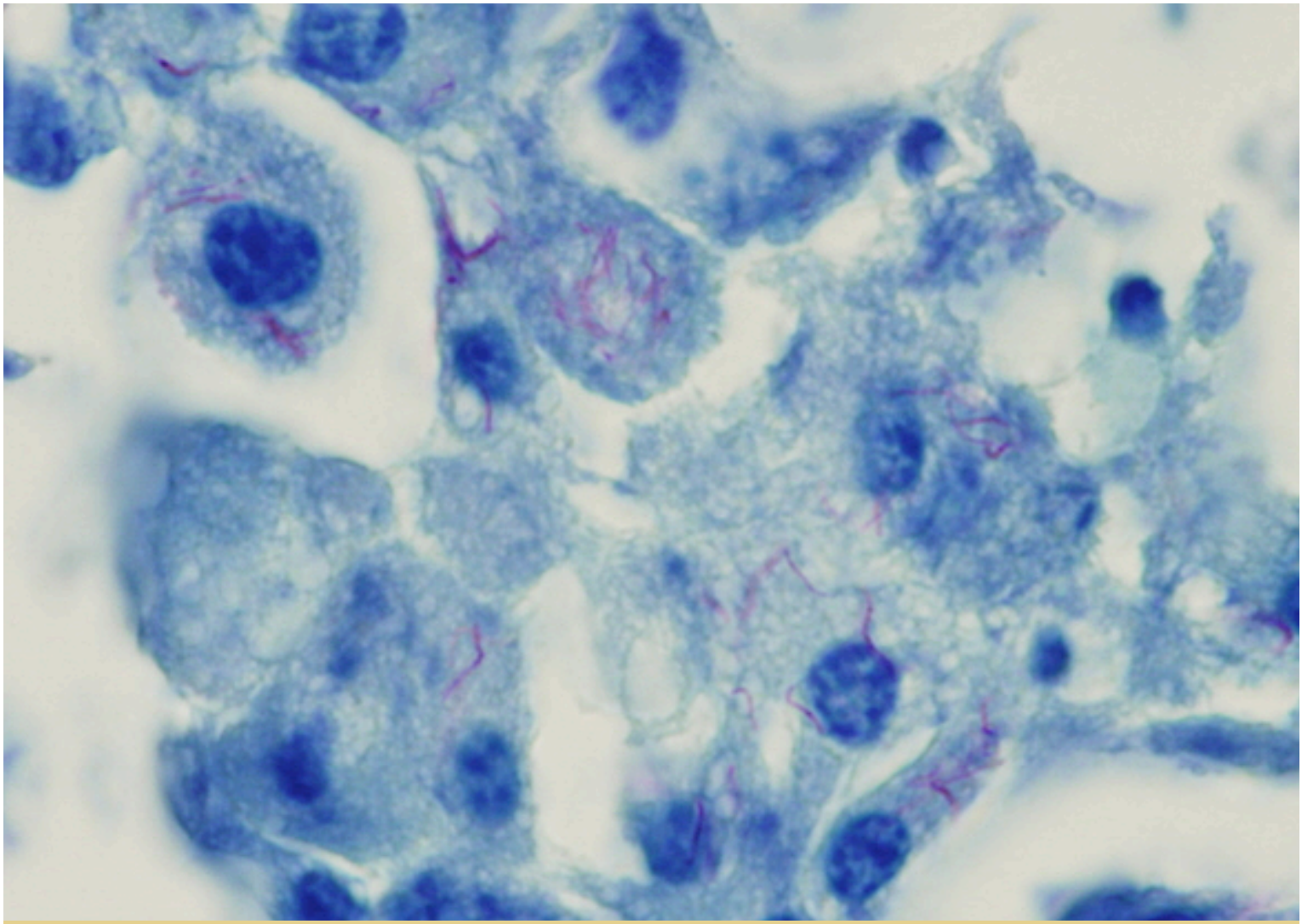

Manuel António Afonso Pereira Barroso

THE IMMUNE RESPONSE TO MYCOBACTERIA OF DISTINCT VIRULENCE

2012

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Universidade de Coimbra

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Manuel António Afonso Pereira Barroso

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Dissertação apresentada ao Departamento de Ciências da Vida, da Faculdade de Ciências e Tecnologia da Universidade de Coimbra para a prestação de provas de Doutoramento em Bioquímica, na especialidade de Microbiologia

Orientador: Prof. Doutor Rui Appelberg Gaio Lima, Professor Catedrático no Instituto de Ciências Biomédicas Abel Salazar, da Universidade do Porto.

Co-orientador: Prof. Doutor Milton Costa, Professor Catedrático do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia, da Universidade de Coimbra.

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De acordo com o disposto no nº 2 do Art. 8 Do Decreto-Lei n. 388/70, nesta dissertação foram utilizados resultados das publicações abaixo indicadas. No cumprimento do disposto no referido Decreto-Lei, o autor desta dissertação, declara que interveio na concepção e na execução do trabalho experimental, na interpretação dos resultados e na redação dos manuscritos publicados ou em preparação, sob o nome de **Afonso-Barroso A:**

Lipoarabinomannan mannose caps do not affect mycobacterial virulence or the induction of protective immunity in experimental animal models of infection and have minimal impact on in vitro inflammatory responses.
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The mycobacterial type VII secretion system ESX-5 plays a crucial role in the ability of *Mycobacterium bovis* BCG to survive in mice.
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“Palavras, leva-as o vento”... as minhas palavras de gratidão, deixo-as aqui gravadas para que perdurem ao alcance de quem partilhou este caminho comigo.

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Ao meu “Sol” e às minhas “Asas”

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ABBREVIATION LIST

APC	Antigen Presenting Cells
AG	Arabinogalactan
BCG	Bacillus Calmete-Guérin
BMMø	Bone marrow derived macrophage
CD	Cluster of differentiation
CFU	Colony forming units
CFP-10	10 kDa culture filtrate antigen
DC	Dendritic cells
DC-SIGN	DC-specific intercellular-adhesion molecule-3 grabbing non-integrin
ELISA	Enzyme-linked immunoabsorbent assay
ESAT-6	6kDa early-secreted antigenic target
EEA1	Early endosome autoantigen 1
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.v.	Intravenous
KO	Knockout
LPS	Lipopolysaccharide
LM	Lipomannan
MAC	*M. avium* complex
MMR	Macrophage mannose receptor
ManLAM	Mannose-capped lipoarabinomannan
Myc Ac	Mycolic acids
PIMs	Phosphatidyl-myo-inositol mannosides
PG	Peptidoglycan
Mf	Macrophage

MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NK	Natural killer
NO	Nitric oxide
PGL	Phenolic glycolipid
ROS	Reactive oxygen species
RNI	Reactive nitrogen species
SL	Sulfolipid
TCR	T cell receptor
TDM	Trehalose dimycolate
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WHO	World Health Organization
WT	Wild type

LISTA DE ABREVIATURAS

APC	Célula Apresentadora de Antigénio
BCG	Bacilo de Calmete-Guérin
BMMø	Macrofagos derivados da Medula Ossea
CFU	Unidades formadoras de Colónias
DC	Células Dendriticas
IFN	Interferão
IL	Interleucinas
i.v.	Intravenoso
LPS	Lipopolissacarídeo
LM	Lipomanano
MAC	Complexo *M.avium*
MMR	Receptor de Manose do Macrófago
ManLAM	Lipoarabinomanano com capuz de Manose
Myc Ac	Acidos Micólicos
PG	Peptidoglicano
MHC	Complexo principal de Histocompatibilidade
NO	Óxido Nitrico
PGL	Glicolipido Fenólico
ROS	Especies Reactivas de Oxigénio
RNI	Especies Reactivas de Azoto
TCR	Receptor da Célula T
ESX-1	Sistema de Secreção 1
ESX-5	Sistema de Secreção 5
WHO	Organizaçao Mundial de Saude

Abstract:

Multidrug resistant strains and the HIV epidemic strongly contributed to Tuberculosis (TB) reemerging again as a major health problem worldwide. The cell wall of *M. tuberculosis* is considered a major determinant of virulence. A main component of the cell wall is the mannose-capped lipoarabinomannan (ManLAM), which appears to play a key role in host cell recognition and immunomodulation. However, while mannose caps have been reported to be responsible for various immunosuppressive activities of ManLAM observed in vitro, there is conflicting evidence about their contribution to mycobacterial virulence in vivo. Furthermore, the presence of ManLAM molecules also in the vaccine strain *M. bovis* BCG may account for its failure to induce long-lasting immunity. We used *M. bovis* BCG and *M. tuberculosis* mutants that lack the mannose cap of LAM to assess the role of ManLAM in the interaction of mycobacteria with the host cells, to evaluate vaccine-induced protection and to determine its importance in *M. tuberculosis* virulence. Deletion of the mannose cap did not affect BCG survival and replication in phagocytes and in mice, nor the efficacy of vaccination against a *M. tuberculosis* challenge in mice and guinea pigs. Finally, the lack of the mannose cap in *M. tuberculosis* did not affect its virulence in mice nor in macrophages.

Besides the immunomodulatory molecules that constitute the cell envelope, pathogenic mycobacteria rely also in protein transport systems, essential to secrete virulence factors. The involvement of secretion systems ESX-1 in mycobacteria virulence is clear, with the ESAT-6/CFP-10 complex among the most important antigens secreted by *M. tuberculosis*. ESX-5 was the second ESX system implicated in mycobacteria pathogenicity and several studies show that has an important contribution to *M. marinum* and *M. tuberculosis* virulence. Each one of these ESX systems fails to complement the loss of virulence caused by deletion of the other, suggesting that they have independent contributions during mycobacterial infections, but also, that both ESX systems must be present for full mycobacterial virulence. However, no data exist on mutant mycobacteria lacking both secretion systems. Using the natural ESX-1 mutant BCG, we show that the presence of ESX-5 is crucial to BCG maintain its virulence, with depletion of ESX-5 leading to a strong attenuation of BCG even in immunocompromised mice.

In this work we show that the absence of the mannose caps of LAM does not affect mycobacterial virulence, and we demonstrated the fundamental contribution of the protein export system ESX-5 into mycobacteria acquiring full virulence. We hope the knowledge generate in this work can contribute for the development of better tools against tuberculosis.

RESUMO

Apesar dos progressos científicos, a tuberculose continua a ser a segunda maior causa de morte por doença infecciosa no mundo, tendo o aparecimento de estirpes multirresistentes e a coinfecção com HIV contribuído de uma forma significativa para que se mantenha um grave problema de saúde à escala global.

A parede celular do *M. tuberculosis* contribui de forma determinante para a sua virulência. Um dos seus principais constituintes é o lipoarabinomanano (LAM), um glicolípido complexo, onde a presença de um "capuz" de resíduos de manose (Man) parece ser fundamental para a interação com o hospedeiro e efeitos imunossupressores que exerce. No entanto, muitos dos efeitos descritos para o ManLAM foram observados em experiências in vitro, pelo que a sua contribuição para a virulência da micobactéria in vivo é ainda duvidosa. Para além disso, a presença de ManLAM no *M. bovis* BCG, poderá ser responsável pela baixa eficácia que a vacina apresenta na indução de uma resposta protetora contra a tuberculose.

De modo a testar a importância do ManLAM in vivo, foram construídos mutantes (*M. bovis* BCG e *M. tuberculosis*) incapazes de sintetizar o "capuz" de manose, e avaliado o modo como estas bactérias interagem com as células do hospedeiro, a capacidade que têm para gerar uma resposta imunitária protetora e a forma como a sua virulência é afectada. Observámos que a ausência do "capuz" de manose do LAM não afectou a sobrevivência e replicação do BCG em células fagocíticas e ratinhos, nem a sua eficácia na indução de uma resposta protetora contra a infeção por *M. tuberculosis* em ratinhos e porquinhos-da-índia. A ausência do "capuz" de manose também não afectou a virulência do *M. tuberculosis* em ratinhos ou macrófagos.

Para além das moléculas imunomoduladoras que constituem a parede celular, as micobactérias patogénicas possuem também sistemas de secreção essenciais para exportar factores de virulência. A contribuição do sistema de secreção ESX-1 para a virulência do *M. tuberculosis* é incontestável, com o complexo ESAT-6/CFP-10 entre os antigénios exportados mais importantes. Recentemente, vários estudos demonstraram que existe um segundo sistema de secreção, o ESX-5, que tem também uma importante contribuição para a virulência de *M. marinum* e *M. tuberculosis*. O facto de cada um destes sistemas ser incapaz de compensar a perda de virulência que ocorre aquando da remoção de um deles sugere que os dois ESX têm contribuições diferentes no decorrer da infeção. Estes factos sugerem ainda que ambos ESX têm de estar presentes para que a micobactéria possua uma virulência completa. Contudo, para validar esta hipótese é necessário a remoção dos dois sistemas. Deste modo, e utilizando a estirpe BCG, que já é um mutante natural para o sistema ESX-1, verificámos que a deleção do segundo

sistema, ESX-5, resultou numa forte atenuação da virulência do BCG, que se manteve inclusive em ratinho imunocomprometidos.

Em conclusão, neste trabalho, mostrámos que o “capuz” de manose do LAM parece não ser um factor determinante na virulência da micobactéria. Demonstrámos ainda a contribuição fundamental do sistema ESX-5 para a virulência da micobactéria. Esperamos que o conhecimento gerado neste trabalho possa contribuir no futuro para o desenvolvimento de melhores ferramentas no combate à tuberculose.

THESIS OUTLINE:

The present dissertation is organized in three parts: Introduction, Results and General Discussion.

In part I a general introduction to the subject of this thesis is presented. An overview of several aspects of mycobacteria is made, going through the diversity of mycobacterium genus and recognition and immune response by the host, until the constitution of the mycobacteria cell wall and they immunomodulatory molecules. In the end, the aims of the thesis are presented.

In part II, the results are presented in the form of chapters. The work entitle: "Lipoarabinomannan mannose caps do not affect mycobacterial virulence or the induction of protective immunity in experimental animal models of infection and have minimal impact on in vitro inflammatory responses." was published in Cellular Microbiology journal.

The work entitle: "The mycobacterial type VII secretion system ESX-5 plays a crucial role in the ability of *Mycobacterium bovis* BCG to survive in mice" is waiting for submission.

In part III, the general discussion and concluding remarks of the work are presented.

ESTRUTURA DA TESE:

A presente dissertação está organizada em três partes: Introdução, Resultados, Discussão e Conclusões finais.

Na primeira parte, uma Introdução geral ao tema da tese é apresentada. Vários aspectos sobre as microbactérias são descritos, que passam pela sua diversidade, reconhecimento e resposta do sistema imunitário do hospedeiro, até à constituição da parede da micobacteria e das suas moléculas imunomoduladoras. No fim da Introdução geral, são apresentados os objectivos da tese.

Na segunda parte, são apresentados os resultados obtidos, sob a forma de capítulos.
O trabalho intitulado: "Lipoarabinomannan mannose caps do not affect mycobacterial virulence or the induction of protective immunity in experimental animal models of infection and have minimal impact on in vitro inflammatory responses." foi publicado na revista Cellular Microbiology, 2012

O trabalho intitulado: "The mycobacterial type VII secretion system ESX-5 plays a crucial role in the ability of *Mycobacterium bovis* BCG to survive in mice" está em preparação para submissão.

Na terceira parte, é apresentada a Discussão geral e as Conclusões finais.

PART I

INTRODUCTION

MYCOBACTERIAL INFECTION

Mycobacterium Genus:

Mycobacterium is a genus of Actinobacteria, characterized by the presence of mycolic acids in their cell wall and the characteristic acid and alcohol fast staining in histological preparations. The vast majority of mycobacteria are environmental saprophytes that rarely cause diseases in humans, but a small subset is able to cause a varied spectrum of human disease with distinct clinical and pathological features. The *M. tuberculosis* complex is composed of *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. bovis* and *M. microti* that can cause tuberculosis in humans and animals (Cosma *et al.*, 2003). Additional pathogens include *M. leprae,* the etiologic agent of leprosy (Suzuki *et al.*, 2012), *M. ulcerans,* the cause of the Buruli ulcer (Einarsdottir *et al.*, 2011), *M. marinum*, a fish pathogen that can also cause skin infections in humans (Linell *et al.*, 1954) and the *M. avium*-complex*,* comprising *M.avium*, an opportunistic microbe that infect immunocompromised patients such as AIDS patients (Herzmann *et al.*, 2011) and *Mycobacterium avium paratuberculosis* (MAP), the causative agent of Johne's disease or paratuberculosis, a gastrointestinal inflammatory condition in ruminants and other animals, very similar to the Crohn's disease (CD) in humans and where this pathogen may also be involved (Hermon-Taylor, 2009).

Mycobacterium tuberculosis and Bacille Calmette-Guérin (BCG)*:*

The most important Human pathogen is undoubtedly *M. tuberculosis,* which is believed to infect one third of humankind, albeit only a fraction develops disease. The WHO estimated that there were 5.4 million new cases of tuberculosis in 2010 that leads to 1.4 million deaths worldwide each year (WHO, 2011) making TB one of the three leading causes of death from a single infectious agent (Blanc L, 2010). Person-to-person transmission occurs by inhalation of aerosolized droplets that carry *M. tuberculosis* bacilli, from a person with active disease. Infection occurs in the lungs, but the organism can seed any organ via hematogenous spread. The majority of infected individuals develop latent infection, a state of equilibrium in which the host is able to control the infection and does not show any clinical signs or symptoms of disease, but is not able to completely eradicate the bacteria, representing a reservoir for potential transmission and reactivation later in life.

Multidrug resistant strains and the HIV epidemic strongly contributed to Tuberculosis (TB) reemerging again as a major health problem worldwide (Espinal *et al.*, 2001, Corbett *et al.*,

2003). TB control has been based on a vaccination strategy and in long chemotherapeutical regimens. Bacille Calmette-Guérin (BCG) is currently the only available vaccine against TB (WHO, 2004, (CDC), 1996). Albert Calmette and Camille Guérin developed BCG between 1908 and 1921, by performing continuous passages of *M. bovis* on bile-containing medium. After 230 passages during 13 years, the bacterium showed decreased virulence in a number of experimental animals and provided protection against a lethal challenge of *M. tuberculosis* in calves and guinea-pigs. Since 1974, BCG has been included in the WHO expanded program on immunization, with over 100 millions dose of BCG administered annually. BCG induces effective protection against childhood disseminated TB and tuberculous meningitis, but has a limited effectiveness in conferring immunity against pulmonary tuberculosis in adults, which is the most prevalent form of the disease (Fine, 1995). The exact reasons for BCG protection variability are unknown, but several factors are pointed to contribute to the heterogeneity in vaccine efficacy: the use of different substrains of BCG in vaccination that exhibit phenotypic and biochemical differences, the exposure of populations to environmental mycobacteria or helminthes, nutritional or genetic differences in human populations and variations among clinical *M. tuberculosis* strains. Despite this variable performance, BCG remains the most widely used human vaccine in the world, due to its partial efficacy in TB prevention and safety.

Recognition of the mycobacteria by phagocytes and microbial killing mechanisms:

The primary route of infection by *M. tuberculosis* involves the lungs. Infection is initiated by inhalation of droplets containing the bacillus, expectorated by patients with active pulmonary TB. The small size of the droplets (1–5 *μ*m in diameter), allows the diffusion of the bacillus into the terminal alveoli, where the first encounter with alveolar resident phagocytes (macrophages and dendritic cells) occurs. Macrophages are a pivotal cell and constitute the first line of defense against mycobacterial invasion. Macrophages are involved in phagocytosis and killing of mycobacteria as well as in the initiation of adaptive cell immunity.

Phagocytosis of mycobacteria by the host cells involves an array of different receptors. Mycobacteria bind to the complement receptor CR3, but also engage the mannose-binding receptors such as the soluble mannose-binding lectin (MBL), surfactant proteins A and D (Sp-A, Sp-D), the membrane-bound macrophage mannose receptor (MMR), DC-specific intercellular-adhesion molecule-3 grabbing non-integrin (DC-SIGN; CD209) and probably Dectin-1 (Akira *et al.*, 2006, Jo, 2008, El-Etr *et al.*, 2001). Many of these receptors recognize important glycolipids in the mycobacterial cell wall, like lipomannan (LM), phosphatidyl-*myo*-inositol mannoside (PIM) and mannose-capped lipoarabinomannan (ManLAM) (Schlesinger *et al.*, 1994, Maeda *et al.*,

2003, Torrelles *et al.*, 2006, Schlesinger, 1993 , Dao *et al.*, 2004). Recently, the monocyte-inducible C-type lectin (Mincle) was show to be a key receptor for mycobacterial glycolipid trehalose-6,6'-dimycolate (TDM) (Ishikawa *et al.*, 2009), also known as cord factor, but its role in phagocytosis is still in debate. The phagocytosis process results in phagosome formation. Phagosome maturation is a highly complex process that ultimately leads to the formation of the phagolysosome, as a result of fusion with endosomes and lysosomes. Acidification by the Vacuolar ATPase proton pumps together with the hydrolytic enzymes from the lysosome, support the necessary environment for denaturation and degradation of the engulfed material in the phagolysosome structure. Besides the low pH and hydrolytic enzymes, other defense mechanisms are targeted to the phagosome (Schekman, 1994, Rothman *et al.*, 1996) in order to help restrain mycobacterial growth.

In a reaction that is often referred to as 'oxidative burst', the enzyme phagocyte oxidase transfers electrons from cytosolic NADPH to phagosomal oxygen to form superoxide anions, which rapidly dismutate into hydrogen peroxide and hydroxyl radicals, two toxic molecules and members of the reactive oxygen species (ROS) family (Bedard *et al.*, 2007). Furthermore, activation of macrophages with the cytokines TNF or IFNγ induces the expression of the inducible nitric oxide synthase (iNOS) enzyme, capable of generating reactive nitrogen species (RNS), namely nitric oxide, nitrogen dioxide and peroxynitrite (Beckman *et al.*, 1990, Bogdan, 2001). The mechanisms dependent of the iNOS enzyme play an essential role in mycobacterial growth control, since iNOS deficient mice succumb to *M. tuberculosis* and BCG infections (Garcia *et al.*, 2000, North *et al.*, 2004, MacMicking *et al.*, 1997).

At the site of infection, immature DCs (IDC) are present in high numbers. As professional Antigen Presenting Cells (APC), they are specialized in antigen uptake and processing (Austyn, 1996) and play a major role in establishing an effective adaptive response (Austyn, 1992). Like macrophages, they are able to recognize and uptake mycobacteria, with DC-SIGN receptor playing an important role in ManLAM recognition (Maeda *et al.*, 2003). After bacterial uptake, they reduce their phagocytic and/or endocytic capability and migrate to the lymph nodes (LN) where they continue their maturation process, up-regulating the expression of MHC class II-peptide complexes and co-stimulatory molecules and efficiently presenting antigens to T cells (Cumberbatch *et al.*, 1992, Larsen *et al.*, 1990, Xu *et al.*, 1995, Randolph *et al.*, 2005).

As intracellular pathogens, pathogenic mycobacteria developed multiple strategies to manipulate the host cell mechanisms responsible for their killing. The mycobacterial enzyme catalase peroxidase converts hydrogen peroxide into water and oxygen, preventing the toxicity caused by

ROS and NOS (Ng *et al.*, 2004). For most microbes, the acidic, hydrolytically competent environment of the phagolysosome is sufficient to kill them. However*, M. tuberculosis* interferes with phagolysosome acidification by excluding the phagosomal proton ATPase and secreting a mycobacterial urease, an enzyme capable of producing neutralizing molecules of ammonia from urea (Reyrat *et al.*, 1995, Sturgill-Koszycki *et al.*, 1994, Grode *et al.*, 2005). Also, the cell wall-associated mycobacterial lipoglycan ManLAM, considered an important virulence factor in *M. tuberculosis*, is able to interfere with the phagosome–lysosome fusion in macrophages (Chua *et al.*, 2004, Kang *et al.*, 2005, Welin *et al.*, 2008, Beatty *et al.*, 2000) and recently it was shown that *M. tuberculosis* is able to escape from phagosome/phagolysosome making use of its early-secreted antigenic export system (ESX-1), that secrets the proteins 6kDa early-secreted antigenic target (ESAT-6) and the 10 kDa culture filtrate antigen CFP-10 (Smith *et al.*, 2008, van der Wel *et al.*, 2007). All these strategies to evade the immune system support the survival of the bacillus inside the host.

Toll Like Receptors:

Apart from phagocytosis, macrophages and DC express pathogen recognition receptors (PRRs) that can recognize conserved molecular structures in pathogens (pathogen-associated molecular patterns -PAMP). The best characterized are the Toll-like receptors (TLRs), and they play a central role in linking innate and adaptive immunity to microbes (Pasare *et al.*, 2004). Several mycobacterial constituents can be recognized by TLR. Mycobacterial DNA CpG can be recognized by TLR9 (Hemmi *et al.*, 2000), while TLR2 in association with TLR1 and TLR6 mediates responses to mycobacterial lipoproteins, lipomannan (LM), phosphatidyl-*myo*-inositol mannoside (PIM) and certain forms of lipoarabinomannan (LAM) (Quesniaux *et al.*, 2004, Gilleron *et al.*, 2006, Nigou *et al.*, 2008).

While the LAM capped with phospho-inositol residues (PILAM), present in fast-growing mycobacteria, can activate TLR2, ManLAM from BCG failed to stimulate it (Stenger *et al.*, 2002). Also, viable *M. tuberculosis* is able to activate TLR2 and TLR4, but the exact components in the cell wall extract that activated TLR4 are still unknown (Means *et al.*, 1999). Most TLRs signal by recruiting the adaptor protein MyD88. MyD88 initiates a cascade that results in the activation of NF-KB and the expression of pro-inflammatory cytokines and antimicrobial mechanism (Underhill *et al.*, 1999, Karin *et al.*, 2005, Akira *et al.*, 2004).

