 2002). The binding of IL-4 to the IL-4 receptor in the surface of naive Th-cells activates STAT6 (signal transducer and activation of transcription 6) protein, which efficiently increases the expression of a zinc-finger transcription factor called GATA3 (Zheng & Flavell, 1997). GATA3 is crucial to some key attributes of Th2 cells (Pai, 2004), acting directly on certain cytokine promoters, or indirectly trough *cis* elements, to instruct the cell to revert chromatin-based loci repression of Th2 cytokines (Murphy & Reiner, 2002). The IL-4/STAT6 signalling pathway can

mediate selective proliferation of Th2 cells by inducing a new transcription factor, the growth factor-independent 1 (Gfi-1) (Zhu, 2002).

Appropriate induction of a Th1 response is required for effective control of intracellular pathogens; in contrast, disproportionate Th1 responses could ultimately lead to immunopathology and organ-specific autoimmunity (Robinson & O'Garra, 2002). Th1 cells produce IL-2, lymphotoxin-α3 and IFN-γ (Roitt, 2001), the latter being essential for activity and maintenance of Th1 responses. Transcription factor T-bet has been identified as a Th1-specific transcriptional regulator that could induce production of IFN-γ even by Th2 cells (Szabo, 2000). T-bet is strongly induced in naive T cells by IFN-γ signalling trough STAT1 and can promote expression of IL-12 receptor β2-subunit in such cells (Afkarian, 2002; Szabo, 2003). An onset scenario for Th1 responses would involve, between other factors, the engagement of the TCR by antigen in a milieu of IFN-γ produced by natural killer (NK) cells, favouring T-bet expression and initiation of Th1 cell-differentiation.

Other cytokines are also reportedly involved in the development of the Th1 responses. IL-18, a cytokine produced by both immune and non-immune cells, is an important later player in Th1 development, synergizing with IL-12 for the enhancement of IFN-γ secretion by Th1 cells and NK cells, even in the absence of TCR engagement (Robinson, 1997; Nakanishi, 2001). IL-18 KO mice are less resistant to infection by *M. tuberculosis* than wild type (Sugawara, 1999). IL-23, another pro-inflammatory cytokine, is composed of the specific p19 subunit (IL-23α) attached to the p40 subunit of IL-12 (Oppmann, 2000). IL-23 induces secretion of IFN-γ in memory T cells, dendritic cells and macrophages (Cua, 2003). A recently discovered cytokine, IL-27 (Pflanz, 2002), acts together with IL-12 to induce early IFN-γ production and proliferation of naive but not memory CD4+ T cells and also

help Th1 development. IL-27 has been detected in human tuberculous granulomas (Larousserie, 2004).

Polarised Th1 and Th2 responses are also seen *in vivo*, after chronic immunisation or disease. In acute immune responses particularly, distinct bipartition is not always found; clones making simultaneously type 1 and type 2 cytokines (**Th0** clones) can be seen *in vivo* (Polakos, 2001). **Th3**-CD4+ lymphocytes, are regulatory T cells producing transforming growth factor-β (TGF-β) and involved in mechanisms of oral tolerance (Weiner, 2001).

Dendritic cells (DC) are the initiators, the modulators, the major antigen-presenting cell generating adaptive immune responses. They perform multiple tasks with high efficiency, and may determine the specificity, magnitude and nature (Th1 versus Th2) of the immune response (Moser & Murphy, 2000).

At an immature stage of development, DCs have higher phagocytic capacity. They also perform receptor-mediated adsorptive endocytosis and macropinocytosis (Banchereau & Steinman, 1998). DCs act as sentinels in peripheral tissues, continuously sampling the antigenic environment. Encounter with microbial products initiates the migration and the activation of DCs. There is up-regulation of co-stimulatory and MHC molecules coupled with increased processing of peptides for presentation for T cells and effective initiation of immune responses. (Moser & Murphy, 2000). Dendritic cells can also cross-present antigens to T cells, and this could be important in generating cytotoxic responses (Kaufmann, 2001; Schaible, 2003). Matured DCs usually express moderate to high levels of MHC-II, which become very stable complexes with peptide in the surface of these cells (Shortman & Liu, 2002). DC maturation also triggers production of cytokines such as IL-12 and IL-10 and they play important roles in the polarisation of T cell responses (Reis e Sousa, 2001). Maturing DCs produce under tight regulation, the bioactive p70 form

of IL-12 (p40 and p35 components), which is a potent inducer of Th1 responses as explained before (Moser & Murphy, 2000). IL-12 signalling is mutually antagonistic with GATA3 expression, providing a mechanism of regulation on lineage fate decision (Ouyang, 1998). IL-10 down-regulates the production of IL-12 by murine and human DCs (Maldonado-Lopez & Moser, 2001).

DCs can be subdivided in different subsets according to patterns of expression of surface molecules such as CD11b, CD8α and CD4 and by cytokines they produce. A number of studies have suggested that different DC subsets induce T cells to differentiate either into Th1 or Th2 (Maldonado-Lopez & Moser, 2001; Szabo, 2003). Other reports show dendritic cells to be quite flexible in driving either Th1 or Th2 responses (Boonstra, 2003). TLRs on DCs provide the link between the innate and adaptive immune responses (Medzhitov, 1997). These conserved, germline encoded co-receptors expressed in macrophages and DCs, recognise specific microbial products such as LPS, peptidoglycan, hypomethylated CpG motifs and double-stranded RNA (Barton & Medzhitov, 2002). Besides up-regulation of MHC and co-stimulatory molecules and the secretion of proinflammatory cytokines TNF-α, IL-1 and IL-6, TLR signalling also lead to high IL-12 production by some DCs, driving differentiation of T cells to Th1 pattern (Barton & Medzhitov, 2002). TLR receptor-signalling pathways controlling Th2 responses are less well characterised (Schnare, 2001; Jankovic, 2002).

1.5 Tuberculosis immunity: of mice and man

Pathogenic *Mycobacterium* spp. survives within the macrophages of their host, contrasting with the accepted view that these cells afford an effective initial barrier to bacterial infection. Multiple receptor molecules can mediate the uptake of

mycobacteria into macrophages (fig.1.3). Complement receptors (CR1 and CR3) are among the most widely used receptors for mycobacteria, for both opsonized and non-opsonized entry (Hu, 2000). Although in some cases not altering significantly the outcome of infection *in vivo* (Hu, 2000), triggering of complement receptors leads to the activation of Rho, a member of the small GTPases family, and this pathway downregulates bactericidal mechanisms in macrophages (Pieters & Gatfield, 2002). Mannose receptor (Kang & Schlesinger, 1998), transferrin receptor, CD14 scavenger receptor, fibronectin (Pasula, 2002) and leukosialin CD43 (Fratazzi, 2000), all promote the attachment of mycobacterial ligands to the macrophage surface and may be involved in bacterial uptake. Interestingly, cholesterol appears to be important for the mycobacterial invasion, since cholesterol-depleted macrophages are unable to specifically internalise mycobacteria (Gatfield & Pieters, 2000). In summary, it appears that the bacilli can gain entry into the cell through ligation of many phagocytic receptors. Mycobacteria can also be phagocytosed by other cell types such as dendritic cells (Bodnar, 2001). Both human and murine dendritic cells have a bacteriostatic but not bactericidal activity against mycobacteria *in vivo* (Bodnar, 2001; Jiao, 2002). In DCs, *M. tuberculosis* targets the dendritic cell-specific C-type lectin DC-SIGN (DC-specific intercellular-adhesion-molecule-3-grabbing-nonintegrin), leading to inhibition of immunostimulatory function by the DC and, hence, promoting pathogen survival (Tailleux, 2003).

As early as 1971, Armstrong and D'Arcy Hart discovered that phagosomes containing live *M. tuberculosis* do not fuse with lysosomes. By contrast, dead mycobacteria were readily transported to lysosomes (Armstrong & Hart, 1971). Pathogenic mycobacteria have evolved a strategy to arrest the normal maturation process of phagosomes after uptake by macrophages, living in a vacuole with characteristics of an early endosome (Russell, 2001). How the bacillus achieves this

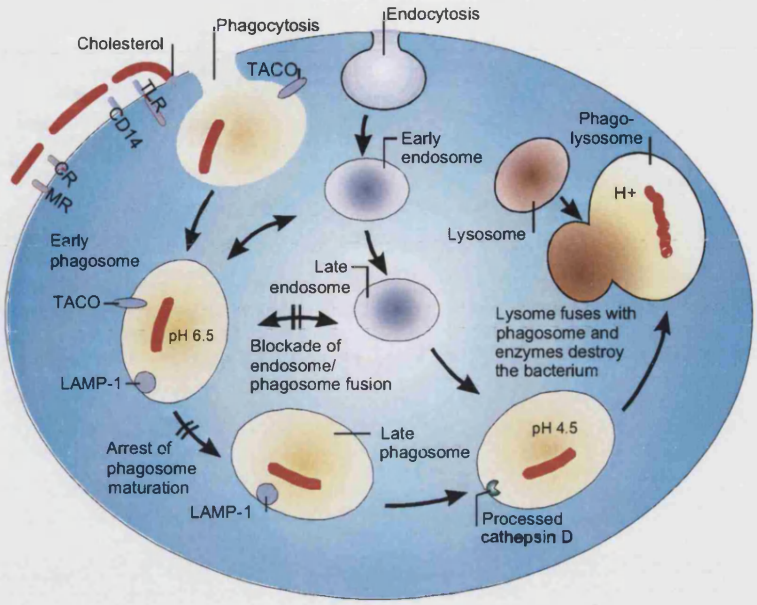


Figure 1.3 Intracellular pathways for *M. tuberculosis*. Macrophages can promote phagocytosis of large particles. Both endosomal and phagosomal pathways undergo interconnected maturation processes that merge at a later stage, prior to lysosomal fusion. Various receptors interact with MTB at the surface of the macrophage. Cholesterol serves as a docking site, facilitating the interactions between mycobacteria and surface receptors (Gatfield & Pieters, 2000). Once inside, MTB ends up in a phagosome, the maturation of which is arrested at an early stage. Infected, the organelle characteristically retains TACO, which apparently prevents its further maturation (Ferrari, 1999). MTB inhibits phagosomal acidification (Russel, 2001) and prevents fusion with the endosomal pathway. This last arrest is incomplete, and some phagosomes mature to form phagolysosomes, particularly in activated macrophages. LAMP-1 and Cathepsin D are lysosomal markers. TLR, Toll-like receptor; CR, complement receptor; MR, mannose receptor; TACO, tryptophane, aspartate-containing coat protein; LAMP-1, lysosomal-associated membrane protein 1. Modified from Kaufmann (2001).

effect is still unclear. A molecule termed tryptophan-aspartate-containing coat protein (TACO) has been found in phagosomes harbouring living mycobacteria and seems to prevent lysosomal fusion by a yet unknown mechanism (Ferrari, 1999). There are also evidence to suggest that *Mycobacterium* spp. survive in phagolysosomes within macrophages in granulomas (Cosma, 2003). Mycobacteria-containing vacuoles are less acidic than their neighbouring lysosomes (Russell, 2001); mycobacterial porins could contribute in setting a favourable pH within the vacuole (Raynaud, 2002).

In professional APCs, MHC-II molecules bind peptides derived from soluble proteins, particulate antigens, and microbes that are internalised by endocytosis or phagocytosis and degraded in the endocytic pathway. In both human and murine systems, the MHC-II antigen processing machinery is inhibited in *M. tuberculosis*-infected APCs, by either reduced synthesis of MHC-II molecules or by sequestering these molecules intracellularly in an immature state (Ramachandra, 1999). In contrast, antigens present in the cytosol are degraded to antigenic peptides by proteasomes and enter the endoplasmic reticulum via the transporter for antigen presentation (TAP) to bind MHC-I molecules. It has been recognised that CD8+ T cells play an important role in immunity to tuberculosis (Tascon, 1998; Lewinsohn, 1998); uptake of mycobacterial antigen from extracellular vesicles from apoptotic cells by APCs resulting in MHC-I presentation has been reported (Schaible, 2003) and is important in the process of specific anti-mycobacterial CD8+ T cell priming.

In the initial steps of infection, a fine-tuned interaction between innate and adaptive mechanisms of immunity would be essential for the host response. Mycobacterial antigens such as 19-kDa lipoprotein and lipoarabinomannan (LAM), a glycolipid that dominates the mycobacterial cell wall, are recognised by TLR2 with induction of TNF-α and nitric oxide (NO) production (Brightbill, 1999; Means, 1999). Whereas most purified mycobacterial ligands activate macrophages via TLR2,

whole killed or live mycobacteria seem to interact with TLR2, TLR4 and other unknown TLRs (Stenger & Modlin, 2002). Only a high-dose aerosol challenge with MTB was able to show defective responses in $TLR2^{-/-}$ mice (Reiling, 2002). Recent work in C3H/HeJ, a strain of mice mutant in the *tlr4* locus, demonstrated that TLR4 might not be critical for MTB control in mice (Kamath, 2004). Finally, MyD88-deficient mice, which are unable to signal internally through TLRs, failed to control mycobacterial replication and showed a definitive participation of a TLR (or a combination of TLRs) in the immune response against the tubercle bacilli (Scanga, 2004).

M. tuberculosis induces the up-regulation of several important host genes in the process of initial resistance to the bacteria; most are chemokines, others are cytokines such as IL-1β and TNF-α (Ragno, 2001). Recruitment of macrophages and T cells to the infection site is essential for protection. Chemokines are potent leukocyte activators and chemoattractants, and are important in granuloma formation *in vivo* (Van Crevel, 2002). The β-chemokines macrophage inflammatory protein-1-β (MIP-1β) and RANTES (regulated upon activation, normal T cell expressed and secreted) are induced by and can suppress intracellular *M. tuberculosis* growth (Saukkonen, 2002). Significantly, C-C chemokine receptor 2 (CCR2) deficient mice are very susceptible to MTB infection (Peters, 2001). Cellular migration to MTB invasion sites in the lung is also affected in intracellular adhesion molecule 1 (ICAM-1) deleted mice, which do not form granulomas (Johnson, 1998). Interestingly, protective immunity against MTB infection is not affected in this model, although long-term survival of the host may ultimately depends upon the production of a stable mononuclear granuloma (Saunders, 1999).

TNF (TNF-α) is a pleiotropic cytokine produced by monocytes and macrophages after *M. tuberculosis* infection and Th1 T cells (Giacomini, 2001). This

cytokine synergizes with IFN-γ to activate murine infected macrophages (fig.1.4), and is essential for the protection against murine tuberculosis (Flynn, 1995). A critical role in humans has been demonstrated by reactivation of tuberculosis in patients treated with anti-TNF antibodies (Keane, 2001). Neutralisation of TNF in a low-dose latent disease model induces death with severe histopathological changes, suggesting a role for TNF in ameliorating pathology of mycobacterial disease (Mohan, 2001). This cytokine also has a pathological facet. TNF appears to be linked to cachexia and increased multiplication of virulent bacilli in human macrophages (Engele, 2002). Absence of synergizing effects of TNF and IFN-γ has been observed in infected human monocytes (Engele, 2002). A tentative explanation for these contrasting effects could be related to dose-dependency: low levels of TNF *in vivo* mediating protection, whereas high levels provoking tissue damage (Bekker, 2000). In mice, TNF is crucial for the formation of tuberculous granulomas (Roach, 2002).

Although not intensively investigated, natural killer (NK) cells and T cells that express the gamma-delta (γδ) TCR may play significant roles in TB immunity. Killing of intracellular *M. tuberculosis* by unstimulated human NK cells is not mediated by granule exocytosis or Fas/Fas ligand interaction but by an unknown apoptotic mechanism (Brill, 2001). In mice, γδ T cells have been shown to protect partially against a high but not low inocula of MTB and are involved in granuloma formation (Kaufmann, 2001). Human γδ T cells are stimulated by a unique group of non-proteinaceous antigens that contains phosphate and they express a specific combination of TCR variable chains (Vγ2δ2); these cells appear to contribute to control mycobacterial infections in non-human primates (Kaufmann, 2001; Shen, 2002). As described before, IL-12 is a critical cytokine connecting innate with the adaptive host response to mycobacteria (van Crevel, 2002).

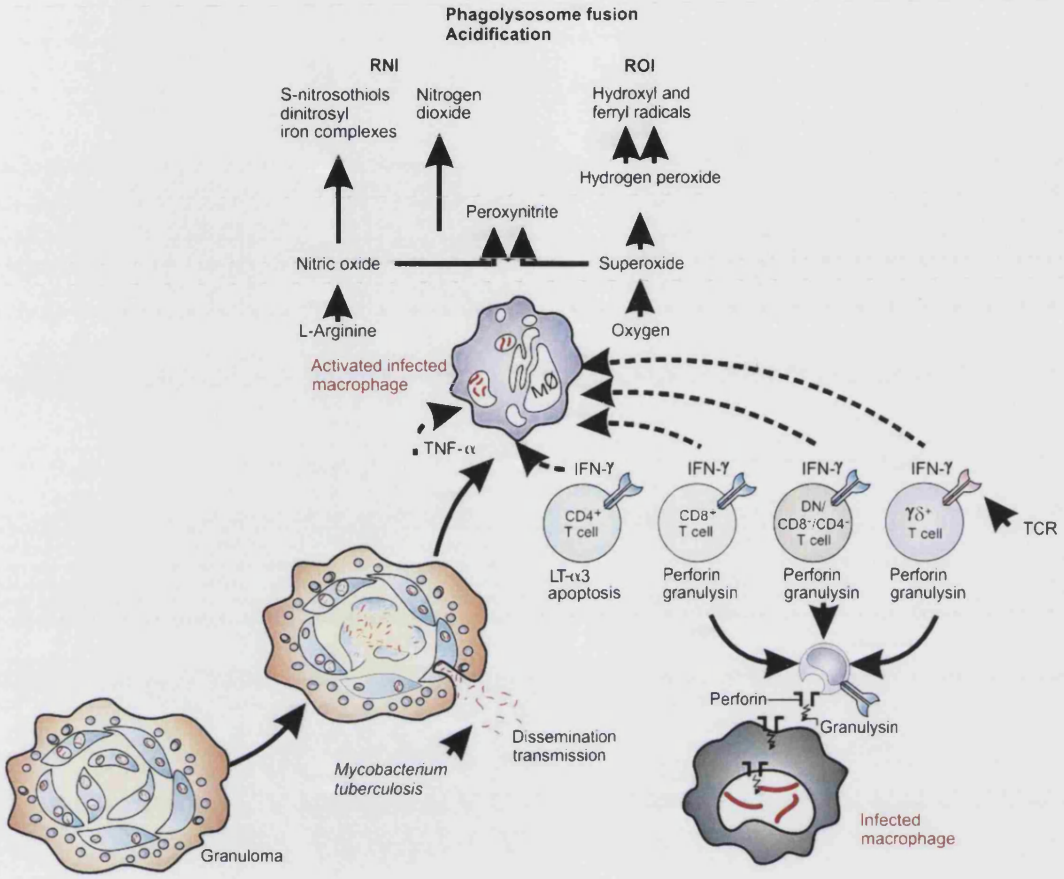


Figure 1.4 **Host defenses against tuberculosis**. In most cases, *M. tuberculosis* is initially contained and disease develops later. The granuloma can be the site of infection, persistence, pathology and protection. Effector T cells (conventional CD4+ and CD8+ T cells and unconventional T cells, such as γδ T cells, double-negative and CD1-restricted cells) and macrophages participate in the control of tuberculosis. IFN-γ and TNF-_ are important macrophage activators. Macrophage activation permits phagosomal maturation and the production of antimicrobial molecules such as reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). LT-_3, lymphotoxin-α3. Modified from Kaufmann (2001).

Mycobacteria are very strong IL-12 inducers; they can even skew the immune response to a secondary antigen towards a Th1 phenotype (Flynn & Chan, 2001). It has been recently reported that IL-12 is induced after phagocytosis of mycobacteria by dendritic cells but not by resting macrophages (Hickman, 2002). This inhibitory effect on IL-12 production by macrophages is probably caused by MTB-induced IL-10 and is reversible with cell-activation by IFN-γ (Giacomini, 2001; Hickman, 2002). Inappropriate IL-12 production or signalling is implicated in increased susceptibility to mycobacteria in mice and humans (Flynn & Chan, 2001; Casanova & Abel, 2002).

Cell-mediated immunity is of critical importance to combat tuberculosis; mice genetically deficient in αβ-TCR T cells are highly susceptible to infection and succumb rapidly to acute tuberculosis infection (Mogues, 2001). The role of B cells in protection against tuberculous disease has been more difficult to demonstrate. Nonetheless, Teitelbaum and colleagues (1998) had reported increased survival in wild type and immunocompromised mice injected with a monoclonal antibody specific for surface arabinomannan and challenged with the tubercle bacilli.

Since *M. tuberculosis* resides primarily in a vacuole and mycobacterial antigen presentation by MHC class II molecules takes place, is not surprising that CD4+ T cells are the most important in protective responses against MTB. Murine studies with antibody depletion of CD4+ T cells, adoptive transfer and gene-disrupted mice support this concept (Flynn & Chan, 2001). MHC-II-/- (Tascon, 1998) and CD4-/- (Saunders, 2002) are very susceptible to infection, the former more than the latter. In humans, the importance of the CD4+ T cell subset is easily demonstrated with HIV infection, which increases the susceptibility to tuberculosis from an averaged 10% lifetime risk (PPD+ HIV- individuals) to a 10% annual risk in PPD+ HIV+ subjects (de Jong, 2004). The primary known effector function of CD4+ T cells would be to produce IFN-γ to activate macrophages, which then can combat infection

by *M. tuberculosis* (Flynn & Chan, 2001). However, other roles controlling tuberculosis have been also attributed to this subset; in CD4-/- mice infected with MTB, IFN-γ production is eventually compensated by CD8+ and NK cells, but these animals show delayed, non-typical granulomas and ultimately succumb to infection (Saunders, 2002). Involvement in disease persistence is shown by depletion of CD4+ in a low dose latency mouse model, which results in reactivation of tuberculosis despite expression of IFN-γ (Scanga, 2000). Structural integrity of granulomas is compromised in humans with HIV/TB co-infection (Raupach & Kaufmann, 2001). Granulysin is a molecule expressed by human T cells and other cell types which can direct kill intracellular *M. tuberculosis* (Stenger, 1998). Canaday and colleagues (2001) had reported a yet unknown mechanism of human CD4+ (and CD8+) T cell-mediated restriction of intracellular MTB growth that is perforin-, Fas/Fas-ligand- and granulysin-independent. CD4+ T cells are important for cytotoxic activity, and possibly to support memory functions of CD8+ T cells in the lungs of MTB infected mice (Serbina, 2001). CD4+ T cells also produce homotrimeric lymphotoxin-α3 (LTα3) and this molecule has been found to be essential to control MTB infection in mice; in its absence, lymphocytes fail to migrate into infected tissues (Roach, 2001).

CD8+ T cells (Tascon, 1998) and macrophages (Frucht, 2001) also participate in IFN-γ production. IFN-γ knockout animals have demonstrated the critical need for this cytokine in the outcome of murine tuberculosis; in its absence, the animals died rapidly of disseminated disease with increased bacterial numbers and purulent necrotic granulomas (Flynn, 1993; Cooper, 1993; Cooper, 2002). Human mutations in either chain of the IFN-γ receptor IFN-γR1 and IFN-γR2 (Casanova & Abel, 2002), as well as mutations on the STAT1 IFN-γ-signal transducer (Dupuis, 2001), can lead to partial or complete deficiency of response to the cytokine and these subjects show

increased susceptibility to mycobacterial disease (Casanova & Abel, 2002). IFN-γ is a major activator of macrophages and activated macrophages produce elevated reactive oxygen and nitrogen intermediates (fig.1.4). The former has only a few contributory effects in controlling tuberculosis in mice (Cooper, 2000). The latter, however is essential for protection (Cooper, 2002). IFN-γ activates expression of inducible nitric oxide synthase (NOS2), which produces NO· and other nitrosative intermediates from L-arginine; all major killer molecules for *M. tuberculosis* (Raupach & Kaufmann, 2001). Of interest, as well as participating in granuloma formation, both IFN-γ and NO· seem to be involved in limiting extensive inflammation in MTB lung sites (Cooper, 2002). Interestingly, mycobacteria are able to disrupt association of transcription activator STAT1 with CREB binding protein and p300, restricting macrophage activation (Ting, 1999).

On the other hand, IL-4 and other Th1-supressive cytokines could be playing a role in limiting the actions of IFN-γ. However, a clear dichotomy in Th1/Th2 responses is difficult to demonstrate in both murine and human models of tuberculosis; a depressed Th1 response rather than a strong Th2 response is more likely seen (Flynn & Chan, 2001; García, 2002). IL-4, IL-10, IL-4/IL-13, IL-4Rα or STAT6 KO mice are not more resistant to *M. tuberculosis* infection than wild type (North, 1998; Jung, 2002; North & Jung, 2004). IL-4 is not always detected in lung granulomas of human patients (Aung, 2000). However, IL-10 production may be important in both late/chronic stages of disease in mice (Turner, 2002a) and in regulating mycobacterial infections in humans (Weir, 2004; Demissie, 2004). TGF-β1, another pleiotropic cytokine, has been detected in patients with active TB (Toossi, 1995; Bonecini-Almeida, 2004).

In addition to MHC-II, MHC class-I restricted T cells contribute importantly to immunity against TB infection. Early adoptive-transfer experiments (Orme & Collins, 1984) and cell-depletion studies *in vivo* (Muller, 1987) have demonstrated that CD8+ T cells were involved in protective immunity against murine TB. Mice deficient in β_2-microglobulin (Flynn, 1992), a molecule that participates in the structure of MHC-I and other non-classical MHC molecules (Behar, 1999), show a rapid lethal outcome in response to MTB infection. CD8+ T cells could contribute to the immune response against MTB by at least three pathways: the release of IFN-γ, lysis of infected targets, and direct antimicrobial activity. Tascon and colleagues (1998) had demonstrated the importance of IFN-γ production by CD8+ T cells to mediate partial protection of athymic mice. *Mycobacterium*-specific CD8+ cytotoxic T cell clones recognising defined specific antigens and producing IFN-γ have been isolated from mice (Silva & Lowrie, 2000) and humans (Lalvani, 1998; Cho, 2000; Lewinsohn, 2001). Although not well defined, cytotoxicity mechanisms against MTB-infected cells by CD8+ T cells have been reported to be at least partially perforin-dependent in mice (Serbina, 2000; Sousa, 2000). CD95/CD95L also seem to participate later in control of murine chronic tuberculosis (Turner, 2001a); but in humans, alternative mechanisms of lysis by CD8+ T cells do exist (Canaday, 2001). Granulysin, a member of the saposin-like protein family, is present in human MHC class I-restricted CD8+ T cells and directly kills MTB (Stenger, 1998). Studies have shown that CD8+ T cells migrate to the lung with similar kinetics to CD4+ T cells in young but not in old mice (Serbina & Flynn, 1999; Turner, 2002b), and interestingly, infection in elderly mice seems to activate a phenotypically different CD8+ T cell population, expressing several NK markers (Turner, 2002b).

CD8α- and MHC-I deficient mice are less susceptible to MTB infection than β_2-microglobulin (β_2m) KO (Sousa, 2000; Mogues, 2001), therefore other β_2m-dependent mechanisms of resistance are suspected. Interestingly, β_2m is involved in iron metabolism, as it stabilises surface expression of a gene that regulates iron uptake (Kaufmann, 2001); β_2m KO mice suffer from iron overload and correction of this imbalance by lactoferrin restored immunity to MTB infection to levels comparable to MHC-I KO mice (Schaible, 2002).

Evidence for the participation of unconventional subsets of T cells in the immune response to the tubercle bacillus is well supported. Formylation of proteins represents a characteristic feature of prokaryotic origin (Kaufmann, 2001) and a non-classical MHC class Ib molecule (H2-M3) presenting N-formylated peptides from *M. tuberculosis*, elicited cytotoxic responses in C56BL/6 mice (Chun, 2001). In humans, a group of β2-microglobulin-associated, non-MHC locus-encoded proteins (CD1a, CD1b, CD1c) were found to present hydrophobic lipids and glycolipids to T cells (Beckman, 1994). CD8+ and CD4-CD8- with specificity for mycobacterial glycolipids presented by CD1 molecules were demonstrated (Stenger, 1997). The CD1-restricted CD8+ T cells appeared to specifically kill *M. tuberculosis* via the newly discovered granulysin (Stenger, 1998). Only homologous CD1d molecules are found in mice and natural killer T cells (NKT) are a subset of CD1d-restricted T cells (Chackerian, 2002). The absence of CD1d molecules did not impair survival of mice towards a challenge with MTB (Behar, 1999). However, activation of the NKT population with α-galactosylceramide, a heterologous antigen, has been reported to protect susceptible mice against infection with *M. tuberculosis* (Chackerian, 2002).

1.6 Vaccination against tuberculosis

1.6.1 Bacillus Calmette-Guérin

In 1798, Edward Jenner, an English country doctor, employed effectively the concept of vaccination that would save millions. Robert Koch knew about vaccination and hoped to find a treatment against tuberculosis. In August 1890, he hinted he had found a promising substance (later called tuberculin), a solution prepared by killing MTB, then filtering and concentrating the liquid. In a published paper, Koch stated that tuberculin induced strong reactions in people with tuberculosis, protecting and helping to heal tubercles in the lungs of animals with TB (Reichman & Tanne, 2002). He was mistaken; clinical trials soon demonstrated the ineffectiveness of his therapy; nonetheless, the substance he discovered became the precursor of modern PPD (Kaufmann, 2001). The quest for a TB vaccine continued and in the beginning of the twentieth century, two French scientists, Albert Calmette and Camille Guérin, started passaging in culture a virulent *M. bovis* strain. With astonishing dedication, they transferred the bacteria from one test tube to another during approximately thirteen years. After 231 passages, the bacillus was unable to infect but protected experimental animals (Reichman & Tanne, 2002). Called bacille Calmette-Guérin (BCG), it was given by the oral route to an infant with high risk of tuberculosis infection in 1921; the newborn remained healthy during its childhood (Reichman & Tanne, 2002).

The encouraging initial trials lead BCG vaccination to spread worldwide and it has been used for over six decades. More than three billion doses were administered. It is considered very safe (is usually administered to neonates). The risk of disseminated disease (BCG-itis) is about 1 in a million of vaccinees (Horwitz, 2000). Nonetheless, there is a progressive infection in BCG-vaccinated SCID (severe combined immunodeficiency) mice (Mills, 2001); hence, the vaccine is not

recommended for the immunocompromised or those with established HIV infection (Horwitz, 2000). WHO has included BCG on its expanded program of immunisation (WHO-EPI). For many years it was administered orally, but now is used intradermally, due to decreased side effects (Lagranderie, 2000). Several experimental studies for more efficient routes have been undertaken: aerosol (Goonetilleke, 2003), intragastric (Lagranderie, 2000), rectal (Lagranderie, 2002) and intravenous (Palendira, 2002a). However, a study in the mouse model has suggested the protective efficacy of BCG to be independent of the route of immunisation (Palendira, 2002a).

Mice usually show less susceptibility to tuberculosis than humans, but it is the animal of choice for initial vaccine testing and infection studies because it is less expensive and has a large research database (Orme, 2001; McMurray, 2001). BCG can protect BALB/c and C57BL/6 against TB, but the quality of the immune responses seems to be different. BALB/c strain shows decreased levels of IFN-γ, IL-12 and TNF-α cytokines, low-magnitude cellular responses and atypical granulomas, in comparison with the C57BL/6 strain. In terms of colony counts, both strains control infection with BCG (Wakeham, 2000).

The classical animal model for mycobacterial infection is the guinea pig, because of its exquisite susceptibility and some remarkable similarities with human infection (McMurray, 2001). Infected guinea pigs commonly present similar DTH reactions to humans and also caseous necrosis in its granulomas (Orme, 2001). Vaccination of guinea pigs with BCG increases resistance to MTB challenge; after vaccination, increased levels of CD8+ T cells have been noted in the spleen (Klünner, 2001).

BCG is the only vaccine available against human tuberculosis. It performs very well in animal models and remains the "gold-standard" against which candidate

TB vaccines with improved efficacy are measured. It is strikingly efficient in protecting children against early and severe forms of tuberculosis such as miliary and tuberculous meningitis, as well as extrapulmonary forms and glandular tuberculosis (Jason, 2002). It exerts excellent levels of heterologous protection against leprosy (Fine, 1995). Nonetheless, BCG has produced more than acceptable variance in protection against pulmonary TB, as seen in several human trials (Fine, 1995). A famous major trial in schoolchildren from the United Kingdom, run by the Medical Research Council (MRC), showed more than 75% of protection, on a twenty-years follow-up (Hart & Sutherland, 1977). In remarkable contrast, trials in Georgia and Alabama (USA) and Puerto Rico, all demonstrated levels of protection below 30% (Fine, 1995). Even worse, 0% protection to infection has been found in trials in South India/Chingleput (Fine, 1995) and Malawi (Ponnighaus, 1992). Even in successful trials, the efficacy of BCG was predicted to wane over time, imparting protection for a decade or so (Sterne, 1998). Although human trials are underway (Dourado, 2003), there is no clear evidence that BCG revaccination would improve protection and this procedure is not recommended by the WHO (WHO-Global Tuberculosis Programme and Global Programme on Vaccines, 1995).

Several hypotheses have been proposed to explain the observed failures in BCG vaccination; genetic variability in the population and vaccination of previously infected individuals are possibilities (Fine, 1995; Andersen, 2001). There have also been suggestions of over-attenuation of BCG substrains (Behr & Small, 1997). After 1921, cultures of BCG were delivered for propagation in laboratories all over the globe (Oettinger, 1999). Continuous passage in different ways of culturing may have lead the original BCG strain to a profusion of phenotypically and genotypically different daughter strains. Work by Mahairas and colleagues (1996) have found that BCG was missing blocks of DNA from its genome in comparison to *M. tuberculosis*.

The deletions were called regions of difference (RD): RD1 and RD3 were missing from all substrains of BCG examined and contained virulence-associated genes such as ESAT-6 and CFP10; RD2 contained, between others, the mycobacterial protein tuberculosis 64 (MPT64) gene (Mahairas, 1996) and affected BCG substrains obtained from Institut Pasteur after 1926 (Oettinger, 1999). In comparison with *M. bovis*, all BCG vaccines lack RD1, which may indicate it was lost during the 1908-1921 attenuation (Behr, 1999). Knock-in studies have shown that RD1 contributes importantly to the loss of virulence observed in BCG (Pym, 2002). Reintroduction of this region made BCG a more effective vaccine against MTB in mice (Pym, 2003). Loss of virulence in BCG appears specifically linked to the lack of cytolytic activity and tissue invasiveness mediated by secreted ESAT-6 (Hsu, 2003). In addition, by using microarray technology, Behr and collaborators (1999) have found further deletions and named them RD1-RD16, four of them (RD2, RD8, RD14, RD16) deleted only in specific substrains. Besides these major deletions and other less conspicuous changes (Behr, 2000), there is little evidence that certain strains would be more immunogenic than others (Oettinger, 1999), and the same vaccine substrain has also been found to provide good protection in one region while performing badly in another (Fine, 1995).

A prevailing hypothesis to explain the variation in BCG efficacy has been related with the heterologous infection of the host with environmental mycobacteria. In animals, a recent study (Brandt, 2002) has complemented previous observations initiated by Palmer & Long (1966). In this study, two strains of the *M. avium* complex, isolated from the Karonga district in Malawi (absence of BCG protection), were found to block BCG activity; prior sensitisation with *M. avium* resulted in a broad immune response which controlled the multiplication of BCG and avoided protective immunity to MTB in mice (Brandt, 2002). Evidences in humans:

correlation of strong efficacy of BCG in trials in which skin-test positive individuals were excluded (Hart & Sutherland, 1977); the success of BCG in neonates, before sensitisation with non-tuberculous mycobacteria occurs (Colditz, 1995). Importantly, poor IFN-γ and DTH responses are associated with no protection by BCG in individuals from areas rich in environmental mycobacteria (Malawi) when compared with individuals living in areas with good records of BCG efficacy and less exposure to atypical mycobacteria (Black, 2002).

Independent of this variation, researchers have tried to improve upon the immunogenicity of BCG with some success (Dietrich, 2003; Pym, 2003; Horwitz, 2003). Expression of cytokines (IL-2, IFN-γ, GM-CSF) has been attempted (Murray, 1996; Biet, 2002). By overexpressing the extracellular antigen 85B in BCG, Horwitz and colleagues (2000) have obtained for the first time a recombinant bacterium that was better than the original in reducing significantly organ associated pathology in the guinea pig model of tuberculosis infection. This vaccine (rBCG30) performed better than BCG in enhancing the survival of guinea pigs in long term infection studies and is being prepared to start human trials soon (Horwitz, 2003).

BCG has little or no effect on the predominant adult pulmonary disease and there is a pressing need for improved vaccines against tuberculosis (Young, 2003). *M. microti* is a natural pathogen of wild voles and causes a progressive and lethal disease in these animals; however, other rodents and humans are relatively resistant even to high doses (Manabe, 2002). In the fifties, the MRC started a comparative trial to test *M. microti* as a better vaccine against tuberculosis (Hart & Sutherland, 1977). Over 50000 children participated. A 20 year follow up showed 77% protective efficacy for both BCG and the vole bacillus (Hart & Sutherland, 1977). In mice, when given orally, *M. microti* was actually better protective than BCG given subcutaneously (Manabe, 2002). Although offering important advantages over BCG such as genomic

stability (the bacillus can be maintained naturally in voles), *M. microti* was abandoned as a vaccine against tuberculosis for unclear reasons (Manabe, 2002). *M. vaccae*, an environmental mycobacteria, is being investigated in a heat-inactivated form as a TB preventive vaccine for HIV+ individuals (Vuola, 2003).

