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INTERACTION OF THE IMMUNE RESPONSE TO BCG AND TO ENVIRONMENTAL MYCOBACTERIA INFECTION

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Interaction of the immune response to *Mycobacterium bovis* BCG and to environmental mycobacteria infection

Interacção da resposta imune à infecção por *Mycobacterium bovis* BCG e por micobactérias ambientais

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Abstract

Tuberculosis is in our days a severe problem of public health, affecting billions of individuals worldwide. Chemotherapy is not sufficient to prevent new cases. The AIDS epidemic led to immunodeficient individuals that are highly susceptible to *M. tuberculosis* infection. In addition the development of new multi-drug resistant strains in consequence of impaired treatments contributes largely to the occurrence of novel infected individuals.

The only licensed vaccine against tuberculosis is the "Bacille Calmette-Guérin". Although this vaccine has been used worldwide since 1921, BCG has shown to induce variable degrees of protection against TB in different regions, ranging from 80% vaccine efficacy to no detectable effect.

Some hypothesis have been pointed out to explain the failure of the BCG vaccine. The most acceptable one correlates with data suggesting the interference of environmental mycobacteria with BCG. In regions near the equator where the incidence of environmental mycobacteria is high the efficacy of BCG is low whereas in areas with higher latitudes the vaccine confers protection against TB. The exact mechanism by which the interaction between environmental mycobacteria and the BCG vaccine occurs it is not well known. The work described in this thesis has the purpose of finding out the specific functional interaction between BCG and environmental mycobacteria and the consequence of that interaction in a following *M. tuberculosis* infection.

Protection to TB is based in cellular immunity, mediated by T $CD4^+$ lymphocytes (T helper) and $TCD8^+$ (T cytotxic). The activation of T $CD4^+$ lymphocytes by pathogen antigens induces IFN-gamma production. In this context, the first part of this study focuses in the antigen specificity of T-cell response to *Mycobacterium avium* infection. *Mycobacterium avium* is an environmental mycobacterium usually found in the water and soil in regions near the equator where the BCG efficacy is lower.

The identification of the key antigenic targets of the immune response to *M. avium* was based in the quantification of T-cell IFN-gamma production induced by three distinct sources of antigens from the bacteria. Cytosolic, envelope and culture filtrate proteins were obtained by extraction and purification methods. In order to find out unique immunogenic proteins an electroeluition method was used to obtain panels of defined molecular weight antigens. T-cells from *M. avium* infected C57BL/6 mice

reacted to secreted, envelope and cytosol proteins and to fractions obtained from these crude extracts. Multiple targets were recognised in the culture filtrate panel, such as antigens with a MW below 21 kDa and with a MW between 29-45 kDa. Antigens around 30 kDa (the MW of Ag85 complex) in the envelope and cytosol, and 45 to 116 kDa proteins in the envelope were recognised by T-cells from infected mice. It was demonstrated by this study that T-cells respond to all classes of mycobacterial antigens shifting in the repertoire of these antigens during the course of infection.

Since the T-cell immune responses to antigens from *M. avium* were well known, the work described in this thesis proceeded by analysing the effect of a prior exposure to environmental mycobacteria in a subsequent BCG vaccination. The studies demonstrate that prior exposure to live environmental mycobacteria or secreted proteins from the bacteria can result in a broad immune response that is recalled rapidly after BCG vaccination and controls the multiplication of the vaccine. In these sensitised mice, BCG induces only a secondary and transient immune response and no protective immunity against a later *M. tuberculosis* infection. In addition, a sub-unit vaccine based in recombinant proteins, such as ESAT-6 and Ag85, was unaffected by prior exposure to environmental mycobacteria.

The latter results point out that pre-existing immune responses to antigens that are common to environmental mycobacteria and to *M. bovis* BCG seem to be responsible for the failure of this vaccine. Using electroelution techniques a panel of secreted antigens from *M. intracellulare*, BCG and *M. tuberculosis* was obtained. The stimulation of T-cells from infected mice with each of those panels of antigens has shown that there are several cross-reactive antigens between these three strains. Ag85 was identified as being one of such cross-reactive antigens. This cross-reactivity at the antigen recognition level inhibits BCG replication in vaccinated C57B76 mice and consequently induces low T-cell response to subsequent T lymphocytes stimulation with *M. tuberculosis* antigens.

The results suggest that prior exposure to environmental mycobacteria induces a secondary immune response against subsequent BCG vaccine. This transient immune response occurs due the existence of cross-reactive antigens between these two mycobacterial strains. As a result the BCG do not replicate and consequently there is no induction of memory immune response essential for protection against later *M. tuberculosis* infection.

In contrast to BCG, vaccines based on recombinant proteins known to be immunogenic, such as Ag85 complex and ESAT-6, and that need no replication in the host are new improvements in the TB vaccination strategy.

Resumo

A tuberculose é nos dias de hoje um grave problema de saúde publica afectando milhões de indivíduos por todo o mundo. O tratamento da tuberculose através de quimioterapia por si só não é suficiente para que o número de novos casos diminua. Existem várias razões que contribuem para este facto, entre as quais, as immunodeficiências associadas com o número crescente de casos de SIDA e o surgimento de novas estirpes multiresistentes resultantes de tratamentos incompletos por parte dos pacientes.

A única vacina licenciada e em uso corrente nos nossos dias é o " Bacille Calmette Guérin". Embora esta vacina seja utilizada a nível mundial desde 1921 não apresenta protecção consistente contra tuberculose pulmonar em indivíduos na fase adulta em populações de diferentes pontos do globo. A sua eficácia é bastante controversa e tem dado origem a extensos trabalhos no sentido de se analisar as razões de tal incapacidade. Várias hipóteses têm sido propostas no sentido de tentar explicar as razões pelas quais a vacina do BCG tem uma variação de eficácia tão grande conforme é aplicada em diferentes latitudes. Existem dados consistentes que indicam que uma maior eficiência desta vacina está associada a locais próximos dos pólos, enquanto que zonas próximas do equador estão associadas a uma baixa e por vezes nula eficácia. A hipótese que prevalece atribui esta variação a interacções entre a vacina e micobactérias ambientais presentes predominantemente em zonas húmidas e quentes como o equador. No entanto, o mecanismo preciso pelo qual ocorre esta interacção não se encontra ainda desvendado. Os estudos descritos nesta tese tiveram por objectivo decifrar quais as interacções especificas entre o "Bacille Calmete Guérin" e as micobactérias presentes no ambiente e ainda quais as consequências de tais interacções numa posterior infecção por *Mycobacterium tuberculosis*.

A protecção contra a infecção por *Mycobacterium tuberculosis* ocorre essencialmente por acção de imunidade celular. Os linfócitos T $CD4^+$ ("Thelper") e os $TCD8^+$ ("Tcytotoxic") são as células que participam activamente na protecção através da produção de IFN-gamma. A activação destes linfócitos ocorre pelo reconhecimento de antigénios do patogéneo por parte das células do sistema imunitário do hospedeiro. Neste contexto a primeira parte deste estudo, focou a especificidade antigénica da resposta de células T á infecção por *Mycobacterium avium*, uma micobactéria ambiental presente na água e solo nos locais onde a eficácia da vacina do BCG é

baixa. A identificação dos antigénios de *Mycobacterium avium* capazes de estimular células T de ratinho C57BL/6 infectado, baseou-se na quantificação de IFN-gamma produzido por estas células em resposta a diferentes extractos celulares da bactéria. Estes três extractos foram obtidos por várias técnicas de extracção e purificação e são ricos respectivamente em proteínas secretadas, proteínas do citosol e proteínas da parede celular. No sentido de encontrar proteínas únicas e especificas, reconhecidas por células T a utilização de um método de electroeluição fraccionada permitiu obter painéis de proteínas de diferentes pesos moleculares. Cada fracção electroeluída contém proteínas de determinado peso molecular. No extracto de proteínas secretadas múltiplas fracções são reconhecidas, especialmente as de peso molecular entre 29 e 45 kDa e as de peso molecular menor que 21 kDa. Antigénios do citosol e da parede celular com peso molecular por volta dos 30 kDa (peso molecular do complexo Ag85) são bastante imunogénicos. Estudou-se a evolução em termos de reconhecimento de antigénios específicos durante o curso da infecção. Nos tempos iniciais de infecção a resposta das células T é desviada para as proteínas secretadas pela micobactéria. Á medida que a infecção evolui esta resposta passa a ser centrada em proteínas da parede celular e por fim em proteínas do citosol. Uma vez conhecidos os antigénios predominantemente imunogénicos na infecção por *Mycobacterium avium*, os estudos prosseguiram no sentido de analisar qual o efeito que uma pre-exposição a estas micobactérias teria na posterior vacina do BCG. A pré-exposição a micobactérias ambientais resulta numa resposta imune secundária aquando da vacinação com BCG. Esta resposta bloqueia a multiplicação do *Mycobacterium bovis* BCG no ratinho, não permitindo que esta vacina confira protecção contra uma posterior infecção por *Mycobacterium tuberculosis*. Por outro lado uma vacina, baseada em proteínas recombinantes altamente imunogénicas, como ESAT-6 e o complexo antigénio 85, que não necessita de se multiplicar no hospedeiro, não é afectada pela pré-exposição a micobactérias ambientais.

No encadeamento destas ideias a última parte dos estudos desta tese focou os antigénios responsáveis pela protecção cruzada dada pelas estirpes ambientais à vacina do BCG. Utilizando técnicas de electroeluição e de estimulação de linfócitos identificaram-se inúmeros antigénios como tendo reactividade cruzada entre as três estirpes de micobactérias, *M. intracellulare*, BCG e *M. tuberculosis*. No entanto, existe um proeminente reconhecimento de antigénios de *M. intracellulare* por animais

infectados com BCG, tendo sido o Ag85 identificado como um dos antigénios mais imunogénicos.

Em conjunto os referidos estudos sugerem que a pré-exposição a micobactérias ambientais induz uma resposta immune contra a subsequente vacina do BCG, pelo facto de estas duas estirpes partilharem os mesmos antigénios. Desta forma a multiplicação do BCG no hospedeiro, essencial para a geração de protecção contra uma posterior infecção por *M. tuberculosis*, é bloqueada. O desenvolvimento de novas vacinas baseadas em proteínas, conhecidas como sendo altamente imunogénicas, como o caso do complexo ag85 e ESAT-6 é de facto um método a seguir no sentido de se obter uma vacina eficaz para a tuberculose.

Sommaire

La tuberculose est de nos jours un problème grave de santé publique, affectant des milliards d'individus dans le monde entier. La chimiothérapie n'est pas suffisante pour prévenir de nouveaux cas. L'épidémie de SIDA a généré des individus immunodéficiants fortement susceptibles de l'infection par *M. tuberculosis*. De plus, le développement de souches multi résistantes, dû à des traitements inefficaces, contribue en grande partie à l'occurrence de nouveaux individus infectés.

Le seul vaccin autorisé contre la tuberculose est le "Bacille de Calmette-Guérin". Bien que ce vaccin ait été employé dans le monde entier depuis 1921, le BCG a été montré comme induisant des degrés variables de protection contre TB dans différentes régions, allant de 80% d'efficacité vaccinale à un effet indétectable.

Certaines hypothèses ont été proposées pour expliquer l'échec du vaccin du BCG. La plus vraisemblable est corrélée à des données suggérant l'interférence des mycobactéries environnementales avec le BCG. Dans les régions proches de l'équateur où l'incidence des mycobactéries environnementales est forte, l'efficacité du BCG est limitée, tandis que dans des régions de plus hautes latitudes le vaccin confère une protection contre TB. Le mécanisme exact par lequel l'interaction entre les mycobactéries environnementales et le vaccin du BCG se produit est mal connu. Le travail décrit dans cette thèse se propose de mettre en évidence l'interaction fonctionnelle spécifique entre le BCG et les mycobactéries environnementales, et la conséquence de cette interaction dans une infection à *M. tuberculosis* postérieure.

La protection à TB est basée sur l'immunité cellulaire médiée par des lymphocytes T $CD4^+$ (T helper) et T $CD8^+$ (T cytotoxique). L' activation des lymphocytes T $CD4^+$ par des antigènes de pathogènes induit la production d'IFN-gamma. Dans ce contexte, la première partie de cette étude se focalise sur la spécificité antigénique de la réponse des cellules T à l'infection par *Mycobacterium avium*. *Mycobacterium avium* est une mycobactérie environnementale habituellement trouvée dans l'eau et le sol dans les régions près de l'équateur où l'efficacité du BCG est faible.

L'identification des cibles antigéniques principales de la réponse immune à *Mycobacterium avium* a été basée sur la quantification de la production d'IFN-gamma par les cellules T induites par trois sources distinctes d'antigènes bactériens. Des protéines cytosoliques, d'enveloppes et de filtrats de culture ont été obtenues par des méthodes d'extraction et de purification. Afin de mettre en évidence les protéines

immunogènes uniques, une méthode d'électroélution a été employée pour obtenir des échantillons d'antigènes de poids moléculaire défini. Les cellules T des souris C57BL/6 infectées par *Mycobacterium avium* ont réagi aux protéines sécrétées d'enveloppe et de cytosol et aux fractions obtenues à partir d'extraits bruts. Des cibles multiples ont été reconnues dans l'échantillon de filtrat de culture, tels que des antigènes avec une masse moléculaire en dessous de 21 kDa et entre 29 et 45 kDa. Des antigènes autour de 30 kDa (la masse moléculaire du complexe Ag85) dans l'enveloppe et le cytosol, ainsi que des protéines de 45 à 116 kDa dans l'enveloppe ont été reconnus par les cellules T des souris infectées. Cette étude a montré que les cellules T répondent à toutes les classes d'antigènes mycobactériens en se décalant dans le répertoire antigénique pendant l'infection.

La réponse immune des cellules T aux antigènes de *M. avium* étant bien connue, le travail décrit dans cette thèse a procédé en analysant l'effet d'une pré exposition aux mycobactéries environnementales sur une vaccination BCG postérieure. Les études démontrent que l'exposition antérieure à des mycobactéries environnementales vivantes ou à des protéines bactériennes sécrétées peut avoir comme conséquence une large réponse immune qui est réactivée rapidement après la vaccination BCG et controle la multiplication du vaccin. Chez ces souris sensibilisées, le BCG induit seulement une réponse immune secondaire et passagère, et aucune immunité protectrice contre une infection tardive par *M. tuberculosis*. De plus, un vaccin basé sur des protéines recombinantes, telles que Esat-6 et Ag85, n'est pas affecté par une pré exposition aux mycobactéries environnementales.

Les derniers résultats indiquent que les réponses immunes pré existantes aux antigènes communs aux mycobactéries environnementales et à *M. bovis* semblent être responsables de l'échec du vaccin BCG. En utilisant des techniques d'électroélution, des échantillonnages d'antigènes sécrétés par *M. intracellulare*, BCG et *M. tuberculosis* ont été obtenus. La stimulation des cellules T des souris infectées avec chacun de ces échantillons d'antigènes a prouvé qu'il y a plusieurs réactivité croisée entre les antigènes de ces trois souches. Ag85 a été identifié comme étant un de ces antigènes. Cette réactivité hétérospécifique au niveau de la reconnaissance de l'antigène empêche la réplication du BCG chez les souris C57B76 vaccinées, et induit par conséquent une réponse faible des cellules T à la stimulation suivante des lymphocytes T par les antigènes de *M. tuberculosis*.

Les résultats suggèrent qu'une pré exposition aux mycobactéries environnementales induit une réponse immune secondaire contre une vaccination BCG postérieure. Cette réponse immune passagère est due à l'existence d'antigènes à réactivité croisée entre ces deux souches mycobactériennes. En conséquence, le BCG ne se réplique pas et il n'y a donc aucune induction d'une réponse immune mémoire essentielle pour la protection contre une infection postérieure par *M.tuberculosis* .

Contrairement au BCG, les vaccins basés sur des protéines recombinantes connues pour être immunogènes, comme le complexe Ag85 et ESAT-6, et qui ne nécessitent aucune réplication dans l'hôte, sont de nouvelles améliorations de la stratégie de vaccination contre TB.

Abbreviations

AIDS	Acquired immune deficiency syndrome
AG	Arabinogalactan
APC	Antigen-presenting cells
BCG	Bacilli Calmette Guérin
CD	Cluster defined
CFP	Culture filtrate protein
CFU	Colony forming units
CR	Complement receptor
CTL	Cytotoxic T lymphocytes
CF	Culture filtrate
CTLA	Cytotoxic T-lymphocyte-associated antigen
DC	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule grabbing nonintegrin
DDA	Dioctadecylammonium bromide
DTH	Delayed-type hypersensitivity
EM	Environmental mycobacteria
ESAT	Early secretory antigenic target
Fc	Constant fragment
HIV	Human immunodeficiency virus
Hsp	Heat shock protein
ICAM	Intercellular adhesion molecules
ICD	Isocitrate dehydrogenase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IRAK	Interleukin-1-receptor-associated kinase
KPTG	Karonga prevention trial group
KO	Knock-out
LAM	Lipoarabinomannan
LM	Lipomannan
LPS	Lipopolisacharide

MAC	*M. avium* complex
MBL	Mannose binding lectin
MCP-1	Monocyte chemo-attractant protein 1
MHC	Major histocompatibility complex
MOTT	Mycobacteria other than *tuberculosis*
MPL	Monophosphoryl lipid A
MR	Mannose receptor
MSMD	Mendelian susceptibility to mycobacterial disease
MyD88	Myeloid differentiation protein 88
NFkB	Nuclear factor kB
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
NTM	Non tuberculous mycobacteria
ORF	Open reading frame
PBS	Phosphate buffered saline
PG	Peptidoglican
Phox	phogocytic oxidase
PID	Primary immunodeficiency
PIMs	Phosphatidyl-*myo*-inositol mannosides
PPD	Purified protein derivative
RGM	Rapidly growing mycobacteria
RD	Region of difference
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGM	Slowly growing mycobacteria
SOD	Superoxide dismutase
Sp-A	Surfactant protein A
Sp-D	Surfactant protein D
ST-CF	Short-term culture filtrate
TB	Tuberculosis
TCR	T cell receptor
TGF-β	Transforming growth factor-beta

Th	T helper
TNF	Tumor necrosis factor
TLR	Toll-Like receptor
WHO	World health organization

Chapter 1

Introduction

Introduction

1. Tuberculosis - A global burden

1.1 History of the disease

Tuberculosis is a chronic infection caused by the obligate aerobe intracellular bacterium *Mycobacterium tuberculosis*, even though *Mycobacterium bovis* can cause the disease. Mycobacterial DNA has been found in South American (Chile, Peru) mummies showing an overall 1% prevalence rate for TB between 2000 B.C. and A.D 1500 (Arriaza et al., 1995).

Currently, one-third of the world's population is infected with *M. tuberculosis* and is at risk of developing active tuberculosis, either 1 or 2 years after infection (primary tuberculosis) or thereafter (post primary tuberculosis). TB may develop anywhere in the body, but usually presents as pulmonary infection. Miliary tuberculosis is the most serious disease manifestation and is characterized by the haematogenous dissemination of large numbers of mycobacteria throughout the body. The disease development or not and the different manifestations of infection with *M. tuberculosis* depends on the balance between the bacteria and the host defences.

TB was a major health problem in the eighteenth and nineteenth centuries, and the disease was known as the white plague. For the first time, in 1722, Benjamin Marten suggested the infectious nature of the disease. Many years later, in 1882, Robert Koch isolated the causative agent of Tuberculosis, *Mycobacterium tuberculosis* from tubercles. The event known as Koch phenomenon, was discovered when he inoculated previously infected guinea pigs with tubercle bacilli and the produced ulcer became a non-healing ulcer or just red nodules that healed several weeks later.

In 1908, Albert Calmette and Camille Guérin used an avirulent form of *M. bovis* originally isolated from a cow to immunize, for the first time, a child, against TB. This vaccine, BCG (bacille Calmette-Guérin) is one of the most well known vaccines today.

In the first half of the twentieth century TB had apparently declined due to the improvement of living conditions, the introduction of antibiotics such as streptomycin (discovered in 1943 by Selman Waksman) and isoniazid, as well as the introduction of a BCG vaccination program. By 1960 the mortality caused by TB was so low in

many countries that the disease was considered close to eradication. However, in the 1980's, in developed countries the number of TB cases increased. Some reasons have been suggested to explain the re-emergence of this public health problem, such as the appearance of multi-drug resistant strains of *M. tuberculosis*; the increase of Human Immunodeficiency virus (HIV) infections and thus a high susceptibility to TB by these persons; the immunosuppressive drugs used in chemotherapy and organ transplantation, and also the tainted social and economic conditions in some regions that give rise to groups with high risk of transmission (Andersen, 1997).

A report by the World Health Organization had predicted that by this year, 2005, TB would kill 4 million people annually. This is a significant increase from an estimated 3 million deaths worldwide caused by TB in 1992 (Fenton and Vermeulen, 1996). An important reason for the current failure to control TB is that even when the best available chemotherapy is used, treatment must be continued for at least 6 months. This treatment regimen is not a realistic proposition in most developing countries, because the patients feel well after a few weeks and stop taking the drugs. However this six-months regimen is truly required. The chemotherapy kills the vast majority of the bacteria within a few days, but "persisters" that are possibly not metabolising are not killed, and may persist in stationary phase or may be replicating extremely slowly in old lesions or at sites of fibrosis or calcification where oxygen availability may be low. Therefore, if chemotherapy is stopped at 3 months, despite the absence of mycobacteria in the sputum, and in spite of the fact that there are very few live organisms in the patients tissues at this time, it is possible that occurs a relapse of the disease (Rook and Hernandez-Pando, 1996).

1.2 Immunity to Tuberculosis

Mycobacterium tuberculosis enters the Human body by the inhalation of droplet nuclei that may contain one to three viable bacteria; particles larger than these ones are retained by cilia and mucus. In the lung, mycobacteria are taken up by alveolar macrophages (first stage). Mycobacteria, which escape the initial intracellular destruction, will multiply, and this will lead to disruption of the macrophage. When this happens, blood monocytes and other inflammatory cells are attracted to the lung (second stage). These monocytes will differentiate in macrophages, which again

readily ingest but do not destroy the mycobacteria. In this symbiotic stage, mycobacteria grow exponentially, and blood-derived macrophages accumulate with little tissue damage. Two or three weeks after infection antigen specific T lymphocytes arrive, proliferate within the early lesions of tubercles and activate macrophages to kill intracellular mycobacteria (third stage) (Crevel et al., 2002).

M. tuberculosis may persist in macrophages within a granuloma. The granuloma consists of macrophages and giant cells, T cells, B cells and fibroblasts.

Endocytosis of *M. tuberculosis* involves different receptors on the phagocytic cell, which bind either to nonopsonized *M. tuberculosis* or recognize opsonins on the surface of the mycobacteria.

This uptake may occur through complement activation. Complement receptor 1, CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) bind molecules from the alternative complement pathway, respectively C3b, C4b and C3bi, once the bacteria have been opsonized with complement proteins (Aderem and Underhill, 1999; Bermudez and Sangari, 2001; Hirsch et al., 1994; Schlesinger, 1993). Nevertheless, nonopsonized *M. tuberculosis* can bind directly to CR3 and CR4. However, the best characterized receptor for non-opsonin-mediated phagocytosis of *M. tuberculosis* is the macrophage mannose receptor (MR), which recognizes terminal mannose residues on mycobacteria (Schlesinger, 1993; Schlesinger LS, 1996; Underhill and Ozinsky, 2002).

When uptake by MR and CRs is blocked, macrophages may also internalise *M. tuberculosis* through the type A scavenger receptor.

Fcγ receptors, which facilitate phagocytosis of particles coated with antibodies of the immunoglobulin G class, seem to play little role in TB.

Other receptors may be involved in *M. tuberculosis* entrance within the macrophage.

Surfactant protein A (Sp-A), a member of the collectin family, that is present in lung surfactant, interacts with macrophages to enhance phagocytosis of *M. tuberculosis* (Gaynor et al., 1995). Surfactant protein D (Sp-D) has been found to block the uptake of pathogenic strains of *M. tuberculosis* in macrophages.

Another member of the collectin family, the plasma factor MBL (Mannose Binding Lectin) may be also involved in the uptake of mycobacteria by phagocytic cells, by recognising carbohydrates in a wide range of pathogens (Crevel et al., 2002).

M. tuberculosis may also interact with non-professional phagocytic cells, such as alveolar epithelial cells. This binding may involve fibronectin, a glycoprotein found in plasma and in the outer surface of many cells. The production and secretion of antigen 85 complex (30 to 32 kDa proteins), a member of the fibronectin-binding protein family, by *M. tuberculosis* leads to further interaction with epithelial cells. In addition, a 28 kDa heparin-binding adhesin, produced by *M. tuberculosis*, will bind to sulphated glycoconjugates on host cells types (Crevel et al., 2002)

Several circulating factors and receptors are involved in the recognition of the major mycobacterial cell wall component, lipoarabinomannan (LAM). Plasma LPS-binding protein enhances macrophage responses to LPS and LAM by transferring these microbial products to the cell surface receptor CD14 (Underhill and Ozinsky, 2002) .

Toll-Like receptors (TLRs) are also involved in cellular recognition of mycobacteria. They are mediators of innate immunity, which are essential for microbial recognition on macrophages and dedritic cells. Through TLRs, *M. tuberculosis* lysate or soluble mycobacterial cell wall-associated lipoproteins induce production of IL-12. In the context of CD14, TLR2 binds lipoarabinomannan, a heterodimer of TLR2 and TLR6 binds a 19 kDa *M. tuberculosis* lipoprotein, TLR9 binds to *M. tuberculosis* DNA (Janssens and Beyaert, 2003). TLRs are expressed not only in cell surface but also in phagosome (Heldwein and Fenton, 2002). TLR1, TLR2, TLR4, TLR5 and TLR6 reside mainly in the plasma membrane whereas those TLR recognizing nucleic acid derivatives, such as TLR3, TLR7, TLR8 and TLR9, are localized in intracellular compartments (Dunne and O'Neill, 2005). After binding to TLR, common signalling pathways lead to cell activation and cytokine production.

The intracellular behavior of *M. tuberculosis* inside dendritic cells differs compared to macrophages and has been linked to a different portal of entry. Dendritic cells express surface lectin receptors, like DC-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN/CD209), which is absent in macrophages (Cambi et al., 2005). The ligand of DC-SIGN is the mannose capped LAM characteristic from virulent mycobacteria such as *M. tuberculosis* and absent from atypical mycobacteria such as *M. fortuitum* and *M. chelonae*. These findings attribute to DC-SIGN a role in the differentiation between pathogenic and non-pathogenic mycobacteria (Herrmann and Lagrange, 2005).

DC-SIGN plays an important role in the function of dendritic cells by mediating resting T cell interactions through ICAM-3 as well as by mediating the DC-specific ICAM-2 dependent migration process.

Mycobacterium tuberculosis and other pathogens such as human immunodeficiency virus 1 (HIV-1) subvert dendritic cell functions to escape immune surveillance by targeting DC-SIGN. Namely by evade antigen processing or change TLR-mediated signalling and consequently bias T-cell responses (van Kooyk et al., 2003; van Kooyk and Geijtenbeek, 2003)

There are multiple routes for the uptake of *M. tuberculosis*, involving a number of different host cell receptors. This may lead to differences in signal transduction, immune activation and intracellular survival of the pathogen.