In macrophages, activation by mycobacterial ligands through TLR2 leads to IL-12, TNF, IL-1 and IL-6 secretion and iNOS induction (Quesniaux *et al.*, 2004, Knutson *et al.*, 1998, Gilleron *et al.*, 1997), while in DC, promotes maturation by inducing the upregulation of the costimulatory

molecules CD80 and CD86 (Hertz *et al.*, 2001)

Interestingly, despite the clear involvement of TLR in the initiation and establishment of a coordinated immune responses to mycobacteria, mice deficient in TLR2 (Sugawara *et al.*, 2003, Drennan *et al.*, 2004), TLR4 (Kamath *et al.*, 2003, Shim *et al.*, 2003, Abel *et al.*, 2002), TLR6 (Sugawara *et al.*, 2003) or TLR9 (Bafica *et al.*, 2005) do not show any differences in resistance to low-dose *M. tuberculosis* infection when compared to wild type mice, although in some cases, defects in antimycobacterial responses are observed during the chronic phase. These results suggest compensatory mechanisms by the other TLRs in the immune response. This is corroborated by the result obtained in the TLR2/TLR9 double knockout mice, that are more susceptible to *M. tuberculosis* infection than either single knockout (Bafica *et al.*, 2005), demonstrating the importance of collaboration between TLR in the immune response to *M. tuberculosis*.

Adaptive immune response:

Besides macrophages and dendritic cells, protective immunity against mycobacteria requires also efficient cell mediated immune response, with CD4 and CD8 T cells playing a central role in protection. Infected phagocytes present bacterial antigens in association with Class II and Class I major histocompatibility complex (MHC) molecules to CD4+ and CD8+ T cells respectively, that become activated and secrete type I cytokines, most importantly interferon-γ (IFN-γ) and tumor necrosis factor (TNF) (Flynn *et al.*, 2001).

It is clear that CD4 T cells play a crucial role in the control of mycobacterial infection. CD4 T cell-deficient mice rapidly succumb to uncontrolled bacterial replication (Caruso *et al.*, 1999) and CD4+ T cells depletion was associated with the reactivation of infection in chronically infected mice (Scanga *et al.*, 2000). Furthermore, HIV patients are highly vulnerable to *M. tuberculosis,* with the susceptibility to developing TB increasing with the decrease of the numbers of CD4 T cells (Hammond *et al.*, 2008, Geldmacher *et al.*, 2008). CD4 T cells are the major producers of IFN-γ (Orme *et al.*, 1993). IFN-γ secretion leads to *M. tuberculosis*–infected macrophages activation, inducing iNOS expression and RNS production, that contribute to the early control of the pathogen (Ismail *et al.*, 2002, Chan *et al.*, 1995, MacMicking *et al.*, 1997). Additionally, it was also shown that CD4+T cells can control the intracellular growth of *M. tuberculosis* by a nitric oxide-dependent mechanism that is independent of IFN-*γ* production (Cowley *et al.*, 2003).

CD8 T cells also play an important role in the immune response to TB. Mice genetically disrupted in the genes for β2 microglobulin or TAP, and thus deficient in MHC class I molecules

and CD8 T cells, were quite susceptible to *M. tuberculosis* infection (Flynn *et al.*, 1992, Sousa *et al.*, 2000). Additionally, depletion of CD8 T cells resulted in latent infection reactivation in the mouse model (van Pinxteren *et al.*, 2000) and the presence of *M. tuberculosis*-specific CD8+ T cells in latently infected individuals suggests that the CD8+ T cells also have a role in the control of latent infection (Lewinsohn *et al.*, 2001). Also, after CD8 depletion, an impaired immunity against acute *M. tuberculosis* infection was observed in BCG vaccinated rhesus macaques (Chen *et al.*, 2009), suggesting that CD8 T cells also play an important role in the protective response. CD8 T cells can also produce IFN-γ and TNF (Lalvani *et al.*, 1998) and have the ability to be cytotoxic for *M. tuberculosis* infected cells. CD8 T cells secrete perforin that allows pore formation into the cellular membrane of infected cells and delivery of granulysin, a cytotoxic granule protein that is responsible for killing of the intracellular bacillus (Cho *et al.*, 2000, Lalvani *et al.*, 1998).

Besides CD4 and CD8 T cells, unconventional T cells that recognize nonpeptidic antigens in an MHC-independent fashion are also implicated in immunity to *M. tuberculosis* (Brigl *et al.*, 2004, Sieling *et al.*, 1995, Beckman *et al.*, 1994). CD1 represents a family of nonpolymorphic MHC class I molecules that present lipid-associated structures to CD1-restricted T cells (Brigl *et al.*, 2004, Ulrichs *et al.*, 2003). CD1 is conserved in most mammals, with size, number and isoforms varying widely among species. According to the current system of classification, Group I CD1 molecules include CD1a, b, and c isoforms whereas Group II includes CD1d (Brigl *et al.*, 2004). Group 1 CD1 molecules mainly present lipid antigens to clonally diverse T cells that mediate adaptive immunity to the vast range of microbial lipid antigens. By contrast, CD1d (group 2) molecules present lipid antigens to natural killer T (NKT) cells, a potent effector of innate immunity (Barral *et al.*, 2007). *M. tuberculosis* cell wall is a rich source of diverse lipid antigens for immune recognition (Gilleron *et al.*, 2004, Ulrichs *et al.*, 2003). The first described nonprotein antigen presented by CD1 molecule was the mycolic acid (Beckman *et al.*, 1994) cell wall component. Since then, other mycobacterial lipid related antigens presented by CD1 have been reported. While Group 2 CD1d has been reported to only bind lower-order PIMs (PIM2 and PIM4) (Zajonc *et al.*, 2006, Fischer *et al.*, 2004) group 1 CD1b binds several mycobacterial lipid including PIM2 and Man-LAM (Sieling *et al.*, 1995, Moody *et al.*, 1997).

Although the exact role and importance of CD1 antigen presentation for *M. tuberculosis* immunity remains to clarify, evidences that CD1 antigen presentation may play a role in resistance to mycobacterial infections grow. The expression of group 1 CD1 on human myeloid APC is upregulated to high levels after M. tuberculosis infection or activation by mycobacterial lipids (Roura-Mir *et al.*, 2005, Felio *et al.*, 2009) and CD1-restricted T cells specific for a

mycobacterial isoprenoid lipid antigen can be found in individuals recently infected with *M. tuberculosis*, but not in naive donors (Moody *et al.*, 2000). Also, CD1-restricted T cells specific for mycobacterial antigens have also been derived from the lesions of leprosy patients, and effective cell-mediated immunity is correlated with expression of group 1 CD1 molecules by the dendritic cells (DCs) in the leprosy lesions (Sieling *et al.*, 1999). More recently a study showed that CD1-restricted T cell population contributes to acute and memory immune responses in human tuberculosis infection (Montamat-Sicotte *et al.*, 2011).

Cytokines:

From the interaction between Mycobacteria and cells of the innate and adaptive immune system results the secretion of cytokines and chemokines that have a serious impact in the expression of immunity.

IFN-*γ* is mainly produced by CD4+, CD8+ T cells, and the NK cells and plays a key role in the protective immune response against *M. tuberculosis*. Humans and mice defective in IFN-*γ* or IFN-*γ* receptor genes are more susceptible to *M. tuberculosis* infection (Cooper *et al.*, 1993, Flynn *et al.*, 1993, Doffinger *et al.*, 2002, Jouanguy *et al.*, 1999). IFN-*γ* induces macrophage activation, promoting phagolysosomal fusion, nitric oxide and other reactive nitrogen intermediates (RNI) production, that result into mycobacterial growth control. Furthermore, IFN-*γ* induces the upregulation of MHC class II expression in macrophages, improving antigen presentation to T cells (Cooper, 2009)

TNF produced by macrophages, dendritic cells, and T-cells, is another cytokine that has a major protective role against *M. tuberculosis* infection in mice. Mice deficient in TNF or TNF receptors succumb rapidly after *M. tuberculosis* infection, with significantly higher mycobacterial outgrowth in different organs compared to wild-type animals. Also, there is a remarkably high incidence of tuberculosis in patients treated with TNF antagonists in autoimmune diseases, suggesting a crucial contribution of TNF for *M. tuberculosis* infection control in humans (Bean *et al.*, 1999, Keane, 2005, Gomez-Reino *et al.*, 2003).

TNF in combination with IFN-*γ* induces antimicrobial activity in murine macrophages via the induction of reactive nitrogen intermediates (Scanga *et al.*, 2001, Chan *et al.*, 2004). TNF is a key player in granuloma formation. TNF deficient mice fail to develop and maintain these structures (Chensue *et al.*, 1995). Furthermore, TNF produced by the infected macrophages contributes to the expression of chemokines, such as IL-8, MCP-1 and RANTES, which provide signals for migration of immune cells to the sites of *M. tuberculosis* infection (Algood *et al.*,

2003).

The importance of Interleukin (IL)-12 is also evident from the increased susceptibility of mice and humans to mycobacterial infections in its absence (de Jong *et al.*, 1998, Doffinger *et al.*, 2002, Cooper *et al.*, 1997). Produced by the antigen-presenting cells, IL-12 is a heterodimeric cytokine, comprised of the p35 and p40 subunits and with a key role in the differentiation of CD4+ T IFNγ producing cells, which are crucial for mycobacterial infection control (Cooper *et al.*, 2002, Flynn *et al.*, 1995, Silva *et al.*, 2001).

The heterodimeric cytokine IL-23 is also from the same family of IL-12. IL-23 is composed by the p19 chain and p40 subunit that it shares with IL-12. IL-23 is not involved in primary resistance to *M. tuberculosis*, but it is required for the generation of an IL-17-producing mycobacteria-specific CD4+ Th17 cell response (Torrado *et al.*, 2010, Khader *et al.*, 2008). IL-17 is an inflammatory cytokine associated with chemokine secretion and neutrophil recruitment. The absence of IL-23 or IL-17 signaling does not significantly impact the ability of mice to control *M. tuberculosis* following a low dose aerosol infection (Khader *et al.*, 2005) but mice deficient in IL-17 are unable to control *M. tuberculosis* growth after a intratracheal high dose infection (Okamoto Yoshida *et al.*, 2010). BCG vaccination induces IL-17-producing CD4+ T cells that lead to the recruitment of CD4+ T IFNγ-producing cells necessary to restrict *M. tuberculosis* bacterial growth (Wozniak *et al.*, 2010, Khader *et al.*, 2007). However, repeated BCG vaccination in *M. tuberculosis*-infected mice results in an enhanced IL-17 response, that leads to extensive lung tissue damage (Cruz *et al.*, 2010). It has been shown that IFN-γ responses can regulate IL-17 producer cells and probably limit IL-17 associated pathology (Cruz *et al.*, 2006). Altogether, although the role of IL-17 protection against *M. tuberculosis* is still unclear, regulation of Th17 responses is essential in order to promote anti-mycobacterial immunity and prevent excessive immunopathology.

Other important regulatory cytokine implicated in *M. tuberculosis* immunity is the anti-inflammatory cytokine IL-10. Initial observations that IL-10 is able to block mechanisms fundamental for mediating immunity to intracellular pathogens, namely decreasing the production of proinflammatory cytokines such as TNF and IL-12 and inhibiting microbial killing on macrophages, lead to the assumption that IL-10 could contribute to persistence of the pathogen in the host (Redford *et al.*, 2011).

However, the role of IL-10 during murine *M. tuberculosis* infection has been controversial, with some groups reporting that $IL10^{-/-}$ mice have identical lung bacterial loads following aerosol infection when compared with wild-type mice (Jung *et al.*, 2003, North, 1998), while others have

reported that in the absence of IL-10, mice display enhanced resistance to aerosol *M. tuberculosis* infection with reduced bacterial loads (Redford *et al.*, 2010, Beamer *et al.*, 2008). Also, Human genetic studies, where the key polymorphisms in the IL-10 gene and the susceptibility to TB have been examined, proved to be inconclusive (Lopez-Maderuelo *et al.*, 2003, Oral *et al.*, 2006, Ates *et al.*, 2008). *M. tuberculosis* can also have an active role in IL-10 regulation. Two of such examples include the *M. tuberculosis* strains "HN878" and "CH." that promote IL-10 production during infection, which limits protective immunity responses and leads to disease exacerbation (Ordway *et al.*, 2007, Manca *et al.*, 2001). The argument that IL-10 prevents pathogen eradication is appealing, but still needs further studies. Integration of all the data suggests that IL-10 from both innate and adaptive sources may inhibit protective responses, probably aiming to limit immunopathology, but on the other hand, this effect may also result in pathogen persistence and chronic infection.

Granuloma:

The protective immune response against most of the pathogenic mycobacteria involves the formation of an organized structure termed granuloma. Several types of cells are involved in granuloma formation, namely macrophages, DCs, T cells, B cells, neutrophils and fibroblasts. The typical model of a granuloma describes a center with activated mononuclear phagocytes (MPs) containing the surviving bacilli, involved by fibroblasts and dendritic cells and an outer ring of activated T cells and B cells, that are further encircled by a fibrotic wall that separates it from surrounding tissue and improves bacterial containment (Ramakrishnan, 2012). Animal models gave important contributions to understand these structures. TNF is a critical cytokine for granuloma formation, with TNF deficient mice having severe deficiencies in developing granulomas (Bean *et al.*, 1999, Roach *et al.*, 2002). Furthermore, in individuals treated with anti-TNF therapy higher rates of reactivation of clinical TB occur, reinforcing the importance of TNF also in humans (Harris *et al.*, 2010).

However, despite the advantage of animal manipulation most of the models used have some limitations to understand what occurs in humans. Mice are unable to reproduce caseous necrosis within the granuloma, while guinea pig and rabbits reproduce granuloma caseation but infection proceeds rapidly and does not reflect the slow development of TB and its chronic character in humans (Dorhoi *et al.*, 2011)

Solid granulomas can contain dormant *M. tuberculosis*, resulting in latent infection without clinical disease. In humans, these granulomas, which contain *M. tuberculosis* can evolve to caseous granulomas during the end-stage or severe TB. The center of the solid granuloma becomes necrotic due to dying cells and the continues weakening of the immune response results in vast cell death, which leads to the formation of a caseous mass and to cavity formation (Fayyazi *et al.*, 2000). The caseous granuloma is no longer able to contain bacteria, and at this point, person to person transmission of pulmonary tuberculosis can occur or dissemination to other organs, with meningeal TB as the most frequent extrapulmonary form of TB.

MYCOBACTERIUM CELL WALL

The mycobacterial cell wall is determinant in the bacterial physiology but also has a central contribution to mycobacterial persistence, conferring resistance against therapeutic agents and interacting with the host (Nigou, 2003, Brennan *et al.*, 1995). The plasma membrane, the cell wall core and the outermost layer compose the Mycobacterial cell envelope.

The plasma membrane has an identical structure to the other plasma membranes, constituted by polar lipids, mainly phospholipids, assemble themselves in association with proteins, into a lipid bilayer (Mamadou *et al.*, 1989, Silva *et al.*, 1983). The main phospholipids are phosphatidyl-inositol-mannosides (PIM), phosphatidylglycerol, cardiolipin and phosphatidylethanolamine, with plasma membrane composition being identical in both slow or rapid- growing mycobacteria (Minnikin, 1982).

Outside the plasma membrane is the cell wall "skeleton", a giant macromolecule that surrounds the bacterial cell and defines their shape (Azuma *et al.*, 1974). It is composed by an arabinogalactan-peptidoglycan layer to which the mycolic acids are covalently attached (Daffe *et al.*, 1990, Chatterjee *et al.*, 1991, Besra *et al.*, 1995, Dover *et al.*, 2004). Except for minor differences, the mycobacterial peptidoglycan is similar to the most common type found in Eubacteria, composed by repeating units of N-acetylglucosamine and N-acetyl/glycollylmuramic acid cross-linked by short peptides (Daffe *et al.*, 1998). The arabinogalactan layer is made up of a polymer of arabinofuranosyl and galactofuranosyl residues and connects the peptidoglycan layer with the mycolic acids (Bhamidi *et al.*, 2008).

The mycolic acids are major and very specific components of the cell wall of mycobacteria. They are high-molecular-weight fatty acids, alpha-branched and beta-hydroxylated, with a large diversity of their chain length and chemical functions. The mycolic acids play a crucial role in cell wall architecture, comprising a network that allows the insertion of additional lipids and contribute to the formation of an impermeable barrier for the penetration of antimicrobial drugs (Daffe *et al.*, 1998, Minnikin *et al.*, 2002). The covalent linkage of mycolic acids results in a hydrophobic layer of extremely low fluidity also referred to as the mycomembrane (Abdallah *et al.*, 2007). Also, mycolic acids are part of the trehalose 6,6'-dimycolate (TDM/cord factor), an abundant glycolipid in the mycobacterial cell wall, responsible for conferring the cord-like appearance to *M. tuberculosis* cells and an important virulence factor (Hunter *et al.*, 2006, Noll, 1956). The outer part of the mycomembrane contains various free lipids, such as phenolic glycolipids, phthiocerol dimycocerosates, cord factor or dimycolyltrehalose, sulpholipids and

phosphatidylinositol mannosides, that are intercalated with the mycolic acids (Brennan *et al.*, 1995).

The outermost portion of the cell envelope is constituted by a diversity of noncovalently attached polysaccharides, lipoglycans, (glyco)lipids and proteins. The surface-exposed material of these outer layers corresponds to the capsule and is mainly composed of polysaccharides and proteins, with only a small amount of lipids (Lemassu *et al.*, 1994, Ortalo-Magne *et al.*, 1995). Three types of polysaccharides are found in the capsular material: α-D-glucan, D-arabino-D-mannan (AM), and D-mannan, with α-D-glucan being the major carbohydrate component (Ortalo-Magne *et al.*, 1995, Lemassu *et al.*, 1994, Dinadayala *et al.*, 2004).

Several mannose-containing molecules are exposed at the surface of *M. tuberculosis*, namely the mannose-capped lipoarabinomannan (ManLAM), the lipomannan (LM), phosphatidyl-myo-inositol mannosides (PIMs), arabinomannan, mannan and manno-glycoproteins. (Venisse *et al.*, 1995, Ortalo-Magne *et al.*, 1995, Crick, 2003)

Lipoarabinomannan and the related glycolipids:

PIM and their multiglycosylated counterparts LM and LAM are ubiquitous mycobacterial envelope lipoglycans that are biosynthetically related. PIMs are glycolipids, composed of fatty acids attached to a glycerol unit, linked by a phosphodiester moiety to myo-inositol (Vilkas *et al.*, 1956, Ballou *et al.*, 1963).

Further substitution of the myo-inositol with α-D-mannopyranosyl (Manρ) units results in the formation of a mannosyl phosphate inositol (MPI) anchor (Lee *et al.*, 1964, Chatterjee *et al.*, 1992) a conserved structure common to LM and LAM. The PIM family comprises phosphatidyl-myo-inositol di, tri, tetra, penta and hexa mannosides (PIM2-PIM6). PIM2 and PIM6 are the most abundant glycoforms present in *M. tuberculosis*, BCG and *M. smegmatis*. PIM2 results from the substitution at position 2 and 6 of the myo-inositol with mannosyl units. Extension of PIM2 with a disacharidyl unit t-α-Man*p* (1→6)- α-Man*p* (1→6) leads to the formation of PIM4, an intermediate structure that can give rise to PIM6 by further elongation with a disacharidyl unit t-α-Man*p* (1→2)- α-Man*p* (1→2), or to LM, by the addition of (1→6)- linked α-Man*p* residues (Ballou *et al.*, 1963, Lee *et al.*, 1964). LM and LAM possess a mannan core composed on average of 20-25 α-(1→6)-linked Man*p* residues. The length of the mannan core of LM and degree of branching varies, depending on the mycobacterial species. (Gilleron *et al.*, 2006, Kaur *et al.*, 2007)

Addition of a D-arabinan domain to LM results in LAM formation. The arabinan domain consists of a linear chain of α-(1→5)-linked Araf which is branched at some points with α-Araf residues. LAM is a tripartite structure, constituted by the mannosyl-phosphatidyl-*myo*-inositol anchor (MPI), a D-mannan and D-arabinan polysaccharide backbone, and finally, the cap. Based on the nature of the motifs that cap the non-reducing termini of the arabinan domain of LAM, three types of LAM have been described. In slow growing mycobacteria, such as *M. tuberculosis, M. bovis, M. bovis* BCG, *M. leprae, M. avium, M. xenopi, M. marinum* and *M. kansasi* the presence of one to three mannopyranosyl residues linked to the arabinan domain constitute the mannose cap (ManLAM), while in fast-growing mycobacteria, such as *M. smegmatis* and *M. fortuitum*, this latter domain is capped with phospho-inositol residues (PILAM) (Nigou, 2003). Also, in some species like *M. chelonae*, no such capping motif is present and is called AraLAM (Guerardel *et al.*, 2002).

LAM is a heterogeneous molecule, differing in terms of the extent of glycosylation and acylation. The amount of LAM that are capped with mannose varies among different species of slow-growing mycobacteria and the number of mannose residues per cap is also variable even within LAM from the same species or strain (Nigou, 2003). The fact that ManLAM is only found in slow growing mycobacteria including most pathogenic species has led to the hypothesis that the mannose caps are important for determining the ability of LAM to act as a virulence factor (Briken *et al.*, 2004). The exact localization of LAM remains unclear. LAM insertion into the outer leaflet of the plasma membrane is the most acceptable hypothesis, based on the cytosolic or membrane anchor localization of the glycosyl-transferases involved in LAM biosynthesis and mannose cap, and on the fact that LAM have never been found in culture supernatants or in the surface exposed material obtained by gentle mechanical treatment (Ortalo-Magne *et al.*, 1995, Daffe *et al.*, 1998, Berg *et al.*, 2005).

However, other studies show that ManLAM and LM from both *M. bovis* BCG and *M. xenopi* are accessible to biotin-hydrazide labeling and are thus likely to be exposed on the cell surface (Pitarque *et al.*, 2005). Also, arabinomannan (AM), a major polysaccharide of the outermost layer, and thought to be derived from LAM has been found in culture supernatants and in the surface expose material (Ortalo-Magne *et al.*, 1996, Nigou *et al.*, 1999). Considering that LAM is not big enough to be anchored in the plasma membrane and be accessible to the outside of the cell wall, these findings suggest the existence of a second pool of LAM. How these lipoglycans reach the surface remains to be elucidated and so far no lypoglycan transporters have been identified. This is a critical question, since mycobacterial virulence depends in part on the

interaction of these molecules with the immune system and their accessibility to host immune receptors.

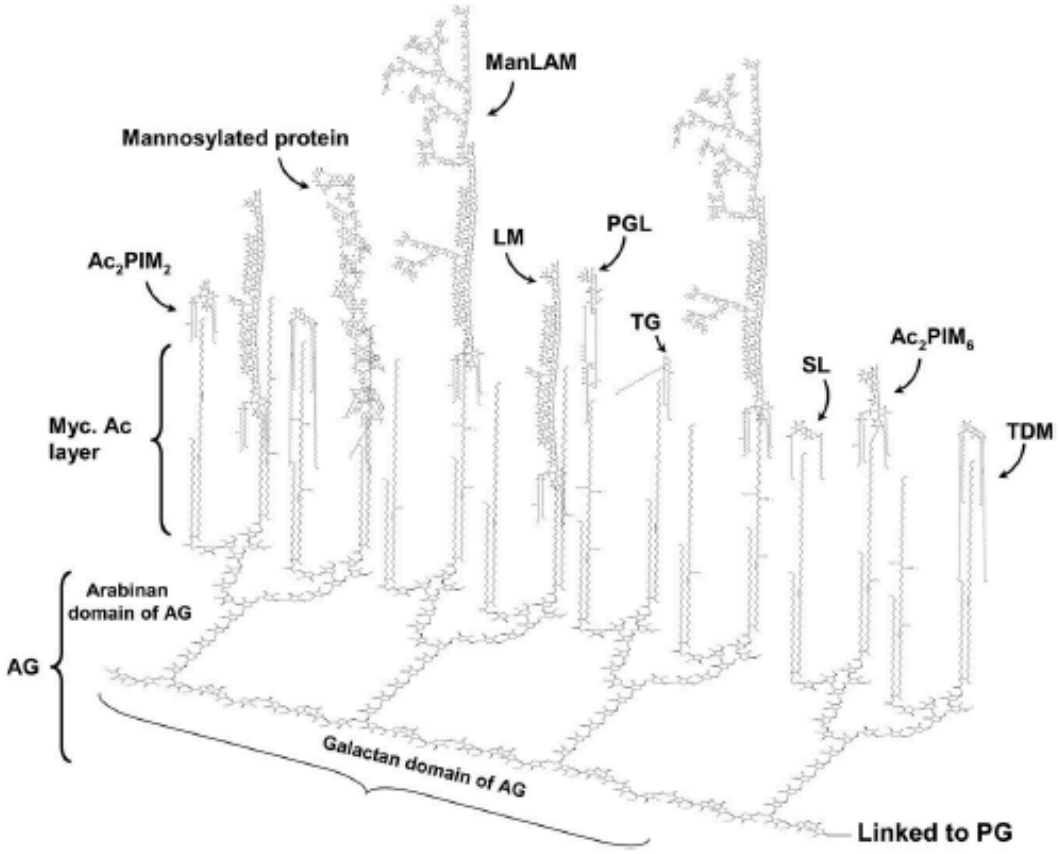

Figure 1: The cell envelope of M. tuberculosis with highlighting the exposed mannosylated cell envelope components. Schematic representation of the cell envelope skeleton elements, showing the distribution of the major mannosylated cell envelope components that is exposed on the M. tuberculosis surface. The arabinogalactan-peptidoglycan layer to which the mycolic acids are covalently attached, are shown perpendicular to the plasma membrane. The polar groups (i.e. carbohydrate domains) of several mannosylated cell envelope components are exposed on the cell surface and their lipid domains are intercalated with the mycolic acids layer. These envelope components include ManLAM, LM, higher- and lower-order PIMs, and lipomannoproteins. Other known virulence factors described for M. tuberculosis that interact with the mycolic acids layer [i.e. TDM, SL; and TGs and PGL, the latter on some M. tuberculosis strains)] are also depicted. For the sake of simplicity the capsule components (arabinomannan, glucan, mannan, and xylan) are not shown, and the relative number of molecules do not accurately reflect experimental data. AG (arabinogalactan); PG (peptidoglycan); Myc Ac (mycolic acids); ManLAM (mannose-capped lipoarabinomannan); LM (lipomannan); PIMs (phosphatidyl-myo-inositol mannosides); TDM (trehalose dimycolate); SL (sulfolipid); TGs (triglycerides); PGL (phenolic glycolipid). Figure removed from Torrelles *et al* (Torrelles *et al*., 2010).

