From The Department of Clinical Microbiology (MTC)
and
The Department of Bacteriology, Swedish Institute
for Infectious Disease Control (SIIDC)

STUDIES ON NEW TUBERCULOSIS VACCINE CANDIDATES IN ANIMAL MODELS

MELLES HAILE

Stockholm 2005

Published and printed by
Intellecta DocuSys

ISBN 91-7140-327-2

I was in jail when they took a specimen

I was diagnosed with TB

When the hospital report came

We sent the specimen before there were holes in the lung !!

After four months treatment I was cured from TB

Today we are calling the world

recognize that we can´t fight AIDS unless we do much more to fight TB as well !!

Remarks by Neslson Mandela
15 July 2004 XV AIDS Conference, Bangkok

Abstract

Background: Tuberculosis (TB) is a major health problem in many countries, especially in low income countries. Globally, TB causes more than 2 million human deaths annually and 1/5 of all adult deaths in developing countries. WHO estimates that one third of the world´s population i.e. 1.9 billion people, are infected with *M. tuberculosis* (Mtb). The most cost effective way to combat infectious diseases is the preventive vaccination. Most of the world's population is vaccinated with the only available TB vaccine, the Bacillus Calmette-Guerin (BCG) vaccine that was developed a century ago. Even though the BCG vaccine protects the young child against disseminated TB disease it has none or little protective effect against adult pulmonary TB (PTB). PTB is the major disease manifestation of TB in adults and it causes death at the most productive age, further adding to poverty in already impoverished countries. Hence, the development of a new more efficient TB vaccine(s) is the highest priority in TB research.

Aims: The aim of this thesis was to evaluate protective efficacy of new TB vaccine candidates with the long term goal to improve the efficacy of primary BCG vaccination against adult PTB by boosting with new vaccine(s).

Results: New vaccine candidates and adjuvant/delivery systems for mucosal applications were developed and tested in animal models. Lipoarabinomannan (LAM) was purified from a virulent Mtb strain and used to prepare novel oligosaccharide-protein conjugate vaccines. To avoid the immuno-suppressive effects of the intact LAM molecule while taking advantage of its carbohydrate antigen epitopes, LAM was de-lipidated and split into oligosaccharides by partial chemical degradation. The arabinomannnan oligosaccharides (AM) were used to prepare AM-protein conjugate (AM-Prot) vaccines and to generate a large number of monoclonal antibodies (MoAbs). Adjuvants can augment the immune response to many vaccine antigens and for some sub-cellular vaccines e.g. Diphteria and Tetanus vaccine, they are essential. There is a vast number of experimental adjuvants but most of them are intrinsically toxic and only few can be considered for use in man. Aluminum salts are so far the only adjuvants approved for large scale human use. In this thesis a new adjuvant L3 was investigated. L3 is non-toxic and approved by the Swedish FDA for human phase I/II trials. The different AM-Prot vaccines and a vaccine based on heat killed whole-cell BCG (H-kBCG) were formulated with L3 and studied for their ability to protect against virulent Mtb challenge in the mouse and guinea pig models - both as primary- and boost-vaccines. Both types of vaccines, when given nasally, evoked specific and robust cellular and humoral immune responses. The vaccines also conferred significant protection, at least equivalent to that induced by the BCG vaccine. Presently the general consensus is that cell mediated immunity (CMI) is decisive for protection against Mtb infections. This, however, has led investigators to neglect the role of the humoral immunity. Importantly, high antibody titers in particular towards LAM are seen in most TB patients but the role of these antibodies is unknown. Therefore, in this thesis the role of antibodies in Mtb infection was re-evaluated in passive protection experiments using an AM-specific MoAb. Mice were infected intravenously with virulent Mtb and the MoAb was added intravenously either prior to or together with the bacteria. The MoAb protected against the infection in terms of a dose-dependent reduction in bacterial load in spleens and lungs, reduced weight loss and, importantly, enhanced long-term survival.

Conclusions: **i)** AM-Prot vaccines can, when formulated with the L3 adjuvant and given nasally, provide as good protection as live BCG, **ii)** The protection is also conferred by conjugates containing Mtb-irrelevant carrier protein (i.e. tetanus toxoid) showing that antigenic epitopes of AM alone are protective. Therefore we propose that AM epitopes should be considered as part of a future new multi-sub-component vaccine, **iii)** The AM-Prot vaccine affords protection both when used as a primary vaccine and as a boost vaccine, **iv)** A H-kBCG vaccine did not protect when administered alone but was protective when given in the L3 adjuvant. The pre-clinical studies of this vaccine candidate are concluded and the vaccine is considered ready for phase I/II trials in man, **v)** None of the vaccines ameliorated the course of TB when used as a therapeutic regimen. Hence, caution is advised in the use of therapeutic vaccines, and in the design of phase I-III trials great care must be taken not to include individuals with active TB disease, **vi)** Certain anti-AM antibodies may confer protection, most likely aiding to the early containment of infection and thus giving time for a protective cellular immune response to develop.

Original Articles

This thesis is based on investigations reported in the following scientific papers, which are referred to by their Roman numerals:

I Hamasur B, **Haile M**, Pawlowski A, Schröder U, Williams A, Hatch G, Hall G, Marsh P, Källenius G, Svenson SB.
Mycobacterium tuberculosis arabinomannan-protein conjugates protect against tuberculosis.
Vaccine 2003; 21 (25-26): 4081-93.

II **Haile M**, Schröder U, Hamasur B, Pawlowski A, Jaxmar T, Källenius G, Svenson SB.
Immunization with heat-killed *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) in Eurocine L3 adjuvant protects against tuberculosis.
Vaccine 2004; 22(11-12): 1498-508.

III Moreira AL, Tsenova L, **Haile M**, Bekker LG, Freeman S, Mangaliso B, Schröder U, Jagirdar J, Rom WN, Tovey MG, Freedman VH, Kaplan G.
Mycobacterial antigen exacerbate disease manifestations in *Mycobacterium tuberculosis*- infected mice.
Infect Immun 2002, 70(4): 2100-7.

IV **Haile M,** Hamasur B, Jaxmar T, D Gavier-Widen, Chambers MA, Sanchez B, Schröder U, Källenius G, Svenson SB, Pawlowski A.
Nasal boost with adjuvanted heat-killed BCG or arabinomannan-protein conjugate improves primary BCG-induced protection in C57BL/6 mice.
Tuberculosis 2005; 85(1-2): 107-114.

V Hamasur B, **Haile M,** Pawlowski A, Schröder U, Källenius G, Svenson SB.
A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab') fragment prolong survival of mice infected with *Mycobacterium tuberculosis.*
Clin Exp Immunol 2004; 138(1): 30-38.

CONTENTS **Page**

INTRODUCTION

ABBREVIATIONS

Ab	antibody
AFB	Acid-Fast Bacilli
Ag85B	antigen 85B
AIDS	Acquired Immunodeficiency Syndrome
AM	arabinomannan oligosaccharides
BCG	Bacille Calmette Guérin
CMI	Cell-Mediated Immunity
CRM_{197}	Mutant diphtheria toxin
CT	Cholera toxin
DCs	dendritic cells
DT	Diphtheria toxoid
DOTS	Directly Observed Treatment Short-course
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunospot
HBHA	Heparin-binding haemagglutinin
Hib	*Haemophilus influenzae* type B
HIV	Human Immunodeficiency Virus
HSP	Heat shock protein
IgA	Immunoglobulin A
IFN-□	Interferon-gamma
i.n.	Intranasal
i.p.	Intraperitoneal
IL	Interleukin
i.v.	Intravenous
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
LT	*E. coli* heat-labile toxin
MDR	Multidrug-resistant
MHC	Major histocompatibility complex
MoAb	Monoclonal antibodies
Mtb	*Mycobacterium tuberculosis*
NMRI	Naval Medical Research Institute
OMP	Outer membrane protein
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
PPP	Private Public Partnership
PTB	pulmonary TB
TB	Tuberculosis
Th	Helper T cell
TLRs	Toll-like receptors
TT	Tetanus toxoid
WHO	World Health Organization
ZN	Ziehl-Neelsen

INTRODUCTION

BACKGROUND

Natural history of tuberculosis (TB)

In 1882, Robert Koch identified the tubercle bacillus, *M. tuberculosis* (Mtb*)*, as the cause of tuberculosis (TB) in humans. This pathogen is still known by many as "*The Koch's bacillus*". In the natural history of tuberculosis infection, susceptible persons are exposed to aerosolized viable tubercle bacilli usually emitted with caugh by a patient with pulmonary tuberculosis (PTB). After the initial infection, intracellular replication of bacilli occurs, and dissemination of organisms may result through lymphatic and hematogenous routes. In a minority of infected individuals, the initial infection may progress to active clinical disease defined as **primary TB**. The vast majority of persons infected with Mtb never develop clinical disease and TB remains latent for many years. Reactivation may occur decades after the initial infection and develops only in a small proportion of latently infected individuals - about 10% of the normal hosts will reactivate TB over their lifetime. Risk factors for **reactivated TB disease** include old age, drug abuse, immuno-suppression (especially by HIV) and malnutrition. Clinically these patients usually present pulmonary disease; prominent symptoms are chronic, productive cough, low-grade fever, night sweats, fatigue, and weight loss. TB may present extra-pulmonary manifestations including lymphadenitis, kidney, bone, or joint involvement, meningitis, or disseminated (miliary) disease. The frequency of such extra-pulmonary manifestations is increased among immuno-compromised individuals such as in elderly, malnourished or HIV-infected individuals.

Global impact of TB

Even today, more than one hundred years after Koch's description of how to stain and culture the tubercle bacillus, TB is still a great health problem worldwide. Globally TB causes 2 million deaths per year and 1/5 of all deaths of adults. It is estimated that today one third of the world population i.e. 1.9 billion people has been infected with TB. The majority of TB cases occurs in the developing countries making TB the number one killer among adults (Kochi A, 1991).

With the advent of TB chemotherapy in the 1950´s many experts calculated that TB would be eradicated in few years using the Bacille Calmette Guérin (BCG) vaccine and anti-

TB drugs, but during the last 20 years increasing evidence has been presented that TB, especially multi-drug resistant TB (MDR), is on the rise in many countries. Today, it is estimated that there are 50 million people infected with MDR strains of Mtb (World Health Organization, 2000). In Sweden about 450 new cases of TB are reported annually. More than half of those are immigrants from non Nordic countries. In the neighboring Baltic countries MDR TB among new cases has now been reported to rapidly increase (Kruuner *et al*., 2001). If immediate measures are not taken to stop the spread of TB, WHO estimates that within the coming twenty years 70 million people will die from infections caused by Mtb.

One important reason for the increase in TB worldwide is the HIV epidemic. Mtb infected, immuno-competent individuals have an estimated 10% lifetime risk of developing the disease. Since the beginning of the 1980´s the number of HIV associated TB cases is increasing in many countries, especially in Africa. Individuals immuno-compromized by HIV infection easily reactivate their previous infections with TB. In these individuals the risk of developing active disease increases from 10% per lifetime to 10% per each year of life. According to the WHO it is estimated that 10% of the 88 million new TB cases worldwide between 1990-1999 and 14 % of the deaths are attributed to co-infection with HIV (Dolin PJ. *et al*., 1994). With the increasing number of HIV positive individuals and the emergence of MDR Mtb strains the incidence of pulmonary TB is increasing. It is obvious that despite the many advances that have been made since the time of Koch new improved methods for diagnosis, detection of drug resistant Mtb strains and most important new better TB vaccines would be of great aid in implementing effective TB control programs.

Mycobacteria and characteristics of *M. tuberculosi*s (Mtb) complex

Mycobacterium is one of the most clinically important and intensively studied pathogens. TB caused by Mtb and leprosy by *M. leprae*, are the most significant diseases caused by mycobacteria and have been recognized by man throughout the recorded times. The oldest examples of TB were found in fossil bone which dates back to about 8000 BC (Ayvazian LF., 1993).

The Mtb complex includes the following species: *M. tuberculosis, M. africanum, M. bovis, M. bovis* BCG (Bacille-Calmette-Guérin), and *M*. *microti*. The first three members of Mtb complex are the cause of human TB; together with *M. leprae* they are the only mycobacterial infection transmissible from person to person. Therefore, identification of these

species in clinical specimens and differentiation from non-TB mycobacteria (NTM) is important to aid the clinician to apply an appropriate therapy.

Members of the Mtb complex are slow growers, with a doubling time of 12-20 h, they grow at a temperature of 34-38^{o}C. Visible colonies of *M. tuberculosis* require 2-6 weeks to appear on Lowenstein-Jensen (L-J) or on 7H10/7H11 Middlebrook agar medium.

In recent years, clinical interest in NTM strains, such as *M. avium* complex and other environmental mycobacteria found in soil and water has increased. The main reason for this is that some of these mycobacteria may cause disseminated infections in AIDS patients. Additionally, the NTM strains were also found to interfere with BCG vaccination, by masking the immune response in individuals pre-sensitized by NTM prior to the administration of the vaccine.

Diagnosis and treatment

According to recent reports from WHO 70% of the world´s TB cases remain undetected. The main reason for the difficulty in detecting the majority of TB cases is lack of adequate methods sensitive enough to identify mycobacteria directly from specimens at an affordable cost. The solid laboratory diagnosis depends on identification of the pathogen in cultures, including the assessment of growth rate, colony morphology, pigment production and biochemical assays (Roberts, 1991).

TB case detection is mainly based on sputum smear microscopy. The detection of acid fast bacilli (AFB) by Ziehl-Neelsen (ZN) method is a highly specific diagnostic tool for pulmonary TB patients. However, the sensitivity of AFB is low and hence only a fraction of cases can be diagnosed by this method. Two methods of staining are commonly used, the ZN and auramine O fluorescence acid-fast stains (Kent, 1985).

TB patients who have been coughing persistently for several weeks are the main source of TB transmission. Diagnosing these patients by smear microscopy, in conjunction with clinical and radiological data and starting adequate treatment have a significant effect on shortening the chain of TB transmission in communities.

Culturing of clinical specimens from TB suspected patients is used to identify the cause of infection. It is currently the golden standard for the diagnosis of TB. The cost and complexity of this technique are unfortunately much higher than those of the ZN smear microscopy. Also, the time needed for primary isolation by culture is very long, as it takes between 3 to 6 weeks to obtain identifiable colonies.

TB diagnosis by sputum smear microscopy remains the widely used method of choice in developing countries. Because of its simplicity, many dispensaries and health centers use it as a case-finding method, even though it identifies only 30-60 % of culture-positive cases and it is 100 times less sensitive than culturing (Levy *et al*., 1989) (Colebunders and Bastian, 2000). Culturing of Mtb in developing country settings is restricted to priority situations such as radiologically suspected TB, when the sample is repeatedly negative for AFB or when patients are not responding to treatment.

There are several new techniques available for detecting mycobacteria rapidly, such as the polymerase chain reaction (PCR), MTD (Mtb direct test) and the Bactec (radiometric method) (Beavis *et al*., 1995) (Aslanzadeh *et al*., 1998) (Salfinger *et al*., 1998). The nucleic acid based PCR techniques are fast compared to culturing in agar medium. Results are often obtained within few hours but these methods are still unavailable for many countries with high number of TB cases, the main reasons being high costs, need for skilled manpower and expensive equipment. Recently, a T-cell based rapid test for Mtb infection has been introduced for routine clinical practice (Liebeschuetz *et al*., 2004). This ELISPOT test detects T-cell antigens (ESAT-6) which are specific for Mtb, but absent from BCG and most environmental mycobacteria (Lalvani *et al*., 2001).

Over 50 years ago the first TB drug streptomycin was introduced, followed by other specific drugs. Recently, to improve the efficacy of TB chemotherapy, a modified regime of treatment with anti-TB drugs, the so-called Directly Observed Treatment Short-course (DOTS) program, has been introduced by the WHO. In this short-course program a three-drug selection of five common TB drugs (isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol) must be taken for 6 months. Although effective when implemented rigorously, this strategy is labor intensive, expensive and demands health care infrastructures that are not always available.