Attenuated strains of *M. tuberculosis* unable for long-term survival in the host have been generated (Hingley-Wilson, 2003). Given the genetic differences between BCG and MTB (Behr, 1999), one would predict that rationally attenuated *M. tuberculosis* would have a more relevant repertoire of species-specific antigens. The matter of safety is a primary concern: the tubercle bacillus must be non-reversibly attenuated and tested in immunocompromised animals (Hondalus, 2000). Along this line, an auxotrophic deletion created by allelic exchange disrupting a gene encoding isopropyl malate isomerase (*leuD*) of MTB generated a mutant dependent of leucine which cannot survive in immunodeficient SCID mice yet providing protection similar to BCG in a mouse challenge model (Hondalus, 2000). Pavelka and colleagues (2003) generated a lysine auxotrophic mutant of MTB able to induce protection equivalent to BCG in the aerosol mouse model of infection. Attenuated MTB mutants have been reported as being ready for phase I trials for safety and immunogenicity soon (Ginsberg, 2002).

Although exposure to certain mycobacteria can provide protection against pulmonary tuberculosis, this protection is not complete; human reactivation and reinfection emphasise that it may not be possible to induce more than about 80% of protection against MTB by exposure to natural mycobacterial antigens (Fine, 1995). Therefore, vaccinologists are left with the difficult task of designing an innovative vaccine, one that induces responses superior to the immune responses raised by the natural infection (Andersen, 2001).

1.6.2 Current vaccine approaches against tuberculosis

The subunit and DNA vaccination approaches are based on the assumption that a few antigens are sufficient to induce and maintain a protective immune response. In addition, they have the advantage to be stable and safe even for immunocompromised individuals (Andersen, 2001). Attention to the vaccine potential of mycobacterial culture filtrate proteins (CFPs) came with the observation that compared to live bacilli, only marginal protection was observed in mice after vaccination with heat-killed tubercle bacilli (Weiss, 1955). Hubbard and colleagues (1992) were the first to show protection against an aerosol MTB challenge in mice immunised with fractionated CFP. Strong protection mediated by CFP plus incomplete Freund's adjuvant was also obtained in the guinea pig model, with the 30-kDa fraction (Ag85B) (Horwitz, 1995).

Several subunit vaccine candidates are currently being tested in animal models. An interesting example is based on the fusion of two immunogenic tuberculosis proteins, Ag85B and ESAT-6; challenge assays in mice demonstrated that this recombinant fusion protein is capable of inducing strong IFN-γ responses and protection levels comparable to BCG even thirty weeks after the last dose (Olsen, 2001). Coler and collaborators (2001) had described a low molecular weight-secreted protein, MTB8.4, which induces IFN-γ production from cells of PPD+ donors and increased protection against MTB in mice when delivered either as a protein or in DNA format. Another fusion protein: recombinant Ag72f+/-Ag85, has been reported as being prepared for human testing soon (Ginsberg, 2002; Reed, 2003). Despite the encouraging results, there is a constant drawback due to the need of a suitable adjuvant to be used in humans to potentiate host responses towards these proteins.

DNA vaccination was first introduced approximately 14 years ago. In his seminal paper, Wolff and colleagues (1990) demonstrated that naked plasmid DNA could be injected into the muscle and the foreign gene that it carried could be transferred and expressed in the muscle cells *in vivo*. Subsequently, Ulmer and colleagues (1993) were the first to report protection induced by a DNA vaccine against an infectious disease. Few years later, Tascon and collaborators (1996) and Huygen and colleagues (1996) simultaneously reported the protective effect of naked DNA vaccination against TB using plasmid DNA coding for a 65-kDa heat shock protein (HSP) from *M. leprae* and the 32-kDa mycolyl transferase or antigen 85A (Ag85A) from *M. tuberculosis*, respectively. Afterwards, the number of reports on the use of DNA vaccines for the immunoprophylaxis of mycobacterial diseases increased steadily; currently, there are several DNA-vaccine candidates under pre-clinical evaluation in the USA and other countries (Huygen, 2003).

The principle of naked DNA vaccination is simple, yet is surprisingly efficacious. Basically, a chosen gene is cloned into a plasmid vector and the purified plasmid injected into the tissue; expression of the coding information is driven by either a strong viral or eukaryotic promoter and the gene of interest is followed by a polyadenylation site for efficient protein production by the eucaryotic cell (Huygen, 2003). The cellular targets are usually myocytes (muscle injection) and keratinocytes (epidermal "gene gun" immunisation) and quantities of antigen produced *in vivo* are usually in the picogram to nanogram range (Gurunathan, 2000). Nonetheless, DNA vaccines trigger efficient humoral and cellular responses, particularly Th1 type and strong activation of CD8+ T cells; both B and T cell memory responses are also generated (Gurunathan, 2000). The antigen encoded by the plasmid DNA may be processed and presented to the immune system by at least three mechanisms (Gurunathan, 2000; Akbari, 1999): (*a*) direct transfection and priming of somatic

cells; (*b*) direct transfection and priming of dendritic cells; and (*c*) cross-priming, a process in which non-transfected dendritic cells take up antigens secreted extracellularly and/or derived from apoptotic bodies of DNA-transfected cells and present the processed peptides to CD4+ and CD8+ T cells. Dendritic cells are fundamental for DNA-induced immune responses (Akbari, 1999). Bacterial DNA has inherent adjuvant properties and can activate dendritic cells and trigger the production of co-stimulatory cytokines such as IL-12, IFN-α and -γ and TNF-α (Gurunathan, 2000), through interaction with the Toll-like receptor 9 (Hemmi, 2000). In one study, treatment of mice with unmethylated oligodeoxynucleotides could increase Th1 responses and reduce MTB growth in the lungs, even if given to infected mice (Juffermans, 2002). However, there are contradictory results to the real protective effect of these oligodeoxynucleotides in tuberculosis (Hsieh, 2004).

After the initial reports on DNA vaccination against TB, a series of new antigens were tested as DNA vaccines with variable degrees of success in different animal models and researchers again concentrated efforts on secreted proteins (CFPs) (Orme, 1997). DNA vaccines based on the extracellular antigen 85 complex, a set of three mycolyl transferases (85A, B and C), had been seen as promising candidates since the initial protective results against MTB obtained by Huygen and colleagues (1996). Successful vaccination with Ag85B in both mice (Kamath, 1999a) and guinea pigs (Baldwin, 1998) was also demonstrated. Interestingly, the protective effect of Ag85A and B vaccines seem to be mediated in mice by CD4+ T cells only (D'Souza, 2000) when administered by the intramuscular route of vaccination (Tanghe, 2000). Vaccination with MPT64 and ESAT-6 DNA also stimulated significant reductions in bacterial load of MTB in the lungs of mice, although not at the same level as BCG (Kamath, 1999a). MPT63, a 17-kDa secreted protein of MTB, conferred increased protection to a MTB challenge in mice when given in DNA form (Morris, 2000).

Potent T cell antigens are not exclusively seen in the CFP but also found in the cytoplasm of *M. tuberculosis*. Dillon and colleagues (1999) using a two-step immunoscreening of a genomic MTB library isolated MTB39, a cytoplasmatic protein containing the proline-proline-glutamic acid motif (PPE protein), showing increased protection in mice immunised with the DNA format. Yet another member of the PPE family, MTB41, induced protection comparable to BCG in mice (Skeiky, 2000). A complete and updated review of experimental DNA vaccines tested against TB can be seen in more detail elsewhere (Huygen, 2003).

Although powerful, DNA vaccines are still of limited efficacy and only rarely comparable to BCG. Several laboratories are testing different protocols to augment the efficiency of DNA vaccination and the prime-boost vaccination approach is one of the most promising strategies found (see below). Meanwhile, Kamath and collaborators (1999a) attempted multi-subunit vaccination with different vectors (MPT64, ESAT-6 and Ag85B), reporting better protection with simultaneous vaccination compared to single vaccines; similar results were obtained using different plasmid sets (Morris, 2000). Combinations up to ten plasmid vectors coding for different MTB antigens have been tested in mice, with improving rates of survival after infection, but still not better than BCG (Delogu, 2002). In this study, the authors fused the antigens either to the signal sequence of the human tissue plasminogen activator, for antigen secretion, or, to ubiquitin proteins, for increased MHC I processing (Delogu, 2002). Co-immunisation with DNA vaccines coding for Ag85B and MPT64 and plasmids expressing cytokines such as GM-CSF (Kamath, 1999b) or IL-12 (Palendira, 2002b) increased T cell immunity but results regarding protection in the murine-MTB challenge model were not very impressive. In addition, DNA coding for Ag85B has been also formulated in cationic lipids in order to improve immunogenicity and the targeting to APCs (D'Souza, 2002).

1.7 The prime-boost approach

Although versatile and being able to generate humoral immunity as well as CD4+ and CD8+ T cell responses, DNA vaccines have not shown impressive results in humans as compared to rodent models (Seder & Hill, 2000). In 1995, Leong and colleagues (Ramsay, 1999) have shown that consecutive immunisations, involving priming with DNA and boosting with poxvirus vectors, both encoding a common vaccine antigen, could generate unprecedented levels of specific immunity against a challenge with the native pathogen (Kent, 1998). This protocol could be defined as the heterologous prime-boost strategy. It involves giving two different vaccines, non-concomitantly, each encoding the same antigen (Li, 1993). Recombinant fowlpox viruses and attenuated vaccinia viruses such as modified Ankara strain (MVA) were considered safe for this protocol, because of their inability to replicate in mammalian cells (Ramsay, 1999). The superiority of this approach was evident against intracellular infections and tumours, enhancing CTL responses which are not easily induced by usual vaccines; Adrian Hill and colleagues at Oxford have obtained high numbers of specific IFN-γ+ CD8+ T cells and protection against malarial challenge using prime-boost of DNA and MVA, encoding for pre-erythrocytic antigens of *Plasmodium berghei* in a murine model (Schneider, 1998). This regimen induced strong cellular responses (although no protection) in a non-human primate malaria model (Schneider, 2001) and partial protection in humans (McConckey, 2003). Interestingly, only the sequential administration of priming with DNA and boosting with MVA appears to induce protective responses (Schneider, 1999); although in terms of mucosal immunity, the reverse order of vaccination seems to work better (Eo, 2001).

Effective results on the use of the prime-boost concept are available for several diseases: successful protection in experimental models is reported for viral diseases such as caused by Ebola virus (Sullivan, 2000) and hepatitis C (Matsui, 2003); protozoal *Leishmania infantum* (Ramiro, 2003) and tumours (Pasquini, 2002). Studies with immunodeficiency virus showed promising results in non-human primates (Amara, 2001; Wee, 2002) and in human phase I trials (Mwau, 2004). Prime-boost including proteins also worked in human papilloma (van der Burg, 2001) and dengue virus models (Simmons, 2001).

The mechanism that generates these high frequencies of CD8 T cells observed after prime-boost protocols still needs better clarification. Antigens carried by viral vectors and DNA vaccines are able to enter in both classical and alternative MHC-I processing pathways (Schneider, 1999). A potential mechanism is immunodominance, where a highly focused but moderate CD8+ T cell response to a few epitopes is induced after priming with DNA; the boost with the virus carrying the same antigen would again prime new cells but would enhance the previously primed "memory" CD8+ T cell population, which can react rapidly to the "danger signals" sent by the viral infection (Schneider, 1999; McShane, 2002). Ramsay and colleagues (1999) had proposed that, because of the low amounts of antigen available, a high avidity CTL population would be preferentially generated.

McShane and collaborators (2001) were the first to report the use of the prime-boost concept against tuberculosis: levels of protection similar to BCG were obtained in a murine model using a polyprotein construct (containing ESAT-6 and MPT63 antigens), cloned in a plasmid DNA vaccine and in MVA (McShane, 2001). Interestingly, in this protocol, IFN-γ +CD4+ T cells were significantly boosted but no evidence of CD8+ T cells producing IFN-γ was seen (McShane, 2001). Only partial protection to MTB infection was seen when using DNA-ESAT-6 plus recombinant

Salmonella typhimurium, instead of MVA as a booster (Mollenkopf, 2001). A DNA-protein prime-boost approach using antigen 85A and B has been able to increase Th1 responses and protection significantly although not equally to BCG in a MTB model (Tanghe, 2001). Similarly, Vordermeier and colleagues (2003) reported significantly better Th1 responses (both humoral and cellular) in a cattle model of tuberculosis, with heterologous DNA prime-protein boost using mycobacterial HSP65 (Vordermeier, 2003); lack of protection in cattle was reported when employing a similar strategy using MPB70 DNA-prime protein-boosting (Wedlock, 2003).

A few studies have evaluated prime-boost strategies including BCG. In a *M. avium* mouse model, prime-boost with recombinant BCG overexpressing the immunodominant 35-kD protein was not more efficient in terms of immune response or protection than the DNA vaccine alone (Martin, 2001). Priming with a DNA vaccine expressing antigen 85B and boosting with BCG improved the protective efficacy of BCG in the murine-MTB aerosol challenge model and depletion of CD8+ T cells clearly impaired the protection reported in this study (Feng, 2001). Finally, boosting BCG-primed mice with purified antigen 85A has significantly improved levels of protection against MTB aerosol challenge when the animals were elderly (Brooks, 2001).

1.8 Immunodominant antigens for vaccination against tuberculosis

1.8.1 Mycobacterial HSP65 and HSP70

Approximately forty years ago, someone inadvertently turned up the temperature of an incubator full of fruit flies; the salivary gland chromosomes from the flies, thus heat-shocked, showed the characteristic puffs, indicative of

transcriptional activity at discrete loci. These loci came later to be known to encode heat shock proteins (HSPs), which were gradually identified in all species tested (Srivastava, 2002a). HSPs are expressed in all cells, in all forms of life and in a variety of intracellular locations: from the cytosol of prokaryotes to several compartments of the eukaryotic cell. They constitute the single most abundant group of proteins inside cells and their expression can be powerfully induced as a result of heat shock and other forms of stress including toxins, oxidative stress and glucose deprivation. A collection of functions such as folding, unfolding and degradation of proteins, assembly of multi-subunit complexes, thermotolerance and others, have been attributed to HSPs (Srivastava, 2002a). These proteins can be classified into ten families; each consisting of one to five closely related proteins (Srivastava, 2002b). There is little homology between families but all families are present in every organism (Srivastava, 2002b).

In the 1980s, it was observed that homogeneous preparations of certain HSPs that were isolated from cancer cells elicited immunity and specific protection, whereas corresponding preparations from normal tissues did not (Srivastava, 1998). The immunogenicity of tumour-derived HSPs resulted from the non-covalent association of molecules such as gp96, HSP70, HSP90, calreticulin and GRP170 with peptides generated by the degradation of proteins expressed by tumour-cells (Srivastava, 2002b). HSP-peptide complexes were also reported to be protective for infectious diseases such as tuberculosis (Zügel, 2001). Further clarification of these effects came in subsequent studies: stripped of chaperoned peptides, HSP70 was ineffective in immunising against cancer cells (Udono, 1993). Exogenously administered HSP-peptide complexes behaved as adjuvants, inducing CD8+ T cell responses (Blachere, 1997). A surface receptor for HSPs in macrophages and dendritic cells has been recently identified: CD91 (which also binds α2-

macroglobulin), binds several HSPs with different affinities (Basu, 2001). Interaction of HSP-complexes with CD91 facilitates the transport of the complex to the cytosol (a proportion of the HSP-peptide complex also ends in acidic compartments for MHC-II processing). Other receptors such as TLR2, TLR4, CD36 and CD14 were suggested to be involved in different immunogenic functions induced by HSPs such as production of cytokines, chemokines and APC activation (Srivastava, 2002a; Srivastava, 2002b). Interestingly, mycobacterial but not human HSP70 bind to CD40 and stimulate production of CC-chemokines MIP-1α, MIP-1β and RANTES in human cell lines (Wang, 2001).

The *M. tuberculosis* HSP70 (MtbHSP70) is homologous to the DnaK protein from *E. coli* and was initially described in 1989 (Mehlert & Young, 1989). It was noticed that this protein had potential for immunomodulation; purified MtbHSP70 plus a powerful adjuvant were able to induce high levels of protection in the guinea pig model (Horwitz, 1995). In the DNA format, mycobacterial HSP70 also elicited a significant prophylactic effect against MTB in outbred and BALB/c mice (Tascon, 1997; Lowrie, 1997), although had lower efficacy when administered therapeutically to infected mice (Lowrie, 1999). Mycobacterial peptides complexed with MtbHSP70 evoked increased DTH reaction in mice (Roman & Moreno, 1997). Interestingly, MtbHSP70 fused with human papilloma virus (HPV) E7 protein and administered in DNA format, improved protection against cervical cancer (Chen, 2000). Gene fusion with MtbHSP70 generates potent CD8+ CTL responses to the fusion partner, independent of CD4+ T cell help (Huang, 2000). Regarding *M. tuberculosis*, overexpression of HSP70 (and other HSPs) is not beneficial for survival of the bacilli, particularly in the chronic phase of infection (Stewart, 2001), supporting the idea of "danger signals", whereby the host immune system recognise high levels of HSPs as

a warning signal for the presence of pathogens (Lowrie, 1998; Colaco, 2001; Srivastava, 2002b).

A DNA vaccine encoding the 65-kDa-heat shock protein (HSP65) of *M. leprae* has demonstrated significant levels of protection against experimental infection with *M. tuberculosis* in mice (Tascon, 1996). The HSP65 protein from *M. leprae* shares 95% of homology with the *M. tuberculosis* protein (Tascon, 1996). Most bacteria encode only one chaperonin 60 (also known as GroEL in *E. coli*), however, in the MTB genome there are two genes: *cpnL1*, encoding the HSP60.1 and *cpnL2* (encoding the known HSP65 or HSP60.2); HSP60.1 from *M. tuberculosis* has been found to be between 10- to 100-fold more active in inducing proinflammatory cytokine synthesis than its counterpart HSP65 (Lewthwaite, 2001). HSP65 DNA vaccine induced substantial protection against intravenous or intraperitoneal MTB challenge; animals immunised with several doses of this antigen expressed significant reductions in bacterial load in target organs after challenge infection, with protection levels comparable to BCG (Tascon, 1996; Lowrie, 1997; Bonato, 1998). This effect appeared to be strictly dependent of the endogenous nature of the delivery process; HSP65 protein alone was ineffective but a murine tumour cell line transfected with the gene and injected in mice exhibited high degree of protection (Silva & Lowrie, 1994). Although HSP65 DNA induced both CD4+ and CD8+ T cell responses, the cell population most effective at transferring protection was associated with activated $CD44^{high}$ CD8+ IFN-γ+ T cells (Lowrie, 1997; Bonato, 1998; Silva & Lowrie, 2000). The HSP65 DNA vaccine was also administered to mice as an immunotherapeutic vaccine (Lowrie, 1999). However, lack of protection and autoimmune pathology has been also reported with the use of DNA vaccines in aerosol models of MTB infection, both in mice and guinea pigs (Turner, 2000;

Taylor, 2003). HSP65 and Ag85A DNA, were also reported to induce failure or severe reactions in both prophylactic and immunotherapeutic modes (Repique, 2002).

1.8.2 MPT83 and MPT70

Mycobacterial protein 70 (MPB70) was initially isolated from culture filtrates of *Mycobacterium bovis* BCG (Nagai, 1981). MPT is used in similar designations for proteins purified from *M. tuberculosis* (Harboe, 1998). Cloning of this 22-kDa protein revealed the sequence of a polypeptide chain preceded by a signal peptide that is typical from secreted proteins and in addition, that this protein was soluble and non-glycosylated (Harboe, 1998). MPB70 structure has been resolved recently (Carr, 2003). There are no differences between *M. bovis* and *M. tuberculosis* in terms of amino acid sequences, for the proteins encoded by the *mpb70/mpt70* genes (Wiker, 1998). MPB70 is an important target for humoral and cellular immune responses during infection with bovine tubercle bacilli and has been exploited in the development of tests for diagnosis of bovine TB (Rhodes, 2000). Cellular *in vitro* immune responses to purified MPB70 were also prominent in human TB (Roche, 1994; Al-Attiyah, 2003). When used as a DNA vaccine, MPT70 was able to induce CD4+ T cell based-proliferative responses in cattle (Vordermeier, 2001). Moreover, given as an immunotherapeutic DNA vaccine to MTB-infected Balb/c mice, MPT70 was able to induce a very significant decline in bacterial counts in both lungs and spleen (Lowrie, 1999). However, vaccination of cattle with DNA coding for MPB70 did not show protection against bovine tuberculosis (Wedlock, 2003).

MPT83 shares 61% homology with MPT70 at the amino acid level; the genes encoding these two proteins are arranged in an operon-like structure and are expressed under identical conditions (Hewinson, 1996; Juarez, 2001). The genes from

M. bovis and *M. tuberculosis* are indistinguishable. Mature MPB83 differs from MPB70 by the presence of a typical lipoprotein consensus motif and also an insert of 35 amino acids at its N-terminus with a putative glycosylation site; the 26-kDa form is an exported lipoprotein associated with the bacillary cell surface while the 23-kDa variant is secreted, glycosylated and non-lipoylated (Wiker, 1998; Harboe, 1998). MPT83 has no identifiable function; it is well recognised by T lymphocytes from *M. bovis* infected- but not BCG-vaccinated cattle, demonstrating potential as a diagnostic tool (Vordermeier, 1999; Vordermeier, 2001). Vaccination with plasmid encoding the MPT83 gene was found to protect mice to a certain extent against a challenge with *M. bovis* (Chambers, 2000) and *M. tuberculosis* (Morris, 2000), but was not able to provide protection against bovine tuberculosis in cattle (Wedlock, 2003). In a *M. bovis* infection model in guinea pigs, DNA vaccination with MPT83 slightly reduced lung pathology but not at the same level as BCG (Chambers, 2002).

1.8.3 Mycobacterial Apa protein

During a series of purification procedures on culture filtrates of *M. tuberculosis*, Nagai and colleagues (1991) identified and purified to homogeneity a secreted protein named by them as MPT32. When injected intradermally in Guinea pigs previously sensitised with dried cells of MTB, this protein induced a lower but quite distinct reactivity (Nagai, 1991). In an independent work, a fraction of 45/47-kDa bands was purified from culture filtrates of BCG (Romain, 1993); it interacted only with the sera from Guinea pigs injected with live but not dead BCG. This fraction showed to be forty times more potent in eliciting DTH responses in animals sensitised with live versus dead BCG, yet it represented less than 2% of the total material excreted by BCG (Romain, 1993).

In 1995, the gene coding for the 45/47-kDa complex (or MPT32) of *M. tuberculosis* was cloned and sequenced (Laqueyrerie, 1995). The gene had an open reading frame of 975bp (base pairs), coding for a protein of 325 amino acids (predicted molecular mass of 28.7-kDa), with a N-terminal signal sequence for secretion of 39 amino acids; the gene, present as a single copy in the genome, was named as *apa* (*a*lanine-*p*roline-rich protein) due to the high percentages of proline (21.7%) and alanine (19%) in the purified protein (Laqueyrerie, 1995). The apparent high molecular mass as determined by SDS-PAGE could be explained from the increased rigidity of molecules due to the high content of proline residues. The function of Apa remains undetermined. Apa protein had sequence homology of 66% with a fibronectin-binding protein of *M. leprae* (43L gene), but no specific consensus sites or motifs had been found to characterise the function of the molecule (Laqueyrerie, 1995). The *apa* gene [(named *ModD*, after the compilation of the *M. tuberculosis* genome (Cole, 1998)], was found in the same operon of a putative ABC transporter system highly similar to the molybdate importer system of *E. coli* (fig.1.5); interestingly, this putative operon, encoded by three genes (*modA-modB-modC*), seems already functionally complete without the *apa* gene itself (Braibant, 2000).

Protein glycosylation is an unusual finding in eubacteria. Initial evidence showed the Apa protein to react with Concanavalin A, demonstrating the presence of mannose (Dobos, 1995; Espitia, 1995). Further characterisation revealed universal O-glycosylation of threonine residues with α-D mannose units only; glycosylation sites were located at threonines 10 and 18 (mannobiose), 27 (single mannose) and 277 (either mannose, mannobiose or mannotriose) (Dobos, 1996).

A more detailed study revealed that the 47-kDa glycoprotein purified from *M. tuberculosis*, *M. bovis* or BCG was composed of nine glycoforms; a small number of

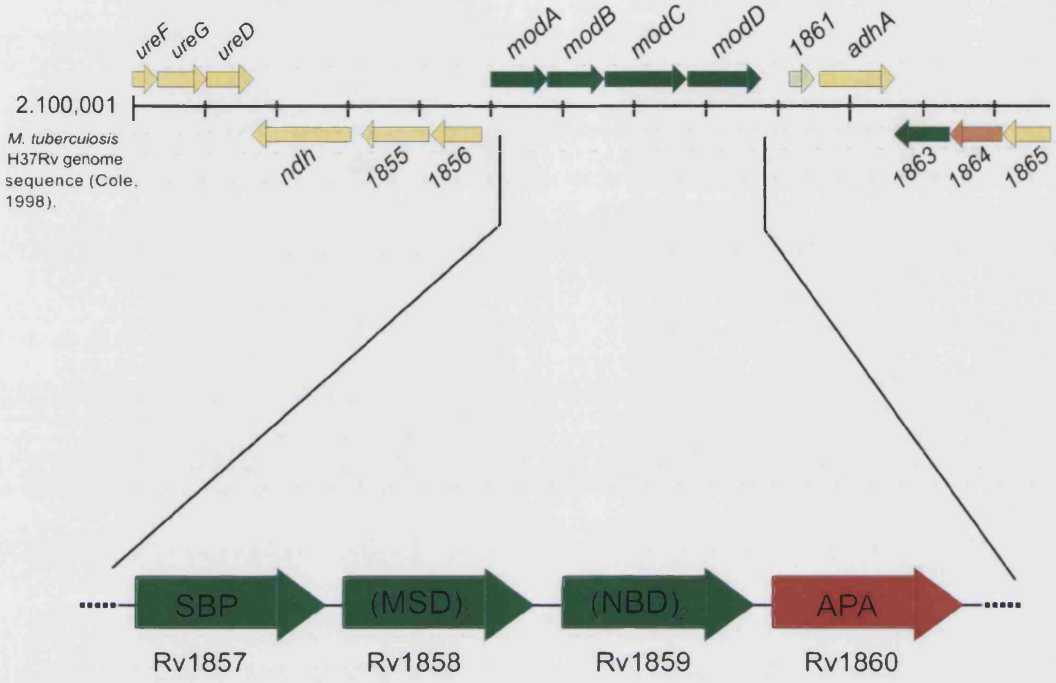


Figure 1.5 Schematic representation of the position of the *apa* gene in the recently annotated *M. tuberculosis* H37Rv genome (Cole, 1998). The *apa* gene is found in the same operon as the putative ABC molybdate transporter. The transporter is composed of a substrate binding protein (SBP-*modA*), two copies of a membrane spanning domain (MSD-*modB*) and two copies of the nucleotide binding domain (NBD-*modC*), according to the prokariotic prototype system in *E. coli* (Braibant, 2000). Modified from Cole and collaborators (1998).

molecules were non-glycosylated, whereas others had up to nine mannose residues per mole of protein (Romain, 1999; Horn, 1999). The 45-kDa band was found to be a COOH-terminal- truncated form of the native Apa (Romain, 1999; Horn, 1999). Interestingly, Apa molecules when deglycosylated, had a significant decrease in the capacity to induce DTH and were also less potent in stimulating *in vitro* proliferation of lymphocytes from BCG-vaccinated Guinea pigs (Romain, 1999; Horn, 1999).

In 1995, Espitia and collaborators presented the first evidences for the potential of Apa for diagnostic use. These authors reported that sera from 70% of individuals with pulmonary tuberculosis recognised the native protein specifically (Espitia, 1995). Using two-dimensional electrophoresis, Samanich and collaborators (1998) demonstrated the native form of Apa to have serodiagnostic potential, being recognised by sera from both early cavitary and non-cavitary TB patients. But further results were not as encouraging. HIV-positive-smear positive TB patients were poor reactors to Apa (Samanich, 2000). The recombinant form of the protein also had reduced reactivity to human TB sera (Samanich, 2000). In addition, tests on patients from Madagascar concluded that imunodiagnosis based on the Apa protein were not sufficiently predictive when compared to routine microscopy (Chanteau, 2000).

Immune responses to Apa protein were reported initially in Guinea pigs to be CD4+ but not CD8+ T cell-dependent (Romain, 1999; Horn, 1999). Besides induction of a significant amount of IFN-γ, a DNA vaccine encoding the *apa* gene was not protective in mice against a challenge with *M. tuberculosis* (Morris, 2000). However, more recent results demonstrated partial protection levels after *M. bovis* BCG infection (Garapin, 2001). Recently, a prime-boost regimen using Apa as DNA vaccine plus live MVA-Apa as the booster, were claimed to be as protective as BCG in an intramuscular MTB infection model in Guinea pigs (Kumar, 2003). In the same article, the authors identified peripheral blood mononuclear cells (PBMC) from

healthy PPD+ human donors proliferating and producing significant amounts of IFN-γ to recombinant Apa *in vitro*, suggesting a role for this particular mycobacterial protein in the protective immunity against tuberculosis (Kumar, 2003).

1.9 Project aims

The aim of this thesis was to study new ways to obtain better protection against tuberculosis in a well-characterised model of infection. I first cloned and expressed the *M. tuberculosis* Apa antigen and then proceeded to investigate the immunogenicity of this antigen when used as DNA vaccine in mice. Importantly, I also tried to improve the efficacy of DNA vaccination against tuberculosis by using a DNA-prime BCG-boost vaccination strategy using different DNA plasmids expressing mycobacterial immunodominant antigens: Apa and other antigens such as HSP65, HSP70, MPT83 and MPT70 were tested alone or in various plasmid cocktail combinations and doses in two mice strains. Concomitantly, humoral and cellular responses with cytokine profiles were dissected, both before and after challenge with *M. tuberculosis*. Ultimately, the final aim of this project was to find a regimen capable of improving BCG protective abilities in mice.

CHAPTER TWO

Material and Methods

2.1 Materials, media, solutions and buffers

All chemicals and reagents used were of analytical grade and were obtained from BDH Chemicals Ltd., Poole, UK, unless otherwise stated. Compositions of media, solutions and buffers are described when necessary in each technique.

2.2 Bacterial strains

2.2.1 Escherichia coli

E. coli One Shot™ TOP10 competent strain (Invitrogen, Groningen, NL) was used as the host for pSecT2C.Apa and pCMV4.Apa vectors and also for the propagation of pHSP65 and pHSP70 vectors. Epicurian Coli™ Supercompetent *E. coli* XL1-BLUE strain (Stratagene, La Jolla, CA) was used to propagate the pQE60.Apa vector. Bacteria were grown in Luria-Bertani (LB) medium (Sambrook, 1989) containing 100μg/ml of ampicillin at 37^0C with rotation. *E. coli* M15 strain (QIAgen, Sussex, UK) was transformed with pQE60.Apa for expression and purification of the recombinant Apa protein. The M15 strain harbours a second plasmid, pREP4, expressing both a Lac-I regulator and a kanamycin resistance gene (see fig.3.3). The M15 strain was grown at 37°C in LB medium containing 100μg/ml ampicillin and 25μg/ml kanamycin.

2.2.2 Mycobacterium bovis - BCG

BCG-Glaxo (Glaxo-SmithKline, UK) vaccine strain was grown in Petri dishes of Middlebrook 7H11 Bacto-agar (Difco, Becton Dickinson, Oxford, UK) plus Bacto Middlebrook OADC (oleic acid, albumin, dextrose, catalase) enrichment (Difco), at 37°C. Single colonies were inoculated and grown statically as a surface pellicule in Sauton's media (for 1L, this medium contains 4g asparagine, 2g citric acid, 0.5g KH_2PO_4, 0.5g $MgSO_4 \cdot 7\ H_2O$, 50mg iron (III) citrate, 1.4mg $ZnSO_4 \cdot 7H_2O$, and 57 mL glycerol; the pH is adjusted to 7.2 with NH_4OH) or dispersed in Difco's Dubos broth (Becton Dickinson) plus 0.2% glycerol and Dubos broth albumin (Difco), and was harvested at $O.D._{500}$ of 0.5-0.8.

2.2.3 Mycobacterium tuberculosis

M. tuberculosis H37Rv and 1424 (streptomycin-resistant) strains were grown in rolling bottles under rotation at 37°C, containing Difco's Dubos broth (Becton Dickinson) plus 0.2% glycerol and supplemented with Dubos broth albumin (Difco). MTB strains were harvested at $O.D._{500}$ of 0.5. For colony counting, serial dilutions of organ homogenates from infected animals in saline were plated in 7H11 Petri dishes and grown for 2-3 weeks at 37°C.

2.3 Preparation of *E. coli* competent cells and bacterial transformation

E. coli M15 cells were grown in LB broth until $O.D._{600}$ reached 0.35 – 0.4. The cells were collected, cooled in ice for 10min, centrifuged at 5000rpm for 10min at 4°C and washed twice with 20ml of cold 0.1M $CaCl_2$ with an incubation of 30

minutes in ice between the washes. The bacterial pellet was finally resuspended in 2ml of 0.1M $CaCl_2$. 0.6ml of glycerol was added and this suspension was frozen in a dry-ice-ethanol mix. Aliquots were kept at –70°C. Transformations were performed by adding 50ng of plasmid to each aliquot of cells and heat-shocking it at 42°C (Sambrook, 1989). Cells were grown with shaking for one hour in SOC medium (0.5% yeast extract, 2% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM $MgSO_4$, 20mM glucose) and then plated in LB-agar containing the appropriate antibiotics for selection.

2.4 Eukariotic cell lines

2.4.1 CV1

CV1 cells (African green monkey kidney cells, a kind gift from Dr. Tony Magee, NIMR) were grown in Iscove's modified DMEM medium (IMDM, Sigma) plus 4mM L-glutamine (Sigma), 80uM of 2-β-mercaptoethanol and 5% fetal calf serum (complete IMDM) in 5% CO_2 at 37°C. Cells were passed by scraping when 50-70% confluent. These cells were used in transient transfections for *in vitro* expression of DNA vaccines.

2.4.2 THP-1

THP-1 cells (human acute monocytic leukaemia, ECACC No. 88081201) was maintained by dilution passage in RPMI 1640 medium (Invitrogen) plus 10% FCS, 80μM β-mercaptoethanol and 2mM L-glutamine. This cell line was used for *in vitro* infection with MTB and RNA purification from the intracellular bacteria.

2.4.3 RAW 264

This cell line (mouse monocyte-macrophage leukaemia, a kind gift from Dr. Ruth Aubrey, NIMR) was grown in complete IMDM. RAW264 cells were also used as host for infection with MTB.

2.4.4 EL-4

This cell line (ECACC No. 85023105) is lymphoblastic in morphology and resulted from a T cell lymphoma induced in C57BL/6. The cells were grown in suspension and were maintained by dilution passage in complete IMDM. This cell line was used to obtain stable transfectants expressing the *apa* gene and as labelled targets in the JAM cytotoxicity assay (Matzinger, 1991).

2.5 Quantification of DNA

DNA concentration and relative purity in solution were determined by measuring the absorbance at 260 and 280nm using a Unican UV/Vis spectrophotometer (A_{260} = 1.00 corresponds to a DNA concentration of 50μg/ml). A ratio A_{260}/A_{280} between 1.8 – 2.0 indicated low amounts of DNAse contamination.

2.6 Agarose gel electrophoresis

Fragments of DNA and RNA were separated by electrophoresis in molecular biology grade agarose (BioRad, Gottingen, The Netherlands) dissolved in TBE buffer (1-1.5% w/v in 89mM Tris-Borate, 2mM EDTA, pH 8.0). The gels contained

0.5µg/ml of ethidium bromide (EtBr) for visualisation of the bands in UV light. Routinely, an 1kb DNA-ladder (Invitrogen) was used. The samples were diluted in loading buffer solution containing 20% Ficoll 400 and 0.1g Orange G.

2.7 Primer design

Primers were designed based on the full-length *apa* gene (Rv1860) sequence (Acession no. x80268 from the NCBI Genbank Database (http://www.ncbi.nlm.nih.gov/)). Primers for the amplification of the entire coding sequence of Apa, including the N-terminal signal peptide, were constructed with a BamH I and a Not I site for directional cloning in both pSecT2C (Invitrogen) and pCMV4 (Tascon, 1997) vectors (Table 2.1).

pSecApa forward	5'-ATTGGATCCGGATGCATCAGGTGGAC-3'
pSecApa reverse	5'-TATGCGGCCGCCCAGGCCGGTAAG-3'
pCMV4.Apa forward	5'-ATTGGATCCGCCATGCATCAGGTGGAC-3'
pCMV4.Apa reverse	5'-TATGCGGCCGCCTCAGGCCGGTAAG-3'

Table 2.1: Primers used for amplification and cloning of *apa* in mammalian vectors.

For cloning into pQE60 vector (QIAgen), primers to amplify the entire coding sequence of the *apa* gene, including the N-terminal signal sequence, were designed to contain a BamH I and a Nco I sites (Table 2.2). All primers described were synthesised by Oswel (Southampton, UK).

pQE60 forward	5'-CATGCCATGGTACAGGTGGACCCCAACTTGACA-3'
pQE60 reverse:	5'- TTAGGATCCGGCCGGTAAGGTCCGCTGCGGTGT-3'

Table 2.2: Primers used for amplification and cloning of *apa* in pQE60 vector.