Mycobacteria protein export systems:

All bacteria, including mycobacteria, have protein export systems to transport proteins synthesized in the cytoplasm across the cytoplasmic membrane. Many of the export systems are involved in essential physiologic processes, but in the case of pathogenic bacteria, they can also be responsible for the export of effector molecules with a role in virulence.

The majority of proteins with a typical signal sequence are exported by two highly conserved protein export systems denominated, general Secretion (Sec) pathway and the Twin-arginine translocase (Tat) pathways, while small proteins without specific signal sequences are exported by the 6kDa early-secreted antigenic target secretion systems (ESX) (Driessen *et al.*, 2008).

The Sec system:

The Sec system is highly conserved and present in all bacteria. The Sec export is a post-translational process dedicated to export unfolded proteins from the cytoplasm to the extracytoplasmatic environment. Proteins destined for Sec export are tagged with a N-terminal signal peptide and are called preproteins. Following their export, the signal peptide is cleaved generating the mature protein (Driessen *et al.*, 2008).

In many gram-negative bacteria, as part of the Sec system, there is a sec export chaperone called SecB, involved in the binding and maintenance of the proteins in the unfolded state and in the delivery of the proteins to SecA, a central component of the Sec system, and responsible for recognizing and binding to the signal peptide. In *M. tuberculosis,* a SecB-like protein and two secA proteins, SecA1 and SecA2, each with distinct functions and involved in the protein export were identified (Bordes *et al.*, 2011, Braunstein *et al.*, 2001). Several *M. tuberculosis* proteins involved in vital functions or with a role in virulence contain Sec signal peptides, making Sec an essential pathway for bacterial viability and virulence (McCann *et al.*, 2011, McCann JR, 2009). Contrary to SecA2, SecA1 is predicted to be essential in *M. tuberculosis* (Sassetti *et al.*, 2003, Griffin *et al.*, 2011) and proven to be essential in *M. smegmatis* (Braunstein *et al.*, 2001). Conditional silencing of *secA1* in *M. smegmatis* (Guo *et al.*, 2007) allowed assessing the contribution of SecA1 to mycobacteria virulence, with an inhibition in the growth and a reduced export of the cell wall porin MspA, observed (Guo *et al.*, 2007, Rigel *et al.*, 2009). The construction of *secA2* mutant in *M. smegmatis*, *M. bovis* Bacille Calmette-Guérin (BCG) and *M. tuberculosis* is possible (Braunstein *et al.*, 2001, Braunstein *et al.*, 2003, Sadagopal *et al.*, 2009) with the SecA2 mutant of *M. tuberculosis* being attenuated for growth in macrophages and mice (Braunstein *et al.*, 2003, Kurtz *et al.*, 2006). Also, it is thought that SecA2 in *M. tuberculosis* is involved in inhibiting the innate immune response, since it was observed that the macrophages

infected with the *M. tuberculosis* SecA2 mutant produce higher levels of proinflammatory cytokines and exhibit more apoptosis than wild-type infected macrophages (Kurtz *et al.*, 2006, Hinchey *et al.*, 2007).

Tat export systems:

Similar to the Sec pathway, the Tat system also exports proteins across the cytoplasmic membrane in a post-translational manner (Driessen *et al.*, 2008). The proteins exported by the Tat pathway are synthesized as preproteins with a characteristic N-terminal signal and distinguished from Sec signal peptides by the presence of a Tat motif that contains twin arginine residues (Berks, 1996, Dilks *et al.*, 2003).

The Tat export machinery consists of two integral membrane proteins, TatA and TatC, and a third protein named TatB that is similar to TatA in terms of amino sequence, but that not all bacteria have. A growing understanding of the Tat export mechanism is occurring. TatB and TatC form a complex for preproteins binding, and TatA is recruited to the complex and believed to form the export channel (Robinson *et al.*, 2011, Tarry *et al.*, 2009, Alami *et al.*, 2003). *M. tuberculosis* and *M. smegmatis* contain genes encoding for all three proteins. Deletion of TatA, TatB or TatC affects *M. tuberculosis* growth under standard laboratory conditions (Saint-Joanis *et al.*, 2006). An *M. tuberculosis* mutant for Phospholipase C, a protein with a Tat signal peptide, shows decreased virulence in mice (Raynaud *et al.*, 2002). Furthermore, *M. smegmatis* tat mutants have increased sensitivity to β-lactam antibiotics due to a reduced export of the β-lactamase (McDonough *et al.*, 2005, Posey *et al.*, 2006). These findings reveal the importance of tat export system in viability, virulence and drug resistance (De Buck *et al.*, 2008, McDonough *et al.*, 2008).

ESX system:

ESX export systems, which are also referred to as Type VII Secretion Systems, were originally identified in mycobacteria. There are five specialized ESX export systems named ESX-1 to ESX-5 with diverse functions in mycobacterial physiology and virulence. The first ESX system secreted substrate identified and responsible for the ESX designation was the 6kDa early-secreted antigenic target (ESAT-6), a small protein with a clear importance in *M. tuberculosis* virulence. Proteins exported via of the ESX systems are characteristically small proteins that lack a Sec or Tat signal peptide. Each of the five distinct ESX loci has genes encoding core components of the secretion machinery as well as genes encoding for the exported ESAT-6-like

proteins (Gey Van Pittius *et al.*, 2001, Abdallah *et al.*, 2007).

ESX-1 was the first ESX system identified. It is the best characterized and has an important role in *M. tuberculosis* and *M. marinum* virulence (MacGurn *et al.*, 2007, Xu *et al.*, 2007, Stanley *et al.*, 2003, Derrick *et al.*, 2007, Hsu *et al.*, 2003, Guinn *et al.*, 2004). The ESX-1 locus is composed of *esxA* and *esxB* genes, which encode ESAT-6 and the culture filtrate protein 10 kDa (CFP-10), respectively (Berthet *et al.*, 1998). ESAT-6 and CFP-10 are two co-secreted proteins with an important role in *M. tuberculosis* and *M. marinum* virulence (MacGurn *et al.*, 2007, Xu *et al.*, 2007, Stanley *et al.*, 2003, Derrick *et al.*, 2007, Hsu *et al.*, 2003, Guinn *et al.*, 2004). Also, genes in the ESX-1 locus encode predicted ATPases (EccA1and EccCb1) and membrane proteins (EccB1, EccCa1, EccD1, EccE1, MycP1) necessary for ESAT-6 and CFP-10 secretion. The ESX-1 locus is located in the genomic region of difference 1 (RD1) (Guinn *et al.*, 2004, Hsu *et al.*, 2003, Pym *et al.*, 2003), present in all fully virulent strains but spontaneously deleted from the attenuated *M. bovis* BCG vaccine strain (Behr *et al.*, 1999, Gordon *et al.*, 1999, Mahairas *et al.*, 1996) and *M. microti* (Brodin *et al.*, 2002). Genetically induced ESX-1 deletion results in loss of secretion of ESAT-6 and CFP-10 and decreased virulence of the affected mycobacterial mutant strains. (Brodin *et al.*, 2004a, Pym *et al.*, 2002, Majlessi *et al.*, 2005, Lewis *et al.*, 2003, Hsu *et al.*, 2003, Guinn *et al.*, 2004).

ESX-3 was the second system study. Contrary to ESX-1, ESX-3 is not involved in virulence and seems to play a critical role in iron and zinc uptake and also in *M. tuberculosis* viability (Serafini *et al.*, 2009, Siegrist *et al.*, 2009). ESX-3 is conserved in all of the mycobacterial genomes and the ESX-3 locus encodes a pair of ESAT-6-like proteins, namely EsxH, whose secretion increases in low iron conditions (Siegrist *et al.*, 2009). Still, the current understanding of the ESX-3 export system is very limited.

ESX-4 represents the most ancestral system as it contains a smaller number of genes than the other ESX loci (Gey Van Pittius *et al.*, 2001), and **ESX-2** is located just downstream of the ESX-1 locus (Bottai *et al.*, 2009). However, the biological role of these systems remains entirely unknown.

More recently, **ESX-5** was also identified as an active protein secretion system. First evidence came from the *M. marinum* model, where an ESX-5 transposon mutant was unable to secrete the *M. tuberculosis* PPE41 protein (Abdallah *et al.*, 2006), that belongs to the proline-proline-glutamic acid (PPE) protein families and also lacks a classical signal sequence (Bottai *et al.*, 2009). The ESX-5 locus contains of a pair of *esx* genes that code for two immunodominant

molecules, the EsxM and EsxN protein (Jones *et al.*, 2010, Alderson *et al.*, 2000), a *ppe25-pe19* gene cluster, coding for members of PE or PPE motifs (Cole *et al.*, 1998, Tekaia *et al.*, 1999) and a block of *ecc* (esx conserved components) genes that encode membrane proteins and ATP-binding proteins from the predicted secretion machine involved in the export (Brodin *et al.*, 2004b, Bitter *et al.*, 2009). The ESX-5 locus is conserved in various pathogenic mycobacteria, but not in the saprophytic species, and is responsible for the transport of several proline-glutamic acid (PE) and PPE proteins, that are highly expanded in the pathogenic mycobacterial species (Cole *et al.*, 1998). Although the exact function of these proteins is unclear, a role in virulence (Li *et al.*, 2005, Ramakrishnan *et al.*, 2000), antigenic variation or immune evasion has been predicted (Cole *et al.*, 1998, Banu *et al.*, 2002, Delogu *et al.*, 2001, Akhter *et al.*, 2012). Recent studies show that ESX-5 disruption in *M. marinum* (Abdallah *et al.*, 2008, Weerdenburg *et al.*, 2012) and *M. tuberculosis* (Iantomasi *et al.*, 2012, Bottai *et al.*, 2012) affects their virulence, strongly supporting a role for ESX-5 system in mycobacterial pathogenesis.

IMMUNOMODULATORY CELL WALL MOLECULES

The inability of the immune system to efficiently eliminate pathogenic mycobacteria results from a wide array of mycobacterial tactics devoted to promote invasion and survival within the host cells.

ManLAM:

The cell envelope of *M. tuberculosis* is considered a major determinant of virulence**.** Initial experiments showed that purified ManLAM was able to reproduce some of the immunomodulatory features of live *M. tuberculosis.* (Chua *et al.*, 2004, Kang *et al.*, 2005, Welin *et al.*, 2008, Beatty *et al.*, 2000). The observation that these effects where mannose cap dependent, lead to the assumption that the ManLAM could play an fundamental role in the manipulation that pathogenic mycobacteria exerted in the host in order to escape de immune system and survive (Briken *et al.*, 2004).

ManLAM is able to interfere with the microbicidal mechanisms of macrophages. The phagosome–lysosome fusion is a crucial microbicidal mechanism, which normally leads to killing and digestion of a pathogen in an acid environment (Armstrong *et al.*, 1971). The formation of this complex structure is dependent on Ca^{2+} influx and the orchestrated participation of several molecules involved in the intracellular trafficking of multiple organelles. Among these molecules, the membrane tethering protein early endosome autoantigen 1 (EEA1) is a crucial player, that in combination with Syntaxin 6, is necessary for the delivery of lysosomal components to the phagosome. ManLAM is able to interfere with the cytosolic Ca^{2+} influx (Vergne *et al.*, 2003) and decrease the amount of EEA1 recruited, leading to phagosome maturation arrest (Fratti *et al.*, 2001, Fratti *et al.*, 2003). ManLAM further interferes with other macrophage microbicidal mechanisms. It can block macrophage activation by IFNy (Sibley *et al.*, 1988), leading to the decrease of nitric oxide, oxygen radicals and inflammatory cytokine production (Torrelles *et al.*, 2010, Strohmeier *et al.*, 1999).

ManLAM seems to exert its immunomodulatory effects through Macrophage Mannose Receptor (MMR) binding (Schlesinger *et al.*, 1994, Maeda *et al.*, 2003, Torrelles *et al.*, 2006, Schlesinger, 1993). The MMR (CD206) is a type I transmembrane monomeric C-type lectin, that along with CR is strongly involved in the phagocytic uptake of mycobacteria by macrophages (Schlesinger, 1993). The MMR is capable of recognizing the mannose residues from mycobacterial glycolipids and is involved in the intracellular trafficking to these molecules to the late endosomal compartments. The fact that MMR is able to bind the Mannose capped LAM (Man-LAM), but not

other types of LAM, namely Ara-LAM or PI-LAM (LS Schlesinger, 1994) suggests that the MMR pathway is associated with the more virulent *Mycobacterium* species.

The interaction between *M. tuberculosis* and host dendritic cells (DCs) is critical for mounting a protective anti-mycobacterial immune response. Contrary to macrophages, in DCs, the uptake of mycobacteria is performed mainly by DC-SIGN (Geijtenbeek *et al.*, 2003a, Driessen *et al.*, 2009) a type II transmembrane tetrameric C-type lectin that recognizes high-mannose-containing structures. Similar to what happens with the MMR, DC-SIGN is able to discriminate between *Mycobacterium* species through selective recognition of the mannose caps on LAM molecules, having higher affinity for the mannosylated LAM. Several studies show that ManLAM, but not non-capped LAM, prevents dendritic cell (DC) maturation, decreases IL-12 production and triggers the production of the immunosuppressive cytokine IL-10 in LPS-stimulated human monocyte-derived DC (Geijtenbeek *et al.*, 2003a), possible contributing to a "deactivation" of the adaptive immune response. Recently it has been shown that ManLAM is able to inhibit murine antigen-specific murine CD4+ T cells and primary human T cell activation, by interfering with very early events in TCR signaling through ManLAM insertion into T cell membranes (Mahon *et al.*, 2012).

In addition to the major Humans pathogens, *M. tuberculosis* and *M. leprae*, also the vaccine strain *M. bovis* BCG has a mannose cap on LAM (Venisse *et al.*, 1993). BCG protects well against disseminated TB, including meningitis in childhood, but protection against adult, namely pulmonary TB, is highly variable ranging from 80% to no protection at all (Colditz *et al.*, 1994). Although some causes have been pointed, the exact reason for this variability is unknown. One possibility that may account for BCG's failure to induce long-lasting immunity is the immumodulatory effect of ManLAM.

ESX-1 and ESX-5:

ESX-1 secreted proteins ESAT-6/CFP-10 complex is among the most important antigens secreted by *M. tuberculosis* (Sorensen *et al.*, 1995). ESAT-6 was found to be a very strong T-cell antigen, with ESAT-6-specific T cells frequently found in TB patients as well as in infected animals (Pollock *et al.*, 1997, Lein *et al.*, 1999, Ravn *et al.*, 1999, Cardoso *et al.*, 2002). Also, ESAT-6 seems to have cytolytic activity, creating pores in host cell membranes that facilitate pathogen escape from the phagosome (van der Wel *et al.*, 2007) and eventually promote dissemination due to host cells lysis.

M. tuberculosis mutants in the ESX-1 secretion pathway exhibit a range of pathogenic defects. They are more attenuated in mice (Guinn *et al.*, 2004, Hsu *et al.*, 2003, Stanley *et al.*, 2003), fail to replicate in cultured macrophages and to inhibit macrophage inflammatory responses, and have a diminished ability to lyse pneumocytes (Hsu *et al.*, 2003). ESX-1 is involved in blocking phagosome maturation (MacGurn *et al.*, 2007, Xu *et al.*, 2007), limiting host cytokine production (Stanley *et al.*, 2003) and promoting apoptosis of the infected cells (Derrick *et al.*, 2007).

Complementation of BCG and *M. microti* with the entire RD1 locus, and thus a full ESX-1 secretion system, results in increased virulence in mice, (Brodin *et al.*, 2004a, Pym *et al.*, 2002, Majlessi *et al.*, 2005) confirming the significance contribution of ESX-1 to mycobacteria pathogenesis.

Recently, a second ESX system has been pinpointed as important for virulence. ESX-5 is confined to the slow-growing mycobacteria, which contains most pathogenic species (Gey van Pittius *et al.*, 2006, Abdallah *et al.*, 2006) and has a distinguishing feature of exporting the PE/PPE protein family, which is unique to mycobacteria and have a role in virulence. Mutation in two PE genes generates *M. marinum* strains incapable of replication in macrophages and with decreased persistence in granulomas (Li *et al.*, 2005, Ramakrishnan *et al.*, 2000). Also, a *M. tuberculosis ppe54* transposon mutant was impaired in its ability to arrest phagosome maturation and trafficked rapidly into acidified compartments (Brodin *et al.*, 2010). A more recently study showed that a *M. tuberculosis* PE_PGRS30 mutant presented an attenuated phenotype in murine and human macrophages, due to the inability to inhibit phagosome–lysosome fusion, and an impaired ability of colonizing lung tissue and causing tissue damage (Iantomasi *et al.*, 2012). Using a more extensive approach, disruption of ESX-5 system in *M. marinum* affects cytokine manipulation in macrophages (Abdallah *et al.*, 2008) and leads to hypervirulence in adult zebrafish or attenuation in zebrafish embryos (Weerdenburg *et al.*, 2012), while in *M. tuberculosis*, ESX-5 plays an essential role for PPE proteins transport, cell wall integrity and full virulence (Iantomasi *et al.*, 2012, Bottai *et al.*, 2012).

AIMS

ManLAM has been considered a key factor in host cell recognition and immunomodulation, and according to the previous data, the mannose cap would play a crucial role in mycobacterial persistence. Most of what is known about the biological activities of LAM stems from *in vitro* work using the purified glycan, which could be different from the effect of LAM as present in its natural form in the bacterial cell wall. In fact, a study where a capless mutant of *M. marinum* and *M. bovis* BCG was created, show that the absence of the mannose cap did not lead to a decreased survival of bacteria *in vitro* or *in vivo*, with the mutants being as virulent as their respective parent strains (Appelmelk *et al.*, 2008). These results contradicted the data obtained earlier with purified LAM and created serious doubts about the importance of the mannose cap of LAM to mycobacteria pathogenesis. The *in vivo* studies have been carried out with BCG and *M. marinum*, but not *M. tuberculosis* itself. Therefore, the use of a *M. tuberculosis* capless mutant would be of great importance to understand the contribution of ManLAM to mycobacteria virulence. Furthermore, it is possible that the vaccine *M. bovis* BCG has a limited effectiveness in conferring immunity to TB, due to a immunosuppressive role of ManLAM, and therefore it would be interesting to see how the protective immune response to *M.tuberculosis* is affected in a capless BCG mutant.

Besides the immunomodulatory molecules that constitute the cell envelope, pathogenic mycobacteria rely also in a collection of secreted virulence factors to manipulate the host**.** The involvement of ESX-1 in mycobacterial virulence is clear (MacGurn *et al.*, 2007, Pym *et al.*, 2002, Majlessi *et al.*, 2005, Hsu *et al.*, 2003, Lewis *et al.*, 2003). The ESX-5 system gained recently further attention due to its importance in the secretion of proteins with a possible role in mycobacterial pathogenesis. Previous studies showed that any of these ESX systems fails to complement the loss of virulence caused by deletion of another, suggesting a strong dependency of both secretory systems for full virulence. Furthermore, the fact that BCG strains became attenuated when compared to their ancestral *M. bovis*, due to ESX-1 loss, suggests the existence of a correlation between the level of virulence and the presence of ESX in the strain. If this correlation exists, BCG virulence would be further affected by the deletion of the ESX-5 system.

Therefore, the main goal of this thesis is to:

1- Understand the contribution of the mannose capping of the glycolipid ManLAM to the development of protective immune responses and in mycobacterial virulence, using a *M. bovis* BCG and *M. tuberculosis* mannose cap mutant.

2- Comprehend the impact of ESX-5 in *M. bovis* BCG virulence.

PART II

RESULTS

Lipoarabinomannan mannose caps do not affect mycobacterial virulence or the induction of protective immunity in experimental animal models of infection and have minimal impact on in vitro inflammatory responses.

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Abstract

Mannose-capped lipoarabinomannan (ManLAM) is considered an important virulence factor of *Mycobacterium tuberculosis*. However, while mannose caps have been reported to be responsible for various immunosuppressive activities of ManLAM observed *in vitro*, there is conflicting evidence about their contribution to mycobacterial virulence *in vivo*. Therefore, we used *M. bovis* BCG and *M. tuberculosis* mutants that lack the mannose cap of LAM to assess the role of ManLAM in the interaction of mycobacteria with the host cells, to evaluate vaccine-induced protection and to determine its importance in *M. tuberculosis* virulence. Deletion of the mannose cap did not affect BCG survival and replication in macrophages, although the capless mutant induced a somewhat higher production of TNF. In dendritic cells, the capless mutant was able to induce the up-regulation of co-stimulatory molecules and the only difference we detected was the secretion of slightly higher amounts of IL-10 as compared to the wild type strain. In mice, capless BCG survived equally well and induced an immune response similar to the parental strain. Furthermore, the efficacy of vaccination against a *M. tuberculosis* challenge in low-dose aerosol infection models in mice and guinea pigs was not affected by the absence of the mannose caps in the BCG. Finally, the lack of the mannose cap in *M. tuberculosis* did not affect its virulence in mice nor its interaction with macrophages in vitro. Thus, these results do not support a major role for the mannose caps of LAM in determining mycobacterial virulence and immunogenicity *in vivo* in experimental animal models of infection, possibly due to redundancy of function.

Introduction

The cell envelope of *M. tuberculosis* is considered a major determinant of virulence in this global pathogen. A major component of the cell envelope of all mycobacteria is lipoarabinomannan (LAM) (Briken *et al.*, 2004, Chatterjee *et al.*, 1998, Gilleron M, 2008, Nigou, 2003), which appears to play a key role in the interaction with the host, and modulation by the bacterium of the host response (Briken *et al.*, 2004, Gilleron M, 2008, Mishra *et al.*, 2011, Nigou, 2003, Nigou *et al.*, 2002). This complex molecule of approximately 17 kDa is the largest member of a series of lipoglycans of varying size based on a conserved mannosyl-phosphatidyl-*myo*-inositol anchor. In slow growing mycobacteria, such as *M. tuberculosis*, the presence of one to three mannopyranosyl residues linked to the non-reducing ends of the arabinan domain constitute the mannose cap of LAM (ManLAM), while in fast-growing mycobacteria, such as *Mycobacterium smegmatis*, this latter domain is capped with phospho-inositol residues (PILAM) (Nigou, 2003) and in some species like *M. chelonae*, no such capping motif is present (Guerardel *et al.*, 2002). The proportion of LAM non-reducing termini that are capped with mannose varies among different species of slow-growing mycobacteria and among strains of *M. tuberculosis*, with fully virulent *M. tuberculosis* laboratory strains having up to 70% capping (Chatterjee *et al.*, 1992, Khoo *et al.*, 1995). The number of mannose residues per cap is also variable even within LAM from the same species or strain (Nigou, 2003).

A large number of studies have assigned a role in virulence to LAM. Initial studies aiming at determining the role of LAM in mycobacterial virulence addressed the *in vitro* effects of the purified molecules. These studies showed that LAM from *M. tuberculosis* is able to alter macrophage functions associated with protective immunity (Chan *et al.*, 1991, Fratti *et al.*, 2003, Kang *et al.*, 2005, Pathak *et al.*, 2005, Sibley *et al.*, 1988). It was additionally shown that LAM from mycobacteria of different virulences have different immuno-modulatory activities (Adams *et al.*, 1993, Bradbury *et al.*, 1993, Chatterjee *et al.*, 1998, Knutson *et al.*, 1998, Roach *et al.*, 1993, Vergne *et al.*, 2003, Yoshida *et al.*, 1997). Numerous studies have so far suggested a role of ManLAM in binding to and in modulating the function of macrophages and dendritic cells (Chan *et al.*, 1991, Fratti *et al.*, 2003, Geijtenbeek *et al.*, 2003a, Kang *et al.*, 2005, Maeda *et al.*, 2003, Nigou *et al.*, 2001, Schlesinger *et al.*, 1994, Schlesinger, 1993 , Sibley *et al.*, 1988, Tailleux *et al.*, 2003, Torrelles *et al.*, 2006, Vergne *et al.*, 2003, Welin *et al.*, 2008).

The studies above have led to the conclusion that ManLAM is an important virulence factor in tuberculosis, and that mannose capping plays an essential role. However, interpretation of these experiments is complex, with the experiments comparing ManLAM from fully virulent *M. tuberculosis* either with ManLAM from strains with a lower proportion

of mannose capping, or with PILAM (Khoo *et al.*, 1995). It is therefore important to carry out experiments directly comparing LAM produced by isogenic strains differing only in their terminal mannosyl decoration.

Enzymatic removal of mannose residues with α-mannosidase revealed that the inhibition of IL-12 secretion by human dendritic cells caused by BCG ManLAM is strictly dependent on an intact molecule (Nigou *et al.*, 2001). However, to unambiguously study the role of mannose capping of LAM in an infectious setting, we have been using genetically engineered mutants in which the cap is not added. A mannosyltransferase encoded by the *M. tuberculosis capA* gene (*rv1635c*) is responsible for the addition of the first mannose residue of the mannose cap in an α(1→5) linkage (Dinadayala *et al.*, 2006). Previously, we identified homologous enzymes in *M. marinum* and *M. bovis* BCG (Appelmelk *et al.*, 2008). Disruption of the *capA* gene resulted in bacteria deficient in the biosynthesis of the mannose cap of LAM (Appelmelk *et al.*, 2008, Dinadayala *et al.*, 2006).