Cellular constituents of Mtb

The outer cell wall of the tubercle bacillus has been extensively investigated. It is built up of a wax and lipid-rich structure with long chain fatty acids, glycolipids, polysaccharides and proteins (Figure 1). According to recent genomic analysis close to 250 genes within the Mtb genome are involved in fatty-acid metabolism (Cole *et al*., 1998). In recent years much progress has been made in elucidating the highly complex cell wall structure of mycobacteria.

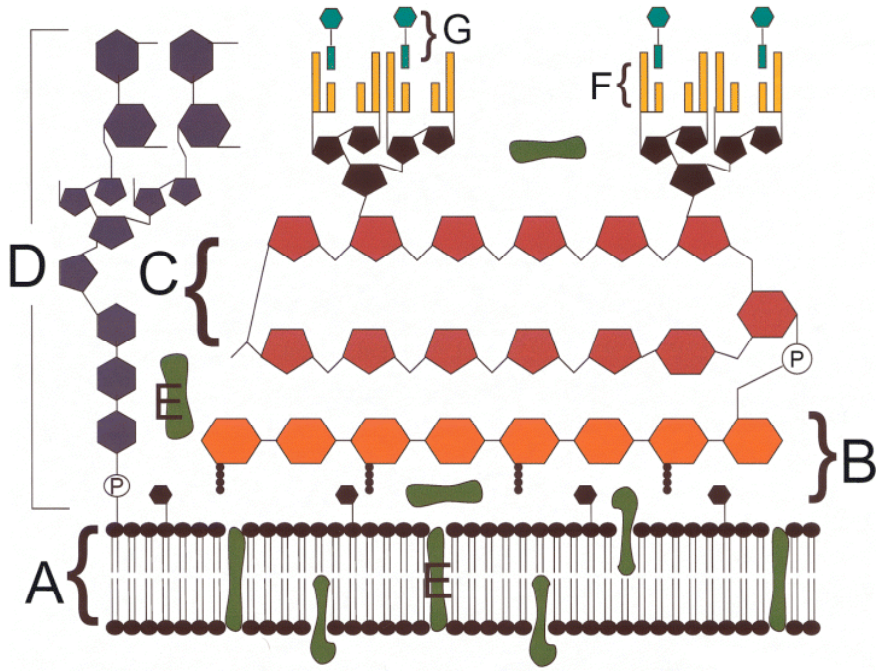


Figure 1. Schematic representation of the cell wall of *M. tuberculosis*. The components include the (A) plasma membrane, (B) peptidoglycans, (C) arabinogalactan, (D) mannose-capped lipoarabinomannan, (E) plasma membrane- and cell-wall associated proteins, (F) mycolic acids and (G) glycolipid surface molecules associated with the mycolic acids. From: Cellular Microbiology 2003; 6(2): 105-116, with permission.

This includes identification, characterization, and purification of a variety of mycobacterial cell wall components (Brennan, 2003) (Chatterjee *et al*., 1992a) (Hamasur *et al*., 1999a).

The most abundant constituents of the mycobacterial cell wall are mycolic acids. Mycolic acids are 1-alkyl branched 2-hydroxy fatty acids with 70 to 90 carbons arranged in a simple alkyl chain. The main chain contains cyclopropyl, methoxyl or keto, and methyl groups.

Another cell wall structure of interest is the peptidoglycan-arabinogalactan which forms a stiff skeleton supporting lipids and other components thought to be involved in virulence/pathogenicity. Mycolic acids and their derivatives, such as trehalose-6,6´-dimycolate (TDM), as well as peptidoglycan, have numerous immuno-modulatory effects affecting both the innate and the adaptive immunity (Ryll *et al*., 2001) (Uehori *et al*., 2003). There is also a large variety of lipids, glycolipids and proteins in the mycobacterial cell wall (McNeil and Brennan, 1991) (Kremer *et al*., 2001). It is believed that identification of cell wall components may yield a wide range of antigens to be used as subunit vaccine candidates (Orme, 1999).

Chemical structure of lipoarabinomannan (LAM)

LAM is a major structurally unique glycolipid component of the outer cell wall of all mycobacterial species (Figure 2). It is the major carbohydrate antigen and accounts for up to 15% of the bacterial weight (Hunter *et al*., 1986). LAM consists of a mannan polysaccharide

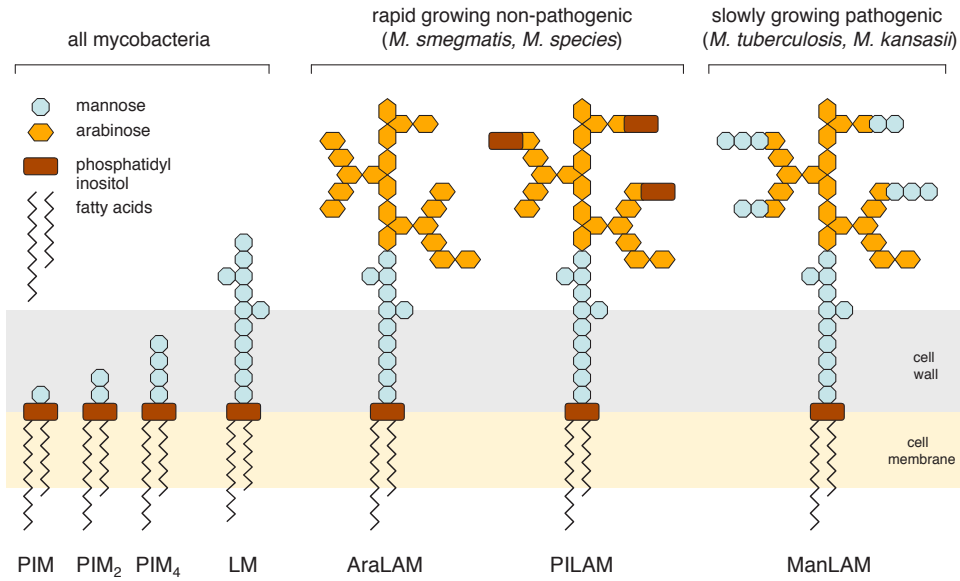


Figure 2. Mycobacterial lipoarabinomannanns (LAMs) and their precursors.

PIM, phosphatidylinositol mannoside; LM, lipomannan; AraLAM, uncapped LAM; PILAM, phosphatidylinositol-capped LAM; ManLAM, mannosyl-capped LAM.

backbone substituted with branched oligoarabinosyl side chains; the former is covalently linked to a phosphatidyl inositol lipid moiety (Chatterjee *et al*., 1991) (Hunter and Brennan, 1990) (Chatterjee *et al*., 1988).

Biological function of LAM

LAM is regarded as an important immuno-modulating compound affecting the immunological network of the host, and hence contributing to the pathogenesis of mycobacterial infections. LAM has been reported to exert a wide range of effects on the immune system of the infected host. LAM isolated from both *M. tuberculosis* and *M. leprae* has been shown to cause suppression of T-cell proliferation (Chatterjee *et al*., 1991). LAM also interferes with gamma-interferon (IFN-γ) mediated activation of macrophages (Sibley *et al*., 1988), is involved in scavenging of free toxic oxygen radicals, inhibits protein kinase activity (Chan *et al*., 1991) and inhibits synthesis of mRNA encoding interleukin 2 (IL-2), interleukin 5 (IL-5), and granulocyte/macrophage colony-stimulating factor (GM-CSF) in human T-cells (Barnes *et al*., 1992). LAM has also been shown to induce the expression of immediate-early genes of macrophages (Chatterjee *et al*., 1992b), enhance the production of tumor necrosis factor alpha (TNF-α) by mononuclear cells (Sibley *et al*., 1988), and mediate complement activation (Hetland *et al*., 1998).

Considering its extensive biological effects, it has been argued that LAM could be a key factor in enhancing the intracellular survival of mycobacteria and inducing much of the pathology seen in TB (Chan *et al*., 1991) (Wallis *et al*., 1990).

Role of cell-mediated immunity in TB

Clinical studies of immuno-deficient patients, who are often susceptible to mycobacterial infection have contributed to the understanding of the importance of cell-mediated immunity (CMI) in TB. This is best illustrated by the susceptibility of HIV-positive individuals to mycobacterial infections. These patients reactivate their latent TB infection at high frequency (50 fold or more).

Numerous animal studies have confirmed that CMI plays a vital role in resistance to mycobacterial infections. Experimental evidence indicates that both CD4+ and CD8+ T cells are important in the control of infection (Flynn, 2004). The role of each lymphocyte subset

and the detailed mechanisms involved are still under investigation. In general it is believed that cytokine production by T cells plays main role in the control of the Mtb infection.

The understanding of the mechanisms involved in the immune response to mycobacteria has advanced dramatically in recent years – in particular different “knock out” mice strains have been valuable tools. Several key elements of the T-cell and macrophage interplay, along with the killing mechanisms used by macrophages to control infection have been elucidated. Cytokines such as IFN-γ and TNF-α were found to play a critical role in macrophage activation leading to killing of intracellular mycobacteria. The phenotypes arising from mutations in genes of the IFN-γ mediated pathway are similar in both mice and humans. It has been shown in studies involving "knock out" mice lacking IFN-γ signaling that such animals succumb early to mycobacterial infections (Orme *et al*., 1993). Accordingly, studies in humans have shown that individuals with defects in IFN-γ and IL-12 receptors are more prone to mycobacterial infections leading to severe disease (Ottenhoff *et al*., 2000). This indicates that IFN-γ mediated macrophage activation plays an important role in controlling mycobacterial growth.

Immunity to TB involves three stages. It starts with the establishment of infection in the lungs, followed by an acquisition of CMI, and a chronic, latent phase which may persist for decades. Eventually a reactivation phase occurs which is followed by uncontrollable growth of TB bacilli, leading to clinical symptoms of TB.

The immune reponses in TB are shown in Figure 3. In the early stage of infection with Mtb, the innate immunity is triggered through interaction of the bacterium with Toll-like receptors (TLR) (Heldwein *et al*., 2003), DC-SIGN and mannose receptors (Nigou *et al*., 2001) on specialized phagocytic cells - dendritic cells (DC) and alveolar macrophages. The binding by TLR2 or TLR4 followed by the uptake of Mtb induces the production of IL-12 and TNF-α (Verreck *et al*., 2004). IL-12 subsequently drives the production of IFN-γ from NK cells and plays a key role in activation, differentiation and expansion of antigen specific T helper-1(Th1) cells. TNF-α and chemokines secreted by DCs and alveolar macrophages are essential for the formation of granuloma (see below) resulting in the early containment of infection.

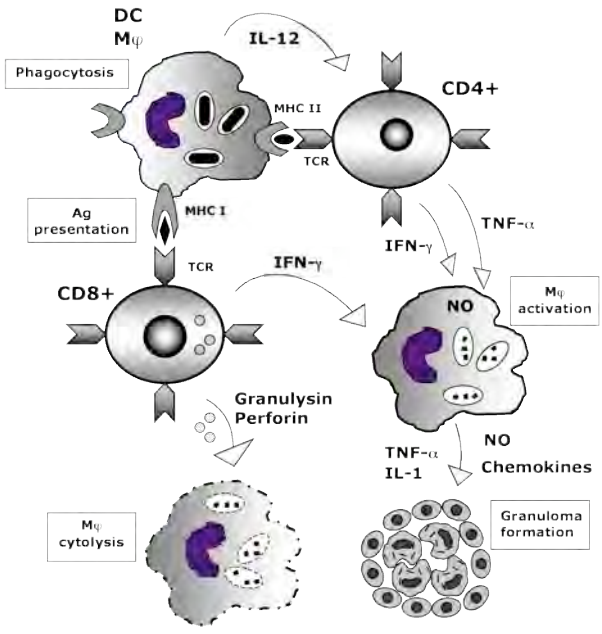


Figure 3. Cellular immune responses in tuberculosis.

DC, dendritic cells; Mφ, macrophage; CD4+, CD4 positive T helper cell; CD8+, CD8 positive cytolytic T cell

Two to three weeks after infection an adaptive immune response develops with expansion of mycobacterium-specific T-helper cells of CD4+ phenotype which produce IFN-γ. These T-cells via IFN-γ activate anti-bacterial defense mechanisms in macrophages resulting in killing of intracellular bacilli or at least containment and halting of the mycobacterial growth (Orme *et al*., 1993) (Cooper *et al*., 1993) (Cooper *et al*., 1997) (Flynn *et al*., 1993). At the later stage another type of T-cells, the cytotoxic T-cells of CD8+ phenotype come into play. These cells contribute to the anti-bacillary response by lysing incapacitated macrophages filled with bacilli and killing bacilli through enzymes and bactericidal compounds released from the cytoplasmic granules (Stenger *et al*., 1999).

Simultaneously with the CMI response a delayed-type hypersensitivity (DTH) reaction is triggered. This leads to the influx of monocytes and lymphocytes into the infected tissues which enables the formation of granuloma containing infected macrophages surrounded by a mantle of activated lymphocytes. The granulomas limit the spread of infection by walling off the micro-organism from the rest of the lung, subsequently making this part of the lung tissue more favorable for the immune cells to contain the bacteria. (Orme *et al*., 1993).

In the mouse the major effector mechanism responsible for the anti- mycobacterial activity of IFN-γ and TNF-α, is the nitric oxide (NO), and other reactive nitrogen intermediates (RNI) produced by macrophages via the action of inducible form of nitric oxide synthethase (NOS2). The production of RNI in vivo is also important for control of TB. Mice deficient in NOS2 activity are very susceptible to acute or chronic Mtb infection, compared to wild-type mice. The involvement of NO in protection in human TB has as yet not been

proved, but there is increasing indirect evidence that it might in fact be so (Chan and Flynn, 2004).

Role of antibodies in TB

There is consensus that cellular immunity to TB is vital for protection against development of the disease. In the Mtb infected host, in addition to CMI, there is also always a vigorous humoral response present but, nevertheless, the role of antibodies and B cells in protection against TB has been neglected for a long time. However, recent studies have shown that B cells as well as certain antibody responses could play an important role in protective immunity to TB. B-cells have been shown to participate in regulating chemokines as well as adhesion molecules. The expression of these molecules leads to recruitment of neutrophils, macrophages and CD8+ T-cells during early Mtb infection (Bosio *et al*., 2000). Stimulation of these types of responses may therefore be an important goal in designing new TB vaccines. Several groups have reported that passive administration of MoAb protected mice against experimental Mtb infection. In a recent paper an IgA MoAb directed against the α-crystallin antigen of Mtb, significantly reduced the CFU in the lungs of the tested animals (Williams *et al*., 2004). In another study the MoAb of isotype IgG2b, when bound to the surface of *M. bovis*, changed the morphology of the granulomas and increased the survival time of the infected mice (Chambers *et al*., 2004a). In still another study, several strains of mice, including IFN-γ and MHC class II deficient mice, when injected with bacilli coated with MoAb specific to LAM survived longer than controls (Teitelbaum *et al*., 1998). Finally, pre-coating of Mtb with anti-HBHA MoAb prior to the intranasal infection, was reported to reduce CFU in the spleen, which has been interpreted in terms of the antibodies interfering with the dissemination of Mtb in the tested animals (Pethe *et al*., 2001).

Immuno-pathology and latency in Mtb infections

Immune responses to Mtb infection in humans can be either beneficial leading to protection or can be detrimental leading to harmful immuno-pathological events. Neither the mechanisms involved in the elimination of the pathogen, which result in protection nor the mechanisms that lead to the disease-associated immuno-pathology are fully understood. It is clear that Mtb, compared to other microbes is a very challenging pathogen for the immune system of the host. The main reason for the successful avoidance of the host´s immune

defenses by Mtb is that the bacilli have cells of the monocyte/macrophage lineage as intracellular habitat and replicative niche. Exactly how the bacteria escape the killing mechanisms of macrophages is not fully understood (Russell, 2001).