For use in Real-Time® reverse transcriptase (RT)–PCR, internal primers specific for the *apa* gene were constructed. In addition, an internal labelled probe specific for the *apa* gene containing both a fluorophore (FAM™: 6-carboxy-fluorescein) and a quencher was also synthesised. Both were designed with the help of the Primer Express™ program (ABI-Prism, Applied Biosystems). Primers and probe were synthesised by Applied Biosystems (Foster City, USA). For the *apa* gene: forward primer, GGAAGCGCGTCGTATTACGA; reverse primer, ATTACGCCCGTCCAGATCTG; FAM™-labelled probe, CAAGTTCAGCGATCCGAGTAAGCCGAAC. For the control gene: *sigA* forward primer, TCGGTTCGCGCCTACCT; *sigA* reverse primer, TGGCTAGCTCGACCTCTTCCT; *sigA* FAM™-labelled probe, TTGAGCAGCGCTACCTTGCCG.

2.8 Amplification of DNA by Polymerase Chain Reaction (PCR)

PCR reactions (50µl reaction) were performed using 32ng of genomic DNA of *M. tuberculosis* H37Rv diluted in PCR buffer [10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris pH 8.74, 0.1% Triton 100, 100µg/ml BSA]. Amplifications were done using 0,5µl of 20µM primers plus 2.5U per reaction of high accuracy cloned *Pfu* DNA polymerase (Stratagene, La Jolla, USA). The annealing temperature was 55°C and

extension time of 1.5min. For the construction of pQE60.Apa, PCR reactions were performed as described above but with an increase in the annealing temperature from 55°C to 60°C. Amplification reactions were run in 0.5ml Eppendorf tubes in a CycleAmp PCR machine (Perkin-Elmer). The resulting PCR product was electrophoresed in agarose gels.

2.9 Isolation of mycobacterial RNA from infected mammalian cells

Bone marrow (BM) derived macrophages were isolated as described previously for BM-derived dendritic cells (Tascon, 2000) and reported in **2.24** of this thesis. Only adherent cells were selected. Approximately 5 x 10^7 cells were seeded to 175cm^2 culture flasks with 30 ml of medium. RAW 264 cells were adjusted at 1 x 10^8 cells/175cm^2 culture flasks. In both cases, to activate the cells, 100U/ml of mouse recombinant IFN-γ cytokine (Roche) was added to the flasks for 16 hours at 37^{0}C. THP-1 cells were distributed at 5 x 10^7 cells in 175cm^2 flasks containing 50ml of media. The cells were stimulated for 24 hours with 12ng/ml of PMA (Sigma), diluted in DMSO. When the cells were adherent, 100U/ml of human recombinant IFN-γ (Roche) was added and incubation proceeded for further 16 hours.

Infection with *M. tuberculosis* was carried out at 10:1 bacteria/cell ratio during 8 hours of incubation at 37^{0}C. BMDCs and THP-1 cells were infected with the 1424 streptomycin-resistant MTB strain. RAW 264 cells were infected with the H37Rv reference strain. Afterwards, the cells were washed 3 times with cold and sterile PBS to remove extracellular bacteria. Lysis of the cells was induced by the addition of saponin (fig.2.1), diluted in media (final concentration of 1%), followed by a period of 40 minutes at RT. The saponin was eliminated by two washes with PBS by centrifugation at 3000rpm. RNA was extracted from the intracellular mycobacteria,

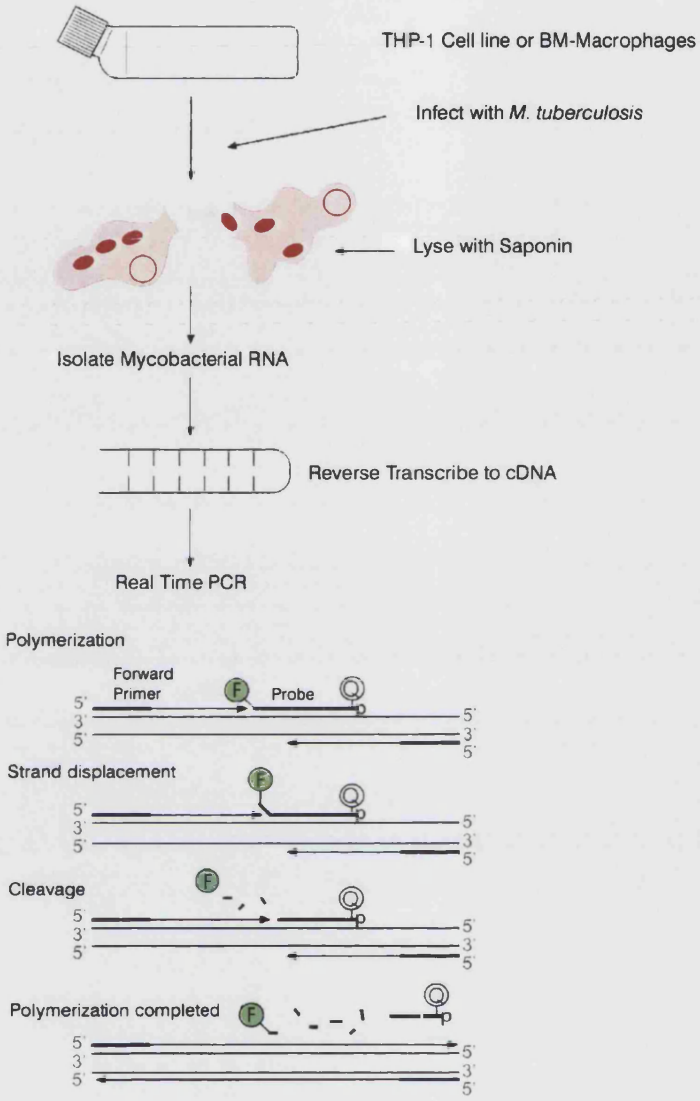


Figure 2.1 Real-time PCR with intracellular bacteria. Intracellular *M. tuberculosis* is partially separated from eukariotic cellular debris by centrifugation. Specific primers and probes for *apa* and *sigA* genes were used. The probes undergoes spontaneous fluorogenic conformational change if hybridised with the specific target but not in solution. Capture of fluorescence allows for the direct measurement of the amount of amplicons when PCR reaction is on logarithmic phase. Q, quencher molecule; p, primer; F, fluorophore.

using a Hybaid RyboLyser apparatus in conjunction with a Hybaid Recovery Blue kit (Hybaid, Little Chalfont, UK), for lysing the cells and isolation of mycobacterial RNA, according with the manufacturer's protocol. The resulting RNA was dissolved with 50µl of RNAse-free water and kept at -70^{0}C until DNAase treatment was performed.

2.9.1 ***Dnase treatment***

This RNA was treated twice with RNAse-free DNAse RQ1 (Promega) in order to avoid carrying contamination of genomic DNA. Briefly, the RNA dissolved in water was mixed with 6U of DNAse, 2µl of RNAsin inhibitor (Promega), 20mM $MgSO_4$, 100mM Sodium Acetate pH 5.2, in a final volume of 150µl. This mixture was initially incubated 1 hour at 37^{0}C, and 6U of enzyme plus RNAse inhibitor added afterwards, followed by another hour of digestion at 37^{0}C. The reaction contents were cleaned of protein contamination with the RNEasy mini kit (QIAgen Ltd, West Sussex, UK), according to the manufacturers protocol. RNA was finally eluted with 50µl of RNAse-free water. A routine PCR reaction was performed using mycobacteria gene-specific primers and the processed RNA as the template, to assure the absence of DNA contamination.

2.10 Real-Time™ reverse transcriptase (RT) – PCR

The mycobacterial RNA was reverse-transcribed to cDNA using specific reverse primers for the *apa* gene and *sigA* factor, the latter showing constitutive expression in *M. tuberculosis*, was considered a good candidate to be the internal

control (Manganelli, 1999). The reverse transcription reaction was performed using Superscript RT II (Invitrogen) reverse transcriptase as described previously (Raynaud, 2002). The resulting cDNAs were used in a quantitative RT-PCR assay determined in real time using fluorescent molecular beacons: oligonucleotide probes homologous to central parts of the gene and coupled to a quenched fluorophore. The fluorescence is restored at the binding of the probe to the target, which in this case is the resulting PCR product (fig.2.1). The intensity of the fluorescence in each amplification step is a direct measure of amplicon concentration (Piatek, 1998). A standard curve was set using serial dilutions at pg/ml of mycobacterial DNA. Samples were assayed in duplicate and to increase the statistical significance of the results, several assays were run for the same sample. In all assays, the 25µl reactions consisted of 12.5µl of PCR master mix (Promega), 4µl of template, 5 picomoles of each primer and 2.5 picomol of the appropriate probe. Reactions were carried out in sealed tubes. The Real-Time PCR reaction was performed in a Taqman™ PCR machine (ABI-PRISM 7700 Sequence detector, Applied Biosystems, Foster City, USA). Results were analysed with the Sequence Detector program (ABI-PRISM, Applied Biosystems).

2.11 Cloning into plasmid vectors

2.11.1 Construction of pCMV4.Apa and pSecT2C.Apa vectors

A 1Kb DNA product was obtained by PCR using *apa*-specific primers, containing the restriction sites; the DNA product was gel-purified for further cloning using the QIAquick gel extraction kit (QIAgen). Vectors pCMV4 and pSecT2C and insert were digested with BamH I and Not I and gel-purified. The ligation reaction

was performed using 1µl of T4-DNA ligase in 10µl of ligation buffer (Boehringer-Mannheim, Sussex, UK) plus purified plasmids and PCR products at 1:2 molar ratio, respectively. A small aliquot of this mixture was used to transform *E. coli* One Shot TOP10 supercompetent cells (Invitrogen). Selection was done in 100mm LB–agar Petri dishes containing 100µg/ml of ampicillin. Colonies were selected at random and plasmids purified using the QIAprep spin miniprep kit (QIAgen). Restriction enzyme mapping identified the recombinant plasmids pCMV4.Apa and pSecT2C.Apa.

2.11.2 Construction of pQE60.Apa vector

The bacterial expression vector pQE60 was obtained from QIAgen. This vector allows fusion of the cloned sequence with a 6xHis tag in the C-terminal part of the recombinant protein. The vector was also gel-purified and double-digested with BamH I and Nco I. PCR fragment and open vector were ligated using T4-DNA ligase in the same conditions described above. *E. coli* XL1-BLUE cells were transformed with the ligation mixture. Recombinant colonies were selected in ampicillin plates and restriction enzyme analysis was used to identify positive clones.

2.12 Purification of plasmidial DNA for vaccination

Plasmids were purified using Endofree QIAfilter Plasmid Giga Kit (QIAgen), according to supplier's protocol. However, in order to improve yields of plasmid DNA per starter culture, the QIAgen plasmid purification columns were re-used, according to a protocol developed by Gregory and colleagues (1999). Briefly, after the initial run, the columns were washed with 100ml of endotoxin-free water and equilibrated with 50ml of buffer QBT, supplied with the kit. The flow-through lysate

from the previous run was saved and passed again on the regenerated column up to three times still yielding significant amounts of plasmid DNA. Volumes of starter bacterial cultures were typically two litres, with yields varying between 5-12mg of purified plasmid DNA per run. Plasmids were dissolved in low-endotoxin sterile saline (Sigma) and kept at -20^{0}C.

2.12.1 Restriction endonuclease mapping

Plasmids	Restriction enzymes
pCMV4.83 (MPT83)	BamH I, Xho I
pCMV.70 (HSP70)	Hind III, Pst I
pCMV4.65 (HSP65)	Bgl II
pBT7 (MPT70)	BamH I, Xba I
pCMV4.Apa (Apa)	BamH I, Not I, Hind III
pCMV4 (vector)	BamH I, Not I, Hind III

Table 2.3: Restriction enzymes used for restriction mapping in mammalian vectors.

To certify for the true type of plasmid being used, a diagnostic panel of restriction enzymes was used (Table 2.3). Digestions were monitored by agarose-gel electrophoresis. All plasmid batches were tested before use in animal vaccination. The enzymes were used according to the manufacturer's protocol (New England Biolabs, Gibco-BRL and Roche).

2.12.2 Endotoxin determination

Plasmids were tested for endotoxin levels with the limulus amebocyte lysate QCL-1000 assay (Biowhittaker, Walkersville, MD), according to the manufacturer's instructions. This is a very sensitive chromogenic test to detect gram-negative bacterial endotoxin (LPS). Basically, the LPS catalyses the activation of a proenzyme in the limulus amoebocyte lysate (LAL) and the rate of activation is dependent on the concentration of LPS present. The activated enzyme catalyses the splitting of p-nitroaniline (pNA) from the colourless substrate supplied with the kit and the pNA is measured photometrically at 405nm in a EL312 microplate ELISA reader (Bio-Tek Instruments, Winooski, USA), after the reaction is stopped with 25% v/v glacial acetic acid in water. Samples were routinely tested in duplicate by the microplate method. Plasmids were used only when endotoxin levels were below 0.1 EU/μg of DNA. All plasmid DNA destined for injection and also, recombinant proteins for *in vitro* cell culture use were evaluated for endotoxin content by this assay.

2.13 DNA transfection

2.13.1 Transient

Transient transfections were performed to certify the ability of the plasmid constructs to express the encoding gene in mammalian cells and for purification of histidine-tagged recombinant Apa. Briefly, 180μg of endotoxin-free plasmids pCMV4.Apa or pSecT2C.Apa, diluted in 9ml of serum-free Optimem™ medium (Invitrogen), were incubated at RT for 45 minutes. 364μl of Lipofectin reagent (Gibco-BRL, Paisley, UK), was diluted in 3.64ml of the same media and also incubated at RT for 45 minutes. DNA and liposome solutions were mixed gently and left undisturbed at RT for further 15 minutes. At the end of this period, 28ml of media

was added to the DNA-liposome mix and this solution transferred to one 175cm^2 bottle containing CV1 cells at 40-50% confluency in 15ml of Optimem®. At t = 16 hours of incubation at 37^{0}C, the cell monolayer was washed and new media added. Supernatants were recovered at t = 21hs and t = 48hs, filtered through 0.2μm membranes and concentrated by lyophilisation. The cells were grown for 48h before being collected for the preparation of a cell extract to test for specific protein expression.

For purification of recombinant Apa, transfection procedures were similar to what is described above, but the plasmid used was pSecTag2C.Apa and the number of 175cm^2 flasks used increased to ten. Supernatants were concentrated by ultrafiltration and dialysed against sterile PBS buffer. A protease-inhibitor cocktail (Roche, Sussex, UK) was added to some batches. All procedures were done at 4^{0}C. Concentrated samples were kept at –70^{0}C until further purification by affinity chromatography.

*2.13.2 **Stable***

For the generation of stable transfectants expressing the Apa antigen, EL-4 cells were plated at a density of 1 x 10^5 cells/well in a 24-wells plate containing 0.5ml of IMDM complete media. For one plate, 30μg of sterile endotoxin-free pCMV4.Apa was diluted in 1.5ml of IMDM media without fetal calf serum (FCS). 62.5μl of Lipofectamine 2000 reagent (Invitrogen) was also diluted in 1.5ml of media without FCS. These components were mixed gently but immediately and incubated at room temperature for 20 minutes. Afterwards, 100μl of the DNA-cationic lipid mix were added per well, and the plate rocked gently. After 4 hours of incubation at 37^{0}C,

1.5ml of complete IMDM was added to each well. After 16 hours, the cells were collected, centrifuged and transferred with new media to 80cm^2 Nunc flasks. After 48 hours, selection reagent Geneticin™ (G418-Gibco) was added to the transfected cultures at 0.8mg/ml. The cells were left incubating for two weeks and the media changed weekly. At the end of this period, survivor clones were selected by limiting dilution at less than 1 cell/well. Apa-expressing clones were identified by Western-blot of cell extracts with monoclonal antibody 6A3 and kept in liquid nitrogen.

2.14 Preparation of protein extracts and culture filtrates

2.14.1 Transfected CV1 cells

The cells were grown for 48h before being scraped off for the preparation of a lysate. After centrifugation for 5 minutes at 1200rpm, the pellet was washed once in Dulbecco's PBS. Both cell lysate and lyophilised supernatant were resuspended in RIPA buffer [PBS, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxicholate, 0.1% SDS)] plus protein inhibitor cocktail [(AEBSF 100μg/ml, 2μg/ml of leupeptin (ICN, Aurora, OH), sodium orthovanadate 100mM and aprotinin 30μl/ml (Sigma, Dorset, UK)]. This mixture was allowed to stand for 30 minutes in ice, transferred to Eppendorf tubes and finally centrifuged for 10 minutes at 10.000xg; supernatants were recovered and kept at –70°C.

2.14.2 BCG-Glaxo

BCG-Glaxo strain was inoculated in Sauton's broth and grown for 2 weeks at 37°C. Acid-fast staining confirmed the purity of the cultures. Cells were harvested by

centrifugation for 40 minutes at 10.000xg, 4°C. The cell-pellet was snap-frozen in dry ice- absolute ethanol mix and kept for 24 hours at -70°C. Approximately 200ml of culture fluid was initially filtered through a GF/F membrane (Whatman, Maidstone, UK) and later concentrated 20X by ultrafiltration in a Stirred Cell model 8400 (Amicon-Millipore, Watford, UK) equipped with a 3000MW regenerated cellulose membrane (Millipore). Afterwards, the concentrated sample was dialysed overnight against water. This material (8ml) was lyophilised and resuspended in 1ml of sample buffer for SDS-PAGE. The cell pellet was thawed and processed according to the method described by Urquhart (1997); briefly, the pellet was resuspended in 10ml of sample washing buffer (**SWB**: KCl, 3mM; KH_2PO_4, 1.5mM; NaCl, 68mM; NaH_2PO_4, 9mM). After washing three times (10.000xg, 50 minutes) in this solution, the pellet was mixed with 0.5g of 150-212 microns glass beads (Sigma) and 1ml of Lysis Buffer (0.11M Chaps; 0.11M Dithiotreitol; 58mM Tris; 10M Urea) plus a protease-inhibitor cocktail (Roche, Sussex, UK). Samples were processed 4 x 45 seconds at speed 6.5 in a Hybaid Ribolyser (Hybaid). Tubes were centrifuged 13.000xg for 10 minutes and supernatant recovered. Samples were subjected to a final centrifugation of 30.000xg for 30 minutes at 4°C to clear up the supernatant, which was kept at -70^0C until further testing by Western-blot.

2.15 Protein separation

10% SDS-PAGE (one-dimension polyacrylamide gel electrophoresis under denaturing 0.1% w/v SDS conditions) was performed using a Mighty Small II Mini-Gel System (Hoeffer Scientific Instruments, Little Chalfont, UK), in order to separate proteins based on their molecular size. Protein samples were denatured in sample buffer (for 10ml: 2.2ml Tris 0.5M pH 6.8, 2.25g SDS, 1.77ml glycerol, 0.4ml 2-β-

mercaptoethanol and 2mg bromophenol blue) and added to the gel alongside prestained Kaleidoscope® molecular weight markers (BioRad). A maximum of 20µl of sample was added per lane. The gel dimensions were 83 x 63 x 0.75mm and electrophoresis was performed at 28mA for approximately 2 hours with cooling. Resolving gel mixes were prepared by mixing Protogel solution (National Diagnostics), a 37.5:1 acrylamide to bis-acrylamide stabilised solution, to a final concentration of 10% in 1.5 M Tris pH 8.8. After addition of 200µl of 10% SDS solution, polymerization was achieved by adding ammonium persulfate and TEMED (BioRad). Stacking gel was overlayed onto the resolving gel. The Protogel mix concentration in the stacking gel was 4%, diluted in 0.5 M Tris pH 6.8. After electrophoresis, gels were either used for immunoblotting or fixed in 25% (v/v) isopropanol, 10% (v/v) acetic acid for 30 min, and stained in 0.006% (w/v) Coomassie Blue R-250 (Sigma) diluted in 10% (v/v) acetic acid, for 1 hour or overnight. The gels were destained with a mixture of 5% methanol and 7% glacial acetic acid.

2.16 Western blot analysis

For the transference, the gel was briefly immersed into transfer buffer (48mM tris, 39mM glycine, 0.037% SDS, 20% methanol) and added to the PVDF membrane previously soaked in the same buffer. Transfer was performed in a semi-dry blotting system (Biometra, Göttingen, DE) for one hour at 60mA. Afterwards, the PVDF membrane was incubated in blocking-buffer (TBS: 10mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20® plus 10% of skim milk and 2mM EDTA) for 2h at room temperature. After washing once in TBS-0.1% Tween 20® for 15 min, the membrane was incubated with the first antibody for one hour. Mouse anti C-terminal 6xHis

antibody (Invitrogen) at 1:2000 dilution in TBS 0.05% Tween 20® (TBS/T) 3% skim milk was used for the detection of 6xHis-tagged proteins. For the detection of Apa protein, a specific monoclonal antibody, mAb 6A3 (Espitia, 1995) was used at 1:250 dilution. After washing 5X for 5 minutes, 1:1500 dilution of the second antibody, anti-mouse IgG alkaline phosphatase (DAKO, Ely, UK) was applied to the membrane for one hour at RT. The membrane was then washed 5X with TBS/T, 2X with TBS only and once with PBS. Bands were visualised after addition of substrate NBT-BCIP (Sigma-FAST) diluted in water. The reaction was stopped with tap water.

2.17 Protein staining with colloidal gold

Staining with stabilised colloidal gold solution (AuroDye forte®, Amersham) was carried out after protein transfer to PVDF membranes. At low pH, the negatively charged gold particles bind to proteins by hydrophobic and ionic interactions. This type of staining is as sensitive as silver staining, although more simple to perform. After blot transfer, the membrane was incubated with an excess of PBS supplemented with 0.3% of Tween 20® at 37^{0}C for 30 minutes with gentle agitation. Afterwards, the membrane was washed intensively with the same solution, at room temperature. The washed blot was drained, washed briefly with distilled water and incubated with an excess of AuroDye forte® solution for 2-4 hours, with gentle agitation. At the end, the blot was washed with tap water. Overstaining did not occur.

2.18 Staining of blotted glycoproteins

Purified recombinant Apa (RecApa) produced in the mammalian cell line CV1 was tested for the presence of carbohydrate groups. Typically, 1μg of purified protein was loaded per well and a 10% SDS-PAGE was performed, followed by transfer of the proteins to a PVDF membrane by semi-dry blotting procedure. CandyCane® glycoprotein molecular weight standard (Molecular Probes, Eugene, USA) was added alongside the samples; this standard contains a mixture of glycosylated and non-glycosylated proteins. The Pro-Q Emerald 300 blot staining kit (Molecular Probes, Eugene, USA) was used to identify the glycoproteins present in the blot according to the instructions provided by the manufacturer. Briefly, the blot was gently agitated immersed in Fix solution (50% methanol + 50% milli-Q water) for 45 minutes, followed by a 2X washing in 3% of glacial acetic acid (Wash solution) for 10 minutes. The carbohydrates were then oxidised by incubation for 30 minutes in periodic acid diluted in wash solution. After a series of 3 washes, the blot was incubated with the fluorescent Pro-Q Emerald 300 staining solution for 2 hours. After washing twice in the dark, the blot was air-dried and exposed to a 300nm ultra-violet light to visualise the fluorescent bands. The blot was photographed and reproduced with the image inverted using an Image Station 440CF (Kodak Digital Science), with assistance of the Kodak 1D 3.5 program (Kodak) and a f-stop of 4.5 for 2 seconds plus multiple 1-second exposures.

2.19 Colony-blots

Plasmid pQE60.Apa was transformed into *E. coli* M15 cells to obtain high levels of recombinant Apa expression; clones expressing high levels of Apa protein were selected by colony blot lifts. Briefly, the transformation mix of *E. coli* M15 and pQE60.Apa was plated in LB agar containing both ampicillin and kanamycin and

incubated 16 hours at 30°C. A replica of this plate was made by touching the agar surface with a nitrocellulose filter disc. The filter with the colonies side-up was transferred to a new LB agar plate containing 1mM of IPTG and incubated 4h at 37°C to induce expression of the recombinant product. The filters were placed sequentially in a serie of solutions: [SDS 10%, denaturing solution (1.5M NaCl, 0.5M NaOH), neutralization solution (1.5M NaCl, 0.5M Tris pH 7.4) and 2 x SSC (0.3M NaCl, 34mM trisodium citrate)] in order to lyse the colonies and induce the binding of proteins to the membrane. The filters were later washed twice in TBS and blocked for one hour with 3% bovine serum albumin (BSA) (Sigma) in TBS buffer. After another series of washes in TBS Tween/Triton (2 x 10 minutes) and TBS (1X), the membranes were incubated for 1 hour in 1:2500 dilution of mouse anti C-terminal 6xHis antibody in blocking buffer, to detect the tagged recombinant protein. After washing, incubation with second antibody was carried out using a 1:1500 dilution of rabbit anti-mouse IgG alkaline-phosphatase (DAKO) in blocking buffer for one hour RT. At the end of incubation, the discs were washed three times with TBS Tween/Triton and developed with NBT-BCIP (Sigma-FAST). Colonies showing the most intense signal were selected and retrieved from the original master plate.

2.20 Purification of 6xHis-tagged recombinant protein

2.20.1 Preparation of periplasmic extract

According to Horn and collaborators (1999), *E. coli* M15 transformed with the pQE60.Apa construct expresses Apa protein into the periplasmic space due to the inclusion of the associated N-terminal secretion sequence. The recombinant bacteria were grown at 37°C in 1 litre of LB medium, supplemented with ampicillin and

kanamycin, until culture reached an OD_{600} of 0.7. Then, 2mM of IPTG was added to the culture and a further 5 hours of incubation was carried out. The cells were harvested by centrifugation at 4000xg for 15 minutes at 4°C. To induce an osmotic shock on the cells, the pellet was resuspended in approximately 500ml of a cold solution of 30mM Tris, 20% sucrose, pH 8.0; 1mM of EDTA was added dropwise and the suspension was incubated on ice for 10 minutes with gentle agitation. The cells were centrifuged at 8000xg for 20 minutes at 4°C and the supernatant was substituted by the same volume of 5mM of ice-cold $MgSO_4$; the preparation was gently agitated for 10 minutes at 4°C. After a new centrifugation at 8000xg, the supernatant containing the periplasmic proteins was collected, filtered through a 0.2μm filter (Millipore), concentrated 50X by ultrafiltration and dialysed extensively at 4^0C against 10mM Tris pH 8.0 with three changes. Dialysis proceeded overnight with buffer-to-sample ratio of 1:60. Sample was kept at -70^0C until further processing.

2.20.2 Ni-NTA affinity chromatography

A nickel-nitrilotriacetic acid agarose (Ni-NTA) column containing 2ml of resin with high affinity for terminal 6-histidines-tagged proteins (Ni-NTA, QIAgen), was equilibrated with 5ml of 10mM Tris pH 8.0. The periplasmic extract containing Apa carrying a His tag was loaded into the column. The column was washed initially with 10 volumes of 10mM Tris pH 8.0 plus 10mM of imidazole (Sigma) and then again with 10 volumes of the same buffer plus 20mM imidazole. Fractions were collected trough the process to evaluate the performance of the procedure. Purified recombinant Apa (RecApa) was eluted four times with 10mM Tris pH 8.0 containing 200mM of imidazole. All steps were carried out in the cold room at 4^0C to diminish

the action of proteases. Eluates were dialysed against water and lyophilised. Western-blotting using the specific mAb 6A3 as described previously, detected the recombinant Apa protein in the eluates.

For purification of RecApa produced in mammalian cells, the routine procedure described above was followed. However, two set of washes with 20mM of imidazole were performed instead of one since mammalian cells are richer in histidine-containing proteins than prokaryotic cells.

2.21 Endotoxin removal by Polymyxin B columns

A support of immobilised Polymyxin B in agarose (Detoxi-Gel Endotoxin Removing Gel, Pierce, Rockford, USA) was used to decrease the levels of endotoxin in the RecApa samples used for *in vitro* cell cultures. Polymyxin B is an antibiotic that contain a cationic cyclopeptide with a fatty acid chain and can bind and neutralise the lipid A portion of bacterial LPS (Morrison & Jacobs, 1976). The procedure used was recommended by the manufacturer's instructions. Briefly, the lyophilised Apa samples were resuspended in 2 ml of ultra pure 0.1M ammonium bicarbonate, pH 7.8 (Fluka, Buchs, Switzerland) diluted in endotoxin-free water (Biowhittaker). This is a volatile buffer, which allows the sample to be lyophilised later as a salt-free powder. The prepacked 1 ml columns were regenerated with 5 washes with sodium deoxicholate (Fluka), followed by 5 washes with pyrogen-free water. Afterwards, the column was regenerated with 5 volumes of 0.1M ammonium bicarbonate buffer. The sample was applied; the column was capped and incubated at room temperature for 30 minutes to increase the binding of LPS to the matrix. The sample was eluted with a gradient of 0.1-0.5 M of NaCl. With this procedure, there was a reduction of approximately 54X in the amounts of endotoxin contamination in the sample, i.e.

RecApa was usually obtained at 5-6 EU/mg of protein and these levels were considered acceptable for use in *in vitro* cell cultures.

2.22 Quantification of total protein

Recombinant Apa derived from mammalian cells, unrelated protein samples and cell extracts obtained in low amounts were quantified by the NanoOrange® protein quantitation kit (Molecular Probes), a sensitive fluorometric method able to detect concentrations of protein in solution of 10ng/ml to 10μg/ml. The protocol was followed according to manufacturer's instructions. Briefly, a standard curve from 10μg/ml to 10ng/ml was prepared diluting bovine serum albumin in the working solution containing the NanoOrange® quantitation agent. Test sample was diluted 1:250 in the same solution. All protein samples were heated at 90^0C for 10 minutes, in the dark, for denaturation. Samples were cooled at room temperature for 20 minutes. Readings were made in quartz cuvettes with a 3mm path-length in a Perkin-Elmer LS50B Luminescence Spectrometer (Perkin-Elmer, Foster City, USA) with an excitation emission at 485nm and capture at 570nm. A standard curve of fluorescence versus protein concentration was generated subtracting the blank from the samples.

2.23 Quantification of recombinant protein

Detection of purified recombinant Apa protein was done by Western-blotting as described previously, using the mAb 6A3. The amount of purified protein in the samples was estimated by measuring absorbance at 260nm and 280nm of the protein solution, using 10mm path-length cuvettes and applying the following formula (Dawson, 1989):

$$\text{Protein conc. (mg/ml)} = 1.55A_{280} - 0.76A_{260}$$

2.24 Isolation of murine bone marrow-derived dendritic cells (BMDCs)

The protocol was adapted from Inaba and collaborators (1992). Briefly, mice were killed by cervical dislocation and both femurs were removed. In a sterile Petri dish (Nunc) containing approximately 10ml of IMDM complete medium, the bone marrow from both femurs was flushed with the help of a 2ml syringe and a 25G needle. The cell suspension was centrifuged once at 1200rpm and after dilution in new media, the cells were distributed in new sterile Petri dishes (up to 5 per mouse). 1:10 dilution of supernatant from a stable cell line expressing murine GM-CSF (granulocyte-macrophage colony growth factor) (a kind gift of Dr. Brigitta Stockinger, NIMR) was added to each plate. After two days of incubation at 37^0C, 5% CO_2, the media and the cells in suspension were discarded. Fifteen millilitres of fresh media plus GM-CSF were added and the cells left undisturbed for additional 4 days. After this period, it was possible to see cells with dendritic morphology in suspension or loosely attached. These cells were collected, counted and used as APCs (antigen-presenting cells).

2.25 Isolation of murine spleen cells

Mice were killed by cervical dislocation and spleens were removed. The organ was homogenised in 10ml of AIM-V serum-free media supplemented with 80μM of 2-β-mercaptoethanol or complete IMDM and a single-cell suspension was obtained after crushing it trough a 70μm cell strainer (Falcon-Becton Dickinson, Le

Pont de Claix, FR). The erythrocytes were lysed osmotically with 1.5 ml (per spleen) of Red Blood Cell Lysis Buffer (Sigma). Spleen cells were washed twice, counted and resuspended at desired concentrations in complete media.

2.26 DNA vaccination

2.26.1 Strains of mice used

Vaccination with plasmid DNA was performed using 6-8 weeks old females. The Institute's Biological Services, National Institute for Medical Research (NIMR) supplied all animals. Food and water were given at will. Strains used were the inbred C57BL/6 (H-2^b) and Balb/c (H-2^d).

2.26.2 Vaccination and challenge procedures

Experiment	Mouse strain	Vaccines used
ApaDNA: immune response and protection studies	C57BL/6	pCMV4.Apa (Apa), pSecT2C.Apa (Apa), pCMV4 (vector), BCG and saline

Table 2.4: Vaccination scheme for ApaDNA groups.

For ApaDNA-vaccination studies, mice were injected with 100μg of plasmid DNA intramuscularly. Control groups were either injected with saline four times or once with 1 x 10^6 BCG-Glaxo, intradermally, at the base of the tail (Table 2.4). The number of DNA injections was 4 with intervals of 3 weeks between inoculations.

Mice were analysed and challenged with MTB at 3 and 6 weeks after the last dose, respectively. Bacterial load was determined 5 weeks later in the lungs and spleen.

For the priming-boosting experiments, 6-8 weeks old Balb/c or C57BL/6 females were injected once with a single antigen or in combinations of three or four antigens at the same time. The amount of DNA injected intramuscularly was 100μg for prime-boost I. For prime-boost II, approximately 50-75μg each of pCMV4.Apa, pCMV4.83, pCMV4.65 and pCMV.70 plasmids were injected simultaneously in the same animal, each plasmid in a different anatomical site (Table 2.5). The muscles from the front and rear limbs were used for injection.

Experiment	Mouse strain	Priming vaccines	Boosting
Prime-Boost I	C57BL/6	pCMV4.Apa, pCMV4, BCG	BCG or pCMV4.Apa
Prime-Boost II	Balb/c	pCMV4.Apa, pCMV4.83 (MPT83), pCMV4.65 (HSP65), pCMV.70 (HSP70), pBT7 (MPT70), pCMV4, BCG	BCG or pCMV4.Apa, pCMV4.83, pCMV.65 and pCMV4.70

Table 2.5: Vaccination scheme for prime-boost I and II.

In the same fashion, another group of animals in this experiment received simultaneously: pBT7, pCMV4.65 and pCMV.70. The boost was performed with approximately 1 x 10^6 CFU of BCG, injected intradermally at the base of the tail, one month after priming with DNA. Groups with initial BCG priming and plasmid DNA boost (one month after priming) were also set (Table 2.5). After approximately 6-12

weeks, the animals were either killed for immune response analysis or challenged with 1 x 10^5 colony-forming units (CFU) of *M. tuberculosis* injected intravenously. Five weeks later, the mice were killed by a Schedule One method and the bacterial load in the lungs and spleens was evaluated. Briefly, the organs were weighted and homogenised in PBS. Serial 10-fold dilutions of the homogenates were plated on Middlebrook 7H11 Bacto Agar (Difco). Colonies were counted 3-4 weeks later and results expressed as CFU/g of tissue. The experiment was repeated twice.

Experiment	Mouse strain	Priming vaccines	Boosting
Prime-boost III	C57BL/6 and Balb/c	pCMV4.Apa, pCMV4.65, pCMV.70, pCMV4, BCG.	BCG

Table 2.6: Vaccination scheme for prime-boost III.

In the prime-boost III studies, the animals received two or three initial doses of combinations of pCMV4.Apa, pCMV4.65 and pCMV.70 plasmids (Table 2.6). The intervals between DNA vaccinations were three weeks and BCG was injected one month after the last dose of DNA. Immune response analysis and infection occurred similarly as described for prime-boost II groups.

In prime-boost IV, a large group of C57BL/6 females (12 per cage) were primed twice or three times with vaccine combinations of pCMV4.65, pCMV4.70 and pCMV4.Apa. Groups of animals primed twice with pCMV4.65 and pCMV4.70 plasmids (no ApaDNA) and boosted or not with BCG were also included. Control groups included animals receiving three doses of saline or vector, and also two and three doses of vector followed by a boost with BCG. 50-75μg each of pCMV4.Apa,

pCMV4.65 and pCMV.70 plasmids were administered simultaneously in the same mouse, each vaccine injected intramuscularly in a different limb (a total per priming dose of 150-200μg of DNA injected per animal). For vector injections, the total amount injected per dose was 200μg. The boost with 1 x 10^6 BCG bacteria was administered intradermally at the base of the tail, one month after the last DNA injection. Interval between DNA injections was three weeks. Animals were infected fifteen weeks after the last vaccination. Bacteriologic burden in the spleen and lungs was measured after six weeks of infection (Table 2.7).

Experiment	Mouse strain	Priming vaccines	Boosting
Prime-boost IV	C57BL/6	pCMV4.Apa, pCMV4.65, pCMV4.70, pCMV4, BCG	BCG

Table 2.7: Description of prime-boost IV groups.

In the prime-boost V experiment, groups of eight Balb/c females per cage received BCG intradermally, as the priming vaccine, and boosting vaccinations were made with a combination of mycobacterial antigens in DNA format. The amounts of DNA and BCG and sites of injection were similar to prime-boost III and IV experiments described above. The Test group was injected once with BCG and eight weeks later received a boosting combination of HSP65 and HSP70 plasmids. A second DNA boosting dose was given three weeks later. Animals were challenged intravenously with 10^5 CFU of *M. tuberculosis* H37Rv three weeks later. The control groups included naive animals, mice receiving a first dose of BCG and then two boosting doses of either saline or pCMV4 empty vector. And, in the last control group, the animals received saline as the priming vaccine and two doses of HSP65

and HSP70 plasmid cocktail as the boosting vaccine. The experiment is described in table 2.8.

Experiment	Mouse strain	Priming vaccines	Boosting
Prime-boost V	Balb/c	BCG, pCMV4.65, pCMV4.70, pCMV4	pCMV4.65, pCMV4.70, pCMV4

Table 2.8: Scheme for prime-boost V protocol.