When we tested capless mutants of *M. marinum* and *M. bovis* BCG *in vitro* and *in vivo* (Appelmelk *et al.*, 2008), we obtained the surprising result that there was no evidence for an altered host-pathogen interaction, and that capless *M. marinum* and BCG mutants were not less virulent than their respective parent strains. Thus, the data obtained with live isogenic strains were discrepant with the mass of data obtained earlier with purified ManLAM.

Here we follow up on our previous study of the role of the mannose cap further using isogenic strains differing only at the *capA* locus: we (1) look at the role of the mannose cap in the protective efficacy of BCG in vaccination studies, and (2) extend our studies on virulence to look at *M. tuberculosis* itself.

M. bovis BCG, the vaccine for tuberculosis protects well against disseminated TB, including meningitis in childhood, but protection against adult pulmonary TB is highly variable, ranging from 80% to no protection at all (reviewed by (Colditz *et al.*, 1994), and a more effective vaccine is therefore required. As ManLAM has been reported as blocking phagolysosome fusion, and/or inducing IL-10, and LAM lacking mannose caps does not exhibit these activities, we hypothesized both that a capless mycobacterium would induce a different type of immune response, being less immunosuppressive and thus more protective. We have therefore tested a capless BCG as a vaccine both in a low-dose challenge model in mice, in contrast to the high-dose challenge model used by (Festjens *et al.*, 2011) and in the guinea pig model.

To date, *in vivo* virulence studies have been carried out with capless mutants of BCG and *M. marinum*, but not *M. tuberculosis* itself. Here we test the virulence of capless *M. tuberculosis* in a low dose aerosol model.

Experimental procedures:

Mycobacterial strains and growth conditions:

The BCG *capA* mutant, lacking the mannose cap of LAM, has been described and characterized before (Appelmelk *et al*., 2008). The *capA* mutant in *M. tuberculosis* H37Rv has not been described before and was constructed in a similar way via a two-step p1NIL-pGOAL19 approach developed by Parish and Stoker (Parish *et al*., 2000), leading to a markerless deletion in the gene of interest (*rv1635c*). In fact, exactly the same plasmids were used to genetically modify BCG and *M. tuberculosis*: the DNA sequences of Rv1635c and its BCG homolog are 100% identical. Briefly, after the second step (sucrose selection) of the p1NIL-pGOAL procedure fourteen colonies were picked. The presence of this deletion was investigated by PCR. Phenotypically, the lack of mannose caps was investigated in immunoblot. In this colony dot blot, eleven of the colonies were non-reactive with the cap-specific Mab 55.921A (Appelmelk *et al*., 2008) and three were reactive. In PCR those three yielded a *rv1635c* product, which was absent in the other eleven colonies. We concluded that of the fourteen colonies picked, three were revertants and eleven were (markerless) mutants lacking Rv1635c. These three mutants were investigated in SDS-PAGE-Immunoblot with monoclonal antibodies (MAbs) F30-5, 183-24, and 55.92.1A1, concanavalinA, and DC-SIGN-Fc and further evidence was obtained they missed the cap (Supplementary Figure 3). Finally, one of the capless colonies was further investigated in capillary electrophoresis again providing evidence the cap is missing (Supplementary Figure 2). A complementant was also made: *rv1635c* was cloned into the shuttle vector pSMT3 and the construct electroporated in the *capA* mutant of *M. tuberculosis* H37Rv. *M. bovis* BCG Copenhagen and mutant CapA were grown in liquid Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80, until the log phase. The H37Rv *M. tuberculosis* wild type, capless mutant and complemented strain were grown in Proskauer-Beck medium (PB) containing 0.05% Tween 80 to mid–log phase. Cultures were aliquoted and frozen at –80°C until the day of use.

Chemical analysis of the mannose caps:

LAM from *M. tuberculosis* wild-type and *M. tuberculosis* capless mutant were analyzed for presence of the mannose cap by the capillary electrophoresis technique described earlier (Nigou et al, 2000). Briefly, purified LAM was partially degraded by controlled acid hydrolysis (0.1M HCl for 20 min. at 110°C), and the oligosaccharides liberated tagged with the fluorescent label 8-aminopyrene-1,3,6-trisulfonate (APTS). During CE, the labeled

oligosaccharides are separated and peaks are detected by laser-induced fluorescence and migration times compared with the appropriate controls.

Analysis of purified PIMs by Mass Spectrometry.

Lipids were obtained by chloroform/methanol extraction of bacteria and subjected to MALDI-TOF MS analysis in the negative ion mode as previously described (Gilleron *et al.*, 2003).

Laboratory mice:

8 week-old female BALB/c and C57/B6 mice were purchased from Charles River (L'Arbresle, France) and housed under specific pathogen-free conditions in our facilities. Sterile chow and tap water ad libitum was given. All experiments were approved by and performed according to the guidelines of the animal ethical committee of IBMC. Five to seven animals were used per experimental group for each time-point.

BCG antigens for cell stimulation:

Mycobacteria antigens were prepared as described elsewhere (Pais *et al.*, 2000). BCG was grown until log phase, at 37°C, in Middlebrook 7H9 medium (Difco) supplemented with 10% albumin/dextrose/catalase (ADC) and 0,05% Tween 80. The culture was centrifuged (10,000 x g, 40 minutes, 4°C) and the remaining pellet washed and re-suspended with phosphate-buffered saline (PBS), containing 0.1% Tween 80 (Sigma), 1 mM $MgCl_2$ (Merck, Darmstadt, Germany) and 1 mM benzamidine (Sigma). The bacteria in suspension were 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 x g), and the supernatant was dialyzed against PBS (molecular weight cut-off of 12,000), followed by ultra-centrifugation for 2 h at 150,000 x g. The remaining pellet, containing the envelope proteins, was re-suspended in PBS, and the supernatant, enriched in cytosolic proteins, was precipitated with 80% ammonium sulfate and dialyzed against PBS. Aliquots were quantified and stored at -80°C until the day of use.

Generation of bone marrow-derived macrophages (BMMØ) and dendritic cells (BMDC):

Bone marrow cells were flushed from the femurs of mice with 5 ml of cold Hanks' balanced salt solution (HBSS; Gibco, Paisley, United Kingdom) using a 26-gauge needle. For macrophage generation, the resulting cell suspension was centrifuged for 10min at

1200rpm, 4ºC; resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 mM HEPES (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Gibco), 10 mM glutamine (Gibco), 10% heat-inactivated fetal bovine serum (Sigma), 10% L929 cell conditioned medium (LCCM, as a source of M-CSF) and cultured for a period of 4 h on cell culture dishes (Nunc, Naperville, IL) in order to remove already differentiated cells. Non adherent cells were then collected with warm HBSS, counted, distributed in 24-well plates at a density of $5x10^5$ cells/well and incubated in 1 ml of similar media at 37°C in a 5% CO2 atmosphere. On day 4 after seeding, 100μL of LCCM was added and on day 7 the medium was renewed. Macrophages were infected at day 10. For dendritic cell differentiation, the cell suspension was cultured at a density of 10^6 cells/ml in RPMI 1640 containing GlutaMAX-I, supplemented with 5% v/v fetal calf serum (FCS), 50 mM β-mercaptoethanol and 10% GM-CSF-containing culture supernatant from transformed J558 cells. Every 2 days, one-half of the media was removed and supplemented with complete medium with GM-CSF. On day 9, the non-adherent cells were cultured at 2 x 10^5 cells/ml in 96-well plate (100 µl/well), and infected at day 10. The purity of the population was determined by FACS analysis of specific surface markers and ranged from 85 to 95%.

Macrophage infection:

A bacterial suspension containing $5x10^6$ CFU/mL was prepared and 200 µl was added to each well to obtain a multiplicity of infection (MOI) of 2 bacteria per macrophage. After 4 h of incubation at 37°C in a 5% CO2 atmosphere, cells were washed with warm HBSS to remove the non-internalized bacteria, and re-incubated in DMEM with 10% LCCM. All treatments (100U IFNγ; 50U TNF) were applied from day zero until day 4. At different time points 100µl of supernatant was collected for subsequent cytokine measurements. For the CFUs assay, the infected cells were lysed with a 10% saponin solution, in sterilized water with 0,05% Tween 80, and serial dilutions of triplicate wells were performed. The number of viable bacteria was assessed by counting the colonies 3–4 weeks after plating on 7H11 Agar medium (Difco) supplemented with 10% oleic acid/ albumin/dextrose/catalase (OADC) and incubated at 37°C.

Dendritic cell infection:

A bacterial suspension containing $5x10^6$ CFU/mL was prepared and 100 µl was added to each well in order to obtain a multiplicity of infection (MOI) of 2 bacteria per DC. Supernatants were collected at different time points for IL-12p70, TNF and IL-10 cytokine

measurements and the levels of expression of co-stimulatory molecules in cells were assessed by flow cytometry.

Cytokine measurement:

Cytokine detection in the supernatants was performed by ELISA. For IFN-γ quantification, affinity-purified monoclonal antibodies (R4-6A2 as capture and biotinylated AN18 as detecting antibody) were used, while commercial kits were used according to the manufacturer's instructions for the detection of TNF, IL-10 (R&D Systems), IL-12p70, and IL-17A (Biolegend).

Replication *of M. bovis* BCG in mice:

Mice were infected intravenously through the lateral tail vein, with $5x10^4$ CFU *M. bovis* BCG, wild type or mutant, in 200 µL of PBS. Bacterial loads in the organs of infected mice were evaluated at different time points post infection. Organs were homogenized in sterile water with 0.05% Tween 80 and ten-fold serial dilutions of organ homogenates were plated in duplicate onto Middlebrook 7H11 agar plates containing OADC. Plates where incubated at 37°C and colonies were counted 21 days later. Results are expressed as log CFU per organ.

Immunization and TB challenge:

Balb/c mice were immunized by a single intradermal injection with $5x10^4$ CFUs of *M. bovis* BCG, wild type or mutant. Two months later, mice were aerogenically infected with *M. tuberculosis* H37Rv using a Glass-col aerosol generation device chamber (Terre Haute, IN, USA). Briefly, mice were exposed for 30min to an aerosol produced by nebulizing 10 mL of PBS-Tween80 containing 10^6 CFU/ml that resulted in the implantation of 10-25 bacilli in the lung of each animal. Bacterial loads in the organs of infected mice were assessed by plating organ homogenates onto Middlebrook 7H11 agar plates, and counting the colonies formed 14 to 21 days after incubation at 37ºC. Results are expressed as log CFU per organ.

Guinea pig immunization and infection:

Groups of 8 Dunkin–Hartley guinea pigs, weighing between 250 and 300 g (and free of infection), obtained from a commercial supplier (Harlan, UK), were used to evaluate the efficacy of capless BCG compared with BCG Danish 1331 (Statens Serum Institute, Copenhagen, Denmark), both delivered subcutaneously in a single dose at a concentration of 5×10^4 CFU and a negative control (PBS vaccinated) group. Individual

animals were identified using subcutaneously implanted microchips (PLEXX BV, The Netherlands). Guinea pig experimental work was conducted according to UK Home Office legislation for animal experimentation and was approved by the local ethics committee.

Animals were infected with a low aerosol dose (10–50 CFU retained dose in the lung) of *M. tuberculosis* H37Rv (Williams *et al.*, 2000) 12 weeks after vaccination. Aerosol challenge was performed using a fully contained Henderson apparatus as previously described (Chambers *et al.*, 2000, Clark *et al.*, 2011, Lever *et al.*, 2000) in conjunction with the AeroMP (Biaera) control unit (Hartings *et al.*, 2004). Fine particle aerosols of *M. tuberculosis*H37Rv, with a mean diameter of 2 μm (diameter range, 0.5–7 μm) (Hartings *et al*., 2004) were generated using a Collison nebulizer and delivered directly to the animal snout. The aerosol was generated from a water suspension containing 5 x 10^6 CFU/ml in order to obtain an estimated retained, inhaled dose of approximately 10–50 CFU/lung. The Henderson apparatus allows controlled delivery of aerosols to the animals and the reproducibility of the system and relationship between inhaled CFU and the concentration of organisms in the nebulizer has been described previously (Chambers *et al*., 2000; Clark *et al*., 2011). The challenge system is controlled by an AeroMP: the aerosol management platform controls, monitors, and records all relevant parameters during an aerosol procedure including air flow rate, temperature and relative humidity (Hartings *et al*., 2004). At 4 weeks post-challenge, guinea pigs were killed humanely by intraperitoneal injection of pentabarbitone (Euthatal). Post-mortem, tissues were aseptically removed for bacteriology analysis. Tissues were homogenized in 5 ml of sterile distilled water using a rotating blade macerator system (Ystral, UK). Viable counts were performed on the macerate by preparing serial dilutions in sterile deionized water and plating 100 μl aliquots onto Middlebrook 7H11 + OADC agar (BioMerieux, UK). Plates were incubated at 37 °C for 3 weeks before counting the number of *M. tuberculosis* colonies (CFU).

Cell preparation and *in vitro* stimulation:

Spleens were gently disrupted with the help of a cell glass homogenizer. The resulting cell suspension was passed through a 70 μm nylon cell strainer, in order to remove large pieces and debris. Lung cell suspensions were obtained as follows: thoracic cavities were opened, and sterile PBS was gently injected into the right heart ventricles to perfuse lungs. Lungs were excised, sectioned, and incubated with digestion media (DMEM supplemented with Collagenase IX (0.7 mg/ml; Sigma) for 30 min at 37°C. The final cell suspension was obtained by passing the digested lung tissue through a 70 μm nylon cell strainer. In all cell suspensions the red blood cells were lysed with a hemolytic solution

(155 mM NH_4Cl, 10 mM $KHCO_3$ (pH 7.2)) during 5 min at room temperature. Cells were then distributed into 96-well plates (2.5 x 10^5 cells/well) and incubated in triplicate with different stimuli: DMEM culture medium and 4 µg/ml of ConA (Sigma-Aldrich) as negative and positive controls, respectively, and depending on the experiment, 4µg/ml of BCG extract or 1.25x10^5 of live *M. tuberculosis* H37Rv bacilli. After 72h of incubation at 37°C in a 5% CO2 atmosphere, the supernatants were collected for cytokine measurement. For intracellular staining, 1 x 10^6 cells/ml were incubated for 4 h at 37°C in the presence of PMA (Sigma-Aldrich) plus ionomycin (Calbiochem) at a final concentration of 25 µg/ml each, followed by an incubation of 2 h in the presence of 0.01 mg/ml brefeldin A (Sigma-Aldrich). Then, cells were fixed, permeabilized and stained with IFNg-specific antibodies.

Flow cytometry:

Cells were labeled with specific antibodies for CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD11b (clone M1/70), CD19 (clone 6D5), DX5 (clone HMα2), CD25 (clone PC61), CD86 (clone GL-1), CD40 (clone 5C3), MHC-II (clone M5/114.15.2) from BioLegend, San Diego, CA) and FOXP3 (e-bioscience). Cell populations were acquired in a FACS Calibur instrument equipped with CellQuest software. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis:

Data were analyzed by using Student's *t* test. Statistical analyses of guinea pig data were performed using Minitab (version 13.32). The CFU data were analyzed by non-parametric Mann–Whitney test comparisons to compare median values of the vaccine group with either the saline or BCG control groups.

Results:

We first studied a capless mutant of BCG. This strain has already been used in an earlier publication where it was fully characterized and shown to synthesize LAM devoid of mannose caps (see supplementary figure 4 from (Appelmelk *et al.*, 2008)). Here we extended the analysis of its interaction with the host cells initially in *in vitro* assays using cultured macrophages and dendritic cells and subsequently in *in vivo* experiments.

Response of mouse macrophages to wild type BCG and capless BCG mutant.

Macrophages are in the first line of cellular defense against mycobacterial infection. However, as intracellular pathogens, mycobacteria have evolved strategies to manipulate the host cell mechanisms responsible for their killing. ManLAM inhibits the maturation of phagosomes (Fratti *et al.*, 2003, Vergne *et al.*,2003) and prevents macrophage activation by IFNγ (Chan *et al.*, 1991, Sibley *et al.*, 1988). To assess the importance of the mannose caps in BCG survival and macrophage activation, we infected bone marrow-derived macrophages (BMDM) from Balb/c mice with either the parental BCG strain or the capless mutant strain and measured mycobacterial growth and the production of TNF and reactive nitrogen species by the macrophages. No differences in phagocytosis were observed between the mutant and parental BCG, with both BCG strains being internalized to the same extent following exposure of the macrophage monolayers to similar multiplicity of infection (MOI=2) leading to similar CFU counts at time 0 of infection, i.e. after 4 hours of contact of the macrophages with the inocula **(Fig 1A).** Both strains replicated in macrophages with similar growth rates **(Fig 1A).** Activation of the macrophages with IFNγ or IFNγ plus TNF caused macrophages to kill BCG but again both BCG strains behaved in the same manner **(Fig 1B)**. Despite the same degree of replication in resting macrophages and the similar bacterial loads found at the end of the experiment, the mutant induced higher amounts of TNF secretion at day 7 of infection **(Fig 1C)**. The production of reactive nitrogen species was also assessed, with both BCG strains inducing the same amount **(Fig 1D).** These data show that the lack of mannose caps in LAM leads to different signaling in infected macrophages but has no consequences on the ability of BCG to proliferate or survive intracellularly.

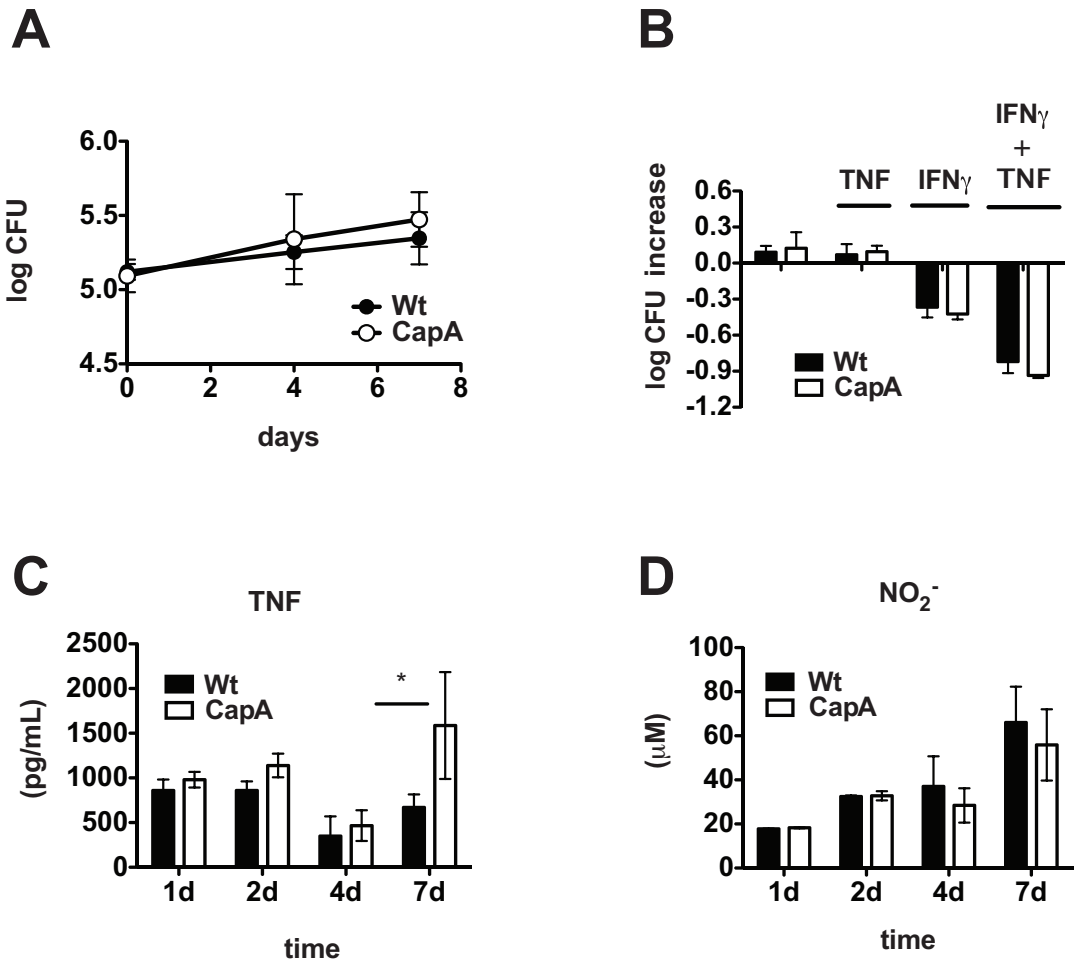


Figure 1: In vitro interaction of BCG with macrophages. A- Growth of wild type (WT) or capless (CapA) mutant BCG in bone marrow derived macrophages (BMDM) from Balb/c mice. B- Survival of WT and capless (CapA) mutant BCG in untreated BMDM or BMDM treated with 50U TNF, 100U IFNγ or both (results expressed as "log CFU increase", that corresponds to the difference of growth, in log CFU, between day 7 and day 0). C- Secretion of TNF into the culture medium by BMDM infected with the BCG strains. D- Release of nitrite into the culture supernatants by BMDM infected with BCG and treated with IFNγ. Data represent the mean ± 1SD of a representative experiment out of a total of 3 experiments. Statistically significant differences are labeled with an asterisk

Response of mouse dendritic cells to wild type BCG and capless BCG mutant

Dendritic cells (DCs) are key players in the induction of cellular immune responses against mycobacteria (Murray, 1999). It has been shown that ManLAM (but not non-capped LAM) prevents human DC maturation, inhibits the production of the pro-inflammatory cytokines IL-12 and TNF (Nigou *et al.*, 2001, Nigou *et al.*, 2002) and triggers the production of the immunosuppressive cytokine IL-10 in lipopolysaccharide (LPS)-activated DCs (Geijtenbeek *et al.*, 2003a). To evaluate the effect of the mannose capping of LAM in the activation and modulation of DC function, we infected bone marrow-derived dendritic cells (BMDC) from Balb/c mice with the parental BCG or the mutant strain and measured the induction of co-stimulatory molecules and the production of cytokines released into the culture supernatants. Both wild type and mutant BCG activated DC and induced the same extent of up-regulation of co-stimulatory molecules (MHC-II, CD40 and CD86) **(Fig. 2A)**. They also induced the secretion of the same amount of IL12p70 and TNF but, interestingly, the mutant strain induced higher amounts of IL-10 **(Fig. 2B)**. Thus both in macrophages and DC, mannose capping of LAM affects cytokine induction.

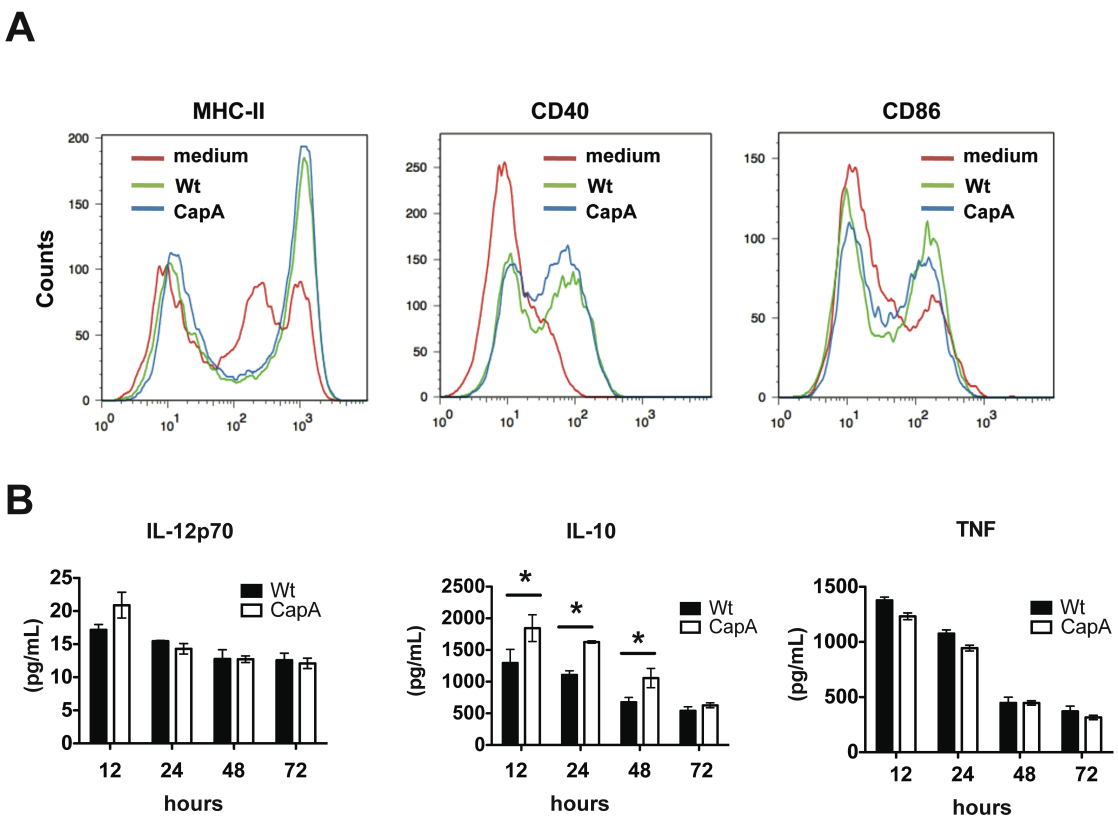


Figure 2: In vitro interaction of BCG with dendritic cells. A- Expression of co-stimulatory molecules in bone marrow-derived dendritic cells infected with WT or capless

(CapA) mutant BCG as evaluated by flow cytometry. B- Secretion of IL-12p70, IL-10 and TNF by dendritic cells infected with WT or capless (CapA) mutant BCG. Data represent the mean ± 1SD of a representative experiment out of a total of 3 experiments. Statistically significant differences are labeled with an asterisk.