LAM has been shown to cause maturation arrest of the mycobacterial phagosome. Hence, LAM was recently proposed to cause long-term Mtb persistence in vacuoles sequestered away from the bactericidal and antigen-processing organelles in infected macrophages (Vergne *et al*., 2003). In another study two lipoproteins derived from mycobacterial lysates were shown to inhibit MHC-II Ag processing in human macrophages. This was proposed to be an important mechanism for Mtb persistence leading to diminished recognition of infected macrophages by CD4+ T cells (Gehring *et al*., 2004). Such mechanisms may allow the TB bacilli to remain latent in the host when they are contained and encapsulated in granulomas in a dormant form.

Many of the clinical symptoms of TB are associated with immuno-pathological events such as delayed type hypersensitivity (DTH) reactions that are triggered during infection with the tubercle bacillus. Koch was first to describe that an extract of a pure culture of the tubercle bacillus, "*tuberculin*", when injected under the skin of guinea pigs, induces a remarkable immuno-pathological response in the infected animal. Twenty-four to 48 hours after injection, the host responded with either a local and/or a severe systemic reaction. The severity of the reaction was determined by the amount of material injected and the immunological status of the host. This reaction was often associated with clinical worsening of TB patients receiving tuberculos therapy and has become known as the *"Koch´s phenomenon*". If the host has never encountered the tubercle bacillus, the reaction to *tuberculin* was absent or very mild.

TB VACCINES

The most cost effective way to combat infectious diseases is the use of effective preventive vaccines. Such preventive vaccines have in the past successfully eliminated one of the most deadly and debilitating human diseases - smallpox - and are currently well underway to eliminate polio. In the case of TB, the BCG vaccine, which has been used for more than a century, is inefficient in controlling the disease. The BCG vaccine protects against childhood TB but this immunity wanes with age, resulting in insufficient protection against adult pulmonary TB. Therefore TB still constitutes a major and even increasing global problem. Hence, the development of a new more efficient TB vaccine(s) than the current BCG vaccine

is one of the top priorities in TB research. The single most important reason why BCG still is widely used is the good protective effect seen against childhood disseminated TB disease and the reported cross protective effect against leprosy (Kaufmann, 2000).

Early attempts: Koch´s *"old tuberculin"*

Robert Koch presented indisputable evidence that the tubercle bacillus caused TB in man and for this Koch was awarded the Nobel prize in 1905 (Sakula, 1979). Koch was able to culture Mtb using ox and sheep serum that was coagulated by heating to 65^{o}C and he tried to use glycerol extracts of the dead tubercle bacilli, the so-called ¨*old tuberculin*¨ as a therapeutic vaccine against TB. Unfortunately, tuberculin was soon proved to have no therapeutic value, however the tuberculin skin test became an instant and very important diagnostic tool in identifying latently infected individuals.

The BCG vaccine

It took over 20 years after the failure of Koch´s tuberculin therapy before another group started to work on a vaccine against tuberculosis. At that time Nocard isolated a virulent *M. bovis* strain from the milk of a heifer, with mastitis. This strain named ¨*lait Nocard*¨ was then sent to the Pasteur Institute and after 230 *in vitro* passages to weaken its virulence, a new live attenuated vaccine named BCG was introduced by Calmette and Guérin. This vaccine showed good protection in children in the early studies performed by its creators. The first report of successful BCG vaccination in 1921 led to the widespread distribution of BCG cultures to laboratories all over the world for further propagation (Behr and Small, 1999).

Since 1923 the BCG vaccines have been used in most countries throughout the world, and they are the most widely used vaccines available through the WHO Expanded programme for Immunization (EPI) (Fine, 1989). Since its introduction, over 3 billion doses of the vaccine have been given worldwide, and 100 million newborn children are still immunized with BCG every year. In Sweden, the routine vaccination of children was stopped in 1975. Only children to immigrant parents and people who work in the medical profession and laboratories or are otherwise regarded as high exposure risk groups are routinely vaccinated with BCG.

BCG strain variation and differences in study populations

Since the introduction of the BCG vaccine the original vaccine strain has been continuously propagated in many laboratories all over the globe resulting in different sub-strains (Hart, 1967). The most widely used BCG vaccine sub-strains include the Connaught, Danish, Glaxo, Moreau, Pasteur and Tokyo strains (Figure 4). Many clinical trials have shown that the efficacy of the BCG vaccines varies greatly between different countries and study populations; figures ranging between 0-94% protection has been reported (Colditz *et al*., 1994; Fine, 1999).

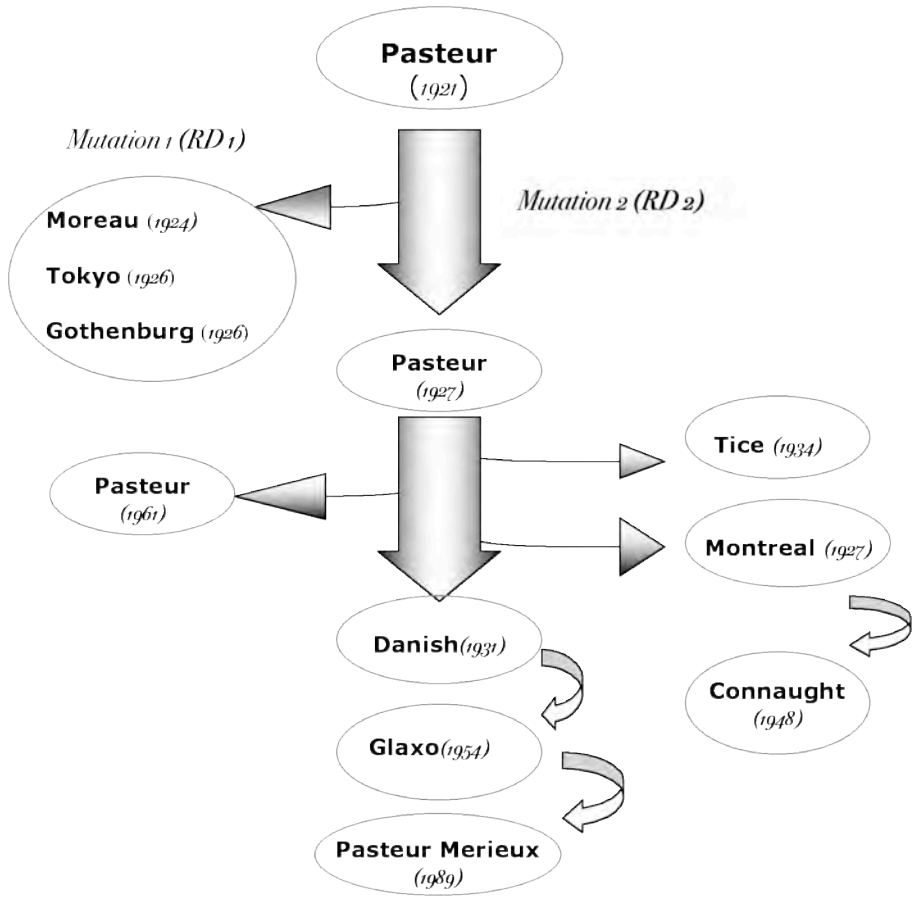


Figure 4. The Genealogy of BCG

One of the largest controlled studies of BCG vaccine efficacy was performed in South India. This study, which included more than 250 thousand individuals, showed that the BCG vaccine gave some protection in younger age groups. However, no protection was seen in middle and older age groups; even increased risk of developing the disease as a result of BCG vaccination was reported in young adults (Chennai, 1999). Recently, a sixty year follow-up study of American Indians and Alaska natives reported the long term efficacy of BCG to be 52% (Aronson *et al*., 2004). The reasons for these variations in efficacy are not clear.

More recent deletion analysis of the genome of different strains of BCG by microarray technology have shown that BCG have lost some genes now thought to be important for the protective immunity. The loss of genes might have occurred during the original attenuation process from the parental strain or during further propagation, before the lyophilization of seed lots was introduced in the 1960´s (Behr *et al*., 1999). Major antigenic proteins were found to be present in the parental strain but either absent (ESAT-6 and CFP-10, MPT64) or not expressed (MPB70 and MPB83) in several BCG vaccines (Vordermeier *et al*., 1999). Even though these findings are spectacular, there is still a need of elucidating whether these strain variations alone can account for the observed variability in the vaccine efficacy (Oettinger *et al*., 1999).

Comparison of epidemiological data from vaccinee cohorts for two strains of BCG (Glaxo and Danish strains) indicated that both strains gave good protection in England, but little protection in Malawi and Chingleput, in South India (Baily, 1980). These observations would indicate that inter-strain differences are of minor importance and some other geographical and/or population-related factors are important for the BCG vacccine efficacy (see below).

Gender-related differences in the vaccine efficacy are highlighted in a recent 60-year follow-up study showing that the overall vaccine efficacy for women was 70%, compared with 29% for men. In this study the reason behind such gender-related differences was not explained (Aronson *et al*., 2004).

Exposure to environmental mycobacteria and chronic parasite infections

It has been proposed that the low protective efficacy of BCG vaccination seen in adults in the tropical regions of the world, could be due to sensitization by environmental mycobacteria prior to BCG vaccination. As some atypical mycobacteria are abundant in certain geographic areas, the early, pre-vaccination exposure to such microorganisms could

induce immune responses that would either mask the responses to ensuing BCG vaccination, or even inhibit the BCG- induced immunity by interfering with persistence of the vaccine strain in the host (Orme and Collins, 1984) (Palmer and Long, 1966) (Fine, 1989) (Rook *et al*., 1981). To test this hypothesis, mice were sensitized with environmental mycobacteria common in tropical regions and then vaccinated with BCG. Such mice were less able to control a subsequent Mtb infection than vaccinated mice which were not subjected to pre-sensitization (Brandt *et al*., 2002). Also, studies in calves have demonstrated that animals with a high IFN-γ response to antigens from an environmental strain of *M. avium* prior to BCG vaccination, were less protected when challenged with virulent *M. bovis* (Brandt *et al*., 2002) (Buddle *et al*., 2002). In contrast to the classical BCG vaccine, the protective efficacy of some new vaccine candidates, comprising of subunit vaccines or attenuated mutants of *M. bovis*, was not affected by pre-sensitization with environmental mycobacteria (Brandt *et al*., 2002) (Buddle *et al*., 2002) (de Lisle *et al*., 2005). These findings suggest that in construction of new vaccine candidates efforts must be taken to avoid the down-modulation of the immune response induced by exposure to environmental mycobacteria.

In a recent collaborative study with others our group have shown that persistent helminth infections shift the immune response towards a Th2 type, thereby significantly reducing the protective efficacy of BCG (Malhotra *et al*., 1999) (Elias *et al*., 2005). This is well in line with earlier observations that de-worming of helminth-exposed BCG-vaccinated individuals improves PPD-specific responses (Elias *et al*., 2001).

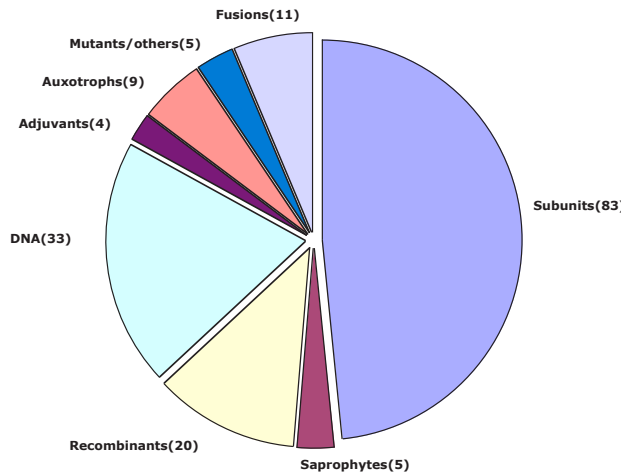
Suboptimal delivery of vaccine

A possible reason for the inability of the current live BCG vaccine to protect against adult pulmonary TB, may be that the immunization by the parenteral route might not be optimal. In order to achieve protection of the lung and the upper respiratory tract, which is the port of entrance of Mtb bacilli, a vaccine should preferably, in addition to a systemic immune response, also elicit a local respiratory mucosal immunity. Presently, some new vaccine strategies are focused on optimizing vaccine efficacy using new adjuvant/delivery systems designed for mucosal administration (Schroder and Svenson, 1999) (Aldwell *et al*., 2003) (Goonetilleke *et al*., 2003) (Lyadova *et al*., 2001) (Doherty *et al*., 2002).

NEW TB VACCINES

The failure of the BCG vaccine to efficiently prevent PTB in the adult populations has encouraged the search for a new TB vaccine(s). Today, using modern techniques, several research groups have developed close to two hundred new vaccine candidates (Figure 5). They include live attenuated vaccines, recombinant virus- and bacteria-vectored vaccines including BCG vector, DNA vaccines, subunit vaccines including fusion proteins as well as killed BCG and *M. bovis* combined with novel adjuvants and delivery systems.

Figure 5. TB vaccines under screening



Live mycobacterial vaccines

Live *M. vaccae* and *M. microti*, have been suggested as TB vaccine candidates, but animal experiments have shown gross variability in their protective efficacy in animal models (de Bruyn and Garner, 2003). Recently, a Mtb mutant strain with defects in mycobacterial lipids (Mtb *drrC*) has been shown to be more protective than BCG when administered in mice (Pinto *et al*., 2004). This and all other types of Mtb attenuated mutants, even though shown to be safe in immuno-deficient SCID mice, still need more investigations to ensure that they would not reverse to a virulent state; for this reason there is a standing safety concern and unsolved liability issue for attenuated Mtb vaccines (Smith *et al*., 2001).

Recombinant BCG (rBCG)

A great deal of work has been done to improve the efficacy of BCG by introducing additional copies of existing genes or by reintroducing some of the genes that were lost during the long *in vitro* attenuation process. Recently, a recombinant BCG (rBCG30) over-expressing the 30kDa major secretory protein of Mtb has shown slightly improved protection in animal models. Guinea pigs immunized with rBCG30 and challenged with aerosolized Mtb had fewer bacilli in their lungs and spleens than animals immunized with the parental, conventional BCG vaccine (Horwitz *et al*., 2000). A phase I clinical trial with rBCG30 was started in 2004. By complementing BCG with ESAT-6, which is missing in BCG, an improved protection was achieved in mice (Pym *et al*., 2003). Other candidates reported to be effective in animal models are rBCGs secreting either the listerolysin molecule (Hess and Kaufmann, 1999) or cytokines (Moreira *et al*., 2000) (Murray *et al*., 1996).

Vectored vaccines

Live vectors, such as the non replicating recombinant vaccinia virus, or recombinant *Salmonella* modified to carry immuno-dominant genes from Mtb are vaccine candidates that have shown good protection in animal models (McShane *et al*., 2001) (Mollenkopf *et al*., 2001). The recombinant vaccinia virus expressing Ag85A (MVA85A) has been tested pre-clinically using different vaccination strategies (homologous or heterologous prime-boost) and was the first vaccine candidate to enter a phase I clinical trial (McShane *et al*., 2004). This trial included both BCG naïve individuals and individuals vaccinated with BCG one month, 12-18 months, and 10-20 years before single MVA85A booster vaccination. The results from this study are so far promising in terms of increased levels of antigen-specific, IFN-γ-secreting T cells in the naïve healthy unvaccinated volunteers and in groups who had been BCG vaccinated earlier. However, this type of vaccines still have the inherent problem in that immunity elicited against the vector itself limits the number of possible immunizations; there are also unsettled safety and liability issues for live viral vaccines.