2.27 Enrichment for murine splenic CD4+ or CD8+ T-cells

For the enrichment of CD4+ and CD8+ T-cell subpopulations, Mouse T Cell Subset Column Kits (R & D Systems, Oxon, UK) were used according to the manufacturer's instructions. Briefly, erythrocyte-depleted spleen cells were resuspended in 2ml of 1X sterile column wash buffer (R & D Systems) and a monoclonal antibody cocktail (1ml) designed to negatively deplete the cell population without interest, thereby enriching for the desired T cells, was added. The mixture was incubated at room temperature for 15 minutes. Following two washes by centrifugation at 1300 rpm, the antibody-treated cells were loaded into the column and let it stand there for 10 minutes at room temperature. Antibody-treated cells bound to glass beads present in the column through Fc and F(ab) interactions. Reasonably pure (up to 90%) CD4+ or CD8+ T cell populations were eluted with 15 ml of column buffer. The cells were harvested by centrifugation (1200rpm, 5 minutes), and after washing, they were counted and plated at the appropriate concentrations. In all cases, the viability of the isolated cells was above 90%, as determined by Trypan Blue (Sigma) exclusion. When isolating cells from *M.*

tuberculosis infected animals, all procedures were carried out in a sterile manner in microbiological class I cabinets in the Containment III unit of the NIMR.

2.28 Anti-Apa enzyme linked immunoabsorbant assay (ELISA)

Nunc Maxisorp ELISA plates (Nunc, Paisley, UK) were coated with 1μg/well of RecApa diluted in 100μl of carbonate buffer (34mM $NaHCO_3$, 15mM Na_2CO_3), pH 9.6. Plates were covered and incubated for 1 hour at 37°C. Afterwards, the plates were left up to 72 hours at 4°C. In the day of the assay, the plates were washed four times with PBS/0.05% Tween 20® (PBS/T) and blocked for two hours at RT with 100μl/well of PBS/T plus 5% of skim milk. The plates were washed again another four times. Serum obtained from vaccinated animals plus controls was serially diluted in blocking buffer and 100μl/well dispensed in the plates. As a positive control, IgG1 anti-Apa mAb 6A3 was used. The plates were left for 2 hours at 37°C and after washing, incubation with the second antibody was carried out. Specific biotinylated anti-mouse IgG1, IgG2a or total IgG (Amersham) was diluted 1:1000 in blocking buffer and dispensed at 100μl per well. The plates were incubated for one hour at 37°C; washed four times and incubated another hour with streptavidin-alkaline phosphatase (Amersham) diluted 1:2500. Plates were washed 3 times and 100μl/well of p-Nitrophenol substrate (SigmaFast) was added for up to one hour. The reaction was stopped with 100μl/well of 3N NaOH. Spectrophotometric readings were obtained at 405nm.

2.29 Cytokine measurements

Duplicate cultures of 3 x 10^6 spleen cells, pooled from 2-4 mice were incubated in 24-well plates in 2-ml of serum-free AIM-V media (GIBCO-BRL) supplemented with 80μM of 2-β-mercaptoethanol or complete IMDM for up to 72 hours at 37°C. A number of different antigens were tested in individual wells, as follows: 6μg/ml of RecApa; 6μg/ml of native Apa (purified from the culture filtrates of *Mycobacterium tuberculosis* H37Rv strain, a kind gift from Dr. John Belisle, Fort Collins, USA); 10μg/ml of concanavalin A (Sigma); 10μg/ml of PPD; 10 micrograms per milliliter of the peptide 42', a sequence of mannosylated aminoacids [DPEPAPPVPT(Manα)TAASPPST(Manα)AAAPPAPAT(Manα)PVAPPPPAAANT PNA] from the N-terminal part of the Apa protein or control peptide 42' (same sequence, non-glycosylated), kind gifts from Dr. Fanny Guzman (Laboratory of Immunology, HSJD, Bogota, Colombia). The supernatants were collected after 24h, 48h and 72h, filtered through 0.2μm filters and kept at –70°C. Cultures of 4 x 10^5 isolated CD4+ cells, pooled from 2-4 mice, were incubated in triplicates in 96-well plates with 250μl of supplemented AIM-V media or complete IMDM. Immature (6^{th} day) BMDCs (8 x 10^4 cells per well) or gamma-irradiated (15000 rads) splenocytes were used as antigen presenting cells. Supernatants were collected after 24, 48 and 72 hours. Using a similar protocol, cultures of isolated CD8+ T-cells were incubated in triplicates with immature BMDCs, in 250μl of complete IMDM. Cells were stimulated with 6μg/ml of RecApa. Supernatants were collected after 24, 48 and 72 hours.

Cytokines released in the supernatants were assayed using commercial sandwich ELISA kits for the presence of interferon-gamma (IFN-γ), interleukin 4 (IL-4), interleukin 10 (IL-10) and interleukin-2 (IL-2) ((Amersham or R&D Systems), and transforming growth factor beta-1 (TGF-β1) (BD-Pharmingen, Oxford, UK),

according to the manufacturer's protocol. In the case of TGF-β1, acid activation of the cytokine was performed before testing: 4µl of 1N HCl per 100µl of supernatant and incubation for 60 minutes at 4°C followed by neutralisation with 4µl of 1N NaOH.

2.30 ELISPOT assays

ELISPOT plates MAIP S45 (Millipore) were coated with 100µl/well of 10µg/ml anti-IFN-γ mAb R4-6A2 (BD-Pharmingen) diluted in calcium–magnesium-free sterile PBS pH 7.4 and incubated overnight at 4^0C. Plates were washed twice with 200µl/well of AIM-V medium (Gibco-BRL), supplemented with 2% of FCS and blocked afterwards with the same volume per well of AIM-V/10% FCS for 2 hours at 37°C. Total spleen cells, CD4+ or CD8+ -enriched cell suspensions were obtained and incubated with or without antigen at approximately 10^7 cells in six-wells plates with 5ml of AIM-V/2% FCS at 37°C, 5% CO_2. Either 6µg/ml of recombinant Apa or HSP65 or 10µg/ml of Purified Protein Derivative (PPD) from MTB (Veterinary Laboratories Agency Weybridge, Surrey, UK), was used to stimulate the cells. After 48 hours of incubation, the cells were resuspended to a single-cell suspension, counted and added in 100µl volumes at various 2-fold serial dilutions to the coated ELISPOT MAIP S45 plates. The maximum number of cells added per well was 1 x 10^6. After adding to the plate, the cells remaining in the suspension were re-counted to increase accuracy. Plates were incubated during 16 hours at 37°C, 5% CO_2 before being washed 2 times with 0.2µm-filtered distilled water; this wash induced osmotic lysis of the cells and reduced background. This was followed by three washes with 0.2µm filtered PBS/T (PBS 0.05% Tween 20). During each wash, the plate was

shaked vigorously for 30 seconds in a microplate shaker. Biotinylated anti-IFN-γ mAb (clone XMG 1.2, BD-Pharmingen) was diluted to a concentration of 1μg/ml in PBS/T plus 0.1% Bovine Serum Albumin (Fraction V, Sigma) and 100μl/well were added to the plates. This was followed by an incubation of 2 hours at room temperature. After washing 5 times, the plates were tapped dry and 100μl/well of streptavidin-alkaline phosphatase (BD-Pharmingen) diluted 1:1000 in PBS/T-BSA was added. The plates were placed for one hour at room temperature, washed 5 times and 50μl/well of NBT-BCIP substrate solution (SigmaFAST, Sigma) added per well. Colour was allowed to develop from 5 to 15 minutes in the dark. Washing the plate extensively with water stopped the reaction. The plates were dried, also in the dark, before spot counting was performed using a dissecting microscope (30X magnification). Results were expressed as the mean number of cytokine-producing cells per 10^6 or 10^5 total cells, subtracted from the controls. Each sample was assayed in triplicate. Controls included wells without cells (background) and non-stimulated cells. A transfected cell line producing constitutive IFN-γ (X63g, a courtesy of Dr. Brigitte Stockinger, NIMR) was used as the positive control to evaluate the efficiency of the assay, which usually ranged from 97% to 100% (all plated cells producing IFN-γ were detected).

2.31 Stimulation of CD8+ T-cells

A protocol described by Chen and collaborators (1993) for promoting presentation of extracellular antigens into the MHC class I presentation pathway was used. Briefly, 200μg of RecApa or recombinant MPT83 (Xue, 2000) was mixed with 50μg of Lipofectin (Gibco-BRL) in 450μl of AIM-V media and incubated for 10

minutes at RT. This solution was then mixed with 1 x 10^6 BMDCs purified from C57BL/6 or H-2^b MHC-II knockout (KO) mice and incubated for a further 15 minutes at RT. These cells were washed, counted with Trypan-Blue exclusion and dispensed at 5 x 10^4 per well in 96-wells plate. Cultures of 5 x 10^5 CD8+ T-cells isolated from the spleens of MTB-infected mice, vaccinated mice or controls were added to the plates containing these liposome-stimulated DCs in a final volume of 250µl. MTB-infected material was always handled in proper cabinets at the Containment III Unit (NIMR). Supernatants were collected after 24-72 hours and sterilised by filtration. Twenty U.I./ml of murine recombinant IL-2 (Boehringer-Mannheim, Sussex, UK) was added to the wells and the cultures maintained for 7-10 days before being subjected to intracellular cytokine staining and FACS analysis.

2.32 The JAM assay

To measure cytolytic (CTL) activity, four weeks after the end of the ApaDNA vaccination, two or three animals were killed from test and control groups; 4 x 10^6 splenocytes, depleted of erythrocytes, were plated in 24-wells plates together with 5 x 10^5 lethally irradiated (20000 rads) EL-4 cells expressing the *apa* gene. Twenty units/ml of mouse recombinant IL-2 (Roche) were added to the cultures. The cells were incubated at 37^0C, 5% CO_2 for 6 days. CTL activity was measured by the JAM assay (Matzinger, 1991). Target EL-4 and EL-4.Apa cells, as well as Concanavalin A (10^4) activated blast cells (H-2^k, CBA mouse spleen cells activated for 48 hours), were labelled with [^{3}H] thymidine (Amersham) at a final concentration of 5µCi/ml. The cells were left undisturbed for 6 hours. The effector cells were washed and plated in round-bottomed 96-wells plates together with labelled target cells at various effector-to-target (E/T) ratios and incubated for further 6 hours. The total volume was

0.2ml. The cells were harvested onto glass fibre mats using a Titertek 96 Mach 2 cell harvester (Tomtec, Orange-CT, USA) and counts were read in a 1205 Betaplate™ liquid scintillator (Pharmacia-LKB, Gaithersburg-MD, USA). The percentage of specific lysis was expressed as:

%Specific killing = Retained DNA in the absence of killers (CPM) – Retained DNA in the presence of killers (CPM)/ Retained DNA in the absence of killers (CPM).

2.33 Intracellular FACS staining

All procedures with the exception of FACS reading, were carried out in containment III facilities. Lungs were dissected from MTB-infected mouse at the day's 38-42 post-infection. The tissues were cut with sterile scissors and forceps into fine pieces and smashed against a 70μm cell strainer (Falcon). Red-blood cells (RBC) were eliminated from the resulting cell suspension using RBC lysis buffer (Sigma). The lung cells were washed twice, counted and resuspended in AIM-V media. For stimulation of cytokine production, 1μg/ml each of anti-mouse CD3ε and anti-mouse CD28 monoclonal antibodies (BD-Pharmingen) were added. To block extracellular secretion, monensin (Golgi-Stop™, BD-Pharmingen) was also added to the cells, and the suspension incubated for 6 hours at 37^0C. At the end of incubation, the cells were washed twice and a single-cell suspension was adjusted to 1 x 10^6 cells per tube. Incubating the cells for 15 minutes at RT with anti-mouse CD16/CD32 antibody (BD-Pharmingen) blocked Fc II/III cell receptors and avoided unspecific staining. For surface receptors, cells were stained for 30 minutes in the dark and in ice with 1:100 dilution of the following antibodies (BD-Pharmingen): fluorescein-isothiocyanate

(FITC)-conjugated anti-CD4+ clone H129.19 and fluorescein-isothiocyanate (FITC)-conjugated anti-CD8+ clone 53-6.7. After washing with PBS 1% FCS, 0.02% N_3Na (FACS buffer), the cells were fixed in ice for 2 hours with 4% of fresh paraformaldehyde diluted in Dulbecco's PBS pH 7.5. After a brief washing with FACS buffer, the cells underwent another round of fixation using Cytofix/Cytoperm™ reagent (BD-Pharmingen), which also contains paraformaldehyde (20 minutes on ice). Cells were washed twice in permeabilisation buffer (0.1% saponin) and stained for 30 minutes on ice with the anti-IFN-γ PE-conjugated clone XMG 1.2 (BD-Pharmingen). The cells were analysed using a fluorescence-activated cell analyser machine FACS Calibur™ and CellQuest™ software (Becton Dickinson).

2.34 Cell Sorting

Spleen cells from two to three DNA-vaccinated mice and controls were enriched for CD4+ T cells using Mouse T Cell Subset Column Kits (R & D Systems). These T cells were then diluted in sterile Dulbecco's PBS (Invitrogen) plus 5% FCS and blocked for 15 minutes at RT with anti-mouse CD16/CD32 antibody (BD-Pharmingen) followed by staining for 30 minutes on ice and in the dark with FITC-conjugated anti-CD4+ clone RMA4.5 (BD-Pharmingen), at 1:100 dilution. The cells were washed with Dulbecco's PBS, passed through a 40μm filter to remove particulates and resuspended at 2 x 10^7 cells/ml. Propidium iodide was added to gate out the dead cells. FITC-positive (i.e. CD4+ T cells) and negative cells were sorted using a MoFlo cytometer FACS sorter (Dako Cytomation, Glostrup, Denmark), at the Flow Cytometry Lab, NIMR. Sterility was preserved throughout the procedure. Levels of purity for the selected population reached 99.9%. After sorting, the cells

were recovered in complete media, counted and plated in a 96-wells plate at 4 x 10^5 cells per well. Immature, 6^{th} day BMDCs were used as APCs. RecApa or native Apa antigens were added in various concentrations. Supernatants were collected and filtered at 24h, 48 and 72 hours.

CHAPTER THREE

Results

3.1 Expression of the *M. tuberculosis apa* gene in infected cells

Previous evidence demonstrated that, in guinea pigs, the Apa protein is recognised by the immune system of animals inoculated with live but not dead BCG and is an immunodominant antigen, even if not expressed in high amounts in culture (Romain, 1993). More importantly, an impressive immune response against the Apa antigen was observed in human patients with active tuberculosis (Espitia, 1995; Samanich, 2000; Kumar, 2003). This evidence would suggest an increase of expression of this specific protein by *M. tuberculosis* during intracellular infection, which could make this antigen more visible for the immune system. To evaluate this hypothesis, transcription levels of Apa mRNA were estimated in infected cells. The murine macrophage-like cell line RAW264 and bone marrow derived macrophage and dendritic cells (BMC) cultured from Balb/c mice were infected at a multiplicity of infection of 10 *M. tuberculosis* bacteria per eukariotic cell. RNA from *M. tuberculosis* was isolated from the infected cells as described in Material and Methods.

The transcription levels of messenger RNA (mRNA) for Apa in MTB infected cells were quantified by Real-time™ PCR using *apa* specific primers. Mycobacterial sigma factor A gene (*sigA*) was used as the reference gene in this study since its expression levels are independent of a variety of changes in growth conditions and also

in *M. tuberculosis* grown in macrophages (Manganelli, 1999; Raynaud, 2002). The levels of Apa-expression in infected cells were compared to levels of Apa-expression in *M. tuberculosis* grown in axenic culture. Apa mRNA expression in infected cells did not seem to vary from the levels of expression obtained in extracellular conditions, either in infected murine RAW264 or BMC cultures (fig.3.1). Interestingly however, a clear increase of transcription in the *apa* gene was seen repeatedly when the cells were previously activated *in vitro* with recombinant IFN-γ (fig.3.1). These results were obtained with two independent RNA samples. In general, four PCR runs were executed for each sample. Similar results were obtained by performing the same experiment with a different type of cell, the human THP-1 monocytic tumour line. A peak on the expression of the *apa* gene was seen only when these cells were previously activated with phorbol-myristate acetate (PMA) and human recombinant IFN-γ and were then infected with *M. tuberculosis* (fig.3.2). Treatment with PMA only did not induce significant up-regulated levels of *apa* expression.

3.2 Expression of the *apa* gene in *E. coli*

The entire coding sequence of the *M. tuberculosis apa* gene, including the N-terminal signalling sequence, was cloned into the pQE60 expression vector in order to obtain a recombinant protein to be used in immunological assays. The expression vector described in fig.3.3 was used in these experiments. The *apa* gene was cloned downstream to a phage T5 promoter under control of two *lac* operators and also in frame with a 6 histidines (6xHis) tagged sequence at the C-terminus, which allowed the fusion protein to be purified from the affinity columns as described.

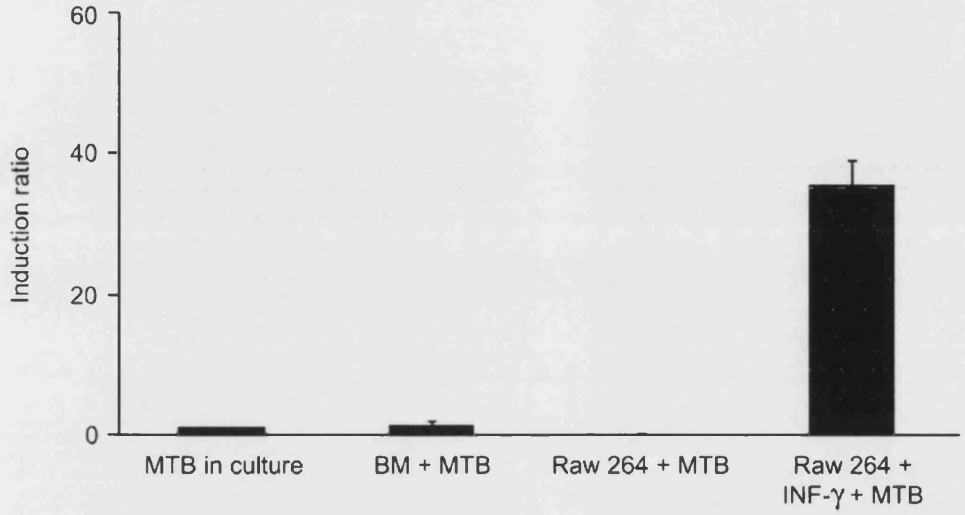


Figure 3.1 Expression of *apa* by *M. tuberculosis* (MTB) infecting RAW264 murine cell line and bone-marrow derived macrophages (BM) from BALB/c mice. The amount of *apa* mRNA relative to that of the normalising gene, *sigA*, was determined by Real-time™ quantitative reverse-transcription (RT) PCR. For activation, 100U/ml of mouse recombinant IFN-γ was added to the cells for 16 hours. The gene-transcription ratio of MTB grown in axenic culture was taken as 1. The values shown are the means of results obtained in several experiments and the error bars indicate the standard errors.

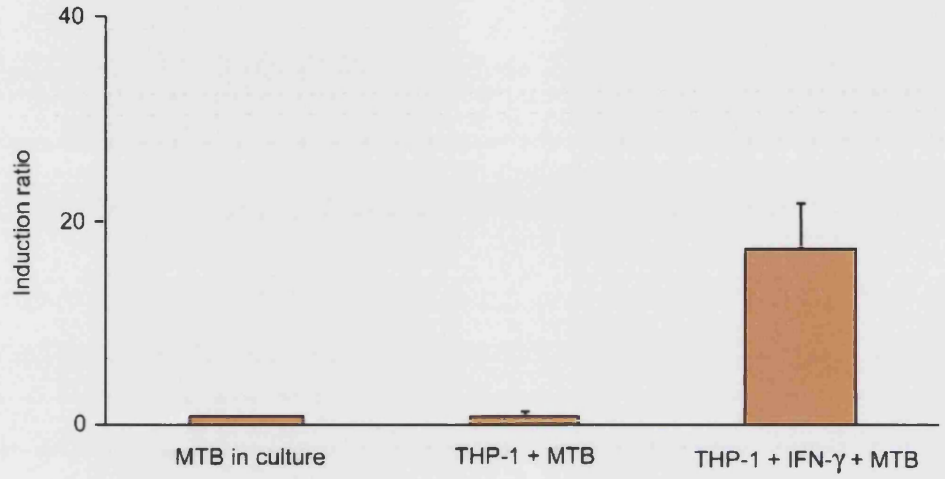


Figure 3.2 Expression of *apa* by *M. tuberculosis* (MTB) in human THP-1 cell line. THP-1 cells were activated with PMA for one day before infection. For activation, the cells were exposed to 100U/ml of human recombinant IFN-γ for 16 hours. The amount of *apa* mRNA was calculated as in fig. 3.1 and measured by Real-time™ RT-PCR. The gene-transcription ratio of MTB grown in axenic culture was taken as 1. The values shown are the means and the error bars indicate the standard errors.

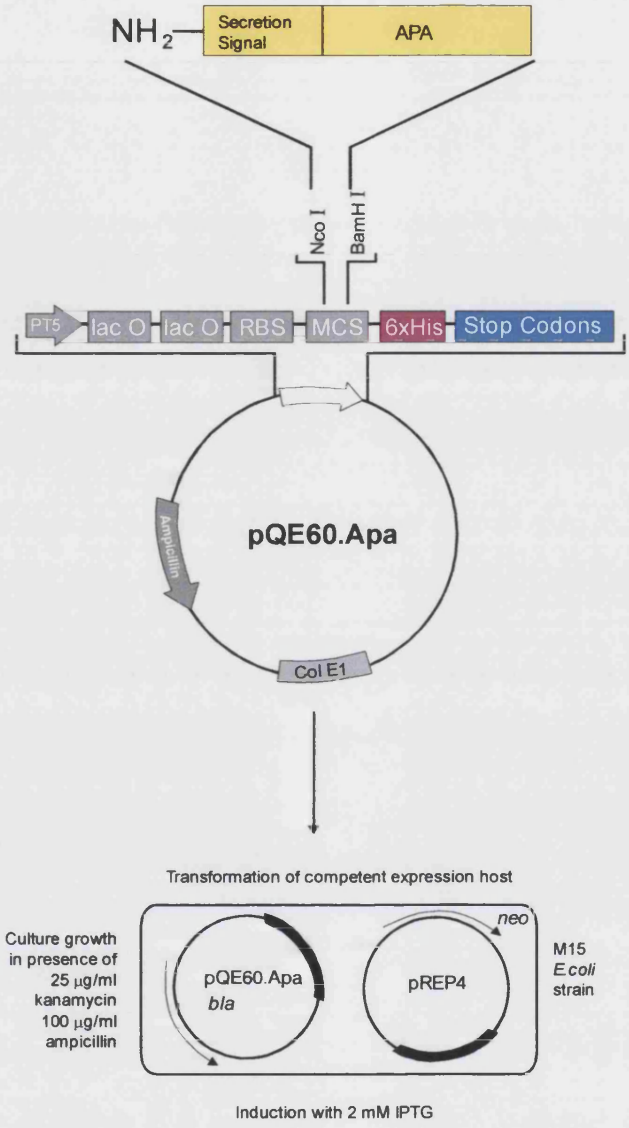


Figure 3.3 The pQE60.Apa plasmid expression system. Apa was cloned into the Nco I and BamH I restriction sites. The secreted Apa protein was fused with a 6xHis-tagged sequence at the C-terminal part. There are stop codons in all 3 reading frames. PT5, T5 phage promoter; lac O, *lac* operator; RBS, ribosome-binding site; MCS, multiple cloning site; 6xHis, 6-histidines tag sequence; Col E1, origin of replication; *bla*, ampicillin resistance gene; *neo*, kanamycin-resistance gene; IPTG, isopropyl-thiogalactoside.

To avoid toxicity by highly expressed recombinant proteins, *E. coli* M15 strain was used as host. This strain contained the pREP4 plasmid in high copy number, which contains the *lacI* repressor gene, for tightly controlled expression in *trans* of the recombinant protein (fig.3.3). Maintenance of both plasmids was achieved by double (ampicillin-kanamycin) antibiotic selection.

Mini-cultures of *E. coli* containing pQE60.Apa were prepared to determine the best amount of IPTG and period of induction. Analysis of whole-cell extracts from these cultures by SDS-PAGE revealed an increased level of expression related to the time of culture. Western-blot analysis of the bacterial-cell extracts with the Apa-specific monoclonal antibody 6A3, revealed a major band of approximately 47-kDa, a minor band of 45-kDa, followed by a couple of lower molecular weight bands, which could probably reflect some degradation occurring in the cell extract (not shown). Comparable findings have been reported using similar expression vectors (Laqueyrerie, 1995; Horn, 1999).

3.2.1 Purification of RecApa with affinity chromatography

Since the fusion protein was cloned with its N-terminal signal sequence, it was expected to find most of the RecApa protein in the periplasmic space of the recombinant *E. coli* M15 (Horn, 1999). This was confirmed by comparing bacterial cell and periplasmic extracts by SDS-PAGE; non-denaturing conditions for the preparation of the extract were also found to provide the best results (not shown). The periplasmic extract was prepared by osmotic shock of the bacterial cultures. RecApa was purified using Ni-NTA affinity chromatography as described. Samples were collected trough all steps of

the chromatographic process and analysed by SDS-PAGE. Sensitive colloidal gold staining showed that almost all RecApa protein was carried out of the column with the first elution. A single band of approximately 47-kDa can be clearly demonstrated (fig.3.4). The 45-kDa band was not found in this fraction and other groups had reported similar findings (Horn, 1999). This result is probably related to the fact that the 45-kDa band represents a C-terminal truncated form of Apa (Romain, 1999; Horn, 1999) and therefore does not contain the affinity tag. A small contaminant band of approximately 31-kDa was also co-purified with RecApa but did not react with the 6A3 monoclonal antibody (fig.3.5). The band was present in very low quantities in the RecApa samples, being only detected with colloidal gold staining, but not Commassie Blue or silver nitrate staining. No further purification has been performed to specifically remove this band.

Purified recombinant Apa samples obtained by the methods described above were found to carry high levels of LPS contamination. Commercial polymyxin B mini-columns were used to decrease LPS levels in purified RecApa samples. The procedure was very efficient and the levels of LPS dropped from 324 EU to 5-6 EU per milligram of protein sample, being considered acceptable for use in all the *in vitro* cellular assays.

3.3 DNA vaccination with the *M. tuberculosis apa* gene

Due to the reported immunodominant characteristics and the up-regulation of expression during infection, the immunogenicity of the Apa antigen in the naked DNA format was evaluated in the murine model.

3.3.1 Construction of the Apa-plasmid DNA vaccines

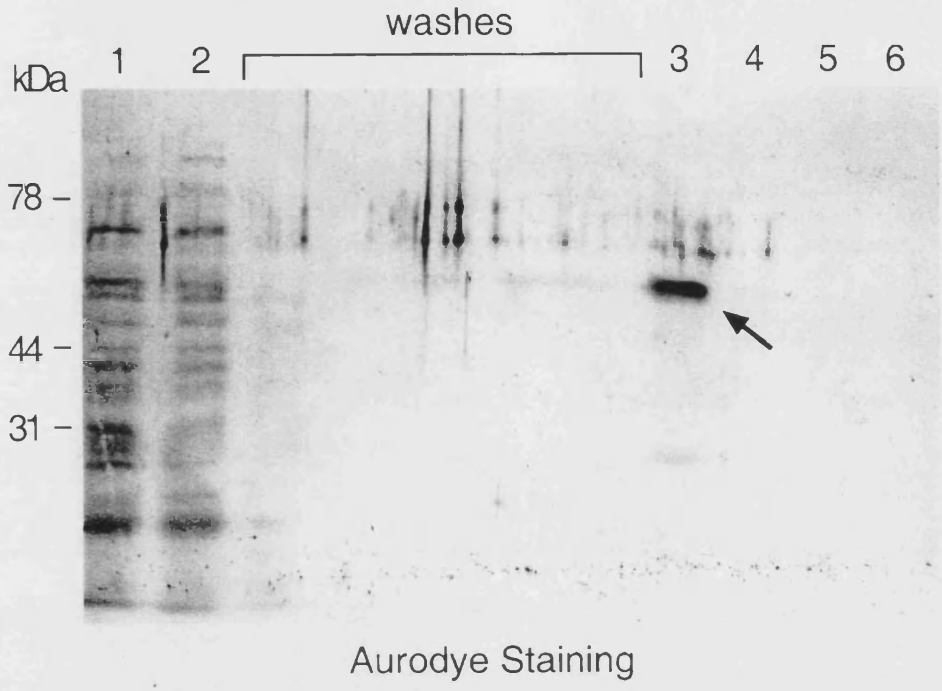


Figure 3.4 Aurodye™ blot staining of RecApa Ni-NTA column eluates. The same volume of sample was collected from all eluates. After protein transfer, the PVDF membrane was stained as described in Material and Methods. RecApa was eluted four times with 200mM of imidazole. 1, periplasmic extract; 2, periplasmic extract after the flowthrough; 3, first elution with 200mM imidazole; 4, second elution; 5, third elution; 6, fourth elution. Arrow indicates the recombinant Apa product.

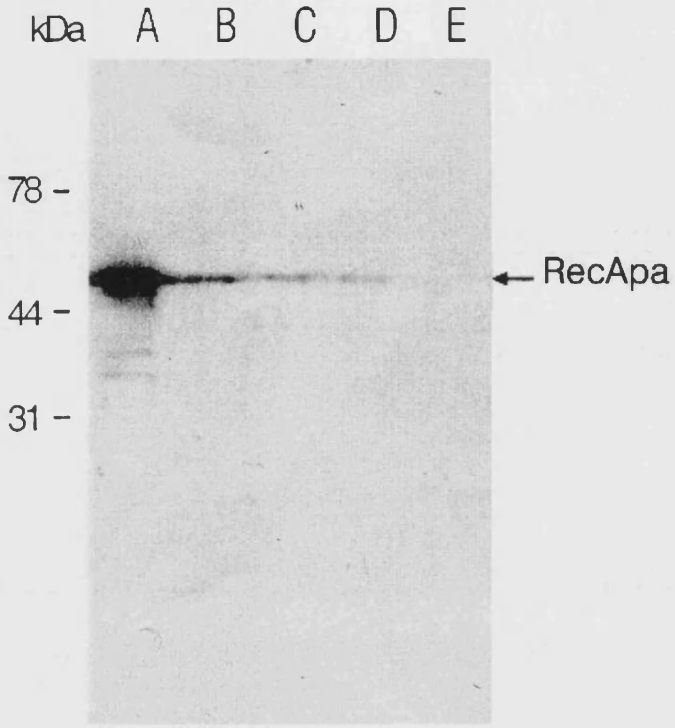


Figure 3.5 Western-blot analysis of RecApa Ni-NTA column eluates. A 10% polyacrylamide running gel was loaded with equal volumes obtained from the Ni-NTA column eluates and transferred for Western-blot analysis as described. A clear 47-kDa RecApa product can be seen. Smaller bands are probably resultant from sample degradation. A, first elution with 200mM imidazole; B, second elution; C, third elution; D, fourth elution; E, lysate of *E. coli* M15 strain, transfected with the pQE60 vector control.

All vectors used contained the promoter of the cytomegalovirus (CMV) immediate early gene, in order to obtain high levels of expression. The *M. tuberculosis* H37Rv *apa* gene coding sequence (including its N-terminal signal sequence), was cloned in frame, downstream of this promoter in the pCMV4 vector (fig.3.6A). An important characteristic of this vector is the presence of the intron A, the largest non-coding sequence from the CMV immediate-early 1 gene, reportedly capable of increasing the expression of cloned genes in this plasmid (Chapman, 1991; Tascon, 1997). A second plasmid expressing Apa, pSecTag2C.Apa, was also constructed (fig.3.6B). This plasmid allowed the extracellular secretion of the cloned product since it contained the murine Ig κ-chain V-J2-C signalling peptide. Between other features, a 6xHis-tag was also fused at the C-terminal part of Apa, allowing further purification of the protein (fig.3.6B). After cloning, mapping of the insert with restriction enzymes confirmed the identity of the gene and the correct orientation of the insert.

3.3.2 In vitro *transfection of cell lines with mammalian vectors*

The ability of these plasmid constructs to express Apa was tested *in vitro*. Both plasmids were transfected into CV1 cells; green-fluorescent protein (GFP)-expressing vectors were used as transfection controls (not shown). *In vitro* expression was detected in cell lysates and supernatants by Western-blot, using the Apa-specific monoclonal antibody 6A3 (Espitia, 1995). Recombinant Apa was included as the positive control. The results demonstrated efficient *in vitro* expression of Apa protein by both plasmids (fig.3.7). No reactivity was observed in the cell extracts or supernatants of CV1 cells (lane A) transfected with pCMV4 vector (lane B).

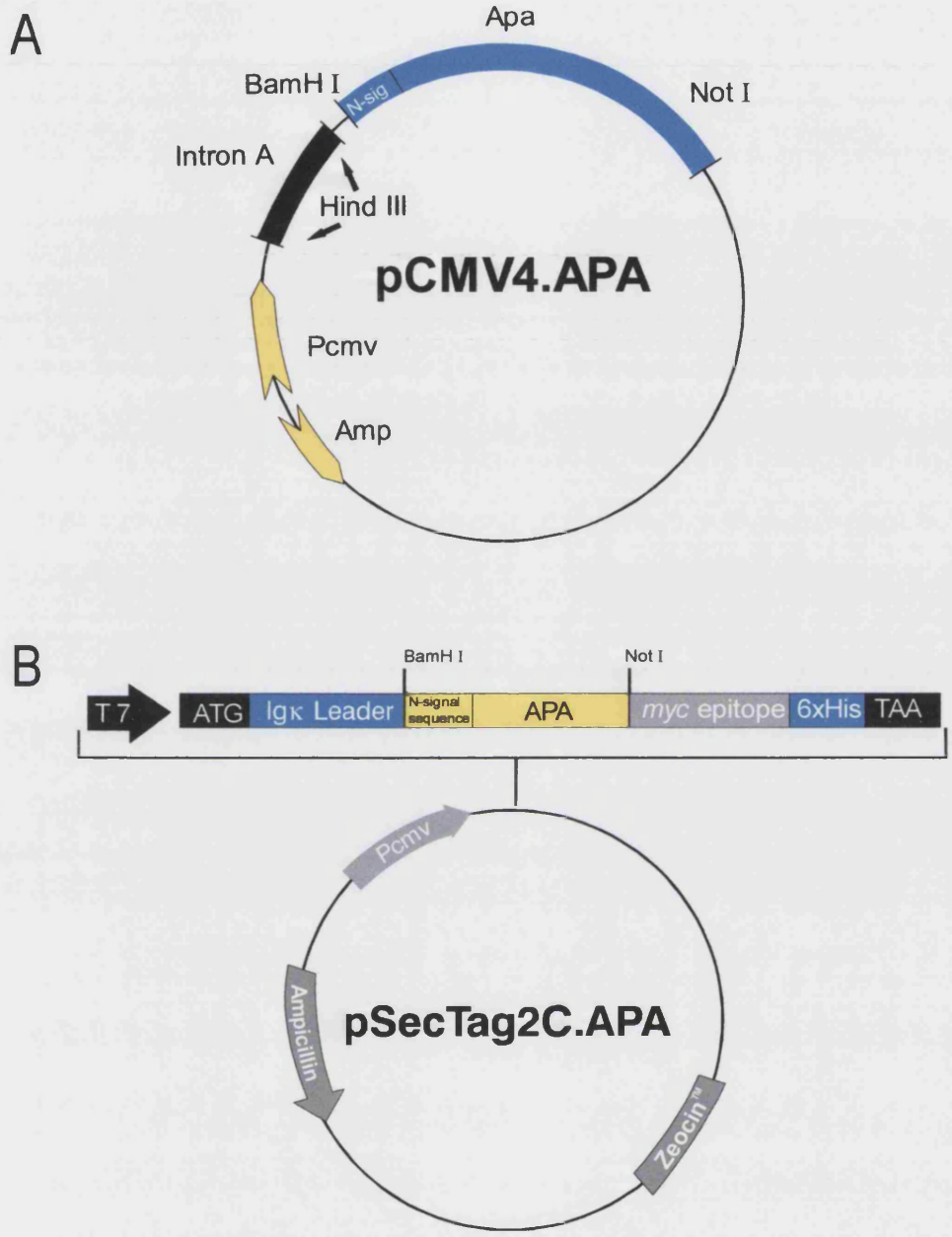


Figure 3.6 Schematic representations of ApaDNA vaccine vectors. The coding sequence of the *apa* gene, including its N-terminal secretion signal, was cloned directionally between the BamH I-Not I restriction sites of pCMV4 (A) and pSecTag2C (B). Plasmids were maintained in *E. coli* One Shot TOP10® cells as described previously.

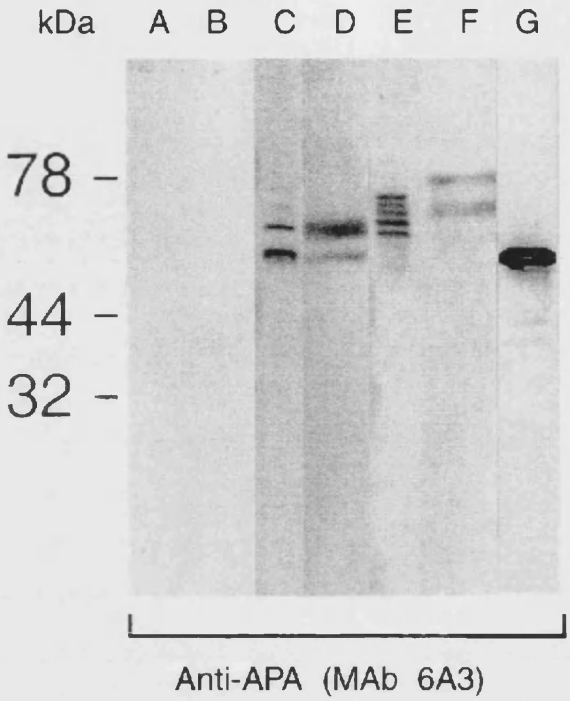


Figure 3.7 Western-blot analysis of cell lysates and supernatant filtrates of CV1 cells transfected with Apa expression plasmids. A 10% polyacrylamide gel was loaded with 5-10μg of total protein from the lysates of CV1 cells transfected with vectors expressing mycobacterial Apa, and processed by Western-blot as described. Lysates of cells transfected with pSecTag2C vector alone did not react with the 6A3 monoclonal antibody (not shown). A, CV1-pCMV4 lysate; B, CV1-pCMV4 supernatant; C, CV1-pCMV4.Apa lysate; D, CV1-pCMV4.Apa supernatant; E, pSecTag2C.Apa lysate; F, pSecTag2C.Apa supernatant; G, RecApa.