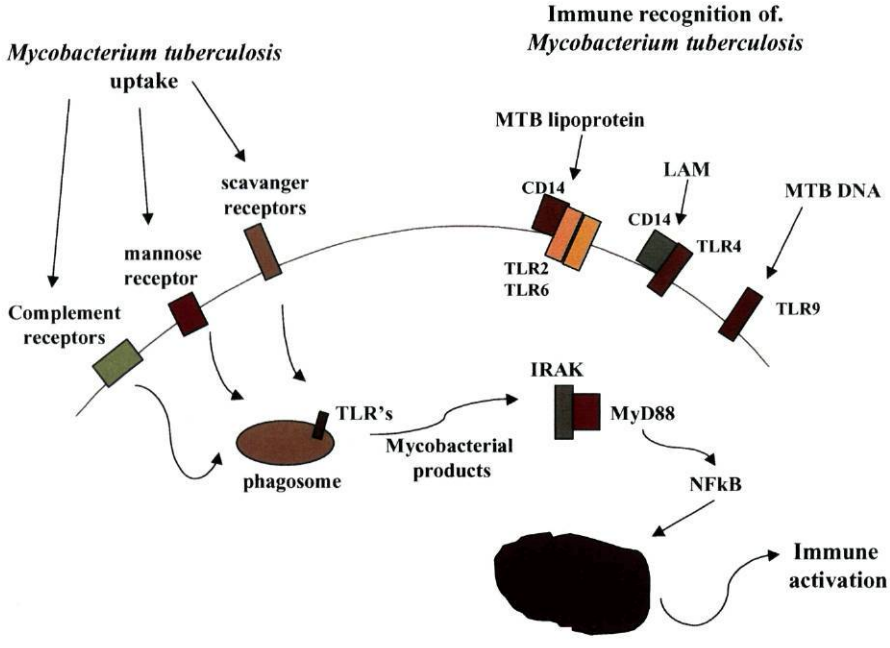

Figure 1. Phagocytosis and immune recognition of *M. tuberculosis* (adapted from (Crevel et al., 2002).

The phagocytosis of the bacteria leads to the activation of the macrophage. The phagosome fuses with the lysosomes leading to an acidification of the compartment, an optimal condition for the action of lytic enzymes from lysosomes. Other mechanisms of defence are activated such as the oxidation of DNA and bacterial

proteins by reactive oxygen intermediates (ROI) such as O_2^-, H_2O_2 and OH, and reactive nitrogen intermediates (RNI) such as NO, NO_2 and HNO_2 (Kaufman, 1993; Shiloh and Nathan, 2000). *In vitro*, murine macrophages activated by IFN-γ, and LAM or TNF-α, are capable of killing *M. tuberculosis*. This antimycobacterial effect is independent of the macrophage capacity to produce ROI but dependent of the L-arginine-dependent generation of RNI (Chan et al., 1992; Denis, 1991). This may be explained by the fact that Mycobacterial products such as LAM have the capacity to scavenge ROI (Chan et al., 1991). However, the role of ROI in the killing of *M. tuberculosis*, has been confirmed in *in vivo* experiments, where mice lacking the $p47^{phox}$ gene (which is essential for effective superoxide production by the NADPH oxidase) had a significant increase in bacterial growth over the early period of infection (Cooper et al., 2000). In other Mycobacterial species such as *M. avium* the role of RNI is quite different, since some experiments have demonstrated that NO is not involved in the mycobacteria killing by *M. avium* infected macrophages (Gomes et al., 1999).

Despite these defence mechanisms, *M. tuberculosis* is capable of surviving inside the macrophage, by preventing phagosome-lysosome fusion (Russell et al., 1996), phagosomal maturation (Clements and Horwitz, 1995) and inhibiting the acidification of phagosomes (Crowle et al., 1991), which in consequence blocks the digestive activity of acidic hydrolases. Other doubtful virulence mechanisms have been proposed: one of these states that virulent *M. tuberculosis* may also elude the microbicidal mechanisms of the macrophages by escaping from fused phagolysosomes into nonfused vesicles or the cytoplasm (McDonough et al., 1993).

Immune recognition of *M. tuberculosis* by macrophages and dendritic cells is followed by an inflammatory response with a crucial role for cytokine production. Initial events in this cellular response include non-specific host defense mechanisms, which may lead to early killing or containment of infection. In addition, various cellular products, including cytokines and cell surface markers are involved in these processes.

After phagocytosis of the bacteria, mycobacterial peptides will be presented by the Major Histocompatibility Complex (MHC) class II molecules on the surface of the macrophages, to the $CD4^+$ α/β T cells, (T helper cells).

The activated T cells start to produce cytokines necessary to activate the cells involved in the immune response against TB. $CD4^+$ T cells can be divided into two sub-populations, Th1 and Th2, based in their different cytokines profiles. Th1 produce IL-2, TNF-α and IFN-γ and are involved in the protection against intracellular infections. Th2 cells secrete IL-4 and IL-5 and promote production of IgG1 and IgE antibodies, which is not believed to contribute to protection against intracellular infections (Abbas et al., 1991; Mosmann et al., 1986).

$CD8^+\alpha/\beta$ T cells are also important in resistance to mycobacterial infections. They recognize antigens presented by MHC class I molecules and have cytolytic activity. Such cytotoxicity could release organisms from macrophages that were failing to kill them and enable uptake of the organisms by fresh activated cells.

$CD4^-/CD8^-$ T cells which express a T cell receptor composed of γ and δ chains, and are activated by *M. tuberculosis*, secrete a pattern of cytokines similar to the secretion pattern of Th1 cells and are cytotoxic. The role of these cells in TB is not clear although it has been suggested that gamma/delta T lymphocytes contribute to protection and may have a regulatory function. Mice in which the Cdelta gene of the gamma/delta TCR has been disrupted control *M. tuberculosis* infection as well as wild type mice. However mutant mice exhibited a pyogenic form of the granulomatous response compared with the lymphocytic response seen in control animals, suggesting a role for gamma/delta T cells in cellular traffic. Promoting the incursion of monocytes and lymhocytes and delaying the influx of inflammatory cells that are responsible for tissue damage (D'Souza et al., 1997).

Both γ/δ T cells and CD1 restricted T cells produce IFN-γ early during TB infection, and both do not react with mycobacterial protein antigens in the MHC class I or II context. Instead, γ/δ T cells may directly recognize small mycobacterial proteins and non-protein ligands in the absence of antigen presenting molecules, and CD1 restricted T cells react with mycobacterial lipid and glycolipid antigens bound to CD1 on antigen presenting cells. This mechanism of antigen presentation enables the activation of a larger fraction of T cells at an earlier point in the infection.

The antigen presentation only leads to T-cell stimulation in the presence of co stimulatory signals, such as CD80, CD86, that are expressed on macrophages and dendritic cells and bind to CD28 and CTLA-4 on T cells

Stimulation of monocytes, macrophages and dendritic cells with mycobacteria or mycobacterial products induces the production of TNF-α, a pro-inflammatory cytokine. TNF-α plays a key role in granuloma formation, induces macrophage activation and has immunoregulatory properties. This cytokine is required for control of acute *M. tuberculosis* infection, namely because TNF-α is required for induction of apoptosis of infected cells (Keane et al., 1997). TNF-α in synergy with IFN-γ induces NOS2 (nitric oxide synthase) expression.

IFN-γ is a key cytokine in control of *M. tuberculosis* infection. Both CD4 and CD8 T cells as well as NK cells produce this cytokine, in tuberculosis. IFN-γ knockout mice are the most susceptible to virulent *M. tuberculosis* (Cooper et al., 1993; Flynn et al., 1993). Although IFN-γ production alone is insufficient to control *M. tuberculosis* infection, it is required for the protective response to this pathogen (Flynn et al., 1993).

IL-1β is a secondary pro-inflammatory cytokine produced by monocytes, macrophages and dendritic cells during TB infection at the site of disease. IL-1R type 1-deficient mice display an increased mycobacterial outgrowth and also defective granuloma formation after infection with *M. tuberculosis* (Juffermans et al., 2000).

IL-4 is a controversial anti-inflamatory cytokine since it is not well known if this cytokine causes or merely reflects disease activity in human tuberculosis. There are studies pointing an increase in IL-4 production during progressive disease (Ordway et al., 2004; Ordway et al., 2005) and reactivation of latent infection in mice infected with *M. tuberculosis* (Howard and Zwilling, 1999). On the other side IL4/KO mice do not display increased susceptibility to mycobacterium tuberculosis (North, 1998).

IL-6, which has both pro and anti-inflammatory properties, has also been implicated in early host response to *M. tuberculosis* at the site of infection (Leal et al., 2001). A controversial duality has been pointed to this cytokine: IL-6 has a protective role related to induction of production of IFN-γ early in the infection and on the other side may be harmful in mycobacterial infections as it inhibits the production of TNF-α and IL-1β (Crevel et al., 2002; Leal et al., 1999; Orme et al., 1993b).

IL-10 is considered to be primarily anti-inflammatory. This cytokine, produced by macrophages and T cells after *M. tuberculosis* infection and after binding of mycobacterial LAM, possesses macrophage deactivating properties, including down regulation of IL-12 production, which in turn decreases IFN-γ production by T-cells.

Transgenic mice producing increases amounts of IL-10 showed during the chronic phase of infection by *M.tuberculosis*, evidence of reactivation of the disease with a highly significant increase in bacterial numbers in the lungs (Turner et al., 2002). However, IL-10 knockout mice were not more resistant to acute *M. tuberculosis* infection, compared to wild type mice (North, 1998).

IL-12, which is composed of a p35 and a p40 subunit, is a crucial cytokine in controlling *M. tuberculosis* infection. IL-12 is induced following phagocytosis of *M. tuberculosis* by macrophages and dendritic cells, which drives development of a Th1 response with production of IFN-γ. During activation IL-12 is found in small amounts, whereas free p40 is produced in excess. p40 can be also covalently linked to a p35-related protein, the p19. This heterodimer forms a recent discovered cytokine, IL-23, that has activity on memory T cells. Recent works showed that the delivery of replication-defective adenovirus vectors encoding IL-23 in *M. tuberculosis* infected mice, induce IFN-γ and IL-17 production in lung tissues. In addition, *in vitro* host T-cell re-stimulation with *M. tuberculosis* purified protein derivative (PPD) has shown high levels of IFN-γ and IL-17 secretion. IL-23 is probably controling mycobacterial growth by enhancing early pulmonary T-cell immunity (Happel et al., 2005).

Another member of this family IL-27 is also a heterodimer composed of the p40 related protein EBI3 (Epstein-Barr virus-induced gene 3) and the p35-related protein, p28. IL-27 participates in early Th1 initiation (Becker et al., 2005; Brombacher et al., 2003). Recent studies from Ehlers and colleagues with knockout mice for one component of the IL-27R complex, showed an increase production of pro-inflammatory cytokines with concomitant elevation of CD4 T cell activation and IFN-γ production in this mice. IL-27 inhibited in vitro IL-12 and TNF production suggesting a role in the modulation of excessive inflammation for this cytokine (Holscher et al., 2005).

IL-18 is a pro-inflammatory cytokine that resembles IL-1. IL-18 is an IFN-γ inducing factor synergistic with IL-12. This cytokine stimulates the production of other pro-inflammatory cytokines, chemokines and transcription factors. IL-18 KO mice are highly susceptible to BCG and *M. tuberculosis* (Sugawara et al., 1999).

Transforming Growth Factor-β is present in the granulomatous lesions of tuberculosis patients and is produced by human monocytes and dendritic cells after stimulation with *M. tuberculosis* or Lipoarabinomannan. Like IL-10, TGF-β is produced in excess

during TB and is expressed at the site of the disease. TGF-β suppresses cell-mediated immunity: in T cells, it inhibits proliferation and IFN-γ production, in macrophages it antagonizes antigen presentation, pro-inflammatory cytokine production and cellular activation. In addition, it may be involved in tissue damage and fibrosis, as it promotes production and deposition of macrophage collagenases and collagen matrix. Within the anti-inflammatory response IL-10 and TGF-β seem to synergise. TGF-β selectively induces IL-10 production and both cytokines show synergism in the suppression of IFN-γ production (Crevel et al., 2002; Flynn and Chan, 2001).

The inflammatory response during TB may be controlled by the expression of anti-inflammatory cytokines. An uncontrolled pro-inflammatory response may lead to excess tissue damage, while an excess anti-inflammatory response may have as consequence the outgrowth of *M. tuberculosis*. On the other side *M. tuberculosis* may evade protective immune mechanisms by selectively inducing anti-inflammatory cytokines.

A large number of different T cell subsets has shown to suppress responses mediated by other populations of T cells and have been named T regulatory cells. For the majority of these subsets the definition of the cells has been based on their phenotype and their capacity to produce suppressor cytokines, such as IL-4, IL-10 and TGF-β. Some regulatory T cells (Treg) arise after antigen exposure and include, regulatory Th2 cells, Th1 cells, interleukin-10-producing Tr1 cells, transforming growth factor-β secreting Th3 cells, $CD8^+$, natural killer and γδ T cells (Garba et al., 2002; Groux and Powrie, 1999; King and Sarvetnick, 1997; Sonoda et al., 2001).

T regulatory cells modulate and down-regulate immune responses at different times, locations and in various inflammatory circumstances. The work of Sakagushi and colleagues provided a strong evidence for the role of $CD4^+CD25^+$ in the generation and maintenance of peripheral self-tolerance. Adoptive transfer of $CD4^+CD25^+$ depleted T cells induced several organ-specific autoimmune diseases in recipient, immunodeficient animals. In addition $CD4^+CD25^+$ cells prevented autoimmune reactions in mice thymectomized on day 3 of life (Sakaguchi et al., 1995).

Piccirillo and co-workers suggest that there are two types of $CD4^+CD25^+$ T regulatory cells differing principally in their origin but also in antigen-specificity and mechanism of action (Piccirillo and Shevach, 2004). One type, the natural-occurring T regulatory cells (nTreg), develops during T cell maturation in the thymus and lives in the

periphery to prevent autoimmune responses. It is well accepted that the second type, induced T regulatory cells (iTreg), are generated from naïve T cells in the periphery after encounter with antigen and under the direction of DC in a way distinct from those that activate Th1 and Th2 cells (Wakkach et al., 2003). $CD4^+CD25^+$ iTregs cells are induced by antigen exposure or co-stimulation, including antigen in the presence of immunosuppressive cytokines such as IL-10 and TGF-β1 (Barrat et al., 2002; Zheng et al., 2002). These two subsets of Treg cells may act together modulating adaptive immune responses.

$CD4^+CD25^+$ nTreg cells represent 5-10% of peripheral $CD4^+$ T cells and have high immunoregulatory function. They have regulatory function during normal surveillance of self-antigens, exerting their primary function by preventing the activation of auto reactive T cells. However it is not known if the antigen repertoire of nTreg is biased towards self-antigens or is more diverse. Recent studies suggest that nTreg cells participate in the modulation of the immune response to infectious agents thus preventing and down-regulating inflammatory responses.

The induction of cytokines such as IL-10 and TGF-β by the cells of the innate immune system after infection by a large number of pathogens has been reported (McGuirk and Mills, 2002). The production of these immunosuppressive cytokines has been documented in individuals diagnosed with active tuberculosis.

In tuberculosis, 15% of infected individuals do not respond to purified protein derivative (PPD). In a work from Boussiotis and colleagues, T cell stimulation with PPD in these anergic patients induced the production of IL-10. However, T cell from PPD positive individuals produced IL-10, IFN-gamma and proliferated in the presence of the stimulus (Boussiotis et al., 2000; Delgado et al., 2002). In addition transgenic mice producing increased amounts of IL-10 showed evidence of reactivation of tuberculosis and suppression of protective Th1 responses with a highly significant increase in bacterial numbers within the lungs (Turner et al., 2002).

It seems that T regulatory cells play a role in the persistence of *M. tuberculosis* infection. The antigen-specific stimulation of Tregs cells following *Mycobacterium tuberculosis* infection led to protective T-cell anergy and may explain the mechanisms by which this pathogen escapes immune surveillance.

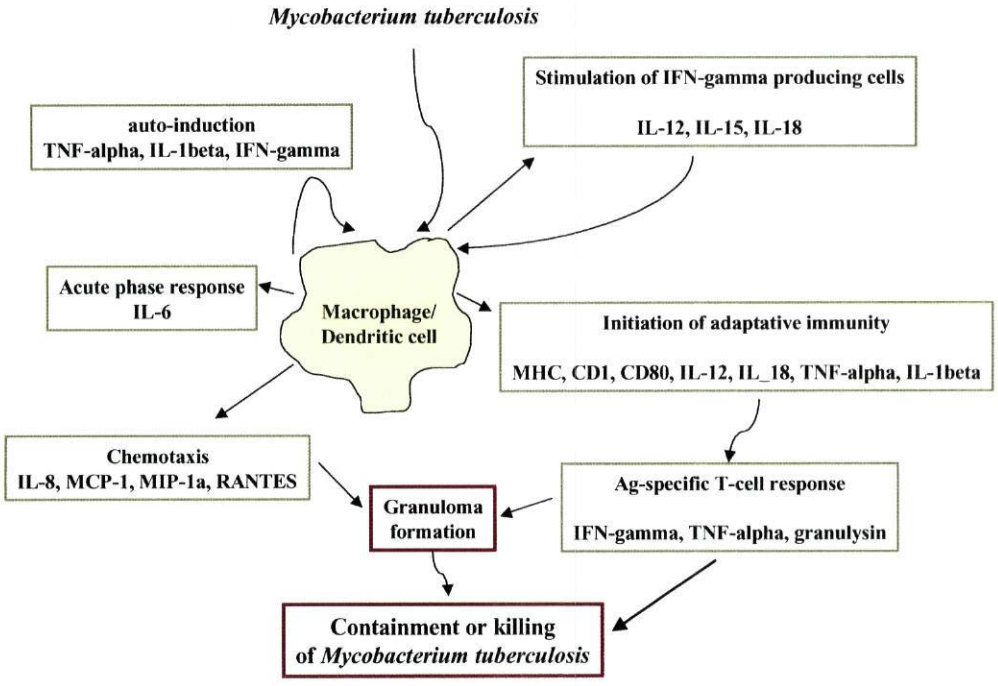

Figure 2. Inflammatory response of phagocytic cells upon activation with *M. tuberculosis* (the anti-inflammatory cytokines are not represented in this picture), (adapted from (Crevel et al., 2002)).

Chemotactic cytokines, such as IL-8, MCP-1 and RANTES are, in TB, responsible for the recruitment of inflammatory cells to the site of infection. Macrophages and pulmonary epithelial cells produce IL-8, which attracts neutrophils, T lymphocytes and monocytes, after *M. tuberculosis* phagocytosis.

Monocyte chemo attractant protein 1 (MCP-1) is a second major chemokine, which is produced by and acts on monocytes and macrophages. In murine models, deficiency of MCP-1 inhibited granuloma formation (Lu et al., 1998). CC chemokine receptor 2-deficient mice, which fail to respond to MCP-1, were found to be highly susceptible to *M. tuberculosis* H37Rv infection (Peters et al., 2001) in a dose-dependent manner (Scott and Flynn, 2002).

RANTES is a third chemokine which is produced by a wide range of cells and which shows promiscuous binding to multiple chemokine receptors. In murine models, expression of RANTES was associated with development of *M. bovis* induced pulmonary granulomas (Chensue et al., 1999).

M. tuberculosis infection causes an increase in the expression of beta chemokines CCL3, CCL4 and CCL5, and their receptor CCR5. CCR5 knockout mice were able to induce a Th1 response and to control the *M. tuberculosis* infection, In addition there were greater numbers of lymphocytes migrating to the lung and higher levels of inflammatory cytokines compared with wild-type mice (Algood and Flynn, 2004).
A number of chemokines have been identified in tuberculosis that may be involved in cell trafficking. Despite that, the contribution of each chemokine is difficult to evaluate due to the redundancy of the chemokine system.

2. The genus *Mycobacterium*

The genus *Mycobacterium*, together with *Corynebacterium* and *Nocardia*, forms a monophyletic taxon within the family of Actynomicetes. It is highly diverse, with 85 different species acknowledged since the identification of *M. leprae* in 1873.
Mycobacteria are aerobic bacteria that exhibit acid-alcohol fastness, some of them being Gram-positive, have genomic DNA with high G/C content and have similar mycolic-acid structures.
Mycobacteria are generally divided into two groups depending on the growth rate in solid media: Slowly Growing Mycobacteria (SGM) that produce visible colonies after more than seven days and Rapidly Growing Mycobacteria (RGM) that produce visible colonies in less than seven days. The SGM include the major mycobacterial pathogens, like *M. tuberculosis* complex, *M. leprae* and *M. avium* complex.
Among the RGM, there are over 40 species, but only few are capable of producing human or animal infections. The main sources of human infections are *M. abcessus*, *M. chelonae*, *M. fortuitum* and *M. ulcerans*. Others like *M. flavescens*, *M. goodii*, *M.mucogenicum*, *M. neoaurum*, *M. peregrinum*, *M. smegmatis*, *M. thermoresistible*, *M. vaccae* and *M. wolinsky* are also source of disease but with a weak pathology (Howard and Byrd, 2000).
The vast majority of mycobacteria are environmental free-living saprophytes. They can live in a variety of natural waters, including fresh and saltwater, and treated water including swimming pools and drinking water, from which they are readily spread via aerosols.

Except for the *M. tuberculosis* complex and *M. leprae*, mycobacteria are referred to collectively as nontuberculous mycobacteria (NTM). Previous names included "environmental mycobacteria" (EM), "mycobacteria other than *tuberculosis*" (MOTT) and "atypical mycobacteria", since it was believed that they were unusual *M. tuberculosis* strains (Falkinham, 1996).

2.1. *Mycobacterium tuberculosis* complex

The *M.tuberculosis* complex consists of *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. bovis* and *M. microti*. Despite having 99% homology at the nucleotide level for some loci, these mycobacteria have different morphology, biochemistry and pathogenesis (Cosma et al., 2003).

All these mycobacteria produce tuberculosis and the pathology of the disease in humans, caused by *M. tuberculosis*, *M. bovis* and *M. africanum* is very similar.

In some world regions like Africa, *M. africanum* causes more cases of tuberculosis than *M. tuberculosis*. On the other side *M. canettii* infection cases are rare.

M. bovis is a pathogen capable of producing tuberculosis in most mammals including humans and cattle and until the regular pasteurisation of milk it was the main cause of human tuberculosis.

M. microti is a pathogen of voles but is avirulent in humans and mice. For this reason the attenuated mycobacteria derived from *M. microti* and also from *M. bovis* are equally efficacious as live tuberculosis vaccines (Casanova and Abel, 2002; Cosma et al., 2003; Manabe et al., 2002).

2.2 *Mycobacterium leprae*

M. leprae is the causal agent of leprosy, 700000 new cases being reported annually worldwide.

M. leprae infects the human host via the nasal mucosa. The existence or not of other routes of infection are not well known. Once inside the host, *M. leprae* has tropism for the nerve and skin, being the Schwann cells of the peripheral nervous system the preferentially infected cells, leading to the characteristic neuropathy of the disease.

Leprosy is a chronic granulomatous disease that presents different possible clinical manifestations, which oscillates between the tuberculoid / paucibacillary type and the lepromatous /multibacillary type (Bermudez and Sangari, 2001; Casanova and Abel, 2002; Cosma et al., 2003)

2.3 Non tuberculous mycobacteria (NTM)

Most NTM are environmentally ubiquitous, and in humans they are low-grade pathogens. The transmission of infection between individuals is rare.

Human disease due to NTM is classified into four distinct clinical syndromes: pulmonary disease, lymphadenitis, cutaneous disease and disseminated disease, being the chronic pulmonary disease the most common (Koh et al., 2002).

Exposure to mycobacteria does not always result in infection. The fate of an infection depends on innate immunity alone or in conjunction with adaptive immunity.

The frequency of infection is, itself, probably underestimated because it is often based on the detection of imunological phenotypes, which reflect an adaptive memory immune response. Innate immunity may itself be sufficient to control the infection, and possible poor memory immune responses may make it difficult to identify the phenotype of interest. Only a small percentage of infected individuals go on to develop the disease, whether it is an environmental mycobacterial infection or it is a virulent species infection such as *M. tuberculosis*. Two other factors contribute to the fate of infection: environmental and host factors. Host factors may be genetic, such as a mutation in a gene involved in immunity to mycobacterial infections, or nongenetic, such as a skin lesion. Environmental factors may be related with mycobacterial virulence or related to the mode of exposure to the mycobacterium. Despite the different contribution of the described factors the occurrence of clinical disease implies that the host defences against mycobacteria have failed (Casanova and Abel, 2002).

There is a markedly geographic variability both in the prevalence of disease and in the mycobacterial species responsible for it. This variety ranges from being *M.avium* complex the most commonly responsible NTM for pulmonary disease in the United States to *M. kansassi* in England (Koh et al., 2002).

2.3.1 *Mycobacterium avium* complex

Mycobacteria included in the *M. avium* complex (MAC) are classified as acid-fast, slowly growing bacilli that may produce a yellow pigment in the absence of light (exposure to light often intensifies pigment production).

This complex is composed of opportunistic pathogens capable of causing disease in humans and in animals.

The *M. avium* complex is a serological complex of 28 serovars of two species *M. avium* and *M. intracellulare*. *M. avium* has three subspecies on the basis of phenotypic properties and nucleic acid studies: *M.avium* subspecies *avium*, *M.avium* subspecies *paratuberculosis* and *M.avium* subspecies *silvaticum* (Inderlied et al., 1993).

The mycobacteria of this complex are widespread in nature, namely in water and soil with low pH, low dissolved-oxygen content and high organic matter content and are often opportunistic pathogens, since the majority of their hosts are immunocompromised, specially HIV infected individuals.

They cause primarily pulmonary infections, although a number of soft tissue infections have been reported. Members of the *M. avium* complex have been isolated from patients with cystic fibrosis, and are also the predominant cause of cervical lymphadenitis in children. MAC infection in HIV-infected children leads to dissemination, rather than being restricted to a local organ (Falkinham, 1996).

The infections by this MAC are often very difficult to eradicate due to the wide diversity in antibiotic susceptibility of *M. avium* complex isolates in addition to the fact that AIDS patients are probably infected with more than one isolate. A large number of different antibiotics such as amikacin, clofazimine, ciprofloxacin, ethambutol, isoniazid and rifampin have been used in an attempt to treat MAC infection. The association between clarithromycin or azithromycin with rifabutin demonstrated remarkably impressive bacteriologic activity (Falkinham, 1996; Inderlied et al., 1993).

M. avium subsp. *paratuberculosis* is the causative agent of Johne´s disease in cattle. Johne´s disease is a slow progressive infection of the intestine that can result in diarrhoea and wasting of the infected cattle.

In addition *M. avium* subsp. *paratuberculosis* has been implicated in Crohn´s disease in humans. Crohn´s disease is a chronic, inflammatory disease of the gastrointestinal

tract, where the presence of granulomatous reaction in infected tissue suggested that the disease could possibly be of mycobacterial origin (Falkinham, 1996).

2.3.2 Other non tuberculous mycobacteria

M. marinum is one of the closest relatives of the *M. tuberculosis* complex bacteria. *M. marinum* causes a tuberculosis-like granulomatous infection in fish and frogs, natural hosts, that are readily studied as laboratory models (Cosma et al., 2003).

Buruli ulcer, caused by environmental *M.ulcerans* is the third most common mycobacterial disease. These mycobacteria secrete a cytotoxic polyketide toxin that produces extensive, painless, necrotic and noninflammatory ulcers where the bacteria grow extracellularly (Casanova and Abel, 2002).

Almost all diseases caused by rapidly growing mycobacteria in humans are due to *M. fortuitum*, *M. chelonae* and *M. abcessus*.

M. fortuitum is responsible for the majority of mycobacterial infections after cardiac bypass surgery or augmentation mammaplasty. In contrast, skin and soft tissue infections are due to *M. chelonae* and *M. abcessus*, and pulmonary infections are most often caused by *M. abcessus*. *M. chelonae* is also responsible for corneal infections. Some cases of presence of *M. chelonae* and *M. fortuitum* in sputum of patients with cystic fibrosis were reported, as well as cases of disseminated infection by those pathogens.