Capless BCG mutant replicates and induces identical immune responses *in vivo* as compared to the wild type strain.

As described above, ManLAM is proposed to be a key molecule in mycobacterial virulence (reviewed by(Nigou, 2003, Briken *et al.*, 2004)) and the mannose capping of LAM may be related to an immunosuppressive activity of this lipoglycan impacting on vaccine efficacy of BCG. We therefore wished to determine the protective efficacy of the capless BCG strain.

Before testing the protective abilities of the parent BCG and its capless mutant we compared their replication *in vivo*, and the immune response they induced. In order to compare the growth of wild type and capless BCG, Balb/c mice were intravenously infected with $5x10^4$ CFU of either the parental strain or the capless mutant and groups of five mice were sacrificed at days 1, 10, 20, 30 and 60 post-infection. No differences in mycobacterial loads were observed for the two strains in either the spleen or the liver **(Fig.3A).** In the spleen, both BCG strains proliferated until around day 20, with mice reducing the bacterial load afterwards, while in the liver, the growth of the parent and mutant strains was slowly controlled after day 10, with bacterial numbers decreasing over time, as has been classically described (Gheorghiu *et al.*, 1985).

We next took spleen cells from infected mice, and stimulated them *in vitro* with BCG antigens to assess cytokine responses. Cells from capless mutant-infected animals released the same amount of IFNγ, TNF and IL-10 as cells from mice infected with the wild type strain **(Fig 3B)**. No differences were found in the number of several different immune cell populations **(table 1).**

These data do not substantiate our hypothesis that a capless BCG would induce a different type of immune response.

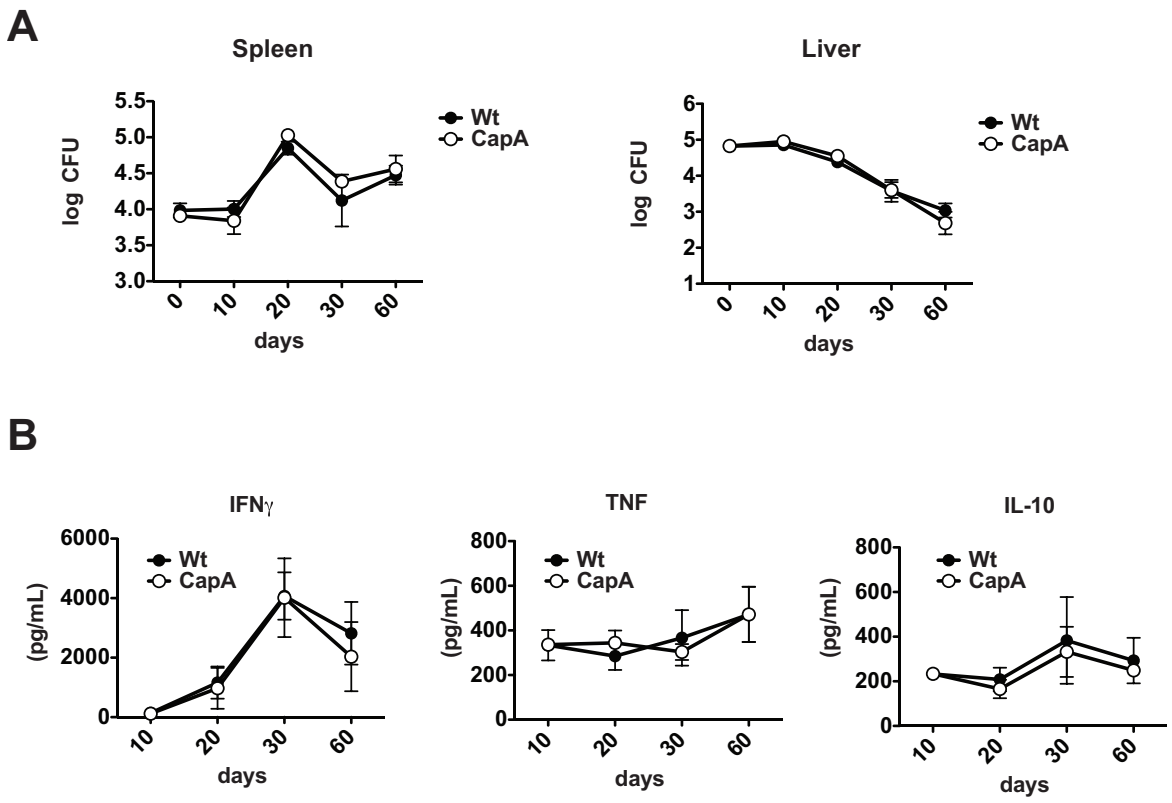


Figure 3: Capless *M. bovis* BCG replicates similarly and induces identical immune responses as compared to the WT strain.

A- Proliferation of WT or capless (CapA) mutant BCG in the liver and spleen of BALB/c mice intravenously infected with 5x104 CFUs. B- Secretion of IFNγ, TNF and IL-10 into the culture supernatants by splenocytes from the infected animals following in vitro re-stimulation with 4µg/ml of *M. bovis* BCG extract for 72h. A total of 5 to 7 mice per time point were used, and all results are representative of at least two independent experiment

Average Cells x10^7 ± SD

	CD4+		CD8+		CD4+Foxp3+		CD19+		CD3+DX5+		DX5+		CD11b+	
Days	Wt	CapA	Wt	CapA	Wt	CapA	Wt	CapA	Wt	CapA	Wt	CapA	Wt	CapA
10	1.2± 0.2	1.2± 0.2	0.6± 0.1	0.6± 0.1	0.2± 0.02	0.2± 0.02	2.4± 0.6	2.1± 0.9	0.1± 0.01	0.1± 0.02	0.2± 0.08	0.2± 0.07	0.3± 0.1	0.3± 0.1
20	1.5± 0.3	1.5± 0.4	0.7± 0.1	0.6± 0.1	0.2± 0.03	0.2± 0.05	3.6± 0.7	3.6± 0.9	0.1± 0.02	0.1± 0.03	0.3± 0.08	0.3± 0.09	0.6± 0.2	0.6± 0.2
30	1.8± 0.4	1.5± 0.1	0.6± 0.2	0.5± 0.1	0.3± 0.06	0.2± 0.03	3.9± 0.6	3.3± 0.7	0.1± 0.03	0.1± 0.02	0.4± 0.04	0.4± 0.07	0.7± 0.2	0.6± 0.1
60	1.6± 0.2	1.4± 0.3	0.6± 0.1	0.5± 0.1	0.2± 0.03	0.2± 0.03	4.1± 0.6	3.4± 0.5	0.1± 0.04	0.1± 0.03	0.3± 0.07	0.3± 0.03	0.8± 0.1	0.6± 0.1

Table 1: *M. bovis* BCG CapA mutant induces identical immune responses as compared to the Wt strain.

BALB/c mice were intravenously infected with 5x10^4 CFUs of *M. bovis* BCG WT or CapA mutant. The animals were sacrificed at different time points and spleen cells were labeled with specific antibodies for flow cytometric analysis of the different splenic cell

populations. A total of 5 to 7 mice per time point were used, and all results are representative of at least two independent experiment.

Wild type and capless BCG induce the same level of protection against a *M. tuberculosis* challenge in a murine low dose aerosol infection model.

We proceeded to carry out a protection study comparing BCG and its capless mutant. Festjens *et al*. (2011) used a high dose ($5x10^4$ CFU intravenous or $2x10^5$ CFU intratracheal) virulent *M. tuberculosis* challenge to assess the protection afforded by wild type or capless BCG. We reasoned that this high dose might overwhelm host immunity and hence mask protective efficacy, and therefore used a low dose exposure aerosol challenge with virulent *M. tuberculosis* to compare the protection afforded by either wild type BCG or its capless mutant.

Balb/c mice were intra-dermally inoculated with $5x10^4$ CFU of the parental or the mutant strain and the dissemination of BCG to the lung and spleen was determined 70 days post-vaccination. Very low numbers of bacteria were detected in the spleen and no CFUs could be detected in the lung of both vaccinated groups (**Fig 4A**), indicating a poor dissemination of BCG after intradermal inoculation. We then determined if a specific immune response could be observed in the lung of these vaccinated mice. Seventy days post-vaccination, leukocytes isolated from the lungs of both immunized groups produced the same amounts of the protective cytokines IFNγ, TNF and IL-17 after *in vitro* re-stimulation with BCG antigens (**Fig 4B**). Furthermore, both BCG strains induced the same number of $CD4^+IFN\gamma^+$ producing cells (**Fig 4C**), showing that both the parental strain and the capless mutant induce a similar type of immune response in the lung. No differences in cytokine production by spleen cells from either group were detected (data not shown).

Seventy days post-vaccination, the two groups of immunized mice and a control group of non-immune animals were subjected to a low dose aerosol challenge with a virulent strain *M. tuberculosis* (strain H37Rv) leading to the implantation of <100 CFU in the lung. The lungs and spleens were harvested at different time points and the numbers of CFU were determined. As expected, 30 days after the challenge with *M. tuberculosis*, a significantly reduced bacterial growth was observed in the lungs and spleens of vaccinated mice when compared to unvaccinated controls (**Fig 4D, $p<0.001$**). The capless mutant induced the same level of protection as the wild type strain.

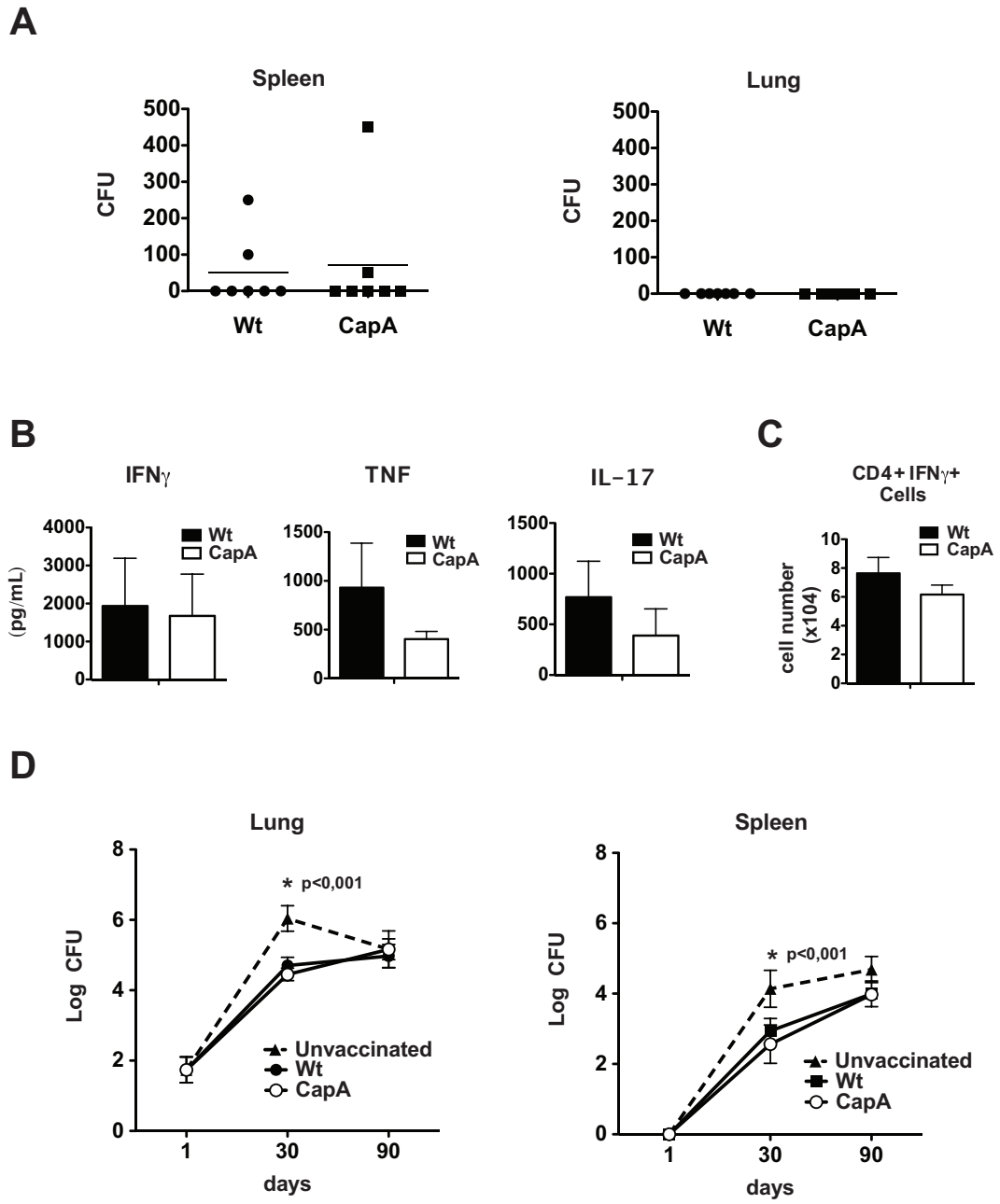


Figure 4: Capless and wild type *M. bovis* BCG induce the same level of protection to a *M. tuberculosis* challenge in the low dose aerosol mouse model.

A- Dissemination of WT and capless (CapA) mutant BCG, after subcutaneous immunization with $5x10^4$ CFU. The number of CFU in the spleen and lung were assessed 70 days after immunization. B- Quantification of cytokine responses in the lungs of vaccinated mice. 70 days post immunization, lung cell suspensions were re-stimulated in vitro with 4µg/ml of BCG extract for 72h and cytokines measured in the supernatants. C-

Number of CD4+IFNγ+ cells in the lungs of vaccinated mice at day 70 post immunization, determined by intracellular cytokine staining of lung cell suspensions re-stimulated in vitro with PMA and Ionomycin. D- Protective efficacy of WT versus capless (CapA) mutant BCG in a M. tuberculosis challenge. Balb/c mice were immunized with $5x10^4$ CFU of parental or mutant CapA BCG. Two months later, mice were aerogenically infected with approximately 100 CFU of M. tuberculosis H37Rv. Mice were sacrificed at days 1, 30, and 90 post-infection and the number of bacteria in lungs and spleens determined. In all experiments a total of 5 to 7 mice per time point were used and all results are representative of at least two independent experiments. Significant values are labeled by an asterisk.

Wild type and capless BCG induce the same level of protection against a *M. tuberculosis* challenge in a low dose guinea pig aerosol model.

Guinea pigs are a key TB vaccine model, as they present pathological features resembling the human disease and, like humans, express CD1b an antigen presenting molecule which is not expressed by mice and that is known to present LAM to antigen-specific T cells (Prigozy *et al.*, 1997, Sieling *et al.*, 1995). Thus, potential LAM-mediated immunity may not be evident in mice, making tests in the guinea pig model prone to show differences in protection not seen in mice. We thus tested capless vs. WT BCG in a low dose aerosol challenge (10-50 CFU in the lung upon infection) in the guinea pig model. **Figure 5** shows that the capless BCG is not more protective as a capped BCG.

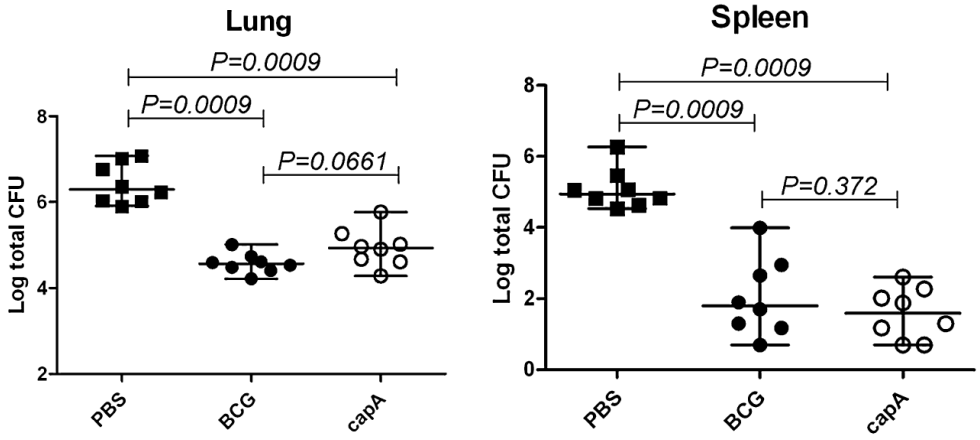


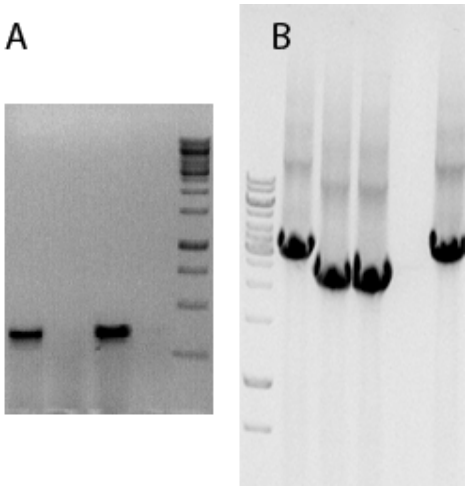
Figure 5: Capless and wild type *M. bovis* BCG induce the same level of protection against a *M. tuberculosis* challenge in the low dose aerosol guinea pig model. Bacterial load of viable *M. tuberculosis* in spleen and lung of guinea pigs was determined.

Adult guinea pigs were infected, sacrificed (8 per group) after 4 weeks and lung and spleen homogenates were plated for enumeration of bacilli (total CFU). Horizontal bars indicate medians after log transformation; error bars indicate range and p values represent statistical comparisons (Mann-Whitney test) between BCG WT (closed circles) and PBS (closed squares) control groups and capless BCG (open circles).

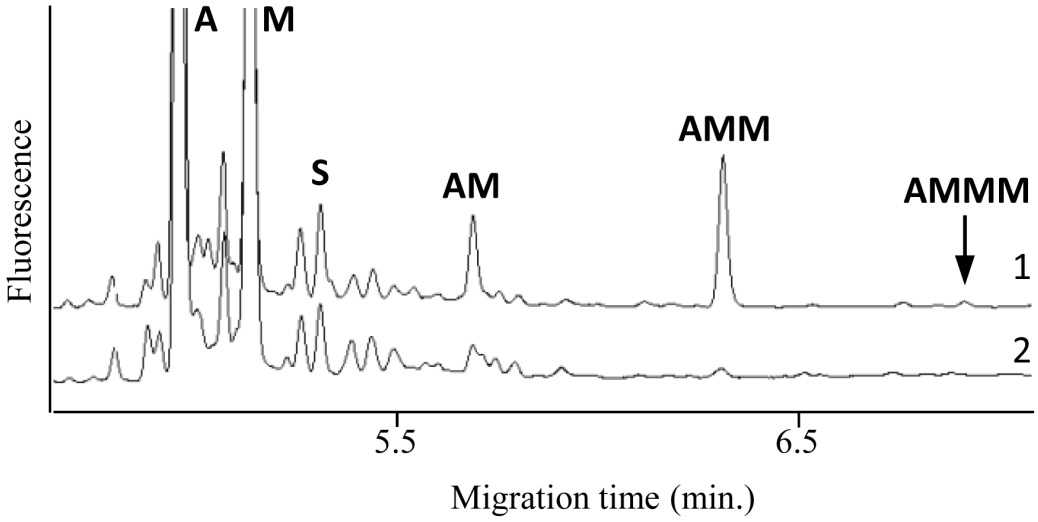
Isolation of a capless *M. tuberculosis* mutant.

The results obtained here as well as the already published information (Appelmelk *et al*., 2008; Festjens *et al*., 2011) point to a redundancy of the mannose cap in BCG and *M. marinum* virulence. However, BCG and *M. tuberculosis* differ drastically in terms of virulence and one could argue that the mannose cap in *M. tuberculosis* may have a more essential role for its virulence than in BCG. We thus studied a *M. tuberculosis* mutant lacking the ability to synthesize the mannose cap to evaluate the role of mannose-capped LAM in *M. tuberculosis* virulence. A capless mutant of *M. tuberculosis* H37Rv was constructed using the pGOAL-pNIL procedure (Parish *et al.*, 2000) exactly as described for *M. bovis* BCG, including isolation of the complementant (Appelmelk *et al,* 2008). **Supplementary Figure 1** provides genetic evidence that indeed *Rv1635c* was disrupted in the mutant and that in the complementant we succeeded in the reintroduction of an intact copy of this gene.

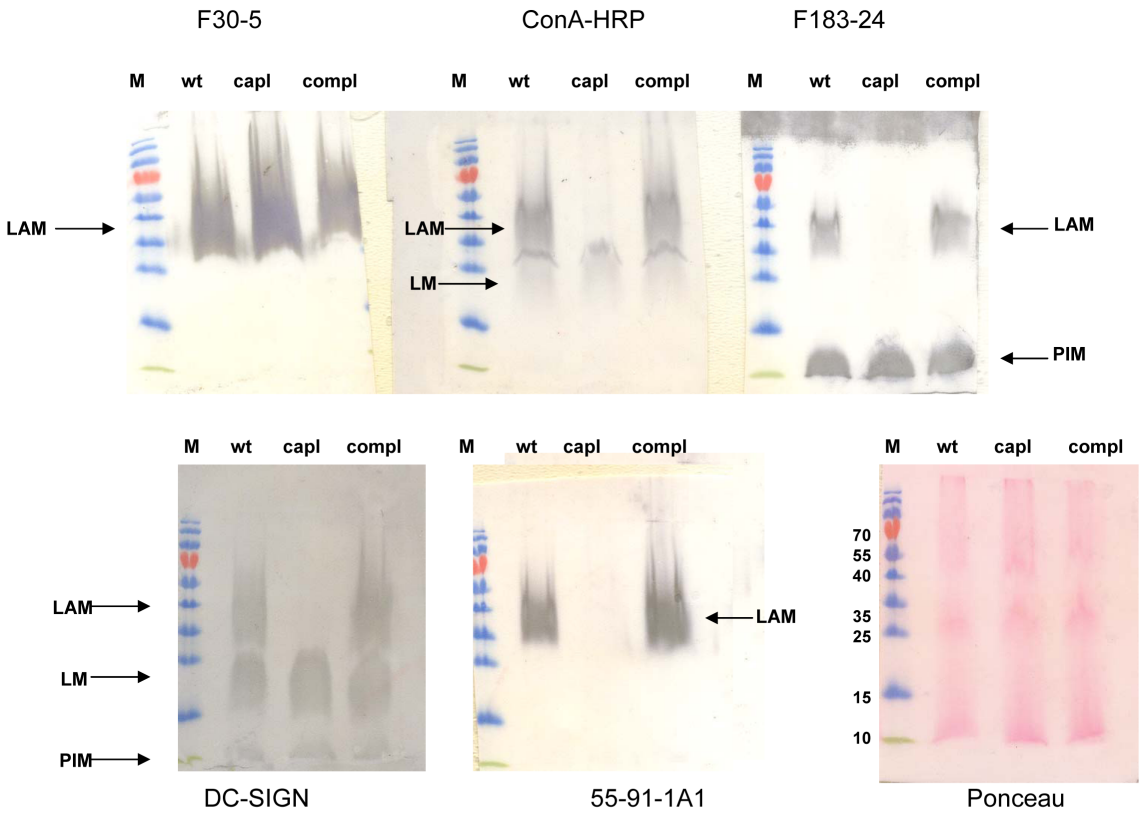
Mild acid hydrolysis of purified LAM followed by analysis of liberated oligosaccharides by capillary electrophoresis (Nigou *et al.*, 2000) provided evidence that all cap motifs were missing (see **Supplementary Figure 2**). We investigated if differences in glycosylation, other than the absence of the cap, were present in the knockout by two independent approaches, i.e. SDS-PAGE followed by immunoblotting with glycosylation-specific probes (**Supplementary Figure 3**) and, secondly, mass spectrometric analysis of isolated PIMs (**Supplementary Figure 4**). No evidence for differences in glycosylation other than in the cap was found.



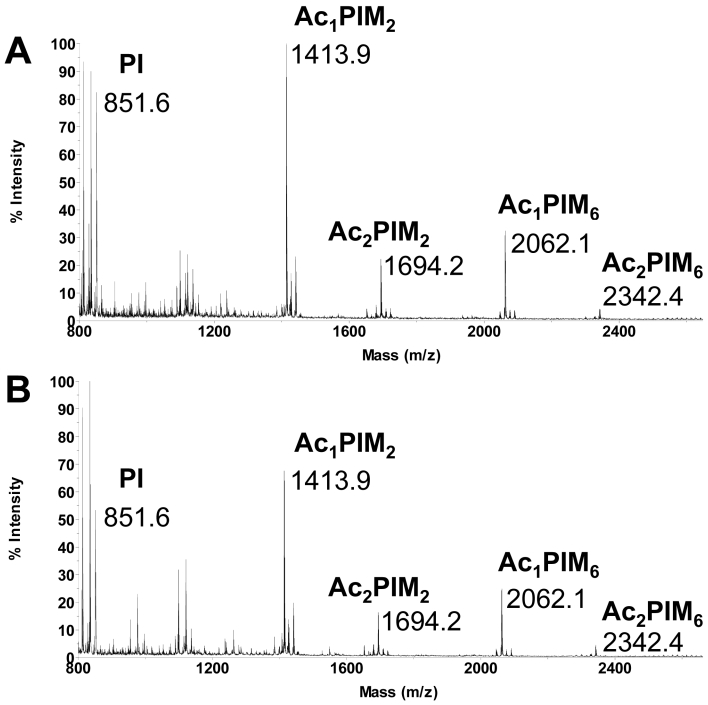
Supp. Figure 1: Genetic evidence for the deletion and complementation of gene *Rv1635c*. The left panel shows the PCR with deletion primers GGCGAACGGAACACTGTGAT and CCGTCTGGCTGACCGTATTA in the deleted region of *Rv1635c*. Lane 1: *M. tuberculosis* H37Rv WT; Lane 2: *M. tuberculosis* H37Rv ΔRv1635c ; Lane 3: Complemented *M. tuberculosis* ΔRv1635c; Lane 4: Negative control; Lane 5: Molecular Weight marker. The predicted product size is 347bp. As expected parent strain and complementant show a product which is of the correct seize, while the knockout does not yield a product. The right panel shows the PCR with flanking primers GTGCCGGTGTGGTCGCTATT and ACCTGGCAACCTGCCGACTT in the regions upstream and downstream of gene *Rv1635c*. Lane 1: *M. tuberculosis* H37Rv WT; Lane 2: *M. tuberculosis* H37Rv ΔRv1635c; Lane 3: Complemented *M. tuberculosis* ΔRv1635c; Lane 4: Negative control; Lane 5: *M. bovis* BCG; Lane 6: Molecular Weight markers. The predicted size of the product in *M. tuberculosis* H37Rv and *M. bovis* BCG is 3256bp; predicted size in H37Rv ΔRv1635c is 2492bp. As expected the complemented strain still yields the knockout product size as the complementation plasmid carries a full-length copy of Rv1635c but no flanking regions. Together the two PCRs prove the identity of parent strain, knockout and complementant.