DNA vaccines

Various DNA vaccines containing plasmids coding for mycobacterial antigens, such as members of the mycolyl-transferase family (Ag85 complex) (Huygen, 1998) and heat shock proteins 60, 65, 70 (Hsp60, 65, 70) (Lowrie and Silva, 2000) (Ferraz *et al*., 2004) (Lowrie,

2003) (Johansen *et al*., 2003), have been extensively tested for their efficacy against TB in laboratory animal models. To further increase the potency of these DNA vaccines, co-immunization with cytokines or formulation of DNA in cationic lipids or other types of adjuvants, has been explored. DNA vaccines not only generate specific Th1 type of T-cell responses, but also CD8+ T cell-mediated cytotoxicity, which is considered to be important for the protection against Mtb challenge. However, so far the DNA vaccines appear to need protein boosting to provide a robust protection. In addition DNA vaccines present safety concerns as there is no definite evidence that the plasmids used would not integrate into the host genome.

Subunit and fusion proteins formulated in novel adjuvants

Mtb expresses cytoplasmic, cell wall-associated, and secreted proteins. Some of these proteins such as ESAT-6, Ag85B, HSP60, and a hybrid protein (fusion of ESAT-6 and Ag85B) are currently being evaluated as vaccine candidates. These proteins formulated in appropriate adjuvant or delivery system have been shown to elicit CMI responses (Brandt *et al*., 2000). Likewise, HSP65 from *M. leprae* have been shown to stimulate both the humoral and T cell immune responses (Silva, 1999). Another vaccine candidate, consisting of a 72-kDa polyprotein, a fusion protein based upon the Mtb32 and Mtb39 antigens of Mtb (designated Mtb72F), has shown protective capacity as an adjunct to the BCG vaccine in mouse and guinea pig models (Skeiky *et al*., 2004) (Evans *et al*., 2004). This vaccine also entered clinical trials in 2004.

Killed BCG and *M. bovis*

Early studies in the beginning of the 19th century with vaccines prepared from killed tubercle bacilli showed little or no protection. These experiments were repeated over and over again throughout the century with similar results. This eventually led to the dogma that any effective vaccine against TB must be live (such as BCG) with the retained ability to propagate or at least survive in the host for a prolonged period in order to induce protective immunity (Weiss, 1959). However, recent evidence suggests that killed whole-cell vaccines indeed can evoke protective immunity, provided they are delivered in a proper adjuvant/delivery system (Chambers *et al*., 2004b).

Conjugate vaccines

In 1929, Avery and Goebel showed that polysaccharides conjugated to carrier proteins could stimulate the immune system to produce specific antibodies. They demonstrated that the poor immunogenicity of purified *S. pneumoniae* type 3 polysaccharide in rabbits could be overcome by conjugating a related hapten disaccharide to a protein. These observations laid the foundation for modern human carbohydrate-protein conjugate vaccines. (Goldblatt, 2000). Later, Svenson *et al*. showed that *Salmonella O*-antigen specific oligosaccharides conjugated to proteins resulted in protective *Salmonella* vaccines (Svenson *et al*., 1979). Carbohydrate-protein conjugate vaccines have now been shown to be efficient against pneumococcal, meningococcal, and *Shigella* infections (Poolman *et al*., 1999).

The first glyco-conjugate vaccine licensed for human use was the *Haemophilus influenzae* type b (Hib) conjugate vaccine. This vaccine was approved for national childhood vaccination programs. Studies done in small children confirmed the immunogenicity of the conjugated Hib capsular polysaccharide poly-ribosylribitol phosphate (PRP). Formulations of the Hib conjugates with different protein carriers including tetanus toxoid (TT), diphtheria toxoid (DT), mutant diphtheria toxin (CRM_{197}) and an outer membrane protein preparation (OMP) were developed and all have contributed to a dramatic decrease of the incidence of Hib pediatric disease. The success of the Hib conjugate vaccines in reducing Hib disease in children has accelerated the interest in developing conjugate vaccines targeting other bacteria. So far, however, there has been no attempt to employ the concept of conjugate vaccine or to utilize mycobacterial carbohydrate antigens in new TB vaccines.

The target populations for a new TB vaccine(s)

In TB endemic countries where the TB incidence is high there is also a great variation in TB status among people. A fraction is naïve and non-infected, some already have active TB, others are latently infected with Mtb, still others are co-infected with Mtb and HIV or carry HIV that makes them more susceptible to either reactivation of latent TB or new infection. Moreover, most of these individuals have already been vaccinated with BCG. It is unlikely that a single vaccine can effectively cover all these groups (Figure 6). Therefore clinical studies of new TB vaccines must be designed to cover one or more of these different populations in a defined way, taking into account the varying immune status, latent infection and disease status.

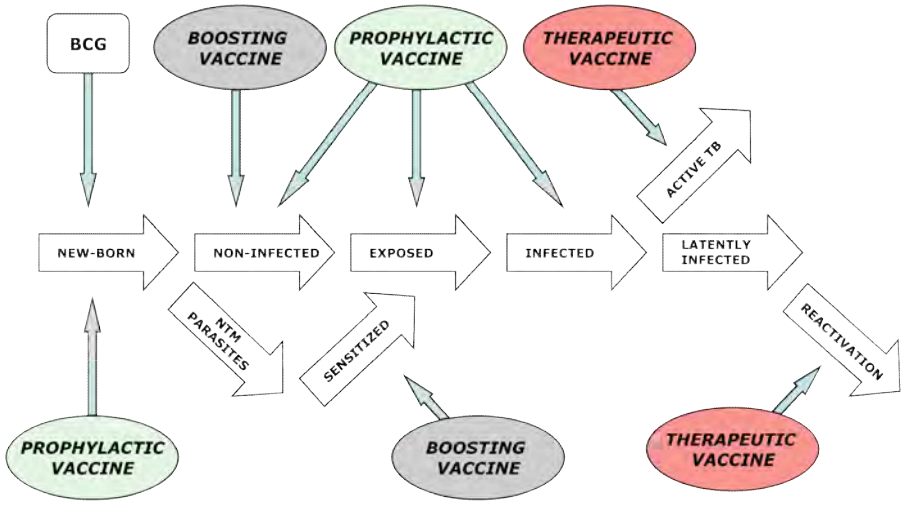


Figure 6. Vaccination strategies for TB (rationale for experimental design in the thesis)

The largest target population consists of those already primed by BCG vaccination; here the most suited vaccine candidate would be a booster vaccine which would reconstitute a balanced protective immunity engaging both the cellular and humoral arms at a systemic as well as local mucosal level.

HOW COULD WE IMPROVE WANING IMMUNITY IN BCG PRIMED ADULTS?

The priority target of TB vaccination - to boost young adults already vaccinated with BCG - calls for a new boosting type of vaccine. There is evidence that repeated BCG vaccination does not increase the protective efficacy of the primary vaccination probably due to accelerated elimination of BCG in the already BCG primed individual (Barreto *et al*., 2002).

A working hypothesis is that new vaccines, in particular those of subunit type, may prove useful in effectively boosting BCG-induced primary immune responses. Therefore, pre-clinical testings of new vaccine candidates in animal models should include BCG priming followed by boost with the new vaccine. Such prime-boost vaccination, using BCG followed by recombinant virus expressing mycobacterial antigen has been shown to induce strong cellular immune responses in mice, guinea pigs and cattle (Vordermeier *et al*., 2004). This vaccination strategy is attained in clinical trials by boosting previously BCG vaccinated individuals (McShane *et al*., 2005).

Oral and nasal vaccination

Oral and nasal vaccination have been reported to be the best ways of attaining robust immune responses in the lung, which is the site of entry of the pathogen (Doherty *et al*., 2002) (Lagranderie *et al*., 2000) (Lagranderie *et al*., 2003). The preferred route to elicit such mucosal immunity would probably be through nasal vaccination. Up to date, only few studies reporting nasal vaccination against TB have been published. However, all these studies indicate that intranasal immunisation with either live BCG (Lyadova *et al*., 2001) (Falero-Diaza *et al*., 2000), or subcomponent (Doherty *et al*., 2002) or recombinant adenovirus-based vaccines (Goonetilleke *et al*., 2003) (Wang *et al*., 2004) can provide protection against challenge with Mtb.

ADJUVANTS/DELIVERY SYSTEMS/ROUTES FOR VACCINES

Adjuvants

An adjuvant is an agent which, when administered with an antigen, enhances the immune response to that particular antigen. Nature itself provides us with a number of delivery systems in the form of diverse microbes (viruses and bacteria) with inherent adjuvant properties. These properties include ability to activate DCs through Toll- like receptors (TLRs), induction and expression of pro-inflammatory cytokines and targeting intracellular MHC processing compartments. Aluminum salts are the only adjuvants currently approved for human use. However, they are mainly driving Th2 type of responses while in protection against TB a strong involvement of Th1 response is required.

Presently, a large number of adjuvants are being evaluated in phase I and/or phase II studies in man (Moingeon *et al*., 2001). Recombinant cytokines (IL-6, IL-12) are included in adjuvant trials because they probably constitute direct proximal mediators of most if not all "classical" adjuvants (Boyaka *et al*., 1999) (Rincon *et al*., 1997).

Studies on the mechanisms of action of toxins from enteric bacteria have led to investigation of bacterial exo-toxins/toxoids as possible adjuvants for mucosal delivery. These include cholera toxin (CT) and *E. coli* heat-labile toxin (LT) (Freytag and Clements, 1999). Other examples of adjuvants being evaluated in phase I or II studies include monophosphoryl lipid A (a sub-derivative of bacterial endotoxin (LPS)), a microfluidized emulsion of oil and

surfactants that is licensed for use as part of an influenza vaccine (MF59), an oil-based emulsion containing muramyl dipeptide from mycobacterial cell wall and non-ionic block co-polymers (SAF-1), saponin derivatives (such as QS21), and polymers such as polyphosphazene (Kenney *et al*., 2002).

Delivery systems

Genetically engineered intracellular bacteria, such as *M. bovis* BCG, *Salmonella*, and *Listeria* have been evaluated as delivery systems for TB vaccines (Ohara and Yamada, 2001). (Miki *et al*., 2004) (Mollenkopf *et al*., 2001) (Hess *et al*., 1998). Viruses such as vaccinia, related to smallpox virus, are highly immunogenic and non-virulent in immuno-competent individuals and therefore have been explored as delivery system for antigens. Accordingly, replication-deficient recombinant vaccinia virus type Ankara has been tested for the efficacy as TB vaccine and was found to generate strong immune responses when compared to antigens mixed with standard adjuvants (Frota *et al*., 2004). An important weakness of such vaccines lies in the presence of neutralizing antibodies in previously exposed or vaccinated individuals. These antibodies will generally interfere with and neutralize vaccine vectors quickly before sufficient multiplication and ensuing response to the encoded Mtb antigen can be attained. Therefore, differential viral vectors in prime-boost vaccination strategies have been tested. Heterologous (different virus vaccines such as fowlpox followed by vaccinia) strategies in prime-boost vaccination have been found to enhance the immune response relative to the homologous (same vaccine) strategies (McShane *et al*., 2001).

Routes of vaccination

Like the majority of human pathogens, Mtb uses the mucosal surface to enter the body. Most often Mtb entry occurs through the upper respiratory route followed by colonization and establishment of primary infection of the lungs. It is conceivable that delivering a new TB vaccine(s) to this mucosal site would result in stimulation of local immunity and thereby hinder primary colonization with TB bacilli.

Our working hypothesis presented in this thesis is that new vaccines given nasally together with an appropriate adjuvant might bring about such a local and systemic immunity. Another advantage of mucosal vaccination is the simplicity of application. This should positively affect the compliance. The best example of the practical success of mucosally administered vaccines is the “worldwide polio vaccination”. Using the oral route millions of

people were immunized with polio vaccine in a single day (Bloom and Widdus, 1998). Such massive vaccination efforts are only possible in terms of both cost and feasibility when oral or nasal vaccines are used.

In this thesis **(papers I, II, III, IV)** a new adjuvant and delivery system based on lipids (L3-phase) that has been developed by Eurocine™ AB, was tested together with several new TB vaccines (Schroder and Svenson, 1999). This new adjuvant is appropriate for mucosal immunization, especially for intranasal immunization. In addition, it has been shown to be non-toxic, as it comprises natural oil based lipids already used in humans. This last notion is of particular practical importance, because among plethora of adjuvants studied, only one so far (aluminum), is non-toxic and certified for human use (Moingeon *et al*., 2001).

ANIMAL MODELS OF TB

The mouse model of Mtb infection is the most frequently used animal model to test new vaccine candidates. This model has several advantages; mice are inexpensive, can be effectively vaccinated with BCG and treated with ant-TB drugs, and there is also a wide variety of immuno-reagents available. As shown in all the papers which are included in this thesis, the C57BL/6, BALB/C and B6D2/F1 inbred mice are relatively resistant to Mtb infection. They can survive for more than one year after being challenged with a low dose of virulent Mtb via the aerosol or intranasal route. A chronic disease follows with extensive granuloma formation in the lungs. During the course of the disease mice generate both strong Th1 response and relatively strong DTH. The bacterial loads remain constant for about 200 days in the lungs and spleens of the infected animals. As mice grow older, the disease severity increases, paralleled by an increase in the bacterial load and progressive degeneration of infected tissues, leading to death of the animal at about 300 to 350 days post-infection (Orme, 1999).

The pathology that develops in mice following infection with tubercle bacilli is different from that encountered in humans, nevertheless due to low cost and availability of inbred and "knock out" strains, mice are commonly used for first stage-vaccine screening.

The other animal TB model that was used in this thesis **(paper I)** is the guinea pig. This animal model is more expensive but has certain advantages compared to the mouse model. Firstly, guinea pigs generate potent skin–test reactions (DTH) and secondly infected guinea pigs develop progressive lung pathology and respiratory distress similar to those observed in human TB. After ten to fifteen weeks post infection the lung granulomas in guinea pigs

become substantially necrotic and eventually cause extensive damage to the lung structure (Orme, 1999).

In a recently published study primates have been shown to develop clinical symptoms similar to human disease upon infection with Mtb (Walsh *et al*., 1996). In this study animals were challenged intra-tracheally with variable doses of a virulent Mtb strain and showed dose dependent disease manifestation. High infection doses caused an acute and rapidly progressing disease with similar pathology as that seen in humans, while low doses of infection resulted in chronic, slowly progressive form of TB.

The non-human primate species, especially Rhesus *(Macaca mulatta*), and cynomolgus species *(Macaca fasicularis*) are susceptible to TB. When the efficacy of BCG vaccination was evaluated in both species only the *Macaca fasicularis* primates were protected (Good R.C., 1984, The Mycobacteria: A Sourcebook, 903-923). Even though the *Macaca mulatta* in this study showed less protection by BCG vaccination, they can still be a valuable model for studies addressing why today large numbers of human populations show no protection with BCG.

CLINICAL TRIALS OF NEW TB VACCINES

Once the immunogenicity, protective efficacy and safety of the new vaccine candidates have been assessed in pre-clinical studies, the facilities must be found which will, under Good Manufacturing Practice (GMP) procedures, prepare vaccine batches for clinical phase I, II, and ultimately phase III trials. For the clinical trials to commence a number of fundamental issues must be addressed, such as an identification of the trial site (geographical regions and specific populations including prevalence of latent infection and active TB), development of standardized protocols, ethical issues and potency measures as well as specific endpoints measuring vaccine efficacy. The trial sites must include necessary infrastructure in the form of on-site laboratories capable of performing simple diagnostic tests and immunological analyses. To meet these challenges, public and private partnerships (PPP) have merged to evaluate the most promising vaccine candidates. For example, the Aeras Global TB Vaccine Foundation, European & Developing Countries Clinical Trials Partnership (EDCTP) and the National Institutes of Health (NIH), in combination with pharmaceutical companies, are together supporting some of the safety studies of the promising vaccine candidates (Rowland *et al*., 2005) (Izzo *et al*., 2005).