The pattern of expression, i.e., a complex of two bands, was similar to that observed in culture filtrates of MTB or BCG (see fig.3.17). Bands of 58- and 59-kDa were also detected in the filtered cell supernatants from CV1 cells transfected with pCMV4.Apa (lane D), suggesting that the secretion machinery of CV1 cells was able to recognise the mycobacterial N-terminal leader sequence cloned as part of the *apa* gene.

An increase in band size for the recombinant Apa protein was noticed after transfecting the cell line with both plasmids. In the case of pSecTag2C.Apa lysates (lane E) and supernatants (lane F), the increase in protein size could be partially, but not fully attributed to the fact the *apa* gene is fused to additional sequences present in this vector. In both cases, some degree of glycosylation in the Apa protein induced by the mammalian cell could be involved. The anti-Apa monoclonal antibody 6A3 was also able to identify several high molecular weight bands in the lysate of CV1 cells transfected with pSecTag2C.Apa (lane E); and these products could possibly be truncated (devoid of N-signal leader for secretion), post-translationally modified forms of recombinant Apa protein with different degrees of glycosylation (Horn, 1999). Glycosylation of Apa has been reported as critical for immune responses generated against this protein *in vivo* (Romain, 1999; Horn, 1999). Since glycosylation of Apa could also play a role in *in vivo* immune responses associated with ApaDNA vaccination, recombinant Apa protein was purified by Ni-NTA affinity chromatography from the supernatant of CV1 cells transfected *in vitro* with pSecTag2C.Apa. This purified protein was tested for the presence of sugar moieties. A specific fluorescent dye reacting with periodate-oxidised carbohydrate groups in the purified protein suggested that the recombinant Apa produced in mammalian cells was indeed glycosylated (fig.3.8), however the specific nature of the sugar moieties was not further investigated.

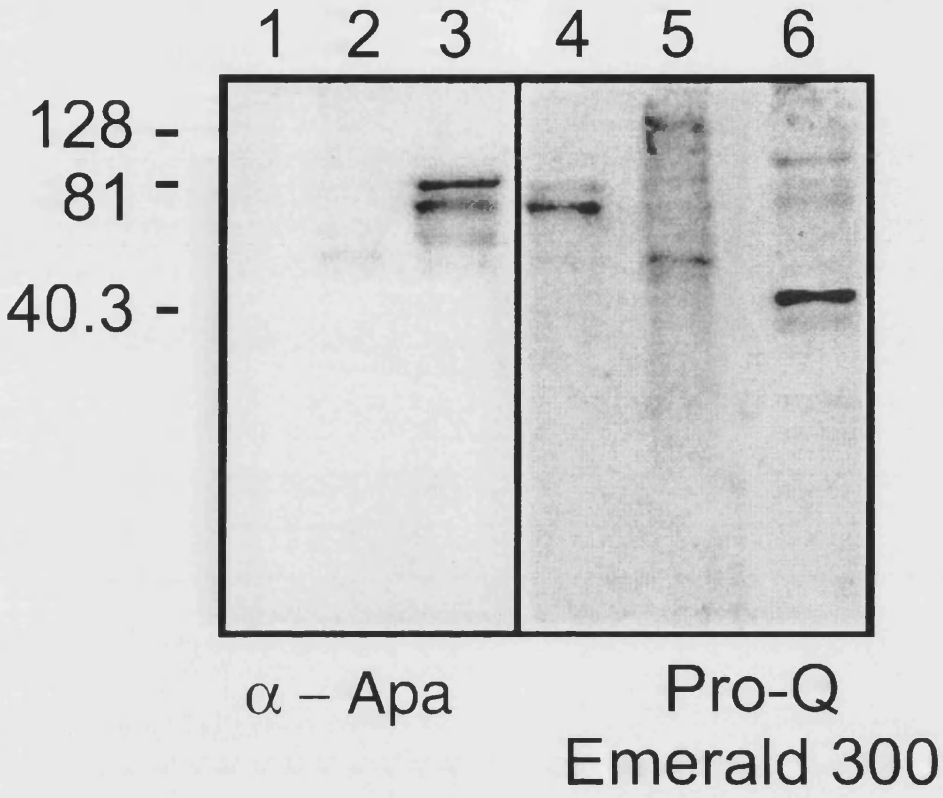


Figure 3.8 Fluorescent glycoprotein staining of Apa. Recombinant Apa purified from CV1 cells transiently transfected with pSecTag2C.apa showed increased molecular weight. Samples were purified by affinity chromatography and loaded in a 10% SDS-PAGE. After blotting, sugar moieties were oxidised by periodic acid and reacted with the Pro-Q Emerald 300® fluorescent stain. The blot was exposed to 300nm light and photographed. Half-blot was probed with anti-Apa 6A3 monoclonal antibody (α-Apa) for comparison. Lanes 2 and 5, CV1-cell extract; lanes 3 and 4, purified CV1-Apa; lanes 1 and 6, CandyCane® glycosylated molecular weight.

In order to obtain stable transfectants expressing Apa for later use as targets in CTL assays, the murine EL-4 tumour cell line was transfected in bulk with circular or linear pCMV4.Apa vectors. As a control, EL-4 cells were also transfected with pCMV4.GFP. After an initial period of two weeks for selection with G418 antibiotic, the surviving cells were cloned by limiting dilution. Several clones were obtained and re-cloned at less than one cell per well. No significant differences in the rates of transfection were observed between circular or linear vectors. Apa-expressing clones were selected by Western-blot, using the Apa-specific 6A3 monoclonal antibody, as described. A clone (AJF.26) stably expressing high levels of Apa protein, represented by a complex of two bands of approximate molecular weight of 59- and 60-kDa (fig.3.9), was expanded, aliquoted and kept in N_2 for future use.

3.3.3 Antibody responses from ApaDNA-vaccinated mice

In order to characterise antigen-specific antibody responses, groups of C57BL/6 were either vaccinated with 4 doses of plasmids expressing the mycobacterial *apa* gene, empty vector, BCG or saline, as described in Material and Methods.

As expected, no significant differences were observed in humoral or cellular immune responses produced by animals vaccinated with pCMV4.Apa compared to the secretion-vector pSecTag2C.Apa (not shown). They are collectively called here as ApaDNA vaccine. In all chosen dilutions, the sera obtained from ApaDNA-vaccinated animals showed elevated specific anti-Apa IgG levels (fig.3.10A), and showing consistency with the results of *in vitro* gene expression. In contrast, control groups or BCG vaccinated mice sera were essentially negative for anti-Apa IgG-antibodies.

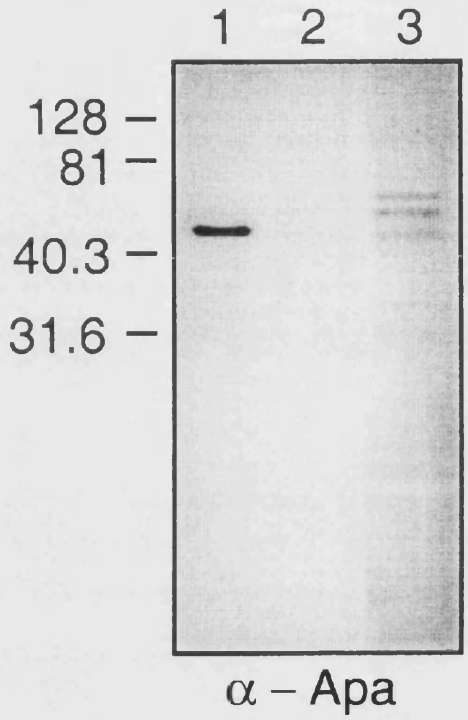


Figure 3.9 Western-blot of Apa-expressing EL-4 clone. Among EL-4 clones under drug selection, Apa-positive clones stably transfected with pCMV4.Apa, were identified according to the reactivity with anti-Apa 6A3 monoclonal antibody (α-Apa). Equal amounts of cell extract proteins (10μg) from EL-4 stable transfectants were loaded in 10% polyacrylamide SDS-PAGE and blotted. The clone AJF.26 reacted more expressively with this antibody and was selected for future use. The Apa-complex of two bands of approximately 59 and 60-kDa can be seen in lane 3. Lane 1, RecApa produced in *E. coli*, 2μg; lane 2, EL-4 cell extract; lane 3, EL-4 clone AJF.26.

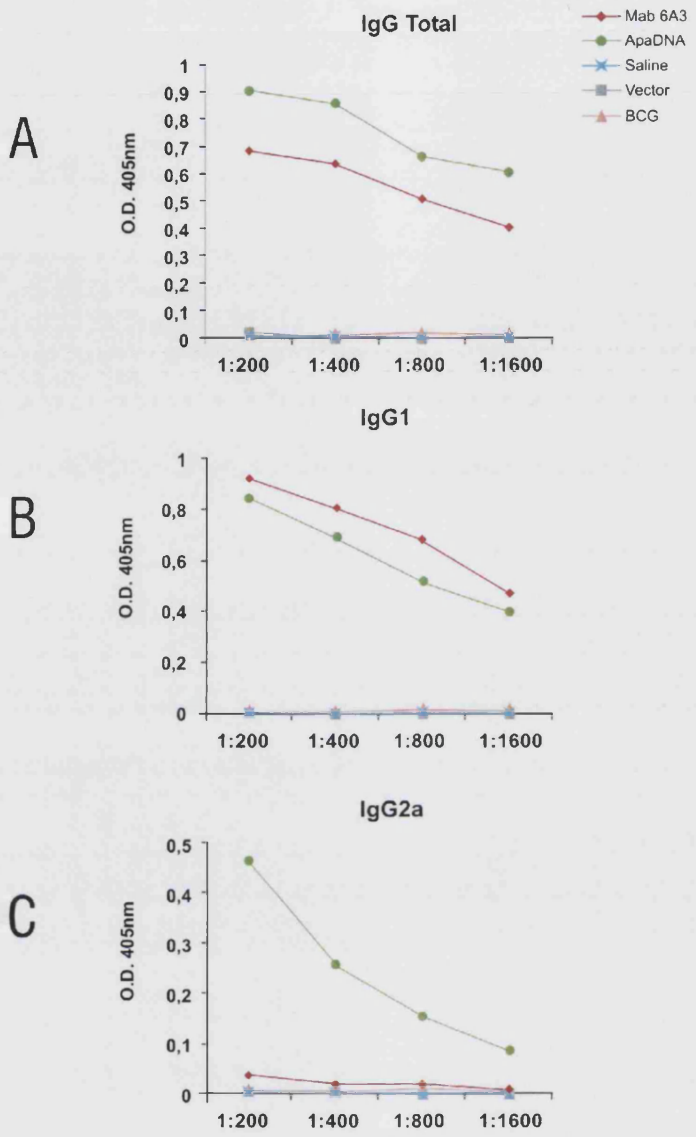


Figure 3.10 Serological responses to RecApa by ApaDNA vaccinated mice. C57BL/6 mice were immunised intramuscularly four times, three weeks apart, with saline, vector, ApaDNA or once intradermally with BCG. Serum samples were collected three weeks after the last injection and analysed by ELISA for the presence of anti-Apa total IgG (A), IgG1 (B) and IgG2a (C) antibodies. Monoclonal antibody 6A3 (mAb 6A3, IgG1 isotype, Espitia, 1995), specific for the Apa protein was used as the positive control. Results show the mean data of four mice.

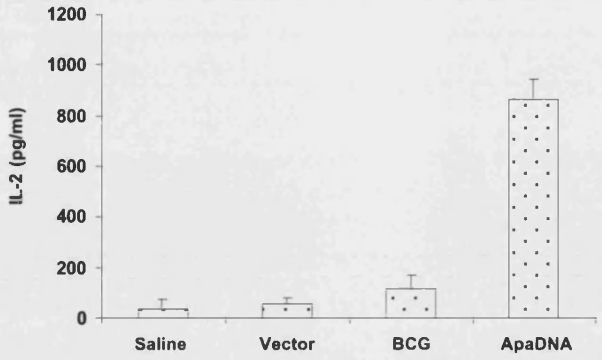
Investigation on the IgG isotypes involved during ApaDNA vaccination revealed that both anti-Apa IgG1 and IgG2a isotypes were present (fig.3.10B & C). These results then suggested the presence of a specific Th1/Th2 mixed type of immune response in Apa-DNA vaccinated mice and are also consistent with findings reported by other groups (Morris, 2000; Garapin, 2001).

3.3.4 Production of cytokines by spleen cells from Apa-DNA vaccinated mice

To study how cellular immune responses are affected by the ApaDNA construct, spleen cells from vaccinated C57BL/6 mice and controls were stimulated with 6μg/ml of purified RecApa, native Apa (purified from culture filtrates of *M. tuberculosis*), 10μg/ml of PPD, control antigens or medium alone. Cytokines were detected in filtered supernatants of the cultures by ELISA collected in a period of 72 hours. Measurement of supernatants collected at 24 hours demonstrated that splenocytes from ApaDNA-vaccinated mice had the ability to produce significant amounts of IL-2 *in vitro*, even if stimulated with non-glycosylated RecApa (fig.3.11A). In comparison, the levels of IL-2 produced by spleen cells from animals injected with saline, vector or BCG were significantly lower. In addition, when splenocytes were highly enriched for CD4+ T cells, the production of IL-2 *in vitro* from ApaDNA mice (but not controls) could also be demonstrated (fig.3.11B).

Production of IFN-γ is a critical requirement for protective immunity against *M. tuberculosis* (Cooper, 1993; Flynn, 1993). To assess the role of the ApaDNA vaccine at inducing IFN-γ production, spleen cells from vaccinated mice and controls were cultured *in vitro* with either RecApa, native Apa, PPD, Concanavalin A (Con A) or a synthetic

A



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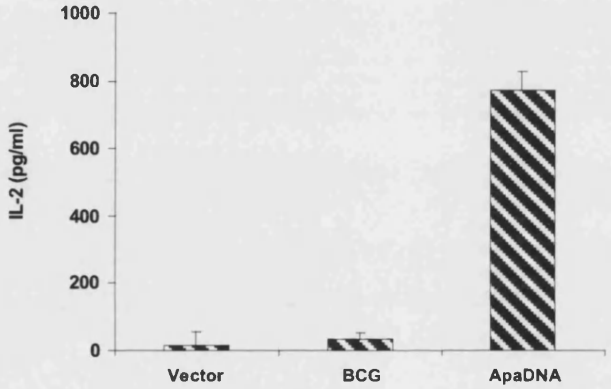


Figure 3.11 Production of IL-2 by ApaDNA vaccinated mice. C57BL/6 mice were immunised intramuscularly four times, three weeks apart, with saline, vector, ApaDNA or once intradermally with BCG. Spleen cells were cultured with or without (not shown) 6μg/ml of purified RecApa. 24h-supernatants were assayed for the presence of IL-2. Total spleen cells (A) or CD4+ T cells (B) were cultured as described. Results are expressed as the mean plus the standard error (SE) number from triplet wells. The experiment was repeated twice.

peptide comprising the first 42 amino acids from the N-terminal part of the mature Apa protein. Cells obtained from mice vaccinated with ApaDNA (but not controls) secreted high levels of IFN-γ *in vitro*, when stimulated with either recombinant or native Apa protein; glycosylation did not seem to play a role in increasing cytokine secretion (fig.3.12B). BCG- but not ApaDNA-vaccinated mice produced significant amounts of IFN-γ when exposed *in vitro* to PPD (fig.3.12C). Cells from ApaDNA vaccinated mice, however, did respond significantly to PPD *in vitro* (1.001ng/ml ± 0.14), if compared to saline and vector groups (0.201 ± 0.01 and 0.293 ± 0.06 ng/ml, respectively), confirming the presence of Apa protein in the antigenic mixture present in PPD preparations. Interestingly, the Apa-peptide 42'(mannosylated) did not induce responses from spleen cells from vaccinated mice, suggesting that at least one specific T cell epitope should be localised further downstream in the Apa sequence (preliminary data).

CD4+ T cells are among the most important in the protective response against *M. tuberculosis* (Flynn & Chan, 2001). To investigate the CD4+ T cell responses from vaccinated animals, spleen cell suspensions were enriched for CD4+ T cells by negative depletion of other spleen subpopulations in affinity columns and stimulated *in vitro* with RecApa plus either BMDCs or irradiated macrophages as antigen-presenting cells. As expected, T cells derived from ApaDNA- but not vector-vaccinated mice were able to specifically produce increased levels of IFN-γ *in vitro* (not shown). The purity of these CD4+ preparations could be increased further up to 99.9% when FACS-sorting was performed. Spleen cells from either ApaDNA- or vector-vaccinated mice, previously stained with anti-CD4+ FITC-monoclonal antibody, were sorted under sterile conditions. The recovered cells were cultured *in vitro* for up to 72 hours, in the presence of RecApa or medium and APCs.

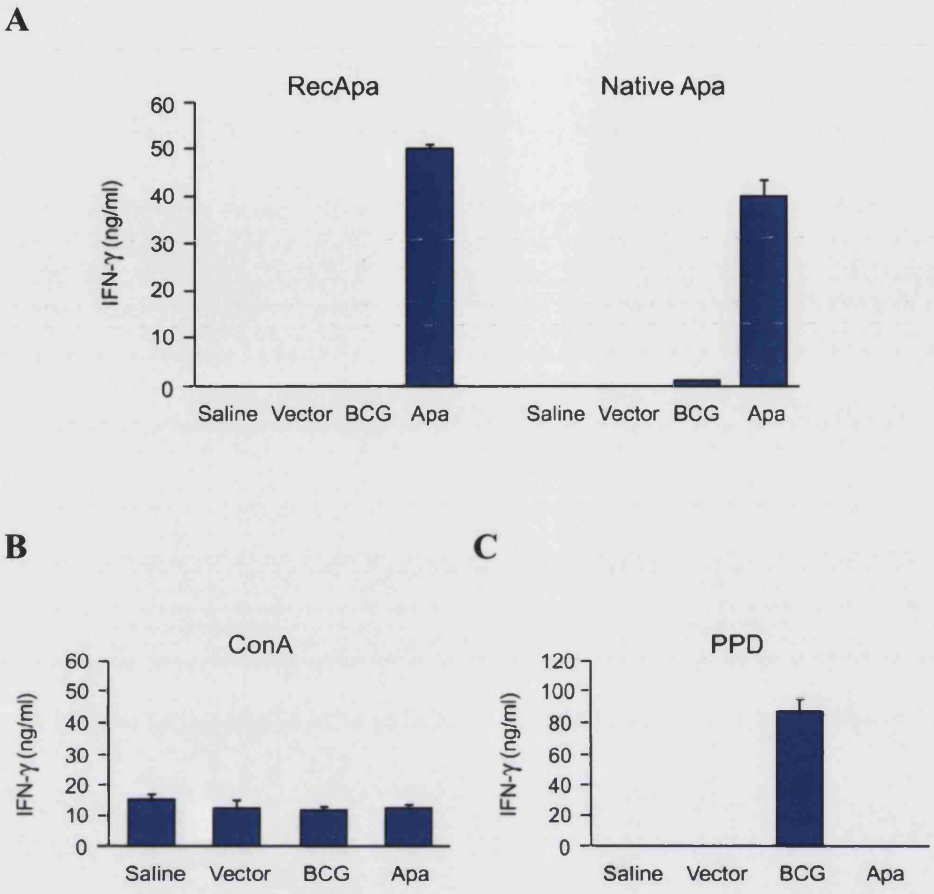


Figure 3.12 Specific IFN-γ levels produced by spleen cells from ApaDNA vaccinated mice. IFN-γ levels were determined by ELISA in 72-hours stimulated cultures. Cells were collected three weeks after last dose and stimulated either with RecApa or native Apa (A), Concanavalin A (B) or PPD (C). Data representing a typical experiment repeated three times are expressed as means plus standard errors for three mice in each group.

Again, only CD4+ but not CD4- cell population from ApaDNA-vaccinated mice were able to show increased levels of IFN-γ in the supernatant (fig.3.13). Bone marrow-derived dendritic cells or macrophages, in the presence of RecApa in culture, were not capable of producing IFN-γ (fig.3.13 and preliminary data). Cytokines such as IL-4, IL-10 and TGF-β1 have either an inhibitory effect on Th1 cell responses or in cellular responses in general and could possibly suppress protective anti-mycobacterial responses (Turner, 2002; Toossi, 1995). The same supernatants collected to measure IFN-γ levels were also used to analyse the levels of these cytokines. Neither total spleen nor CD4+ T cells from DNA vaccinated mice (or controls) were able to show measurable levels of any of these cytokines in response to *in vitro* RecApa stimulation (not shown). These measurements were repeated three times in two different vaccination experiments.

3.4 CD8+ T cell responses to Apa

Control of tuberculosis infection in mice involves the participation of CD8+ T cells. Besides cytotoxic functions, secretion of cytokines derived from CD8+ T cells, particularly IFN-γ, is regarded as highly beneficial, if not critical to the protective response against MTB (Tascon, 1998). To investigate the CD8+ T cell response in ApaDNA vaccinated mice, spleen cell cultures were enriched for CD8+ T cells by negative selection during column purification. Isolated CD8+ T cells from vaccinated mice and controls were cultured *in vitro* with RecApa antigen and immature BMDCs as antigen-presenting cells. In order for the MHC-restriction to be preserved, BMDCs were isolated from MHC-II knockout ($MHC^{-/-}$) mice and cultivated *in vitro*, as described in Material and Methods.

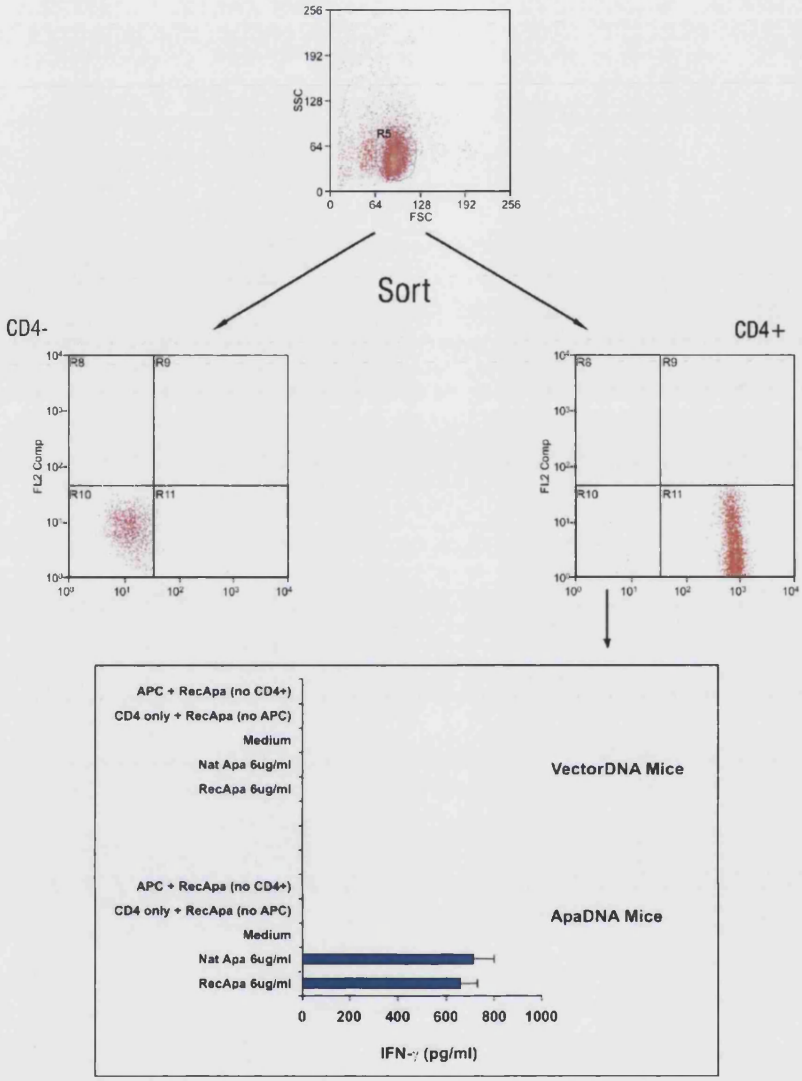


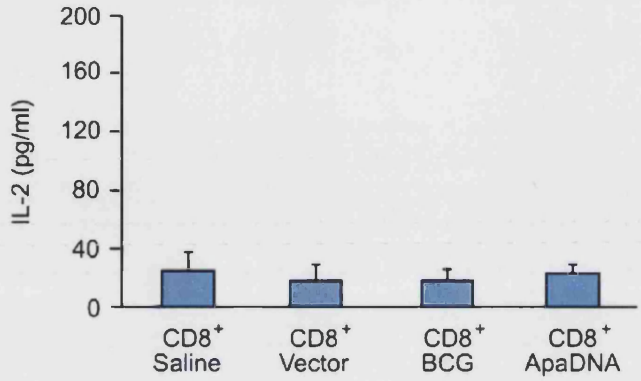
Figure 3.13 CD4+ T cells from ApaDNA vaccinated mice produce IFN-γ in response to Apa. Spleen cell preparations from ApaDNA vaccinated mice or control vector were enriched for CD4+ T cells by affinity column purification; the isolated population was submitted to another round of purification by FACS-sorting, as described. The CD4+ T cells were co-cultured with bone-marrow derived dendritic cells as APCs, plus RecApa or native Apa for 72 hours. IFN-γ was measured by ELISA in the supernatant. Data represents the mean + standard error for three mice in each group. The experiment was repeated twice.

Supernatants were collected at intervals to measure IL-2 and IFN-γ cytokines by ELISA. The results demonstrated that when CD8+ T cells from ApaDNA-vaccinated mice were antigenically re-stimulated *in vitro*, they secreted very low amounts of either IL-2 or IFN-γ (fig.3.14A & B).

CD8+ T cells do not respond well to antigens provided in the extracellular format. As an extra control for the experiment described above, immature bone marrow dendritic cells were induced to process a complex of cationic liposomes and RecApa. These complexes have been demonstrated to increase loading into the class I processing pathway (Chen, 1993). CD8+ T cells were isolated by column purification from the spleen of C57BL/6 mice which have been infected intravenously with *M. tuberculosis* H37Rv for 30 days. The isolated lymphocytes were transferred to U-bottom sterile plates containing dendritic cells exposed to either RecApa-liposomal complexes, liposomes alone or recombinant MPT83-liposomal complexes, as described previously. MPT83 was able to stimulate cytotoxic T cell responses in mice and was detected during mycobacterial infection (R.E. Tascon, unpublished results). The results demonstrated that IFN-γ levels from CD8+ T cells were increased when activation with MPT83 occurred (fig.3.15A). After intracellular FACS-staining of CD8+ IFN-γ+ T cells, a significant percentage of CD8+ T-cells from *M. tuberculosis* infected mice were stained positively for IFN-γ in response to MPT83 protein (fig.3.15B). However, only a small fraction of CD8+ T cells from the infected animals showed a similar phenotype in response to liposome-RecApa or liposome-control.

The ability of CTL to lyse infected targets is thought to facilitate control of the infection by releasing the bacilli so they can be taken up at low multiplicity by freshly activated macrophages and destroyed (Serbina, 2000). To address whether specific Apa-

A



B

Figure 3.14 Production of IL-2 and IFN-γ in Apa-stimulated CD8+ T cells from ApaDNA vaccinated mice. C57BL/6 mice were immunised intramuscularly four times, three weeks apart, with saline, vector, ApaDNA or once intradermally with BCG. CD8+ T cells were isolated as described in Material and Methods and cultured with 6μg/ml of purified RecApa. 24h-supernatants were assayed for the presence of IL-2 (A) and 72h-supernatants assayed for IFN-γ (B). Results are expressed as the mean plus the standard error (SE) from triplet wells. The experiment was repeated three times.

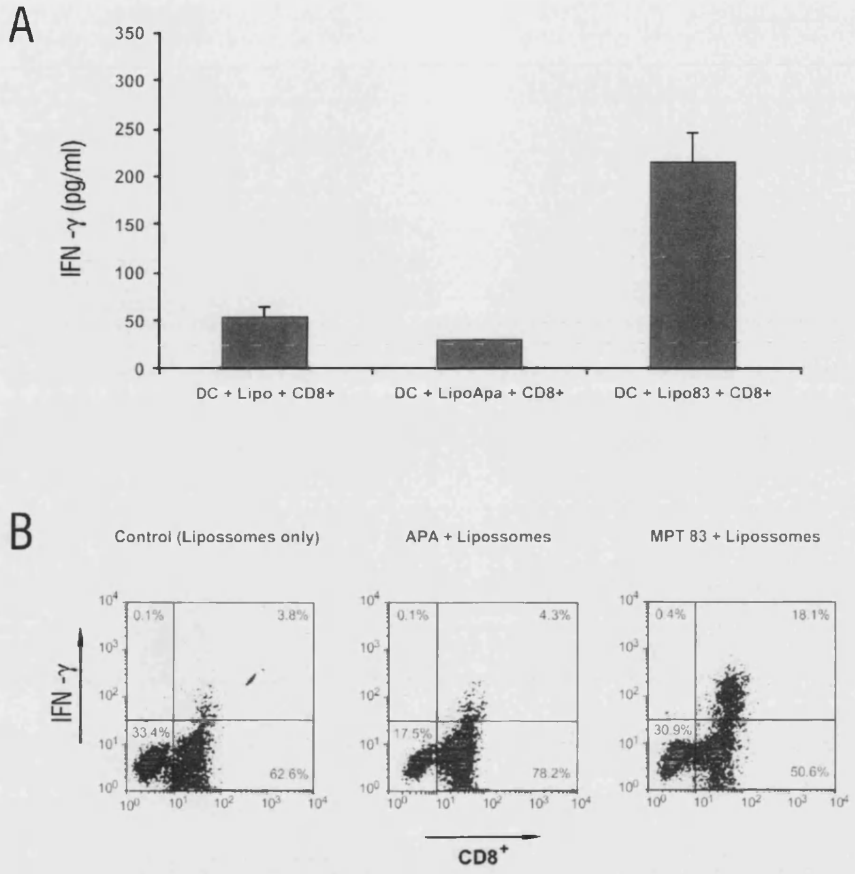


Figure 3.15 Dendritic cell processing of exogenous antigen for CD8+ T cells. Immature BMDCs were loaded with mixtures of liposomes and RecApa or MPT83 recombinant proteins. These APCs were immediately washed and then co-cultured with isolated CD8+ T cells from the spleen of C57BL/6 mice infected with *M. tuberculosis*. (A) IFN-γ as measured by ELISA in 72 hours supernatants. Data represents the mean + standard error from four mice in each group. (B) Representative flow cytometry dot plots depicts the percentage of cells labeled with anti-CD8 and intracellular anti-IFN-γ from the co-cultures of liposome-induced dendritic cells and CD8+ cells maintained for 10 days. The experiment was done twice.

CTL responses were occurring in ApaDNA vaccinated mice, a murine tumour cell clone with stable Apa expression was generated *in vitro* by transfecting EL-4 cells (H-2^b) with the pCMV4.Apa plasmid. Spleen cells from mice either vaccinated four times with ApaDNA or empty vector were isolated as described and incubated with lethally irradiated EL-4.Apa cells for six days. EL-4, EL-4.Apa or positive control Con A-activated blasts, derived from spleen cells of CBA mice (H-2^k) were used as targets in a ^{3}H JAM cytotoxicity assay (Matzinger, 1991). As demonstrated in fig. 3.16A & B, alloreactive spleen cells from C57BL/6 mice (H-2^b) vaccinated either with vector or ApaDNA, exhibited high cytotoxicity against Con A blast targets from CBA mice (H-2^k) in all effector-to-target (E/T) ratios, as expected; on the other hand, the cytotoxicity levels against EL-4.Apa targets was surprisingly similar between both mice vaccinated with ApaDNA or empty vector (fig.3.16C), suggesting the lack of Apa-specific subpopulations of cytotoxic cells in ApaDNA vaccinated mice.

3.5 Prime-boost vaccination with ApaDNA vaccine and BCG (Prime-boost I)

3.5.1 Expression of Apa antigen by BCG-Glaxo

Heterologous prime-boost is a vaccination strategy able to generate unprecedented levels of cellular immunity and afforded significant levels of protection to several diseases, including tuberculosis (McShane, 2001; Feng, 2001, Skinner, 2003a). The heterologous prime-boost protocol involves administering two different vaccines, non-concomitantly, each encoding the same antigen (Li, 1993). In the strategy used here, the ApaDNA vaccine was combined with the BCG Glaxo strain (Glaxo-SmithKline, UK) in

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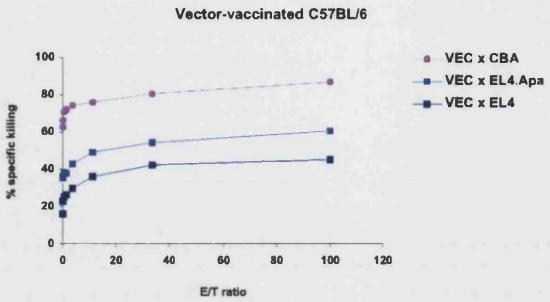

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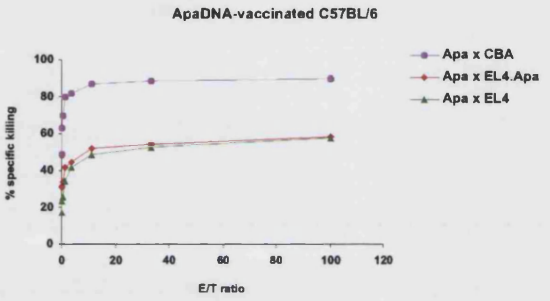

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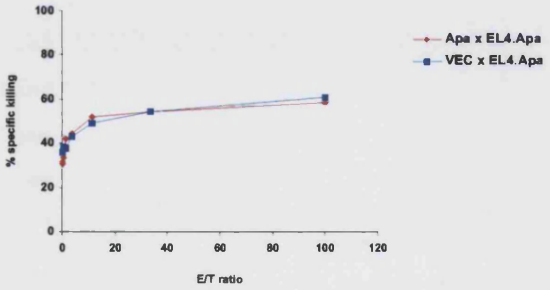

Figure 3.16 Cytotoxic responses induced by ApaDNA and vector-vaccinated mice. C57BL/6 mice were injected four times, with 100μg of either ApaDNA or pCMV4 vector; spleen cells from three mice per group were cultured with irradiated EL-4.Apa cells and IL-2. CTL activities from vector (A) and ApaDNA (B) mice were tested against ^{3}H-thymidine-labelled EL-4, EL-4.Apa and Con A-blasts from CBA mice (H-2^{k}), using the JAM assay (Matzinger, 1991). Calculations for specific killing are described in chapter two. (C) CTL curves for vector- and ApaDNA-effectors against EL-4.Apa-targets compared closely.

a prime-boost regimen using the mouse model of intravenous *M. tuberculosis* infection. BCG strains sometimes exhibit differential expression for certain antigens (Oettinger, 1999). In order to comply with the prime-boost principle, it was important to address whether BCG-Glaxo expressed significant levels of the Apa protein. BCG-Glaxo growing as a surface pellicule in a non-protein based medium (Sauton medium) were harvested during logarithmic phase, for the preparation of protein extracts from BCG-cell lysate (lane 1) and culture filtrate (lane 2, fig.3.17). *M. tuberculosis* lysate (lane 3) and purified recombinant Apa (lane 4) were used as positive controls. The results demonstrated that when detection was performed using the anti-Apa specific monoclonal antibody 6A3, it was possible to visualise the characteristic doublet band from the Apa complex in all four fractions described above. The approximate molecular weight for the Apa bands were 47- and 48-kDa; stronger bands were clearly seen in the culture filtrate of BCG-Glaxo (lane 2). No reactivity was detected in the negative control, *E. coli* lysate (lane 5). BCG-Glaxo viable bacteria were utilised in all prime-boost experiments.

3.5.2 Immune responses in animals receiving the prime-boost I vaccination protocol

Besides being capable of inducing excellent levels of cell-mediated immune responses, prime-boost strategies also raise significantly the humoral responses, particularly the IgG2a isotype, normally associated with the production of IFN-γ (Ramshaw & Ramsay, 2000; Amara, 2001). The prime-boost I protocol (fig.3.18) was designed to analyse the potential of ApaDNA priming plus BCG boosting to improve protective responses. C57BL/6 mice were primed once with ApaDNA, intramuscularly and boosted one month later with BCG-Glaxo, as described.