M. fortuitum, *M. chelonae* and *M. abcessus* are widely distributed in the environment. They have been isolated from freshwater rivers and lakes, seawater, from animal drinking troughs, from soils and even from drinking-water samples.

Because of the widespread presence of *M. fortuitum*, *M. chelonae* and *M. abcessus* in the environment and drinking-water systems, everyone is exposed to these rapidly growing mycobacteria.

Like the slowly growing nontuberculous mycobacteria, *M. fortuitum*, *M. chelonae* and *M. abcessus* are resistant to many antimycobacterial drugs but all three species are susceptible to clarithromycin and azithromycin (Falkinham, 1996; Howard and Byrd, 2000)

2.4 The mycobacterial structure

Mycobacteria are very resistant to chemical and mechanical stress due to its unique protective surface, which consists of two distinct layers, the inner plasma membrane and the external cell wall.

The plasma membrane is a typical phospholipid bilayer, which lies underneath a rigid peptidoglycan (PG). A number of proteins are found in association with the membrane, with PG and between the membrane and the PG and some of these may be immunogenic (Barnes et al., 1989).

Peptidoglycan is covalently linked via phosphodiester bonds to arabinogalactan (AG), a polymer of two sacharides arabinose and galactose. These arabinogalactan molecules are attached to large (C60-C90) branched chain fatty acids, the mycolic acids. Associated with the mycolic acids are a number of phenolic and other types of glycolipids.

Lipoarabinomannan (LAM), as well as its related precursors, lipomannan (LM) and phosphatidyl-*myo*-inositol mannosides (PIMs) are also found in the mycobacterial cell wall. PIMs, LM and LAM are major lipoglycans that are non-covalently attached to the plasma membrane through their phosphatidyl-*myo*-inositol anchor and extend to the exterior of the cell wall. The fatty acids, typically palmitate and tuberculostearate, occur in the form of diacylglycerol, linked to the branched arabinose and mannose containing polysaccharide via phosphatidyl-*myo*-inositol.

The LAM molecule in *M. tuberculosis* and *M. bovis* BCG is capped with mannose residues constituting the named manLAM, in contrast to the LAM of fast-growing non-pathogenic mycobacteria which is capped with inositol phosphates. These differences in LAM capping play an important role in the pathogenesis of the mycobacterium, shaping different factors such as macrophage uptake and cytokine induction (Briken et al., 2004; Vermeulen, 1996).

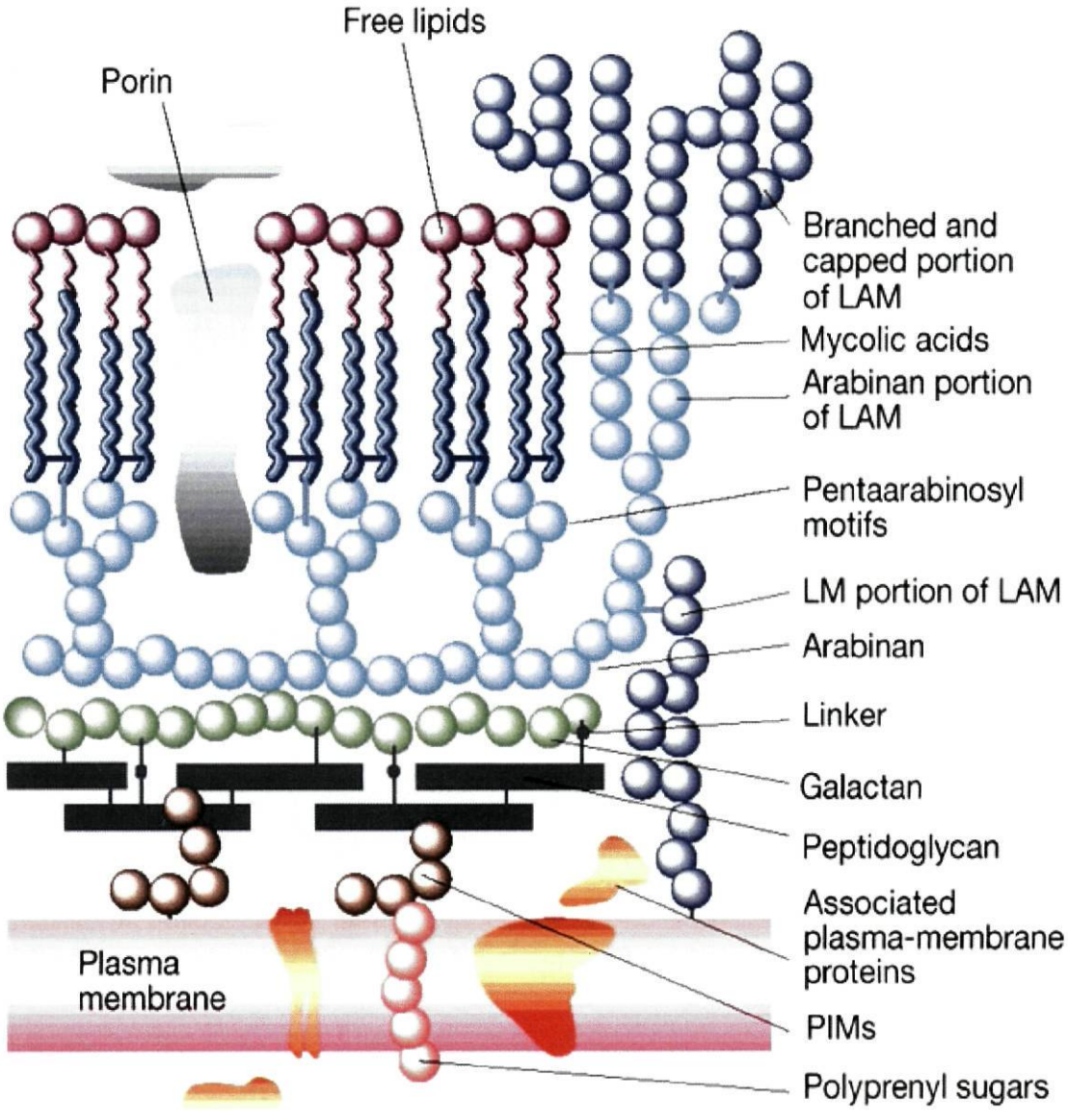

Figure 3. Schematic model of the mycobacterial cell wall (reproduced from (Park and Bendelac, 2000)

3. Mycobacterial proteins

The proteins of *M. tuberculosis* have been isolated and described by classical separation techniques or molecular biological approaches, due to their capacity to stimulate or suppress the human immune response during infection (Collins et al., 1988). These antigens were identified by their abundance in culture supernatants or by the specific recognition by available monoclonal antibodies. Abou-Zeid and

colleagues were the first to propose that a specific subset of antigens is produced by *M. tuberculosis* during the first phase (short-term culture-3 days) of bacterial growth. These secreted proteins have been suggested as protective antigens responsible for the rapid recognition of bacteria by host lymphocytes (Abou-Zeid et al., 1988).

This hypothesis was supported by the next findings: a) whereas immunization with live bacilli efficiently generated protective T lymphocytes, killed preparations did not (Orme, 1988c); b) there is a strong correlation between bacterial multiplication in the host and T-cell reactivity against secreted proteins (Andersen et al., 1991b).

3.1 The components of *M. tuberculosis* – Secreted, cell wall and cytoplasmic proteins.

Some laboratories focused on proteins from *M. tuberculosis* and suggested the division of mycobacterial proteins into three major classes which differ in rate of release and sub cellular localization:

a) Secreted proteins, produced in large quantities by *M. tuberculosis* during the first days of culture. They accumulate in CF but are present in few amounts in the entire bacilli.

b) Cell wall proteins, gradually released from the outer cell wall during growth of the bacilli. Detectable both in CF and in cell wall preparations.

c) Cytoplasmic proteins, released from dead bacteria, appear suddenly in high concentrations during the late logarithmic growth phase (Andersen et al., 1991a; Wiker et al., 1991).

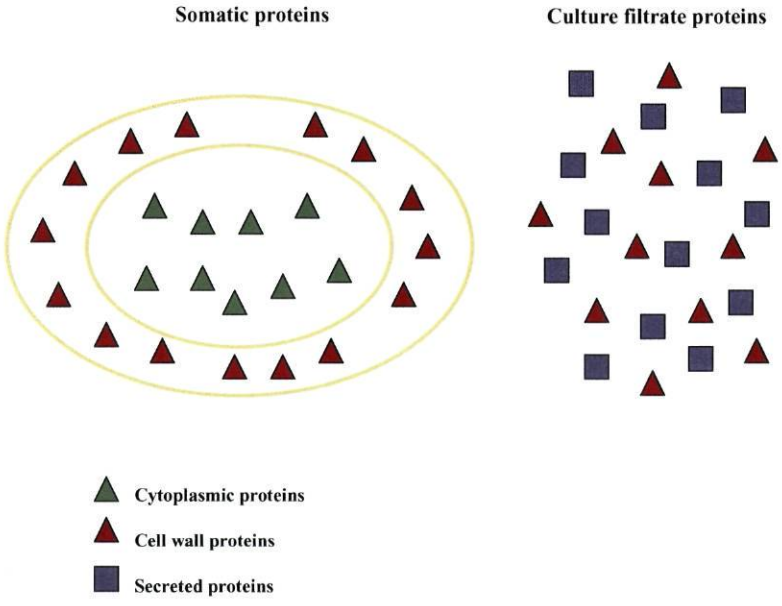

Figure 4. Schematic representation of protein release during growth of *M. tuberculosis*. (Adapted from (Andersen, 1997)

The protein profile of culture filtrate during first 4 days of *M. tuberculosis* growth has been analysed. This culture filtrate was named short-term culture filtrate (ST-CF) and was produced by bacteria in their logarithmic growth phase. ST-CF was enriched in outer cell wall proteins and excreted proteins and poor in autolytic products. The autolysis was monitored by the presence of the metabolic enzyme isocitrate dehydrogenase (ICD), located in the cytoplasm of the bacteria (Andersen et al., 1991a). ST-CF was initially described as containing 33 major protein bands analysed by SDS-PAGE separation. Particularly, the heat shock protein GroES, the MPT64 and the Ag85 complex were found in abundant quantities in ST-CF.

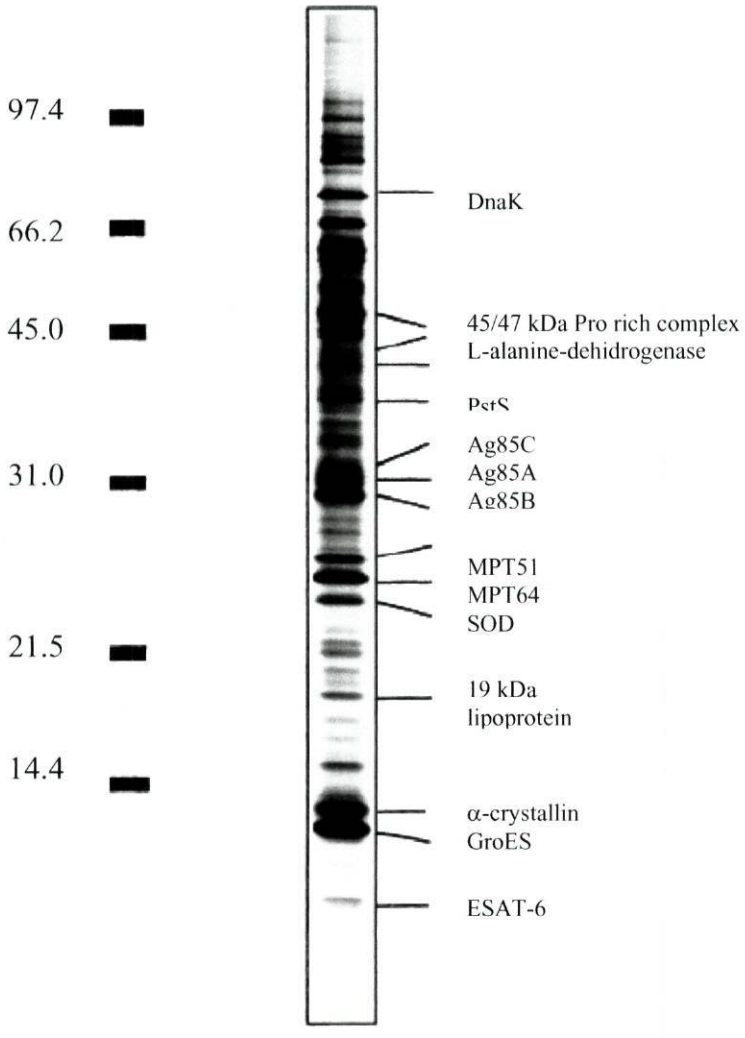

Figure 5. Composition of ST-CF from a logarithmically growing culture of *M. tuberculosis*. (Adapted from (Andersen, 1997)

The secretion of proteins is commonly defined as a passage of these ones across the bilaminar lipid membrane. In prokaryotes it is associated with the presence of a signal peptide at the N-terminus of the protein, which is inserted into the hydrophobic membrane barrier thereby guiding the protein through to the exterior. The signal peptide consists of two or three positively charged amino acids followed by a stretch of 20 to 40 non-polar residues, which is cleaved off from the mature protein by specific signal peptidases. This secretory pathway is known as the Sec-dependent pathway and is probably the major protein export pathway in both Gram-negative and Gram-positive bacteria (Pugsley, 1993). It is suggested that this secretory pathway exist in mycobacteria and many of the cell wall or culture filtrate proteins contain a putative signal peptide (Wiker et al., 1991). The analysis of the *M. tuberculosis*

genome has revealed the presence of genes encoding the referred Sec-proteins (Chubb et al., 1998).

Proteins from the ST-CF, such as ESAT-6, GroES, MPT64, L-alanine dehydrogenase, SOD and DnaK do not contain a putative leader sequence, suggesting that they have a signal peptide-independent secretion (Andersen et al., 1992a; Zhang et al., 1991).

It has been suggested that export of glutamine synthase were directed by the residues sequence and/or the conformation of the protein (Harth and Horwitz, 1997; Harth and Horwitz, 1999). The enzyme superoxide dismutase (SOD), one of the 10 major extracellular proteins of *Mycobacterium tuberculosis*, seems to be exported by information that is contained within the protein but additionally export machinery specific to mycobacteria is required (Harth and Horwitz, 1999).

Heat shock proteins (hsp), **DnaK** (70 kDa), **GroES** (10 kDa) **and GroEL** (65 kDa).

Heat shock proteins were first described and later named to reflect their production by cells exposed to sudden elevations in temperature (Tissieres, 1974). Although cellular stress such as oxygen radicals or ethanol increases the synthesis of heat shock proteins, many are also constitutively expressed and play an important role in normal cell function. They are believed to function intracellularly as molecular chaperons involved in protein folding, unfolding and assembly. A prominent characteristic of these proteins is the highly conserved nature, with extensive sequence homology between different species (Jindal, 1989; Thole, 1990; Young, 1985). The mycobacterial GroES and DnaK are found mutually in the cytoplasm and in the cell surface. The mechanism by which these molecules reach the surrounding media is unknown since the genes encoding these proteins lack a consensus signal sequence (Andersen, 1997; Rocchi et al., 1993).

The 65 kDa protein of *M. tuberculosis* was originally described as a protein antigen of *Escherichia coli* that cross-reacted with an antigen in more than 50 other bacterial species. Homologous proteins are also present in humans with a sequence identity of 40 to 50%.

The L-alanine dehydrogenase (40 kDa) is an enzyme that catalyses the reversible conversion of piruvate to alanine. This enzyme is probably involved in cell wall synthesis, as alanine is one of the three amino acids constituting the repeating peptide subunit of the peptidoglycan layer. The enzyme is present in 4-day-old culture filtrate,

suggesting that is secreted across the cell membrane to participate in the synthesis, although no consensus signal sequence was identified in the gene (Andersen et al., 1992a; Harboe and Wiker, 1992; Wiker and Harboe, 1992).

The phosphate binding protein (38-kDa) is a major constituent of *M. tuberculosis* culture filtrate. It is also present in *M. bovis* BCG culture fluid, but in far lower concentrations.

The 38 kDa is a lipoprotein that binds phosphate and turns it available to the bacteria. This protein is mainly localized in the outer cell wall and only released to the surroundings in limited amounts (Andersen, 1997).

The cloned gene contains a signal sequence that is characteristic of proteins actively secreted from mycobacteria. The sequence contains a lipoprotein consensus element, suggesting that the N-terminal cysteine is acylated with a lipid tail that is probably responsible for partial attachement of the protein to the lipid-rich mycobacterial surface (Harboe and Wiker, 1992).

Ag85 complex (30/32 kDa)

Ag85A (FbpA), Ag85B (FbpB), Ag85C (FbpC2)

Is a complex of three highly related proteins. Although very closely related these three proteins are separate entities encoded by separate genes and transcribed as distinct transcription units (Harth et al., 1996; Wiker and Harboe, 1992). At the DNA and amino acid levels, the 30/32-kDa complex proteins share a high degree of homology not only with each other but also with their counterparts in a number of pathogenic and non-pathogenic mycobacterial species. Homology studies at the genetic level showed only one or five amino acid differences in the Ag85 complex between *M. tuberculosis* and two *M. bovis* BCG strains (Harth et al., 1996).

The molecular masses of the individual components of the antigen 85 complex determined by SDS-PAGE are as follows: antigen 85B, 30 kDa; antigen 85A, 31 kDa, antigen 85C, 31.5 kDa. Antigen 85C appears to be slightly heavier than antigen 85A, and in most SDS-PAGE runs these two components are not properly resolved (Wiker and Harboe, 1992). In Nagai's terminology, MPT44/MPB44, MPT59/MPB59, MPT45/MPB45 correspond to antigens 85A, 85B and 85C, respectively.

These proteins are major secretion products of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. They are expressed in the *M. tuberculosis* phagosome in

infected human monocytes and are mostly localized on the mycobacterial cell wall (Harth et al., 1996). These findings in addition with studies from Belisle revealing that the compex has mycolic acid transferase activity suggests that is involved in the final stages of mycobacterial cell wall assembly (Belisle et al., 1997). The three antigens demonstrate a significant level of fibronectin binding capacity suggesting involvement in complement receptor mediated phagocytosis (Wiker and Harboe, 1992).

CFP29 (29 kDa) is conserved among mycobacterial species, being present in both pathogenic and non-pathogenic bacteria such environmental mycobacteria, suggesting that this protein may play a physiological role in mycobacteria.
Like ESAT-6, CFP29 is released to culture fluids in small amounts.
CFP29 was found in both culture filtrate and in the membrane fraction from *M. tuberculosis*, suggesting that this protein is released from the envelope to culture filtrate during growth of the pathogen. Like other secreted proteins CFP29 does not have a consensus signal sequence (Rosenkrands et al., 1998).

MPT51 (26 kDa) / FbpC
MPT51 (26 kDa) is a protein that has approximately 40% identity with the Ag85 components. The function of this protein remains to be elucidated but it has been suggested that it may represent a new family of non-catalytic alpha/beta hydrolases (Wilson et al., 2004).

The **MPT64** (24kDa) protein belongs to the group of proteins actively secreted by mycobacteria and the gene contains a typical signal sequence (Yamaguchi et al., 1989). The analog MPB64 gene is encoded in the region of difference 2 (RD-2) which is absent in the BCG sub-strains Copenhagen, Pasteur, Glaxo and Tice (Li et al., 1993).
This protein was found to elicit strong skin reactions in *Mycobacterium tuberculosis* infected guinea pigs when compared with BCG infected ones. This results had suggested that MPT64 may be a promising candidate for a specific diagnostic skin test reagent for human TB (Elhay et al., 1998; Haslov et al., 1995).

SOD (23kDa)

Superoxide dismutase exists as a tetramer in its native form with a molecular mass of 88 kDa. This protein appears in very early culture filtrates and contributes to the protection of bacteria from the toxic effect of superoxide radicals during the oxidative burst in the macrophage (Andersen et al., 1991a).

Early secretory antigenic target ESAT-6 family

This family consists of at least 14 low-mass proteins with some homology to ESAT-6 and a defined genomic organization.

ESAT-6 (9.8 kDa) exists in low quantities in ST-CF and can also be detected in cytosol and cell wall of the bacteria. The molecular weight of the purified native protein was found to be 24 kDa, suggesting that this protein exists as a tetramer in its native configuration.

CFP10 is another low-molecular-mass protein found in ST-CF. The gene encoding this protein is encoded together with the *esat-6* gene. Both genes are located in RD-1 (Berthet et al., 1998). **TB10.4, TB10.3 and TB12.9** belong to a subfamily within the esat-6 family, the TB10.4 subfamily.

TB 10.3 and TB12.9 were in general less antigenic than TB10.4 but contain T-cell epitopes, several of which unique to these proteins. The specificity of the T-cell response to these three closely related esat-6 subfamily members is markedly different despite the amino acid homology. This subfamily plus the esat-6 family has been defined as one paralogous gene family meaning that these genes possibly have been duplicated in *M. tuberculosis* during evolution. This duplication of genes encoding major T-cell antigens lead to several copies of proteins that can replace each other functionally but which differ in their immunodominant epitopes. The controlled expression of these genes allows the pathogen to have antigen variation and so escape the host immune defences. The members of the TB10.4 family are present in strains of the *M. tuberculosis* complex, including BCG and *M. kansasii*, and in some species of environmental mycobacteria such as *M. avium*, *M. intracellulare* and *M. marinum* (Skjot et al., 2002).

3.2 Identification of T-cell antigens

Past studies have shown that only vaccination with live mycobacteria can induce long-term specific protective immunity against tuberculosis. On the other hand immunization with dead bacteria and with cell wall proteins elicits a delayed-type hypersensitivity (DTH) response, but are not able to generate protective T cells capable of adoptive immunization against virulent tuberculosis (Orme, 1988b; Orme, 1988c). This findings guide some authors to investigate proteins secreted by live mycobacteria. However, recent work from Andersen and colleagues suggest that experimental vaccines based on killed mycobacteria can confer high levels of protection and that past results would maybe be due deficient adjuvant delivery (Agger et al., 2002).

Living mycobacteria secrete highly immunogenic proteins when compared to those derived from dead mycobacteria. These secreted antigens are the first to be recognized by the immune system during *M. tuberculosis* infection (Orme, 1988a; Orme, 1988c; Orme et al., 1993a). These findings led several studies to analyse the immunogenicity of *M.tuberculosis* culture filtrate proteins. These studies demonstrated that culture filtrate proteins provide high levels of protective immunity in different animal models (Andersen, 1994; Attanasio et al., 2000; Horwitz et al., 1995; Hubbard et al., 1992; Pal and Horwitz, 1992; Roberts et al., 1995; Weldingh and Andersen, 1999).Guinea pigs immunized with extracellular proteins and then challenged with a *M. tuberculosis* aerosol exhibit protective immunity (Pal and Horwitz, 1992). Mice immunized with culture filtrate proteins had a significant reduction in the number of viable bacteria in the spleen and lungs after an aerogenic challenge with *M. tuberculosis* when compared with non immunized controls (Hubbard et al., 1992).

ST-CF is a complex mixture of proteins, and the specific components responsible for the priming of protective T cells needs to be known. To identified these specific antigens several authores using a modified preparative SDS-PAGE technique analyse the cell mediated immunity to different fractions of *M. tuberculosis* culture filtrate. T-cell responses were evaluated by quantifying cellular proliferation and IFN-γ production in stimulated cultures. Two molecular mass regions were reported to induce high levels of IFN-γ: the region of molecular weight below 11 kDa and the

region within 26-35 kDa. Within the 26-35 kDa region four potent single antigens were identified, two of them belonging to the Ag85complex (Andersen et al., 1992b).

In order to continue the identification of immunodominant antigens during *Mycobacterium tuberculosis* infection a suitable method was develop, the multielution technique. The culture filtrate proteins were separated into narrow molecular mass regions by SDS-PAGE followed by elution in the Whole Gel Eluter. This method yields 15 to 30 protein fractions and each pool contains only a few protein bands all in the same molecular mass region. These protein fractions are obtained in a physiological buffer suitable for subsequent analysis in cell cultures (Andersen and Heron, 1993). These fractions were then used to stimulate cells obtained either from *M. tuberculosis* infected mice or from human TB patients. The level of production of IFN-γ by T cells is used to measure the immunological importance of each protein fraction (Andersen et al., 1995; Boesen et al., 1995).

Several studies have been conducted since then in order to find major antigens recognised during *M. tuberculosis* infection by T-cells.

Proteins of the Ag85 complex have been demonstrated to be immunoprotective in the guinea pig model of pulmonary tuberculosis (Horwitz et al., 1995) and to be major target antigens of the long-lived immunity mediated by highly reactive memory effector $CD4^+$ T cells in mice (Andersen et al., 1995).

The low molecular mass secreted antigen ESAT-6 is an early and dominant T cell target during TB infection in experimental animal models (Andersen et al., 1995; Brandt et al., 1996; Sorensen et al., 1995) and in TB patients (Mustafa et al., 1998).

Antigens from the esat-6 family have been thoroughly studied. CFP-10 is strongly recognized by TB patients, but not by BCG-vaccinated donors, with levels of IFN-γ comparable to or higher than those of ESAT-6 (Skjot et al., 2000). TB10.4 protein is strongly recognized and induced IFN-γ production, at an even higher level than ESAT-6, by T-cells from human TB patients and *M. bovis* BCG vaccinated donors (Skjot et al., 2000) .

Five proteins that do not belong either to the 30/32-kDa regions or the low mass molecular region were identified as molecules which can promote protective immunity to TB. CFP17, CFP20, CFP21, CFP25 and CFP29 were strong IFN-γ inducers in *M. tuberculosis* infected mice and in tuberculosis patients (Weldingh and Andersen, 1999; Weldingh et al., 1998).

Two new proteins, MTSP17 (17 kDa) and MTSP11 (11 kDa), have been reported to induce strong IFN-γ production in cells isolated from healthy tuberculin reactors, comparable to those induced by Ag85 complex (Lim et al., 2004).

4. Bacille Calmete Guérin

4.1 History of BCG vaccine

The search for a vaccine against TB began 115 years ago. In 1882, Robert Koch discovered that tuberculosis was caused by *Mycobacterium tuberculosis* and 8 years later he produced a heat inactivated culture filtrate, known as Koch old tuberculin (Brewer and Colditz, 1995; Grange, 2000). The second attempt in developing a TB vaccine was initiated later by two French scientists of the Institute Pasteur, Albert Calmette and Camille Guérin. It was Nocard the first that had isolated the virulent strain of *Mycobacterium bovis* from the udder of a tuberculous cow. In 1908 Calmette and Guérin started a series of subcultures of this mycobacterium on glycerol-potato-bile medium. Thirteen years and 230 transfers later, they had a mycobacterium that they believed was harmless to animals. This vaccine was named BCG (bacille Calmete Guérin), and is used worldwide since then.

After some BCG vaccine experiments in animal models, in 1921 Weill-Hallé and Turpin gave a dose of oral BCG to a child whose mother had died from tuberculosis. The results were great, since the child did not develop tuberculosis and no secondary effects occurred. Between 1921 and 1924 the vaccine was given to more than 300 children, and since then it was distributed worldwide. Although Calmette and Guérin claimed that BCG had only one avirulent strain type, others, such Petroff and colleagues showed that BCG colonies develop two different morphologies when cultered on egg medium and that one of this was able to cause progressive disease in animals. In 1930, a tragedy took place in Lubeck, Germany, among 249 infants that had received what was thought to be BCG vaccine, 67 developed tuberculosis or even died. The BCG vaccine used was contaminated with a virulent strain of the tubercle bacillus. Since then many trials were conducted to evaluate the efficacy and the safety of the BCG vaccine (Brewer and Colditz, 1995).