Supp. Figure 2: Mannooligosaccharide cap analysis of LAM. Purified LAM was analyzed for presence of the mannose caps by capillary electrophoresis monitored by laser-induced fluorescence after mild acid hydrolysis and 8-aminopyrene-1,3,6-trisulfonate tagging. Shown are the profiles of LAM from *M. tuberculosis* wild type (trace 1) and *M. tuberculosis* capless mutant (trace 2). A, Ara-APTS; M, Man-APTS; S, internal standard, mannoheptose-APTS; AM, Man*p*-(α1→5)-Ara-APTS (monomannoside cap); AMM, Man*p*-(α1→2)-Man*p*-(α1→5)-Ara-APTS (dimannoside cap); AMMM, Man*p*-(α1→2)-Man*p*-(α1→2)-Man*p*-(α1→5)-Ara-APTS (trimannoside cap).



Supp. Figure 3: Evidence for a LAM-specific defect in mannosylation. *M. tuberculosis* H37Rv, capless mutant and complementant were grown on plates, suspended to 50 mg/ml wet-weight, disrupted by beat-beating and 10 µl samples subjected to SDS-PAGE (12% acrylamide), blotted to PVDF membranes and probed with various monoclonal antibodies or lectins at 1-2 mg/ml and immunostained. Mab F30-5 recognizes the arabinan domain of LAM; conA and DC-SIGN are lectins specific for mannosyl residues; Mab 183-24 recognizes tri-mannosyl residues in both the mannose cap of LAM and PIMs; Mab 55.92.1A1 recognizes mannose residues in cap only. Arrows indicate the migration of the indicated molecules. Ponceau staining proves adequate transfer to PVDF. The five immunoblots together show that only the mannose cap of LAM is affected in the capless mutant with no other changes in mannosylation visible. Molecular weight markers (expressed in kDa) are indicated along the Ponceau staining.



Supp. Figure 4: MALDI-TOF Mass Spectrometry analysis of PIMs from *M. tuberculosis* wild-type (A) and capless mutant (B) strains. Lipids were obtained by chloforform/methanol extraction of bacteria and subjected to MALDI-TOF MS analysis in the negative ion mode as previously described (Gilleron *et al*., 2003).

Wild type and capless *M. tuberculosis* show similar survival in murine macrophages and induce similar amounts of TNF and NO in vitro.

We tested the interaction of wild type and capless *M. tuberculosis* with Balb/c macrophages. As shown in **Figure 6A** the phagocytosis and growth rate of the two strains in resting BMDM was similar. The restriction of their growth in macrophages following activation by cytokines was also of the same extent (**Figure 6B**). The induction of TNF secretion and nitrite production did not differ significantly between the two strains (**Figure 6C and D**). Hence, the difference in TNF production observed in capless BCG (see Figure 1 C) was not found for capless *M. tuberculosis*.

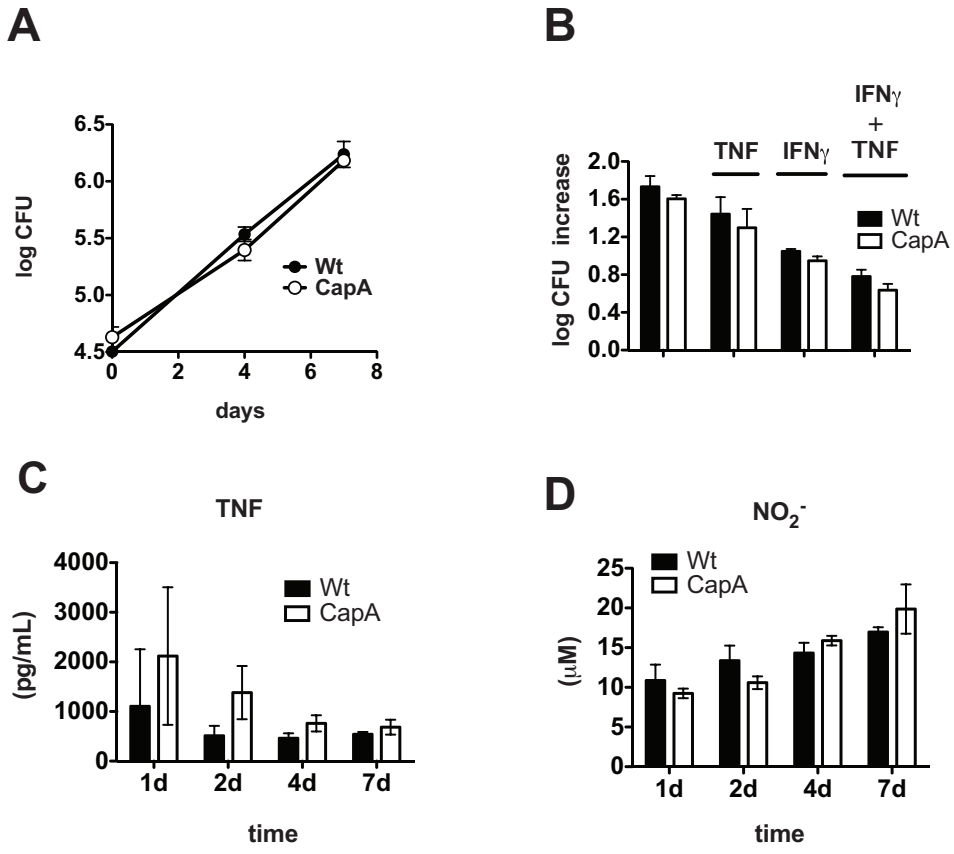


Figure 6: In vitro interaction of *M. tuberculosis* with macrophages.

A- Growth of wild type (WT) or capless (CapA) mutant *M. tuberculosis* in bone marrow derived macrophages (BMDM) from Balb/c mice. B- Survival of WT and capless (CapA) mutant *M. tuberculosis* in untreated BMDM or BMDM treated with 50U TNF, 100U IFNγ or both (results expressed as "log CFU increase", that corresponds to the difference of growth, in log CFU, between day 7 and day 0). C- Secretion of TNF into the culture medium by BMDM infected with the M. tuberculosis strains. D- Release of nitrite into the culture supernatants by BMDM infected with *M. tuberculosis* and treated with IFNγ. Data represent the mean ± 1SD of a representative experiment out of a total of 3 experiments. No statistically significant differences were found.

Wild type and capless *M. tuberculosis* behave similarly in vivo with regards to replication and cytokine induction.

The *M. tuberculosis* wild type strain, capless mutant, as well as a complemented strain were then used for *in vivo* infection studies. Balb/c mice were infected by the low-dose aerosol route. Infection resulted in the implantation of 10 to 25 bacilli in the lungs of each animal. Mice were sacrificed at days 1, 60 and 120 post-infection and the bacterial loads in the lung and spleen determined. The course of the infection is presented in **Figure 7A** and shows that all strains replicated to similar extents in both organs, with mice stabilizing the infection after day 60 post-infection in both the lung and the spleen at similar bacterial loads. In addition, an identical immune response was observed for all the strains, with the production of similar amounts of IFNg, TNF and IL-17 by spleen cells of mice with 60 days of infection **(Fig. 7B)**. No IL-10 was detected in culture supernatants (data not shown). We extended this study to the widely used C57/BL6 mouse strain. Mice were infected as previously described and sacrificed at days 1 and 120 post-infection. Results are expressed as "log CFU increase" corresponding to the difference of growth, in log CFU between day 1 and day 120**.** Like in the Balb/c mouse model, there were no significant differences in growth between the parental and the capless strain **(Fig. 7C)**.

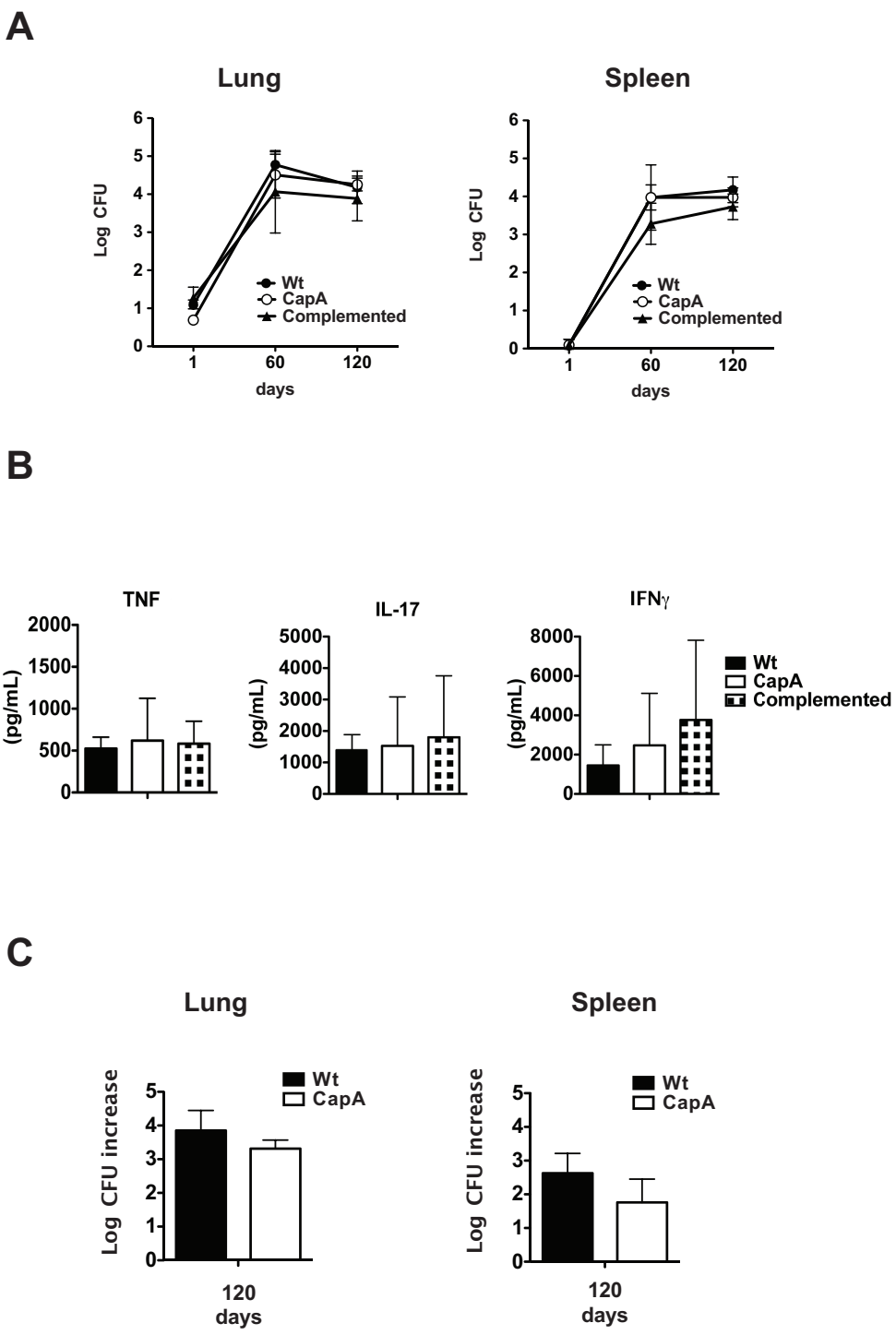


Figure 7: Capless and wild type *M. tuberculosis* have the same virulence. A- Proliferation of WT, capless (CapA) mutant and Complementant strains of *M. tuberculosis* in the lung and spleen of Balb/c mice following an aerogenic infection leading to the implantation of 10 to 25 bacilli in the lung of each animal. B- Secretion of IFNg, TNF and IL-17 into the culture supernatants by lung leukocytes from Balb/c mice infected for 60 days and following in vitro re-stimulation with live M. tuberculosis bacilli for 72h. C- Proliferation of M. tuberculosis WT or capless (CapA) mutant in C57BL/6 mice following an aerogenic infection. Mice were sacrificed at days 1 and 120 post-infection and the

bacterial loads in the lung and spleen determined. Results are expressed as "log CFU increase", that corresponds to the difference of growth, in log CFU, between day 1 and day 120. A total of 5 to 7 mice per time point were used.

Discussion:

The cell envelope of *M. tuberculosis* plays an important role in the pathogenesis of tuberculosis. For years, the mannose-capped lipoarabinomannan (ManLAM) has been considered a key factor in host cell recognition and immunomodulation. The present work questions the importance of mannose capping of LAM as a requirement for virulence by showing that the *in vivo* growth in mice of mycobacteria (*M. bovis* BCG and *M. tuberculosis*) that are deficient in the biosynthesis of the mannose cap is not affected, and that a BCG mutant lacking mannose caps is as effective as a vaccine as its capped parent strain in low-dose (<100 CFU) aerosol models in mice and guinea pigs. These data are surprising since mannose capping of LAM is found in slow growing mycobacteria, amongst which are many pathogenic species, but is (almost completely) lacking in fast growing, environmental non-pathogenic species.

It has been shown *in vitro* that, at the level of the macrophage, purified ManLAM interferes with phagosome maturation (Fratti *et al*., 2003; Vergne *et al*., 2003) and IFN-γ dependent activation (Chan *et al*., 1991; Sibley e*t al*., 1988). ManLAM inhibits IL-12 secretion by LPS-stimulated human dendritic cells (Nigou *et al*., 2001). In addition, ManLAM also prevents DC activation as measured by CD80, CD83 and CD86 expression and triggers secretion of IL-10 by LPS primed human DCs through DC-SIGN binding (Geijtenbeek *et al*., 2003). Together these data demonstrate an immunosuppressive activity of this lipoglycan and suggest that a capless *Mycobacterium* would be attenuated and would replicate less well in macrophages. Importantly, these studies were all done with purified ManLAM, not live bacteria.

Our *in vitro* findings comparing BCG with isogenic capless BCG showed that mannose capping of LAM is not crucial for mycobacteria survival in macrophages (Fig. 1A,B), with the capless BCG mutant growing exactly as the wild type strain both in non-activated and in activated bone marrow-derived primary murine macrophages (Fig. 1B) Our data are in agreement with two other studies where capless BCG replicated as well as its parent in either human THP-1 macrophages (Appelmelk *et al*., 2008) or in the murine macrophage cell line Mf4/4 (Festjens *et al*., 2011). Hence, the prediction that the mannose cap of LAM would influence mycobacterial survival does not come true in mice. With regard to cytokine induction *in vitro*, no difference was seen for IL-12p70 (Fig. 2B); for TNF, a difference was seen after 7 days only (Fig. 1C and 2B); in contrast to data obtained with purified ManLAM and non-capped LAM (Geijtenbeek *et al*., 2003), BCG with capless LAM induced more IL-10 than parent BCG (Fig. 2B). Also *in vivo*, after intravenous injection in C57/Bl6 mice (Fig. 3), capless BCG was not attenuated as compared to parent strains, confirming earlier data following intranasal challenge in C57/Bl6 mice (Appelmelk *et al*.,

2008) or intravenous injection in Balb/c mice (Festjens *et al*., 2011). Altogether these data show that studies with live capless LAM mutant bacteria yield data conflicting with those obtained with purified LAM. This could be due to several reasons. First, to show enhanced IL-10 (Geijtenbeek *et al.,* 2003) or decreased IL-12 (Nigou *et al*., 2001; Pathak *et al.,* 2005) production by purified ManLAM as compared to LAM without mannose caps, DC and macrophages were primed by the TLR4 ligand LPS; however, live mycobacteria are poor triggers of TLR4 signaling, (Reiling *et al*., 2008) and hence, not surprisingly, live mycobacteria do not recapitulate the behavior of purified ManLAM. Second, we assume there is redundancy for the role of ManLAM. For example, binding of BCG to DC is dominated by DC-SIGN ligand interactions (Appelmelk *et al*., 2008; Geijtenbeek *et al*., 2003, (Geurtsen *et al*., 2009) and when the cap, i.e. one of the ligands, is removed, binding to DC stays fully intact as a sufficient number of back-up ligands remains available e.g., PIMs (Driessen *et al*., 2009), lipomannan and glycoproteins (Pitarque *et al*., 2005). We conclude that ligands other than the mannose cap determine binding to DC, and assume that a similar redundancy exists for other effects of ManLAM, such as the inhibition of phagolysosome fusion.

A major goal of our studies was to evaluate the potency of capless BCG as a vaccine in a low dose *M. tuberculosis* aerosol challenge model. Based on the ability of ManLAM to induce immunosuppressive IL-10 (Geijtenbeek *et al*., 2003) one could expect that a capless BCG might be a more effective vaccine than parent BCG. Using a high dose murine *M. tuberculosis* challenge models ($5x10^4$ CFU intravenously or $2x10^5$ CFU intratracheally), Festjens *et al.* (2011) showed that prior immunization with a capless BCG (10^5 CFU subcutaneously) indeed appeared to be more protective than parent BCG: mean survival time increased from 26.5 to 27.5 wk (intravenous challenge) and from 48 to 52 wk (intratracheal challenge); also after capless BCG immunization and i.v *M. tuberculosis* challenge, the weight loss was delayed as compared to immunization with parent BCG. However, the differences in protection between capless BCG and parent strain were small and not statistically significant (N. Festjens, pers. communication). We reasoned, based on early experience, that differences in immune protection could become more evident in a low dose challenge model (Appelmelk *et al.*, 1986). The low dose aerosol *M. tuberculosis* infection model is currently considered to be the golden standard to evaluate protective efficacy of tuberculosis vaccines. In the aerogenic model, infectious doses are as low as 10-25 CFU. The outcome of our studies (Fig. 4A) is that BCG and capless BCG hardly disseminate to the spleen or lung after intradermal injection, that (Figs. 4B and C) immune parameters following immunization were similar

for both vaccines, and most importantly, the capless BCG had a protective efficacy identical to its parent strain (Fig. 4D).

A second goal was to evaluate capless BCG in a particularly susceptible host, the guinea pig, an animal species which is also able to present the glycolipid LAM to T cells. Specialized CD1b lipid antigen presenting molecules are present in humans, but are lacking in mice. ManLAM has long been known to be presented by CD1b to antigen-specific T cells (Sieling *et al*., 1995), and hence in mice protection mediated by LAM-specific CD1b-restricted T cells will not be evident. Guinea pigs in contrast express CD1b. In addition, unlike in mice, experimental tuberculosis in the guinea pigs causes caseating granulomas. Hence, as compared to mice the guinea pig model is seen as more representative of human disease and is an essential step in human tuberculosis vaccine development. To mimic natural disease, the aerogenic challenge route was again chosen. Fig. 5 shows that no differences in protection to *M. tuberculosis* infection were seen in this model following vaccination with the capless BCG or the parent strain.

A final goal was to test the role in virulence of the mannose cap of *M. tuberculosis*, a species not tested hitherto. In vitro, we found no differences between wild type and capless *M. tuberculosis* with regards to replication rate in mouse macrophages, susceptibility to cytokine activated macrophages, and induction of TNF and nitrite secretion. To maximize the possibility to observe potential LAM-mediated immunomodulatory effects, we challenged via the aerogenic route two immunologically contrasting mouse strains, i.e., Balb/c (a Th2-skewed strain) and C57Bl/6 (a Th1-skewed strain) (see Fig. 7A and 7B, respectively). However, in both mouse strains capless *M. tuberculosis* and parent strain proliferated equally well.

Altogether our data suggest that the dominant role attributed to the mannose cap of LAM, which was predominantly based on *in vitro* studies with purified ManLAM (Mishra *et al.*, 2011) cannot be confirmed by in vivo studies in mice with isogenic pairs of mutants. The cap does not affect immunoprotection by BCG, nor does it affect virulence of pathogenic mycobacteria in mice. One explanation for this lack of effect is that the role of the cap might be redundant as discussed above. Still, the outcome of our studies is puzzling: we investigated, within the taxonomic tree of the genus *Mycobacterium*, which species expressed a mannose-capped LAM and we found it almost exclusively in slow growing species, many of which are pathogens; in fast growing, often environmental species, the cap was mostly lacking (Driessen and Appelmelk, unpublished). This suggests evolutionary pressure to preserve the cap. It cannot be excluded that the cap albeit not relevant in the biological assay systems tested so far with live mycobacteria, may still be important for example for transmission of *M. tuberculosis* from one person to another. As

the DC-SIGN system in humans differs strongly from that of mice (Park *et al.*, 2001), this aspect of tuberculosis is not accessible to animal experimentation; possibly, non-human primates mimic the human DC-SIGN system more accurately.

In short, together with two earlier studies, our novel data provide overwhelming evidence that the mannose cap of lipoarabinomannan does not dominate the interaction of mycobacteria with the three experimental animal hosts (zebrafish, mice and guinea pigs) tested.

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The mycobacterial type VII secretion system ESX-5 plays a crucial role in the ability of *Mycobacterium bovis* BCG to survive in mice.

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Abstract

Pathogenic mycobacteria secrete virulence proteins that can facilitate colonization and persistence in the host. The transport of these proteins across the mycobacteria cell wall requires the presence of specialized machinery. A set of homologous secretion systems, named ESX-1 to ESX-5 and belonging to type VII secretion systems (T7SS) has been identified in mycobacteria. ESX-1 is required for secretion of the ESAT-6 and CFP-10, two proteins very important in *M. tuberculosis* and *M. marinum* pathogenicity, and ESX-5 is involved in the transport of proteins PE and PPE, with a possible role in virulence. The presence of two ESX systems dedicated to virulence in the pathogenic strains suggests a distinct role of each one during mycobacteria infection but also a strong dependency of both secretory systems for full virulence. Further more, BCG attenuation due to ESX-1 loss indicates a correlation between the level of virulence and the number of ESX present. Using the natural ESX-1 mutant BCG, we show that the presence of ESX-5 is crucial to BCG maintain its virulence, with depletion of ESX-5 leading to a strong attenuation of BCG even in immunocompromised mice. These results support the strong dependency from each ESX systems for full mycobacteria virulence acquisition.

Introduction

Pathogenic mycobacteria rely on a multitude of factors in order to survive and multiply in their hosts. These range from structural components of their complex cell walls, to pathways of metabolic adaptation e.g. to nutrient limitation or hypoxia, to true virulence factors, i.e. molecules whose only function appears to be the modification of host cells that allow an increase in the ability of the microorganism to replicate inside its host. Recent years have seen the identification of such secreted virulence factors and their secretion apparatuses.

Due to the complex architecture of the mycobacterial cell wall (Brennan *et al.*, 1995), the transport of proteins across the cell envelope requires the presence of a specialized secretion machinery. The majority of proteins bear a typical signal sequence and are exported by two highly conserved protein export systems, the general secretion (Sec) and the Twin-arginine translocase (Tat) pathways (Bronstein *et al.*, 2004). However, the finding of small highly immunogenic proteins that lack specific signal sequences in the culture filtrates of *Mycobacterium tuberculosis* (Sorensen *et al.*, 1995) led to the assumption that other types of secretion systems would be involved in the transport of these molecules. A set of homologous secretion systems, encoded by distinct gene clusters named ESX-1 to ESX-5 and belonging to the type VII secretion systems (T7SS) family, has subsequently been identified in mycobacteria (Gey Van Pittius *et al.*, 2001, Abdallah *et al.*, 2007).

ESX-1 was the first one of such systems to be identified and is the best characterized so far. It is required for the secretion of the 6 kDa early secreted antigenic target (ESAT-6) (Sorensen *et al.*, 1995) and the 10 kDa culture filtrate protein (CFP-10) (Berthet *et al.*, 1998), two proteins that are responsible for inducing a strong T cell mediated immune response and very important in *M. tuberculosis* and *M. marinum* virulence. In macrophage infection, ESX-1 effectors are involved in blocking phagosome maturation (MacGurn *et al.*, 2007, Xu *et al.*, 2007), limiting host cytokine production (Stanley *et al.*, 2003) and promoting apoptosis of the infected cells (Derrick *et al.*, 2007). ESX-1-secreted ESAT-6 seems to be involved in pore formation in host cell membranes (Smith *et al.*, 2008), facilitating pathogen escape from the phagosome (van der Wel *et al.*, 2007) and

eventually promoting dissemination due to host cells lysis (Hsu *et al.*, 2003, Guinn *et al.*, 2004)

The first hint that the ESX-1 system contributes to mycobacterial virulence came from genomic studies which defined a region of difference 1 (RD1), present in all fully virulent species/strains of the *M. tuberculosis* complex (Brosch *et al.*, 2002), that was spontaneously deleted from the genome of the attenuated *Mycobacterium bovis* BCG vaccine (Behr *et al.*, 1999, Gordon *et al.*, 1999, Mahairas *et al.*, 1996) and was absent in the low virulence *Mycobacterium microti* (Brodin *et al.*, 2002). The RD1 locus was later found to encode for the proteins that constitute the secretion system ESX-1 (Guinn *et al.*, 2004, Hsu *et al.*, 2003, Pym *et al.*, 2003). The contribution of ESX-1 to virulence became clear when complementation of BCG and *M. microti* with the entire RD1 locus resulted in increased virulence in mice (Brodin *et al.*, 2004a, Pym *et al.*, 2002, Majlessi *et al.*, 2005) and when deletion of RD1/ESX-1 in *M.tuberculosis* caused its attenuation (Lewis *et al.*, 2003, Hsu *et al.*, 2003, Guinn *et al.*, 2004).