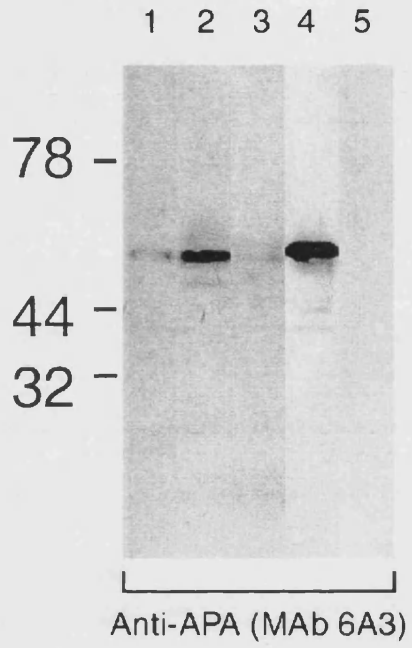

Figure 3.17 Expression of Apa in BCG-Glaxo strain. 8-10μg of total protein extracts were loaded in a 10% polyacrilamide denaturing gel and electroblotted into PVDF membranes for Western-blot analysis, as described. The blot was probed with anti-Apa mAb 6A3 diluted 1:250. Lane 1, BCG-Glaxo cell lysate; lane 2, BCG-Glaxo culture filtrate; lane 3, *M. tuberculosis* cell lysate; lane 4, RecApa; lane 5, *E. coli* cell lysate (control).

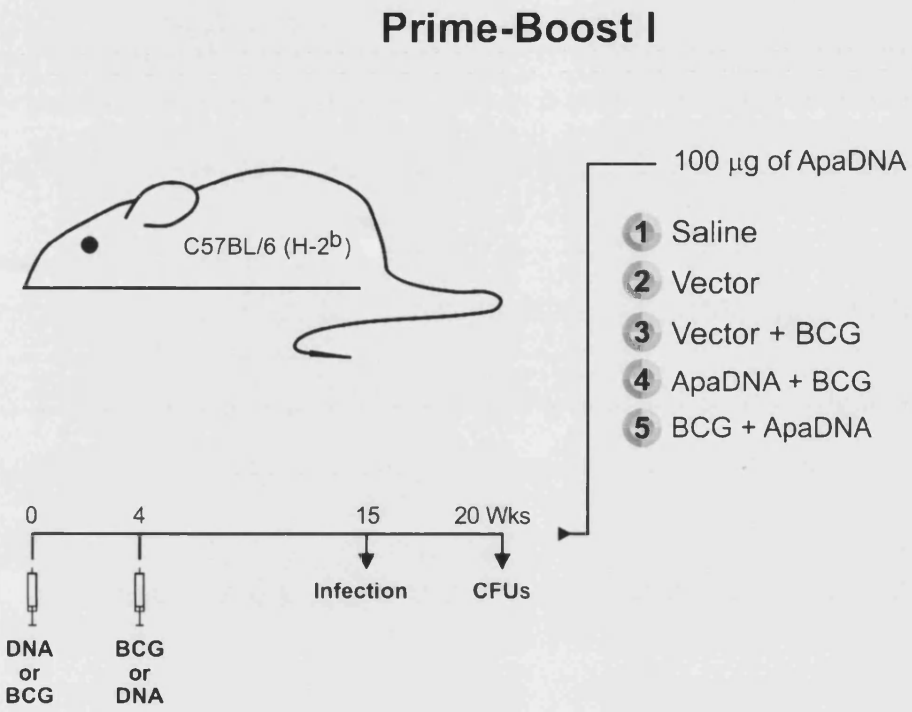

Figure 3.18 Schematic representation of the vaccination protocol prime-boost I. 6-8 weeks old C57BL/6 females were injected intramuscularly with plasmid DNA or intradermally with BCG, as either the priming or boosting depending on the group. Control groups included saline, vector and vector plus BCG. CFUs, colony forming units.

Groups of animals where BCG was given once as the priming vaccine and ApaDNA as a booster were also included. Approximately six weeks after the boosting injection, serum was obtained and anti-Apa antibody titres were determined. Both groups of animals receiving either ApaDNA as priming or boosting vaccine, showed raised specific IgG antibody levels (fig.3.19A). Mice receiving vector only or vector plus BCG did not show significant reactivity. Interestingly, when IgG isotypes were analysed separately, only animals receiving BCG as the priming vaccine showed measurable IgG2a antibodies (fig.3.19B). It was not possible to detect anti-Apa IgG2a antibodies in ApaDNA primed-BCG boosted mice at the time of screening. On the other hand, IgG1 titres were of very low magnitude for all animals analysed (fig.3.19C).

The production of IL-2, IFN-γ and IL-4 cytokines by spleen cells from prime-boosted vaccinated mice and controls, in response to RecApa stimulation *in vitro*, were also analysed. Animals were killed by Schedule one protocol in the sixth week after boosting and spleen cells isolated and stimulated *in vitro* with recombinant Apa protein. The amount of IL-2 secreted by prime-boost groups was considered low but still notably increased in comparison with controls either receiving vector alone or vector-BCG combination (fig.3.20A). There was no significant difference in the amount of IL-2 produced between the animals primed either with ApaDNA or BCG. In the case of IFN-γ, groups of mice receiving BCG as the priming agent exhibited a significantly higher production of IFN-γ ($P < 0.05$) when compared with animals receiving ApaDNA first (fig.3.20B). It is interesting to note that the trend was repeated when cells from the same group were analysed for the production of IL-4 cytokine (fig.3.20C); although the overall levels of IL-4 produced were very low, significantly higher levels of this Th2 cytokine were produced in the group of mice primed with BCG and boosted with DNA.

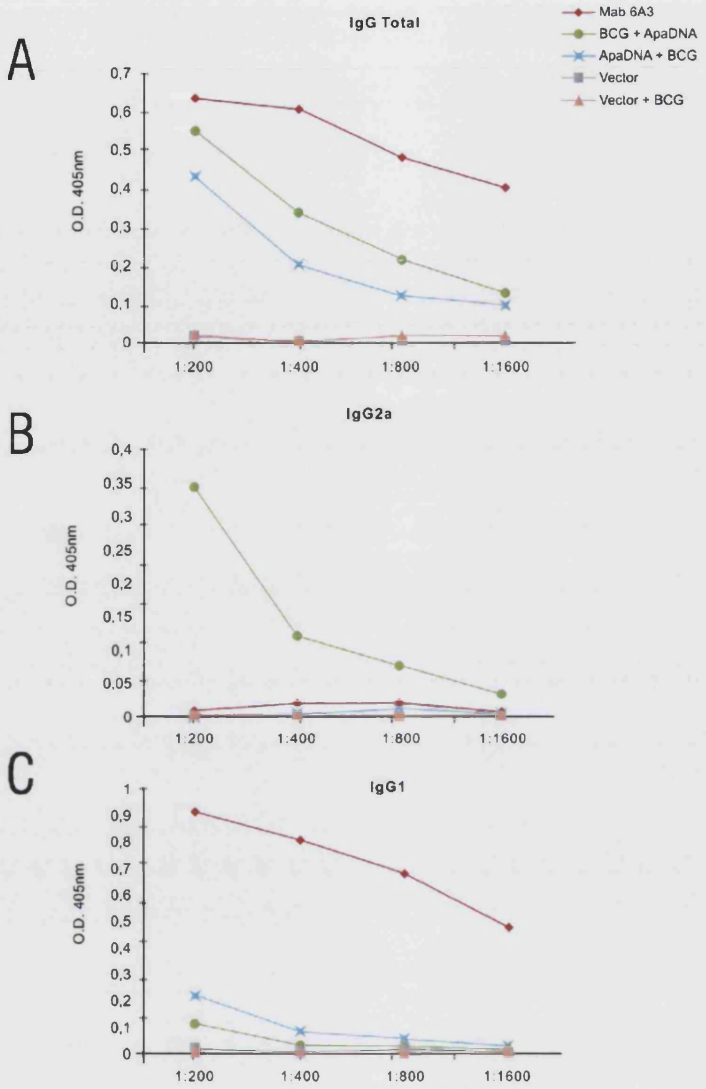

Figure 3.19 Serological responses to RecApa in prime-boosted vaccinated mice. C57BL/6 mice were immunised once with 100μg of ApaDNA and boosted one month later with BCG-Glaxo or vice-versa, as described. Controls received empty pCMV4 vector only or pCMV4 empty vector added to BCG. Titres of total anti-Apa IgG (A), IgG2a (B) and IgG1 (C) antibodies, were determined six weeks after boosting. The monoclonal antibody 6A3 (IgG1 isotype) was used as the positive control. Each point represents the mean data for four mice.

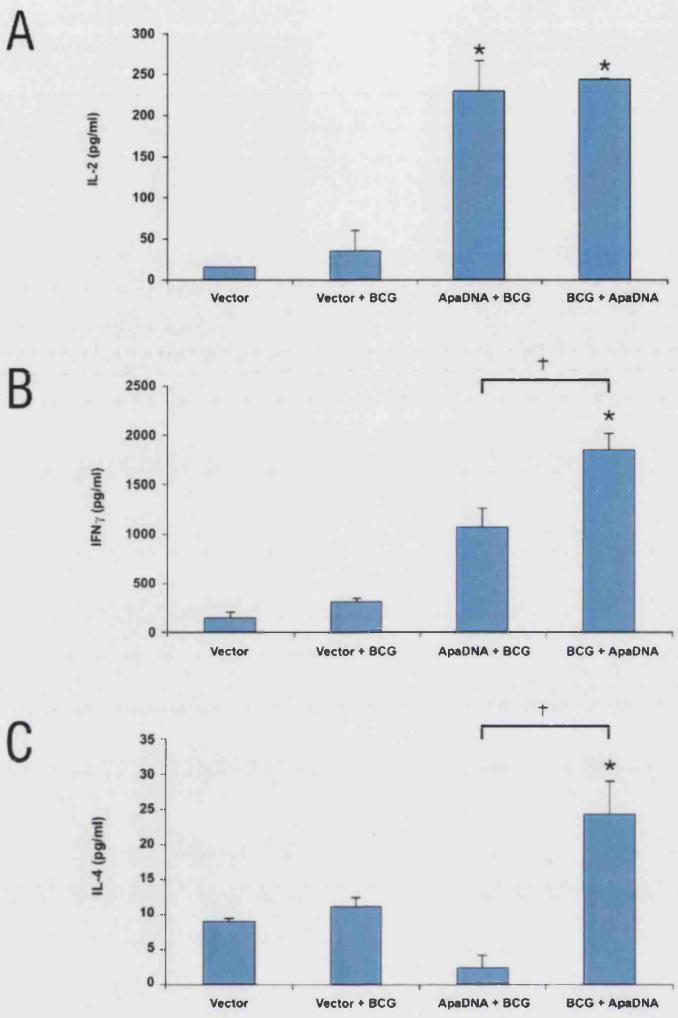

Figure 3.20 Cytokine production by spleen cells from prime-boosted I vaccinated mice. Spleen cells were cultured and stimulated for 48-72 hours with RecApa or medium. The levels of IL-2 (A), IFN-γ (B) and IL-4 (C) secreted in the supernatant were analysed by ELISA, as described. Asterisks indicate that the amount of cytokine secreted is statistically significant compared to the vector or vector plus BCG group, as determined by Student *t* test ($P < 0.05$). The significance of the difference between BCG + ApaDNA group and ApaDNA + BCG group was $P < 0.05$ (□). Error bars indicate standard error of the means. The experiment was repeated twice

In both cases, controls stimulated with RecApa *in vitro* did not show detectable levels of either IL-4 or IFN-γ.

3.5.3 Protective responses in animals receiving the prime-boost I vaccination protocol

The type of immune response induced by the prime-boost strategy is particularly dependent on the nature of the boosting agent (Ramsay, 1999). In these terms, it has been demonstrated that BCG is a more effective boosting agent than viral carriers in prime-boost protocols against murine tuberculosis (Feng, 2001). Since the specific immune responses were up-regulated by the prime-boost I vaccination protocol, vaccinated mice were then challenged with *M. tuberculosis* H37Rv. In the experiments described here, BCG was tested both as priming or boosting agent, combined with one injection of 100μg of ApaDNA plasmid. C57BL/6 mice were infected ten weeks after the boosting dose and killed five to six weeks later to analyse the bacteriologic burden in the spleen and lungs. The use of combinations of ApaDNA and BCG, independent of the order, did not confer better protection than BCG itself, both in the lungs and spleen (fig.3.21). All BCG-vaccinated groups showed significant protection in both lungs and spleen ($P < 0.05$).

3.6 Prime-boost with DNA combination vaccine and BCG (Prime-boost II)

An usual strategy to increase the potency of DNA vaccines against tuberculosis is demonstrated by the use of multisubunit vaccination with different DNA vectors (Huygen, 2003).

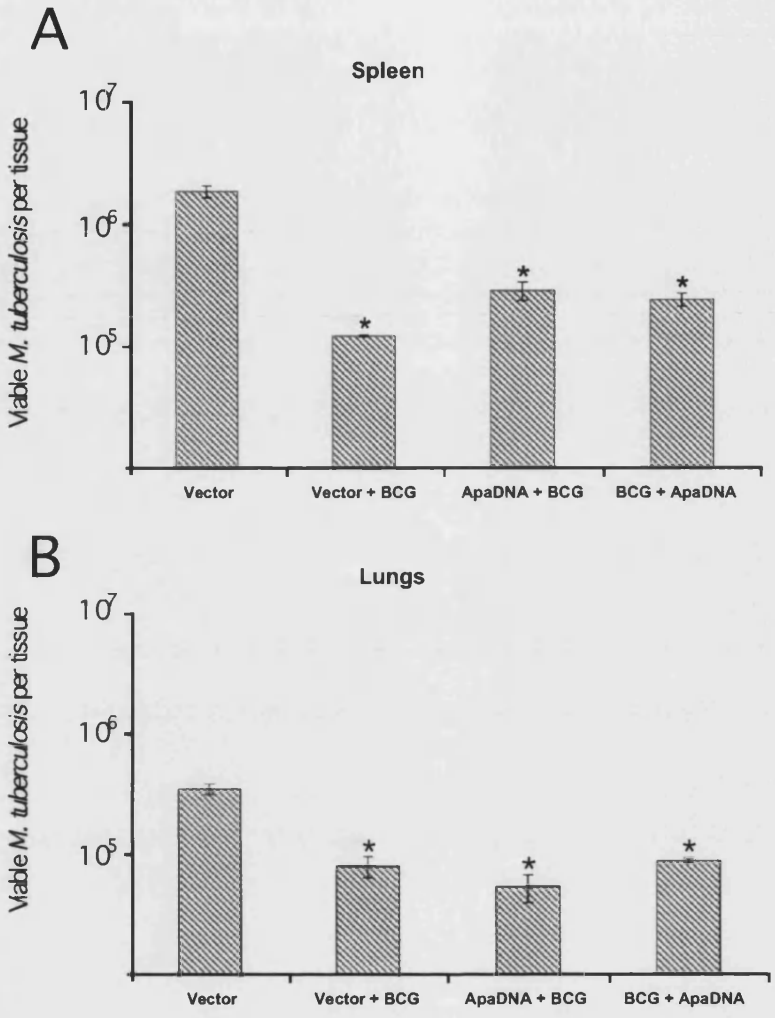

Figure 3.21 Protection provided by prime-boost I immunisation. C57BL/6 mice were vaccinated intramuscularly with ApaDNA, empty vector, or BCG and boosted one month later with BCG or ApaDNA. The mice were challenged intravenously with approximately 10^5 CFU of *M. tuberculosis* H37Rv. Protection was assessed thirty-eight days later in the spleen (A) and lungs (B). Asterisks indicate protection statistically significant compared to vector group ($P < 0.05$), as determined by the Student *t* test. Error bars indicate standard errors of the means.

Improved IFN-γ responses, reduced bacterial burden and prolonged survival time were reported when these multisubunit regimens were tested in mice (Kamath, 1999; Delogu, 2002). The prime-boost II immunisation experiment was designed to examine the immune responses and to investigate whether the simultaneous injection of ApaDNA vaccine, in combination with other DNA gene constructs expressing different immunodominant mycobacterial genes could induce resistance against tuberculosis (fig.3.22). Groups of mice were vaccinated once with DNA combinations comprising the HSP65, HSP70, Apa, MPT83 and MPT70 encoding plasmids. These MTB antigens were selected for evaluation together with ApaDNA, due to their previously documented immunogenicity in the context of DNA vaccination against tuberculosis (Tascon, 1996; Tascon, 1997; Lowrie, 1999; Chambers, 2000). Each plasmid was injected separately in a different limb. BCG, as a booster, was injected intradermally one month later. Groups receiving BCG as the priming vaccine, instead of DNA, were also evaluated.

3.6.1 Immune responses in animals receiving the prime-boost II vaccination protocol

In order to assess whether the prime-boost with DNA Combos I (Apa/HSP65/HSP70/MTP83) and II (HSP65/HSP70/MTP70) plus BCG stimulated appropriate cell-mediated immunity, spleen cell cytokine responses from vaccinated animals were evaluated as described. Since IFN-γ is a critical component of the anti-mycobacterial protective response, a special focus was taken on analysing the specific IFN-γ responses from these vaccinated mice. PPD stimulation of spleen cells induced an increase in the frequency of IFN-γ+ cells from all groups of animals vaccinated with BCG (fig.3.23).

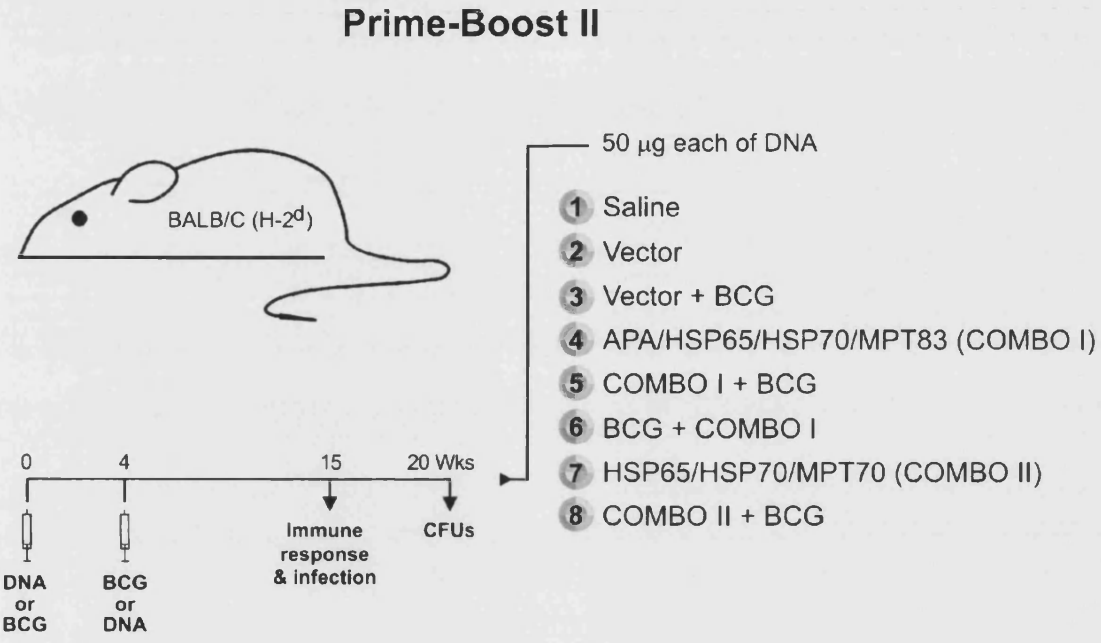

Figure 3.22 Schematic representation of the vaccination protocol for prime-boost II. 6-8 weeks old Balb/c mice were vaccinated with fifty micrograms of each specific DNA vaccine as described in Material and Methods. Vector control groups received 200µg of empty plasmid. CFUs, colony forming units.

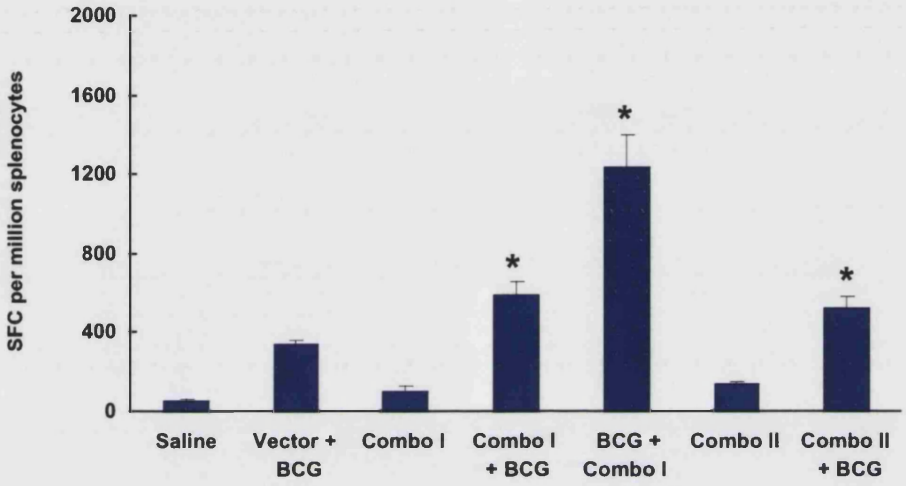

Figure 3.23 IFN-γ-secreting spleen cells from prime-boost II vaccinated mice. Spleen cells (4 mice/group) were cultured with PPD. Viable and counted cells were serially diluted in ELISPOT plates and processed as described. Results are expressed as the mean number of spot-forming cells per million, minus spots from non-stimulated and background control wells. Errors bars indicate standard errors. SFC, spot-forming cells; Combo I, Apa, HSP65, HSP70 and MPT83; Combo II, HSP65, HSP70 and MPT70. Assays were performed in triplicate and duplicate. *, $P < 0.05$ versus vector + BCG or Combo I and II groups.

Groups of mice primed with Combo I and Combo II vaccine combinations plus BCG, all had significant increases ($P < 0.05$, Student's t test) in IFN-γ+ cell frequencies when compared with vector + BCG control. Robust, but not statistically significant IFN-γ responses were generated in mice primed with BCG and boosted with Combo I plasmids when compared to DNA priming (fig.3.23). Stimulation of the spleen cells with recombinant HSP65, generated increased IFN-γ+ cell frequencies in all groups injected with the HSP65 DNA vaccine, but particularly in groups primed with Combo I and boosted with BCG ($P < 0.05$) in comparison with mice injected with vector plus BCG (preliminary results). Although with its inhibitory role still to be better clarified during the immune response in the tuberculous host, induction of IL-4 and IL-10 by anti-mycobacterial vaccines is not a desired feature for a future vaccine against human tuberculosis. IL-4 and IL-10 levels produced *in vitro* by spleen cells were also evaluated by ELISA. Again, in response to PPD, groups that received BCG produced levels of IL-4 and IL-10 always increased in comparison to groups that did not receive BCG as part of their vaccination protocol (fig 3.24A & B). In addition, the groups of mice producing high levels of Th1-IFN-γ were also producing increased levels IL-4 and IL-10 cytokines. Animals receiving saline or DNA Combos without boost did not produce important amounts of IL-4 and IL-10.

3.6.2 Protective responses in animals receiving the prime-boost II vaccination protocol

In order to evaluate the ability of the prime-boost II protocol to confer levels of protection superior to BCG, vaccinated animals were infected intravenously with *M. tuberculosis* H37Rv after twelve weeks of post-vaccination resting.

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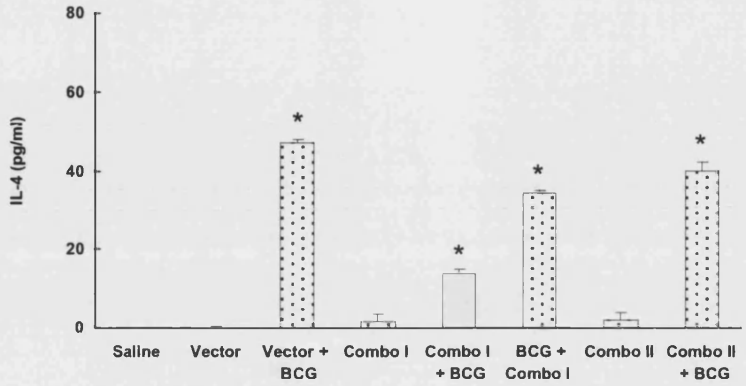

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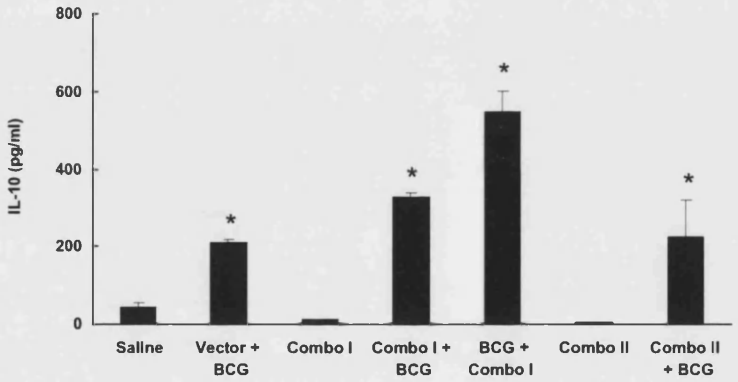

Figure 3.24 Specific IL-4 and IL-10 cytokine responses produced by spleen cells from prime-boosted II vaccinated mice. Spleen cells from vaccinated mice were collected as described and cultured *in vitro* with 10μg/ml of PPD. IL-4 (A) and IL-10 (B) were measured in the supernatants by ELISA. Results show the mean plus SE of cells pooled from four mice. All groups receiving BCG produced significantly more (*, $P < 0.05$) cytokine than saline or Combo I and II groups. The experiment was repeated twice.

Spleen and lungs were removed to evaluate the bacteriologic burden present in these organs 5-6 weeks after challenge. As expected, the results show that the animals receiving BCG were protected in comparison to saline control (fig.3.25A & B). In addition, mice receiving one injection of any combination of plasmids (without BCG) did not significantly decrease the CFU counts in both organs. More importantly, there was no significant additional protective effect with one priming dose of Combo I or II DNA cocktails, on the protection induced by BCG. The mice primed with BCG and boosted with Combo I also did not demonstrate better protection than BCG alone (fig.3.25A & B).

3.7 Priming with two or three doses of DNA combination vaccines and boosting with BCG (prime-boost III)

Whereas a single vaccination with DNA can induce a detectable humoral and CTL response in several model systems, cellular and humoral responses are boosted by successive additional immunisations (Gurunathan, 2000). Repeated injections of DNA vaccines, whether using a single antigen (Tascon, 1996) or a cocktail of different plasmids (Delogu, 2002), have demonstrated the ability to protect animals as well as BCG. In this context, C57BL/6 and Balb/c mice strains were primed repeatedly with DNA combinations composed of ApaDNA, HSP65 and HSP70 plasmids (Combo III), and were boosted or not with BCG (fig.3.26). Groups of Balb/c mice received either two or three priming doses of DNA Combo III cocktail vaccine, on intervals of three weeks, and were boosted or not with BCG, intradermally, one month after the last DNA vaccination.

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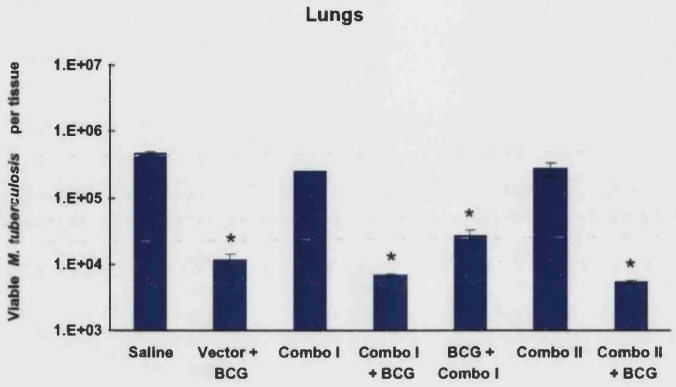

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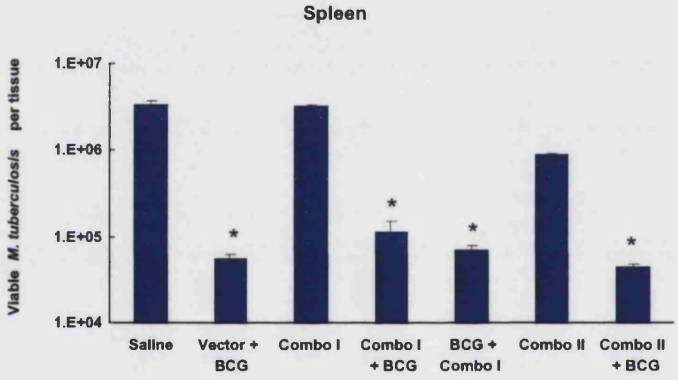

Figure 3.25 Protection provided by prime-boost II immunisation. Balb/c mice were primed once with the described combination of plasmids or BCG, followed by a boosting dose one month later. Challenge was performed intravenously with *M. tuberculosis* H37Rv sixteen weeks later. Bacteriologic burden in the lungs (A) and spleen (B) was expressed as the mean number of live bacteria ± SE. All animals injected with BCG were protected in both organs (*, $P < 0.05$), in comparison with mice not receiving BCG. The experiment was repeated twice. Combo I, Apa/HSP65/HSP70/MPT83; Combo II, HSP65/HSP70/MPT70.

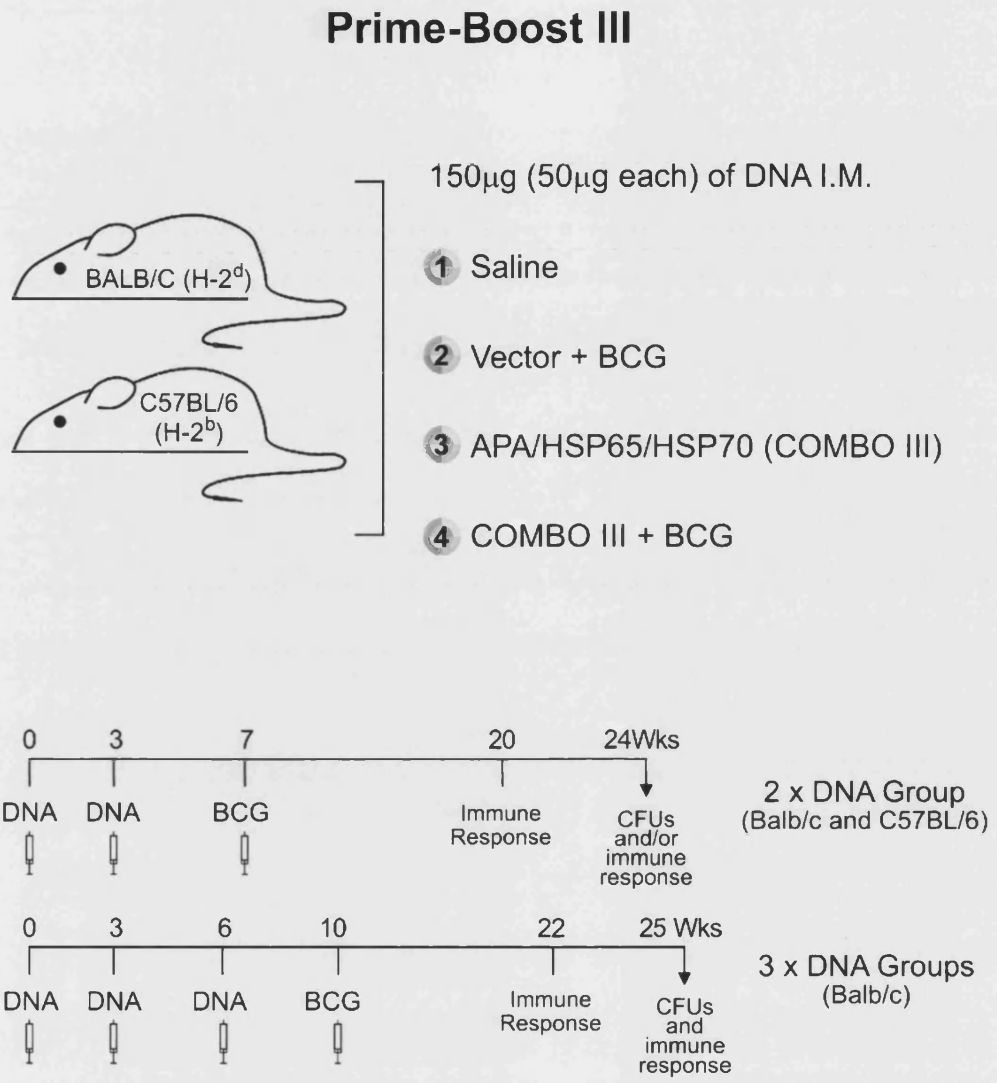

Figure 3.26 Schematic representation of the vaccination protocol prime-boost III. 6-8 weeks-old Balb/c or C57BL/6 mice were injected intramuscularly with fifty micrograms of each specific DNA vaccine. The plasmids were injected separately in the posterior and anterior limbs. Vector groups received 150μg of empty plasmid. CFUs, colony-forming units.

C57BL/6 mice received two doses of DNA Combo III plus/minus BCG boost. Cytokine responses were studied both before and after intravenous infection with *M. tuberculosis* H37Rv.

3.7.1 Immune responses of animals receiving the prime-boost III vaccination protocol

In order to investigate specific immune responses after the prime-boost III protocol, CD4+ and CD8+ T cells were isolated from the spleen of vaccinated animals and were co-cultured *in vitro* with antigen-presenting cells and either PPD, RecApa or medium. Frequencies of IFN-γ+ cells were evaluated by ELISPOT assay and levels of IFN-γ produced *in vitro* were analysed by ELISA as described previously. Fifteen weeks after boosting, stimulation of CD4+ T cells from Balb/c mice vaccinated twice with the plasmid cocktail and boosted with BCG, did not differ significantly from controls vaccinated with vector + BCG or DNA only (fig3.27A). The same trend was observed when the cells were stimulated with RecApa (fig3.27B). Analysis of ELISA results showed a different picture: CD4+ T cells from DNA-primed BCG-boosted mice were capable of producing higher amounts of specific IFN-γ *in vitro* than its counterparts, when stimulated with PPD (fig.3.28). On the other hand, the analysis of CD8+ T cell frequencies from prime-boosted mice by ELISPOT showed a significant increase in the numbers of IFN-γ+ CD8+ T cells in response to PPD antigen, in Balb/c mice primed with DNA twice and boosted with BCG (fig.3.29). Controls and vector plus BCG groups did not show elevated numbers of CD8+ T cells producing IFN-γ. The increase in IFN-γ+ cell frequencies found was also correlated with an increase in the levels of IFN-γ cytokine, as determined by ELISA (data not shown).

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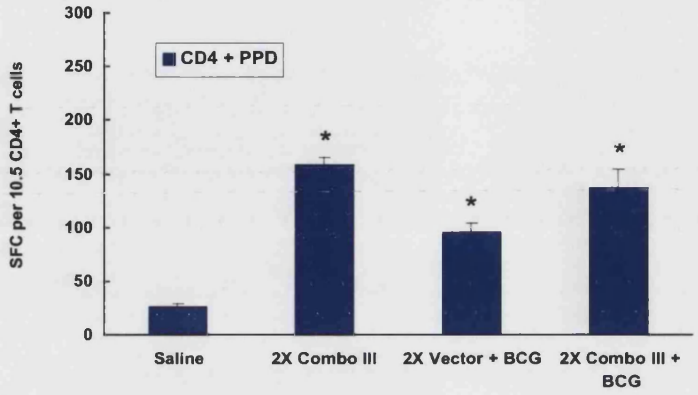

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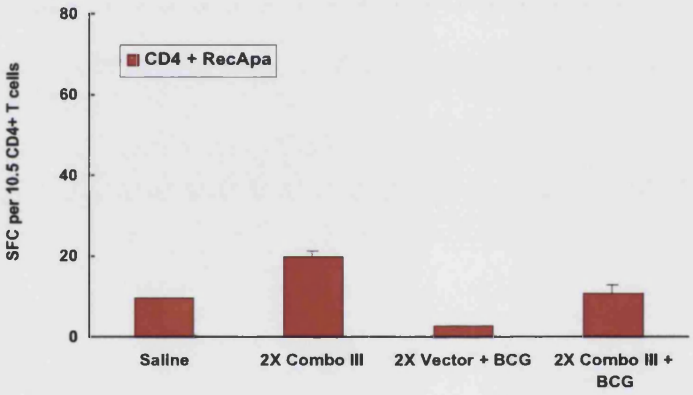

Figure 3.27 IFN-γ+ CD4+ T cells induced by prime-boost III protocol. Balb/c mice were primed by two doses of Combo III (Apa, HSP65 and HSP70) plasmids and boosted or not by BCG. CD4+ T cells were purified from the spleen and cultured *in vitro* with PPD (A) or RecApa (B) and the number of IFN-γ+ CD4+ T cells determined by the ELISPOT assay. Three mice per group were used and the number of spot-forming cells (SFC), are expressed as the mean plus SE of triplicates. *, $P < 0.05$ versus saline group.

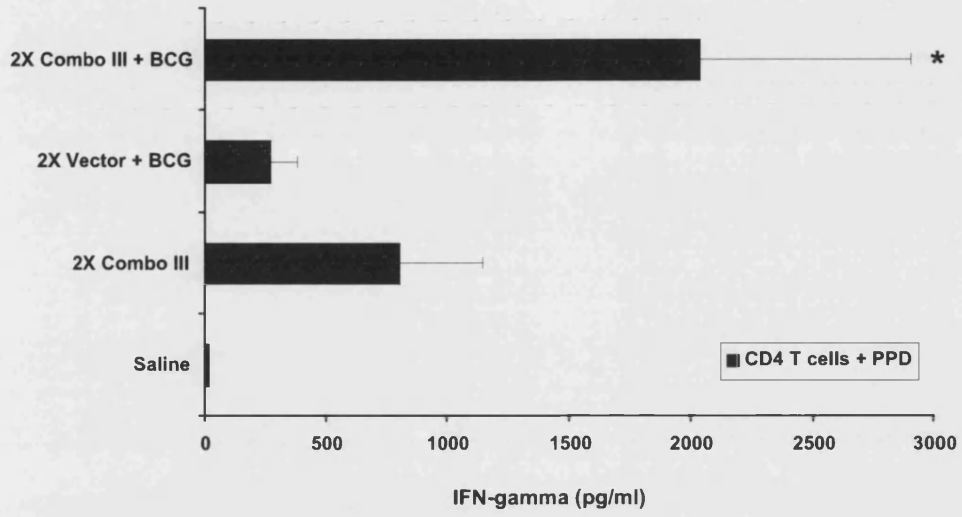

Figure 3.28 IFN-γ production by PPD-stimulated CD4+ T cells. Groups of Balb/c mice were immunised twice with the Combo III cocktail (Apa, HSP65, HSP70 plasmids) and were boosted with BCG. Controls received saline, DNA plasmids only or were primed with empty vector and boosted with BCG. CD4+ T cells were purified from the spleens of vaccinated mice and cultured *in vitro* with 10μg/ml of PPD and BMDCs as antigen presenting cells. Supernatants were collected after two days and measured by ELISA as described. Data shown are the mean determinations plus SE of three mice per group. *, $P < 0.05$ versus other groups.