AIMS OF THIS STUDY

The specific aims of the present studies were:

1. To evaluate prophylactic efficacy of a new vaccine based on mycobacterial arabinomannan (AM) conjugated to a carrier protein tetanus toxoid (AM-TT) formulated in Eurocine™ L3 adjuvant for mucosal administration **(paper I)**

2. To evaluate prophylactic efficacy of a new vaccine comprising heat killed BCG (H-kBCG) formulated in the L3 adjuvant **(paper II)**

3. To evaluate several new vaccine candidates for their therapeutic efficacy in animals already infected with Mtb **(paper III)**

4. To evaluate the ability of AM-TT and HK-BCG vaccine candidates to boost the immunity in mice which were primed at young age with BCG **(paper IV)**

5. To evaluate the role of antibodies in protection against TB, using monoclonal antibodies with specificity to AM epitopes of LAM in passive protection studies **(paper V)**

MATERIALS AND METHODS

CONSTRUCTION AND FORMULATION OF VACCINE CANDIDATES

Preparation of LAM specific oligosaccharides

LAM from *M. tuberculosis* H37Rv and LAM-derived oligosaccharide fragments were prepared as previously described (Hamasur *et al*., 1999a). LAM was delipidated and the polysaccharide portion of delipidated LAM was fragmented to oligosaccharides by weak acidic hydrolysis after partial periodate oxidation. The reaction mixture was fractionated by gel exclusion filtration on a Sephacryl S-100 column. Carbohydrate and reducing terminal contents were measured by the phenol sulphuric acid method (Dubois M, 1956) (Park JT, 1949) respectively.

Preparation of Mtb culture filtrate proteins

The clinical isolates Mtb strain Harlingen and Mtb H37Rv were grown in Proskauer-Beck medium for six weeks at 37 °C. Thereafter bacterial growth was terminated by centrifugation of the culture at 3000 g for 20 min. The culture supernatant containing secreted proteins was sterilized by filtration and concentrated. Two proteins, Ag85B and 75 kDa protein were identified in the culture supernatant. After separation and chromatographic purifications, the proteins were concentrated, desalted and their identity verified using Western blot.

Preparation of AM oligosacaccharide-protein conjugates

AM oligosaccharides (AM) of average molecular weight of 28 kDa were pooled, concentrated and coupled to TT or Mtb proteins via thioether linkages as previously described (Hamasur *et al*., 1999b) using the conjugation method described by Pawlowski *et al* (Pawlowski *et al*., 1999).

Preparation of L3 adjuvant

The basic constituents of the L3 adjuvant were monooleate and oleic acid. Two formulations of adjuvants were used, without soy bean oil (suspension) and with soy bean oil (emulsion). For vaccine formulation in L3 suspension, the appropriate antigen was admixed with the adjuvant. Vaccine formulation in L3 emulsion comprised short sonication of the antigen and adjuvant.

Preparation of anti-LAM MoAbs

The antigen for immunizations comprised purified LAM from *M. tuberculosis* H37Rv mixed with heat-killed *M. tuberculosis* H37Rv bacteria or AM-protein conjugates. Mice (female NMRI) were injected intra-peritoneally with this mixture emulsified in complete Freund´s adjuvant at a ratio of 1:1. The mice were then boosted twice, with 2 weeks interval. Three days prior to spleen cells harvest for hybridoma production, the mice were given the antigen intravenously without adjuvant. Spleen cells were fused with SP2/AG64G14 myeloma cells according to the method of Köhler and Milstein (Kohler and Milstein, 1992). The hybridoma cell lines that secreted, LAM-specific antibodies were identified by LAM

ELISA. The MoAbs were purified, from the culture supernatants by affinity chromatography over protein G- Sepharose. Isotype identification of the MoAbs were done by ELISA using LAM as coating antigen. $F(ab')_2$ fragments from MoAb were prepared by pepsin treatment using standard methods.

ANIMALS AND IMMUNIZATION PROTOCOLS

Eight to ten week old female C57/BL, BALB/C and B6D2/F1 mice, female guinea pigs (250-300 g) and New Zealand female rabbits (2.5-3 kg) were used in experiments. Immunization of mice was either subcutaneously in the flanks with the vaccine in 100 □*l*, or intranasally with the vaccine in 10 □ *l* (5 *µl* into each nostril). BCG vaccination was performed by subcutaneous injection of $5x10^5$ CFU in 100 *µl* PBS in the hind flank.

Rabbits were immunized with: i) LAM, ii) AM-TT conjugate, iii) AM-Ag85B conjugate, iv) AM-75 kDa protein conjugate. The animals received 50 *µg* of LAM or 20 *µg* (on carbohydrate basis) of conjugate in 100 *µl* PBS emulsified with 100 *µl* FIA and injected intramuscularly. Booster immunizations with the same dose of LAM or conjugate were given two and four weeks after primary immunization. Blood samples were drawn at day 0 and 48, and serum antibody titers were evaluated by ELISA.

Guinea-pigs were immunized subcutaneously on day 0 and boosted nasally on day 24 with AM-TT conjugate in L3 emulsion using varying doses of antigen and different concentrations of L3. For protection studies guinea-pigs were immunized with 15 *µg* of AM-Ag85B (per carbohydrate basis) in 10% L3 emulsion using the same regimen. BCG vaccination was performed by subcutaneous injection of $5x10^4$ CFU in 100 *µl* PBS in the nape. Blood samples were drawn from test animals on day 0, 14, and 38, and serum antibody levels were determined by ELISA method.

INFECTION OF MICE AND GUINEA PIGS

The C57/BL and BALB/C mice were infected by triple nasal inoculation of total 10^5 Mtb Harlingen cells in 30 □l of PBS containing 0.5% Pluronics detergent **(papers I-V)**.

Intranasal challenge of guinea-pigs was performed by instillation into the nostrils of $5x10^4$ *M. tuberculosis* Harlingen cells in 2x25 □l of PBS containing 0,5% Pluronics detergent **(paper I)**.

Using an infection model mimicking the human pulmonary disease, where the bacteria are inhaled into the lung and cause disease via the natural aerogenic way have several advantages. To this end an aerosol generating system is used for infecting mice and guinea pigs with low dose of Mtb. In this thesis the aerosol infection of the B6D2/F1 mice **(paper III)** was carried out at the Rockefeller University in Gilla Kaplan´s laboratory utilizing the Nose-Only aerosol system (In-Tox Products, Albuquerque, NM) according to the protocol developed there (Tsenova *et al*., 1997). This method resulted in implantation of approximately 100 organisms into the lungs of mice. Guinea pig aerosol infections **(paper I)** were performed at CAMR, United Kingdom.

SURVIVAL EXPERIMENTS

The body weights of vaccinated and infected animals were monitored weekly **(papers I-V)**. Mice with overt symptoms of the disease and significant weight loss were killed by cervical dislocation. The last body weight was recorded and included in subsequent mean weight of respective group of mice. Survival of the mice was followed daily until all animals in the control group (unvaccinated but challenged) were dead **(papers I-V)**.

CFU COUNTS OF LUNGS AND SPLEENS

The tissues (both lung and spleen) were homogenised in sterile deionised water using a rotating blade macerator system. Viable counts were performed on serial dilutions of the homogenate, plated onto Middlebrook 7H11 selective agar and examined after 3 weeks for growth of Mtb **(papers I-V)**.

HISTOPATHOLOGY OF LUNGS AND SPLEENS OF GUINEA PIGS

Blinded semi-quantitative histological analysis of lungs and spleens of all animals was performed **(papers I, III and IV)**. In all animals 3 lung lobes per animal were examined. Features analyzed were granulomas (percent consolidation, caseation, calcification (number of foci), encapsulation and lymphocytes (subjective score). Spleen lesion severity of the vaccinated animals were estimated by the number, size, caseation, calcification, haemorrhage and necrosis of the granuloma in the organ.

IMMUNOGENICITY OF VACCINE CANDIDATES

Rabbit and guinea-pig serum antibodies against the AM portion of the AM-protein conjugates and against LAM were measured using 96-well ELISA plates coated with LAM. Antibodies against the protein moiety of the respective conjugates were measured using microtiter plates coated with protein.

In the experiments exploring the prophylactic mode of vaccination **(papers I and II)**, proliferation of T-cells from spleen cells from immunized mice was studied 2 weeks after subcutaneous priming and 3 weeks after nasal booster. Three different AM protein conjugates (AM-Ag85B, AM-TT, and AM-75 kDa) in two different doses (0.2, and 1.5 □*g*) were used for vaccination. Mouse spleen cells were stimulated with PPD and H37Rv extract *in vitro*, and incorporation of ^{3}(H) thymidine was measured by □-scintillation counting.

In the experiments using therapeutic mode of vaccination **(paper III)**, cytokine mRNA levels in the infected lung of the vaccinated mice were analyzed. Proliferation of spleen and draining lymph node T cells derived from vaccinated mice were analyzed for the incorporation of ^{3}(H) thymidine after *in vitro* stimulation with *M. tuberculosis* H37Ra.

STATISTICS

Data were analyzed using the unpaired two-sided Student´s *t*-test for spleen CFU and comparison of mean weights and by Kaplan-Meier survival plots followed by the log-rank (Mantel Haenszel) test **(papers I, II, III, IV and V)**, chi-square test (at the end of the experiment) for comparison of survival curves **(paper I)**.

In the guinea pig protection experiment the median values of viable Mtb in the vaccine and control groups were compared by Mann Whitney U-test **(paper I)**.

Pathology scores were analyzed using non-parametric ANOVA (Kruskal-Wallis Test) with Dunn´s Multiple Comparison post-test **(paper IV)**.

RESULTS AND DISCUSSION

PAPER I. *Mycobacterium tuberculosis* arabinomannan-protein conjugates protect against tuberculosis

Mycobacterial lipoglycans such as LAM have been reported to induce CD1 restricted T-cell responses that can promote the clearance of Mtb (Porcelli *et al*., 1992). On the other hand LAM in its native form has also been reported to exert several immunosuppressive functions such as down-regulation of T-cell proliferation (Kaplan *et al*., 1987) (Moreno *et al*., 1988) (Molloy *et al*., 1990) and inhibition of IFN □ mediated activation of macrophages (Chan *et al*., 1991) (Sibley *et al*., 1990).

In the present studies, in order to avoid the immunosuppressive effect of the whole LAM molecule and to induce immune response against the carbohydrate portion of LAM, the lipid was removed by treating the molecule by mild alkaline hydrolysis. Purified arabinomannan (AM) derived oligosaccharides were obtained by weak acidic hydrolysis of partially oxidized AM and were covalently linked to different purified *M. tuberculosis* proteins (Ag85B, 75kDa), or tetanus toxoid (TT). The resulting conjugates were tested as prophylactic TB vaccines. The capability of AM oligosaccharide-carrier protein conjugates to elicit cell mediated, and humoral responses as well as their protective efficacy against *M. tuberculosis* infection in mice and guinea pigs were analyzed.

Immunization of rabbits with purified LAM did not induce any LAM specific IgG antibodies, while all arabinomannanprotein conjugates induced high AM-specific serum IgG antibodies. A dose dependent serum specific IgG response was also seen with AM-TT conjugate (1, 5, 15 μg in 10% L3 adjuvant) in guinea-pigs.

Increased proliferation of spleen cells from mice immunized with this vaccine was obtained following stimulation with PPD in vitro. The mice immunized with AM-TT conjugate vaccine in the presence of L3 adjuvant survived longer than controls and as long as BCG vaccinated groups, when challenged with a virulent *M. tuberculosis* strain. In another experiment in which mice were immunized with AM-Ag85B conjugate in 1% alum some increase in survival was seen when compared to non-vaccinated animals, however the protective effect was less pronounced than that obtained with AM-protein conjugate in L3 adjuvant.

As the present data show, vaccination with a carbohydrate-protein conjugate vaccine administered in an appropriate adjuvant formulation (L3) confers significant protection

against infection with Mtb. The AM-TT conjugate vaccine in L3 adjuvant administered in s.c. priming-i.n. boost regime, protected the mice to the same level as BCG. Additionally, when guinea-pigs were immunized with varying amounts of AM-TT conjugate, a dose dependent IgG response was seen to both the AM and the protein moiety of the conjugate. In general these conjugate vaccines showed good protective efficacy in mice and guinea pigs in terms of prolonged survival and reduced pathology. We hypothesize that the protective immunity afforded by the AM-protein conjugate vaccine could at least partly rely on T helper cell-dependent B-cell activation and induction of AM-specific antibodies which interefere with the immunologically detrimental effects that LAM from the cell wall of Mtb exerts during infection.

PAPER II. **Immunization with heat-killed *Mycobacterium bovis* bacilli Calmette-Guerin (BCG) in Eurocine™ L3 adjuvant protects against tuberculosis**

The development of a new vaccine for human use from laboratory bench to a final GMP product takes about 20 years. Great part of this delay is due to a long and tedious regulatory process required to procure safety approval for new technologies and materials to be used in humans. Today, there is no room for waiting 20 years for a new TB vaccine.

Having that in mind we asked if it would be possible to construct an improved vaccine based exclusively on existing and approved materials in order to facilitate the safety approval and thereby cut the development time. Accordingly, the vaccine candidate studied here consisted of conventional BCG Copenhagen reformulated by gentle heat-inactivation followed by mixing with the L3 adjuvant that recently has been approved for vaccinating humans in Sweden. Besides simplicity and safety this heat-killed (H-kBCG) vaccine would have several other desirable features. Firstly, unlike subunit vaccines that display a single antigen, the whole bacteria in H-kBCG vaccine would present broad spectrum of antigens that might be relevant in stimulating a strong immune response. Secondly, being heat-inactivated, the H-kBCG vaccine would be safe in immuno-compromised individuals such as HIV infected people in whom conventional live BCG may cause disseminated infections. Thirdly, the H-kBCG in the L3 adjuvant would be suitable for mucosal application.

The non-adjuvanted H-kBCG did not give any protection when compared to sham-vaccinated controls. However, the same vaccine given together with two different formulations (emulsion and suspension) of L3 adjuvant afforded significant levels of

protection. The level of protection was at least as good as that of the live attenuated BCG vaccine.

All H-kBCG formulations in L3 adjuvant elicited mycobacterial antigen-specific serum IgG and IFN-γ responses. In general, among the different vaccine formulation(s) those that produced a relatively high Th2 response, as measured by IgG1/IgG2a ratio and IFN-γ production in vitro, were the most protective.

Overall, this study shows that non-live whole cell vaccine can efficiently protect against TB in experimental system, providing the appropriate adjuvant is used. Recently another group reported that formalin-killed BCG in another type of lipid adjuvant was protective in guinea pigs (Chamber MA *et al*, 2004). Of note, when a number of novel adjuvants were explored in combination with subunit vaccines, the best adjuvant among those tested was the classic incomplete Freund's adjuvant (FIA) which consists of mineral oil. This adjuvant, unlike the L3 used in this study is, however highly toxic and therefore not approved for humans.

PAPER III. **Mycobacterial antigens exacerbate disease manifestation in *Mycobacterium tuberculosis*-infected mice**

Vaccination of adults already having active TB disease i.e. therapeutic vaccination could today possibly be a complimentary therapy to antimicrobial therapy. This strategy was first tested by Robert Koch when he used the so-called “old tuberculin” in 1890. Unfortunately, the tuberculin immunotherapy caused strong systemic reaction in patients with relatively mild disease symptoms. Among patients with advanced cavitary disease almost 10% died as a result of the treatment.

To re-evaluate the therapeutic vaccination, B6D 2F1 mice were infected through the aerosol route with a very low dose of a virulent Mtb strain and five weeks later the infected mice were treated with various vaccine candidates. The first immunization started 5 weeks after low dose challenge and booster vaccine was given 3 weeks later. The course of the infection was followed by enumeration of bacilli in infected tissues, histological examination, quantitation of cytokine mRNA induction and peripheral T cell proliferation in response to mycobacterial antigens.