4.2 How BCG works

Orme and colleagues demonstrated that after immunization of mice with BCG a population of $CD4^+$ T lymphocytes emerge. These cells secrete IFN-γ when stimulated in vitro with purified secreted proteins from the bacillus (Orme, 1988c). These activated $CD4^+$ T cells develop a memory T cell phenotype that remains long lived and re-circulating (Orme, 1988a). In subsequent challenge with *M. tuberculosis* the progression of the infection by this pathogen is slowed down and there is a rapid granuloma formation in the lungs. This granuloma occurs due to the rapid recognition of the primary lesion by memory T cells that cross the inflamed local blood vessel and recognize antigen being presented by infected macrophages. Vaccinated individuals have this earlier response when compared with unvaccinated ones. This accelerated response prevents dissemination of the bacteria from the primary lesion to other sites of the lung and to other organs, such as spleen and liver where secondary lesions can be established. However, this earlier generation of immunity in immunized mice does not lead to the resolution of infection. Recent studies have shown that although immunized mice generate Th1 immunity to an *M. tuberculosis* infection sooner than naïve mice, there is no motive to consider that the secondary response is quantitatively or qualitatively superior to the primary response (Jung et al., 2005).

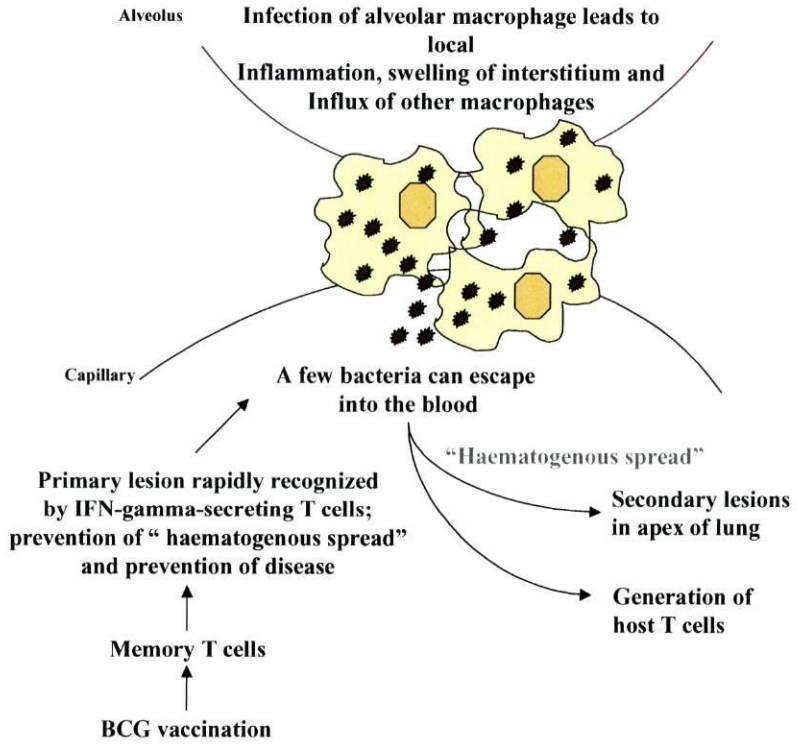

Figure 6. Schematic representation of the role of BCG vaccine in the fate of *M. tuberculosis* infection (adapted from (Orme, 1999))

4.3 Efficacy of BCG vaccine

Although BCG is highly efficacious in laboratory models of disease (Smith, 1985) , it has varied tremendously in protective efficacy in field trials, and in some geographical regions the vaccine has not shown any efficacy at all (Fine, 1995; Ponninghaus et al., 1992).

The debate about the effectiveness of BCG vaccine is reflected in the different national vaccination strategies around the world. Only the USA and the Netherlands have not promoted universal BCG vaccination against TB, although both countries recommended BCG vaccine for high-risk groups. In Europe the WHO recommendation concerning BCG vaccination is a single dose at birth, however there are some northern countries that are moving towards selective vaccination (KPTG, 1996).

The use of BCG vaccine has been controversial for decades due to the contrasting results from clinical trials that evaluate its efficacy and the debate related to these

differences. From 1927 to 1968, 21 controlled clinical trials of the efficacy of BCG were initiated in 10 countries, 19 were completed and evaluated the protection conferred by the vaccine which ranged from 0% to 80% with different vaccines in different settings (Cohn, 1997).

Striking differences had emerged between a major trial in the United Kingdom by the Medical Research Council, which showed more than 75% protection, and trials by the United States Public Health Service in Georgia, Alabama, and Puerto Rico, which all recorded less than 30% protection (Fine, 1995).

A study in Karonga district, northern Malawi, analysing the protective efficacy of BCG (Glaxo, freeze dried) vaccine against tuberculosis and leprosy showed that there was no statistically significant protection by BCG against tuberculosis in this population, despite a protection of 50%, or more, against leprosy (Ponninghaus et al., 1992).

In a trial performed in Chingleput, India, with more than 200,000 participants, BCG showed no efficacy at all (Fine, 1995).

In a meta-analysis of 14 prospective trials and 12 case-control studies measuring the efficacy of BCG vaccination in preventing TB cases and/or deaths, the authors conclude that the overall protective effect of BCG was 50%. In addition the BCG vaccine protected against pulmonary TB as well as against disseminated TB (78% protective effect), tuberculous meningitis (64% protective effect) and death (71% protective effect) (Colditz et al., 1994).

Evaluation of household contacts of known cases of tuberculosis also showed a protective efficacy of 53% to 74% in those contacts that received the BCG vaccine (Fine, 1995).

The reasons suggested for the wide variation in efficacy of BCG in those trials include: differences in vaccine strains, methods of vaccine administration, virulence of *M. tuberculosis*, risk of primary infection versus reactivation, susceptibility determined by genetic background of the population, age at vaccination and even study methodologies (Cohn, 1997; Fine, 1995). Besides all these possible explanations one that has been most generally accepted relates the efficacy of BCG with the exposure to nontuberculous mycobacteria.

The BCG strains used in clinical trials differ from the original Bacille Calmete Guérin and differ between each trial. These differences may be related to colony morphology, biochemical characteristics, DNA restriction fragments, and degree of virulence.

Although differences among BCG strains have been suggested as being one source of efficacy variation, the fact that similar vaccines perform very differently in different settings indicates that this cannot be the entire explanation. The Glaxo freeze-dried BCG is a good example, when analysing the good protective efficacy conferred by this vaccine in England but not in Malawi (Ponninghaus et al., 1992). In addition the Danish BCG which performed well in the original British trial, provided very little protection in the South India/Chingleput trial (Cohn, 1997; Fine, 1995).

Meta-analysis suggests that the type of BCG strain is not significant in determining the overall efficacy of the vaccine (Brewer and Colditz, 1995; Cohn, 1997). Recent studies suggest that different BCG strains have evolved and differ from each other and from the original BCG first used in 1921, however these mutations have yet to be shown to affect BCG associated protection (Behr, 2002).

The variation of efficacy of the BCG vaccine has been suggested to be related to differences in risk of infection, to differences in *M. tuberculosis*, or to differences in pathogenesis of the disease. The correlation between BCG efficacy and risk of infection has been refuted by studies in Britain where the vaccine appears to be constantly effective over a period when the risk of infection fell by more than 95% (Fine, 1988).

Studies in guinea pigs have shown that the protection imparted by BCG vaccines may differ according to the strain of *M. tuberculosis*, but they have shown no evidence for poor protection against the low virulence strains (Hank et al., 1981).

There are significant observations that suggest that BCG would protect better against systemic disease than against pulmonary disease. A meta-analysis of all randomised controlled trials and case-control studies of the effect of BCG vaccine against TB since 1950 to 1993 led to the conclusion that BCG protects against miliary and meningeal TB, while it has a heterogeneous protective effect against pulmonary TB (Rodrigues et al., 1993).

There is no evidence that the variation in the protection imparted by BCG is related to genetic factors in human populations. Protective efficacy was higher among the white than black race in one trial in the USA, but the difference was not statistically significant. On the other hand studies on vaccinated Asians and British infants indicate equal efficacy of the BCG vaccine in both populations (Fine, 1988).

Differences in geographic latitude were a significant determinant in variance between trials. Colditz and colleagues concluded that latitude could explain 41% of the

variance between published studies Those studies performed closest to the equator (warm and moist climates), where exposure to environmental mycobacteria is very high, showed a diminished efficacy of the BCG vaccine when compared with trials in other latitudes (Colditz et al., 1994).

Study	Latitude	Urban/Rural	Vaccine efficacy
Norway, gen pop	65	U+R	81%
Sweden, gen pop	62	U+R	80%
Sweden, military	62	U+R	55%
Denmark, school	56	U	94%
Ireland, school	55	R	82%
Canada, indians	55	R	81%
Canada Alberta, indians	55	R	57%
Canada Manitoba, indians	55	R	70%
UK, school children	53	U+R	77%
UK, gen pop 1973	53	U+R	79%
UK, gen pop 1978	53	U+R	74%
UK, gen pop 1983	53	U+R	75%
UK, Asians	53	U	49%
UK, Birminghan Asians	52	U	64%
UK, Birminghan	52	U	88%
USA, Indians	52	R	79%
USA, Chicago infants	42	U	72%
USA, New York infants	41	U	7%
Korea, Seoul	38	U	74%
Aegentina, Buenos Aires	35	U	73%
USA, Georgia school	33	U+R	-56%
USA, Georgia Alabama gen pop	33	U+R	16%
Israel, children	31	U+R	38%
South Africa, miners	27	U+R	62%
Australia, Queensland	20	U+R	41%
Puerto Rico	18	R+U	29%
Haití	18	R+U	80%
Burma, Rangoon	17	U	38%
Thailand, Bangkok(1)	14	U	74%
Thailand, Bangkok(2)	14	U	83%
Thailand, Bangkok(3)	14	U	47%
India, Madanapalle	13	R	20%
India, Chingleput	13	R	-19%
Papua New Guinea	10	U+R	41%
Malawi, Karonga	10	R	-11%
Indonesia, Jakarta	6	U	37%
Togo Lome	6	U	66%
Columbia, Cali	4	U	16%
Cameroun, Yaounde	4	U	66%
Kenya, Kisumu	0	R	22%

Figure 7. Estimate efficacy of BCG against pulmonary tuberculosis in trials and observational studies, by latitude (reproduced from (Fine, 1995))

There are many species of mycobacteria in the environment and individuals exposed to these bacteria develop a sensitivity to them as detected by specific reactivity to skin tests with antigens from various mycobacteria or by moderate levels of non-specific

reactivity to tuberculin. Some data reveal that in the tropics there is a high prevalence of individuals reacting to antigen from *Mycobacterium avium intracellulare*. In the South India/Chingleput trial area 90% of individuals with 10-14 years age and 95% of older individuals were strongly positive to Purified Protein Derivative B (PPD-B), a complex mixture of proteins from *M. avium intracellulare*.

Other studies have found the prevalence of non-specific tuberculin sensitivity to be higher in the rural than in the urban areas in the same region. In the Puerto Rico trial it was found that in rural areas where the non-specific tuberculin sensitivity was elevated, the observed efficacy of BCG was lower, in contrast to the urban areas (Fine, 1995).

Palmer and colleagues were the first to suggest that the pre-sensibilization with the environment mycobacteria could impart some protection against TB, and that BCG could do little to improve such naturally acquired protection. They exposed guinea pigs to *M. avium*, *M. fortuitum*, *M.gause* and *M.kansasii* and then gave them BCG. After this intradermal infection the animals were challenged with *M. tuberculosis* H37Rv. The protection imparted by BCG, administered after exposure to the environmental mycobacterial species, was not independent of the previous exposure, but represent only the difference between the protection imparted by each one alone (Palmer and Long, 1966).

Experiments done by Edwards et al, where vaccination with *M. avium-intracellulare* protected against H37Rv, and against a low-virulence strain of *M. tuberculosis*, isolated from the Chingleput/South India population, gave similar protection as those provided by Danish BCG (Edwards et al., 1982).

Some other experiments found out that prior aerosol infection with *M. avium* was as effective as intravenous BCG in protecting mice against aerosol challenge with H37rv (Orme and Collins, 1984).

Brown and colleagues had shown that exposure of mice to *M. vaccae* in drinking water before BCG vaccination influenced the response of splenic cells to mycobacterial antigens in a way (can either enhance, mask or interfere) dependent of dose and timing of exposure to that environmental mycobacteria (Brown et al., 1985).

In the same way, there are studies showing some evidence that exposure to environmental mycobacteria can protect against tuberculosis in man and interfere with BCG vaccination. Studies of US Navy personnel, where the individuals were tested

with PPD-B, the lowest tuberculosis risks were found in individuals whose reactivity to PPD-B was greater than to human tuberculin (Edwards et al., 1973).

In BCG trials, where individuals with any prior tuberculin sensitivity were excluded, a high efficacy of the vaccine was found (Fine, 1995).

The typical decline of BCG efficacy in some trials such as the British Medical Research Council and the South India/Madanapale trial, in which the estimated efficacy fell from 80% to nil over a period of 20 years after vaccination, may be predictable as a result of continued exposure of the trial population to environmental mycobacteria (Fine, 1995). There are consistent studies that show the success of BCG vaccination in neonates in preventing tuberculosis, before any significant sensibilization with environmental mycobacteria occurs. BCG vaccination of newborns and infants significantly reduces the risk of tuberculosis by over 50% (Colditz et al., 1995). This neonatal BCG vaccination confers protection and induces a type 1 immune response (Marchant et al., 1999), older children and adults are primed for either protective or tissue-necrotizing (Koch-type) immune responses by prior contact with different populations of saprophytic mycobacteria in the environment, and that BCG vaccination boosts the pre-selected response (Rook et al., 1981; Stanford et al., 1981).

Globally these results suggest that environmental mycobacterial exposure may influence BCG protection by providing a degree of partial protection on which BCG cannot improve (masking hypothesis) or by "immunizing" the vaccinee against the vaccine bacilli, which need to multiply to induce effective immunity (Andersen and Doherty, 2005; Fine, 1995; Orme and Collins, 1984).

These two prevailing hypothesis are based on the close relationship of species within the genus *Mycobacterium*. Environmental mycobacteria besides having markedly different lifestyles from the intracellular pathogens *M. tuberculosis* and *M. bovis*, have several characteristics in common, such as a lipid-rich outer cell wall, genomes with a high GC content and many similar gene families. There are many cross-reactive antigens between different mycobacterial species, being some of the immunodominant antigens, such as Ag 85 complex, highly conserved.

These cross-reactive antigens are responsible for the success of the BCG vaccine and it is also because of the existence of these cross-reactive epitopes that the efficacy of this vaccine is affected. BCG vaccination is a double-edged sword in areas of high

prevalence of environmental mycobacteria, due to the close relationship between different mycobaterial species.

It was suggested that those cross-reactive epitopes present in environmental mycobacteria might induce either protective responses or responses that increase susceptibility to *M. tuberculosis*, these latter ones associated with a Th2-like immune response, which is boosted by the BCG vaccine (Hernandez-Pando et al., 1997).

The differences in protection conferred by BCG against leprosy and tuberculosis, correlate with some studies where the vaccine is more effective in preventing miliary tuberculosis and tuberculosis meningitis than against pulmonary disease (Rodrigues et al., 1993). It seems that BCG is more consistently effective against systemic mycobacterial infections (including leprosy and miliary and meningitic tuberculosis) than against local pulmonary disease. The protection conferred by BCG, as with most vaccines, is not life-long. There are studies suggesting that BCG is protective for only 10 to 20 years (Sterne et al., 1998). The period in which BCG induced protection wanes, between childhood and young adult, coincides with the gradual increase in TB. For that reason it seems reasonable to suggest that the main reason for BCG ineffectiveness against adult pulmonary TB is that newborn/childhood vaccination only provides protection for that limited period of time (Andersen and Doherty, 2005).

4.4 Safety of BCG vaccine

BCG is considered a safe vaccine. Nevertheless, BCG vaccination by itself may cause a variety of infectious adverse effects. The majority of the reactions occur often at the site of the intradermal injection and are characterized by a local superficial ulcer, or pustule, which results in a scar with occasional keloid formation. Severe or prolonged ulceration may occur in a small percentage of vaccinees. Regional, cervical or axillary lymphadenitis (BCG-itis) may also occur, but more frequently in newborns, and varies by strain, number of viable bacteria in the vaccine, dose and method of administration. Rare complications such as erythema multiform, iritis, lupus vulgaris and osteomyelitis may also occur. Finally the most acute complication of the BCG vaccine, the disseminated infection (BCG-osis) and consequent death, is generally considered to be rare but may be frequent in certain epidemiological contexts, such as

immunodeficiences (AIDS, hairy cell leukaemia and bone marrow and organ transplantation) and human inherited disorders (cystic fibrosis; Mendelian immune disorder in its two forms, classical primary immunodeficiency, PID, and Mendelian susceptibility to mycobacterial disease, MSMD) (Casanova and Abel, 2002).

4.5 Tuberculin skin testing (TST)

Tuberculin or Purified Protein Derivative (PPD) has been used for the past 50 years to support diagnosis of tuberculosis in the clinic. PPD, which is a complex mixture of denaturated proteins from *M. tuberculosis*, induces a delayed-type hypersensitivity response (DTH), detectable as swelling and reddening at the site of intradermal injection after 24-48 hours, in patients with mycobacterial sensitisation. This mycobacterial sensitisation was assumed to be due to *M. tuberculosis* infection. However, PPD is not specific to *M. tuberculosis* since many mycobacterial proteins are conserved among the genus *Mycobacterium*. The consequence of this fact was the emergence of false positives, in the diagnosis of TB, since BCG vaccinated individuals and individuals exposed to NTM may react to a PPD skin test. The magnitude of this "shared" mycobacterial proteins, in the efficacy of BCG vaccination will be further discussed.

The BCG vaccine results in positive conversion of tuberculin skin tests in most individuals and the extent of this hypersensitivity is variable. However there is little correlation between the skin test conversion rates, size of induration, and protective immunity. The revaccination of negative skin test individuals has minimal risk, but on the other side no evidence shows that repeated BCG vaccination confers additional protection against TB (Cohn, 1997; Fine, 1989). In Malawi, a study in BCG-scar positive persons revaccinated with BCG or killed *M. leprae* vaccine revealed no evidence that those vaccines gave further protection against TB (KPTG, 1996). For these reasons, the World Health Organization (WHO) recommended that tuberculin skin testing should be used to select persons for possible repeat BCG vaccination.

A species-specific reagent is required that distinguish BCG vaccinated individuals or individuals exposed to EM from patients infected with *M. tuberculosis*.

The identification of regions in the *M. tuberculosis* genome that are not present in both *M. bovis* BCG and in environmental mycobacteria affords the opportunity to develop new specific diagnostic tools.

Two antigens from *M. tuberculosis*, ESAT-6 and MPT-64 are capable of inducing a delayed-type hypersensitivity skin response in guinea pigs infected with *M.tuberculosis* but not in those sensitised with *M. bovis* BCG or *M. avium* (Elhay et al., 1998). Another species-specific antigen is the CFP-10 that is found in the same strongly stimulatory fraction of culture filtrate as ESAT-6 and with the same limited distribution outside the *M. tuberculosis* complex. These results introduce novel candidates to a new skin test for TB (Andersen et al., 2000).

Strain tested	Antigens		
	ESAT-6	CFP 10	MPT 64
Tuberculosis complex			
M. tuberculosis	+	+	+
M. africanum	+	+	+
M. bovis	+	+	+
BCG substrains			
Gothenburg	-	-	+
Moreau	-	-	+
Tice	-	-	+
Tokyo	-	-	+
Danish	-	-	-
Glaxo	-	-	-
Montreal	-	-	-
Pasteur	-	-	-
Environmental strains			
M. abcessus	-	-	-
M. avium	-	-	-
M. branderi	-	-	-
M. celatum	-	-	-
M. chelonae	-	-	-
M. fortuitum	-	-	-
M. gordonii	-	-	-
M. intracellulare	-	-	-
M. kansasii	+	+	-
M.malmoense	-	-	-
M. marinum	+	+	-
M.oenavense	-	-	-
M. srofulaceum	-	-	-
M. smegmatis	-	-	-
M. szulgai	+	+	-
M. terrae	-	-	-
M. vacae	-	-	-
M. xenopi	-	-	-

Figure 8. Distribution of diagnostic antigens in mycobacterial species (adapted from (Andersen et al., 2000)

4.6. The need for a more-effective vaccine.

Besides all efforts in understanding the biology and pathogenesis of tuberculosis, this disease remains one of the most important threats to world health. Current vaccination and prevention strategies are inadequate and there is an urgent need for a new vaccine

and new diagnostic reagent. Although BCG is cheap, safe and widely used, it is not ideal due to the variation of protection in different populations around the world.

In general, an efficient vaccine must elicit a protective and memory immune response, be focussed against conserved antigenic regions, be immunogenic in a genetically heterogenous population and induce immune responses that can be recalled by natural infection. An ideal vaccine against tuberculosis may have to contain different antigenic epitopes to control infection at different stages of pathogenesis. Immunogenicity and feasibility studies and controlled trials will necessitate careful planning to avoid some mistakes of the past. In addition, HIV infection, geography and exposure to NTM are variables that will need to be considered in the design of future vaccines.

During the past decade there has been a significant increase in the development of improved vaccines against TB. These advances result mainly from the completed *Mycobacterium tuberculosis* genome and the progress in molecular biology, that have made possible the discovery and characterisation of genes and antigens of *Mycobacterium tuberculosis*.

The leading TB vaccine candidates are live mycobacterial vaccines, subunit vaccines and DNA vaccines.

Live mycobacterial vaccines are achieved by attenuated forms of *M. tuberculosis* and also by genetically modifying the current BCG vaccine. One strategy is to start from virulent *M. tuberculosis* and to reproduce the attenuation efforts of Calmette and Guérin using modern genetic tools to remove selected antigens. A second strategy is to build on the existing BCG vaccine, boosting expression of selected antigens.

The loss of important genes in the continuous attenuation of BCG vaccine, may be the main reason for the loss of efficacy of this vaccine (Behr and Small, 1997). It is calculated that near 129 ORF's are absent from various BCG strains. The proteins encoded by these ORF's may play an important role in T cell recognition of *M. tuberculosis* infection, and are not provided by current BCG vaccination. One of the most studied regions that was deleted during the first phase of BCG attenuation process between 1908 and 1921 is the Region of Difference-1 (RD-1). Recent studies in guinea pigs show that *M. bovis* strains that hold knockout mutations in the RD1 region are much less virulent (Wards et al., 2000). RD1 encodes at least three well know antigens, ESAT-6, CFP-10 and PPE68 (RV3873). These antigens are recognized by the majority of individuals carrying potential latent/early *M.*

tuberculosis infection, suggesting that these proteins are present during the early stages of infection and play an important role in the interaction with the host immune system.

Recombinant BCG exporting ESAT-6 (RD-1) induced an increased protection against tuberculosis compared to BCG (Pym et al., 2003). This vaccine shows the capacity to trigger an activation/inflamation program comparable to that induced by H37Rv (Majlessi et al., 2005).

A new vaccine using *Mycobacterium microti* where the complete region of difference 1 (RD-1) was reintroduced displayed better efficacy against disseminated disease in the mouse and guinea pig models of tuberculosis than BCG or *M. microti* alone (Brodin et al., 2004).

A recombinant BCG vaccine (rBCG30) that over expresses Ag85B protein from *M. tuberculosis* induced stronger protective immunity against aerosol challenge with *M. tuberculosis* than conventional BCG vaccine (Horwitz et al., 2000). This vaccine is currently in clinical trials. These results show that supplementing deleted genes back to BCG or increasing the expression of immunodominant genes might improve the effects of current BCG vaccination.

Recent studies lead to the construction of novel BCG recombinant strains expressing Ag85B and ESAT-6 fusion proteins of *M. tuberculosis* (Shi et al., 2005). They are new vaccine candidates for preventing tuberculosis. Works from Dhar and colleagues with recombinant BCG over expressing Ag85A and Ag85C demonstrate that these strains elicit an increased humoral response and Ag85A an increased Th1-like response in mice, when compared to wild-type BCG (Dhar et al., 2004).

Another recombinant vaccine that had already entered clinical trials, MVA85A, is based on recombinant modified *vaccinia virus Ankara* expressing Ag85A from *M. tuberculosis*. This kind of vaccine that uses live vectors such as recombinant *vaccinia* or adenovirus needs no adjuvant, because the vectors themselves elicit strong cell-mediated immune responses. MVA85A significantly boosted BCG-primed and naturally acquired anti-mycobacterial immunity in humans (McShane et al., 2004).

Another attempt to optimise BCG vaccination has been the development of a recombinant BCG strain (rBCG::ΔureC-llo^{+}) that is a urease-deficient mutant, unable to arrest phagosome maturation and that expresses the *lysteriolysin O* gene from *Lysteria monocytogenes*. The *lysteriolysin O* is thought to damage the phagosome

membrane, potentially increasing the amount of bacterial-derived antigen available for presentation to CD8 T cells through the cytosolic scavenger pathways (Conradt et al., 1999; Grode et al., 2002). The rBCG::ΔureC-llo^{+} is expected to enter clinical testing soon.

Sub-unit vaccines are based on selected antigens involved in the protective immunity against the pathogen of interest, in this case against *M. tuberculosis*.

In addition to the selected antigens, this kind of vaccine must have additional factors to stimulate a cell-mediated immune response. Until recently, there has been no effective adjuvants or antigen delivery systems for inducing effective Th1/or CTL responses for use in humans. Adjuvants used with licensed vaccines include aluminium salts and the squalene-based adjuvant, MF-59, neither of these being particularly effective in TB vaccination. This situation is changing, as different vaccination strategies have entered clinical trials. Recent developments in research brought out some new adjuvants, such as IC31 from Intercell and the AS02 from GlaxoSmithKline, both with proven efficacy and safety in animal models (Lingnau et al., 2002; Skeiky et al., 2004). Corixa´s Monophosphoryl Lipid A (MPL) is another currently used adjuvant that is a chemically modified and biologically attenuated derivative of lipopolysaccharide (LPS) from *Salmonella minessota*. MPL is able to stimulate antigen-presenting cells (APC) through binding of the Toll-like receptor 4, leading to the production of TNF-α, IFN-γ and IL-12. These cytokines have all been implicated in protective immune responses against TB. Currently the most effective adjuvants inducing protective immune responses with protein-based subunit vaccines, such as AS02, contain MPL.

Other adjuvants like those which associate liposomes and immunostimulating complexes are now being developed (Fonseca et al., 2000; Holten-Andersen et al., 2004).

Subunit vaccines can be purified native proteins, synthetic peptides or recombinant proteins. The Early Secreted Antigen Target (ESAT-6) was found to be an immunodominant target for IFN-γ producing T-cells from infected mice (Andersen et al., 1995). A particular attribute of ESAT-6 is that the gene encoding this protein is missing from the BCG vaccine, and it can therefore be used to distinguish *M. tuberculosis* infection from BCG vaccination (Harboe et al., 1996). ESAT-6 as a sub-unit vaccine was shown to elicited a strong specific T-cell response and protective

immunity comparable to that achieved with *Mycobacterium bovis* BCG (Brandt et al., 2000).

The members of the Ag85 family, both Ag85A and Ag85B have been shown to be major targets of human T cell responses to *M. tuberculosis* and leading vaccine candidates. In guinea pigs vaccination with Ag85B induces protective immunity against aerosol challenge with *M. tuberculosis* (Olsen et al., 2004). From some works it has become clear that a subunit vaccine containing multiple antigenic epitopes may be necessary to guarantee a complete coverage of a genetically heterogeneous population. A fusion protein consisting of ESAT-6 and Ag85B was shown to promote a strong immune response which was highly protective against TB in the mouse, guinea pig and non-human primate models (Langermans et al., 2005; Olsen et al., 2001; Olsen et al., 2004). This vaccine was more protective in both mouse and guinea pig animal models than either of the single components (Doherty et al., 2004). Clinical trials to test the Ag85B-ESAT-6 started running in 2005.