Recently, a second ESX system has been pinpointed as important for virulence in mycobacteria. The expression of ESX-5 is confined to the slow-growing mycobacteria, including most pathogenic species (Gey van Pittius *et al.*, 2006, Abdallah *et al.*, 2006), and is involved in the transport of different members of the proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) protein families (Abdallah *et al.*, 2006, Abdallah *et al.*, 2009, Daleke *et al.*, 2011). The PE and PPE genes are highly expanded in the pathogenic mycobacterial species, with about 10% of the total coding potential of the genome of *M. tuberculosis* dedicated to them (Cole *et al.*, 1998). PPE proteins seem to be located at the cell surface (Sampson *et al.*, 2001) and although the exact function of these proteins is unclear, a role in virulence (Li *et al.*, 2005, Ramakrishnan *et al.*, 2000), antigenic variation or immune evasion (Cole *et al.*, 1998, Banu *et al.*, 2002, Delogu *et al.*, 2001, Akhter *et al.*, 2012) has been predicted. Recent studies show that disruption of ESX-5 in *M. marinum* affects its ability to interfere with cytokine production by macrophages (Abdallah *et al.*, 2008), lead to hypervirulence in adult zebrafish or attenuation in zebrafish embryos (Weerdenburg *et al.*, 2012). ESX-5 plays an essential role in transport of PPE proteins, cell wall integrity and full virulence of *M. tuberculosis* (Iantomasi *et al.*, 2012, Bottai *et al.*, 2012).

From the five ESX systems present in mycobacteria, ESX-1 and ESX-5 are the only two that seem to be involved in virulence. Each one of these ESX systems fails to complement the loss of virulence caused by deletion of the other, suggesting that they have independent contributions during mycobacterial infections (Abdallah *et al.*, 2011) but also that both ESX systems must be present for full mycobacterial virulence. However, no data exist on mutant mycobacteria lacking both secretion systems.

Although large genomic losses occurring during the prolonged culture of the BCG vaccine strain were responsible for the attenuation of the virulence of the parent *M. bovis* strain, it is accepted, based on complementation experiments, that the loss of ESX-1 played a major role in such attenuation (Pym *et al.*, 2002). Despite having lost the virulence of *M. bovis*, BCG still conserves a certain degree of virulence, as shown by its ability to induce pathology and death in immunocompromised mice (Garcia *et al.*, 2000, Ladel *et al.*, 1995). The presence of an ESX-5 secretion system in BCG may contribute to this remaining virulence if we accept that ESX-1 and ESX-5 act in additive or synergistic ways. If this is the case, deletion of the ESX-5 should further reduce BCG's ability to survive in a host.

In this report we show that disruption of ESX-5 from the ESX-1-lacking BCG results in further attenuation of this strain, even in immunocompromised mice, highlighting the unique role of each secretion system for full virulence of *M. bovis*.

Experimental procedures:

Mycobacterial strains:

For the ESX-5 mutant, deletion in the *M. bovis* BCG homologue of Rv1783 was made using the procedure developed by Parish and Stoker (Parish *et al.*, 2000), which utilize the flexible p1NIL and the GOAL19 cassette. *M. bovis* BCG Copenhagen and the ESX-5 mutant were grown in liquid Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80, until the log phase. Cultures were aliquoted and frozen at –80°C until the day of use.

Laboratory animals:

6 to 8 week-old female BALB/c and C57/B6 mice and were purchased from Charles River (Spain). B6.IFNg$^{-/-}$ and B6.p47phox$^{-/-}$ mice were purchased from the Jackson Laboratories (Bar Harbor, ME), B6.Rag2$^{-/-}$ mice were purchased from CDTA (Orleans, France), and B6.iNOS2-deficient mice were kindly provided by Drs. J. D. MacMicking and C. Nathan (Cornell University, New York, NY) and J. Mudgett (Merck Research Laboratories, Rahway, NJ). All animals were housed under specific pathogen-free conditions in our facilities and sterile chow and tap water *ad libitum* was given. All experiments were approved by and performed according to the guidelines of the animal ethical committee of IBMC. Five to seven animals were used per experimental group for each time-point.

Generation of bone marrow-derived macrophages (BMMØ):

Bone marrow cells were flushed from the femurs of mice with 5 ml of cold Hanks' balanced salt solution (HBSS; Gibco, Paisley, United Kingdom) using a 26-gauge needle. For macrophage generation, the resulting cell suspension was centrifuged for 10min at 1200rpm at 4ºC, resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 mM HEPES (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Gibco), 10 mM glutamine (Gibco), 10% heat-inactivated fetal bovine serum (Sigma), 10% L929 cell conditioned medium (as a source of M-CSF) and cultured for a period of 4 h on cell culture dishes (Nunc, Naperville, IL) in order to remove already differentiated cells. Non adherent cells were then collected with warm HBSS, counted, distributed in 24-well plates at a density of 5x10^5 cells/well and incubated in 1 ml of similar media at 37°C in a 5% CO2 atmosphere. On day 4 after seeding, 100μL of L929 cell conditioned medium was added and on day 7 the medium was renewed. Macrophages were infected at day

10. For dendritic cell differentiation, the cell suspension was cultured at a density of 10^6 cells/ml in RPMI 1640 containing GlutaMAX-I, supplemented with 5% v/v fetal calf serum (FCS), 50 mM β-mercaptoethanol and 10% GM-CSF-containing culture supernatant from transformed J558 cells. Every 2 days, one-half of the media was removed and supplemented with complete medium with GM-CSF. On day 9, the non-adherent cells were cultured at 2 x 10^5 cells/ml in 96-well plate (100 µl/well), and infected at day 10. The purity of the population was determined by FACS analysis of specific surface markers and ranged from 85 to 95%.

Macrophage infection:

A bacterial suspension containing 5x10^6 CFU/mL was prepared and 200 µl was added to each well to obtain a multiplicity of infection (MOI) of 2 bacteria per macrophage. After 4 h of incubation at 37°C in a 5% CO_2 atmosphere, cells were washed with warm HBSS to remove the non-internalized bacteria, and re-incubated in DMEM with 10% LCCM. All treatments (100U IFNγ; 50U TNFα) were applied from day zero until day 4. At different time points 100µl of supernatant was collected for subsequent cytokine measurements. For the CFUs assay, the infected cells were lysed with 10% saponin solution, in sterilized water with 0,05% Tween 80, and serial dilutions of triplicate wells were performed. The number of viable bacteria was assessed by counting the colonies 3–4 weeks after plating on 7H11 Agar medium (Difco) supplemented with 10% oleic acid/ albumin/ dextrose / catalase (OADC) and incubated at 37°C.

Cytokine measurement:

Cytokine detection in the supernatants was performed by ELISA. For IFN-γ quantification, affinity-purified monoclonal antibodies (R4-6A2 as capture and biotinylated AN-18 as detecting antibody) were used, while commercial kits were used according to the manufacturer's instructions for the detection of TNFα and IL-10 (R&D Systems). The amount of nitrite released was quantified by the Griess Reagent System kit from Promega.

Replication *of M. bovis* BCG in mice:

Mice were infected intravenously through the lateral tail vein, with 5x10^4 CFU *M. bovis* BCG Wt or mutant, in 200 µL of PBS. Bacterial loads in the organs of infected mice were evaluated at different time points post infection. Organs were homogenized in sterile water

with 0.05% Tween 80 and ten-fold serial dilutions of organ homogenates were plated in duplicate onto Middlebrook 7H11 agar plates containing OADC. Plates were incubated at 37°C and colonies were counted 21 days later. Results are expressed as log CFU per organ.

BCG antigens for cell stimulation:

Mycobacterial antigens were prepared as described elsewhere (Pais *et al.*, 2000). BCG was grown until log phase at 37°C in Middlebrook 7H9 medium (Difco) supplemented with 10% albumin/dextrose/catalase (ADC) and 0,05% Tween 80. The culture was centrifuged (10,000 x g, 40 minutes, 4°C) and the pellet was washed and re-suspended with phosphate-buffered saline (PBS), containing 0.1% Tween 80 (Sigma), 1 mM $MgCl_2$ (Merck, Darmstadt, Germany) and 1 mM benzamidine (Sigma). The bacteria in suspension were 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 x g), and the supernatant was dialyzed against PBS (MWCO of 12,000), followed by ultra-centrifugation for 2 h at 150,000 x g. The pellet, containing the envelope proteins, was re-suspended in PBS, and the supernatant, enriched in cytosolic proteins, was precipitated with 80% ammonium sulfate and dialyzed against PBS. Aliquots where quantified and stored at -80°C until the day of use.

Cell preparation and *in vitro* stimulation:

Spleens were gently disrupted with the help of a cell glass homogenizer. The resulting cell suspension was passed through a 70 μm nylon cell strainer in order to remove large pieces and debris. The red blood cells in the cell suspensions were lysed with a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$ (pH 7.2)) during 5 min at room temperature. Cells were then distributed into 96-well plates (2.5 x 10^5 cells/well) and incubated in triplicate with DMEM or with 4 µg/ml Con A (Sigma-Aldrich) or 4µg/ml BCG extract. After 72h of incubation at 37°C in a 5% CO_2 atmosphere, the supernatants were collected for cytokine measurement.

Flow cytometry:

Cells were labeled with specific antibodies for CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD11b (clone M1/70), CD19 (clone 6D5), DX5 (clone HMb2), CD25

(clone PC61) from BioLegend and FOXP3 (e-bioscience). Cell populations were acquired in a FACS Calibur instrument equipped with CellQuest software. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis:

Data were analysed by using Student's *t* test.

Results:

The ESX-5 mutant BCG is attenuated in Balb/c mice:

In order to assess the importance of the ESX-5 secretory system in BCG virulence, Balb/c mice were intravenously infected with $5x10^4$ CFU of either the parental strain or the ESX-5 mutant, and the number of bacteria in the spleen and liver was determined during 60 days. The ESX-5 mutant showed an attenuated phenotype when compared to the parental strain (**Fig. 1A**). Whereas the wild type BCG was able to survive and multiply in the spleen until day 20 post-infection and maintain the bacterial load until day 60, the mutant strain was readily eliminated and it was already possible to observe a statistically significant difference in bacterial loads in the spleen between the mutant and wild type BCG at day 10 post-infection. Both strains were eliminated from the liver from day 10 onwards but, again, elimination of the mutant strain was faster than that of wild type BCG.

To assess the immune response during infection, spleen cells from infected mice were stimulated in vitro with BCG antigens and cytokine production was analyzed. Cells from mice infected with wild type BCG or ESX-5 mutant released the same amounts of TNF, but cells from ESX-5 mutant-infected animals released less IFNγ than the cells from animals infected with wild type BCG (**Fig. 1B**). When the populations of immune spleen cells were analyzed, small differences in some populations were observed at some time points, with a lower cell number of $CD19^+$ (B cells) and $CD11b^+$ (phagocytes) in mice infected with ESX-5 mutant BCG **(table1).**

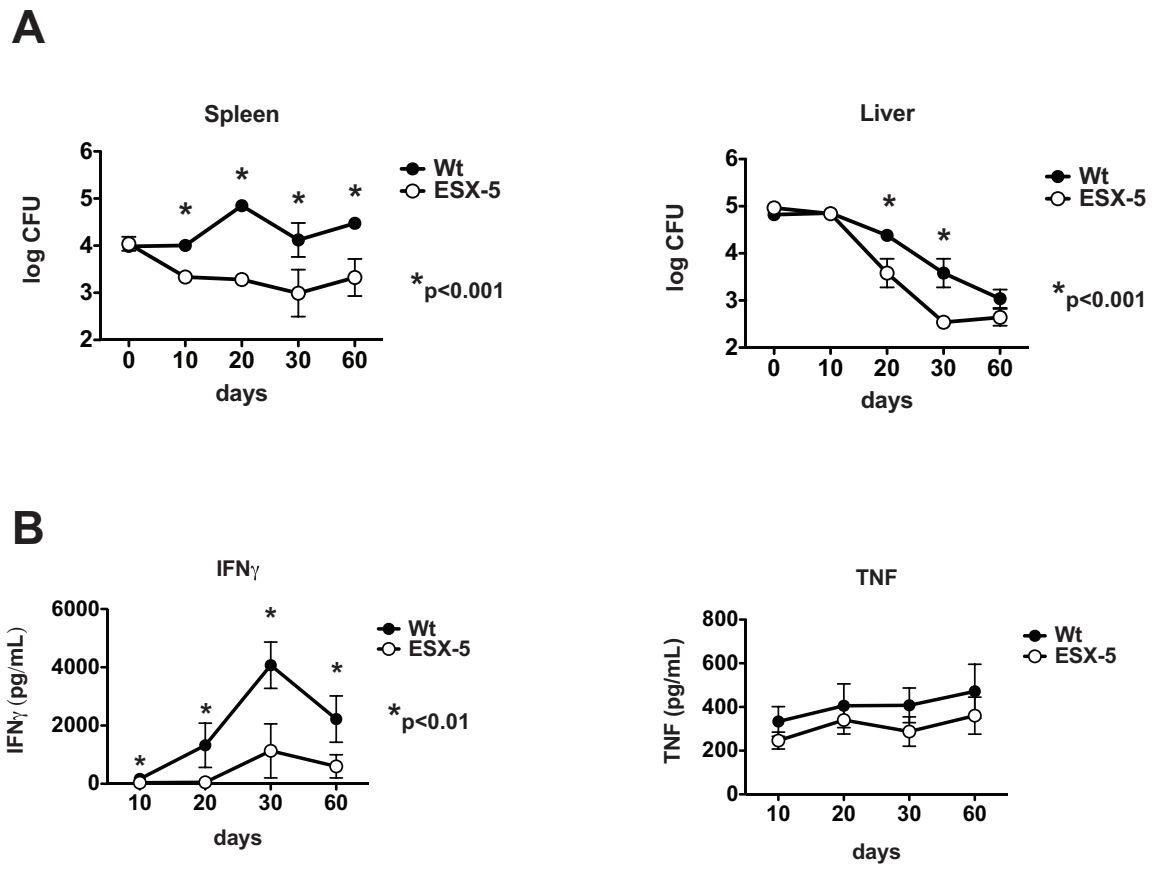

Figure 1: ESX-5 deletion leads to BCG attenuation in mice.

A- BALB/c mice were intravenously infected with $5x10^4$ CFUs of *M. bovis* BCG WT or ESX-5 mutant. The animals were sacrificed at different time points and the spleen and liver removed for plating onto 7H11 agar and CFU counting. The results are expressed as log CFU. **B-** Splenocytes from the intravenously infected animals were stimulated *in vitro* with 4µg/ml of *M. bovis* BCG antigens for 72h and the levels of IFNγ and TNF in the supernatant quantified by ELISA. In all experiments, a total of 5 to 7 mice per time point were used and data represent the mean ± SD of a representative experiment out of a total of 3 experiments. Statistically significant differences are labeled with an asterisk.

Average cells x 10^7 ± SD														
	CD4+		CD8+		CD4+Foxp3+		CD19+		CD3+DX5+		DX5+		CD11b+	
Days	Wt	ESX-5	Wt	ESX-5	Wt	ESX-5	Wt	ESX-5	Wt	ESX-5	Wt	ESX-5	Wt	ESX-5
10	1.2±0.2	1.3±0.2	0.6±0.1	0.6±0.1	0.2±0.02	0.2±0.04	2.4±0.6	2.6±0.6	0.1±0.01	0.1±0.01	0.3±0.02	0.3±0.05	0.3±0.07	0.3±0.02
20	1.5±0.3	1.1±0.2	0.6±0.2	0.4±0.1	0.3±0.03	0.2±0.04	3.6±0.7	2.3±0.1	0.1±0.02	0.1±0.02	0.3±0.08	0.3±0.06	0.6±0.16	0.4±0.07
30	1.8±0.4	1.4±0.3	0.6±0.1	0.6±0.1	0.3±0.06	0.2±0.05	*3.9±0.6	3.0±0.1	0.1±0.03	0.1±0.02	0.4±0.04	0.3±0.07	*0.7±0.1	0.5±0.04
60	1.6±0.2	1.3±0.3	0.6±0.1	0.5±0.1	0.2±0.03	0.2±0.03	*4.1±0.6	2.4±0.6	0.2±0.04	0.1±0.02	0.3±0.07	0.2±0.03	*0.8±0.0	0.5±0.06

Table 1: *M. bovis* BCG ESX-5 immune responses as compared to the Wt strain.
BALB/c mice were intravenously infected with 5x10^4 CFUs of *M. bovis* BCG WT or ESX-5 mutant. The animals were sacrificed at different time points and spleen cells were labeled with specific antibodies for flow cytometric analysis of the different splenic cell populations. A total of 5 to 7 mice per time point were used, and all results are representative of at least two independent experiment. Statistically significant differences are labeled with an asterisk.

The ESX-5 mutant is attenuated in immunocompromised mice:
From the first in vivo experiment it was clear that the virulence of the ESX-5 mutant BCG is compromised in immunocompetent mice. We decided to further test the impact of the inactivation of ESX-5 on BCG virulence by infecting mice that lack important cells or molecules for mycobacterial control. C57BL/6 control mice and B6.Rag$^{-/-}$, B6.iNOS$^{-/-}$, B6.IFNg$^{-/-}$ and B6.p47phox$^{-/-}$ mice were infected intravenously with 5x10^4 CFU of either the parental strain or the ESX-5 mutant, and the number of bacteria in the spleen and liver determined at days 0, 20 and 50 of infection (**Fig.2**). Wild type BCG proliferated in the spleen of control mice until day 20 after which the infection became controlled. This control was not observed in lymphocyte-deficient B6.Rag$^{-/-}$ mice or in B6.iNOS$^{-/-}$ animals which showed around 100-fold more bacteria at day 50 than control B6 mice or in B6.IFNg$^{-/-}$ animals which had around 1000-fold more bacteria at day 50 as compared to control mice. A transient exacerbation of the infection in the spleen was observed at day 20 in B6.p47phox$^{-/-}$ mice which was no longer evident at day 50. In the liver, control of BCG growth was observed in all mouse strains until day 20 but B6.Rag$^{-/-}$ and B6.IFNg$^{-/-}$ mice were unable to kill the mycobacteria past that time point exhibiting increases in bacterial loads of 2.0 and 2.5 log, respectively, when compared to the control mice. In contrast, the ESX-5 mutant was readily eliminated from the spleen of all mouse strains except B6.IFNg$^{-/-}$ mice and was eliminated from the livers of all mouse strains.

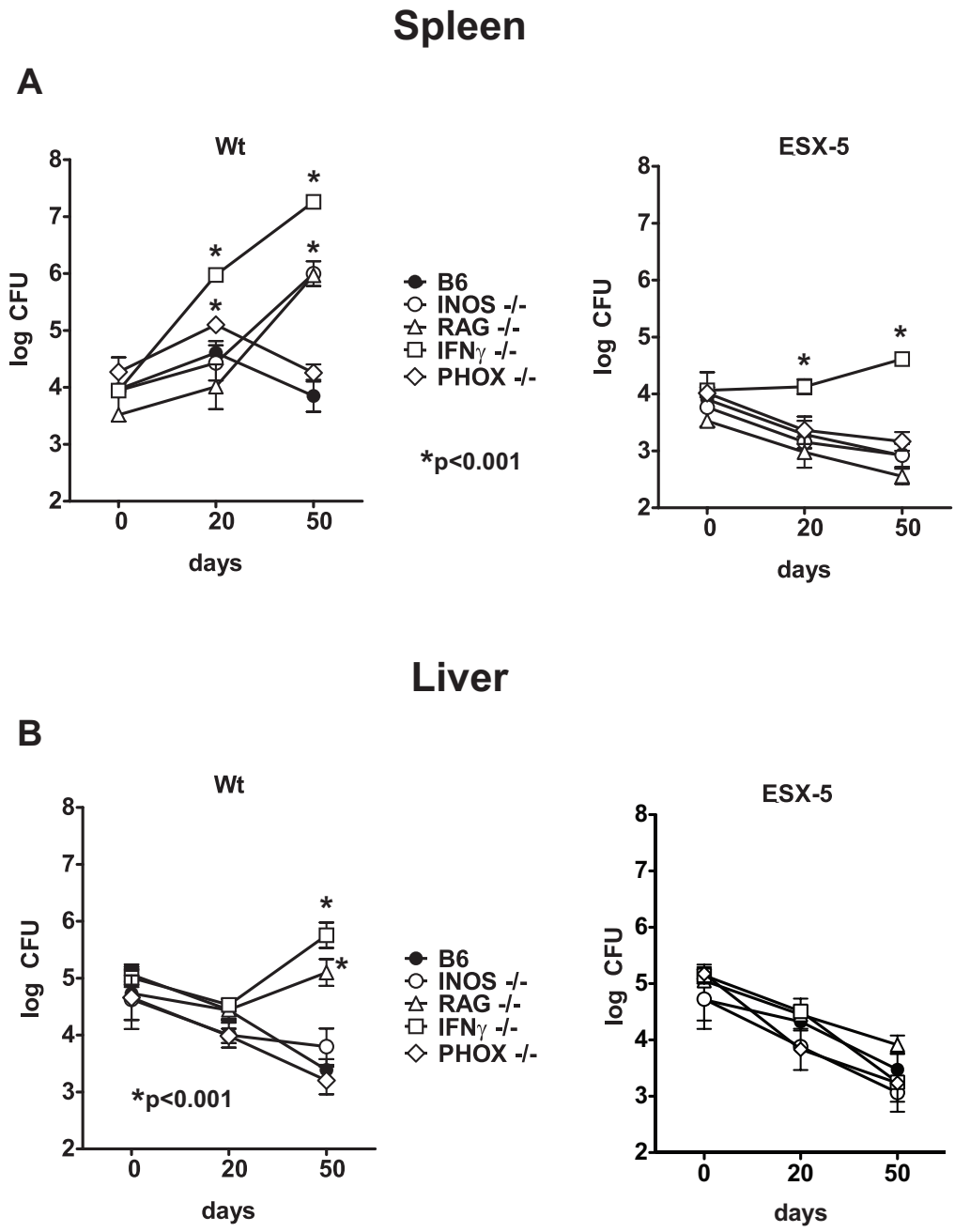

Figure 2: ESX-5 BCG mutant is attenuated in immune-compromised mice.

Female C57BL/6J, B6.IFNg$^{-/-}$, B6.Rag2$^{-/-}$, B6.INOS2$^{-/-}$ and B6.p47phox$^{-/-}$ mice were intravenously infected with 5x10^4 CFUs of *M. bovis* BCG WT or ESX-5 mutant BCG. At different time points mice were sacrificed and spleen (**A**) and liver (**B**) homogenates plated onto 7H11 medium. After 21 days at 37ºC, the number of CFUs was determined. A total of 5 mice per time point were used and data represent the mean ± SD of a

representative experiment out of a total of 2 experiment. Statistically significant differences are labeled with an asterisk.

Growth of the ESX-5 mutant in macrophages:

Macrophages are the first cells to mediate host resistance against mycobacterial infection. In this way, the early control of ESX-5 mutant growth observed in vivo might result from a diminished capability of this strain to survive and proliferate within the phagocytic cells. To investigate the importance of the ESX-5 secretion systems in BCG survival, bone marrow-derived macrophages (BMDM) were infected with the parental or the ESX5 mutant BCG strains and the mycobacterial growth and macrophage activation was measured. In 3 out of 5 experiments, it was observed that mutant ESX-5 BCG grew less than the parental strain in C57/BL6 derived macrophages. Activation of the macrophages with IFNγ and TNF led to killing of both strains (**Fig.3A**). Again, the mutant was more susceptible to killing by activated macrophages than the wild type strain. Both strains induced the production of the same amount of TNF and nitrite (**Fig.3B,C**).

Two of the most important antimicrobial systems of phagocytic cells are the NADPH phagocyte oxidase (also known as phox) and the inducible nitric oxide synthase (iNOS) pathways, which are responsible for the generation of reactive oxygen (ROS) and reactive nitrogen species (RNS), respectively. In an attempt to further elucidate the mechanism involved in ESX-5 mutant growth control, BMDM from p47phox$^{-/-}$ and iNOS$^{-/-}$ mice were differentiated and infected with either the wild type or the ESX-5 mutant BCG. In the p47phox$^{-/-}$ macrophages, the behavior of the two strains of BCG was identical to the one observed in control macrophages, with 3 out of 5 experiments showing a lower growth of the ESX-5 mutant in both non-activated and activated macrophages. In the iNOS$^{-/-}$ macrophages, the ESX-5 mutant also grew less than the Wt BCG but activation of macrophages with IFNγ and TNF failed to restrict the growth of either BCG, providing in vitro evidence of the importance of RNS in BCG control during the adaptive phase of immunity.