The vaccine candidates tested were: i) heat killed BCG in L3 adjuvant formulation, ii) recombinant BCG secreting murine cytokines such as TNF-α, IFN-γ and IL-2 and iii) mixture of dead and live bacteria strain Erdman. These therapeutic vaccine candidates given post

infection induced antigen-specific T-cell proliferation but did not decrease the bacterial load in the lungs or the granuloma size. In fact, some of the treatments induced slight exacerbation of pathology (increased granuloma size) associated with the enhanced production of TNF-α. These results show how difficult it is to change the course of the disease once the infection has been established in the lung. Similar discouraging results with immunotherapy were obtained by others, using subunit vaccines, such as culture filtrate proteins in combination with an adjuvant or a DNA vaccine comprising the gene for *M. tuberculosis* Ag85 (Turner *et al*., 2000). So far only immunotherapeutic vaccination with *M. leprae* Hsp65 resulted in some decrease of bacterial load in the lungs of infected mice (Lowrie *et al*., 1999). It is, however, worth noting that this vaccine was efficient only when the animals were pre-treated with anti-TB drugs before vaccination. In such setting, the treatment with antibiotic followed by repeated post-exposure vaccination, gave good protection against reactivation of disease (Lowrie *et al*., 1999). Accordingly, in a recent study by another group, the concurrent immunotherapy with plasmid DNA encoding mycobacterial HSP65 and chemotherapy could shorten treatment duration of MDR-TB (Silva *et al*., 2005).

The inability of the tested vaccine candidates, which were effective as pre-exposure vaccines, to reduce pre-existing infection is clearly associated with increased immune activation. This indicates that developing a therapeutic vaccine for already exposed individuals should be done with great caution.

PAPER IV. **Nasal boost with adjuvanted heat-killed BCG or arabinomannan-protein conjugate improves primary BCG-induced protection in C57/BL/6 mice**

Today, almost 80% of the world population is already vaccinated with BCG. The BCG vaccine protects against childhood TB but this immunity wanes with age, resulting in insufficient protection against adult pulmonary TB. Hence, the simplest strategy to improve the protective efficacy of the BCG vaccine would be to boost in adulthood, but unfortunately repeated vaccination with BCG is proven to be ineffective (Barreto *et al*., 2002) (Fine, 1996)

In this study, using the mouse model, we evaluated the ability of two new TB vaccine candidates, the H-kBCG and AM-TT, given intranasally in L3 adjuvant, to boost a primary BCG-induced immune response and to improve protection. Young C57BL/6 mice were vaccinated with conventional BCG and, 6 months later, boosted intranasally with adjuvanted H-kBCG or AM-TT, or subcutaneously with live BCG. Ten weeks after the booster, mice were challenged intravenously with *M. tuberculosis* strain H37Rv. In spleens, there was a

significant reduction of CFU counts in mice boosted with either H-kBCG or AM-TT vaccines compared to the non-boosted BCG vaccinated mice. None of the boosting regimens significantly reduced bacterial loads in lungs, compared to non-boosted BCG vaccination. However, the extent of granulomatous inflammation was significantly reduced in the lungs of mice that received two of the booster vaccines (AM-TT and conventional BCG), as compared with sham-vaccinated mice. All boosted groups, except for mice boosted with the AM-TT vaccine, responded with a proliferation of spleen T cells and IFN-γ production comparable to that induced by a single BCG vaccination. In a recent publication by others with a similar approach involving boosting BCG with a heterologous vaccine using both DNA vaccine and protein vaccines in suitable adjuvants improved the protective efficacy of BCG against challenge with *M.bovis* in a cattle model (Buddle *et al*., 2005).

PAPER V. **A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab´)$_2$ fragment prolong survival of mice infected with *Mycobacterium tuberculosis***

The antibody responses induced by different bacterial carbohydrates have been shown previously to play a vital role in protection against many infectious diseases. In active TB, high antibody titres against mycobacterial lipoarabinomannan (LAM) are often seen. The role of such LAM-specific antibodies in the immune response against TB is unknown. In this study a monoclonal antibody to mycobacterial LAM and its F(ab´)$_2$ fragment were evaluated for their capability to protect against *M. tuberculosis* infection.

The monoclonal antibody (MoAB) SMITB14 used in this study was shown by immunofluorescence to bind to whole cells of the clinical isolate *M. tuberculosis* strain Harlingen as well as to *M. tuberculosis* H37Rv. The binding of MoAB SMITB14 to LAM was inhibited by arabinomannan (AM) and oligosaccharides (5.2 kDa) derived from AM, showing that this MoAB binds specifically to the AM carbohydrate portion of LAM. In passive protection experiments, mice were infected intravenously with *M. tuberculosis* Harlingen. MoAb SMITB14 was added intravenously either prior to, or together with, the bacteria. The antibody proved to be protective against the *M. tuberculosis* infection in terms of a dose-dependent reduction in bacterial load in spleens and lungs, reduced weight loss and, most importantly, increased long-term survival.

Mtb is capable of inducing a vast array of antibodies of different specificities and isotypes - the characteristics known to decide which role a particular antibody plays in

immune responses. Among those antibodies probably only a fraction is truly protective. This has resulted in contradictory data from studies exploring the function of antibody in TB, ranging from having no effect at all, to being able to prevent bacillary dissemination, to prolonging survival of infected animals (Glatman-Freedman and Casadevall, 1998) (Pethe *et al*., 2001) (Teitelbaum *et al*., 1998).

Data agreeing with our findings has recently also been published by others. Teitelbaum *et al*. showed that a MoAB specific to LAM administered prior to intratracheal Mtb infection prolonged the survival of TB infected animals. By contrast to our findings, they did not however find any reduction in bacterial loads (Teitelbaum *et al*., 1998). Williams *et al*. found that a MoAB directed against α-crystalin significantly reduced the CFU in the lungs of infected animals (Williams *et al*., 2004) and Chambers *et al*. found that a MoAb directed against the MPB83 surface antigen of M.bovis increased survival of mice (Chambers *et al*., 2004b). Moreover, in a recent study IgA deficient mice ($IgA^{-/-}$) showed increased susceptibility to BCG infection which was paralleled by a reduction in lung IFN-γ and TNF-α production, suggesting that IgA antibodies may have a regulatory role also in the cell-mediated responses (Rodriguez *et al*., 2005).

Considering the data in **paper V** and the data of Teitelbaum's et. al it can be speculated that one possible mechanism through which AM/LAM-specific antibodies may act is scavenging of soluble/released LAM and hence interfering with its potent immuno-regulating activities (i.e. effects on cytokine levels/regulation, effects on cells of the T cell and macrophage cell lineages, etc). In addition AM/LAM-specific antibodies may interfere with the homing of tubercle bacilli to different tissues or with interaction of bacilli with dendritic-/monocyte/macrophage cells. One possible mechanism for the infection-ameliorating effect of the antibodies could be to interfere with the receptor-mediated phagocytosis and thereby to change the pathway by which the bacilli reach the intracellular milieu of the macrophage. The differential Mtb entry pathways were suggested to play an important role in the outcome of disease (Caron and Hall, 1998) (Schlesinger *et al*., 1990).

The protective effect of the passively transferred AM-specific antibodies indicates that antibody-mediated protection should be considered as at least one of the possible mechanisms contributing to protection seen with the novel TB vaccine candidates.

GENERAL SUMMARY

Research on future new TB vaccines should not only concentrate on testing the vaccines for their ability to protect naïve, healthy individuals, but should also take in consideration the large number of individuals (80%) of the world´s population who are already BCG vaccinated in childhood and who need a new booster vaccine to avoid adult PTB. Additionally, in TB endemic countries there is also a need of a new effective therapeutic vaccine which along with implementation of DOTS programs can aid in controlling the spread of the disease.

PROPHYLACTIC VACCINES

In humans, TB is most often caused by inhalation of the pathogen leading to the establishment of infection in the lung. Immunizing through the nasal mucosal surface should therefore have advantage over that using other routes of immunization, as it will elicit protective immunity in the lung i.e. at the site of infection. In this thesis several new vaccine candidates were evaluated for their protective efficacy in animal models using the mucosal route of immunization. In formulating such vaccines, the adjuvants and delivery systems are crucially important. In the studies included here, the L3 adjuvant was used which is non-toxic and could be directly applied to the mucosal surface of the nostril of the tested animals. Recently, the L3 adjuvant was approved for human use as a component of nasal diphtheria vaccine after a successful phase I clinical trial. Two new vaccine candidates, the AM-Ag85B conjugate-vaccine and the H-kBCG formulated in L3 adjuvant were tested in a prophylactic mode of vaccination in conjunction with L3 adjuvant **(papers I and II)**. Both vaccines afforded good protection approaching that seen with the conventional BCG vaccine.

BOOSTER VACCINES

Despite the wide coverage of the BCG vaccine, the protective efficacy of the BCG shows great variations between study populations. One important observation that is shared by most of the efficacy studies, and today generally accepted as valid is that BCG vaccination protects against childhood forms of TB, but this immunity wanes with age, resulting in none, or insufficient, protection against adult pulmonary TB. Therefore, a booster vaccination of

people already vaccinated with BCG might restore immunity and prevent reactivation of disease.

Based on this assumption a study was performed where the vaccine candidates that earlier proved effective on their own **(papers I and II)**, were tested for the ability to boost earlier BCG vaccination **(paper IV)**. In this model, animals were first vaccinated at young age with live BCG vaccine and boosted later with the AM-TT and H-kBCG vaccines in the L3 adjuvant. The groups which were boosted with the two vaccine candidates, when compared to the non-boosted group which received BCG prime showed improved protection in terms of reduced bacterial load in the spleens and the AM-TT vaccine improved the lung pathology in terms of reduced granulomatous inflammation.

The results obtained with the AM-TT conjugate vaccine aid to the growing evidence that it is possible to boost primary BCG vaccination using a heterologous prime/boost strategy this time using carbohydrate antigen from Mtb. The fact that non-live BCG was effective as booster further underscores **(paper II)** the importance of the proper adjuvant in TB vaccination. We conclude that both new TB vaccine candidates may prove useful for boost vaccination in man.

THERAPEUTIC VACCINES

In **paper III** several vaccines were tested for their therapeutic effect in mice pre-infected with a low dose of a virulent strain of Mtb. In this study animals were vaccinated with BCG, H-kBCG in L3, BCG plus other antigens, recombinant BCG secreting murine cytokines, and heat-killed Mtb; none of these treatments reduced the CFU in the lungs of the infected mice.

Characteristically, the same cytokine-secreting BCG strains and H-kBCG afforded good protection when used as pre-exposure vaccines. Similar discouraging results with immunotherapy were obtained by others using different subunit vaccines.

In summary the inability of the present vaccine candidates, which have been effective as pre-exposure vaccines, to reduce pre-existing infection is clearly associated with increased and detrimental immune activation. This indicates that developing a therapeutic vaccine for already diseased individuals will be difficult if the vaccine is to be used alone. However, as indicated by studies of others such a vaccine might be valuable as a possible complement to aggressive DOTS chemotherapy. Therefore the new vaccine candidates, Hk-BCG and AM-

TT in L3 adjuvant studied here, should be further evaluated in the therapeutic mode of vaccination in conjunction with chemotherapy.

PUTATIVE ROLE OF ANTIBODIES IN VACCINE INDUCED IMMUNITY

TB infected individuals usually exhibit strong antibody responses, but the possible contribution of antibody mediated immunity to protection against TB still remains controversial.

In this thesis **(paper V)** a MoAb directed against the AM part of LAM was studied for its protective efficacy in the murine TB model. Upon passive administration of this monoclonal antibody significant protection was seen (prolonged survival, reduced bacterial load, and reduced weight loss) in mice infected with virulent Mtb. Data agreeing with this finding have recently also been published by others.

Our data considered in conjuction with data from others strongly indicate that at least some antibodies are beneficial for protection. It can be speculated that (dependent on antigen specificity and isotype) some antibodies might be beneficial, some neutral while others might be even detrimental to the host. The question whether the overall humoral immune response induced in vaccinated, TB exposed, latently infected or actively diseased individuals is beneficial remains to be further investigated and much more extensive research on the role of the humoral immune arm in TB is needed to address this issue.

CONCLUSIONS

From the investigations presented in this thesis it can be concluded that:

i) AM-protein conjugate vaccines can, when formulated with the L3 adjuvant and administered nasally, provide at least as good protection as live BCG.

ii) The protection is achieved also when Mtb-irrelevant carrier protein is used (ie. tetanus toxoid) showing that immunity to antigen epitopes contained in the AM portion of the LAM molecule alone is protective. This proves that AM epitopes should be considered important as part in a future new sub-component vaccine.

iii) The AM oligosaccharide protein conjugate vaccine affords protection both when used as a primary vaccine and as a boost vaccine (after primary BCG vaccination)

iv) The protective effect of passively transferred AM-specific MoAb shows that antibodies alone may confer protection, most likely helping the animal to limit the early infection and or aid in the mounting of a protective cellular immune response.

v) A heat-killed BCG vaccine did not protect when administered alone but was protective when given in the L3 adjuvant. The preclinical studies of this vaccine candidate are concluded and the vaccine is considered ready for phase I/II trials in man.

vi) None of the vaccines tested in these studies (AM-protein conjugate, H-kBCG, or live recombinant BCG/murine-TNF-□, IL-2 and IFN-□vaccines) ameliorated the course of experimental TB in pre-infected animals. In fact the effects of these vaccines were rather negative in therapeutic settings. Hence, great caution is advised in development of therapeutic vaccines and, furthermore, in the design of phase I-III trials great care must be taken not to include individuals with active TB disease. If so, aggressive implementation of DOTS therapy must be adjunct.

ACKNOWLEDGEMENTS

I would like to express my gratitude to people who have contributed to this thesis. I would like to thank

At the **Swedish Institute for Infectious Disease Control**

Andrzej Pawlowski my supervisor who, from the first day of your involvment in this project, invested all your time and genuine effort until the day the thesis work became a reality. Thank you again for all your excellent scientific guidance, constant support and encouragment!!

Gunilla Källenius and **Stefan Svenson** my assistant supervisors for introducing me to the world of mycobacteriolgy, for giving me endless support and motivation and all the kindness I have received from you in the past years.

Reneé Norberg former head of our department, for being my mentor, for your good wishing and encouragement. I want to thank you for giving me the courage to take further step and finalize this thesis.

Ulf Schröder for sharing your knowledge in the field of vaccinology, adjuvants and delivery systems and for all other help.

Beston Hamasur for all help during the years, for introducing me to the chemistry of carbohydrates, on how to purify mycobacterial antigens and its applications.

Doleres Gavier-Widen and **Mark Chambers** for good collaboration and contributions to the paper included in this thesis.

Emma Huitric for your efforts in reading my thesis critically.

To all dear **colleagues at TB lab** especially **Sven**, **Tuija**, **Lisbeth, Solomon, Ramona, Juan Carlos, Pontus, Jim, Nancy, Terese, and Begoña.**

To all **EMB** colleagues especially **Mats, Christel, Monica, Sara, Gunilla**, and **Ingegard** and all others.

At **Public Health Research Institute**

Gilla Kaplan for the fruitful time I had in your lab. For introducing me to the immunology of TB and for all your kindness and hospitality I received while I stayed in New York.

Liana and **Sherry** for not only being nice friends, but also being good teachers during my stay in your lab. I want also to thank all members of the lab for your help and kindness!

To all members of my family, for your encouragements and support during the past years.

Last but not least to my beloved wife **Asmeret**, for all love and encouragements, for my children **Miriam**, **Lidia** and **Aman**, for all patience and understanding especially when I am not with you at home!!

REFERENCES

Aldwell, F. E., Keen, D. L., Parlane, N. A., Skinner, M. A., de Lisle, G. W., & Buddle, B. M. (2003). Oral vaccination with Mycobacterium bovis BCG in a lipid formulation induces resistance to pulmonary tuberculosis in brushtail possums. *Vaccine*, *22(1)*, 70-6.