ESAT-6 is widely used as a diagnostic reagent, so the replacement of this antigen in the Ag85B-ESAT-6 vaccine seems to have great value. Recent studies using the Ag85B and TB 10,4 fusion vaccine show a high level of protection against TB in the mouse model comparable to both Ag85B-ESAT-6 and BCG and superior to the individual antigen components (Dietrich et al., 2005). This vaccine is a potent candidate as a leading vaccine against tuberculosis.

Finally, another recombinant vaccine is a fusion molecule of two proteins, the Rv1196 (PPE family) and Rv0125 (serine protease) from *M. tuberculosis*, the Mtb72F. Mtb72F immunization resulted in the protection of mice and guinea pigs against aerosol challenge with a virulent strain of *M. tuberculosis*. In addition, immunization of guinea pigs with Mtb72F, delivered either as DNA or as a rAg-based vaccine, resulted in longer survival when compared with BCG after aerosol challenge with virulent *M. tuberculosis*. Mtb72F in AS02A formulation is currently in phase I clinical trial, making it the first recombinant tuberculosis vaccine to be tested in humans (Brandt et al., 2004; Skeiky et al., 2004).

First steps are being taken in the development of an epitope-driven tuberculosis vaccine. Epitope-driven vaccines are created from selected sub-sequences of proteins, named epitopes, derived by scanning the protein sequences of *M. tuberculosis* for patterns of amino acids that permit binding to human MHC molecules. A prototype

vaccine that contains epitopes from secreted proteins from *M. tuberculosis* elicited epitope-specific T-cells responses in mice (De Groot et al., 2005).

DNA vaccines consist of the foreign gene of interest cloned into a bacterial plasmid. The plasmid DNA is engineered for optimal expression in eukaryotic cells. Plasmid DNA vaccines can induce both strong cellular and humoral immune responses against the encoded antigen, without the need of any adjuvant or delivery vehicle ("naked DNA"), in a variety of murine and primate disease models. Several mycobacterial antigens delivered as "naked DNA" have shown to be effective in reducing bacterial counts in mice following *M. tuberculosis* aerosol challenge. Examples of antigens that protect mice when delivered as DNA are, Ag85 (Baldwin et al., 1999; Huygen et al., 1996), Mtb8.4 (Coler et al., 2001), Mtb39 (Dillon et al., 1999), Mtb41 (Skeiky et al., 2000), MPT-63 and MPT-83 (Morris et al., 2000), hsp65 (Lowrie et al., 1999), PstS-3 (Tanghe et al., 1999), ESAT-6 and MPT-64 (Brandt et al., 2000; Kamath et al., 1999) and the 38 kD lipoprotein (Fonseca et al., 2001).

Another effort to solve the problem of 4 billion people already vaccinated with BCG is boosting these individuals with a vaccine capable of significantly enhancing the level of protective immunity induced by BCG. A booster vaccine for BCG, comprising the purified recombinant *Mycobacterium tuberculosis* 30-kDa proteins had greatly increased cell-mediated and humoral immune responses to that protein. This vaccine had significantly enhanced protective immunity against aerosol challenge with highly virulent *M. tuberculosis* in the guinea pig model (Horwitz et al., 2005).

5. Aim of the thesis

The aims of this thesis were:

1) To characterize the T-cell response to environmental mycobacteria protein antigens using a mouse model of infection.
2) To study the influence of pre exposure to environmental mycobacteria in the efficacy of subsequent BCG vaccination.
3) To find out the specific cross-reactive antigens between environmental mycobacteria and *Mycobacterium bovis* BCG that are responsible for the block of *M. bovis* BCG multiplication.

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Chapter 2

Antigen specificity of T-cell response to *Mycobacterium avium* infection in mice.

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Antigen Specificity of T-Cell Response to *Mycobacterium avium* Infection in Mice

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T cells from *Mycobacterium avium*-infected C57BL/6 mice reacted to culture filtrate, envelope, and cytosol proteins and to fractions obtained from these proteins. Multiple targets were recognized, such as 29- to 45-kDa and <21-kDa antigens of the culture filtrate, antigens of around 30 kDa in the envelope and cytosol, and 45- to 116-kDa proteins in the envelope.

The identification of the key antigenic targets of the immune response to mycobacteria is of pivotal importance in the design and testing of new vaccine candidates against mycobacterial pathogens, most notably *Mycobacterium tuberculosis*. Work from several laboratories has identified secreted/exported proteins from *M. tuberculosis* as the major targets of a protective immune response to experimental tuberculosis infections (2, 3, 5, 12, 13, 17, 18, 20). These antigens are also favored targets during an immune response in human patients infected with the tubercle bacilli (17). However, other proteins believed not to be excreted by the mycobacterial cells have also been identified as important targets, namely in the induction of protective immune responses in experimental animals (14, 23), suggesting that the complete picture of the immune response to mycobacterial infections may be more complex with regard to the antigenic repertoire recognized in vivo. *Mycobacterium avium* is an opportunistic pathogen that is thought to interfere, in certain areas of the world, with the efficacy of the only tuberculosis vaccine in use today, the attenuated *Mycobacterium bovis* strain bacille Calmette-Guérin (BCG) (9). The reason for the failure of vaccination trials is not known, but it has been speculated that sensitization of human beings to antigens from environmental mycobacteria might affect BCG efficacy, most likely because all mycobacteria would share common antigens. Sharing of antigens between nontuberculous mycobacteria and BCG was already shown to occur at the immunological level in mice (15). However, except for two defined antigens, the latter study used crude extracts that combined many different antigens. The sharing of common antigens between *M. avium*, BCG, and *M. tuberculosis* will be understood in the near future thanks to efforts in the area of genomics. However, we still need studies in the fields of proteomics and immunology to generate functional data and therefore to be able to critically analyze the genomic information, namely studying native proteins rather than recombinant ones, as the latter may lack the immunogenicity of the former (1, 21). Thus, we initiated the characterization of the T-cell response to *M. avium* protein antigens using a mouse model of infection.

We isolated antigens from *M. avium* 2447, an AIDS patient isolate obtained from F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium) that forms smooth transparent colonies when cultured on solid media. Mycobacteria from log-phase cultures were inoculated into Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose (Sauton P+G) (8) and with no detergent, at a final concentration of approximately 5×10^6 CFU/ml (according to the absorbance measured at 600 nm), and grown at 37°C without shaking. The number of viable bacteria and the smooth transparent morphotype were confirmed by plating serial dilutions of the cultures on solid Middlebrook 7H10 medium (Difco, Sparks, Md.). At the end of log phase (i.e., at day 15 as evaluated from the previous growth curves), cultures were centrifuged for processing of bacterial antigens. Culture filtrate proteins were obtained from the filter-sterilized supernatant of the culture by ultrafiltration in a stirred cell (Millipore, Bedford, Mass.) with an Amicon YM membrane (molecular weight cut-off [MWCO] of 3,000) (Millipore). The concentrate was precipitated with 80% ammonium sulfate, and the precipitate was washed in a Centriprep (MWCO of 3,000) (Millipore). Cytosolic and envelope proteins were obtained after the pellet was washed twice with phosphate-buffered saline (PBS), resuspended in PBS containing 0.1% Tween 80 (Sigma), 1 mM $MgCl_2$ (Merck, Darmstadt, Germany) and 1 mM benzamidine (Sigma) (10), and disrupted through sonication with pulses of 1 min at maximum power, with the sample kept in ice during the whole procedure. The sonicate was centrifuged to discard intact mycobacteria (30 min at $2,700 \times g$), and the supernatant was dialyzed against PBS (MWCO of 12,000). The suspension was then ultracentrifuged for 2 h at $150,000 \times g$. The pellet containing the envelope proteins was resuspended in PBS, and the supernatant enriched in cytosolic proteins was precipitated with 80% ammonium sulfate and dialyzed against PBS.

Following the preparative procedures described above, we obtained crude extracts which were analyzed to assess if they were distinct sources of antigens. Cytosolic, envelope, and culture filtrate proteins (20 μg) were separated in a 10 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and analyzed either by silver staining or by Western blotting after transfer to a PROTON nitrocellulose (Schleicher and Schuell, Dassel, Germany)

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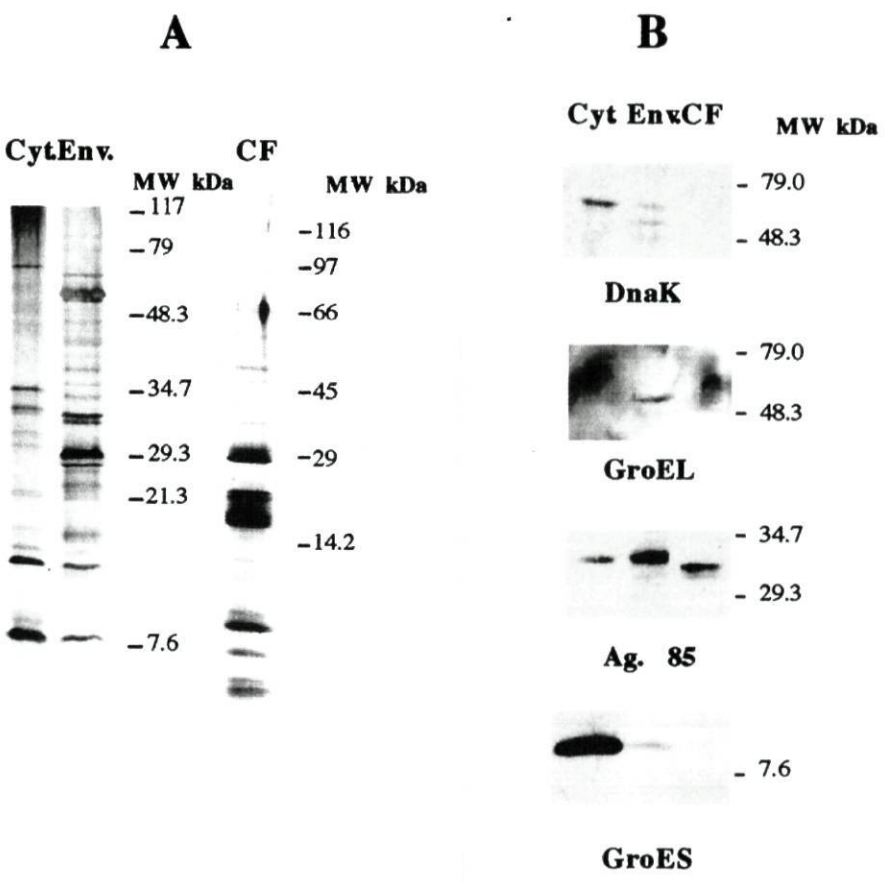

FIG. 1. Analysis of crude extracts of *M. avium* 2447 proteins. Cytosol (Cyt.), envelope (Env.), and culture filtrate (CF) preparations were analyzed by gradient (10 to 20%) SDS-PAGE followed by silver staining (A) or transferred to nitrocellulose and incubated with antibodies against DnaK, GroEL, Ag85 complex, or GroES followed by ECL-dependent detection of antigen-positive bands (B).

membrane in a semidry electrophoretic transfer cell (Bio-Rad, Richmond, Calif.). During the latter procedure, the membrane was blocked in PBS containing 0.5% Tween 20 (Sigma), incubated for 2 h at room temperature with the antibodies diluted (1:50) in PBS–0.05% Tween–0.37 M NaCl, and washed in the dilution buffer. The primary antibodies used were specific for DnaK (clone HÅT 3), GroEL (clone HÅT 5), GroES (clone HYB 76-1), and Ag85 (clone HYT 27), and they were kindly provided by Peter Andersen (Statens Seruminstitut, Copenhagen, Denmark). The secondary antibody, a horseradish peroxidase-coupled sheep anti-mouse antibody (Amersham-Pharmacia Biotech, Little Chalfont, United Kingdom) was incubated at a 1:500 dilution for 2 h at room temperature. The specific protein complexes were detected using the ECL reagents (Amersham-Pharmacia Biotech).

The protein profiles observed in the silver-stained gels for the three fractions were different, with distinct, different major bands obtained with the three preparations (Fig. 1A). The immunochemical analysis by the Western blotting with the panel of monoclonal antibodies against well-defined mycobacterial proteins (DnaK, GroEL, Ag85 complex, and GroES) showed that the three protein preparations corresponded to distinct cellular compartments. Thus, the DnaK and GroES proteins were mostly found in the cytosolic preparations, whereas the GroEL protein was found exclusively in the envelope fraction. Although we could find Ag85 in all three compartments, the molecular weight (MW) pattern varied, with higher-MW forms being found in the cytosol and envelope and a lower-MW form being found in the culture filtrate. We thus conclude that the culture filtrate and envelope preparations have negligible contamination with cytosol proteins. These preparations were then studied in terms of their ability to stimulate T cells isolated from infected mice. Six-week-old C57BL/6J female mice (Harlan Iberia, Madrid, Spain) were infected intravenously with 10^6 CFU of *M. avium* 2447 (smooth transparent morphotype) through a lateral tail vein at different time points and sacrificed on the same day at different times of infection to perform the in vitro stimulation of splenic cells. At days 15, 30, 60, and 90 after infection, spleens were removed and single-cell suspensions were prepared. Cells were washed with RPMI–2% fetal calf serum (FCS), and the erythrocytes were lysed with a hemolytic solution (155 mM NH_4Cl–10 mM $KHCO_3$, pH 7.2). The cells were cultured in Dulbecco's modified Eagle medium (Life Technologies, Paisley, United Kingdom) supplemented with HEPES buffer, pyruvate, and 10% FCS. The cells were cultured in 96-well, round-bottom, microtiter tissue culture plates

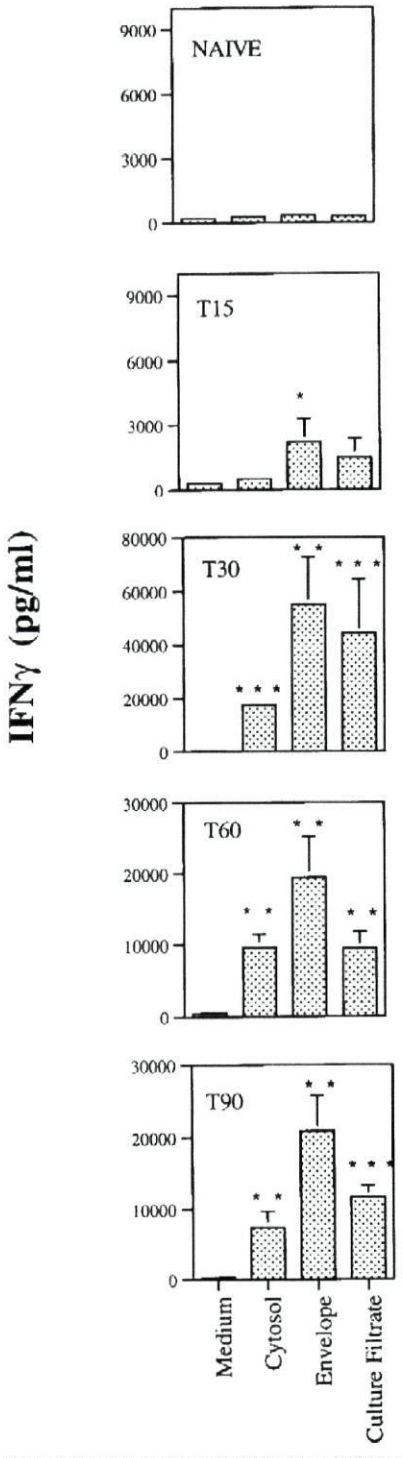

FIG. 2. Antigenicity of the crude extracts of *M. avium* 2447 proteins. Single-cell suspensions were prepared from spleens of mice infected for 15 (T15), 30 (T30), 60 (T60), or 90 (T90) days with *M. avium* 2447 or noninfected (naive) animals and stimulated in vitro with 4 μg of cytosol, envelope, or culture filtrate proteins per ml. IFN γ release was quantified by ELISA in the 72 h culture supernatants. Results are expressed as means of values of triplicate samples ±1 standard deviation performed on cells pooled from four mice. Statistically significant differences compared to values for nonstimulated cells are labeled: •, $P < 0.05$; ••, $P < 0.01$; •••, $P = 0.00$.

(Nunc, Roskilde, Denmark), each well containing 2×10^5 cells in a volume of 200 μl with no stimulus or incubation in the presence of antigen at a final concentration of 4 μg of crude extract/ml. Culture supernatants from triplicate wells were harvested 72 h later for the detection of gamma interferon (IFN-γ) as a readout of the response of those T cells to the different antigens using an enzyme-linked immunosorbent assay (ELISA) as previously described (22). As shown in Fig. 2, the envelope proteins were strong stimulators of IFN-γ production from day 15 of infection. The three preparations had antigens that stimulated T cells from mice infected for 30, 60, or 90 days, with the highest amount of IFN-γ being produced at day 30 of infection.

To obtain panels of MW fractions from the culture filtrate, envelope, and cytosol preparations, the multielution technique (4) was used. Briefly, 7 mg of culture filtrate or cytosolic proteins or 9 mg of envelope proteins was separated by SDS-PAGE (with a gradient gel of 10 to 20% acrylamide), and the gel was prepared for electroelution as described before (4). The proteins were electroeluted (40 V) for 20 min into a 2-mM phosphate buffer in a whole-gel eluter (Bio-Rad) in a cold room. The fractions were collected and analyzed (40 μl of each fraction) in a gradient SDS-PAGE (10 to 20% acrylamide) after fixation and silver staining (16). Protein concentration was quantified by the Micro BCA method (Pierce, Rockford, Ill.). The fractions were stabilized with 0.5% FCS in PBS. The SDS-PAGE analysis of the fractions obtained showed that most of them contained proteins in a very narrow range of MWs (Fig. 3), thereby greatly reducing the complexity of the crude extracts. The fractions obtained were then used to study the antigenic specificity of the T cells from infected mice, as detailed above (Fig. 4). The in vitro stimulation of spleen cells with the MW fractions obtained from the three different compartments at a final concentration of 2 μg/ml showed that the response to the culture filtrate fractions emerged at day 15, earlier than the response to the cytosol or envelope fractions. At day 30 of infection, the peak of the response, the strongest stimulators of T cells were found among fractions of culture filtrate in the 29,000-to-45,000 MW range. Fraction 13 from the culture filtrate, which was enriched in proteins in the 30-kDa region (where proteins such as Ag85 complex, a group of proteins that are highly immunogenic among mycobacteria (11, 24), are expected to locate), induced the strongest IFN-γ production. Fractions 12 of the cytosol and envelope, which contain proteins in the same region of MW, were also very immunogenic. Fractions from the envelope between 45 and 116 kDa contained highly stimulatory proteins that were absent from the corresponding MW fractions of the culture filtrate. On the other hand, there was a group of fractions in the culture filtrate below 21 kDa (fractions 17 to 21), which did not include the lowest-MW fractions, that were also inducers of IFN-γ production.

The antigen repertoire recognized by IFN-γ-secreting T cells during an *M. avium* infection in mice revealed a highly diverse set of protein antigens. The antigen targets were not confined to the secreted/exported proteins but rather were promiscuous among all compartments of the mycobacterial cell when grossly dissected into secreted/exported (culture filtrate), envelope, and cytoplasmic proteins. Several groups have

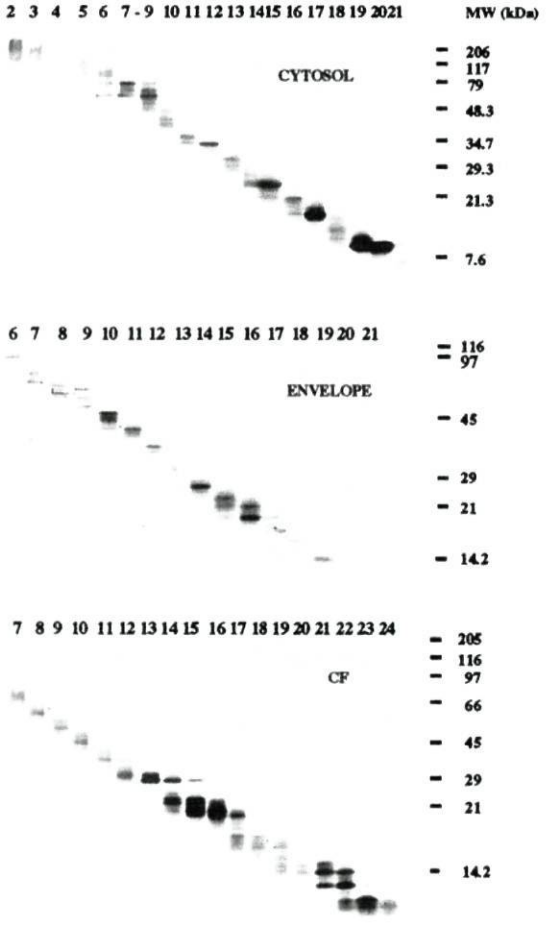

FIG. 3. Fractionation of cytosol, envelope, and culture filtrate (CF) proteins. Proteins were separated by SDS-PAGE and electroeluted as described in the text. The different MW fractions were analyzed on a silver-stained SDS-PAGE gel.

favored secreted/exported proteins as the major targets of the protective immune response to *M. tuberculosis* (2, 3, 5, 12, 13, 17, 18, 20). In addition, cell wall-associated proteins have also been shown to evoke an immune response during infection (17), suggesting their shedding from the cell wall during growth of mycobacteria inside the vacuoles of the infected macrophage. Finally, the response to cytoplasmic antigens would, according to some, appear later in infection as a result of the killing of the mycobacteria, and this response would be associated not with a protective IFN-γ-mediated immune response but most likely with a type 2 immune response, which is putatively associated with removal of the dead mycobacteria (19). Our data confirm the above paradigm by showing important reactivity towards secreted/exported as well as envelope proteins. However, IFN-γ-secreting T cells were also found to respond to the cytosolic proteins, namely to fractions of around 30 to 32 kDa, characteristic of the Ag85 complex. Although it is believed that this is a typical secretion antigen (24, 25), we detected its presence in both the somatic (cytosol and envelope) and secreted fractions. Curiously, the former forms had a higher MW than the secreted form. The fact that Ag85 is found in the envelope is not surprising due to its function in the synthesis of the cell wall (7). Its presence in the cytosol may be due to contamination during preparation of the antigens, e.g., by its release from the cell wall during sonication of the bacilli, and it could justify reactivity to the cytosol. Otherwise, we are rather confident that cross-contamination represents negligible components of each fraction, since we found no traces of DnaK, GroEL, or GroES in the culture filtrates, whereas their presence in the cytoplasm or envelope was clearly detectable by Western blotting. This finding contrasts with the data reported for *M. tuberculosis*, where the heat shock proteins DnaK, GroEL, and GroES were found in the short-term culture filtrate (3). Although GroES has been described as a major T-cell antigen recognized by cells from tuberculosis patients and infected animals (6, 18), we failed to observe any reactivity to the fractions of the cytosol expected to contain this antigen. This might be explained by the fact that only the molecule isolated from culture filtrates is able to stimulate T cells, as elegantly shown by Rosenkrands and colleagues (21).

The fractionation techniques utilized in this work are not precise enough to identify single antigens but are suitable for an initial screening of the responses against the whole proteome. They are adequate for the kind of kinetic study presented here, which would be extremely laborious with other methods, such as those relying on two-dimensional separation procedures. They also have the advantage of using native antigens, which may differ from the recombinant products. The data generated here may, on the other hand, guide us to select groups of antigens separated through more potent techniques. Our data also raise interesting speculations regarding the field of vaccine development by suggesting that antigens from different compartments may be adequate candidates for the generation of protection. Also, it will be important to understand whether an improvement on the protective efficacy of subunit vaccines based on culture filtrate protein can be obtained by adding proteins from other sources of the mycobacterial cell. The fact that the responses to the antigens showed distinct kinetics raises the possibility that antigens from different compartments may be involved in protection at distinct stages of the disease. In this context it should be mentioned that vaccination with HSP60-expressing DNA vaccines had a major impact on experimental tuberculosis when performed during infection as a therapeutical vaccine, whereas a similar construct expressing a secreted antigen had no effect (14); on the other hand, both antigens prevented infection when given prior to bacillary challenge (3, 23). It would be interesting to follow up the present observations and reanalyze the antigenic repertoire in experimental tuberculosis. Finally, it should be stressed that the reactivities against *M. avium* proteins observed here were determined by the proteins expressed during culture in a particular medium. Other proteins may be expressed in other media and, more importantly, other proteins may be expressed in vivo and not in vitro. It is therefore still necessary to broaden this type of analysis to fully understand the nature of the immunogenic proteome of *M. avium* in the context of infection.

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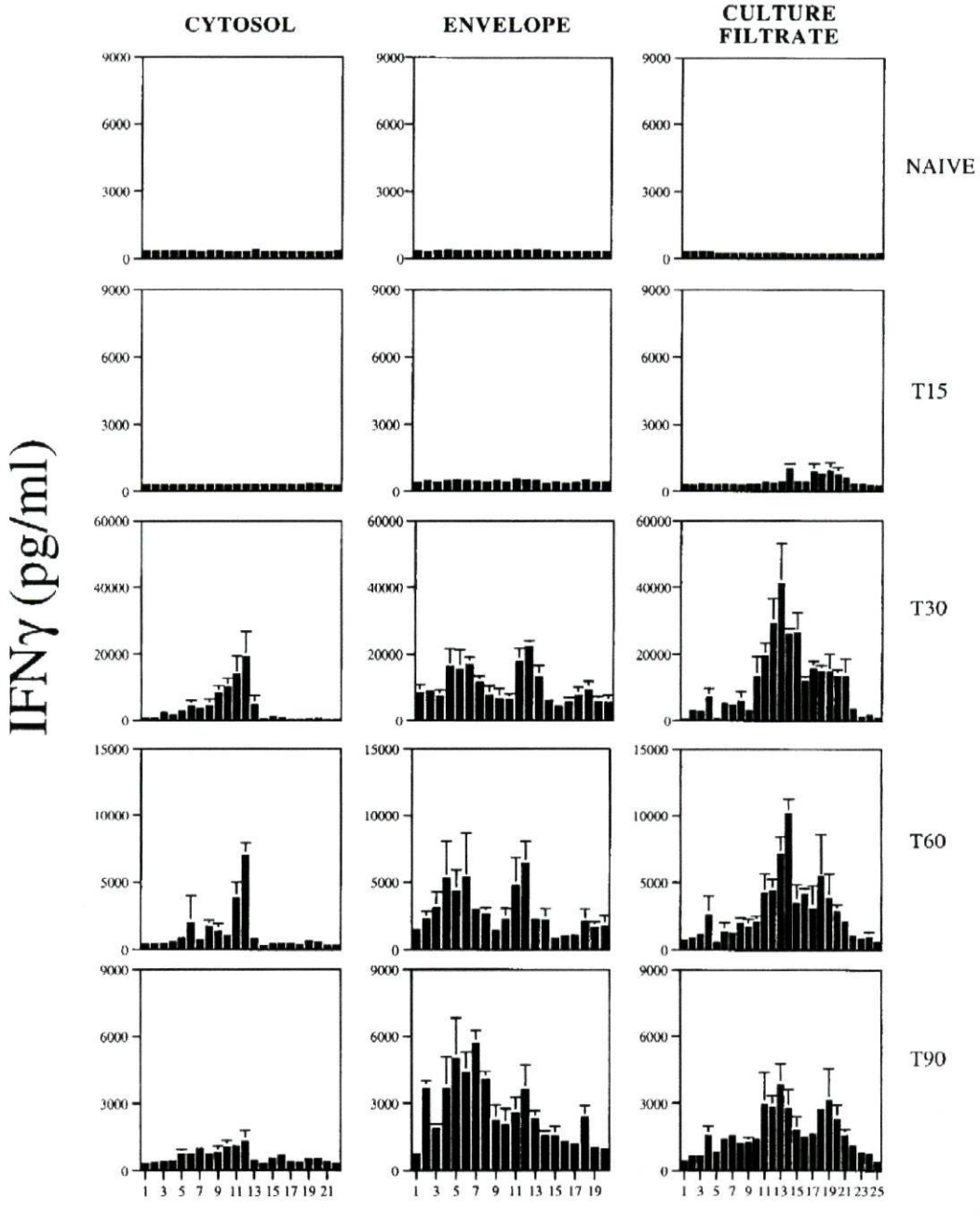

FIG. 4. Antigenicity of the fractions obtained from cytosol, envelope, and culture filtrate proteins (the fraction numbers on this figure correspond to the numbers in Fig. 3). Spleen cells of noninfected mice or mice infected with *M. avium* 2447 were stimulated in vitro with 2 μg of each fraction per ml. IFN-γ release was quantified by ELISA with culture supernatants.