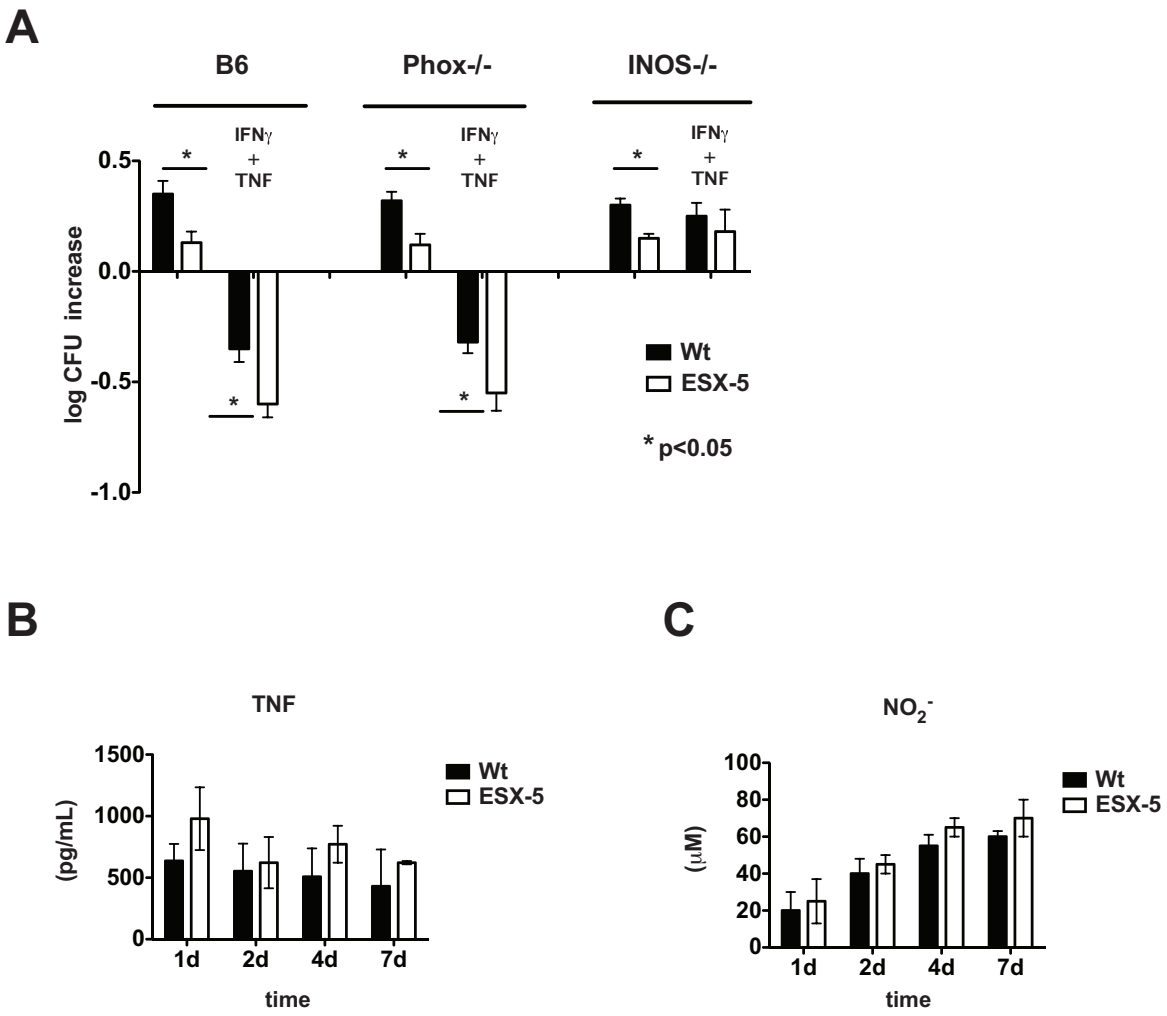

Figure 3: Role of macrophages in ESX-5 mutant BCG growth control

A- Bone marrow derived macrophages (BMDM) from C57BL/6J, B6.p47phox$^{-/-}$ and B6.IFNg$^{-/-}$ mice were infected with wild-type (Wt) and ESX-5 mutant BCG (ESX-5) to a multiplicity of infection of 2 bacteria per macrophage (MOI 2:1) and left untreated or treated with both 50U TNF and 100U IFNγ. The number of viable bacteria was assessed and the results expressed as "log CFU increase", that corresponds to the difference of growth, in log CFU, between day 7 and day 0. **B**- Supernatant from BMDM C57BL/6J infected with the BCG (MOI 2:1) was collected at different time points and the production of TNF quantified by ELISA. **C-** Culture supernatants from BMDM C57BL/6J infected with BCG and treated with TNF plus IFNγ were collected and the amount of nitrite released measured by the Griess test. Data represent the mean ± SD of a representative experiment out of a total of 6 experiment. Statistically significant differences are labeled with an asterisk.

Discussion:

The involvement of ESX-1 in mycobacterial virulence has been extensively studied (MacGurn *et al.*, 2007, Pym *et al.*, 2002, Majlessi *et al.*, 2005, Hsu *et al.*, 2003, Lewis *et al.*, 2003) but the interest in the ESX-5 system is recent. ESX-5 was shown to be responsible for the secretion of proteins with a possible role in mycobacterial pathogenesis and its disruption affects *M. marinum* and *M. tuberculosis* virulence (Weerdenburg *et al.*, 2012, Bottai *et al.*, 2012). The presence of both ESX-1 and ESX-5 in pathogenic mycobacteria seems to be crucial for conserving full virulence, as evident from the loss of ESX-1 in BCG and its contribution to diminished virulence (Lewis *et al.*, 2003, Pym *et al.*, 2002, Hsu *et al.*, 2003). With our results we further confirm this hypothesis by showing that deletion of a second ESX system in BCG, the ESX-5 system, further compromises the virulence of BCG, already lacking the ESX-1 system, as assessed in the mouse model.

Wild type BCG was able to proliferate in the spleen of mice during the first 20 days of infection leading to the induction of an adaptive T cell response characterized here by the ability of in vitro cultured spleen cells to secrete IFNγ in response to specific antigenic challenge. This T cell-mediated immune response was required for the elimination of BCG from the organs of the infected animals as shown by the progressive infection that developed in lymphocyte-deficient B6.Rag$^{-/-}$ mice and in mice lacking IFNγ. Killing of wild type BCG in the spleen as well as in *in vitro* cultured bone-marrow derived macrophages was dependent on the induction of iNOS. However, the elimination of wild type BCG in the liver did not require this NO-generating system. The respiratory burst dependent on the NADPH oxidase complex (NOX2) played a minor role in the control of wild-type BCG as only a small increase in splenic bacterial loads was observed early in infection of B6.p47phox$^{-/-}$ mice as compared to control animals. These results are in accordance with previous observations (North *et al.*, 2004).

In contrast, the ESX-5 mutant of BCG was readily eliminated from the spleen and the liver in a T cell-, NADPH oxidase-, and iNOS-independent way. The lack of replication of this strain in the tissues likely explains the lower induction of IFNγ responses in spleen cells during infection. However, IFNγ produced by cells other than T cells was required for the elimination of the mutant in the spleen. Killing of this strain in the liver was independent of all the molecules studied here.

Our data show that the two type VII secretion systems, ESX-1 and ESX-5, work in an additive way to provide mycobacteria with survival properties in their interaction with the host. Both systems appear necessary to resist the activity of T cells, IFNg and, to a lesser degree, nitric oxide-mediated killing. Mice that are deficient in these pathways are extremely susceptible to *M. tuberculosis* and even to BCG infection. The level of vulnerability correlates with the virulence of the mycobacterium.

It is widely accepted that the ESX-1 system is a major determinant of virulence in mycobacteria. Mice infected with *M. tuberculosis* develop pathology earlier and die faster than mice infected with BCG (Boom, 1996, Flynn *et al.*, 1993, Sambandamurthy *et al.*, 2005, Garcia *et al.*, 2000, Ladel *et al.*, 1995) and BCG complementation with ESX-1 resulted in an increased persistence in immune-competent mice and a more vigorous growth and extensive splenomegaly in immune-deficient mice. (Pym *et al.*, 2002, Hsu *et al.*, 2003)

The role of ESX-5 is still not clear as controversial data have been generated in *M. marinum* where disruption of this system may enhance virulence [38]. Here we show that deletion of Rv1783, essential for an functionally active ESX-5 in BCG, compromises the virulence of the mycobacterium to such an extent that the mutant becomes attenuated even in immune-compromised mice.

The observation that the control of the growth of the ESX-5 mutant occurs in an early phase of the infection and is independent of lymphocytes suggests an essential contribution of innate immune mechanisms. Our *in vitro* data suggest that macrophages might fulfill this function of early control of proliferation. Both the *in vitro* and the *in vivo* results show that neither the respiratory burst nor the iNOS system are required for the control of the ESX-5-lacking strain by macrophages although strong activation of macrophages with cytokines may induce extensive killing via NO-dependent mechanisms. In contrast, the NADPH oxidase is not required for killing by cytokine-activated macrophages *in vitro*. The possible involvement of this system *in vivo* was only seen for the wild type mycobacterium at the early stages of infection consistent with the transient increase in susceptibility to mycobacterial infection seen by others (Cooper *et al.*, 2000).

In conclusion, we show that the presence of ESX-5 system in BCG is crucial to maintain its virulence, highlighting the importance and dependency of each ESX system for the acquisition of full virulence.

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PART III

GENERAL DISCUSSION AND CONCLUDING REMARKS

GENERAL DISCUSSION

Redundancy of the mannose cap in mycobacterial virulence.

For years, the lipoarabinomannan (LAM) has been considered a key factor in host cell recognition and immunomodulation. Numerous studies suggested a role for the mannose cap of LAM (ManLAM) in the modulation of the function of macrophages and dendritic cells, and probably contributing to persistence of pathogenic mycobacteria. The work presented questions the importance of mannose capping as a requirement for LAM virulence. We show that the growth and virulence of mycobacteria (*M. bovis* BCG and *M. tuberculosis*) that are deficient in the biosynthesis of the mannose cap is not affected.

Both MR and DC-SIGN have a high-affinity for α(1→2)-linked Manp residues, the predominant type of mannose cap present in *M. tuberculosis* and *M. bovis* BCG (Pitarque *et al.*, 2005, LS Schlesinger, 1994) and binding of these receptors is associated with the ManLAM immunomodulatory effects in phagocytic cells. Interestingly, a *M. bovis* BCG mutant lacking the mannose cap of LAM, binds equally well to DC-SIGN and dendritic cells (Appelmelk *et al.*, 2008). This highlighted the hypothesis that other ligands, in addition to ManLAM, could be involved in the receptor binding and cell modulation. LM and the PIMs are other important mannose-containing biomolecules present on *M. tuberculosis*. Higher-order PIMs (PIM5 and PIM6), that also have terminal α(1→2)-linked Manp residues similar to the mannose cap of Man-LAM also exhibit a high affinity for MR and DC-SIGN binding (Driessen *et al.*, 2009). PIM6 participates in the phagocytosis events through the MR and limits phagosome-lysosome fusion events (Torrelles *et al.*, 2006). Interestingly, a double knockout mutant for PIM6 biosynthesis and mannose capping of LAM bound equally well to DC-SIGN and DCs and induced similar levels of IL-10 and IL12p40 as the parent strain (Driessen *et al.*, 2009). LM associates with DC-SIGN but not with the MR (Torrelles *et al.*, 2006) and induces apoptosis and a proinflammatory responses through TLR2 (Quesniaux *et al.*, 2004, Nigou *et al.*, 2008, Vignal *et al.*, 2003).

More recently, alpha-glucan, the dominant capsular polysaccharide was also shown to be capable of binding DC-SIGN and to induce IL-10 secretion in LPS-activated monocyte-derived dendritic cells (Geurtsen *et al.*, 2009). Therefore, recognition of the whole bacterium seems to result from the cooperative binding of several ligands, and the absence of one, in this case ManLAM, seems not to strongly affect the mycobacterial binding and host cell modulation.

In vivo studies by other groups have been carried out with BCG and *M. marinum*, in order to understand the importance of ManLAM to mycobacterial pathogenesis (Appelmelk *et al.*, 2008). However, BCG is an attenuated strain, which differs drastically in terms of virulence from the *M. tuberculosis* species and therefore, the mannose cap in *M. tuberculosis* might have a more essential role for its virulence than in BCG. However, this was not the case. We show that the lack of the mannose cap in *M. tuberculosis* did not affect its virulence, with the mutant strain surviving and replicating in mice and macrophages exactly like the parent strain and inducing the same type of cytokine production. These data are surprising, since mannose capping of LAM is found in slow growing mycobacteria, amongst which are many pathogenic species, but is (almost completely) lacking in fast growing, environmental non-pathogenic species, suggesting an evolutionary pressure to preserve the cap. Nonetheless, from an evolutionary perspective, it is also interesting to observe that *M. bovis* BCG and its ancestor *M. bovis* have ManLAM molecules exhibiting the same acyl-forms and in similar amounts, despite being very different in terms of virulence. Similarly, ManLAM from the avirulent strain of *M. tuberculosis*, H37Ra, shows the same acyl-forms and in similar amounts as ManLAM of the virulent strain H37Rv (Nigou, 2003).

Altogether, these facts do not support a major role for the mannose cap of LAM in determining mycobacterial virulence and immunogenicity, possibly due to redundancy of function. However, it cannot be excluded that the cap can have some importance in human infection, being necessary to study other models, such as non-human primates, which mimic more accurately human biology.

Redundancy of the mannose cap of LAM in a protective immune response.

Despite the several hypotheses proposed, the exact reason for BCG's variability in conferring immunity against pulmonary tuberculosis is still unknown. BCG vaccination promotes activation of MHC class II and I restricted CD4+ and CD8+ T cells respectively, that recognize mycobacteria-derived protein antigens and promotes a protective immune response dependent on interferon-γ (IFN-γ) and tumor necrosis factor (TNF) production. BCG survives and replicates in the phagosomes of macrophages and DCs by inhibiting phagosome acidification and phagosome-lysosome fusion (Sturgill-Koszycki *et al.*, 1994, Fratti *et al.*, 2001), a process that can to some extent hamper an optimal immunization response by limiting the easy access of protein antigens to the cytosol for MHC class I

presentation or to the lysosome MHC class II compartment (MIIC) for MHC class II presentation (Nasser Eddine *et al.*, 2005). As an evidence for this, the construction of a recombinant BCG strain that improves access of mycobacterial antigens to the MHC-I pathway results in a more efficacious protection against *M. tuberculosis* than the parental BCG strain (Grode *et al.*, 2005).

Also, BCG vaccination has been associated to an increase in infant survival that cannot be explained just by the prevention of tuberculosis (TB) deaths. The beneficial effects of BCG vaccine could be the result of either strengthening of pro-inflammatory mechanisms, helping neonates to fight infections, or the induction of an immune-regulatory network restricting overt inflammation and intense pathology. This last argument was based on the observation that monocyte-derived DCs that matured in the presence of BCG showed enhanced IL-10 and diminished IL-12 production, and primed naive T cells to develop into IL-10-producing T cells (Madura Larsen *et al.*, 2007). However, the development of an IL-10 anti-inflammatory response can counteract the protective IFNγ response needed for Tuberculosis protection. Therefore, in this context, ManLAM seemed to be an important player responsible for BCG failure in inducing long-lasting immunity, since it has been reported that ManLAM is able to block phagolysosome fusion and modulate dendritic cells, decreasing IL-12 and increasing IL-10 production (Fratti *et al.*, 2001, Fratti *et al.*, 2003, Geijtenbeek *et al.*, 2003b).

The hypothesis that a capless mycobacterium would be less immunosuppressive and thus more protective, was warranted. However, this was not observed. The capless mutant induced the same level of protection as the wild type strain against a *M. tuberculosis* challenge, evidencing that the mannose cap of LAM has a redundant role in the induction of a protective immune response to *M. tuberculosis*. Probably, this redundancy is also related to what was discuss before; the presence of other ligands in the mycobacterial cell wall with identical immunomodulatory properties to ManLAM. However, since BCG mutants for these molecules are also available, with some strains being mutant for more than one molecule, it would be interesting to test their efficacy in a *M. tuberculosis* challenge and understand how the level of protection correlates with absence of one or more immunomodulatory molecules of the cell wall.

Lipid-specific T cell responses are known to be an important component of the host defense against tuberculosis and recent studies show that BCG vaccination also induces responses to lipid antigens, which can be involved in protection against Tuberculosis

(Ulrichs *et al.*, 2003, Watanabe *et al.*, 2006). Contrary to mouse, guinea pigs express CD1b molecules necessary to present ManLAM to antigen-specific T cells (Sieling *et al.*, 1995, Moody *et al.*, 1997). Similar to what was observed in mice, capless BCG induce the same level of protection as the parent strain, against a *M. tuberculosis* challenge. Despite the possible role observed for ManLAM CD1b-restricted T-cells in tuberculosis protection (Porcelli *et al.*, 1999), the mycobacterial cell wall is a rich source of diverse lipid antigens for immune recognition. Several distinct lipid-specific, CD1-restricted T-cells have been found after *M. tuberculosis* infection, and although their exact function is unknown, a possible role in protection was also attributed (Beckman *et al.*, 1994, Moody *et al.*, 2000, Sieling *et al.*, 1999)

Mannosylation, a possible strategy for host adaptation.

Tuberculosis (TB) is one of the oldest known human diseases, being possible to identify individuals with bone TB, who died more than 4,000 years ago (Smith, 2003). The success of this long-term interaction between *M. tuberculosis* and its human host may reside, not only in the collection of virulence factors important to manipulate the immune system, but also from possible structural modifications that allowed the pathogen to gain access to the host in a controlled way. The surface of *M. tuberculosis* is predominantly rich in structures containing mannose residues (ManLAM, LM, PIM, arabinomannan, mannan (AM) and manno-glycoproteins). Torrelles *et al* (Torrelles *et al.*, 2010) proposed that *M. tuberculosis* is adapting to the human host by cloaking its cell wall molecules with terminal mannosylated (Man-α-(1→2)-Man) oligosaccharides. By increasing surface mannosylation with mannans that resemble the glycoforms of eukaryotic mannoproteins, heavily mannosylated *M. tuberculosis* strains are optimally phagocytosed by human macrophages using the MR, favoring an intracellular bacterial survival program associated with latency. These strains grow more slowly in the macrophage and cause less tissue damage during infection (Martinez-Pomares *et al.*, 2001, Torrelles *et al.*, 2008).

In contrast, *M. tuberculosis* strains with a poor surface mannosylation associate instead with CR3 pathway for entry, with a rapid intracellular growth and marked tissue damage (Manca *et al.*, 2001, Tsenova *et al.*, 2005). These strains have a “hypervirulent” phenotype which favors progression from latency to active TB disease. Supporting this model are the studies which show that alteration of the amount of mannosylated molecules on the *M. tuberculosis* surface has relevant biological consequences. Inactivation of PimB, a key enzyme in the biosynthesis of PIMs, LM, and ManLAM in *M.*

tuberculosis (Schaeffer *et al.*, 1999), results in a quantitative decrease of the production of LM and ManLAM and infection of human macrophages by the pimB mutant leads to an alteration in macrophage phenotype concomitant with a significant increase in the rate of macrophage death (Torrelles *et al.*, 2009). Also, overexpression of *M. tuberculosis* manB, a phosphomannomutase involved in the biosynthesis of GDP-mannose (a major mannose donor in ManLAM biosynthesis), led to a hypermannosylated *M. smegmatis* strain, with greater ability to bind human monocyte-derived macrophages, as a result of increased abundance of higher order PIMs with mannose cap structures (McCarthy *et al.*, 2005). On the other hand, the analysis of clinical isolates that were found associated with large cluster outbreaks (Gutacker *et al.*, 2006) in geographical areas of high TB incidence, like HN885 and HN1554, showed alterations in the cell wall with a truncation in the ManLAM molecules and a marked reduction of higher order PIM. These strains show a low association with the macrophage mannose receptor, and lower internalization by macrophages, when compared to the heavily mannosylated standard laboratory strains (i.e. *M. tuberculosis* H37Rv and Erdman strains) and despite the reduced level of entry via phagocytosis, both HN885 and HN1554 strains, grow rapidly inside macrophage cells and remain highly virulent once inside these cells (Torrelles *et al.*, 2008).

Therefore, the capability to identify a relationship between a genetic lineage of *M. tuberculosis* and their phenotype with regard to cellular interactions is crucial to understanding how *M. tuberculosis* is evolving to adapt to the human host. However, this task is only beginning. Contrary to what was believed, members of the Mycobacterium tuberculosis Complex (MTBC) are more genetically diverse than generally recognized, and the degree to which this genetic variation influences disease phenotype is still being uncovered. High-throughput sequence analysis has allowed to differentiate MTBC into six main phylogenetic lineages (Hershberg *et al.*, 2008, Comas *et al.*, 2010): three lineages, which branched off from a common ancestor at an early stage of evolution, that include *M. africanum* and are referred to as evolutionarily ‘‘ancient’’ lineages; and three separate evolutionarily ‘‘modern’’ lineages, that diverged at a later time points, as a result of human migration from Africa to other continents. Compared to ancestral strains, “Modern” lineages are pointed to have higher virulence and shorter latency time and a high degree of diversity in the molecular mechanisms of pathogenicity and virulence (Portevin *et al.*, 2011). This variability is observed intra-lineage, with Modern lineage Vietnam, East Asian/Beijing and Indo-Oceanic strains being significantly more likely to cause disseminated tuberculosis with meningitis than those from the modern Euro-American lineage (Hernandez Pando *et al.*, 2010), but also sub-lineage, with different M.

tuberculosis strains within the Beijing genotype demonstrating variability in virulence (Aguilar *et al.*, 2010).

So far, despite the studies performed it was not possible to establish a relation between genetic background structures and phenotype. Examination of *M. tuberculosis* isolates from wider geographic distributions using whole genome scanning approaches revealed that early interactions between *M. tuberculosis* and host are determined by the lineage of the infecting strain, but they were unable to show that these differences are driven by a lineage-specific cell-surface expressed lipids (Krishnan *et al.*, 2011). Furthermore, and also contributing to the variability observed, recent findings suggest that the ability of *M. tuberculosis* to cause disease is not only dependent on its own genetic diversity, but may also be associated with a genetic predisposition from the host (Hershberg *et al.*, 2008). Apart from the already known very rare cases of Mendelian disorders, recent studies reported associations between particular human genetic variants in immunity-related genes and different MTBC lineages (Caws *et al.*, 2008, Intemann *et al.*, 2009, Herb *et al.*, 2008, van Crevel *et al.*, 2009) showing that individual TB-associated genetic loci can also contribute to the development of the disease.

Thus, a new period is emerging. Full genetic and structural characterization of the diverse *M. tuberculosis* clinical strains is needed as a crucial step to understand which factors defined by strain genotype are important for the clinical spectrum of tuberculosis and to predict a possible *M. tuberculosis* evolution in terms of virulence and adaptation to the host.

Contribution of ESX systems to virulence and to Tuberculosis prevention and treatment.

Besides the structural components that constitute the cell wall, secreted proteins are also among the most important molecules involved in host–pathogen interaction of *M. tuberculosis*. The involvement of ESX-1 secretion systems in mycobacterial virulence is clear (MacGurn *et al.*, 2007, Pym *et al.*, 2002, Majlessi *et al.*, 2005, Hsu *et al.*, 2003, Lewis *et al.*, 2003), with the ESAT-6/CFP-10 complex among the most important antigens secreted by *M. tuberculosis* (Sorensen *et al.*, 1995). ESX-5 was the second ESX system implicated in mycobacterial pathogenicity and several studies show that it has an important contribution to *M. marinum* and *M. tuberculosis* virulence. The presence of two out of five ESX systems involved in virulence, suggested that both ESX probably have independent contributions during mycobacterial infections. ESX-1 and ESX- 5 have been

found to secrete different effector proteins that differentially affect subcellular localization and macrophage cell responses. While ESX-1 is involved in the translocation of bacteria into the cytosol, ESX-5 is responsible for the release of secreted effectors into the cytosol triggering cytotoxicity and inducing inflammasome activation (Abdallah *et al.*, 2011). Also, they fail to complement the loss of virulence caused by deletion of the other, suggesting that the presence of both ESX is necessary for full mycobacterial virulence. We show that disruption of ESX-5 from the ESX-1-lacking BCG results in further attenuation of this strain, even in immunocompromised mice, highlighting the unique role of each secretion system for full virulence of *M. bovis* and demonstrating that a correlation between the presence of ESX system and level of virulence can be established.

Vast progress has been made in these last years in the characterization of the ESX systems that can have an important contribution for tuberculosis control. As an example, the design of an improved BCG vaccine against tuberculosis has benefited from all the knowledge generated so far. Reintroduction of RD1 in BCG confers enhanced protection against tuberculosis and an RD1-deleted *M. tuberculosis* mutant is currently in pre-clinical trials as a possible future vaccine (Sambandamurthy *et al.*, 2006). Furthermore, antigens Rv3875 (ESAT-6) and Rv3874 (CP-10) are also under investigation for a potential BCG vaccine boost (van Dissel *et al.*, 2010). Recently, also the ESX-5 has been explored for this purpose. The expression of MPT64, a highly immunogenic *M. tuberculosis* antigen, enhances BCG's protective activity against *M. tuberculosis* in mice (Sali *et al.*, 2010). The surface expression of MPT64 was achieved by fusion with the PE Domain of PE_PGRS33, where the export is dependent of the ESX-5 secretion system.

Additionally, the exported proteins and their respective protein export systems can be considered as potential drug targets. There are no known homologs of ESX systems in eukaryotic organisms, which make unlikely the occurrence of off-target effects on the host by the possible inhibitors designed. Furthermore, an inhibitor that targets a conserved core component of the ESX pathways has the potential to disturb all ESX systems simultaneously, increasing the therapeutic efficiency and probably preventing the development of potential drug resistance.

CONCLUDING REMARKS

The mycobacterial cell wall is a determinant factor in bacterial virulence and characterization of their constituents is crucial to understand the key players that contribute to the evasion from the immune system and survival within the host cells. LAM, a major glycolipid in *M. tuberculosis* cell wall, has been considered an important virulence factor, with the terminal mannose residues seeming to be responsible for its immunomodulatory activity. In this work, we show that the absence of the mannose caps of LAM does not affect mycobacterial virulence, possibly due to redundancy of function. Additionally, we demonstrated the fundamental contribution of the protein export system ESX-5 for the acquisition of full virulence by mycobacteria. In a time when Tuberculosis continues to be a major burden on society and when the current strategy of prevention and treatment has limited effectiveness, we hope that the knowledge generated in this work can contribute in the future for the development of new drugs and more efficient vaccines.

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