Aronson, N. E., Santosham, M., Comstock, G. W., Howard, R. S., Moulton, L. H., Rhoades, E. R., *et al*. (2004). Long-term efficacy of BCG vaccine in American Indians and Alaska Natives: A 60-year follow-up study. *Jama*, *291(17)*, 2086-91.

Aslanzadeh, J., de la Viuda, M., Fille, M., Smith, W. B., & Namdari, H. (1998). Comparison of culture and acid-fast bacilli stain to PCR for detection of Mycobacterium tuberculosis in clinical samples. *Mol Cell Probes*, *12(4)*, 207-11.

Ayvazian, L. F. (1993). History of tuberculosis;. *Tuberculosis. New York, Dekker*,

Baily, G. V. (1980). Tuberculosis prevention Trial, Madras. *Indian J Med Res*, *72 Suppl*, 1-74.

Barnes, P. F., Chatterjee, D., Abrams, J. S., Lu, S., Wang, E., Yamamura, M., *et al*. (1992). Cytokine production induced by Mycobacterium tuberculosis lipoarabinomannan. Relationship to chemical structure. *J Immunol*, *149(2)*, 541-47.

Barreto, M. L., Rodrigues, L. C., Cunha, S. S., Pereira, S., Hijjar, M. A., Ichihara, M. Y., *et al*. (2002). Design of the Brazilian BCG-REVAC trial against tuberculosis: a large, simple randomized community trial to evaluate the impact on tuberculosis of BCG revaccination at school age. *Control Clin Trials*, *23(5)*, 540-3.

Beavis, K. G., Lichty, M. B., Jungkind, D. L., & Giger, O. (1995). Evaluation of Amplicor PCR for direct detection of Mycobacterium tuberculosis from sputum specimens. *J Clin Microbiol*, *33(10)*, 2582-6.

Behr, M. A., & Small, P. M. (1999). A historical and molecular phylogeny of BCG strains. *Vaccine*, *17(7-8)*, 915-22.

Behr, M. A., Wilson, M. A., Gill, W. P., Salamon, H., Schoolnik, G. K., Rane, S., *et al*. (1999). Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science*, *284(5419)*, 1520-23.

Bloom, B. R., & Widdus, R. (1998). Vaccine visions and their global impact. *Nat Med*, *4(5 Suppl)*, 480-84.

Bosio, C. M., Gardner, D., & Elkins, K. L. (2000). Infection of B cell-deficient mice with CDC 1551, a clinical isolate of Mycobacterium tuberculosis: delay in dissemination and development of lung pathology. *J Immunol*, *164(12)*, 6417-25.

Boyaka, P. N., Marinaro, M., Jackson, R. J., Menon, S., Kiyono, H., Jirillo, E., *et al*. (1999). IL-12 is an effective adjuvant for induction of mucosal immunity. *J Immunol*, *162(1)*, 122-28.

Brandt, L., Elhay, M., Rosenkrands, I., Lindblad, E. B., & Andersen, P. (2000). ESAT-6 subunit vaccination against Mycobacterium tuberculosis. *Infect Immun*, *68(2)*, 791-95.

Brandt, L., Feino Cunha, J., Weinreich Olsen, A., Chilima, B., Hirsch, P., Appelberg, R., *et al*. (2002). Failure of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect Immun*, *70(2)*, 672-8.

Brennan, P. J. (2003). Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. *Tuberculosis (Edinb)*, *83(1-3)*, 91-97.

Buddle, B. M., Wards, B. J., Aldwell, F. E., Collins, D. M., & de Lisle, G. W. (2002). Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine*, *20(7-8)*, 1126-33.

Buddle, B. M., Skinner, M. A., Wedlock, D. N., de Lisle, G. W., Vordermeier, H. M., & Glyn Hewinson, R. (2005). Cattle as a model for development of vaccines against human tuberculosis. *Tuberculosis (Edinb)*, *85(1-2)*, 19-24.

Caron, E., & Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science*, *282(5394)*, 1717-21.

.

Chambers, M. A., Gavier-Widen, D., & Hewinson, R. G. (2004a). Antibody bound to the surface antigen MPB83 of Mycobacterium bovis enhances survival against high dose and low dose challenge. *FEMS Immunol Med Microbiol*, *41(2)*, 93-100.

Chambers, M. A., Wright, D. C., Brisker, J., Williams, A., Hatch, G., Gavier-Widen, D., *et al*. (2004b). A single dose of killed Mycobacterium bovis BCG in a novel class of adjuvant (Novasome) protects guinea pigs from lethal tuberculosis. *Vaccine*, *22(8)*, 1063-71.

Chan, J., & Flynn, J. (2004). The immunological aspects of latency in tuberculosis. *Clin Immunol*, *110(1)*, 2-12.

Chan, J., Fan, X. D., Hunter, S. W., Brennan, P. J., & Bloom, B. R. (1991). Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages. *Infect Immun*, *59(5)*, 1755-61.

Chatterjee, D., Bozic, C., Aspinall, G. O., & Brennan, P. J. (1988). Glucuronic acid- and branched sugar-containing glycolipid antigens of Mycobacterium avium. *J Biol Chem*, *263(9)*, 4092-97.

Chatterjee, D., Bozic, C. M., McNeil, M., & Brennan, P. J. (1991). Structural features of the arabinan component of the lipoarabinomannan of Mycobacterium tuberculosis. *J Biol Chem*, *266(15)*, 9652-60.

Chatterjee, D., Hunter, S. W., McNeil, M., & Brennan, P. J. (1992a). Lipoarabinomannan. Multiglycosylated form of the mycobacterial mannosylphosphatidylinositols. *J Biol Chem*, *267(9)*, 6228-33.

Chatterjee, D., Roberts, A. D., Lowell, K., Brennan, P. J., & Orme, I. M. (1992b). Structural basis of capacity of lipoarabinomannan to induce secretion of tumor necrosis factor. *Infect Immun*, *60(3)*, 1249-53.

Chennai., T. R. C. I. C. M. R. (1999). Fifteen year follow up of trial of BCG vaccines in South India for tuberculosis prevention. *Indian J Med Res 1999*, *110*, 56-69.

Colditz, G. A., Brewer, T. F., Berkey, C. S., Wilson, M. E., Burdick, E., Fineberg, H. V., *et al*. (1994). Efficacy of BCG vaccine in the prevention of tuberculosis. Meta- analysis of the published literature. *Jama*, *271(9)*, 698-702.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., *et al*. (1998). Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*, *393(6685)*, 537-44.

Colebunders, R., & Bastian, I. (2000). A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. *Int J Tuberc Lung Dis*, *4(2)*, 97-107.

Cooper, A. M., Callahan, J. E., Keen, M., Belisle, J. T., & Orme, I. M. (1997). Expression of memory immunity in the lung following re-exposure to Mycobacterium tuberculosis. *Tuber Lung Dis*, *78(1)*, 67-73.

Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G., & Orme, I. M. (1993). Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med*, *178(6)*, 2243-7.

de Bruyn, G., & Garner, P. (2003). Mycobacterium vaccae immunotherapy for treating tuberculosis. *Cochrane Database Syst Rev*, *1*, CD001166.

de Lisle, G. W., Wards, B. J., Buddle, B. M., & Collins, D. M. (2005). The efficacy of live tuberculosis vaccines after presensitization with Mycobacterium avium. *Tuberculosis (Edinb)*, *85(1-2)*, 73-79.

Doherty, T. M., Olsen, A. W., van Pinxteren, L., & Andersen, P. (2002). Oral vaccination with subunit vaccines protects animals against aerosol infection with Mycobacterium tuberculosis. *Infect Immun*, *70(6)*, 3111-21.

Dolin PJ., Raviglione MC., & Kochi A. (1994). Global tuberculosis incidence and mortality during 1990-2000. *Bulletin of the World Health Organization*, 213-20.

Dubois M, G. K. A., Hamilton JK, Rebers RA,Smith F. (1956). Colorimetric method for the determination of sugars and related substances. *Anal Biochem*, *28*, 350.

Elias, D., Akuffo, H., Pawlowski, A., Haile, M., Schon, T., & Britton, S. (2005). Schistosoma mansoni infection reduces the protective efficacy of BCG vaccination against virulent Mycobacterium tuberculosis. *Vaccine*, *23(11)*, 1326-34.

Elias, D., Wolday, D., Akuffo, H., Petros, B., Bronner, U., & Britton, S. (2001). Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. *Clin Exp Immunol*, *123(2)*, 219-25.

Evans, J. T., Ward, J. R., Kern, J., & Johnson, M. E. (2004). A single vaccination with protein-microspheres elicits a strong CD8 T-cell-mediated immune response against Mycobacterium tuberculosis antigen Mtb8.4. *Vaccine*, *22(15-16)*, 1964-72.

Falero-Diaza, G., Challacombea, S., Banerjeeb, D., Doucec, G., Boyda, A., & Ivanyia, J. (2000). Intranasal vaccination of mice against infection with Mycobacterium tuberculosis. *Vaccine*, *18(28)*, 3223-29.

Ferraz, J. C., Stavropoulos, E., Yang, M., Coade, S., Espitia, C., Lowrie, D. B., *et al*. (2004). A heterologous DNA priming-Mycobacterium bovis BCG boosting immunization strategy using mycobacterial Hsp70, Hsp65, and Apa antigens improves protection against tuberculosis in mice. *Infect Immun*, *72(12)*, 6945-50.

Fine, P. E. (1996). Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed Mycobacterium leprae vaccine for prevention of leprosy and tuberculosis in Malawi. Karonga Prevention Trial Group. *Lancet*, *348(9019)*, 17-24.

Fine, P. E., Carneiro A.M.I.,Milstien BJ. (1999). Issues relating to the use of BCG in immunization programmes A discussion document. *WHO bulletin*, *23*.

Fine, P. E. (1989). The BCG story: lessons from the past and implications for the future. *Rev Infect Dis*, *11 Suppl 2*, S353-9.

Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A., & Bloom, B. R. (1993). An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med*, *178(6)*, 2249-54.

Flynn, J. L. (2004). Immunology of tuberculosis and implications in vaccine development. *Tuberculosis (Edinb)*, *84(1-2)*, 93-101.

Freytag, L. C., & Clements, J. D. (1999). Bacterial toxins as mucosal adjuvants. *Curr Top Microbiol Immunol*, *236*, 215-36.

Frota, C. C., Hunt, D. M., Buxton, R. S., Rickman, L., Hinds, J., Kremer, K., *et al*. (2004). Genome structure in the vole bacillus, Mycobacterium microti, a member of the Mycobacterium tuberculosis complex with a low virulence for humans. *Microbiology*, *150(Pt 5)*, 1519-27.

Gehring, A. J., Dobos, K. M., Belisle, J. T., Harding, C. V., & Boom, W. H. (2004). Mycobacterium tuberculosis LprG (Rv1411c): a novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. *J Immunol*, *173(4)*, 2660-68.

Glatman-Freedman, A., & Casadevall, A. (1998). Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against Mycobacterium tuberculosis. *Clin Microbiol Rev*, *11(3)*, 514-32.

Goldblatt, D. (2000). Conjugate vaccines. *Clin Exp Immunol*, *119:1-3*, 1-3.

Goonetilleke, N. P., McShane, H., Hannan, C. M., Anderson, R. J., Brookes, R. H., & Hill, A. V. (2003). Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J Immunol*, *171(3)*, 1602-09.

Hamasur, B., Kallenius, G., & Svenson, S. B. (1999a). A new rapid and simple method for large-scale purification of mycobacterial lipoarabinomannan. *FEMS Immunol Med Microbiol*, *24(1)*, 11-7.

Hamasur, B., Kallenius, G., & Svenson, S. B. (1999b). Synthesis and immunologic characterisation of Mycobacterium tuberculosis lipoarabinomannan specific oligosaccharide-protein conjugates. *Vaccine*, *17(22)*, 2853-61.

Hart, P. D. (1967). Efficacy and applicability of mass B. C.G. vaccination in tuberculosis control. *Br Med J*, *1(540)*, 587-92.

Heldwein, K. A., Liang, M. D., Andresen, T. K., Thomas, K. E., Marty, A. M., Cuesta, N., *et al*. (2003). TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG. *J Leukoc Biol*, *74(2)*, 277-86.

Hess, J., & Kaufmann, S. H. (1999). Live antigen carriers as tools for improved anti-tuberculosis vaccines. *FEMS Immunol Med Microbiol*, *23(2)*, 165-73.

Hess, J., Miko, D., Catic, A., Lehmensiek, V., Russell, D. G., & Kaufmann, S. H. (1998). Mycobacterium bovis Bacille Calmette-Guerin strains secreting listeriolysin of Listeria monocytogenes. *Proc Natl Acad Sci U S A*, *95(9)*, 5299-304.

Hetland, G., Wiker, H. G., Hogasen, K., Hamasur, B., Svenson, S. B., & Harboe, M. (1998). Involvement of antilipoarabinomannan antibodies in classical complement activation in tuberculosis. *Clin Diagn Lab Immunol*, *5(2)*, 211-18.

Horwitz, M. A., Harth, G., Dillon, B. J., & Maslesa-Galic', S. (2000). Recombinant bacillus calmette-guerin (BCG) vaccines expressing the Mycobacterium tuberculosis 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc Natl Acad Sci U S A*, *97(25)*, 13853-58.

Hunter, S. W., & Brennan, P. J. (1990). Evidence for the presence of a phosphatidylinositol anchor on the lipoarabinomannan and lipomannan of Mycobacterium tuberculosis. *J Biol Chem*, *265(16)*, 9272-79.

Hunter, S. W., Gaylord, H., & Brennan, P. J. (1986). Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. *J Biol Chem*, *261(26)*, 12345-51.

Huygen, K. (1998). DNA vaccines: application to tuberculosis. *Int J Tuberc Lung Dis*, *2(12)*, 971-78.

Izzo, A., Brandt, L., Lasco, T., Kipnis, A. P., & Orme, I. (2005). NIH pre-clinical screening program: overview and current status. *Tuberculosis (Edinb)*, *85(1-2)*, 25-28.

Johansen, P., Raynaud, C., Yang, M., Colston, M. J., Tascon, R. E., & Lowrie, D. B. (2003). Anti-mycobacterial immunity induced by a single injection of M. leprae Hsp65-encoding plasmid DNA in biodegradable microparticles. *Immunol Lett*, *90(2-3)*, 81-85.

Kaplan, G., Gandhi, R. R., Weinstein, D. E., Levis, W. R., Patarroyo, M. E., Brennan, P. J., *et al*. (1987). Mycobacterium leprae antigen-induced suppression of T cell proliferation in vitro. *J Immunol*, *138(9)*, 3028-34.

Kaufmann, S. H. (2000). Is the development of a new tuberculosis vaccine possible. *Nat Med*, *6(9)*, 955-60.

Kenney, R. T., Regina Rabinovich, N., Pichyangkul, S., Price, V. L., & Engers, H. D. (2002). 2nd meeting on novel adjuvants currently in/close to human clinical testing. World Health Organization-Organization Mondiale de la Sante Fondation Merieux, Annecy, France, 5-7 June 2000. *Vaccine*, *20(17-18)*, 2155-63.

Kent, P. T., and G. P. Kubica. (1985). *Public Health Mycobacteriology: a Guide for the Level III Laboratory. Centers for Disease Control, Atlanta*. Atlanta Georgia 30333: U.S. Department of Health and Human Services.

Kochi, A. (1991). The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle*, *72*, 1-6.

Kohler, G., & Milstein, C. (1992). Continuous cultures of fused cells secreting antibody of predefined specificity. 1975. *Biotechnology*, *24*, 524-26.

Kremer, L., Dover, L. G., Morehouse, C., Hitchin, P., Everett, M., Morris, H. R., *et al*. (2001). Galactan biosynthesis in Mycobacterium tuberculosis. Identification of a bifunctional UDP-galactofuranosyltransferase. *J Biol Chem*, *276(28)*, 26430-0.