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Chapter 3

Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis.

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Failure of the *Mycobacterium bovis* BCG Vaccine: Some Species of Environmental Mycobacteria Block Multiplication of BCG and Induction of Protective Immunity to Tuberculosis

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The efficacy of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine against pulmonary tuberculosis (TB) varies enormously in different populations. The prevailing hypothesis attributes this variation to interactions between the vaccine and mycobacteria common in the environment, but the precise mechanism has so far not been clarified. Our study demonstrates that prior exposure to live environmental mycobacteria can result in a broad immune response that is recalled rapidly after BCG vaccination and controls the multiplication of the vaccine. In these sensitized mice, BCG elicits only a transient immune response with a low frequency of mycobacterium-specific cells and no protective immunity against TB. In contrast, the efficacy of TB subunit vaccines was unaffected by prior exposure to environmental mycobacteria. Six different isolates from soil and sputum samples from Karonga district in Northern Malawi (a region in which BCG vaccination has no effect against pulmonary TB) were investigated in the mouse model, and two strains of the *Mycobacterium avium* complex were found to block BCG activity completely.

Tuberculosis (TB) is one of the most prevalent causes of death from infectious diseases in the world. As is the case for many intracellular pathogens, cell-mediated immunity plays an important role in host protection against TB (25, 29). In particular, gamma interferon (IFN-γ)-secreting T cells have been shown to be important for the protective immune response (17). The only vaccine currently available against TB is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG). The efficacy of this vaccine varies from 0 to 80% in different populations, with a consistently low efficacy in many tropical regions of the world where the vaccine is most needed (15, 16, 35, 38). The reason for the failure of BCG in some populations has been a subject of debate since the 1950s, and many different hypotheses have been suggested to explain the observed variation. Some investigators have suggested that differences in the strain of BCG (23), the age at vaccination (40), or methodological differences are important factors for the variation reported (8). The most widely accepted hypothesis relates the efficacy of BCG to geographic location, with low to nondetectable levels of protection against pulmonary TB seen in tropical regions such as Africa and India, where exposure to nontuberculous mycobacteria is common (15). One exception from this general rule is the consistent high efficacy when BCG is used to vaccinate newborns. Neonatal vaccination with BCG imparts protection against the childhood manifestations of TB (in particular, meningitis) (1, 9, 24), but the efficacy wanes over a period of 10 to 15 years, and therefore it does not prevent against the later breakdown with pulmonary TB in the adult population in the third world (37).

There is convincing evidence that exposure of laboratory animals to environmental mycobacteria can provide some protection against infection with *M. tuberculosis* (7, 14, 20, 30, 33). The influence of such cross protection on the efficacy of subsequent BCG vaccination is not yet clarified, but based on animal experiments, it has been suggested that the protection provided by environmental mycobacteria may partly mask the effect of a subsequent BCG vaccination (33, 42) or that environmental mycobacteria have a direct antagonistic influence on subsequent BCG vaccination (34, 36). Our study demonstrates that prior sensitization with environmental mycobacteria can inhibit BCG multiplication and thereby prevent the induction of an efficient BCG-mediated immune response and protection against TB challenge. Interestingly, different species isolated from soil and sputum in Karonga, Malawi, an area in which BCG has been shown to provide no protection against TB (22), differed in their ability to inhibit BCG multiplication. In contrast, a TB subunit vaccine had the same protective effect in naive and sensitized animals.

MATERIALS AND METHODS

Animals. These studies were performed with pathogen free 6- to 12-week old CBA/J and C57BL/6J female mice, purchased from Bomholtegaard, Ry, Denmark, or, in some of the experiments, purchased from Harlan UK, Ltd., Belton, England, or Harlan Interfauna Ibérica, Barcelona, Spain.

Bacteria. *Mycobacterium avium* (ATCC 15769), *Mycobacterium scrofulaceum* (ATCC 19275), and *Mycobacterium vaccae* (ATCC 15483) were grown in 7H9 broth until the mid log phase of the bacterial growth. *Mycobacterium tuberculosis* (Edman) was grown at 37°C on Löwenstein-Jensen medium or in suspension in modified Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose. In prepa-

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TABLE 1. Sensitization with environmental mycobacteria blocks the protective effect of BCG

Group of mice[a]	Result in[b]:			
	Spleen		Lung	
	CFU[c]	Log_{10} resistance[d]	CFU	Log_{10} resistance
Naive	4.44 ± 0.13		6.34 ± 0.11	
BCG	3.76 ± 0.16	0.68*	5.21 ± 0.08	1.13*
Sensitization	4.36 ± 0.17	0.08	6.14 ± 0.11	0.20
Sensitization + BCG	4.33 ± 0.17	0.11	6.25 ± 0.06	0.09

[a] Naive or sensitized mice were BCG vaccinated (5×10^4 CFU) followed by aerosol challenge with virulent *M. tuberculosis*.

[b] The experiment was repeated twice with similar results.

[c] Bacterial numbers determined by growth of individual whole organ homogenates 6 weeks postinfection.

[d] Protective effect expressed as the log_{10} reduction in bacterial loads compared to those of naive mice. Bacterial numbers significantly different ($P < 0.05$) from those seen in naive mice are indicated by an asterisk.

ration for immunization of mice, frozen aliquots of the bacterial strains were thawed and sonicated for 5 min with a Branson 2210 ultrasonifier, and the viability of each strain was enumerated on 7H11 plates. *Mycobacterium fortuitum* (S78/2) and *M. fortuitum* (S160/5) are soil isolates from the north and south of Karonga, Malawi, respectively. We used standard decontamination of samples with 4% sodium hydroxide and culture at 37°C on nutrient agar-based medium to isolate the organisms from soil. *M. fortuitum* (Sp2001), *Mycobacterium chelonae* (Sp2015), and two strains of the *M. avium* complex (Sp1891) and (Sp2011) were sputum isolates from donors with suspected TB in the Karonga district. The organisms were isolated with acidified Löwenstein Jensen medium in Malawi, and their identity was confirmed at the Mycobacterium Reference Unit, Dulwich, United Kingdom, by standard biochemical identification tests for mycobacteria. Frozen aliquots of these strains were prepared for animal inoculation as described above.

Sensitization with environmental mycobacteria. Mice were immunized subcutaneously (s.c.) in the back three times at 2-week intervals with 2×10^6 CFU of each of three ATCC strains of environmental mycobacteria (*M. avium*, *M. scrofulaceum*, and *M. vaccae*). To clear the remaining mycobacteria, sensitization was followed, 3 weeks after the last inoculation, by 1 month of treatment with rifampin (Sigma; 100 mg/liter), ethambutol (Sigma; 200 mg/liter), and clarithromycin (Abbott Laboratories, Solna, Sweden; 200 mg/liter) added to the drinking water.

To assess virulence of the strains isolated from Karonga, Malawi, mice were infected with 10^5 CFU of each environmental mycobacterial strain in a volume of 0.2 ml of phosphate-buffered saline by intravenous (i.v.) injection via a lateral tail vein. At the appropriate time points, mice were killed (four in each group), and the organs were removed for bacterial enumeration. Whole organs were homogenized in a 0.04% Tween 80 (Sigma) solution in distilled water, serial 10-fold dilutions were plated on Middlebrook 7H10 medium at 37°C, and the numbers of CFU were determined.

Vaccinations. A single dose of BCG Danish 1331 (5×10^4 CFU) was injected s.c. at the base of the tail. There were no significant differences in the protection obtained with doses ranging from 5×10^4 to 10^7 BCG bacteria (results not shown). In one experiment, an i.v dose of 5×10^6 CFU of BCG was used to determine growth of BCG in naive versus sensitized mice. For subunit vaccination, the mice were immunized s.c. three times at 2-week intervals with 10 μg (per dose) of either ESAT 6 or the Ag85B–ESAT 6 fusion protein emulsified in dioctadecylammonium bromide (DDA; 250 μg/dose; Eastman Kodak, Inc., Rochester, N.Y.) plus 25 μg of monophosphoryl lipid A (MPL; Corixa, Hamilton, Mont.) as described recently (5).

***M. tuberculosis* infections.** Animals were infected with approximately 100 CFU of *M. tuberculosis* (Edman) per lung by the aerosol route in a Glas Col inhalation exposure system. The mice were sacrificed 6 weeks after infection, and bacterial numbers in the lung and spleen were determined as described before (5).

The protective effect of BCG or subunit vaccination was expressed as the log_{10} reduction of the bacterial counts compared to that in the unvaccinated control mice. All results are based on five or six animals per group.

Mycobacterial antigens. A crude BCG antigen preparation (BCG Ag) was produced as an ammonium sulfate-precipitated culture filtrate from cultures at week 6 as described in reference 2. In one of the experiments (see Fig. 4), the BCG responses to an ammonium sulfate-precipitated extract of the cell wall were measured as described elsewhere (31). These two preparations were found to give similar responses in vitro.

Protein concentration was quantified by the Micro bicinchoninic acid method (Pierce, Rockford, Ill.).

Recombinant ESAT 6 was produced as described previously (18). The LPS content was below 0.3 ng/μg of protein and had no influence on cellular activity. The fusion protein Ag85B–ESAT-6 was produced as described recently (26). The proteins were kept at −80°C until use.

Lymphocyte cultures. Lymphocytes from spleens and blood were isolated and cultured as described previously (5). Briefly, cells from individual mice were cultured in microtiter wells (96 well; Nunc, Roskilde, Denmark) containing 2×10^5 cells in a volume of 200 μl of RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, penicillin-streptomycin, 1 mM glutamine, and 5% (vol/vol) fetal calf serum. Based on previous dose response investigations, BCG Ag and ESAT 6 were each used at 5 μg/ml in the cultures. Phytohemagglutinin at a concentration of 1 μg/ml was used in all experiments as a positive control for cell viability. IFN-γ, interleukin 4 (IL-4), and IL-5 were detected in 72-h culture supernatants by duplicate enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunospot (ELISPOT) analyses were conducted with cells from individual mice or, when blood was analyzed, with cells pooled from groups of mice, as described in reference 6. The detection level was 10 spots.

Statistical methods. Because all of the data show a normal distribution, the assessment of experiments was carried out by analysis of variance. Differences between means were assessed by Dunnett's test (Tables 1 and 2) or Student's *t* test (see Fig. 2 and 4). A *P* value of <0.05 was considered significant.

RESULTS

The multiplication of BCG is inhibited in mice sensitized with certain environmental mycobacteria. We inoculated CBA/J mice s.c. three times at 2-week intervals with a mixture

TABLE 2. Bacterial numbers in organs of naive and sensitized mice after vaccination and aerosol challenge with virulent *M. tuberculosis*

Vaccine group[a]	Result in:			
	Lung		Spleen	
	Log_{10} CFU[b]	Log_{10} resistance[c]	Log_{10} CFU	Log_{10} resistance
Expt 1				
Naive				
Control	6.36 ± 0.08		4.71 ± 0.05	
BCG	5.83 ± 0.06	0.53*	4.12 ± 0.12	0.59*
DDA-MPL	6.34 ± 0.09		4.94 ± 0.12	
ESAT-6	5.76 ± 0.09	0.60*	4.39 ± 0.09	0.32*
Sensitized				
Control	6.18 ± 0.08		4.82 ± 0.16	
BCG	6.27 ± 0.07	<0.05	4.79 ± 0.05	<0.05
DDA-MPL	6.39 ± 0.05		4.73 ± 0.11	
ESAT-6	5.74 ± 0.16	0.44*	4.43 ± 0.05	0.39
Expt 2				
Naive				
Control	6.88 ± 0.12		5.10 ± 0.18	
DDA-MPL	7.19 ± 0.05		5.48 ± 0.11	
Ag85B–ESAT-6	6.03 ± 0.12	0.85*	4.40 ± 0.08	0.70*
Sensitized				
Control	6.30 ± 0.08		4.39 ± 0.09	
DDA-MPL	6.49 ± 0.05		4.27 ± 0.08	
Ag85B–ESAT-6	5.37 ± 0.14	0.93*	3.89 ± 0.07	0.50*

[a] Naive or sensitized mice were immunized s.c. with BCG or injected three times with a subunit vaccine emulsified in DDA MPL.

[b] Bacterial numbers are given as log_{10} CFU of *M. tuberculosis* isolated from the lung and spleen 6 weeks after aerosol challenge with virulent *M. tuberculosis*.

[c] Protective effects of the two vaccines are expressed as log_{10} reductions in bacterial numbers compared to those in unvaccinated control mice. Bacterial numbers significantly different from those seen in control mice are indicated by an asterisk.

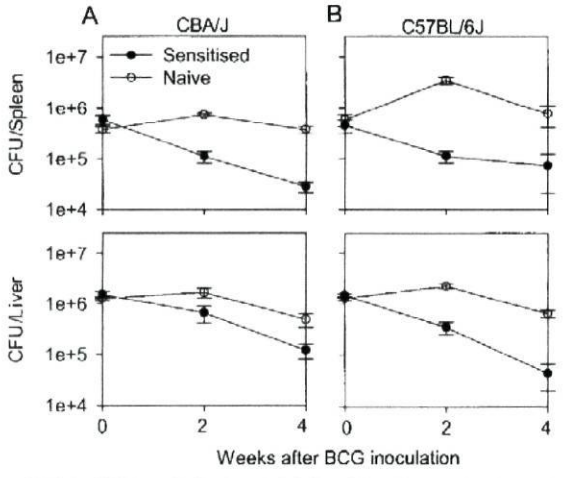

FIG. 1. BCG multiplication is inhibited in mice previously sensitized with environmental mycobacteria. (A) CBA/J mice. (B) C57BL/6J mice. The growth of BCG was compared in naive mice (open symbols) and in sensitized mice (solid symbols). The data shown are the means of BCG CFU ± standard errors. For both groups, five animals were sacrificed for each time point. The experiment was repeated twice with similar results.

of the mycobacterial strains *M. avium*, *M. scrofulaceum*, and *M. vaccae*. These species have repeatedly been isolated from soil and water samples in tropical regions (21). Three weeks postinoculation, a low but significant mycobacterium-specific recall response was measured in the spleen, with detectable levels of IFN-γ release in response to BCG Ag. (1.26 ± 0.01 ng/ml) (data not shown). The BCG Ag preparation gave no IFN-γ release (<0.05 ng/ml) from splenocytes isolated from naive mice. No IL-4 or IL-5 was detected in any of the supernatants. Three weeks after the last inoculation with environmental mycobacteria, we subjected the mice to 4 weeks of chemotherapy to clear remaining live mycobacteria. After the end of chemotherapy treatment, no environmental mycobacteria were detected in any of the target organs (liver, spleen, and lymph nodes).

We inoculated groups of sensitized and age-matched naive CBA/J mice i.v. 1 week after the end of chemotherapy treatment with 5×10^6 BCG and monitored the growth in the spleen and liver over time. Sensitization with environmental mycobacteria resulted in inhibition of the initial multiplication of BCG in the spleen and liver (Fig. 1A). In naive mice, the initial multiplication of BCG resulted in 10- to 30-fold more bacteria in the spleen postinoculation than in sensitized mice. A difference was also seen after a conventional s.c. vaccination, although the bacterial numbers were at lower levels (data not shown). Similar data were obtained with C57BL/6J mice, which are more susceptible to BCG (11). In this strain, larger differences in BCG numbers were found between sensitized and nonsensitized mice (Fig. 1B).

Immune responses induced by BCG vaccination in sensitized and naive mice. We continued by investigating the immune response induced by BCG in sensitized and age-matched naive control CBA/J mice. ELISPOT was used to monitor frequencies of BCG-specific T cells before and 3, 5, 8, and 11 weeks after the s.c. vaccination with BCG (Fig. 2). Before BCG vaccination, no mycobacterium-specific IFN-γ-producing T cells were detected in any of the mice. Three weeks after BCG inoculation, the number of BCG-specific IFN-γ-producing cells in the draining lymph nodes had increased and reached the same level in sensitized and in naive mice (Fig. 2A). The response in sensitized mice was, however, transient, and from 5 weeks after BCG inoculation and onwards, a higher frequency of mycobacterium-specific cells was found in naive vaccinated mice. At the termination of the experiment (week 11), a 10-times-higher frequency of BCG-specific T cells was found in the naive vaccinated group than in the sensitized vaccinated group ($P = 0.032$). A similar dynamic development of responses was found in the blood, although it was delayed so

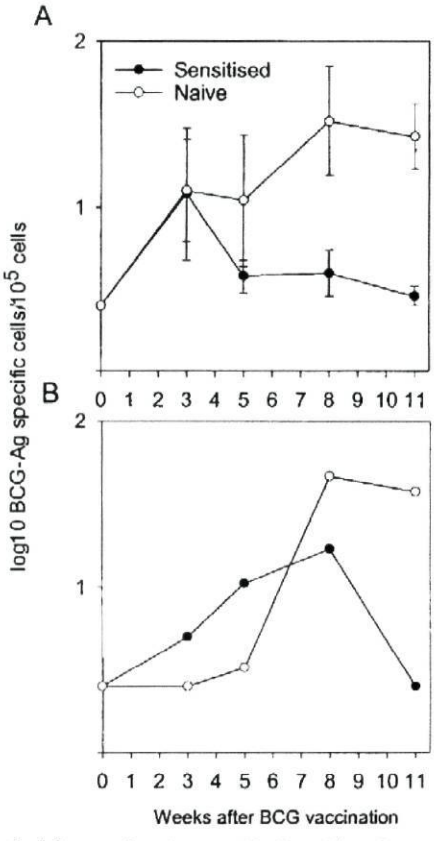

FIG. 2. Influence of previous sensitization with environmental mycobacteria on the BCG-specific immune responses. BCG was administered s.c., and the frequencies of IFN-γ-producing cells isolated from the draining lymph nodes (A) and the blood (B) in naive mice (open symbols) and sensitized mice (solid symbols) were detected by the ELISPOT assay postvaccination after in vitro stimulation with BCG-Ag. The data presented here represent the logarithmic mean of results obtained from lymph node cells from three individual mice per group ± standard errors. The responses in the blood were analyzed on cells pooled from three animals for each time-point. A pilot experiment conducted on weeks 2, 4, and 6 supported the overall difference in the response profiles of the two groups of animals.

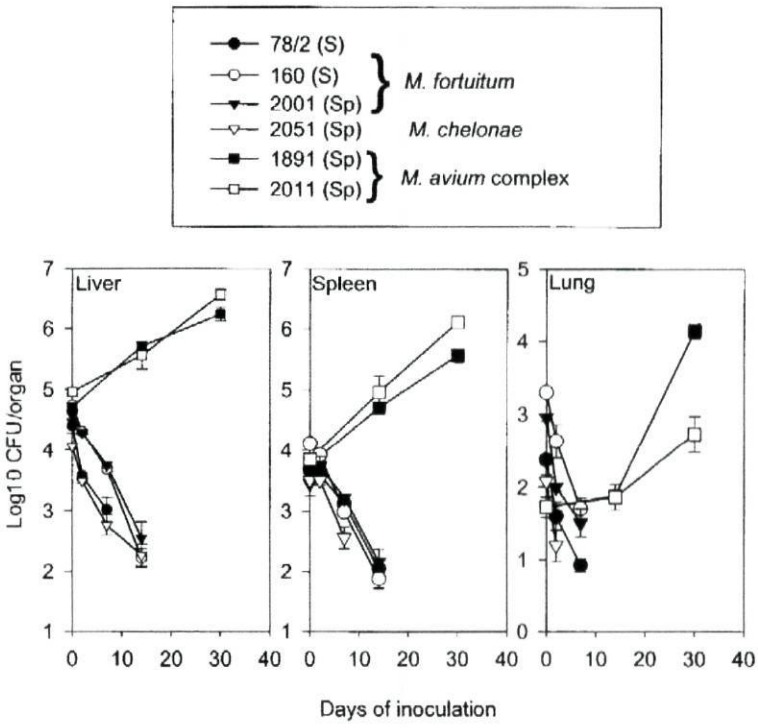

FIG. 3. Growth of isolates from Karonga, Malawi, in the mouse model. To evaluate the virulence of the isolates, mice were infected i.v. with *M. fortuitum* strains S160 (open circles), S78/2 (solid circles), and 2001 (solid triangles); *M. chelonae* strain 2015 (open triangles); and *M. avium* complex strains 1891 and 2011 (solid and open squares, respectively). The mycobacterial loads were determined in the liver, spleen, and lung at the time points indicated. *M. chelonae* and *M. fortuitum* were all below the level of detection from day 14 onwards. Data are given as means with standard errors ($n = 4$).

that higher frequencies of specific T cells were found from week 8 onwards in naive vaccinated mice (Fig. 2B). At no time point after vaccination was IL-4 or IL-5 detected in the supernatants of the stimulated cultures (results not shown).

Sensitization with environmental mycobacteria blocks the protective effect of BCG, but not a TB subunit vaccine. We continued by vaccinating sensitized and naive age-matched control CBA/J mice 4 to 5 weeks after the end of chemotherapy-treatment, followed 2 months later by an aerosol challenge with *M. tuberculosis*. The mice were killed 6 weeks post-TB infection, and *M. tuberculosis* CFU were enumerated in the lungs and spleens. The BCG vaccine imparted appreciable protection to naive mice against the TB challenge, with significantly reduced bacterial numbers in the organs (0.68 to 1.13 $\log_{10}$ reduction; Table 1). Sensitization with environmental mycobacteria on its own, or followed by BCG vaccination, failed to induce a statistically significant level of protection against TB (Table 1).

We also asked if a previous sensitization with environmental mycobacteria would influence protection induced by a subunit vaccine. Groups of naive and sensitized CBA/J mice were vaccinated with BCG or injected (three times at 2-week intervals) with recently developed TB subunit vaccines based on the immunodominant antigens ESAT-6 and Ag85B mixed with a DDA-MPL adjuvant emulsion (5, 26). ESAT-6-vaccinated animals mounted a very strong recall immune response (5 to 7 ng of IFN-γ/ml) to the homologous preparation 1 week postvaccination in the blood (data not shown). The protection obtained by BCG in control mice was log 0.53, and as in the previous experiment, BCG did not protect presensitized mice (Table 2, experiment 1) The ESAT-6 subunit vaccine, in contrast, induced a similar degree of protection in both naive and sensitized mice. A subunit vaccine based on a fusion protein of Ag85B and ESAT-6 has recently been demonstrated to induce levels of protection similar to those of BCG in the mouse model (26), and this vaccine also protected against TB challenge at the same level in naive and sensitized mice (Table 2, experiment 2).

Mycobacterial species isolated in Karonga, Malawi, differ in their ability to block BCG activity. We investigated six different isolates from soil and sputum samples from Karonga District in Northern Malawi in the mouse model. Three of these isolates were typed as *M. fortuitum*, one was a strain of *M. chelonae*, and two were classified as belonging to the *M. avium* complex (Fig. 3). The growth of these isolates in spleen, liver, and lung was investigated with C57BL/6J mice over a period of 30 days. Most of the isolates were rapidly cleared to below the level of detection, but the strains from the *M. avium* complex multiplied and reached bacterial numbers 3 logs above those of *M. chelonae* and *M. fortuitum* after day 14 (Fig. 3). The mice

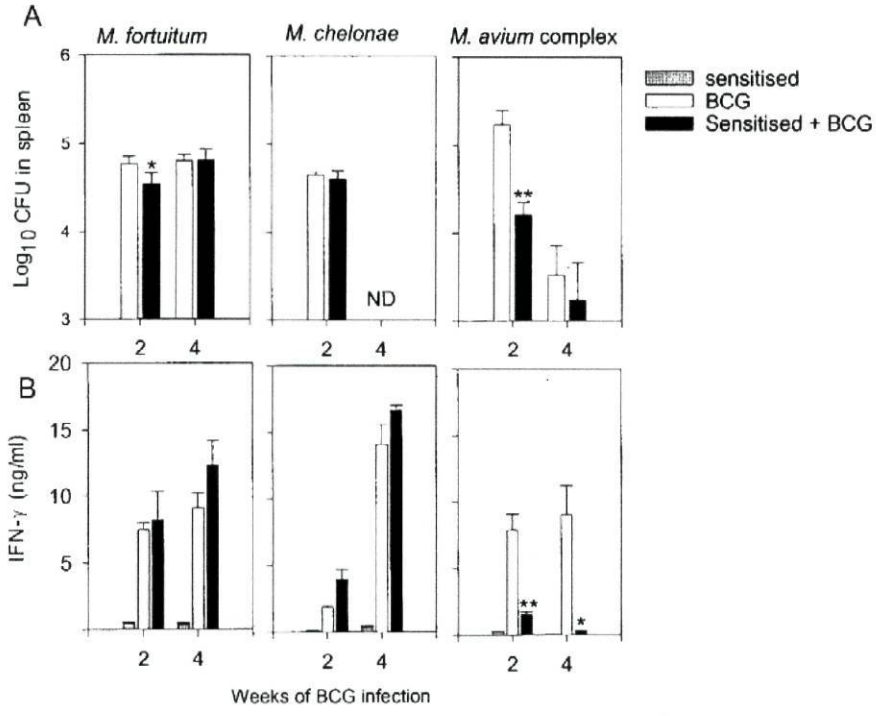

FIG. 4. (A) Growth of BCG in sensitized and naive animals. Mice were infected s.c. with 2×10^6 CFU of either *M. fortuitum*, *M. chelonae*, or *M. avium* or were left untreated. After chemotherapy, mice were infected with BCG Pasteur. BCG CFU in the spleen are shown as means with standard errors ($n = 4$). Statistically significant differences between sensitized (solid bars) and naive (open bars) mice are indicated: *, $P < 0.05$; **, $P < 0.01$, according to Student's t test. ND, not done. (B) Effects of sensitization on the IFN-γ response to BCG. Spleen cells were pooled from four individual mice sensitized with the environmental strains (shaded bars), naive mice inoculated with BCG (open bars), or sensitized mice inoculated with BCG (solid bars). IFN-γ production was assessed in the supernatants of spleen cell cultures stimulated in vitro with BCG Ag and are given as means with standard errors. Statistically significant effects of sensitization on the response to BCG infection are labeled: *, $P < 0.05$; **, $P < 0.01$, according to Student's t test.

were treated with chemotherapy, followed by an injection of BCG according to our standard protocol. BCG counts in the spleen of these mice were quantified at week 2 postinoculation (Fig. 4A). *M. fortuitum* and *M. chelonae* did not inhibit the growth of BCG, whereas bacteria from the *M. avium* complex reduced BCG numbers by 1 to 1.5 log ($P < 0.01$). This difference correlated with the immune responses induced by the BCG vaccine. There was no influence on the level of IFN-γ responses to BCG Ag by sensitization with *M. chelonae* or *M. fortuitum*, whereas the previous inoculation with bacteria from the *M. avium* complex completely ablated BCG immune responses (Fig. 4B). All strains, on the other hand, induced low and variable responses to antigens extracted from the homologous strain of environmental mycobacteria (results not shown).