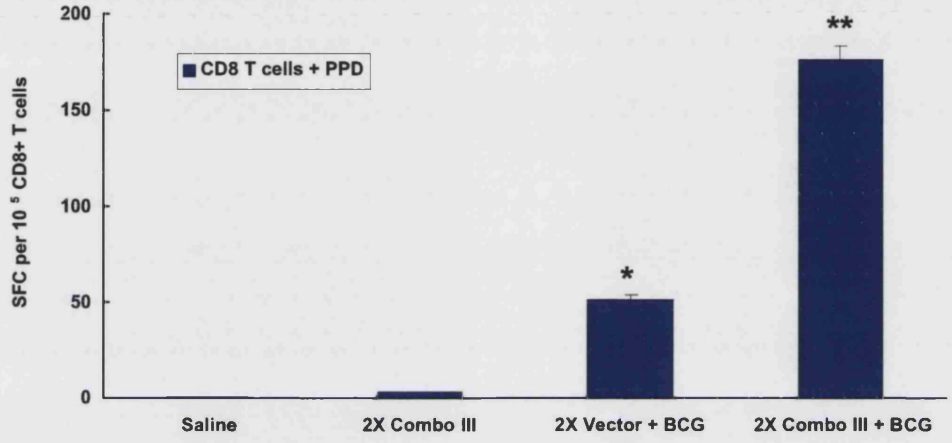

Figure 3.29 Specific IFN-γ+ ELISPOT responses of CD8+ T cells from prime-boost III vaccinated mice. Groups of Balb/c were vaccinated twice with Combo III cocktail and boosted with BCG. Controls received saline, DNA plasmids only or vector plus BCG. CD8+ T cells were purified from the spleen of three mice per group and co-cultured with BMDCs and 10μg/ml of PPD. The frequency of CD8+ T cells producing IFN-γ was determined by ELISPOT as described. The numbers of SFC are expressed as the mean plus SE of triplicates. *, $P < 0.05$ versus saline or 2X Combo III. **, $P < 0.05$ versus vector + BCG group.

Priming of Balb/c mice with three doses of Combo III (Apa/HSP65/HSP70) plasmid cocktail supplemented by boosting one month later with BCG, induced an apparently higher but not statistically significant number of CD4+ IFN-γ+ T cells, when compared with the BCG-vaccination group, in the cultures grown in the presence of PPD (fig.3.30A). A small increase in Apa-specific CD4+ IFN-γ+ T cells from prime-boosted Balb/c mice was also demonstrated (fig.3.30B). Consistent with previous results, the levels of IFN-γ secretion in the supernatant were determined (fig.3.31), and CD4+ T cells from DNA-primed BCG-boosted groups, showed higher IFN-γ levels than cells from mice vaccinated with BCG alone in response to PPD antigen ($P < 0.05$).

The augmented number of CD8+ T cells producing IFN-γ in response to PPD was maintained in Balb/c mice receiving three doses of DNA Combo III priming plus BCG boosting. The IFN-γ+ CD8+ T cells from these mice showed higher frequencies and secreted more IFN-γ in response to PPD, in the culture supernatants, when compared to control groups (fig.3.32A & B). On the other hand, CD8+ T cells isolated from prime-boost vaccinated mice did not respond to stimulation with 6μg/ml of recombinant Apa *in vitro* (not shown).

The analysis in C57BL/6 mice demonstrated that the frequency of CD4+ T cells producing IFN-γ in response to PPD, from mice vaccinated with empty vector plus BCG or Combo III plasmid cocktail plus BCG, was increased in comparison to saline and DNA Combo III cocktail controls (fig.3.33A). In addition, a significant ($P < 0.05$, Student's *t* test) increase in Apa-responsive CD4+ T lymphocytes producing IFN-γ was also demonstrated in mice receiving Combo III plus BCG prime-boost, when compared to vector plus BCG group (fig.3.33B).

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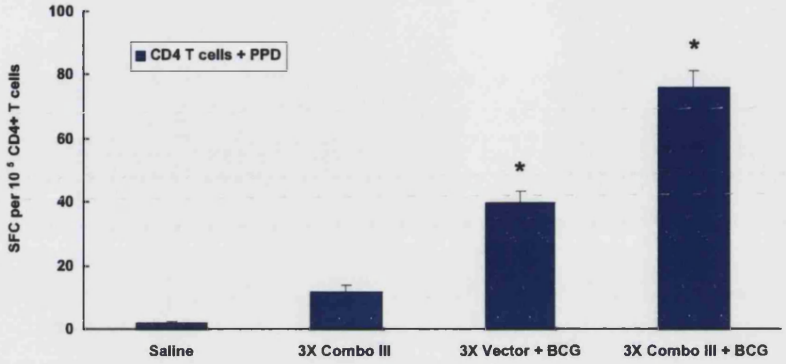

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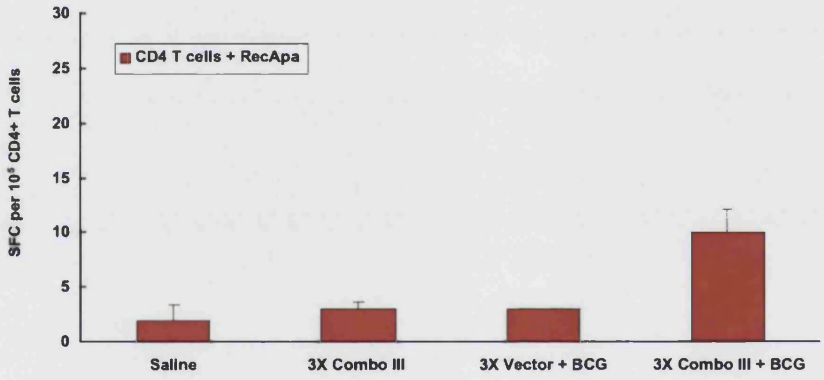

Figure 3.30 Specific IFN-γ+ ELISPOT responses of CD4+ T cells from prime-boost III vaccinated Balb/c mice. Mice were injected three times with Combo III DNA vaccine and boosted with BCG, as described. Controls received saline, DNA vaccines or vector plus BCG. CD4+ T cells were co-cultured with BMDCs and 10μg/ml of PPD (A) or 6μg/ml of RecApa (B). The frequency of CD4+ T cells producing IFN-γ was determined by ELISPOT. The mean number of SFC is expressed plus standard error of three mice per group. *, $P < 0.05$ versus 3X Combo III or saline group.

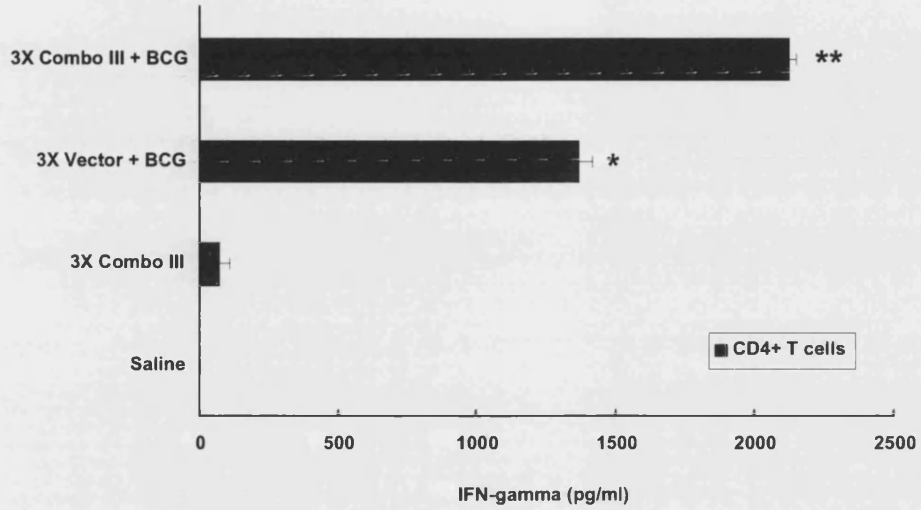

Figure 3.31 Specific IFN-γ production by CD4+ T cells from prime-boost III vaccinated Balb/c mice. Groups of mice received three immunisations with Combo III cocktail (Apa, HSP65, HSP70 plasmids) and were boosted later with BCG. Controls received saline, DNA vaccines only or vector plus BCG boost. CD4+ T cells were purified from spleen cell suspensions and cultured *in vitro* with 10μg/ml of PPD and BMDCs as antigen presenting cells. Supernatants were collected after 48 hours and assayed for IFN-γ by ELISA. Data shown represents the means plus SE of three mice per group. *, $P < 0.05$ versus 3X Combo III or saline. **, $P < 0.05$ versus vector + BCG group, as determined by Student *t* test.

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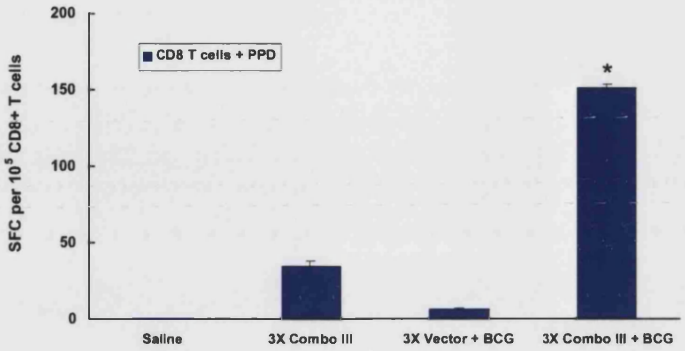

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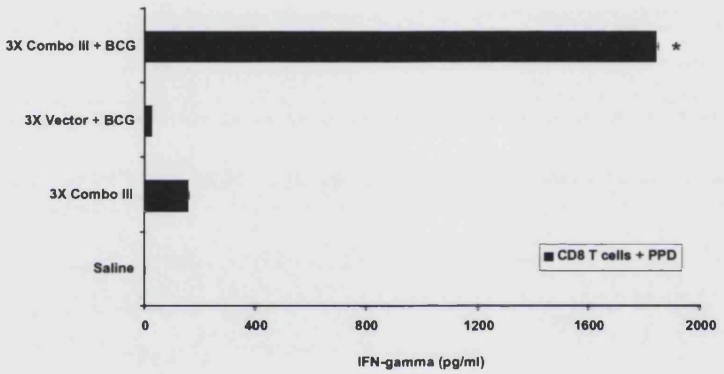

Figure 3.32 Specific IFN-γ production by CD8+ T cells from prime-boost III vaccinated Balb/c mice. Groups of mice were primed three times with Apa, HSP65 and HSP70 plasmids (Combo III) and were boosted with BCG. Controls received saline, DNA only or vector plus BCG. (A) CD8+ T cells were purified from the spleens of vaccinated mice and co-cultured with BMDCs and 10μg/ml of PPD. The frequency of CD8+ T cells producing IFN-γ was determined by the ELISPOT assay as described. The number of SFC are expressed as mean plus SE of triplicates. (B) Supernatants from the cell cultures were assayed for IFN-γ by ELISA. Bars represent mean values plus standard errors from duplicate wells. *, $P < 0.05$ versus other groups.

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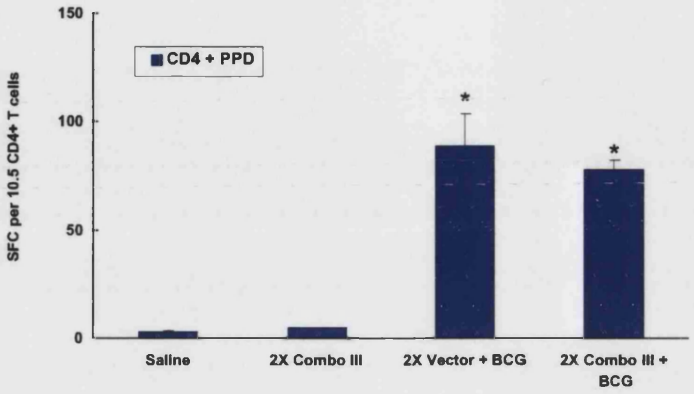

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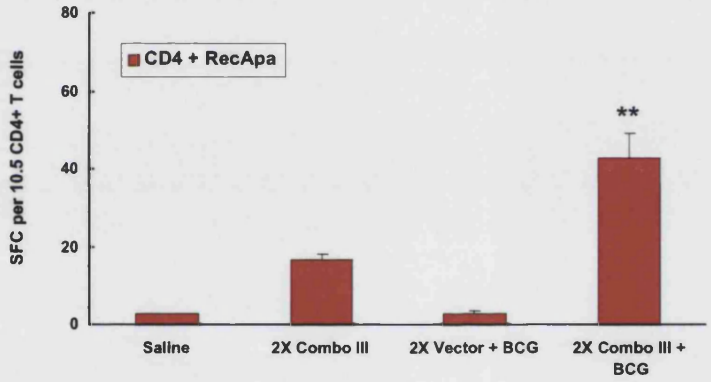

Figure 3.33 IFN-γ+ CD4+ T cells induced by prime-boost III protocol in C57BL/6 mice. Animals received two doses of Combo III (Apa, HSP65, HSP70) plasmids and were boosted or not by BCG. CD4+ T cells were purified from the spleens and cultured *in vitro* with PPD (A) or RecApa (B) and the resulting number of IFN-γ+ CD4+ T cells in the cultures was determined by ELISPOT assay as described. Three mice per group were used. The number of SFC is expressed as the mean plus standard error of triplicates. *, $P < 0.05$ versus 2X Combo III or saline. **, $P < 0.05$ versus 2X Vector + BCG group.

3.7.2 *Intracellular cytokine staining of lung T cells from mice receiving the prime-boost III vaccination protocol and challenged with* M. tuberculosis

Mycobacterial antigen-specific CD4+ and CD8+ T cell populations producing IFN-γ are necessary for an effective immune response against an infection by the tubercle bacilli (Flynn & Chan, 2001; Tascon, 1998). Analysis of these subpopulations in the lungs of infected and vaccinated mice is important to observe a possible correlation with an increase in IFN-γ+ CD4+/CD8+ population frequencies and significant decreases in bacterial burden from vaccinated animals. After 5 weeks of infection, lung cells were isolated from groups of Balb/c mice receiving two priming doses of DNA Combo III cocktail and controls, for performing intracellular cytokine staining as described previously. At the time of the screening, there was no difference in the numbers of IFN-γ+ CD4+ or CD8+ T cells, between any of the groups tested (fig.3.34). Total percentages of IFN-γ+ CD4+, and particularly CD8+ T cells, were elevated in the lungs of all groups examined. A similar picture was also observed in the lungs of infected C57BL/6 challenged under the same conditions (not shown).

3.7.3 *Protective responses in animals receiving the prime-boost III vaccination protocol*

In this experiment, prime-boost-inducing protective responses were evaluated in groups of Balb/c mice only. Animals were infected fifteen weeks after boosting with *M. tuberculosis*, as described previously. The results demonstrated that all animals receiving BCG as part of their regimen were protected in comparison with controls.

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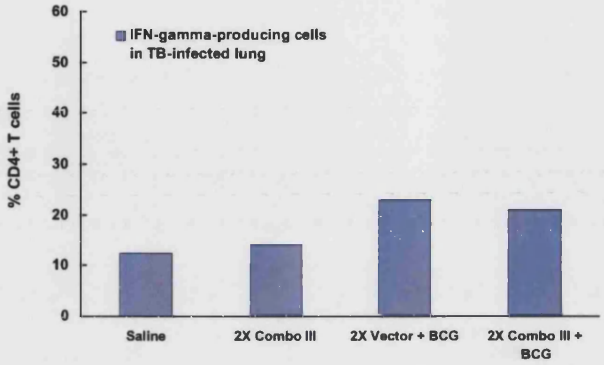

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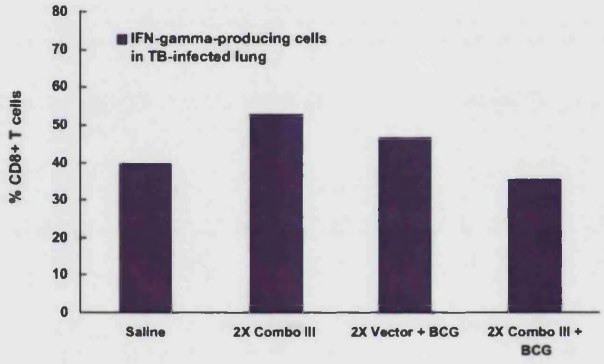

Figure 3.34 Intracellular IFN-γ staining of lung cells from infected prime-boosted III vaccinated mice. Intravenously-infected Balb/c mice from prime-boost III protocol had their lungs harvested 38 days post-infection. The cells were pooled and stimulated for 6 h with anti-CD3 and anti-CD28 antibodies plus monensin, as described in Material and Methods. The cells were stained with anti-CD4, anti-CD8 and anti-IFN-γ and were analysed by two-colour flow cytometry as described. Cells were further gated on CD4 (A) and CD8 (B), and the percentages of IFN-γ-producing cells within each gate were plotted for each one of the groups.

In addition, Balb/c mice receiving two doses of Apa/HSP65/HSP70 DNA combinations (Combo III) boosted with BCG did not show significant reduction in CFU numbers in both lungs and spleen, when compared to mice primed with empty vector boosted with BCG (fig.3.35A & B). Importantly, Balb/c mice vaccinated with three doses of Combo III mycobacterial vectors displayed a significantly increased protection in both spleen and lungs ($P < 0.05$) than animals injected with saline (fig.3.36A & B). Further, for mice primed three times with the plasmid cocktail and boosted with BCG, the protection induced in the lungs was significantly better than that observed in mice vaccinated with vector plus BCG ($P < 0.05$) (fig.3.36B), demonstrating an additive effect for the protective immunity offered by the two vaccination strategies.

3.8 Confirmation of the DNA-prime BCG-boost vaccination efficacy against tuberculosis (prime-boost IV)

The challenge experiments described above were repeated with a larger number of animals. Groups of twelve mice per cage, were primed two or three times with the DNA Combo III cocktail (Apa/HSP65 and HSP70 plasmids) and were boosted or not with BCG, as described previously for prime-boost III experiment. C57BL/6 is usually the standard strain adopted for tests of new vaccines against tuberculosis (Orme, 2001) and was selected for this experiment. Groups of mice receiving three doses of vector alone were also included as controls. The mice were challenged intravenously with *M. tuberculosis* H37Rv, fifteen weeks after the BCG-boost administration. The results demonstrated that the animals receiving two doses of DNA combination containing Apa, HSP65 and HSP70 plasmids, controlled bacillary replication at levels achieved by BCG

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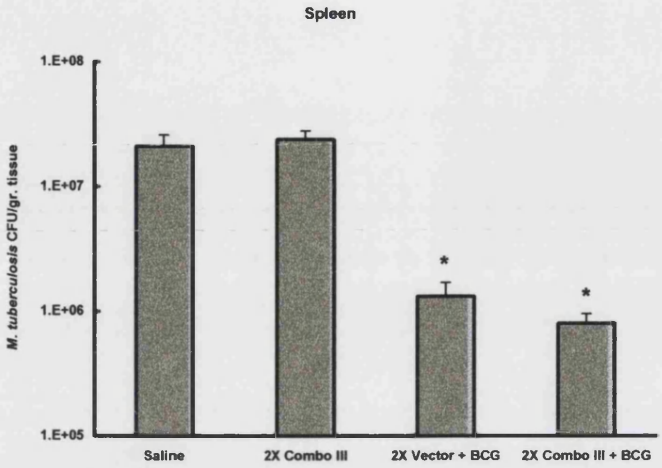

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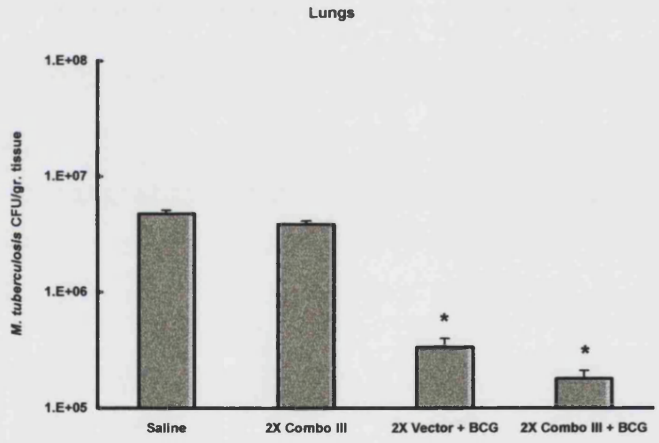

Figure 3.35 Challenge experiments in Balb/c mice from prime-boost III groups. Mice were injected twice with Combo III (Apa, HSP65 and HSP70 plasmids) or empty vector, and boosted or not with BCG. The animals were then infected intravenously with MTB fifteen weeks later as described. Spleen (A) and lungs (B) from 3-5 mice per group, were harvested after 38 days and the mean number of viable bacteria per gram (gr.) of tissue was calculated. Error bars represent standard errors. *, $P < 0.05$ versus 2X Combo III or saline groups.

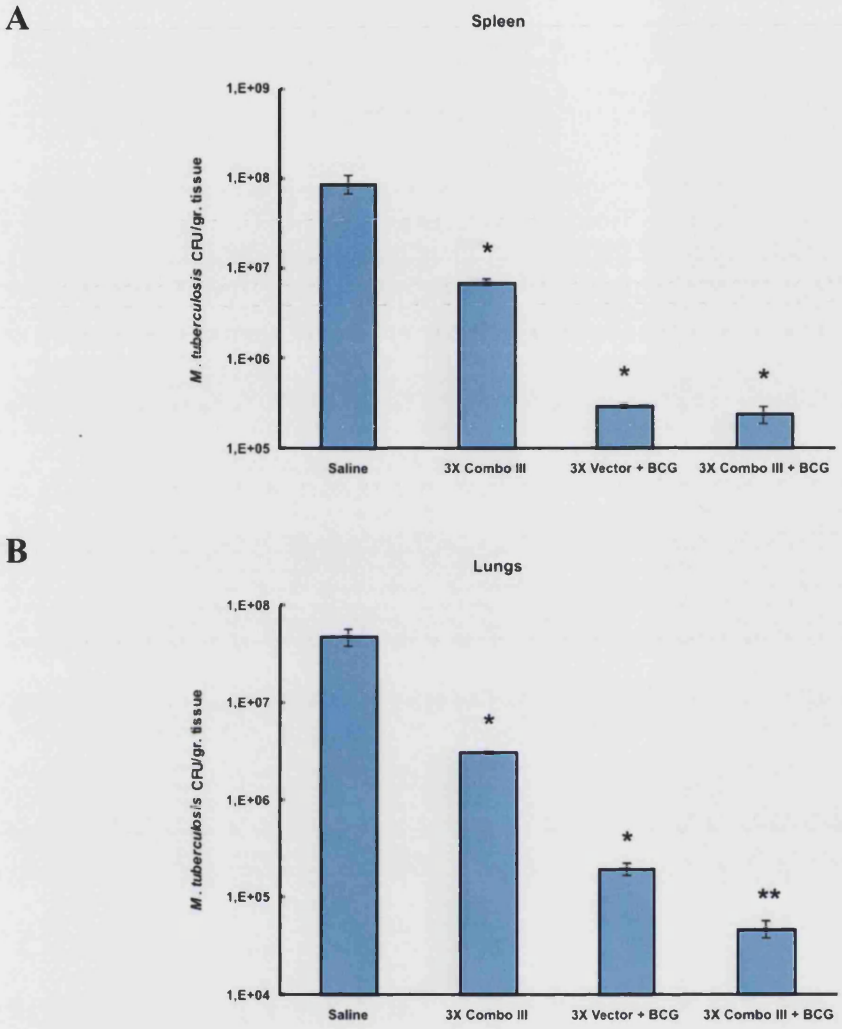

Figure 3.36 Protection provided by prime-boost III immunisation in Balb/c mice. Animals received three intramuscular doses of the Combo III (Apa, HSP65 and HSP70 plasmids) or empty vector, and were boosted or not with BCG. The mice were challenged intravenously with 10^5 CFU of *M. tuberculosis*. Protection was assessed forty-two days later by enumerating the mean number of live bacteria in the spleen (A) and lungs (B). *, $P < 0.05$ versus saline group; **, $P < 0.05$ versus 3X vector + BCG group, as determined by the Student *t* test. Error bars indicate standard errors of the means.

vaccination, in both the lungs and spleen (fig.3.37A & B). Importantly, mice receiving this plasmid DNA combination, boosted one month later by BCG showed a significantly better control of the infection when compared with the animals that received vector plus BCG ($P < 0.001$), in both lungs and spleen (fig.3.37A & B). In addition, groups of twelve C57BL/6 mice were set with the intention to evaluate the degree of contribution of the Apa antigen for the protective efficacy of the plasmid cocktail. The animals received two priming doses of HSP65, HSP70 and empty vector (as a substitute for the Apa plasmid) and were boosted or not, one month later with BCG. When compared, the protection induced by these combinations appeared to be intermediary. Groups receiving the DNA combination excluding the Apa plasmid, had significant decrease in bacterial loads versus saline control ($P < 0.001$); however, the protection achieved was not better than that exhibited by animals receiving the complete Apa/HSP65/HSP70 (Combo III) cocktail. In addition, mice that were vaccinated with the full plasmid Combo III cocktail and boosted with BCG, performed better in both lungs and spleen than mice injected with the combination lacking the Apa plasmid boosted with BCG ($P < 0.001$, fig.3.37A & B). Nonetheless, the group of mice lacking ApaDNA boosted with BCG was still able to afford significantly better protection than the control group vector + BCG ($P < 0.001$), in both organs. These results demonstrated the beneficial and critical effect of the Apa antigen in the protective response induced by this plasmid mixture.

For groups of C57BL/6 mice receiving three vaccination doses of Combo III plasmids, protection against *M. tuberculosis* challenge was highly significant in both lungs and spleen ($P < 0.001$, Student's *t* test) when compared to vector control group, and also reached similar levels as the animals vaccinated with three doses of empty vector plus BCG (fig.3.38A & B).

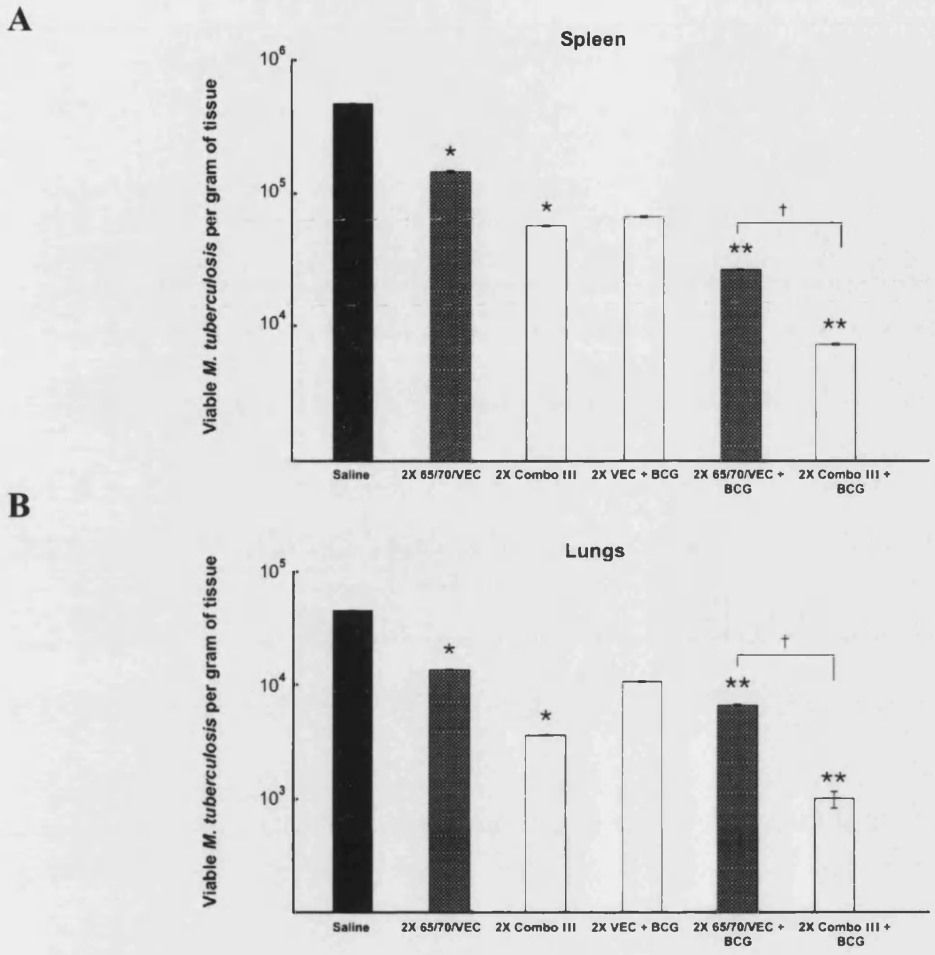

Figure 3.37 Protection experiment for prime-boost IV (first group). C57BL/6 mice were primed with two doses of Combo III (Apa/HSP65/HSP70 plasmids) and boosted or not with BCG as described. Groups not receiving ApaDNA were primed twice with HSP65, HSP70 DNA and empty vector (65/70/VEC). Controls received saline. Intravenous challenge with MTB was performed fifteen weeks after boosting. Six weeks later, CFU counts in spleen (A) and lungs (B) were compared. Data are expressed as the CFUs mean ± SE of twelve animals per group. *, $P < 0.05$ versus saline group (Student *t* test); **, $P < 0.001$ versus 2X vector + BCG group (ANOVA); †, $P < 0.001$ (ANOVA).

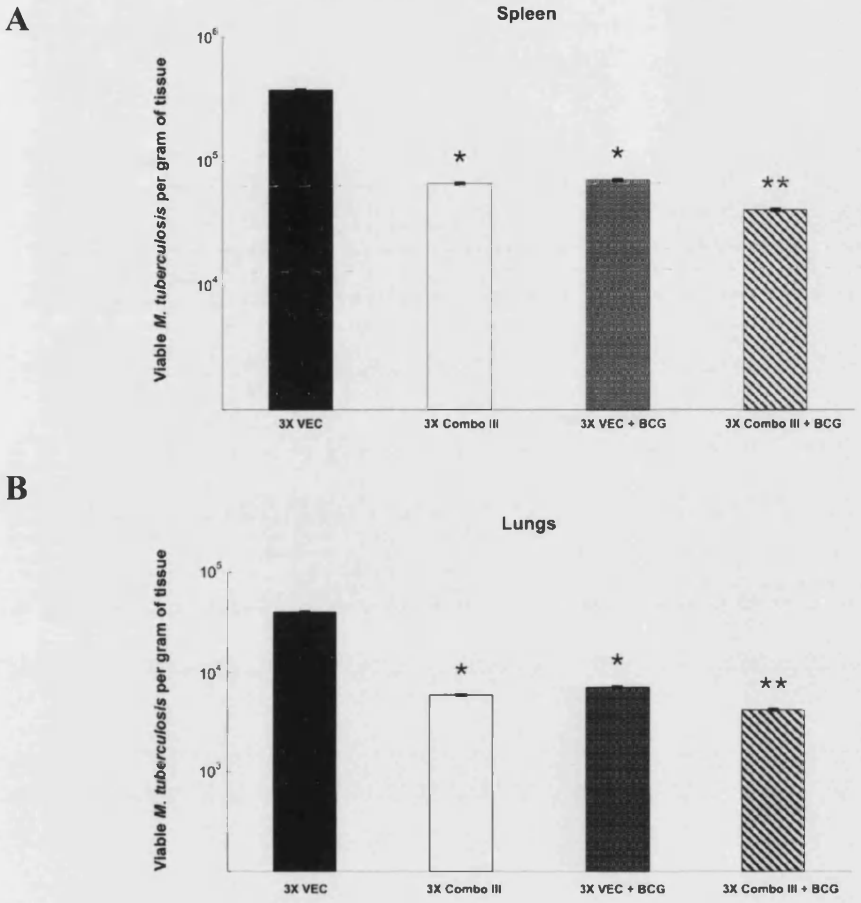

Figure 3.38 Protection experiment for prime-boost IV (second group). C57BL/6 mice was primed with three doses of Combo III (Apa/HSP65/HSP70 plasmids) and boosted or not with BCG. Controls received three injections of empty vector (VEC) and were boosted or not with BCG. Mice were infected intravenously with MTB fifteen weeks after boosting. Six weeks later, CFU counts in spleen (A) and lungs (B) were compared. Data is expressed as the mean ± SE of CFUs from twelve animals per group. *, $P < 0.001$ versus 3X VEC group, as determined by Student *t* test; **, $P < 0.001$ versus 3X VEC + BCG group, as determined by Student *t* test.

As in the results shown in figure 3.37, mice receiving three doses of Combo III plasmids plus BCG boosting were also able to significantly improve ($P < 0.001$, Student's *t* test) protection obtained by vaccination with vector plus BCG, in both lungs and spleen (fig.3.38A & B). When animals from groups receiving three doses of saline or vector were compared, analysis of CFU counts in the lungs revealed that the group of mice receiving injections of empty vector behave similarly as the saline control mice; however in the spleen there was statistical significance ($P < 0.05$) showing a possible beneficial effect resultant from the immunogenic CpG islands present in the pCMV4 plasmid backbone (not shown).

3.9 Protective responses of animals receiving the ApaDNA vaccine

To evaluate the protective effect exhibited by the ApaDNA vaccine alone, groups of C57BL/6 mice were immunised four times, intramuscularly, three weeks apart, with 100μg of ApaDNA plasmid. As negative controls, mice were injected with empty vector. As the positive control, groups of mice were immunised once, intradermally, with BCG. Six weeks later, the animals were challenged intravenously with *M. tuberculosis* H37Rv, as described previously in Material and Methods. Bacteriological burden was measured in the lungs and spleen, five weeks after challenge. The results of these experiments demonstrated that, in contrast to the protective effect induced in mice by the ApaDNA vaccination when associated with the HSP65 and HSP70 vaccines, the level of protection induced by the ApaDNA vaccine injected alone was not significantly better when compared to the protection conferred by the empty vector control, at the time of screening (fig.3.39).

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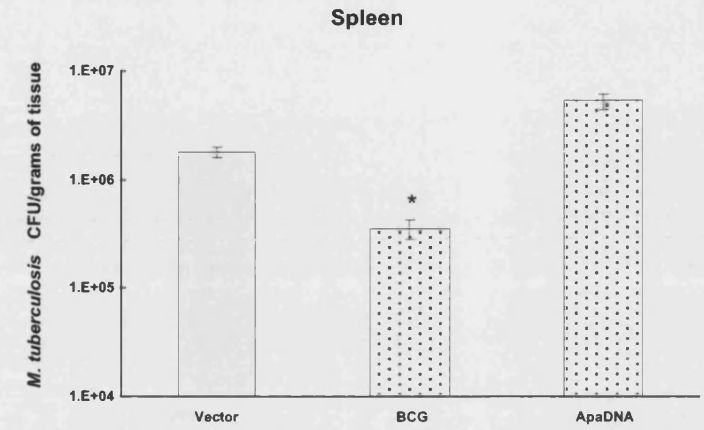

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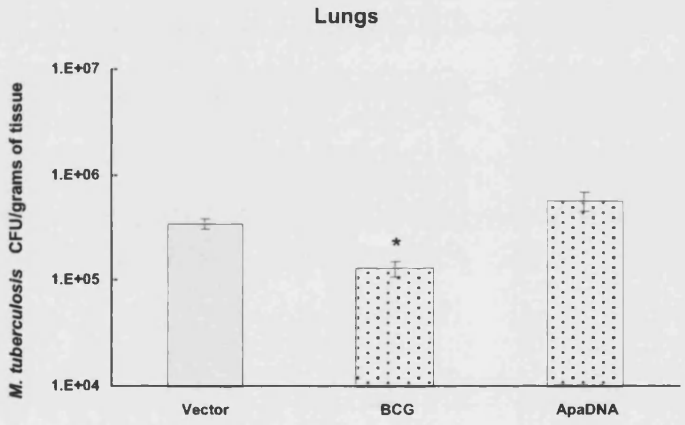

Figure 3.39 Protection provided by ApaDNA immunisation. Groups of C57BL/6 mice were immunised either with ApaDNA, empty vector or BCG. Six weeks later, the mice were challenged intravenously with 10^5 CFU of *M. tuberculosis* H37Rv. Protection was assessed by determining the number of live bacteria (mean ± SE) in the spleens (A) and lungs (B). Compared to the other groups, protection in the BCG group was significant (*, $P < 0.05$) for both organs (Student *t* test). The experiment was repeated twice.