Kruuner, A., Hoffner, S. E., Sillastu, H., Danilovits, M., Levina, K., Svenson, S. B., *et al*. (2001). Spread of drug-resistant pulmonary tuberculosis in Estonia. *J Clin Microbiol*, *39(9)*, 3339-45.

Lagranderie, M., Chavarot, P., Balazuc, A. M., & Marchal, G. (2000). Immunogenicity and protective capacity of Mycobacterium bovis BCG after oral or intragastric administration in mice. *Vaccine*, *18(13)*, 1186-95.

Lagranderie, M., Nahori, M. A., Balazuc, A. M., Kiefer-Biasizzo, H., Lapa e Silva, J. R., Milon, G., *et al*. (2003). Dendritic cells recruited to the lung shortly after intranasal delivery of Mycobacterium bovis BCG drive the primary immune response towards a type 1 cytokine production. *Immunology*, *108(3)*, 352-64.

Lalvani, A., Pathan, A. A., McShane, H., Wilkinson, R. J., Latif, M., Conlon, C. P., *et al*. (2001). Rapid detection of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells. *Am J Respir Crit Care Med*, *163(4)*, 824-28.

Levy, H., Feldman, C., Sacho, H., van der Meulen, H., Kallenbach, J., & Koornhof, H. (1989). A reevaluation of sputum microscopy and culture in the diagnosis of pulmonary tuberculosis. *Chest*, *95(6)*, 1193-7.

Liebeschuetz, S., Bamber, S., Ewer, K., Deeks, J., Pathan, A. A., & Lalvani, A. (2004). Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. *Lancet*, *364(9452)*, 2196-203.

Lowrie, D.B., Tascon, R.E., Bonato, V.L., Lima, V.M., (1999). Therapy of tuberculosis in mice by DNA vaccination. *Nature, 400*, 269-71

Lowrie, D. B. (2003). DNA vaccination: an update. *Methods Mol Med*, *87*, 377-90.

Lowrie, D. B., & Silva, C. L. (2000). Enhancement of immunocompetence in tuberculosis by DNA vaccination. *Vaccine*, *18(16)*, 1712-16.

Lyadova, I. V., Vordermeier, H. M., Eruslanov, E. B., Khaidukov, S. V., Apt, A. S., & Hewinson, R. G. (2001). Intranasal BCG vaccination protects BALB/c mice against virulent Mycobacterium bovis and accelerates production of IFN-gamma in their lungs. *Clin Exp Immunol*, *126(2)*, 274-79.

Malhotra, I., Mungai, P., Wamachi, A., Kioko, J., Ouma, J. H., Kazura, J. W., *et al*. (1999). Helminth- and Bacillus Calmette-Guerin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. *J Immunol*, *162(11)*, 6843-48.

McNeil, M. R., & Brennan, P. J. (1991). Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information. *Res Microbiol*, *142(4)*, 451-63.

McShane, H., Brookes, R., Gilbert, S. C., & Hill, A. V. (2001). Enhanced immunogenicity of CD4(+) t-cell responses and protective efficacy of a DNA-modified vaccinia virus Ankara prime-boost vaccination regimen for murine tuberculosis. *Infect Immun*, *69(2)*, 681-86.

McShane, H., Pathan, A. A., Sander, C. R., Goonetilleke, N. P., Fletcher, H. A., & Hill, A. V. (2005). Boosting BCG with MVA85A: the first candidate subunit vaccine for tuberculosis in clinical trials. *Tuberculosis (Edinb)*, *85(1-2)*, 47-52.

McShane, H., Pathan, A. A., Sander, C. R., Keating, S. M., Gilbert, S. C., Huygen, K., *et al*. (2004). Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-

primed and naturally acquired antimycobacterial immunity in humans. *Nat Med*, *10(11)*, 1240-44.

Miki, K., Nagata, T., Tanaka, T., Kim, Y. H., Uchijima, M., Ohara, N., *et al*. (2004). Induction of protective cellular immunity against Mycobacterium tuberculosis by recombinant attenuated self-destructing Listeria monocytogenes strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51. *Infect Immun*, *72(4)*, 2014-21.

Moingeon, P., Haensler, J., & Lindberg, A. (2001). Towards the rational design of Th1 adjuvants. *Vaccine*, *19(31)*, 4363-72.

Mollenkopf, H. J., Groine-Triebkorn, D., Andersen, P., Hess, J., & Kaufmann, S. H. (2001). Protective efficacy against tuberculosis of ESAT-6 secreted by a live Salmonella typhimurium vaccine carrier strain and expressed by naked DNA. *Vaccine*, *19(28-29)*, 4028-35.

Molloy, A., Gaudernack, G., Levis, W. R., Cohn, Z. A., & Kaplan, G. (1990). Suppression of T-cell proliferation by Mycobacterium leprae and its products: the role of lipopolysaccharide. *Proc Natl Acad Sci U S A*, *87(3)*, 973-77.

Moreira, A. L., Tsenova, L., Murray, P. J., Freeman, S., Bergtold, A., Chiriboga, L., *et al*. (2000). Aerosol infection of mice with recombinant BCG secreting murine IFN- gamma partially reconstitutes local protective immunity. *Microb Pathog*, *29(3)*, 175-85.

Moreno, C., Mehlert, A., & Lamb, J. (1988). The inhibitory effects of mycobacterial lipoarabinomannan and polysaccharides upon polyclonal and monoclonal human T cell proliferation. *Clin Exp Immunol*, *74(2)*, 206-10.

Murray, P. J., Aldovini, A., & Young, R. A. (1996). Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines. *Proc Natl Acad Sci U S A*, *93(2)*, 934-39.

Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M., & Puzo, G. (2001). Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J Immunol*, *166(12)*, 7477-85.

Oettinger, T., Jorgensen, M., Ladefoged, A., Haslov, K., & Andersen, P. (1999). Development of the Mycobacterium bovis BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. *Tuber Lung Dis*, *79(4)*, 243-50.

Ohara, N., & Yamada, T. (2001). Recombinant BCG vaccines. *Vaccine*, *19(30)*, 4089-98.

Orme, I. M. (1999). Beyond BCG: the potential for a more effective TB vaccine. *Mol Med Today*, *5(11)*, 487-92.

Orme, I. M., Andersen, P., & Boom, W. H. (1993). T cell response to Mycobacterium tuberculosis. *J Infect Dis*, *167(6)*, 1481-97.

Orme, I. M., & Collins, F. M. (1984). Efficacy of Mycobacterium bovis BCG vaccination in mice undergoing prior pulmonary infection with atypical mycobacteria. *Infect Immun*, *44(1)*, 28-32.

Orme, I. M., Roberts, A. D., Griffin, J. P., & Abrams, J. S. (1993). Cytokine secretion by CD4 T lymphocytes acquired in response to Mycobacterium tuberculosis infection. *J Immunol*, *151(1)*, 518-25.

Ottenhoff, T. H., de Boer, T., Verhagen, C. E., Verreck, F. A., & van Dissel, J. T. (2000). Human deficiencies in type 1 cytokine receptors reveal the essential role of type 1 cytokines in immunity to intracellular bacteria. *Microbes Infect*, *2(13)*, 1559-66.

Palmer, C. E., & Long, M. W. (1966). Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. *Am Rev Respir Dis*, *94(4)*, 553-68.

Park JT, J. M. J. A. (1949). A submicrodetermination of glucose. *J Biol Chem*, *181*, 149-51.

Pawlowski, A., Kallenius, G., & Svenson, S. B. (1999). A new method of non-cross-linking conjugation of polysaccharides to proteins via thioether bonds for the preparation of saccharide-protein conjugate vaccines [In Process Citation]. *Vaccine*, *17(11-12)*, 1474-83.

Pethe, K., Alonso, S., Biet, F., Delogu, G., Brennan, M. J., Locht, C., *et al*. (2001). The heparin-binding haemagglutinin of M. tuberculosis is required for extrapulmonary dissemination. *Nature*, *412(6843)*, 190-94.

Pinto, R., Saunders, B. M., Camacho, L. R., Britton, W. J., Gicquel, B., & Triccas, J. A. (2004). Mycobacterium tuberculosis defective in phthiocerol dimycocerosate translocation provides greater protective immunity against tuberculosis than the existing bacille Calmette-Guerin vaccine. *J Infect Dis*, *189(1)*, 105-2. Epub 2003 Dec 22.

Poolman, J., Laferriére, C., & Svenon, S. B. (1999). *Carbohydrate-based bacterial vaccines*. Perlman and Wigsell.

Porcelli, S., Morita, C. T., & Brenner, M. B. (1992). CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen. *Nature*, *360(6404)*, 593-97.

Pym, A. S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., *et al*. (2003). Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat Med*, *9(5)*, 533-39.

Rincon, M., Anguita, J., Nakamura, T., Fikrig, E., & Flavell, R. A. (1997). Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp Med*, *185(3)*, 461-69.

Roberts, G. D. (1991). Manual of clinical microbiology. Mycobacterium. Washington DC. *Book*, 304-39.

Rodriguez, A., Tjarnlund, A., Ivanji, J., Singh, M., Garcia, I., Williams, A., *et al*. (2005). Role of IgA in the defense against respiratory infections IgA deficient mice exhibited increased susceptibility to intranasal infection with Mycobacterium bovis BCG. *Vaccine*, *23(20)*, 2565-72.

Rook, G. A., Bahr, G. M., & Stanford, J. L. (1981). The effect of two distinct forms of cell-mediated response to mycobacteria on the protective efficacy of BCG. *Tubercle*, *62(1)*, 63-68.

Rowland, S. S., Mayner, R. L., & Barker, L. (2005). Advancing TB vaccines to Phase I clinical trials in the US: regulatory/manufacturing/licensing issues. *Tuberculosis (Edinb)*, *85(1-2)*, 39-46.

Russell, D. G. (2001). Mycobacterium tuberculosis: here today, and here tomorrow. *Nat Rev Mol Cell Biol*, *2(8)*, 569-77.

Ryll, R., Kumazawa, Y., & Yano, I. (2001). Immunological properties of trehalose dimycolate (cord factor) and other mycolic acid-containing glycolipids--a review. *Microbiol Immunol*, *45(12)*, 801-11.

Sakula, A. (1979). Robert Koch (1843--1910): founder of the science of bacteriology and discoverer of the tubercle bacillus. A study of his life and work. *Br J Dis Chest*, *73(4)*, 389-94.

Salfinger, M., Hale, Y. M., & Driscoll, J. R. (1998). Diagnostic tools in tuberculosis. Present and future. *Respiration*, *65(3)*, 163-70.

Schlesinger, L. S., Bellinger-Kawahara, C. G., Payne, N. R., & Horwitz, M. A. (1990). Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. *J Immunol*, *144(7)*, 2771-80.

Schroder, U., & Svenson, S. B. (1999). Nasal and parenteral immunizations with diphtheria toxoid using monoglyceride/fatty acid lipid suspensions as adjuvants. *Vaccine*, *17(15-16)*, 2096-103.

Sibley, L. D., Adams, L. B., & Krahenbuhl, J. L. (1990). Inhibition of interferon-gamma-mediated activation in mouse macrophages treated with lipoarabinomannan. *Clin Exp Immunol*, *80(1)*, 141-48.

Sibley, L. D., Hunter, S. W., Brennan, P. J., & Krahenbuhl, J. L. (1988). Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. *Infect Immun*, *56(5)*, 1232-36.

Silva C:L:, Bonato, V:L:, Coelho-Castelo, A:A:, De Souza, A.O. (2005). Immunotherapy with plasmid DNA encoding mycobacterial hap65 in association with chemotherpy is a more rapid and efficient form of treatment for tuberculosis in mice. *Gene Ther*, 12, 281-7

Silva, C. L. (1999). The potential use of heat-shock proteins to vaccinate against mycobacterial infections. *Microbes Infect*, *1(6)*, 429-35.

Skeiky, Y. A., Alderson, M. R., Ovendale, P. J., Guderian, J. A., Brandt, L., Dillon, D. C., *et al*. (2004). Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. *J Immunol*, *172(12)*, 7618-28.

Smith, D. A., Parish, T., Stoker, N. G., & Bancroft, G. J. (2001). Characterization of auxotrophic mutants of Mycobacterium tuberculosis and their potential as vaccine candidates. *Infect Immun*, *69(2)*, 1142-50.

Stenger, S., Rosat, J. P., Bloom, B. R., Krensky, A. M., & Modlin, R. L. (1999). Granulysin: a lethal weapon of cytolytic T cells. *Immunol Today*, *20(9)*, 390-94.

Svenson, S. B., Nurminen, M., & Lindberg, A. A. (1979). Artificial Salmonella vaccines: O-antigenic oligosaccharide-protein conjugates induce protection against infection with Salmonella typhimurium. *Infect Immun*, *25(3)*, 863-72.

Teitelbaum, R., Glatman-Freedman, A., Chen, B., Robbins, J. B., Unanue, E., Casadevall, A., *et al*. (1998). A mAb recognizing a surface antigen of Mycobacterium tuberculosis enhances host survival. *Proc Natl Acad Sci U S A*, *95(26)*, 15688-93.

Tsenova, L., Moreira, A., Esmeralda P.,Freedman V. H. (1997). Aerosol Infection of mice with Mycobacteria using a nose-only exposure device. *Journal of the American Biological Safety Association* 2, 20-31

Turner, J., Rhoades, E. R., Keen, M., Belisle, J. T., Frank, A. A., & Orme, I. M. (2000). Effective preexposure tuberculosis vaccines fail to protect when they are given in an immunotherapeutic mode. *Infect Immun*, *68(3)*, 1706-09.

Uehori, J., Matsumoto, M., Tsuji, S., Akazawa, T., Takeuchi, O., Akira, S., *et al*. (2003). Simultaneous blocking of human Toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by Mycobacterium bovis bacillus Calmette-Guerin peptidoglycan. *Infect Immun*, *71(8)*, 4238-49.

Vergne, I., Chua, J., & Deretic, V. (2003). Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade. *J Exp Med*, *198(4)*, 653-59.

Verreck, F. A., de Boer, T., Langenberg, D. M., Hoeve, M. A., Kramer, M., Vaisberg, E., *et al*. (2004). Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci U S A*, *101(13)*, 4560-65.

Vordermeier, H. M., Cockle, P. C., Whelan, A., Rhodes, S., Palmer, N., Bakker, D., *et al*. (1999). Development of diagnostic reagents to differentiate between Mycobacterium bovis BCG vaccination and M. bovis infection in cattle. *Clin Diagn Lab Immunol*, *6(5)*, 675-82.

Vordermeier, H. M., Rhodes, S. G., Dean, G., Goonetilleke, N., Huygen, K., Hill, A. V., *et al*. (2004). Cellular immune responses induced in cattle by heterologous prime-boost vaccination using recombinant viruses and bacille Calmette-Guerin. *Immunology*, *112(3)*, 461-70.

Wallis, R. S., Amir-Tahmasseb, M., & Ellner, J. J. (1990). Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins: the monocyte western blot. *Proc Natl Acad Sci U S A*, *87(9)*, 3348-52.

Walsh, G. P., Tan, E. V., dela Cruz, E. C., Abalos, R. M., Villahermosa, L. G., Young, L. J., *et al*. (1996). The Philippine cynomolgus monkey (Macaca fasicularis) provides a new nonhuman primate model of tuberculosis that resembles human disease. *Nat Med*, *2(4)*, 430-36.

Wang, J., Thorson, L., Stokes, R. W., Santosuosso, M., Huygen, K., Zganiacz, A., *et al*. (2004). Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. *J Immunol*, *173(10)*, 6357-65.

Weiss, D. W. (1959). Vaccination against tuberculosis with nonliving vaccines. I. The problem and its historical background. *Am Rev Respir Dis*, *80*, 676-88.

Williams, A., Reljic, R., Naylor, I., Clark, S. O., Falero-Diaz, G., Singh, M., *et al*. (2004). Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs. *Immunology*, *111(3)*, 328-33.

W.H.O. (2000). Global tuberculosis control:. *Who Report 2000. Geneva: World Health Organization(2000)*,