DISCUSSION

This study demonstrates that animals exposed to certain environmental mycobacteria raise an immune response that controls the multiplication of BCG, thereby curtailing the vaccine-induced immune response before it is fully developed. The finding is important for the long-held discussion on the failure of BCG vaccination against TB in some parts of the world (15, 16, 38). One hypothesis to explain the failure of BCG was presented in 1966 by Palmer and Long, based on large-scale guinea pig experiments. They argued that because contact with nontuberculous bacteria offers some level of protective immunity to TB, the protective effect of a superimposed BCG vaccine would be masked (33). The present study confirms the classical observation that priming with environmental mycobacteria promotes some levels of protective immunity to other mycobacteria (7, 10, 14, 33), in this case to BCG. However, this effect was not sufficient to significantly reduce the growth of *M. tuberculosis*, which multiplied at an almost unchanged rate in these sensitized animals. The difference from the partial protection imparted by environmental mycobacteria in the guinea pig model (14, 33) may be related to the fact that the earlier studies made no effort to clear the environmental mycobacteria by chemotherapy before challenge with *M. tuberculosis*, as well as the different genetic makeup and susceptibility of mice versus guinea pigs. The differences in these models and their relevance to human disease are the subject of an ongoing study.

That prior sensitization to environmental mycobacteria interferes in a similar way with human BCG vaccination is

strongly suggested by a number of classical epidemiological observations: (i) the finding of strong efficacy of BCG in trials in which tuberculin skin test-positive (and therefore sensitized) donors have been vigorously excluded (19); (ii) the consistent success with BCG in neonates vaccinated before any significant sensitization from environmental mycobacteria occurs (1, 9, 24); and, (iii) finally, the observation of a lower rate of skin test conversion, much smaller average diameter, and rapidly waning responses after BCG vaccination in areas with environmental sensitization (India and Egypt), compared with those in areas with minimal environmental exposure (Denmark) (4, 32). This observation was recently confirmed and extended by the observation of only minimal in vitro IFN-γ responses to purified protein derivative (PPD) induced by BCG vaccination in donors from Karonga, Malawi, compared to those from the United Kingdom (P. E. Fine and H. Dockrell, personal communication). Taken together, these findings are in agreement with the low and transient immune response in the group of animals sensitized with environmental mycobacteria before vaccination, whereas the naive animals developed strong and sustained responses (Fig. 3). Our experimental model is therefore relevant to the many tropical regions where BCG is not protective against pulmonary TB and where the high incidence of TB indicates that any partial protection provided by exposure to environmental mycobacteria is insufficient for the prevention of TB.

Our main conclusion is that BCG, as a live vaccine, is particularly sensitive to the influence of preexisting immune responses to antigens shared with certain environmental strains. In this regard, a recent study has demonstrated the cross-recognition of a large number of antigens shared between *M. avium* and BCG (T. Pais and R. Appelberg, unpublished results). Multiplication is a precondition for the induction of immunity by BCG and killing of BCG by chemotherapy after administration has been demonstrated to abrogate subsequent immunity completely (13, 39). In the present study, this blocking is achieved by immunological control instead of chemotherapy, but the outcome in both cases is interference with the protective immune response, which would normally develop in response to the growing BCG. The requirement for BCG multiplication can be explained as a simple consequence of dosage, but more likely is due to the fact that only live BCG secretes many antigens of importance for the induction of a protective immune response (3, 28). Interestingly, our data from the animal model also suggest that only environmental strains, which are capable of an initial multiplication in the host, block the activity of BCG. A detailed evaluation of a large number of different soil isolates from Karonga, Malawi, and of their interactions with BCG is ongoing. In the future, information on the geographical distribution of such strains would be a valuable resource when trying to understand the huge variation in BCG efficacy in human trials.

This inhibitory effect of the environmental mycobacteria on the growth and activity of BCG provides an important argument in the ongoing discussion of live attenuated vaccines versus nonviable subunit vaccines against TB (12, 27, 44). In comparison with live attenuated vaccines, the present study suggests that subunit vaccines may be much less influenced by prior contact with environmental mycobacteria. As mentioned above, neonatal BCG vaccination consistently imparts protection against the childhood manifestations of TB (mostly extrapulmonary disease), but as its efficacy wanes over a period of 10 to 15 years (37), the adult pulmonary manifestations of TB are prevented neither by neonatal vaccination, by vaccination in adolescence after exposure to environmental mycobacteria (41), nor by a BCG revaccination strategy (22, 43). A TB subunit vaccine could therefore fulfill the criterion of having consistently high efficacy in different populations and may have a particularly important use for revaccination of third world children in adolescence.

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Chapter 4

Functional cross-reactivity among antigens from *Mycobacterium intracellulare*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*.

Functional cross-reactivity among antigens from *Mycobacterium intracellulare*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*.

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Abstract

The protective efficacy of *Mycobacterium bovis* BCG vaccine against tuberculosis seems to be strongly influenced by prior exposure to environmental mycobacteria. Pre-existing immune responses to antigens that are common to environmental mycobacteria and *Mycobacterium tuberculosis* seem to be responsible for the failure of this vaccine. Our study demonstrates that there are several cross-reactive antigens between *Mycobacterium avium intracellulare*, an environmental mycobacterium isolated from sputum donors from Karonga district in Malawi, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. We identified the Ag85B as being one of such cross-reactive antigens. This cross-reactivity at the antigen recognition level inhibits BCG replication in vaccinated C57BL/6 mice and consequently induces low T-cell response to subsequent T lymphocyte stimulation with *M. tuberculosis* antigens.

Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, is a re-emerging disease that remains one of the most important infectious diseases, accounting for several million deaths per year worldwide. Currently, one-third of the world population is infected with *Mycobacterium tuberculosis* and is at risk of developing active tuberculosis. The immune response to this intracellular pathogen is based in cell-mediated immunity (Crevel et al., 2002) in particularly IFN-secreting T-cells play an essential role in the protective response to *M. tuberculosis* infection (Flynn et al., 1993). Several works have identified the secreted proteins of *M. tuberculosis* as the major target antigens of the protective immune response to that pathogen in experimental models of infection, such as the guinea pig model (Pal and Horwitz, 1992), the mouse model (Andersen, 1997; Andersen et al., 1995; Andersen et al., 1992; Andersen et al., 1991; Hubbard et al., 1992) and also in tuberculosis patients (Demissie et al., 1999; Weldingh and Andersen, 1999). Two molecular mass regions (6 to 10 kDa and 24 to 36 kDa) of the panel of *M. tuberculosis* secreted proteins have been repeatedly recognized as immunodominant antigens (Haslov et al., 1995).The Antigen 85 complex in the 30/32 molecular mass region is a complex of three highly related proteins encoded by separate genes and transcribed as distinct transcriptional units (Wiker and Harboe, 1992). At the genomic and amino acid level these proteins share a high degree of homology not only with each other but also with their counterparts in other mycobacterial species. Studies from Harth et al showed differences only in 1 or 5 amino acids in the Ag85 complex proteins between BCG and *M. tuberculosis* (Harth et al., 1996).

The only vaccine currently available against tuberculosis is BCG (Bacilli Calmette Guérin) and although BCG is highly efficacious in laboratory models of disease (Smith, 1985), it has varied tremendously in protective efficacy in field trials, and in some geographical regions the vaccine has shown no efficacy at all (Fine, 1995; Ponninghaus et al., 1992; Rodrigues et al., 1993). The reason for the failure of the BCG vaccine has been the subject of debate since the 1950´s and although many explanations have been speculated the most accepted hypothesis is that sensitisation to antigens from environmental mycobacteria might interfere with the BCG vaccine. According to this there are consistent studies that show the success of BCG vaccination in neonates in preventing tuberculosis before any significant sensitisation

with environmental mycobacteria occurs (Colditz et al., 1995; Marchant et al., 1999). The prior sensitisation to environmental mycobacteria might have one of two different effects in the BCG vaccine. One hypothesis, *the masking hypothesis,* proposed by Palmer and Long, point out that exposure to environmental mycobacteria offers some level of protective immunity to tuberculosis and that BCG cannot improve it (Palmer and Long, 1966). The *blocking hypothesis* came out from our previous works and suggests that pre-existing immune responses to antigens common for the mycobacteria block the replication of BCG (Brandt et al., 2002). As a live vaccine BCG needs to multiply to induce and sustain protective immunity against tuberculosis, the pre-sensitisation to atypical mycobacteria blocks BCG replication by inducing an immune response to antigens that are cross-reactive with BCG antigens. The sharing of common antigens between environmental mycobacteria, BCG and *M. tuberculosis* is the key to understand the failure of the BCG vaccine. Our study demonstrates that there are cross-reactive antigens between mycobacteria from the *M. avium* complex, a strain that we had shown to inhibit BCG replication in pre-sensitised mice, and BCG. Antigens from the same molecular mass region than the Ag 85 complex are predominantly recognised by these strains. In addition, prior sensitisation either with the live environmental mycobacteria or the culture filtrate proteins led to the recognition of Ag85 complex from *M. tuberculosis.* Finally, sensitisation to secreted antigens from the atypical mycobacteria inhibits BCG multiplication more efficiently than sensitisation to *M. tuberculosis* Ag85B peptide.

Materials and Methods

Animals. These studies were performed with pathogen-free 6 to 8-week-old C57BL/6J female mice, purchased from Harland Interfauna Ibérica, Barcelona, Spain.

Bacteria. *Mycobacterium intracellulare* a strain from the *Mycobacterium avium* complex was sputum isolated from sputum of suspected cases TB in the Karonga district, Malawi. *Mycobacterium intracellulare* was isolated with acidified Lowenstein-Jensen medium in Malawi and the identity of these bacteria was confirmed at the Mycobacterium Reference Unit, Dulwich, United Kingdom, by standard biochemical identification tests.
Mycobacterium bovis BCG Danish 1331 was from the Statens Serum Institut, Copenhagen (Denmark).

Infections and Immunizations. Groups of four mice were immunized intravenously (i.v.) through a lateral tail vein either with 10^6 CFU of *Mycobacterium intracellulare* or with 5×10^4 CFU of BCG Danish.
For sensitisation mice were immunized subcutaneously (s.c.) three times at 2-weeks intervals with 50 μg (per dose) of either *Mycobacterium tuberculosis* Ag85B (MTb Ag85B) or the *Mycobacterium intracellulare* culture filtrate proteins emulsified in dioctadecylammonium bromide (DDA; 250 μg /dose; Eastman Kodak, Inc., Rochester, N.Y.).

Mycobacterial antigens. Mycobacteria from log-phase cultures were inoculated into Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose (Sauton P + G) (Collins et al., 1988) and with no detergent, at a final concentration of approximately 5×10^6 CFU/ml (according to the absorbance measured at 600 nm), and grown at 37°C without shaking. At the end of log phase (i.e., at day 15 as evaluated from previous cultures), cultures were centrifuged for processing of bacterial antigens. Culture filtrate proteins were obtained from the filter-sterilized supernatant of the culture by ultrafiltration in a Vivaflow 200 filtration module (Milipore). Finally the concentrates were precipitated with 80% ammonium sulphate. Panels of molecular weight (MW) fractions from the culture filtrate preparations were

obtained using the multielution technique, as described elsewhere (Andersen and Heron, 1993). Briefly, 7 mg of culture filtrate proteins was separated by SDS-PAGE (with a gradient gel of 10 to 20% acrylamide), and the gel was prepared for electroelution as described before (Andersen and Heron, 1993). The proteins were electroeluted (40V) for 20 minutes into a 2-mM phosphate buffer in a whole-gel eluter (Bio-Rad) in a cold room. The fractions were collected and analysed (40 µl of each fraction) in a gradient SDS-PAGE (10 to 20% acrylamide) after fixation and silver staining (Morrissey, 1981). Protein concentration was quantified by the Micro BCA method (Pierce, Rockford, Ill.). The protein fractions were stabilized with 0.5% fetal calf serum (FCS) in PBS.

The *Mycobacterium tuberculosis* antigens, TB10,4 (CFP7), MtbAg85A, MtbAg85B and MtbAg85B.P63 were prepared at the Statens Serum Institute, Copenhagen (Denmark).

Lymphocyte stimulation. Lymphocytes from spleens were isolated and cultured. Spleens were removed and single-cell suspensions were prepared. Cells were washed with RPMI-2% fetal calf serum (FCS), and the erythrocytes were lysed with a haemolytic solution (155 mM NH_4CL-10 mM $KHCO_3$, pH 7,2). The cells were cultured in Dulbecco´s modified Eagle medium (Life Technologies, Paisley, United Kingdom) supplemented with HEPES buffer, pyruvate, and 10% FCS. Cells pooled from groups of mice were cultured in 96 well, round-bottom, microtiter tissue culture plates (Nunc, Roskild, Denmark), each well containing 2×10^5 cells in a volume of 200 µl with no stimulus or incubation in the presence of antigen at a final concentration of 2 µg/ml or 4 µg/ml. Culture supernatants from triplicate wells were harvested 72 h later for the detection of gamma interferon (IFN-γ) as a readout of the response of those T cells to the different antigens using a two-site sandwish enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Silva et al., 1998). Briefly, an anti- IFN-γ-specific, affinity purified mAbs (R4-6A2 as capture and biotinylated AN-18 as detecting antibody) and a standard curve was generated with known amounts of IFN-γ (Genzyme, Cambridge, CA). The sensitivity of the assay was 30 pg/ml.

To determine the frequency of IFN- γ producing cells, an ELISPOT assay was performed. Briefly, microtiter plates were covered with 0.25 µg/well monoclonal

anti-mouse IFN-γ (cell line R4-6A2), incubated at 4°C, and then plates were emptied and blocked for 2 h with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 0.05% Tween 20 and washed four times with PBS/Tween 20. Cells from individual mice were cultured in the microtiter plates in duplicates in the presence of 4 μg/ml of the antigens for 24 h at 37°C in 7% CO2 atmosphere. Six serial twofold dilutions from a starting concentration of 4×10^5 cells were done from each cell sample. Cells were removed by washing the plates and IFN-γ was detected using 0.25 μg/well biotin-labeled rat anti-mouse mAb (cell line AN18) and 0.1 μg/well phosphatase–conjugated streptavidin. The enzyme reaction was concluded with the addition of 0.9 mg 5-bromo-4-chloro-3-indolylphophate per ml substrate buffer (0.74mM MgCl2, 0.1% Triton-X405, 9.6% 2-amino-2-methyl-1-propanol, pH 10.25) in 0.6% agarose. The development of blue spots was analysed mycroscopically. The relation between the number of develop spots and the number of input cells was calculated.

BCG growth in target organs. The BCG growth was monitored by counting BCG colony forming units (CFU) in spleen and liver of immunized mice. Briefly, at different intervals after immunization, the organs were homogenized and suitable dilutions were plated on Middlebrook 7H11 medium. The plates were incubated at 37°C for 2 to 3 weeks.

Results

Mycobacterium intracellulare culture filtrate fraction 11 is recognised by BCG vaccinated mice.

We inoculated groups of four C57BL/6J female mice intravenously with either 10^6 CFU of *Mycobacterium intracellulare* or 5×10^4 CFU of BCG Danish. At two different time points, 30 and 60 days, we investigated the immune response induced by stimulation with 2 μg/ml and 4μg/ml (results not shown) of *Mycobacterium intracellulare* (Figure 1), BCG (Figure 2), and *Mycobacterium tuberculosis* (Figure 3) culture filtrate proteins and culture filtrate protein fractions. Thirty days post inoculation spleen cells from BCG infected mice produced significant levels of IFN-γ in response to fraction 11 from *Mycobacterium intracellulare* culture filtrate (Fig.4). In contrast, low levels of IFN-γ were measured in response to *Mycobacterium tuberculosis* antigens (Fig.4). Fraction 11 from *Mycobacterium intracellulare* culture filtrate was analysed by mass spectroscopy and it was identified as the *Mycobacterium avium* Ag85B in this molecular mass region (Fig.5). The BCG antigen preparation gave high levels of IFN-γ release from splenocytes isolated from *M. intracellulare* infected mice (Fig.4). The spleen cells from mice infected with either of the two strains, the environmental mycobacteria or the BCG strain strongly recognised the homologous culture filtrate proteins (Fig. 4). The three antigenic preparations induced no IFN-γ production from splenocytes isolated from naïve mice (results not shown). Similar data were obtained for 60 days after infection (data not shown). Experimental data obtained by stimulation with 4μg of antigen gave the same pattern, albeit while with higher levels of IFN-γ production (results not shown).

Mycobacterium tuberculosis Ag85A, Ag85B and Ag85B.P63 are recognised in mice immunised with live *Mycobacterium intracellulare.*

We inoculated groups of four C57BL/6J female mice, intravenously with either 10^6 CFU of *M. intracellulare* or 5×10^4 CFU of BCG Danish. Thirty days post inoculation, the levels of IFN-gamma in response to *M. tuberculosis* antigens such as TB10,4 (CFP 7), Ag85A, Ag85B, Ag85B.P63 were measured. The Ag85B.P63 peptide pools belong to Peppool 5 in the recombinant Ag85B genetic sequence (Fig.9).

Spleen cells isolated from mice immunized with the environmental mycobacteria produced higher levels of IFN-γ in response to Ag 85A, Ag 85B and Ag85B.P63 but not in response to the low molecular weight protein TB 10,4, when compared to mice vaccinated with BCG (Fig.6).

Influence of previous sensitisation with environmental mycobacteria culture filtrate proteins on antigen specific immune responses and on the BCG multiplication

We continued by immunizing groups of four C57BL/6J female mice, subcutaneously (s.c.) three times at 2-weeks intervals with 50 μg (per dose) of either *M. tuberculosis* Ag85B or the *M. intracellulare* culture filtrate proteins, emulsified in DDA (250 μg /dose). Two weeks after the last immunization we proceed to BCG vaccination (5×10^4 CFU). Three weeks after the vaccine we analysed the BCG growth in the spleen and liver of infected mice (Table 1). Prior immunization with *M. intracellulare* CF proteins caused the inhibition of BCG growth in sensitised mice when compared with control mice. A similar albeit smaller inhibition was observed in MtbAg85B sensitised mice. Three weeks after the vaccination, the MtbAg85B immunized mice immune response measured by IFN-gamma production in response to Ag85B and to Ag85B.P63 is significantly higher than those from mice immunized with environmental mycobacteria culture filtrate proteins (Fig.7A). Three weeks after the vaccine the frequency of IFN-γ producing cells in response to Ag85B. P63 was found to be higher in MTbAg85B sensitised mice (Fig.8). These differences in responses were maintained till eight weeks after BCG inoculation (Fig. 7B), along with this, the frequency of Ag85B.P63-Ag specific cells is at this time significantly higher in MTbAg85B sensitised mice (Fig.8).

Discussion

Differences in BCG efficacy against pulmonary tuberculosis in different geographical areas have been suggested to be due to the influence of the exposure of individuals to environmental mycobacteria. Studies performed closest to the equator, where exposure to environmental mycobacteria is highest, showed diminished efficacy of the BCG vaccine when compared with trials in other latitudes (Colditz et al., 1994; Fine, 1995). The two prevailing hypothesis to explain this variance in BCG efficacy are based on the close relationship of species within the genus *Mycobacterium*. Bacteria from the genus *Mycobacterium* besides having markedly different lifestyles, that span from non-pathogenic environmental mycobacteria to intracellular pathogens, have several characteristics in common such as the outer cell wall and many gene families (i.e. *esat-6* family, Ag85 complex). Thus there are many cross-reactive antigens between the genus *Mycobacterium*, being some of them at the same time highly immunogenic and highly conserved. This finding explains the mode of action of the BCG vaccine (Orme, 1988; Orme, 1999) and at the same time the protection conferred to TB by other mycobacteria such as *Mycobacterium microti* in humans and in animal models (Brodin et al., 2004; Dannenberg et al., 2000; Manabe et al., 2002). BCG as a live vaccine needs to multiply to generate long-lived immunity and is particularly sensitive to the influence of pre-existing immune responses to antigens shared with some environmental mycobacteria.

In line with this we analysed, in more detail, which secreted antigens from *Mycobacterium intracellulare*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* are recognised by animals infected with each of these strains. We have noted that there is a prominent recognition by BCG infected animals of different molecular mass secreted proteins from *M. intracellulare*, especially of antigens from the Ag85 complex. On the other hand antigens from *M. tuberculosis* are recognised in lower extent by BCG than by *M. intracellulare* infected mice. This suggests that there is more homology between the environmental mycobacteria and the BCG vaccine in terms of antigen recognition. These results are in accordance with other reports that suggest the homology of Ag85 complex between the genus mycobacteria (Launois et al., 1994).

In addition our results suggest that secreted antigens are effectively responsible for these cross-reactivity since the pre-sensitisation with culture filtrate proteins inhibited

the BCG growth, as well as the pre-immunization with the environmental mycobacteria secreting those proteins elicits a strong T-cell response against antigens from *M. tuberculosis*. This is in accordance to other works that reveal the importance of secreted antigens in the early cell-mediated immune response (Abou-Zeid et al., 1988; Andersen et al., 1991; Pais et al., 2000).

The fact that *M. intracellulare* infected mice recognise more intensely the secreted antigens, Ag85A, Ag85B and Ag85B.P63 from *M. tuberculosis* than BCG may contribute to clarify the *masking hypothesis* proposed by Palmer and colleagues. The *masking hypothesis* states that in some regions of the world, exposure to environmental mycobacteria confer some degree of protective immunity to TB due to the recognition of cross-reactive antigens. In the way that there is a close relationship between the genus mycobacteria and that BCG vaccine is effective in protecting against infection by *M. tuberculosis* and its counterparts such as the distantly related *M. leprae* (Fine et al., 1986), the possibility that other mycobacteria can also confer some cross-protection against TB must be acknowledged.

Our data contribute to explain the interference of pre sensitisation to *M. intracellulare* in the BCG vaccine, suggested in our previous work by the so called *blocking hypothesis* (Brandt et al., 2002). There are antigens, especially the Ag85 complex, that are recognised by T-cells from mice previously exposed to *M. intracellulare,* when BCG vaccine is delivery. The recognition by memory T-cells of cross-reactive antigens triggers an immune response directed against those antigens, blocking BCG replication and consequently the effectiveness of the vaccine. In this way there are some works such as the results of Buddle and colleagues that showed that cattle with reactivity to PPD from *M. avium* have no BCG induced protection (Buddle et al., 2002).

The pre-sensitisation with the secreted proteins from the environmental mycobacteria inhibited the BCG growth due to the wide range of antigens that are present in the culture filtrate that are possibly cross-reactive with the BCG proteins. This inhibition resulted in the development of a minor T-cell response when these cells were stimulated with antigens from *M.tuberculosis*. On the other hand the boost with *M.tb* Ag85B primes the T-cell response to *M.tb*. Ag85A and *M.tb*. Ag85B. These results are in accordance to our proposed *blocking hypothesis* in the way that prior exposure to environmental mycobacteria indeed inhibits the BCG replication essential to the

establishment of an effective immune response to subsequent *M. tuberculosis* antigens challenge.

Figure legends

Fig. 1. Fractionation of *Mycobacterium intracellulare* culture filtrate (CF) proteins. Proteins were separated by SDS-PAGE and electroeluted as described. The different MW fractions were analysed on silver staining SDS-PAGE gel.

Fig. 2. Fractionation of *Mycobacterium bovis* BCG culture filtrate (CF) proteins. Proteins were separated by SDS-PAGE and electroeluted as described. The different MW fractions were analysed on silver staining SDS-PAGE gel.

Fig.3. Fractionation of *Mycobacterium tuberculosis* culture filtrate (CF) proteins. Proteins were separated by SDS-PAGE and electroeluted as described. The different MW fractions were analysed on silver staining SDS-PAGE gel.

Fig.4. Antigenicity of the *Mycobacterium intracellulare*, BCG and *M. tuberculosis* culture filtrate fractions (the fraction numbers on this figure correspond to the numbers in Fig. 1, Fig.2 and Fig.3 respectively). Spleen cells of noninfected mice or mice infected for 30 days with *Mycobacterium intracellulare* (A) or BCG (B) were stimulated in vitro with 2 μg/ml of each fraction per ml. IFN-γ release was quantified by ELISA in the culture supernatants.

Fig. 5. Analysis of fraction 11 of *Mycobacterium intracellulare* CF. Fraction 11 was analysed by mass spectroscopy and then transferred to nitrocellulose and incubated with an antibody against A85 complex followed by ECL-dependent detection of antigen-positive bands.

Fig.6. IFN-gamma response to specific antigens: TB 10,4 (CFP 7), Ag85A, Ag85B and Ag85B.P63. Spleen cell suspensions were prepared from groups of mice infected for 30 days with 10^6 CFU of *M. intracellulare,* 5×10^4 CFU of BCG or noninfected (naïve) and stimulated in vitro with 4 μg of TB 10,4, Ag85A, Ag85B and Ag85B.P63 per ml. IFN-γ release was quantified by ELISA in the 72-h culture supernatants. Results are expressed as means of values of triplicate samples ± 1 standard deviation

performed on cells pooled from four mice. Statistically significant differences are labelled: * $P< 0,05$ according to Student's *t* test.

Fig. 7. Effects of sensitisation on the IFN-γ response to *M. tuberculosis* antigens
Spleen cells were pooled from groups of four mice sensitised with the culture filtrate proteins from the environmental strain or sensitised with MTbAg85B, three weeks (A) and 8 weeks (B) after BCG vaccination. IFN-γ was measured in the supernatants of spleen cell cultures stimulated in vitro with, Ag85A, Ag85B and Ag85B.P63. The results are given as means of values of triplicate samples with standard errors. Statistically significant differences are labelled: ** $P <0,01$, according to ANOVA.

Fig.8. Frequency of IFN-γ- producing cells isolated from the spleens of individual mice sensitised with culture filtrate proteins from *M. intracellulare* and sensitised with MTbAg85B, in response to Ag85B.P63.
The frequency of IFN-γ- producing cells was detected by the ELISPOT assay post vaccination (3 and 8 weeks) after in vitro stimulation with 4μg of Ag85B.P63 per ml. Data are expressed as means of results obtained from spleen cells from four individual mice per group ± standard errors. Statistically significant differences are labelled: ** $P <0,01$, according to ANOVA.

Fig.9. The peptide pools of MtbAg85B. P63 is located in Peppool 5.

Table 1. Bacterial numbers in spleens of mice sensitised with *M. intracellulare* CF proteins or with MTbAg85B, after vaccination with BCG. Mice were immunized s.c. three times with the crude extract of culture filtrate proteins from *M. intracellulare* or with MTbAg85B, emulsified in DDA. Two weeks following, animals were vaccinated with BCG and three weeks after the vaccine spleens were removed to bacterial count. Four animals of each group were sacrificed for each time point. The data shown are the means of Log BCG CFU ± standard errors. The Log inhibition

represents the Log reduction in bacterial numbers compared to those in unsensitised control mice. Bacterial numbers significantly different from those from control animals are indicated as follows: **, $P<0.01$ according to ANOVA.