3.10 Boosting BCG with DNA vaccines (prime-boost V)

More than one billion humans have already been vaccinated with BCG. It was then relevant to know the efficacy of giving a DNA vaccine to mice that have previously received BCG vaccination. To address this important issue, groups of mice were primed with BCG-Glaxo and then were rested for eight weeks. After this period, the animals were either injected with two doses of saline, empty vector or a combination of HSP65 and HSP70 plasmids. Control groups receiving two doses of DNA only and naive groups were also set in parallel. The animals were then infected intravenously with *M. tuberculosis* H37Rv four weeks after the last injection. As shown in fig.3.40, animals receiving BCG or two doses of plasmid DNA combinations only were all significantly protected when measured against infected naive mice ($P < 0.001$), in both lungs and spleen. However, the groups of mice primed with BCG and then boosted with two doses of HSP65/HSP70 DNA cocktail, did not show significant improvement in protection when compared to mice vaccinated with BCG plus saline or empty vector.

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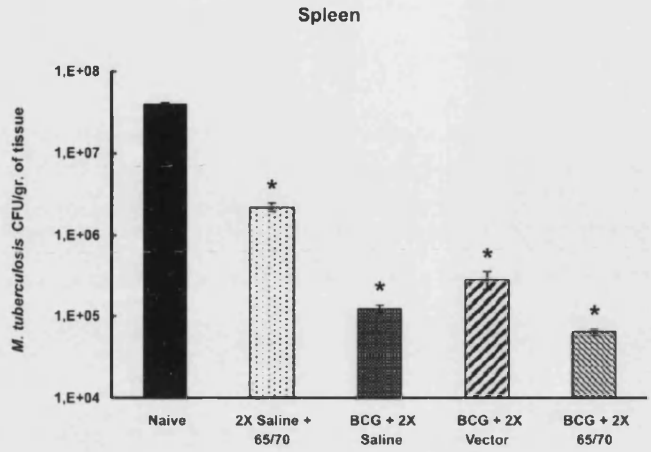

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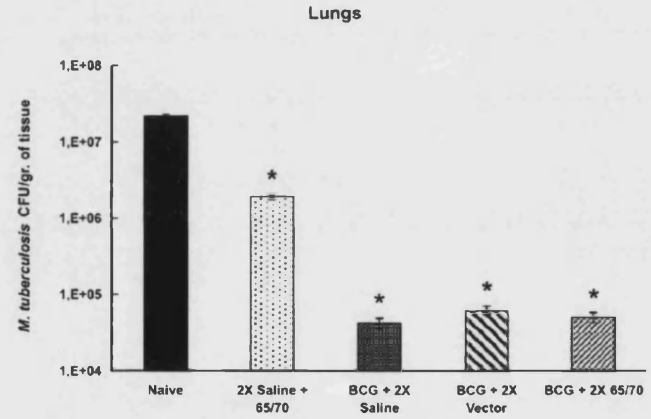

Figure 3.40 Protection provided by boosting BCG vaccination with DNA vaccines. Balb/c mice received either BCG or saline and were boosted eight weeks later by two doses of mycobacterial HSP65 and HSP70 plasmids or empty vector or saline. Plasmids were injected separately in the posterior limbs. Animals were challenged intravenously with MTB, one month after the last dose. CFUs were determined in the spleen (A) and lungs (B) after six weeks. Data is expressed as the mean ± SE. *, $P < 0.001$ versus naive control group.

CHAPTER FOUR

Discussion

4.1 Up-regulation of *M. tuberculosis apa* gene during infection

An increase in *apa* messenger RNA expression from *M. tuberculosis* grown in monocytic cell lines, was detected by Real-Time™ reverse transcription PCR (figs. 3.1 and 3.2). Moreover, this increase was demonstrated in both human- and mouse-derived cells, previously activated with IFN-γ, a situation mimicking the ongoing scenario of an *in vivo* immune response against the bacterium, generated by the host. Like other intracellular bacteria, MTB is expected to alter its program of gene expression in response to intracellular environmental changes. As the immunodominance presented by certain antigens during the anti-mycobacterial response is frequently associated with a proportional increase in their level of transcription, these results are consistent with such observations. Genes such as *fbpB* and *hspX*, coding respectively for the mycolyl transferase antigen 85B and the α-crystallin 16-kDa protein showed increased level of transcription after infection of human monocytes (Wilkinson, 2001; Dubnau, 2002). In addition, studies with other pathogens have shown that genes, which are up-regulated during infection, are often essential for pathogenicity (Chiang, 1999). It would be interesting therefore, to observe the behaviour of a MTB mutant lacking the *apa* gene during infection in mice, to assess the role of Apa in mycobacterial virulence.

A recent article (Schnappinger, 2003) reported that the transcripts of the *apa* gene, as analysed by microarray hybridisation, were repressed during *M. tuberculosis* infection of bone-marrow macrophages. A possible explanation for this discrepancy could be the augmented sensitivity (and specificity) presented by techniques such as quantitative reverse-transcription Real-Time™ PCR (qrtPCR) in comparison with the less sensitive microarray hybridisation. Its is interesting to note, that similarly to *apa*, the transcripts from the genes *fbpB* (Wilkinson, 2001) and Rv0899 (OmpATb) (Raynaud, 2002), previously reported as significantly increased in infected monocytes by qrtPCR, were also considered to have similar expression levels in liquid cultures and in the phagosome of macrophages, when analysed by microarray hybridisation (Schnappinger, 2003).

4.2 Expression of the *M. tuberculosis apa* gene in *E. coli* and mammalian hosts

The Apa protein from *M. tuberculosis* was expressed in *E. coli* and the recombinant product was subsequently purified by affinity chromatography. Only the 47-kDa band was observed and the resulting protein was devoid of glycosylation (Horn, 1999). Although the recombinant clone chosen was selected on basis of higher levels of protein expression, the final yield of recombinant Apa protein (RecApa) in the system described here, was very limited. Approximately eleven litres of bacterial culture were necessary to obtain a maximum of two milligrams of recombinant protein as the end product ready for use in *in vitro* assays. A tempting explanation could be related to the presence of the N-terminal secretion signal, and consequently, the secretion and purification of the protein from the periplasmic space of *E. coli*, which is rich in proteases; these enzymes would then be responsible for the poor yield observed. The native Apa protein has been reported

to be rather unstable and very prone to degradation by proteases (Nagai, 1991). Exogenous addition of protease inhibitors during the purification procedures was avoided to prevent toxicity during cellular assays. One way to overcome this problem in the future would be to remove the N-terminal secretion signal and compare whether purification in denaturing conditions would be more rewarding in terms of protein recovery. The use of *E. coli* host strains that are deficient in periplasmic proteases (Kandilogiannaki, 2001) may also offer an additional alternative to improve the final yield of recombinant Apa obtained.

A second issue related to the purification of recombinant Apa from the *E. coli* host was the finding of lypopolysaccharide (LPS) contamination in eluted samples. Up to 380 endotoxin units (EU) per milligram of recombinant protein were initially present in RecApa samples after Ni-NTA column purification. LPS through its bioactive centre lipid A can promote activation of dendritic cells through Toll-like receptors and other molecules (Triantafilou & Triantafilou, 2002), which in turn could induce the production of a variety of cytokines in mixed lymphocyte cultures (Qi, 2003). The presence of such contaminants in Apa samples would undoubtedly affect the results and make interpretation of RecApa-stimulation of immune cells very difficult. Preliminary testing using recombinant Apa without further LPS removal, induced unspecific production of IL-10 by spleen cells from ApaDNA-vaccinated mice; importantly, this production was not observed with the use of LPS-free RecApa (not shown).

Regarding the expression of DNA vaccine vectors encoding Apa in CV1 mammalian cells, it was observed an increase in the molecular size of the recombinant Apa protein that could not be solely attributed to additional plasmid sequences fused with the *apa* gene (fig.3.7). The product expressed from the pSecTag2C.Apa vector (secreted form)

reached 72-kDa in CV1 cells while the molecular weight of the Apa protein in MTB was 47-kDa. This recombinant secreted protein would carry as part of the vector-fusion sequences, a *myc* epitope and a 6XHis tag sequence, totalling around 27 additional amino acids, an estimated increase of 3-kDa on the predicted molecular weight of the final recombinant product. The amino acid content did not include additional prolines which could possibly influence the migration properties of the protein in SDS-PAGE (Furthmayer, 1971). These observations suggested that the recombinant Apa was very likely being post-translationally modified in the mammalian system to include an unknown content of sugar moieties. Similarly, the Apa protein secreted from CV1 cells transfected with the pCMV4.Apa, which did not include any fused vector sequences, still exhibited a relative increase in size from 47- to 59-kDa (fig.3.7). A number of bands of various molecular weights were also observed in the lysate of CV1 cells transfected with pSecTag2C.Apa vector (fig.3.7, lane E). These could possibly be a result of truncated forms of the protein, but also to glycosylated isoforms of the recombinant Apa. Importantly, staining of the purified Apa produced in the transfected mammalian cell confirmed the glycosylation of the protein (fig.3.8).

Several immunodominant antigens of *M. tuberculosis* and *M. bovis* have been reported to be glycosylated (Dobos, 1996). To date, the unambiguous demonstration of glycosylation in mycobacteria has been shown only for Apa and, recently for the *M. bovis* antigen MPB83 (Michell, 2003). Although similar to MPB83 in its carbohydrate composition (mannose), there are differences in the sugar linkages of Apa; while Apa contains (1→ 2)-linked mannobiose and (1→ 2), (1→ 2)-linked mannotriose, the terminal mannose in MPB83 is (1→ 3)-linked (Michell, 2003). The type of linkage seen in Apa is also observed in the di- and trimannosyl units of the mannose cap of LAM

(Venisse, 1995) and as one of the linkages for the mannose present in the phosphatidylinositol mannoside (PIM) family of mycobacterial phospholipids such as PIM_5 and PIM_6 (Chatterjee, 1992).

Removal of covalently bound mannose from *M. tuberculosis* Apa antigen reduced by 10-fold its ability to elicit a delayed-type hypersensitivity reaction in guinea pigs immunised with BCG (Romain, 1999). The increase on the immunogenicity of mycobacterial Apa because of its glycoproteic nature could be explained through facilitated antigen presentation by better accessibility to surface receptors. Mannose Receptor (MR), is a C-type lectin which binds mannose through its eight Ca^{2+}-dependent carbohydrate recognition domains and is present on the surface of dendritic cells and macrophages (Gordon, 2002); in addition, it is one of the favourite entry ports of MTB in macrophages (Ernst, 1998). MR confers an enhanced ability of the order of 200- to 10.000-fold (Tan, 1997) to concentrate mannosylated antigens for presentation to T cells by MHC class II molecules and CD1b (Prigozy, 1997). DC-SIGN, another C type lectin specific for dendritic cells and a preferred entry port for MTB in human dendritic cells (Tailleux, 2003), binds mannosylated structures such as ManLAM (polymannosylated-capped lipoarabinomannan). These structures could potentially bind the Apa antigen and increase its presentation to CD4+ T cells (Engering, 2002).

It would be important to gain more information on possible host binding molecules and the nature of sugar moieties of the Apa protein produced *in vivo* after DNA vaccination, studying to what extent this could affect the immune responses. Variations in the glycosylation pattern of recombinant Apa produced in *Mycobacterium smegmatis* were implied as responsible for its loss of immunogenicity in DTH responses and lymphocyte proliferation assays (Horn, 1999). Preliminary results of enzymatic

digestion of Apa purified from transfected mammalian cells with α-mannosidase were inconclusive and need repetition, perhaps with additional data provided by Con A binding and mass spectrometry analysis.

4.3 DNA vaccination with the *apa* gene

C57BL/6 mice that received four vaccinations with DNA encoding the mycobacterial Apa protein exhibited specific immune response where significant levels of IFN-γ were produced in response to Apa by stimulated spleen cells. In these experiments, absence of specific production of IL-4 or IL-10 cytokines was also demonstrated, strongly suggesting the existence of a predominant Th1 response. The initial analysis of the humoral response in these animals showed the presence of both IgG1 and IgG2a antibodies, specific for Apa, which is also consistent with an ongoing Th1-Th2 mixed immune response. CD4+ T cells but not CD8+T cells from ApaDNA vaccinated mice secreted IFN-γ in response to Apa, and in addition, these animals presented a specific absence of cytotoxic activity for the Apa antigen.

Upon activation, mature B-lymphocytes may undergo class-switching recombination to produce a single, specific Ig isotype, which could be IgA, IgE, or one of the IgG subclasses (Roitt, 2001). Between other signals, cytokines such as IL-4, IFN-γ and TGF-β appear to play critical roles in this process. For instance, TGF-β may selectively stimulate switching for the IgG2b isotype (McIntyre, 1993). IL-4 directs murine IgE and IgG1 isotype production by activating transcription factors such as STAT6, which bind to and transactivate the germ-line Cε and Cγ1 promoters (Bacharier & Geha, 2000). Finally, IFN-γ regulates IgG2a class switching in B cells, in part through

the transcription factor T-bet (Peng, 2002). These results highlight that antibody isotypes sometimes can help to define which T cell phenotype is acting in the course of an immune response. Other investigators have also shown the presence of a mixed Th1-Th2 antibody response after ApaDNA vaccination. Although not examining IL-4 cytokine production, Morris and collaborators (2000) obtained a balanced ratio of IgG1 and IgG2a antibodies after ApaDNA vaccination in C57BL/6, while Garapin and colleagues (2001) using a non-secretable ApaDNA vaccine found a high percentage of IgG1 and low IgG2a, in vaccinating Balb/c mice. In the results obtained here, the titres of IgG1 were higher than IgG2a after ApaDNA vaccination (fig. 3.10); therefore, although I was not able to detect IL-4 production *in vitro* by immune cells (including purified CD4+ T cells) from ApaDNA vaccinated mice, the analysis of the humoral immune responses is suggestive that some level of IL-4 production does occur *in vivo* after ApaDNA vaccination. However, the role of IL-4 in tuberculosis is still elusive. Both young (North, 1998) and old (Turner, 2001b) IL-4 knockout mice are as resistant as the wild type to MTB infection.

Some reports support an important role for B cells on protection (Teitelbaum, 1998; Glatman-Freedman, 2003) or granuloma formation (Bosio, 2000; Turner, 2001b) in tuberculosis. Monoclonal antibodies recognising mycobacterial arabinomannan (Teitelbaum, 1998), heparin binding hemaglutinin adhesin and MPB83 (Glatman-Freedman, 2003), either enhanced survival or/and had effects on granuloma formation. Interestingly, similarly to Apa, all molecules discussed are glycosylated and contain mannose moieties. B cells and antibodies may have other, not yet investigated roles in anti-tuberculous immunity. Similarly to mycobacteria, in *Listeria monocytogenes* (an intracellular bacteria) model, B cells are not required for protective immunity; however,

the absence of these cells results in increased death of activated CD8+ T cells during the contraction phase, leading to a lower level of Ag-specific CD8+ T cell memory in infected animals (Shen, 2003). In summary, a role for Apa-specific antibodies in the immune response generated after ApaDNA vaccination may deserve further study.

The first data showing protection against *M. tuberculosis* using naked DNA immunisation demonstrated it to be one of the most effective vaccination strategies to be able to stimulate T cell responses, both CD4+ and CD8+, and to induce priming of Th1 immune responses (Tascon, 1996; Huygen, 1996; Huygen, 2003). Several mycobacterial antigens such as Mtb8.4, Ag85, Mtb41, Mtb39, MPT63, MPT83, HSP65, PstS3, ESAT-6, MPT64, 38-kDa protein and others, delivered as naked DNA have shown to be effective at inducing protection in mice following aerosol challenge (reviewed in Reed, 2003; Huygen, 2003). In addition, most of the antigens tested originated from culture filtrate preparations (Orme, 1997). Apa protein is one of such culture filtrate components; cells obtained from guinea pigs vaccinated with live (but not dead) BCG recognised this protein strongly (Romain, 1993; Romain, 1999), suggesting that mycobacterial Apa protein would be a good candidate to test in vaccination experiments against MTB. However, regardless the extensive production of IFN-γ, C57BL/6 mice vaccinated four times with ApaDNA was not better protected than vector-vaccinated animals, after the intravenous challenge with *M. tuberculosis* H37Rv (fig.3.39). Consistent with our results, Morris and collaborators (2000) also tested a similar DNA construct in C57BL/6 mice obtaining a high frequency of IFN-γ-positive cells in the spleens of ApaDNA-vaccinated mice, with lack of protection after *M. tuberculosis* challenge infection. At the same time, other researchers had also reported similar findings using this vaccine in *M. tuberculosis* aerosol-infected animals (I. Orme, USA, personal communication). In contrast, Garapin

and colleagues (2001) demonstrated significant protection (0.5 log unit CFU) against mycobacterial infection in mice using a DNA construct with the *apa* gene, subtracted of the signal peptide. However, the challenge experiments were performed in mice infected with the attenuated *M. bovis* BCG strain rather than the virulent *M. tuberculosis* H37Rv (Garapin, 2001), making their results difficult to compare with the results obtained here. Kumar and colleagues (2003) have also reported recently lack of protection by the ApaDNA plasmid injected as a single vaccine, in the guinea pig model.

In developing countries, as many as 40 to 80% of individuals with AIDS will also develop tuberculosis, indicating a key role for CD4+ T cells in the immune control of TB infection (Dye, 2002; Flynn & Chan, 2001). CD4+ T cells are the main producers of IFN-γ, which then activates macrophages to control bacterial growth. One of the principal anti-mycobacterial mechanisms induced by IFN-γ is the expression of the enzyme NOS2, responsible for restricting *M. tuberculosis* replication via nitric oxide generation (Chan, 1992; Ehrt, 2001). Recently, another IFN-γ-dependent mechanism has been identified (MacMicking, 2003), LRG-47, a member of a newly emerging family 47-kDa guanosine-triphosphatases, provides a NOS2-independent and functionally separated system for the control of MTB replication in macrophages. Supporting the crucial role of IFN-γ in the control of mycobacterial infections by the host, both mice and humans with genetic deficiencies in IFN-γ signalling pathways were showed to be highly susceptible to severe mycobacterial infections (Casanova & Abel, 2002). Tuberculosis-vaccine antigen-candidates have been prioritised based on their ability to stimulate cells from healthy PPD+ donors to proliferate and produce IFN-γ (Reed, 2003). These data and others (Black, 2002) support the use of quantitative assays such as the ELISPOT assay for IFN-γ responses, as promising tests for correlating protective immunity. The initial

observations on the increased production of IFN-γ by cells from ApaDNA vaccinated mice in response to Apa protein *in vitro* were thus very encouraging (figs.3.12 & 3.13). However, after challenge with *M. tuberculosis* H37Rv, the animals demonstrated lack of successful protection in short-term experiments (fig.3.39). Thus, IFN-γ production failed to correlate with effective protection in this model. The importance of the production of IFN-γ in mycobacterial immunity is beyond doubt, but as discussed (Kaufmann, 2001), in certain instances IFN-γ production and protection are not correlated. Interestingly, the lack of ESAT-6-induced IFN-γ after *M. bovis* infection in cattle has been found to be associated with protective efficacy after BCG vaccination (Vordermeier, 2002). Importantly, the timing for detection of IFN-γ-producing cells after vaccination may be crucial. In summary, one explanation for the lack of protection observed in the ApaDNA vaccinated mice would be that the response generated by immune CD4+ T cells was not sufficient in quantitative terms to inhibit bacterial replication.

Even if the IFN-γ response specifically generated by CD4+ T cells after ApaDNA vaccination in mice, could be considered adequate, it is equally possible that other components of an effective anti-tuberculous response were missing. The role of unconventional T cells in anti-mycobacterial responses in mice has been demonstrated, although these cells are not as critical for protection as the conventional T cells (Kaufmann, 2001; Flynn & Chan, 2001). Significant roles have has been attributed for CD8+ T cells in protection against mycobacteria in both mice and humans (Boom, 2003). The IFN-γ produced by activated CD8+ T cells would certainly contribute for a more concerted protective response against the tubercle bacillus (Tascon, 1998; Lalvani, 1998; Smith, 1999). CD8+ T cells from the lungs of MTB-infected old mice produces

significantly more IFN-γ than their counterparts in young mice and are critically involved in the early resistance displayed by old mice to aerosol infection (Turner, 2002a). In a experimental murine model of latent tuberculosis, depletion of CD8+ T cells or neutralisation of IFN-γ had greater impact on the increase of pulmonary bacterial numbers than anti-CD4 treatment (van Pinxteren, 2000). Successful vaccination strategies against mycobacteria aim at inducing a powerful Th1 response as well as an effective cytotoxic response. When investigating CD8+ T cell responses in ApaDNA vaccinated mice, I found that the IFN-γ production from these animals were never significantly different from the cells originated from animals injected with saline or empty vector, when re-stimulated *in vitro* with RecApa; and in addition, there was no specific production of IL-2 in the cultures (fig.3.14). Re-stimulation with the glycosylated native Apa protein also resulted in similar findings (not shown). Therefore, it is tempting to speculate that a more efficient protective response in this model would be induced if an increased number of Apa-specific CD8+ T cells actively secreting IFN-γ could be present. Kumar and colleagues (2003) have reported recently, increased IFN-γ+ secretion by Apa-specific human CD8+ T cells from some healthy PPD+ individuals. A possible explanation for these contrasting findings would be the natural difference in TCR and MHC repertoire between mouse and humans, where the latter, being vaster would allow existing Apa-specific precursors to be expanded. Interestingly, Kumar and colleagues (2001) had used approximately 8-fold more recombinant Apa/ml of *in vitro* culture. This higher antigen concentration could possibly facilitate the Apa-specific primed CD8+ T cells to differentiate and secrete IFN-γ (Hecht, 1983). More importantly, the lack of CD8+ T cell activation demonstrated by failure to produce IFN-γ and lack of cytotoxicity has been reported for the C57BL/6 strain (but not for the Balb/c strain)

vaccinated with Ag85A DNA vaccine; protection in this mice was solely mediated by CD4+ T cells (D'Souza, 2000). However, despite of the absence of CD8+ T cell response in mice, there are several reports demonstrating human CD8+ T cell responses against mycobacterial Ag85A (Smith, 2000a; Smith 2000b). Thus, Apa could be behaving in similar fashion as Ag85A in the murine system but not in humans.

To explain the lack of protection, a crucial observation is the fact that Apa-dependent specific cytotoxicity was not detected (fig.3.16). Although cells such as NKT, NK or CD4+ T cells may have cytotoxic properties against mycobacteria, the CD8+ T cell sub-population play a determinant role in mycobacterial protection in mice (Flynn & Chan, 2001; Kaufmann, 2001). Besides cytokine release, CD8+ T cells can contribute to the control of *M. tuberculosis* infection through other three mechanisms: (1) cytotoxicity via granule-dependent exocytosis pathway, (2) cytotoxicity mediated through Fas/Fas ligand interaction, and (3) direct microbicidal activity, e.g. granulysin (Lazarevic & Flynn, 2002). The first cytotoxic mechanism is perforin-dependent, the second activates a cascade of caspase molecules resulting in apoptosis (programmed cell death) of the infected cell; to date, the third mechanism has not been detected in mice (Lazarevic & Flynn, 2002). Additional evidence for the importance of CD8+ cytotoxic effector functions comes from CD4+ T cell deficient mice which succumbed to infection besides apparently normal IFN-γ responses in the lungs (Scanga, 2000). CD8+ T cells from CD4+ T cell deficient mice actually have impaired cytotoxic functions in the lungs of MTB-infected mice (Serbina, 2001).

In conclusion, these observations suggest that the lack of an appropriate CD8+ T cell response in ApaDNA-vaccinated mice could be more likely responsible for the observed failure in protection against *M. tuberculosis* infection. Thus, strategies of

vaccination designed to induce increased levels of CD8+ T cell responses would be extremely interesting to investigate. ApaDNA vaccination did not elicit improved levels of protection compared to BCG. In fact, there are rare occasions where any single antigen showed better protection than BCG in rodent models (Reed, 2003). Vaccination of mice with one of the single antigens MTB8.4 (Coler, 2001), MTB41 (Skeiky, 2000), HSP65 (Tascon, 1996; Bonato, 1998), 38-kDa (Zhu, 1997) and PstS-3 (Tanghe, 1999), in DNA format, have been reported in the literature to show reductions in MTB bacterial load similar to BCG in either lungs or spleen or both. For broader T cell responses, it would be necessary for a single antigen to contain a sufficient number of MHC class I and MHC class II epitopes to cover the diversity of HLA types in a targeted population. The antigenic complexity of BCG is not comparable to a single gene vaccine. Although increasing in complexity, it is possible to construct vaccines expressing defined epitopes for multiple MHC-class I loci ("polytope vaccines"), which facilitates co-priming of CD8+ T cell populations with a diverse repertoire (Schirmbeck, 2003).

4.4 Prime-boost vaccination using DNA and BCG

Prime-boost vaccination appears to be an effective immunisation strategy against tuberculosis. Here I demonstrated that intramuscular DNA vaccination of C57BL/6 and Balb/c mice with two or three priming doses of a plasmid cocktail encoding the mycobacterial antigens Apa, HSP65 and HSP70, followed by one intradermal injection boost of BCG one month later resulted in highly significant reductions in *M. tuberculosis* bacterial load from either the lungs, spleen or both, in comparison to either vaccine alone (figs. 3.36, 3.37 & 3.38).

In the model described here, protection was critically dependent on the composition of the priming cocktail and the number of priming doses. Priming once was not demonstrated to be effective (figs. 3.21 and 3.25). Removal of the Apa plasmid from the cocktail resulted in significantly diminished protection in comparison with the injection with all components together, although the protection obtained was still superior to BCG injected alone (fig.3.37). This result demonstrates the importance of the Apa antigen (high IFN-γ secretion by CD4+ T cells) in the protective immune response generated by the prime-boosting protocol studied here. In this context, mycobacterial HSP65 and HSP70 were also critical elements of this plasmid cocktail. These stress proteins can chaperone exogenous peptides to MHC class I molecules and thus promote antigen specific CD8+ CTL responses, as previously demonstrated in other systems (reviewed in Pockley, 2003; Audibert, 2003). More importantly, they can also play a role as intercellular signalling molecules, activating innate immune responses: mycobacterial HSP70 has been reported to induce several proinflammatory cytokines and the maturation of dendritic cells (Wang, 2001; Wang, 2002). Signalling and peptide binding in HSP70 are separate entities, localised at the C-terminal domain (MacAry, 2004). Intracellular signalling by HSP70 in dendritic cells seems to be independent of the TLR pathway (MacAry, 2004) and involves several receptors; CD40 for instance, is one important HSP70 receptor (Wang, 2001). Recently, $CD40^{-/-}$ mice has been reported to be susceptible to a low-dose aerosol infection with *M. tuberculosis* due to poor priming of IFN-γ-producing T cells in the lymph nodes, a result of attenuated IL-12 production (Lazarevic, 2003). CD40 activation by mycobacterial HSP70 was implied as the alternative ligand *in vivo* (Lazarevic, 2003). These relevant evidences suggest an important adjuvant effect induced by HSP70 DNA vaccination in the activation of

dendritic cells in our model. Although a carrier effect for promoting CTL-induction by the HSP vaccines for the Apa protein (produced *in vivo* after ApaDNA vaccination) is difficult to consider since the two vaccines were not mixed and administered in different anatomical sites, it is still likely that the APC activation induced by HSP70 proteins, promoted effective priming of protective T cells in this model. It would be certainly interesting to test the carrier adjuvant effect of mycobacterial HSP70 by the production of a fusion protein with Apa, since similar strategies have been described to be very successful in promoting potent CTL responses against tumours (Chen, 2000). Mycobacterial HSP65 has also been reported to help cross-presentation of soluble, free antigen by dendritic cells for CD8+ T cells, although a direct role for DC activation is still controversial (Chen, 2004).

The efficacy of the plasmid combination Apa/HSP65/HSP70 plus BCG was recently tested in cattle with successful results (Skinner, 2003a). In that model, DNA/BCG vaccination resulted in significant enhancement of six pathological and microbiological parameters of protection following *M. bovis* challenge, i.e. number of animals with lung lesions, the mean lung lesion score, the number of animals with lymph node lesions, the mean lymph node lesion score, the mean number of lymph nodes with lesions per animal, and the mean lymph node bacterial count, while BCG alone affected only two (Skinner, 2003a). In contrast to HSP65 and HSP70 antigens, ApaDNA vaccination has been successful only when combined with a live heterologous boost (Skinner, 2003a; Kumar, 2003). Interestingly, prime-boost using single-dose ApaDNA vaccine plus BCG boosting did not improve BCG protection against *M. tuberculosis* in C57BL/6 mouse (fig.3.21). However, Kumar and collaborators (2003) had reported significant protection with a ApaDNA/ApaMVA combination in the guinea pig model,

although heterologous viral boosting does not seem suitable for all antigens, as is the case of Ag85B (Feng, 2001).

One-dose vaccination priming with ApaDNA or Apa/HSP65/HSP70/MPT83 or HSP65/HSP70/MPT70 plasmids followed by BCG boosting did not induce significant protection over BCG alone, in the mice strains tested (figs.3.21 and 3.25). Besides the apparent increase in humoral (fig.3.19) and Th1 responses (fig.3.20A & B and fig.3.23) in prime-boost groups, inhibitory cytokines such as IL-4 and IL-10 (figs.3.20C and 3.24) were also detected in both groups of mice, either receiving a single dose of one or more plasmids, as part of their vaccination regimen. However, the role of these cytokines in this and other models (Jung, 2002) is still uncertain. Nonetheless, IL-10 would probably not be desirable cytokine in terms of vaccine efficiency. IL-10-producing T cells have been found in anergic tuberculous patients; T cells from these patients had defective phosphorylation of the zeta-chain of the TCR and failure in activation of ZAP70 kinase (Boussiotis, 2000).

Although a single vaccination with DNA can induce both antibody and CTL responses in several model systems, both cellular and humoral immune responses are increased by one or two additional immunisations (Gurunathan, 2000). HSP65-plasmid vaccination against murine tuberculosis increases considerably in efficiency with a regimen of four-doses, four weeks apart. Accordingly, in view of the failure in inducing protection against murine tuberculosis with one-dose priming of DNA as described above, I decided to test whether repeated priming doses of plasmid DNA would result in increased protection. The composition of the vaccine cocktail was also altered during the course of my experiments. As predicted, a protocol of two or three priming doses of Apa/HSP65/HSP70 DNA plasmids (Combo III) followed by a boost with BCG generated

unprecedented levels of protection over BCG alone, against *M. tuberculosis* infection in C57BL/6 mice (fig. 3.37 & 3.38). To my knowledge, it is the first time that a prime-boosted vaccination study against tuberculosis is able to afford such a high degree of reduction in bacterial load in both lungs and spleen, in comparison to mice vaccinated with empty vector plus BCG. Interestingly, a similar prime-boosting study using a priming vaccine expressing the mycobacterial antigens ESAT-6 and Ag85A followed by BCG boosting was not successful in obtaining better protection than BCG alone in mice (Skinner, 2003b). In comparison to my results, this study brings an immediate confirmation of how critical is to obtain a good combination of antigens necessary for successful priming. Improved levels of protection over BCG in Balb/c females were demonstrated when using three priming doses of DNA cocktail (fig.3.36). However, similarly to the experiment with C57BL/6 described in figure 3.37, it is possible that a protective effect in Balb/c after a two dose priming plus boosting would be seen more clearly once a large number of animals were examined. Nonetheless, numbers of *M. tuberculosis* CFU in the lungs of Balb/c and C57BL/6 mice are non-uniform depending on the day of infection (Wakeham, 2000).

The improved protection over BCG seen in Balb/c mice from prime-boost III groups, correlated with a very significant increase in the frequency of splenic CD8+ T cells secreting IFN-γ in response to PPD before challenge (fig.3.32). This increased IFN-γ secretion was probably dependent on the HSP65 and HSP70 antigens since stimulation of the cultures with recombinant Apa did not result in significant number of CD8+ T cells producing this cytokine (not shown). Whether this increase explains the successful results of protection is however a matter of speculation. Besides the excellent protection observed in the C57BL/6 strain, no correlation was obtained in terms of increase in

CD8+ T cells; only IFN-γ+CD4+ cells responding to Apa could be seen differentially increased in prime boosted groups (fig.3.33). Importantly, specific IFN-γ secretion was not predictive of protection in most of the prime-boost experiments with C57BL/6 mice.

DNA vaccination is known to induce a Th1-type of immune response and IFN-γ is an essential component of the anti-tuberculous response in the host; it seems logical to expect that high levels of this cytokine, present immediately before challenge would be a sign of successful protective outcome after challenge. However, the levels of this cytokine may fluctuate over time and only a kinetic study with a more detailed follow-up after vaccination and challenge would determine whether a better correlation with protection could be found. No correlation with protection on IFN-γ secretion by either CD4+ or CD8+ T cells was seen in the lung of prime-boosted groups after challenge at the time the bacterial load was assessed (fig.3.34).

Mycobacterial leprae HSP65 plasmid DNA, one of the components of the prime-boost cocktail used in this study has been previously demonstrated to be effective against experimental tuberculosis when administered prophylatically or immunotherapeutically (Tascon, 1996; Lowrie, 1999). Protection associated with this antigen was critically associated with CD8+ T cells (Bonato, 1998; Silva, 2000). The natural infection by tuberculosis in the murine host indicates that CD8+ T cells would be important but only most critical at a later stage of infection (Turner, 2001; Turner, 2002a; Van Pinxteren, 2001). In this context, a strong CD4+ T cell response is more important, particularly at the initial stages and many vaccine strategies have been developed to favour the induction of these responses, although none have been more effective than BCG in animal models (Lazarevic & Flynn, 2002). It is interesting to observe that successes in vaccinology have been mostly obtained stimulating a kind of "unnatural" immunity,

where paradoxically, specific antibodies or cells are induced but are unlikely to be involved in the so called control/clearance of natural infections (Casadevall & Pirosfky, 2003). As one of several examples, detailed immunological studies showing that CD4+ T lymphocytes are critical components of the successful host defence against both *Blastomyces dermatitides* and *Hystoplasma capsulatum* fungi might have predicted that vaccination with a live attenuated *B. dermatitides* strain or live *H. capsulatum* would be ineffective, considering an eventual CD4+ T cell deficiency; yet surprisingly, CD8+ T cells were conclusively shown to mediate protection in the absence of CD4+ T cell lymphocytes (Wuthrich, 2003). It would be interesting to study more deeply how highly potent and specific CD8+ T cell responses, induced early in animal models by vaccination would perform in terms of protection against tuberculosis.

In the current study, the efficacy of BCG priming and DNA boosting was assessed in Balb/c and C57BL/6 strains but no significant improvement in protection was observed. Several DNA-boosting combinations and doses have been used in this strategy: boosting with one dose of ApaDNA or one dose of the combination of mycobacterial genes Apa/HSP65/HSP70/MPT83 resulted in protection similar to BCG (figs.3.21 and 3.25). Furthermore, no additional improvement has been observed in boosting BCG with two doses of HSP65 and HSP70 plasmids (fig.3.40), in contrast with the significant protection seen in the reverse combination (fig.3.37). The studies above underline the importance of the immunisation order. Although many vector agents are able to prime an immune response, not all of them are effective at boosting. Priming the response requires induction of specific T cells, including a population that persists as antigen-specific memory cells, which then undergoes rapid expansion upon re-exposure to the same antigen in a boosting immunisation. Vector agents such as DNA plasmids,

protein-in-adjuvant formulations, virus-like particles and lipopeptides are excellent priming agents but relatively ineffective boosting agents (Dunachie & Hill, 2003). Recombinant viruses including MVA, attenuated fowlpox viruses and non-replicating adenoviruses appear capable of either priming or boosting, however if used alone or in homologous boosting, the increase in cellular immunity is not observed; boosting BCG with BCG in mice resulted in no improvement in protection or immunity over a single BCG dose (not shown). Priming with MVA vector and boosting with DNA vaccine resulted in no improvement in immunogenicity or protection over MVA alone (Schneider, 1998). Part of the explanation for these results may be related to an immunodominance effect; the overall immunogenicity of a recombinant virus or bacteria (BCG) is substantially greater than that of a plasmid DNA vector (Dunachie & Hill, 2003). In addition, BCG priming may activate undesirable components of the anti-tuberculous immunity such as Th2-responses, in contrast to a more focused response resulting from a plasmid DNA vaccine. Nonetheless, it is important to remember that the majority of the world population has received BCG vaccination at birth and therefore, is imperative to develop vaccination strategies able to boost efficiently BCG.

In summary, I have demonstrated that: (1) the mycobacterial *apa* gene is up-regulated during *M. tuberculosis* infection in the host cell and can be used effectively as a component of a plasmid combination vaccine cocktail that was able to induce significant protective responses against murine tuberculosis, despite its inability to stimulate IFN-γ-secreting CD8+ T cells in ApaDNA-vaccinated mice. (2) This DNA-prime BCG-boost approach induced statistically better protection than BCG against *M. tuberculosis* infection in mice. In addition (3), I demonstrated the importance of the nature of the components of the priming vaccine cocktail and also, the order of vaccination, to the

successful outcome of immunisation. As the primary route of infection in humans is the pulmonary route, further testing using a more relevant aerosol model of *M. tuberculosis* challenge would be of great value.

CD8+ T cells are very likely involved in this high improvement in BCG protection, although a more clearly defined answer would be provided by the isolation of CD8+ T cell clones from prime-boosted animals as well as testing the prime boosting protocol in animals devoid of CD8+ T cell populations (Feng, 2001). Also, it would be interesting to employ this effective prime-boost model of protection in search for more reliable correlates of protection against TB in mice.

Recently, some reports on potential autoimmune reactions in rodents due to vaccination with *M. leprae* HSP65 DNA have been published in the literature (Turner, 2000; Taylor, 2003). Despite the fact that, in a decade of research and vaccine testing in our laboratory at the NIMR and others around the world, there have not been reports of any deleterious reaction with the use of the HSP65 and HSP70 plasmid DNA vaccine in mice, it is important that a study investigating these important questions in more detail could be carried in animal models before HSP-vaccines could advance for clinical testing. Evaluation of alternative antigen combinations may also be carried out concomitantly.

CHAPTER FIVE

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