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Figures

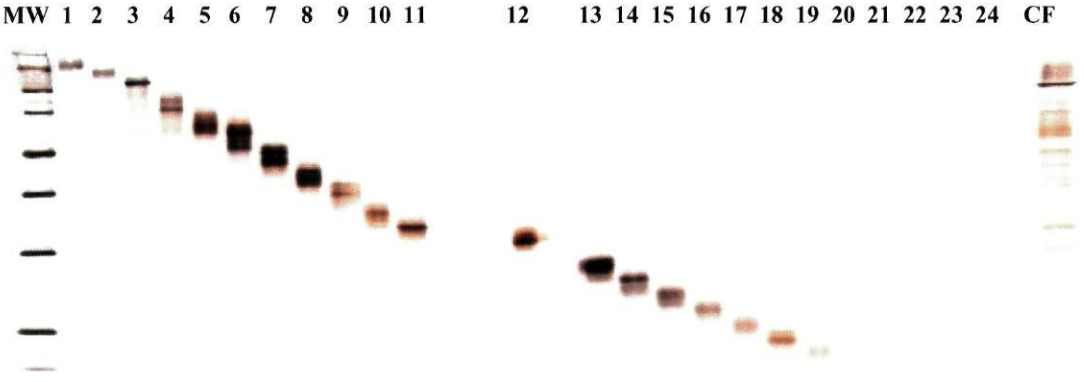


Figure 1

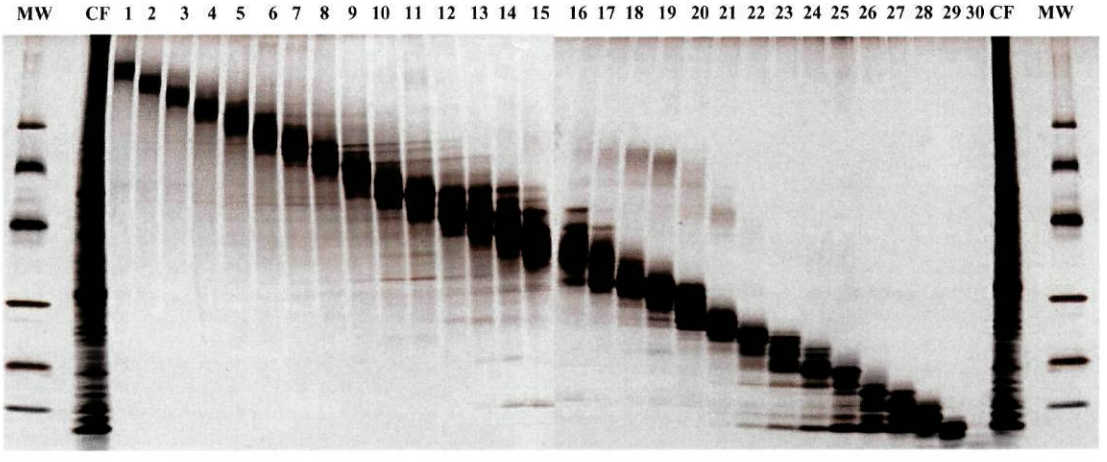


Figure 2

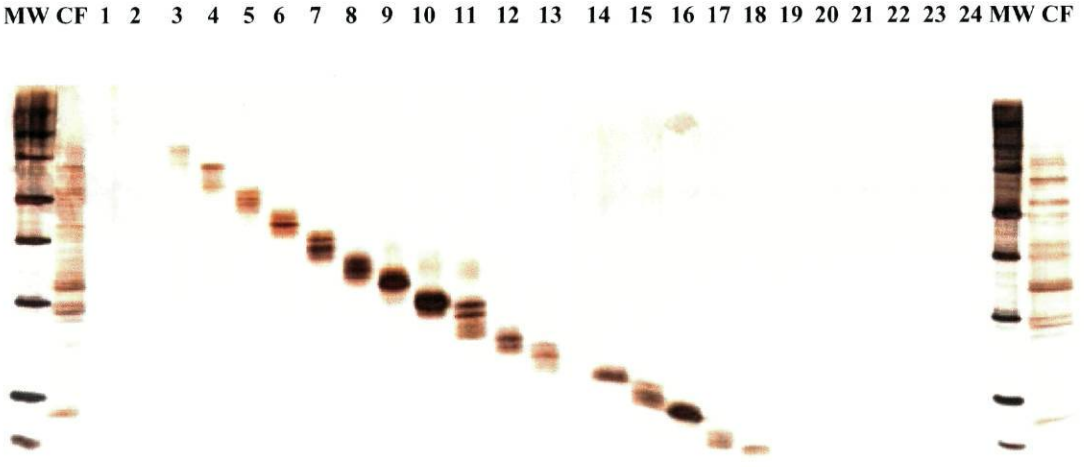


Figure 3

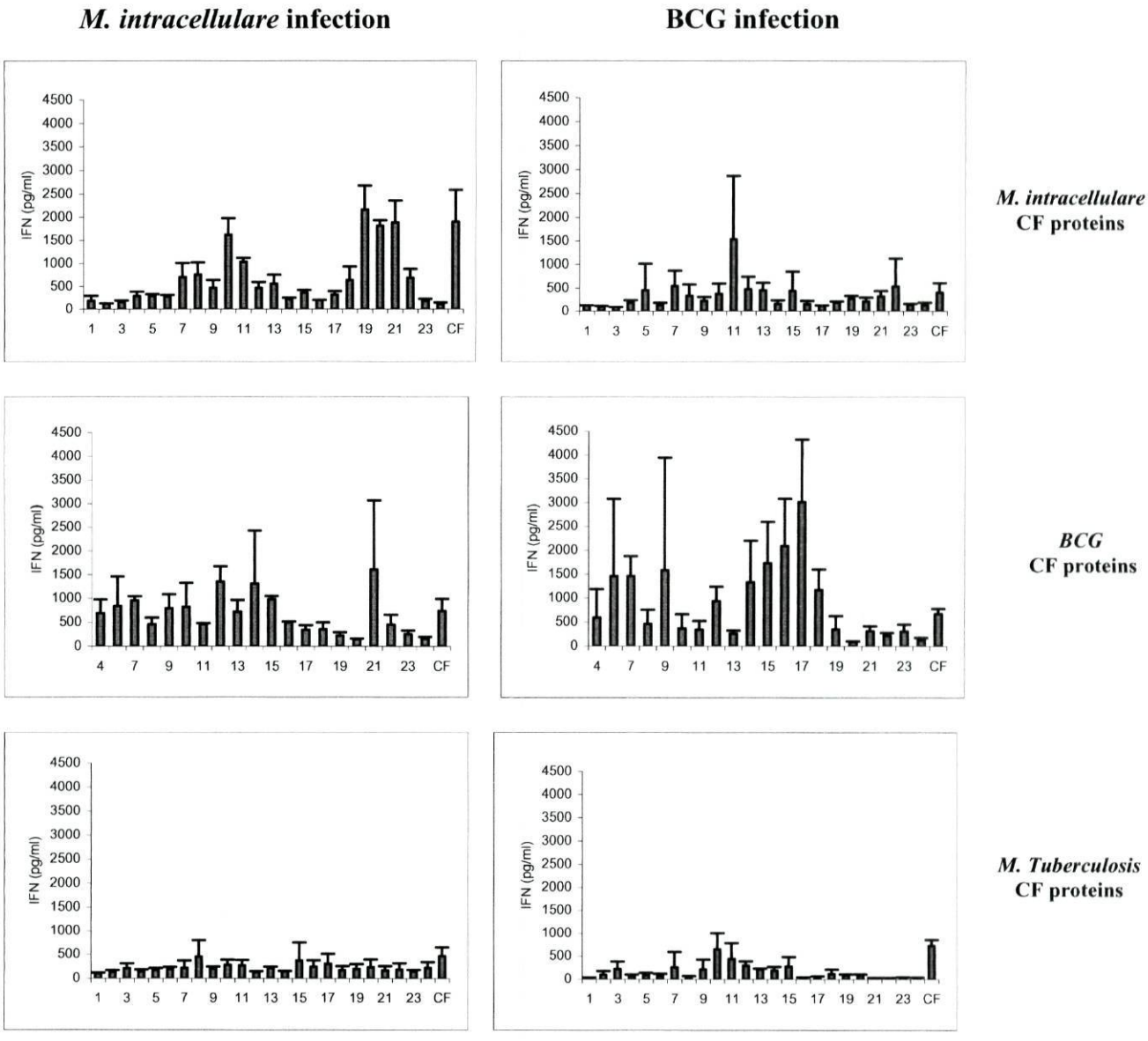

M. intracellulare infection
BCG infection
IFN (pg/ml)
M. intracellulare CF proteins
BCG CF proteins
M. Tuberculosis CF proteins

Figure 4

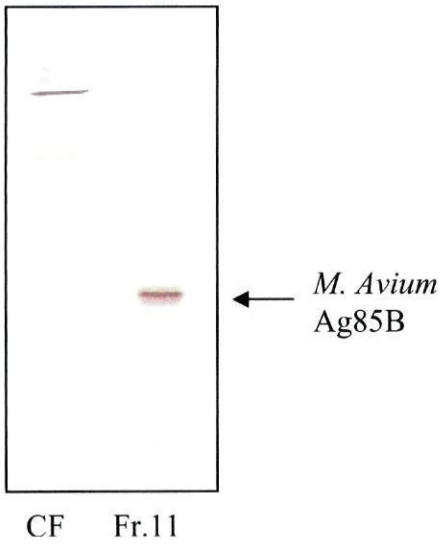


Figure 5

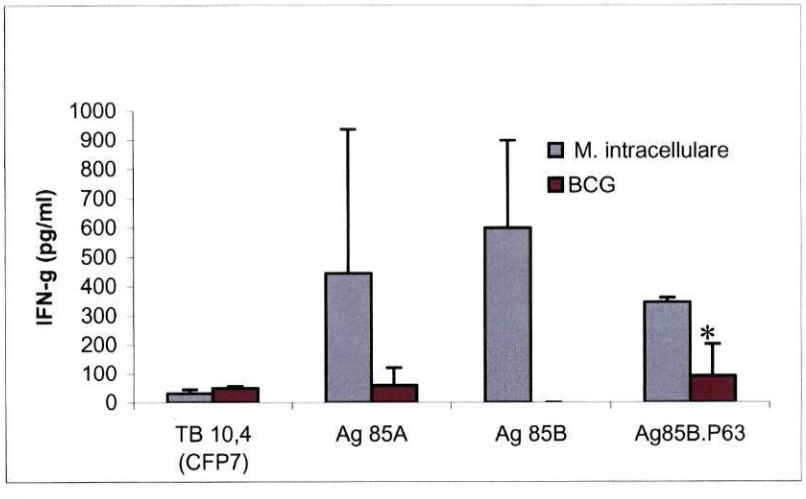
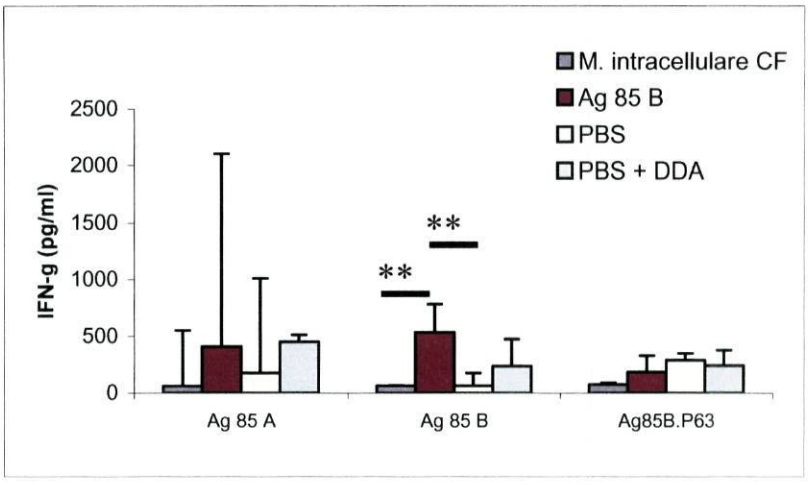
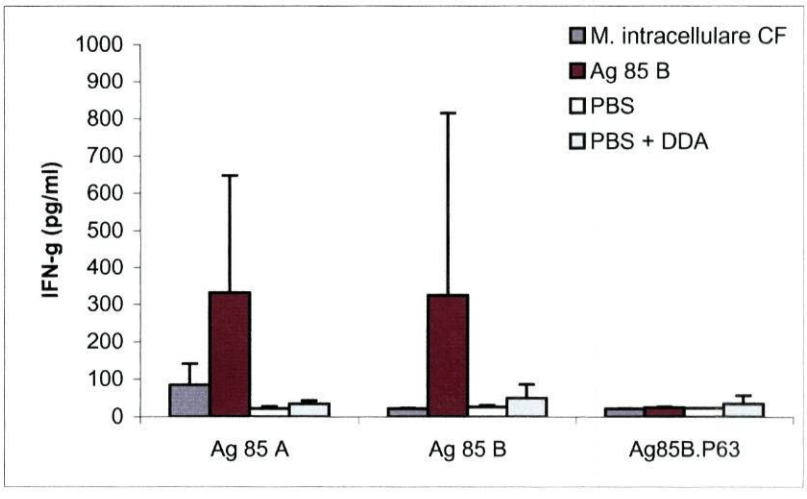


Figure 6

A


M. intracellulare CF
Ag 85 B
PBS
PBS + DDA
IFN-g (pg/ml)
2500
2000
1500
1000
500
0
**
**
Ag 85 A
Ag 85 B
Ag85B.P63

B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Ag 85 B
PBS
PBS + DDA
IFN-g (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
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Ag 85 B
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Figure 7

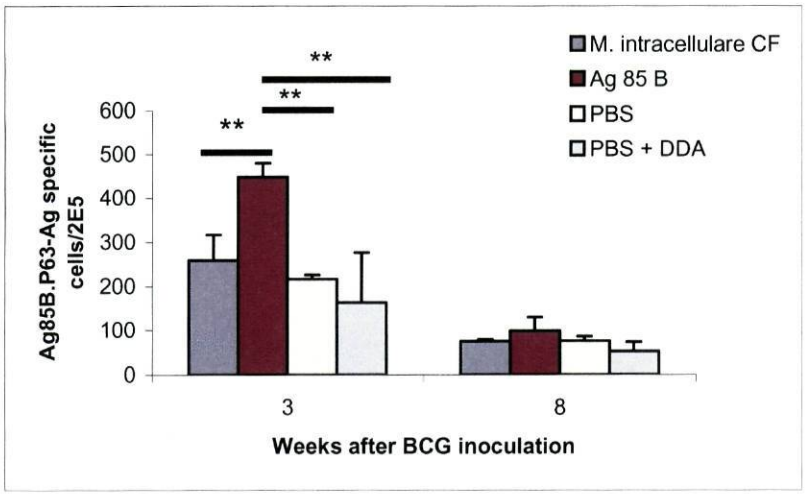

Figure 8

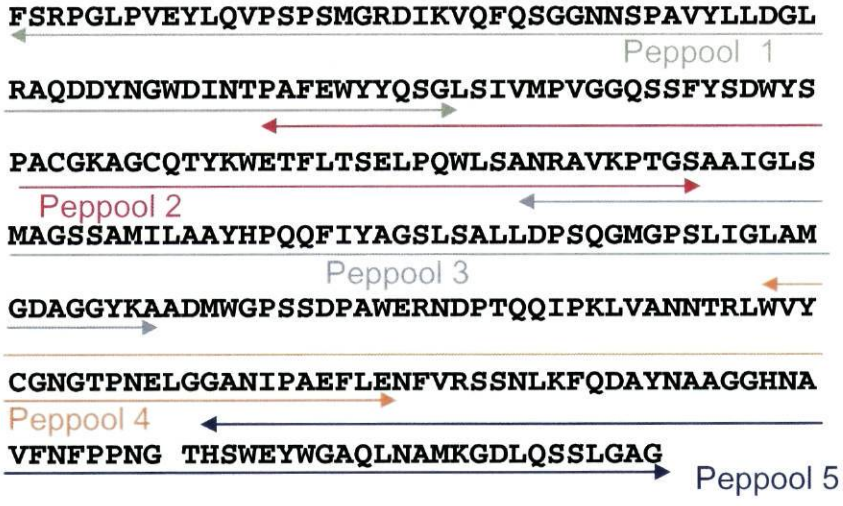

Figure 9

	Spleen		Liver	
Sensitised group	Log CFU	Log inhibition	Log CFU	Log inhibition
Control	3.53 ± 0.02		3.40 ± 0.07	
DDA	3.48 ± 0.03		3.36 ± 0.05	
M. intracellulare CF	3.24 ± 0.05	0.29**	2.82 ± 0.18	0.58**
MTbAg85B	3.39 ± 0.04	0.14**	3.14 ± 0.02	0.27**

Table 1

Chapter 5

Discussion

Discussion

The current and only licensed vaccine against *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, has been widely evaluated and in our days it is calculated that more than 3 billion people have received BCG.

This vaccine, considered as a safe vaccine, induces a level of protection nearing 80% against severe infant TB. Nevertheless, protection against lung TB at all ages is strongly variable and predominantly poor. The failure of the BCG vaccine is consistently found in regions close to the equator where there is a high incidence of bacteria from the genus *Mycobacterium*. This finding led to the first hypothesis proposed by Palmer and Long, to explain the inefficacy of the BCG vaccine. This hypothesis centres on the close relationship between species of the genus *Mycobacterium*. Mycobacteria from the *M. tuberculosis* complex like *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG and environmental mycobacteria such as *Mycobacterium fortuitum*, *Mycobacterium chelonae* and *Mycobacterium intracellulare* despite their different lifestyles have many common features. The outer cell wall rich in peptidoglycan and lipids, the high content in GC in the genome and many similar protein families are some of the important common characteristics. One consequence of this homology is the common antigens shared between these species. These shared and many times immunodominant antigens are the basis of the protection conferred by the BCG vaccine as well as the protection conferred by other mycobacterial species such as *Mycobacterium microti*. It is the memory immune recognition of these specific antigens that induces protection against latter *Mycobacterium tuberculosis* infection. At the same time the immune recognition of shared antigens is responsible for the failure of the efficacy of the BCG vaccine predominantly in areas where environmental mycobacteria are found. The prior exposure to environmental mycobacteria sensitise individuals to antigens that are shared among *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* and stimulate cellular immune responses directed to those antigens. In line with this, there are consistent data from different trials suggesting that: i) there is a high prevalence of skin tested positive individuals to antigens from *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* in tropical areas, (Malawi), where the exposure to atypical mycobacteria is significant, when compared to individuals in higher latitudes

(Fine et al., 2001); ii) PPD related immune responses induced by BCG vaccination are minimal in individuals with high responses to antigens from *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* (MAIS complex) when compared with individuals with low responses to MAIS antigens (Black et al., 2001) ; iii) neonatal BCG vaccination prevents from *Mycobacterium tuberculosis* infection (Colditz et al., 1995; Marchant et al., 1999).

The masking hypothesis suggested by Palmer and colleagues proposes that prior exposure to environmental mycobacteria confers some degree of protection against *Mycobacterium tuberculosis* infection nearly as good as that conferred by BCG. Moreover any additional protection conferred by additional BCG vaccination is insignificant and limited. This hypothesis was based on the guinea pig model of TB infection in which immunization with environmental mycobacteria induced protection to *M. tuberculosis* challenge and the effects of a later BCG vaccine were markedly reduced (Palmer and Long, 1966). In fact this proposed explanation is in accordance with the results of others (Lozes et al., 1997) as well as our own, that suggests that there are cross-reactive antigens between environmental mycobacteria and *Mycobacterium bovis* BCG. Consequently there is the possibility that mycobacterial species other than *M. bovis* BCG confer some cross-protection against tuberculosis. Recent work from Howard and colleagues show that prior exposure to *Mycobacterium avium* induces protection to latter *Mycobacterium bovis* infection in cattle (Hope et al., 2005). One important aspect in the masking hypothesis is that it is assumed that prior exposure to environmental mycobacteria confers a significant level of protection to TB. If that is the case, in some geographical areas where exposure is high the incidence of TB should be lower when compared with regions where environmental mycobacteria are less common (Colditz et al., 1994; Ponnighaus et al., 1993; Ponninghaus et al., 1992). However, reported trials conducted to analize BCG efficacy point to the contrary. Regions, such as Malawi, where there is a high incidence of environmental mycobacteria the occurrence of tuberculosis is also high (Colditz et al., 1994; Fine et al., 1986).

The results shown in this thesis point out to a new hypothesis that may explain the interference of environmental mycobacteria in the BCG vaccine efficacy. The blocking hypothesis suggests that previous exposure to environmental mycobacteria induces an immune response to antigens common for both mycobacteria and block the replication of *Mycobacterium bovis* BCG essential for effective vaccination.

Six different isolates from soil and sputum samples from Karonga District in Northern Malawi were tested in the mouse model of infection. *Mycobacterium intracellulare* was the most virulent species tested and its replication could not be controlled in mice. *Mycobacterium intracellulare* is a "slow grower" mycobacterium, ubiquitous in the environment including soil and water and commonly found in sputum smears and cultures. The environmental mycobacterial strain inhibited the multiplication of BCG and completely ablated BCG induced immune responses in sensitised mice. In addition sensitisation with the environmental mycobacteria by itself or even followed by BCG vaccination failed to induce protection against *Mycobacterium tuberculosis* infection.

In line with these results the proposed hypothesis suggests also that the level of protection conferred by atypical mycobacteria has minimal influence on a more virulent challenge, such as *M. tuberculosis* infection. Related to this subject is the work of Buddle and colleagues who showed that cattle with reactivity to PPD from environmental mycobacteria have no BCG induced protection but a more virulent BCG vaccine was able to confer protection to TB in sensitised animals (Buddle et al., 2002). In the same point there is another work using a recombinant BCG vaccine containing the region of difference 1 (RD-1), that confers more virulence to the strain, that show protection to TB despite prior exposure to atypical mycobacteria (Demangel et al., 2005). The fact that in some regions the BCG vaccine confers protection against the less virulent pathogen *Mycobacterium leprae* and not against *Mycobacterium tuberculosis* (Ponninghaus et al., 1992) may also be explained by the blocking hypothesis.

The two referred hypothesis may seem to be logical and mutually accepted but data from human clinical trials favours the blocking hypothesis. The BCG-induced DTH is significantly smaller and wanes faster in individuals from areas with high levels of sensitisation to environmental mycobacteria (Malawi) when compared to areas with minimal exposure (United kingdom or Denmark) (Fine et al., 1994; Floyd et al., 2002). These transient responses induced by BCG vaccination in sensitised individuals are recall responses due to the previous sensitisation and consequently induce poor protective immunity against tuberculosis.

The main idea of the proposed blocking hypothesis is that BCG as a live vaccine is particularly susceptible to the influence of pre-existing immune responses to antigens common for the mycobacteria In this regard the study on antigen specificity of the T-

cell response to *Mycobacterium avium* infection reveals important data concerning the antigen repertoire recognized by those T-cells. Proteins secreted by growing mycobacteria are major T-cell antigens in the host during live infection as described by several authors (Andersen, 1994; Andersen, 1997; Andersen et al., 1992; Andersen et al., 1991; Demissie et al., 1999; Horwitz et al., 1995; Hubbard et al., 1992; Mustafa et al., 1998; Pal and Horwitz, 1992). In addition some authors had already reported the importance of cell wall (Barnes et al., 1989; Dhiman and Khuller, 1998; Orme et al., 1993) and cytosolic (Agger et al., 2002) proteins in the induction of the immune response during mycobacterial infection. Of importance, however, is the demonstration by the present study that T-cells respond to all classes of mycobacterial antigens shifting in the repertoire of these antigens during the course of infection. In the earlier time points of infection the T-cell response to secreted proteins by living mycobacteria emerged before the response to the cytosol and envelope fractions. Among the recognised secreted antigens, proteins with the same MW as the Ag 85 complex are the strongest IFN-gamma production inducers. Near the same time envelope proteins began to be recognised in what could be interpreted as the detaching from the cell wall during growth of the mycobacteria inside the macrophage. Later in infection and as a consequence of the killing of the bacteria, cytosolic antigens are at this moment recognised by T-cells. The present results suggest that there is a different kinetics related to the T-cell recognition of each class of antigens. It seems that antigens from different compartments in the bacterial cell may be responsible for protection at different stages of mycobacterial disease. However, secreted antigens are responsible for the higher and earlier peak of T-cell mediated immunity in mycobacterial infection. In this regard the work presented in this thesis was conducted to analyse the cross-reactive secreted antigens among one environmental mycobacteria strain, *Mycobacterium intracellulare*, and *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. A wide range of *M. intracellulare* secreted proteins are recognised by T-cells from BCG infected animals. In particular proteins from the Antigen 85 complex mainly the Ag85B are cross-recognized by BCG infected animals.

The protective antigens from *M. tuberculosis* are still not entirely defined, but research in this field has demonstrated that the Ag85complex is predominantly recognised by T lymphocytes (Andersen et al., 1992). The three components of the Ag85 complex, a 30-32 kDa protein family (85A, 85B and 85C) are major secretion

products of *Mycobacterium tuberculosis* and BCG (Harth et al., 1996). Antigen 85 homologues are found in all mycobacterial species (Launois et al., 1994; Wiker and Harboe, 1992). Others have shown that Ag85 complex is a potent inducer of cell-mediated immunity (Roche et al., 1994; Silver et al., 1995). Our data with the Ag85B+ESAT-6 sub-unit vaccine showing protection against a latter TB challenge in sensitised mice, confirms the protective effect of the Ag85 complex. In addition these results suggest that a non-living vaccine based on secreted proteins is not affected by prior exposure to environmental mycobacteria and could effectively protect against subsequent *M. tuberculosis* infection.

The exposure to live *Mycobacterium intracellulare* or to secreted proteins from these mycobacteria prior to BCG vaccination induces an anamnestic response that is biased towards antigens present in the BCG vaccine. This implies that *M. intracellulare* infection primes the immune system and imprints a memory of the exposure onto the T-cell repertoire that affects later BCG vaccination.

Overall, the results presented in this thesis suggest that part of the low efficacy of BCG vaccination against *M.tuberculosis* observed in geographical areas where *M.intracellulare* is found in the environment, could be related to the activation of a cross-reactive immune response against antigens (including Ag85 complex) from BCG, after vaccination. The T cell memory immune response against these cross-reactive antigens from BCG inhibits the essential *M. bovis* BCG replication to the vaccine "take".

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"Interaction of the Immune Response to BCG and to Environmental Mycobacteria Infection"

Erratum

1. Missing

Joana Maria Lencastre Serpa de Castro Feijó is a PhD student belonging to the PhD program GABBA (Programa Doutoral em Biologia Básica e Aplicada da Universidade do Porto).

2. The paragraphs described in each of the following pages should be changed by:

Page 16

Endocytosis of *M. tuberculosis* involves different receptors on the phagocytic cell, which bind either to nonopsonized *M. tuberculosis* or recognize opsonins on the surface of the mycobacteria (Figure 1).

Page 20

The activated T cells start to produce cytokines necessary to activate the cells involved in the immune response against TB (Figure 2).

Page 31

The plasma membrane is a typical phospholipid bilayer, which lies underneath a rigid peptidoglycan (PG). A number of proteins are found in association with the membrane, with PG, and between the membrane and the PG and some of these may be immunogenic (Barnes et al., 1989) (Figure 3).

Page 33

Some laboratories focused on proteins from *M. tuberculosis* and suggested the division of mycobacterial proteins into three major classes which differ in rate of release and sub cellular localization (Figure 4):

Page 34

ST-CF was initially described as containing 33 major protein bands analysed by SDS-PAGE separation (Figure 5).

"Interaction of the Immune Response to BCG and to Environmental Mycobacteria Infection"

Page 43

Orme and colleagues demonstrated that after immunization of mice with BCG a population of $CD4^+$ T lymphocytes emerge. These cells secrete IFN-γ when stimulated in vitro with purified secreted proteins from the bacillus (Orme, 1988c) (Figure 6).

Page 44

Although BCG is highly efficacious in laboratory models of disease (Smith, 1985) , it has varied tremendously in protective efficacy in field trials, and in some geographical regions the vaccine has not shown any efficacy at all (Fine, 1995; Ponninghaus et al., 1992) (Tabela 1).

Page 48

Table 1. Estimate efficacy of BCG against pulmonary tuberculosis in trials and observational studies, by latitude (reproduced from Fine, 1995).

Page 53

These results introduce novel candidates to a new skin test for TB (Andersen et al., 2000) (Table 2).

Page 54

Table 2. Distribution of diagnostic antigens in mycobacterial species (adapted from (Andersen et al., 2000)

3. In any place where the references are described between two brackets, there is an error due to the use of the program "End Note".